

**From Rare Syndromes to DNA Repair Mechanisms:
UBQLN1 Regulates Homologous Recombination and
Exposes Actionable Cancer Vulnerabilities**



Doctoral thesis

for

the award of the doctoral degree

of the Faculty of Mathematics and Natural Sciences

of the University of Cologne

submitted by

Maxim Alexander Hützen

born in Cologne

02.04.2026

I. Table of Contents

II. List of abbreviations.....	VII
III. Abstract	XI
1. Introduction.....	1
1.1 Relevance of Genome Maintenance	1
1.2 The DNA Damage Response	1
1.2.1 DNA damage sources	1
1.2.2 DNA damage repair pathways.....	2
1.2.3 DNA damage sensing.....	3
1.2.4 DNA damage repair and cell cycle checkpoints	3
1.2.5 DNA damage repair and chromatin	3
1.2.6 DNA damage repair and cell-fate outcomes	4
1.3 DNA Double-Strand Break Repair Pathways	4
1.3.1 Sensing DNA double-strand breaks and initiating repair	5
1.3.2 Homologous recombination.....	6
1.3.3 Non-homologous end-joining	7
1.3.4 Alternative homology-mediated pathways	8
1.3.5 Consequences of dysregulated pathway choice	9
1.4 Coordination and Regulation of the DNA Double-Strand Break Repair Response	9
1.5 Genome Instability Syndromes as Models for DNA Repair Research	11
1.5.1 Progeroid syndromes and genome instability	11
1.5.2 Syndrome discovery enables understanding of DNA repair mechanisms	12
1.5.3 Population-level implications of DNA repair variants	12
1.5.4 Utilizing mouse models to study physiological consequences of DNA repair defects.....	13
1.6 DNA Repair Defects and Cancer Development.....	14
1.6.1 Homologous recombination deficiency patterns and tumor characteristics.....	14
1.6.2 Homologous recombination deficiency prevalence and cancer entity distribution	14
1.6.3 Therapeutic vulnerabilities through homologous recombination deficiency	15
1.7 Exploiting DNA Repair Defects for Personalized Cancer Therapy.....	15
1.7.1 Synthetic lethality and application of PARP inhibition	15
1.7.2 Detecting homologous recombination deficiency in the clinic.....	16
1.7.3 Resistance mechanisms limiting PARP inhibitor efficacy	17
1.7.4 Genome instability and immune checkpoint inhibition	17
1.7.5 The KP mouse model for preclinical studies of lung cancer	18

I. Table of contents

1.8 Dissertation Architecture	19
2. Results: Publications and Manuscripts	21
2.1 Manuscript I: Biallelic Variants in <i>CRIP1</i> Cause a Rothmund-Thomson-like Syndrome with Increased Cellular Senescence.....	21
2.1.1 Contributions	21
2.1.2 Peer-reviewed publication	22
2.2 Manuscript II: Optical Genome Mapping Identifies Structural Variants in Potentially New Cancer Predisposition Candidate Genes in Pediatric Cancer Patients	40
2.2.1 Contributions	40
2.2.2 Peer-reviewed publication	40
2.2.3 Relevant supplementary figure (II-Figure S5)	49
2.3 Manuscript III: UBQLN1-mediated Repression of Homologous Recombination is an Actionable Target for Cancer Therapy.....	50
2.3.1 Contributions	50
2.3.2 Manuscript status	52
2.3.3 Manuscript (under review)	52
2.4 Manuscript IV: Targeting a Glutamic Acid in PDEδ with Fluoromethyl-Aryl Electrophiles Impairs KRAS Signaling	72
2.4.1 Contributions	72
2.4.2 Peer-reviewed publication	73
3. Extended Discussion of Individual Manuscripts.....	92
3.1 Manuscript I: Characterization of the <i>CRIP1</i> Syndrome.....	92
3.1.1 Rationale and phenotypic overlap with Rothmund-Thomson syndrome	92
3.1.2 Senescence as a primary phenotype and implications for <i>CRIP1</i> syndrome classification	93
3.1.3 Convergence of Rothmund-Thomson syndrome-associated genes.....	94
3.1.4 Limitations	94
3.1.5 Conclusions and future directions	95
3.2 Manuscript II: Optical Genome Mapping Identifies Structural Variants	96
3.2.1 Rationale and study overview	96
3.2.2 Characterization of a <i>de novo</i> 17p13.3 duplication encompassing <i>RPA1</i>	97
3.2.3 Additional structural variants of uncertain significance.....	98
3.2.4 Limitations	99
3.2.5 Conclusions and future directions	99

I. Table of contents

3.3 Manuscript III: From Syndrome to DNA Repair Mechanism to Actionable Cancer	
Vulnerabilities: UBQLN1 in DNA Repair	100
3.3.1 <i>UBQLN1</i> deficiency syndrome discovery.....	100
3.3.2 The "shielding" model of UBQLN1	101
3.3.3 UBQLN1 within the multi-layered RAD51 regulatory network.....	103
3.3.4 The "domain switch" theory of UBQLN1	104
3.3.5 The UBQLN1/UBQLN4 paradox of opposing HR phenotypes	105
3.3.6 A dosage-dependent model of UBQLN1 function	107
3.3.7 UBQLN1 as a proteostasis regulator in genome maintenance	108
3.3.8 Proteostasis as a regulatory layer in DNA double-strand break repair	110
3.3.9 UBQLN1 dysregulation in cancer	111
3.3.10 <i>UBQLN1</i> overexpression as an oncogenic driver	112
3.3.11 Alternative repair pathway engagement in UBQLN1-dysregulated cells.....	113
3.3.12 UBQLN1 as a candidate therapeutic biomarker	114
3.3.13 Conclusions and future directions	115
3.4 Manuscript IV: Targeting a Glutamic Acid in PDEδ with Fluoromethyl-Aryl	
Electrophiles Impairs KRAS Signaling	116
3.4.1 Advances in direct KRAS ^{G12D} targeting	116
3.4.2 Carboxylate-targeting chemistry in the context of emerging covalent inhibitors.....	117
3.4.3 Therapeutic validity of PDEδ as a surrogate target.....	118
3.4.4 Alternative mechanisms of antitumor efficacy.....	119
3.4.5 KRAS-induced DNA damage response rewiring	119
3.4.6 Implications for combination therapy strategies	119
3.4.7 Limitations	120
3.4.8 Conclusions and future directions	120
4. Cumulative Discussion	121
4.1 From Syndrome Discovery to Therapeutic Vulnerabilities in Cancer	121
4.2 Mechanistic Concepts and Therapeutic Perspectives	122
5. Conclusion	125
6. Publications and Manuscripts	127
6.1 Included in this Dissertation	127
6.2 Not Included in this Dissertation	127
7. Materials and Methods	128
7.1 Materials and Methods of Integrated Manuscripts	128

I. Table of contents

7.2 Software Tools.....	128
8. Eidesstattliche Erklärung	129
9. Curriculum Vitae.....	130
10. Acknowledgements.....	132
11. References	134
12. Appendix	150
12.1 Supplementary Data of Integrated Manuscripts	150
12.1.1 Biallelic Variants in <i>CRIP1</i> Cause a Rothmund-Thomson-like Syndrome with Increased Cellular Senescence	150
12.1.2 Optical Genome Mapping Identifies Structural Variants in Potentially New Cancer Predisposition Candidate Genes in Pediatric Cancer Patients	150
12.1.3 UBQLN1-mediated Repression of Homologous Recombination is an Actionable Target for Cancer Therapy.....	150
12.1.4 Targeting a Glutamic Acid in PDE δ with Fluoromethyl-Aryl Electrophiles Impairs KRAS Signaling.....	174

II. List of abbreviations

Abbreviation	Definition
53BP1	p53-binding protein 1
ABL1	Abelson murine leukemia viral oncogene homolog 1
ADAM9/32	A disintegrin and metallopeptidase domain protein 9/32
AKT	AKT serine/threonine kinase
ALL	Acute lymphoblastic leukemia
alt-NHEJ	Alternative non-homologous end-joining
ANAPC1	Anaphase-promoting complex subunit 1
AT	Ataxia-telangiectasia
ATM	Ataxia-telangiectasia-mutated
ATR	Ataxia-telangiectasia- and Rad3-related kinase
BCR	Breakpoint cluster region
BCR::ABL1	Oncogenic fusion protein between BCR and ABL1
BER	Base excision repair
BLM	Bloom syndrome helicase
BRCA1	Breast cancer susceptibility protein 1
BRCA2	Breast cancer susceptibility protein 2
CDK	Cyclin-dependent kinase
CHEK2	Checkpoint kinase 2
ClinVar	NCBI archive of genomic variation and its relationship to human health
CRIP1	Cysteine-rich interactor of PDZ three
CtIP	CtBP-interacting protein (RBBP8)
DDB2	Damage specific DNA binding protein 2
DDR	DNA damage response
DNA2	DNA replication helicase/nuclease 2
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DR-GFP	Direct repeat-GFP (HR reporter assay)
DSB/DSBs	Double-strand break/s
DSBR	Double-strand break repair
dsDNA	Double-stranded DNA
E3	E3 ubiquitin ligase
EGFR	Epidermal growth factor receptor
EMI1	Early mitotic inhibitor 1 (FBXO5)
ERK	Extracellular signal-regulated kinase
EXO1	Exonuclease 1
FBH1	F-box DNA helicase 1 (FBXO18)

II. List of abbreviations

Abbreviation	Definition
FDA	U.S. Food and Drug Administration
FIGNL1	Fidgetin-like protein 1
FLIP	FIGNL1 interacting protein, alias FIRRM (C1orf112)
GABA _A	Gamma-aminobutyric acid type A
GEMM	Genetically engineered mouse model
gnomAD	Genome aggregation database
GPX4	Glutathione peroxidase 4
GRB2	Growth factor receptor-bound protein 2
GTP/GDP	Guanosine tri-/diphosphate
HCI	High-content imaging
HR	Homologous recombination
HRD	Homologous recombination deficiency
ICB	Immune checkpoint blockade
ICLR	Interstrand crosslink repair
IDR	Intrinsically disordered region
IR	Ionizing radiation
KMT2A	Lysine methyltransferase 2A
KP	<i>Kras</i> ^{LSL.G12D/wt} ; <i>Trp53</i> ^{fl/m} mouse model
KPU1	<i>Kras</i> ^{LSL.G12D/wt} ; <i>Trp53</i> ^{fl/m} ; <i>Rosa26</i> ^{LSL.HA-Ubqln1} mouse model
KRAS	Kirsten rat sarcoma viral oncogene homolog
Ku70	<i>XRCC6</i> (X-ray repair cross-complementing 6)
Ku80	<i>XRCC5</i> (X-ray repair cross-complementing 5)
LIG4	DNA ligase 4
LOH	Loss of heterozygosity
LSL	Lox-Stop-Lox (<i>Cre/lox</i> -system)
LST	Large-scale state transition
LUAD	Lung adenocarcinoma
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MG132	Proteasome inhibitor
MHC	Major histocompatibility complex
MMEJ	Microhomology-mediated end-joining
MMR	Mismatch repair
MRE11	Meiotic recombination 11, double-strand break repair nuclease
MRN	MRE11-RAD50-NBS1 complex
MSI-H	Microsatellite instability-high
mTOR	Mechanistic target of rapamycin

II. List of abbreviations

Abbreviation	Definition
mTORC1	Mechanistic target of rapamycin complex 1
MYC	Myelocytomatosis proto-oncogene
NBS1	Nijmegen breakage syndrome 1
NCS	Neocarzinostatin
NER	Nucleotide excision repair
NHEJ/c-NHEJ	(Classical) non-homologous end-joining
NSCLC	Non-small cell lung cancer
OGM	Optical genome mapping
OMIM	Online mendelian inheritance in man database
OS	Overall survival
PAH	Polycyclic aromatic hydrocarbon
PALB2	Partner and localizer of BRCA2
PAR	Poly(ADP-ribose)
PARP	Poly(ADP-ribose) polymerase
PARP1/2	Poly(ADP-ribose) polymerase 1/2
PD-1	Programmed death-1
PDEδ	Phosphodiesterase δ
PD-L1	Programmed death-ligand 1
PI3K	Phosphatidylinositol 3-kinase
Pol θ / PolQ	Polymerase θ
PTM	Post-translational modification
RAD23B	UV excision repair protein RAD23 homolog B
RAD50	RAD50 double strand break repair protein
RAD51	RAD51 recombinase
RAD52	RAD52 homolog, DNA repair protein
RAD54	RAD54 DNA repair and recombination protein
RAF1	Raf-1 proto-oncogene, serine/threonine kinase
RAP1B	Ras-related protein 1B
RECQL4	RecQ-like helicase 4
RFWD3	Ring finger and WD repeat domain 3
RHEB	Ras homolog, mTORC1 binding
RIF1	Replication timing regulatory factor 1
ROS	Reactive oxygen species
RPA	Replication protein A heterotrimer (RPA1, RPA2, RPA3)
RPA1	Replication protein A 1, alias RPA70
RPA2	Replication protein A 2, alias RPA32
RTS	Rothmund-Thomson syndrome

II. List of abbreviations

Abbreviation	Definition
SASP	Senescence-associated secretory phenotype
SDSA	Synthesis-dependent strand annealing
SERPINF1/2	Serpin family F member 1/2
SMYD4	SET and MYND domain containing 4
SNV	Single nucleotide variant
SSA	Single-strand annealing
SSB/SSBs	Single-strand break/s
SSBR	Single-strand break repair
ssDNA	Single-stranded DNA
STAT3	Signal transducer and activator of transcription 3
STI1	Stress-inducible protein 1-like domain
SV	Structural variant
TAI	Telomeric allelic imbalance
TCGA	The Cancer Genome Atlas
TMB	Tumor mutational burden
TP53	Tumor protein p53 (human)
Trp53	Tumor protein p53 (murine)
UBA	Ubiquitin-associated domain
UBL	Ubiquitin-like domain
UBQLN	Ubiquilin-family protein
UBQLN1	Ubiquilin-1, alias PLIC-1
UBQLN4	Ubiquilin-4
UHRF1	Ubiquitin like with PHD and ring finger domains 1
UV	Ultraviolet radiation
WDR81	WD repeat domain protein 81
WES	Whole exome sequencing
WGS	Whole genome sequencing
WRN	Werner syndrome helicase
XPC	XPC subunit DNA damage recognition and repair factor
XRCC4	X-ray repair cross complementing 4
ZEB1	Zinc finger E-box binding homeobox 1
γH2AX	Phosphorylated histone H2AX
θ-NHEJ	Polymerase θ-mediated end-joining

This list includes and defines all abbreviations and gene/protein names used in this dissertation (excluding integrated publications). To preserve readability, only the most relevant abbreviations are additionally introduced at first use in the text.

III. Abstract

Genomic integrity is continuously challenged by DNA damage. Cells have evolved complex repair mechanisms to maintain tissue homeostasis. The study of rare genome instability syndromes caused by defects in these repair pathways can reveal previously unrecognized DNA repair factors and regulatory mechanisms. Many of these syndromes are associated with cancer predisposition, highlighting the direct link between impaired DNA repair and tumorigenesis. Understanding the mechanisms underlying these syndromes may therefore reveal exploitable tumor-specific dependencies, with implications beyond rare syndromes. This dissertation spans syndrome characterization, mechanistic investigation, and preclinical therapeutic studies. The four integrated manuscripts illustrate this trajectory. Biallelic variants in *Cysteine-Rich Interactor of PDZ Three (CRIPT)* cause a progeroid disorder driven by cellular senescence without detectable DNA damage sensitivity, demonstrating how functional studies distinguish phenotypically overlapping conditions from DNA repair deficiencies. Optical genome mapping in a pediatric cohort with suspected cancer predisposition improves diagnostic capacity for structural variant detection. A *de novo* duplication encompassing *Replication Protein A1 (RPA1)* identified in this cohort is associated with genotoxic hypersensitivity. The central study, discovery and characterization of the *Ubiquilin-1 (UBQLN1)* deficiency syndrome, reveals that UBQLN1 regulates homologous recombination (HR) repair by shielding the recombinase RAD51 from ubiquitin-dependent proteasomal degradation. Loss of *UBQLN1* destabilizes RAD51 and impairs accurate DNA repair via HR. By contrast, *UBQLN1* overexpression promotes degradation of the DNA repair nuclease MRE11 and is associated with excessive DNA end resection and engagement of alternative mutagenic repair pathways. *UBQLN1* is frequently overexpressed in aggressive human cancers. In a *Kras*^{G12D}-driven lung cancer mouse model, *Ubqln1* overexpression emerges as a candidate biomarker for tumor aggressiveness and for sensitivity to poly(ADP-ribose) polymerase (PARP) inhibition and immune checkpoint blockade, with the potential to improve therapeutic stratification in the clinic. Lastly, in a target-directed approach, development of Deltafluorine, a first-in-class covalent phosphodiesterase δ (PDE δ) inhibitor, enables targeting of oncogenic KRAS membrane trafficking. Treatment of the *Kras*^{G12D}-driven mouse model with Deltafluorine reduces tumor burden *in vivo*. Overall, this work demonstrates how investigating rare syndromes alongside target-directed therapeutic development can identify mechanisms and vulnerabilities relevant to cancer therapy.

1. Introduction

1.1 Relevance of Genome Maintenance

Every day, each human cell faces approximately 70,000 DNA lesions from sources such as reactive oxygen species, replication errors, and environmental damage.¹ Yet most cells are capable of maintaining genomic integrity throughout many cell cycles and decades of life.² As the source of all cellular information, DNA integrity is critical for cellular function and requires active maintenance.³ Germline cells preserve genetic information across generations, while somatic cells maintain tissue homeostasis within an individual's lifespan. Nevertheless, germline DNA alterations contribute to shaping evolutionary trajectories and can also cause genetic disease.⁴ When repair is insufficient, cells can activate programmed death or permanent growth arrest to prevent propagation of damaged DNA and uncontrolled cancerous cell growth.⁵ This balance between DNA repair and cell elimination can become increasingly compromised with age.^{2,6}

Ageing is characterized by progressive deterioration of an organism under the extrinsic and intrinsic pressures of life.⁷ Impaired DNA repair can cause genome instability, a hallmark of ageing that creates a vicious cycle: accumulation of unrepaired damage drives the ageing process, while ageing leads to progressive decline of DNA repair capacity over time,⁷⁻¹⁰ as exemplified by premature ageing syndromes frequently caused by DNA repair defects.¹¹ Ageing is a major risk factor for most cancers and approximately 90 % of cancer patients are older than 50 years.¹² The stochastic accumulation of somatic mutations can activate oncogenes or inactivate tumor suppressors, initiating cancer development.^{7,13,14} Once established, cancer cells often exploit genomic instability to increase mutational burden and adaptability.^{15,16} Understanding DNA repair mechanisms can therefore contribute to the development of targeted therapies that exploit tumor vulnerabilities and may help prolong human healthspan.^{17,18} To understand these vulnerabilities, one must first consider the nature of the threats for which DNA repair systems have evolved.

1.2 The DNA Damage Response

1.2.1 DNA damage sources

DNA integrity faces continuous pressure from extrinsic and intrinsic sources, each generating distinct lesion types that require specialized repair responses. Extrinsic damaging factors include ultraviolet (UV) radiation¹⁹⁻²¹, ionizing radiation (IR)²², and environmental chemicals²³. UV radiation contributes to skin cancer development by generating photoproducts that alter the DNA backbone and can stall replication or lead to base substitutions.¹⁹⁻²¹

1. Introduction

IR can induce DNA base modifications, single-, and double-strand breaks (SSB/DSB).²² DSBs are particularly hazardous, as even a single unrepaired break can trigger cell death or drive chromosomal rearrangements.²⁴ Beyond radiation, humans are exposed to numerous DNA-damaging environmental chemicals, either voluntarily or unavoidably. This includes, among others, cigarette smoking, exhaust gases, and chemicals in food.^{23,25,26} Cigarette smoke contains carcinogens that damage DNA, with smokers accumulating thousands of additional mutations per cell in the bronchial epithelium compared to non-smokers, thereby increasing lung cancer risk.²⁵ Environmental air pollutants, particularly polycyclic aromatic hydrocarbons (PAHs) from vehicle exhausts, can cause DNA adducts, oxidative DNA damage, and subsequent genomic instability.²³ The human diet contributes to the DNA damage burden through natural and processing-derived carcinogens.²⁶ By contrast, clinical applications deliberately harness extrinsic damage, where cancer patients are intentionally exposed to DNA-damaging chemotherapy and radiotherapy to eliminate cancer cells by exploiting their faster growth and compromised cellular physiology.²⁷

Intrinsic damaging factors include intracellular reaction products from metabolic reactions like reactive endogenous chemicals.²⁸ DNA undergoes spontaneous chemical alterations including oxidation, alkylation, and deamination.³ While the DNA replication machinery is a complex and highly evolved mechanism with proofreading capabilities, it is not entirely error-free, with approximately 1 in 10^9 errors after proofreading.²⁹ If not accurately repaired, these errors can drive the accumulation of severe mutations over time. Additionally, metabolism can result in intrinsic damage through mitochondrial respiration and buildup of reactive oxygen species (ROS). ROS damage DNA bases, introduce SSBs, and stall the replication machinery.³⁰

Importantly, if genes that encode proteins of the DNA damage response (DDR) are damaged or harbor mutations, the capacity to detect and repair subsequent damage can become compromised, which may accelerate genomic deterioration.³¹ To counteract this threat, interdependent and redundant DNA repair mechanisms have evolved to maintain genomic integrity.³² Defects in these complex processes can lead to genomic instability and subsequent cancer development, neurodegenerative disease, and premature ageing.³³⁻³⁶ The diversity of DNA lesions has driven the evolution of specialized repair pathways, each tailored to recognize and resolve specific types of damage.³⁷

1.2.2 DNA damage repair pathways

The selection among these specialized pathways resembles a resource allocation problem. Response options are constrained by damage type, cell cycle phase, metabolic status, and the availability and integrity of repair and regulatory factors.³¹ DNA repair mechanisms are thus tailored to different types of DNA lesions within these cellular constraints.

1. Introduction

Base excision repair (BER) addresses small, non-helix-distorting modifications caused by oxidation, alkylation, or deamination.³⁸ Nucleotide excision repair (NER) resolves bulky, helix-distorting lesions such as UV-induced pyrimidine dimers.³⁹ Mismatch repair (MMR) repairs base-base mismatches and insertion-deletion loops that escape replication proofreading.⁴⁰ SSB repair (SSBR) addresses backbone discontinuities, which can convert to DSBs during replication if unrepaired.⁴¹ Interstrand crosslink repair (ICLR) resolves covalent linkages between DNA strands.⁴² DSB repair (DSBR) addresses the most cytotoxic form of DNA damage, where both strands are severed.⁴³ While these pathways maintain genomic integrity, the appropriate pathway can only be effectively engaged once damage has been detected and characterized.

1.2.3 DNA damage sensing

Different lesion types are recognized through distinct mechanisms suited to their structural or chemical features. Small base modifications are detected by glycosylases that scan DNA and recognize specific chemical alterations.⁴⁴ Helix-distorting lesions are sensed either through global surveillance mechanisms that detect structural distortion or when they stall the transcription machinery.⁴⁵ Mismatches are identified by recognition complexes that detect thermodynamic instability of improperly paired bases.⁴⁶ SSBs are rapidly bound by sensor proteins that recognize exposed backbone discontinuities.⁴⁷ DSBs, as the most dangerous lesion type, trigger complex signaling cascades. Sensor complexes recognize broken DNA ends and activate kinases that phosphorylate hundreds of substrates, amplifying the damage signal across chromatin domains.⁴⁸ This amplification allows recruitment of repair factors and communication with cell cycle machinery to coordinate DSBR.³¹

Across all pathways, damage sensing serves three key functions. It identifies lesions requiring repair, it recruits the appropriate repair machinery, and it signals to checkpoint pathways that coordinate repair with cell cycle progression.^{31,49}

1.2.4 DNA damage repair and cell cycle checkpoints

The checkpoint pathways engaged by damage sensing halt cell cycle progression to provide time for repair before critical transitions. The G₁/S checkpoint prevents replication of damaged DNA.⁵⁰ The intra-S checkpoint slows replication fork progression when damage occurs during S phase.⁵¹ The G₂/M checkpoint prevents mitotic entry when damage persists.⁵² The efficiency of these signaling cascades, however, depends on the physical accessibility of damage sites within their chromatin context.

1.2.5 DNA damage repair and chromatin

Compacted chromatin regions exhibit delayed damage detection and reduced repair efficiency, as repair factors require physical access to lesion sites.⁵³

1. Introduction

Therefore, damage triggers local chromatin relaxation through histone modifications, which also serves as a recruitment platform for specific repair factors.⁵⁴ Nucleosome remodeling permits access, and following repair completion, the original chromatin state is restored.⁵⁵ When repair succeeds, cells resume normal function, but when damage proves irreparable, alternative cell fates are engaged.

1.2.6 DNA damage repair and cell-fate outcomes

The choice among these alternative fates reflects the DDR's integration of damage severity and repair capacity.⁵⁶ While reparable damage triggers only transient checkpoint activation followed by cell cycle re-entry,⁵⁷ persistent or excessive damage engages terminal outcomes. Severe or irreparable damage can lead to senescence, a state of stable cell cycle arrest that prevents proliferation while maintaining cell viability.⁵⁸ Senescent cells can promote a senescence-associated secretory phenotype (SASP), releasing inflammatory cytokines, growth factors, and proteases that alter tissue microenvironment and lead to tissue inflammation.⁵⁹ Premature or excessive senescence induction is associated with a class of progeroid syndromes, in which constitutional defects in DNA repair, replication, or cell cycle checkpoint regulation lead to accelerated cellular ageing and organism-level features of premature ageing.⁶⁰ Premature ageing syndromes demonstrate that impaired DNA repair and dysregulated senescence signaling can cause systemic tissue deterioration independently of chronological age.⁶¹

When damage is severe and irreparable, cells can activate programmed cell death pathways to eliminate potentially dangerous cells. Apoptosis represents a major mechanism, executing controlled cell dismantling while avoiding inflammatory responses.⁶² Other forms of cell death serve as alternative pathways. Necroptosis can provide a programmed but inflammatory alternative when apoptosis is blocked,⁶³ while ferroptosis is driven by iron-dependent lipid peroxidation and has emerged as a relevant cell death pathway following oxidative DNA damage.⁶⁴

Together, these mechanisms prevent propagation of damaged DNA to daughter cells. Among all lesion types, DSBs present the greatest challenge to genome maintenance.

1.3 DNA Double-Strand Break Repair Pathways

DSBs are among the most genotoxic DNA lesions due to the complete disruption of the double-stranded DNA (dsDNA) and the potential for large-scale insertions and deletions, necessitating timely and accurate repair.⁴³ Cells employ two major DSB repair pathways: *i*) Non-homologous end-joining (NHEJ) directly ligates broken ends and operates throughout the cell cycle but can

1. Introduction

introduce small mutations, including insertions and deletions,⁶⁵ and *ii*) Homologous Recombination (HR), which utilizes the sister chromatid as a template for high-fidelity repair, but is restricted to S and G₂ cell cycle phases when a template is available.⁶⁶ A central step for HR is the end resection of the break into single-stranded DNA (ssDNA) overhangs.⁶⁷ The decision to initiate the resection of broken strands serves as the critical determinant of pathway choice, where resection commits cells to HR, whereas inhibition of resection favors NHEJ (**Figure 1**).^{66,68} Understanding how this decision is reached requires examining each step from initial damage sensing through pathway commitment.

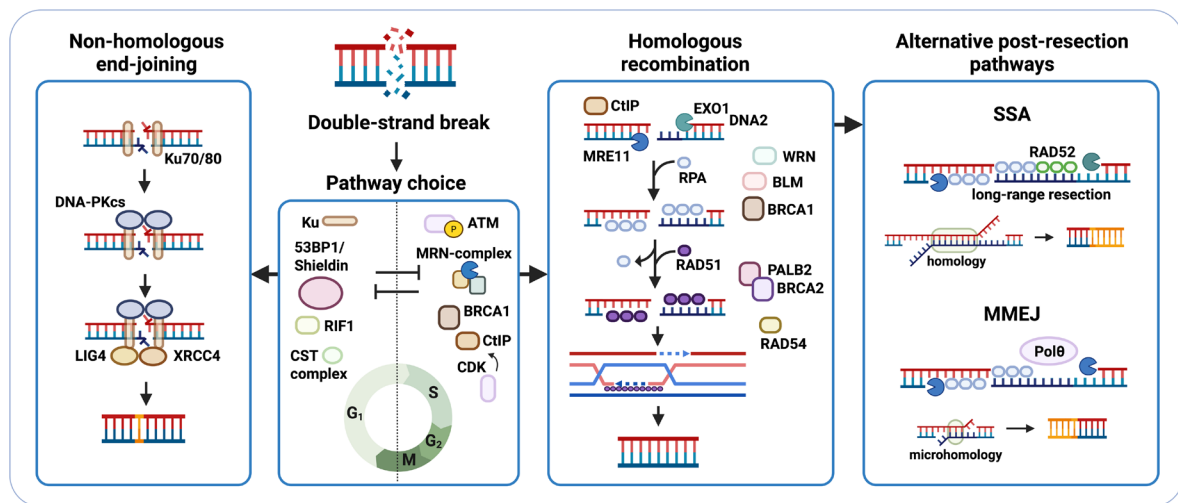


Figure 1: DSB repair pathway choice is governed by end resection and cell cycle stage. In NHEJ, minimally processed DNA ends are rapidly ligated via Ku70/80 and LIG4-XRCC4, frequently introducing small insertions or deletions. In S/G₂ phase, CDK-dependent activation of CtIP and the MRN complex promotes end resection, enabling error-free repair via RAD51-mediated HR. Dysregulated resection can alternatively channel repair into mutagenic single-strand annealing (SSA) or Pol θ-dependent microhomology-mediated end-joining (MMEJ), both of which can introduce large deletions and genomic rearrangements. The balance between resection-blocking (53BP1/Shieldin) and pro-resection (BRCA1/MRN/CtIP) factors at the break site determines pathway commitment. Figure created with BioRender.

1.3.1 Sensing DNA double-strand breaks and initiating repair

The pathway toward this decision begins with damage recognition. DSBs are rapidly recognized by the MRN complex (MRE11-RAD50-NBS1), which serves as the primary DSB sensor.⁶⁹ MRN binding initiates a signaling cascade that enables chromatin accessibility, amplifies the damage signal across megabases of surrounding chromatin,^{54,70} and activates the Ataxia-telangiectasia-mutated (ATM) kinase.⁶⁹ The central role of ATM in DSB signaling was discovered through investigation of Ataxia-telangiectasia (AT), a genome instability syndrome characterized by cerebellar degeneration, immunodeficiency, chromosomal instability, and cancer predisposition.⁷¹ Yosef Shiloh identified the *ATM* gene in 1995 following investigations that began with a visit to a family affected by the syndrome during his graduate studies in 1977.^{72,73}

1. Introduction

The identification of *ATM* transformed understanding of DSB signaling and cell cycle control, and remains one of the clearest examples of syndrome investigation revealing fundamental repair mechanisms.

Activated *ATM* phosphorylates the histone H2AX on serine 139, generating γ H2AX, which promotes chromatin accessibility for DSB signaling.⁷⁴ Downstream signaling recruits two key protein complexes with opposing functions. Ku70/80, 53BP1, and the Shieldin complex, which promote NHEJ, and the BRCA1-A complex, which facilitates HR (**Figure 1**).⁶⁸ Thus, the sensing machinery not only detects damage and amplifies the signal, but also establishes the molecular context for subsequent pathway choice.

1.3.2 Homologous recombination

When conditions favor HR, the pathway is initiated via an endonucleolytic 5' nick by CtIP, which is followed by resection of 50-100 nucleotides by MRE11.⁷⁵ This resection bypasses Ku-blocked ends and represses NHEJ.⁷⁶ Further, long-range resection is executed by EXO1 in the 5'-3' direction, creating kb-sized ssDNA overhangs.^{77,78} In parallel, the Bloom (BLM) and Werner (WRN) helicases unwind dsDNA and enable access for DNA2 nuclease, which degrades the 5' strand and leaves long 3' ssDNA overhangs.^{79,80} The cell cycle restriction of HR is mediated through CDK-dependent phosphorylation of CtIP, which activates its endonuclease activity only in S/G₂.⁸¹⁻⁸³

Subsequently, the ssDNA overhangs resulting from resection are coated by RPA for protection from secondary structures and degradation.⁸⁴ RPA is exchanged with RAD51 via the BRCA1-PALB2-BRCA2 complex.^{85,86} The RAD51-coated nucleoprotein filament facilitates HR by actively searching for a homologous DNA sequence on the undamaged sister chromatid in S/G₂ phase.⁸⁷ RAD54, a chromatin remodeling factor, facilitates this homology search by stabilizing the RAD51 filament and removing nucleosomes to enable access to the template DNA.^{88,89} Upon homology detection, RAD51 mediates strand invasion. The 3' ssDNA invades the homologous dsDNA, displacing one strand and forming a displacement-loop (D-loop).⁹⁰ The ssDNA of the broken strand is then synthesized by DNA polymerases.⁹¹

HR can proceed through two major resolution pathways. In the canonical DSB pathway, both DNA ends are captured, leading to the formation of double Holliday junctions that are resolved by specialized nucleases.^{92,93} Alternatively, HR can proceed via synthesis-dependent strand annealing (SDSA). Here, the invading strand is displaced upon DNA synthesis and re-anneals with the second resected end.^{94,95} DNA synthesis completes repair by filling remaining gaps, restoring DNA integrity.⁹⁵

1. Introduction

SDSA represents the predominant HR mechanism in somatic cells, as it avoids the formation of Holliday junctions and prevents potentially deleterious chromosome crossovers.⁹⁶ Overall, HR repairs DSBs with high fidelity while preserving genetic information.⁹⁶

The critical roles of BRCA1 and BRCA2 in HR emerged from genetic studies of hereditary breast and ovarian cancer families. Hall *et al.* identified chromosomal linkage to *BRCA1* in 1990⁹⁷, with subsequent cloning of *BRCA1* by Miki *et al.* in 1994⁹⁸ and *BRCA2* by Wooster *et al.* in 1995⁹⁹. Molecular characterization revealed that BRCA1 functions in DNA end resection and checkpoint signaling, while BRCA2 loads RAD51 onto resected ssDNA to initiate strand invasion.^{100,101} Given HR's critical role in preserving genomic integrity, heterozygous *BRCA1/2* mutations confer up to 88 % lifetime breast cancer risk,¹⁰² while biallelic null mutations cause embryonic lethality in mouse models.¹⁰³ This discovery contributed to establishing DNA repair defects as a mechanism of cancer predisposition.

Beyond BRCA1/2, RecQ family helicases contribute to HR fidelity by resolving aberrant DNA structures that arise during recombination. Werner syndrome and Bloom syndrome show how helicase dysfunction compromises HR and causes genome instability. Werner syndrome, first described by Otto Werner in 1904, presents with premature ageing features including early graying and hair loss, skin atrophy, cataracts, and type II diabetes, typically manifesting in the second and third decades of life.^{104,105} Cloning of the *WRN* gene in 1996 revealed that WRN possesses both 3'-5' helicase and exonuclease activities essential for replication fork stability, telomere maintenance, and resolution of recombination intermediates. WRN processes D-loops and Holliday junctions during HR, and its loss leads to illegitimate recombination events and chromosomal instability.^{11,106-108} Bloom syndrome, caused by biallelic *BLM* mutations, presents with growth retardation, photosensitivity, immunodeficiency, and markedly elevated cancer predisposition.^{109,110} Loss of *BLM* causes an increase in sister chromatid exchanges, reflecting uncontrolled crossover recombination.¹¹¹ Both WRN and BLM thus ensure that HR proceeds accurately without generating deleterious chromosomal rearrangements, and their associated syndromes underscore the importance of controlled recombination for genome stability.

1.3.3 Non-homologous end-joining

When no homologous template is available, cells rely on NHEJ for DSB repair. This pathway requires active inhibition of DNA end resection, mediated by 53BP1, which blocks access to the resection machinery.⁶⁸ ATM-mediated phosphorylation of 53BP1 enables recruitment of RIF1, the Shieldin complex, and the CST complex.^{112,113}

1. Introduction

The Ku70/80 heterodimer rapidly binds to DNA ends, forming a ring structure that encircles the DNA,¹¹⁴ recruiting the DNA-PKcs kinase to each DNA end.^{115,116} The XRCC4 and DNA ligase IV complex is recruited to ligate the processed DNA ends.¹¹⁷ **(Figure 1)**

Because NHEJ directly ligates DNA ends without a template, it often introduces small insertions or deletions, rendering NHEJ an error-prone repair pathway.¹¹⁸ Despite this mutagenic potential, NHEJ is essential for maintaining genome stability, particularly in G₁ phase, where HR is not possible due to unavailability of the sister chromatid.⁶⁸ In DSB repair pathway choice, NHEJ is a fast and readily available mechanism, where some mutagenicity is tolerated in exchange for speed and cell cycle independence. Together with HR, these canonical pathways handle the majority of DSBs, though additional mechanisms exist for situations where they are unavailable or defective.

1.3.4 Alternative homology-mediated pathways

These alternative pathways are typically more mutagenic but can prevent catastrophic outcomes when resection or ligation processes become dysregulated. When regulation of resection is defective, extended resection can produce extensive ssDNA tracts revealing distant flanking homologous repeat sequences.¹¹⁹ Single-strand annealing (SSA) utilizes these repeats to repair resected DSBs when HR is impaired.¹²⁰ As in HR, RPA coats the ssDNA, but is subsequently exchanged with RAD52 instead of RAD51.^{119,121} RAD52 nucleoprotein filaments allow homology detection and facilitate annealing with complementary ssDNA.¹¹⁹ Extensive ssDNA may additionally overwhelm the RAD51-loading machinery, shifting repair toward SSA.¹²⁰ However, SSA results in deletion of the sequence between two repeats and one of the repeat copies, in a size range of kb to Mb, which leads to significant loss of genetic information.¹¹⁹ Despite its mutagenic nature, this pathway can compensate for HR-defects downstream of DNA end resection.¹²² However, SSA requires the presence of homologous repeat sequences, limiting its applicability. **(Figure 1)**

When such repeats are absent, cells can employ alternative end-joining mechanisms that require only short stretches of microhomology. Alternative NHEJ (alt-NHEJ), microhomology-mediated end-joining (MMEJ), and polymerase θ -mediated end-joining (θ -NHEJ) are terms used variably in the literature to describe overlapping Ku-independent, microhomology-dependent repair pathways.¹²³ Alt-NHEJ/MMEJ/ θ -NHEJ is initiated through limited DNA end resection mediated by MRE11 and CtIP, generating short 3' overhangs.¹²⁴ Unlike canonical NHEJ, MMEJ does not require Ku70/80 or DNA-PKcs. Instead, it relies on PARP1 for initial end recognition and stabilization.¹²⁵ Polymerase θ (Pol θ) plays a central role by unwinding DNA, facilitating microhomology search, and annealing complementary ssDNA through its helicase and polymerase domains.^{126,127}

1. Introduction

However, Pol θ frequently generates deletions between annealing sites.¹²⁸ MMEJ becomes particularly active in HR-deficient cells with shorter resection tracts than required for SSA (**Figure 1**).^{129,130}

When cells become reliant on these error-prone backup mechanisms, the accumulated mutagenic burden can drive disease.

1.3.5 Consequences of dysregulated pathway choice

Dysregulation of DSBR pathway choice is directly linked to human disease.^{131,132} Germline mutations in HR genes predispose to error-prone repair pathways.^{133,134}

The balance between HR, NHEJ, and alternative pathways is critical for maintaining genome integrity, and its disruption underlies numerous hereditary cancer syndromes and therapeutic vulnerabilities.¹³¹⁻¹³³ Understanding the regulatory mechanisms that maintain this balance is therefore essential for both disease characterization and therapeutic intervention.

1.4 Coordination and Regulation of the DNA Double-Strand Break Repair Response

DSBR is coordinated through post-translational modifications (PTMs) and regulated protein turnover.¹³⁵⁻¹³⁷ The crosstalk between addition and removal of different PTMs enables dynamic repair coordination and ensures genome stability.^{135,138,139} Protein abundance, regulated through transcription and protein turnover, determines the pool of available repair factors that can be recruited and assembled into functional complexes at damage sites.^{140,141} Disruption of these regulatory systems can impair DNA repair.^{135,136} PTMs fine-tune DSBR through reversible chemical modifications of repair proteins, regulatory proteins, and chromatin organization. Several classes of modification are particularly important for DSBR coordination.

Phosphorylation by ATM, ATR, and DNA-PKcs kinases initiates signaling cascades through adding negatively charged phosphate groups to serine, threonine, or tyrosine residues, altering protein conformation and activity to regulate checkpoint activation, resection, and pathway choice.¹⁴²⁻¹⁴⁵ Ubiquitination attaches ubiquitin to lysine residues, facilitating chromatin remodeling, protein recruitment, and degradation of repair proteins.¹⁴⁶⁻¹⁴⁸ Ubiquitination is mediated via an E1-E2-E3 cascade. E1, ubiquitin-activating enzyme, hydrolyzes ATP to activate ubiquitin. E2, ubiquitin-conjugating enzyme, receives activated ubiquitin and is recruited by E3, ubiquitin ligase, to the target protein for ubiquitination. E3 ligases can generate differentially linked polyubiquitin chains with distinct signaling characteristics: K48-linked polyubiquitin chains signal proteasomal degradation, while K63-linked chains promote scaffolding and protein recruitment.¹⁴⁹

1. Introduction

For instance, the DSB end resection factor MRE11 is ubiquitinated and degraded by the proteasome during the HR process. However, premature degradation of MRE11 can impair HR initiation and influence pathway choice.¹³⁶ The regulatory importance of ubiquitin-mediated MRE11 turnover was revealed through discovery of the *UBQLN4* deficiency syndrome.

Jachimowicz *et al.* identified children from two consanguineous families harboring biallelic *UBQLN4* mutations with loss of protein expression and presenting with developmental delay, microcephaly, anemia, hearing loss, and ataxia.¹³⁶ Functional characterization demonstrated that *UBQLN4* promotes ATM-dependent proteasomal degradation of MRE11 and thereby regulates DSB pathway choice. When *UBQLN4* is absent, MRE11 accumulates and promotes excessive DNA end resection. When *UBQLN4* is overexpressed, premature MRE11 degradation impairs HR initiation. This discovery illustrates how syndrome investigation can uncover regulatory mechanisms of DSB repair.¹³⁶

UBQLN4 belongs to the Ubiquilin family of UBL-UBA shuttle factors, proteins that bridge substrate recognition and proteasomal delivery through their modular domain architecture.¹³⁶ The ubiquitin-associated (UBA) domain recognizes polyubiquitinated substrates, while the ubiquitin-like (UBL) domain mediates proteasome docking.¹⁵⁰ However, Ubiquilins also possess stress-inducible protein 1-like (STI1) domains capable of ubiquitin-independent protein-protein interactions, raising the possibility that their cellular functions extend beyond canonical substrate degradation.¹⁵⁰

Beyond ubiquitination, additional PTMs contribute to DSB coordination. Acetylation, another PTM type, promotes chromatin decompaction and repair machinery access by neutralizing the positive charge of lysine residues and weakening histone-DNA interactions.^{151,152} Methylation adds methyl groups to lysine or arginine residues, creating binding sites for reader proteins that differentially promote NHEJ or HR.¹⁵²⁻¹⁵⁴ SUMOylation stabilizes repair complexes and modulates protein-protein interactions at DSB sites.^{155,156} PARylation, catalyzed by PARPs, serves as one of the earliest DSB sensors by synthesizing poly(ADP-ribose) (PAR) chains that recruit repair factors, with PAR glycohydrolase subsequently removing these chains to allow repair progression.^{157,158,159}

These PTMs do not function in isolation but create complex regulatory networks through extensive crosstalk.¹⁶⁰ For example, phosphorylation can create binding sites for ubiquitin ligases, linking damage signaling to protein turnover.¹⁶¹ This hierarchical organization ensures that repair proceeds through defined stages with appropriate factor recruitment and release.

1.5 Genome Instability Syndromes as Models for DNA Repair Research

As the preceding sections illustrated, investigation of rare syndromes caused by defects in DNA repair genes has repeatedly led to advances in understanding DNA repair mechanisms. Affected individuals typically present with clinical features including neurodegeneration, growth defects, and premature ageing.¹⁶²

Genetic sequencing can identify candidate mutations in DNA repair genes,^{71,136,163,164} which are then functionally characterized to uncover pathogenic mechanisms and reveal novel aspects of DNA repair biology.¹³⁶

1.5.1 Progeroid syndromes and genome instability

Progeroid syndromes are disorders characterized by accelerated cellular ageing and organism-level features of premature ageing.^{7,60,165} Intriguingly, not all syndromes presenting with features of premature ageing harbor canonical DNA repair defects, and mechanistic characterization is important to distinguish genome instability from other pathogenic mechanisms. DNA repair defects like Werner syndrome demonstrate clear links between genome instability and premature ageing,¹⁶⁶ where the genetic defect directly impairs a DNA repair factor. However, laminopathies such as Hutchinson-Gilford progeria syndrome are caused by mutations in structural nuclear proteins rather than canonical DNA repair genes.^{60,167} Disrupted nuclear lamina organization induces senescence through altered chromatin architecture, gene expression, and mechanotransduction.⁶⁰ Secondary DNA repair defects including accumulated unrepaired DSBs contribute to the phenotype of laminopathies.¹⁶⁸ The primary pathogenic mechanisms, senescence or genomic instability, differ between DNA repair-defective and nuclear envelope-disrupted syndromes.^{60,165-168}

Rothmund-Thomson syndrome (RTS) occupies a complex position within the premature ageing classification.¹⁶⁹ First described by August von Rothmund in 1868 with additional characterization by Matthew Sydney Thomson in 1936, RTS manifests as poikiloderma (mottled skin atrophy) with osteosarcoma predisposition.¹⁷⁰ Kitao *et al.* identified biallelic RecQ-like helicase 4 (*RECQL4*) mutations as causative in 1999,^{171,172} revealing that *RECQL4* belongs to the RecQ helicase family, like *WRN* and *BLM*, and participates in DNA replication initiation and DSBR.^{173,174} Biallelic *RECQL4* helicase mutations, responsible for a subclass of RTS, display features consistent with DNA repair deficiency including chromosomal instability and cancer predisposition. *RECQL4* participates in DNA replication, DNA repair, and mitochondrial DNA maintenance, and RTS patient cells show variable sensitivity to genotoxic agents across studies.^{169,175} This phenotypic variability suggests that progeroid features in RTS-spectrum disorders may arise through multiple pathogenic mechanisms, potentially

1. Introduction

including both DNA damage-dependent and damage-independent routes. Understanding whether a given progeroid disorder results primarily from accumulated DNA damage or from perturbation of senescence programs has important implications for elucidating the fundamental biology of cellular ageing.⁸

Elucidating the mechanistic basis of each syndrome, whether primarily DNA damage-dependent or involving alternative routes to cellular dysfunction, remains essential for understanding genome maintenance and processes contributing to premature ageing and cancer development.⁷

1.5.2 Syndrome discovery enables understanding of DNA repair mechanisms

The syndrome examples integrated throughout this introduction, from ATM-mediated DSB signaling discovered through ataxia-telangiectasia⁷¹, to BRCA-dependent HR revealed by hereditary breast cancer families⁹⁹, and to ubiquitin-regulated pathway choice uncovered through *UBQLN4* deficiency¹³⁶, demonstrate how clinical phenotyping, genetic analysis, and functional characterization of rare conditions can promote mechanistic understanding with potential clinical impact.^{21,60,176}

Modern technologies are accelerating how rare syndromes are discovered. Whole exome sequencing (WES) enables rapid identification of causative variants in affected families,¹⁷⁷ while optical genome mapping (OGM) detects structural variants invisible to short-read sequencing.^{178,179} Together with long-read sequencing and improved bioinformatic pipelines, these technologies have enabled discovery of ultra-rare syndromes affecting individual families worldwide, wherever these technologies are accessible.¹⁸⁰⁻¹⁸² The relevance of DNA repair gene variants extends beyond the rare homozygous mutations that cause syndromes.^{183,184}

1.5.3 Population-level implications of DNA repair variants

Beyond identifying genes causing rare syndromes, population-level studies have revealed that heterozygous variants in DNA repair genes are more common than previously recognized and may contribute to cancer susceptibility across populations.^{183,184} However, establishing cancer risk associations requires large sample sizes due to low allele frequencies.¹⁸⁵ Combinations of heterozygous variants in multiple DNA repair genes may have additive or synergistic effects on cancer risk, highlighting how genetic background and disease susceptibility are interconnected.^{186,187}

Investigating rare syndromes therefore continues to reveal new insights into DNA repair mechanisms and inform clinical practice. However, once a candidate variant has been identified through sequencing, establishing its pathogenicity and understanding its physiological consequences remain challenging.

1. Introduction

Cell-based assays can reveal molecular functions, but recapitulating organism-level phenotypes such as tissue-specific cancer predisposition, developmental defects, or premature ageing requires animal models.^{21,188} A range of organisms, from yeast to mice, have contributed to our understanding of conserved DNA repair pathways.

For modeling human syndrome phenotypes at the organism level, genetically engineered mouse models (GEMMs) offer advantages due to their physiological and genetic properties close to humans.^{21,188}

1.5.4 Utilizing mouse models to study physiological consequences of DNA repair defects

Mouse models recapitulate the genetic basis of human syndromes and allow investigation of both tissue-specific effects and systemic consequences at the organism level.^{21,188} These models provide several critical advantages over patient studies. First, they offer more uniform genetic backgrounds, reducing confounding effects of multiple additional heterozygous variants that are common in syndrome patients, especially from consanguineous families.¹³⁶ This genetic comparability enables attribution of phenotypes to specific DNA repair defects. Second, CRISPR-Cas9 technology has revolutionized the generation of these models,¹⁸⁹ allowing rapid and precise introduction of patient-specific mutations into the mouse genome for functional validation.¹⁹⁰ Mouse models are particularly valuable for the characterization of ultra-rare syndromes where only one or two families are known worldwide, precluding systematic patient studies.¹⁹¹

Constitutive knockout models generate systemic loss of gene function and can recapitulate many features of human syndromes. For instance, *Atm*^{-/-} mice show immunodeficiency and cancer predisposition similar to AT patients.¹⁹¹ However, some human phenotypes are not fully recapitulated. *Atm*^{-/-} mice lack the striking cerebellar abnormalities of human AT in phenotyping experiments, while the underlying Purkinje-cell defects are detectable at cellular level in histology.^{192,193}

Conditional approaches using *Cre/lox* systems enable tissue-specific or temporally inducible transgene modulation, circumventing embryonic lethality seen with constitutive knockouts of essential genes like *Brca1/2* and enabling assessment of physiological consequences in one specific tissue or cell type.^{194,195}

Genome instability syndrome patients are often young and observing later consequences of their condition, for instance cancer predisposition, requires long observation times. Mouse models of genome instability enable shorter observation times and indeed frequently reveal that DNA repair deficiency predisposes to cancer development, establishing a mechanistic link between the syndromes discussed above and oncogenesis.²¹

1.6 DNA Repair Defects and Cancer Development

DNA repair deficiency represents an enabling hallmark of cancer, permitting the accumulation of mutations that drive malignant transformation.^{14,196} Defective DNA repair promotes genome instability by allowing DNA damage to persist through successive cell divisions.¹⁹⁷

When high-fidelity repair pathways like HR are compromised, cells may increasingly rely on error-prone backup mechanisms, which generate mutations, chromosomal rearrangements, and structural variants.^{198,199} This mutational burden creates genetic diversity that may accelerate malignant evolution.²⁰⁰ Together with tumor-promoting inflammation, genome instability is a fundamental hallmark of cancer that enables the manifestation of other hallmarks, such as sustaining proliferative signaling, evading growth suppression, resisting cell death, enabling replicative immortality, and activating invasion and metastasis.¹⁴ Inherited and acquired repair defects can therefore contribute to carcinogenesis.^{201,202} Among these, defects in HR have emerged as particularly important both for tumor biology and therapeutic intervention.²⁰³⁻²⁰⁵

1.6.1 Homologous recombination deficiency patterns and tumor characteristics

HR deficiency (HRD) is one of the most actionable DNA repair defects in cancer. *BRCA1/2* mutations occur in approximately 10-20 % of breast and ovarian cancers,^{203,204} and defects in other HR genes including *ATM*, *PALB2*, *CHEK2*, and *RAD51* paralogs create comparable functional effects.²⁰⁵ Error-prone backup mechanisms engaged upon HRD can generate characteristic genomic scars detectable through multiple modalities,^{198,206,207} including loss of heterozygosity (LOH), telomeric allelic imbalance (TAI), large-scale state transitions (LST), and mutational signatures.²⁰⁸⁻²¹⁰ These genomic features can serve as biomarkers for patient stratification and may inform therapeutic decision-making. The clinical utility of such testing depends on HRD prevalence across cancer entities.

1.6.2 Homologous recombination deficiency prevalence and cancer entity distribution

Genomic analyses indicate that the average overall frequency of HRD in solid tumors is approximately 17-18 %, as measured by genomic scarring scores²⁰⁹ and HR gene mutations²⁰⁵. However, prevalence varies significantly across cancer entities and subtypes. In ovarian cancer, HRD is most prevalent, with 69 % of cases testing positive.²⁰⁹ Triple-negative breast cancer shows HRD at rates of 47-59 %.²¹¹ In lung adenocarcinoma (LUAD), HRD is observed in approximately 36 % of cases when measured by genomic scar scores.²⁰⁹ This heterogeneity highlights the variable nature of HRD across tumor entities and the requirement for patient-specific screening to utilize HRD therapeutically.

1.6.3 Therapeutic vulnerabilities through homologous recombination deficiency

HRD can be therapeutically exploited through two approaches. First, impaired repair capacity can render HRD tumors hypersensitive to DNA-damaging agents such as platinum compounds.²¹² In a study on ovarian cancer patients, HRD-positive tumors with *BRCA1/2* mutations showed 97 % platinum-sensitive response rates compared to 74 % in HRD-negative tumors.²¹³

Second, HRD creates opportunities for synthetic lethality, a therapeutic principle discussed in detail in section 1.7. These observations demonstrate that DNA repair defects fundamentally shape cancer evolution and treatment response. Integration of DNA repair profiling into clinical practice is therefore transforming patient stratification.²¹⁴

1.7 Exploiting DNA Repair Defects for Personalized Cancer Therapy

Several therapeutic strategies now exploit DNA repair defects. Current strategies include synthetic lethality approaches, biomarker-guided patient selection, and immunotherapy exploiting the increased mutational burden of DNA repair-deficient tumors.

1.7.1 Synthetic lethality and application of PARP inhibition

Synthetic lethality describes the principle that simultaneous disruption of two cellular pathways induces cell death, while cells remain viable when either pathway alone is disrupted.^{176,215} The synthetic lethality between HRD and PARP inhibition represents a central discovery in precision oncology and provided the first clinically successful DDR-targeted compounds exploiting this principle.¹⁷

PARP enzymes are among the earliest responders to DNA damage. Upon binding preferentially to SSBs, PARP catalyzes the synthesis of PAR chains, which recruit downstream repair factors to the damage site. PARP inhibitors exert their effects through two mechanisms: catalytic inhibition of PARylation, which prevents repair factor recruitment, and trapping of PARP1/2 on DNA, which obstructs replication fork progression and creates DSBs.²¹⁶ While PARP1/2 trapping has been proposed as the primary cytotoxic mechanism,²¹⁶ recent work suggests that synthetic lethality may instead, or additionally, arise from unresolved transcription-replication conflicts that accumulate when PARP1 function is lost, rather than from trapped PARP-DNA complexes.²¹⁷ Regardless of the precise mechanism, the consequence of PARP inhibition is an increased conversion of unrepaired SSBs into DSBs during replication.^{216,217}

1. Introduction

In cells with intact HR, these replication-associated DSBs are resolved through BRCA1/2-dependent high-fidelity repair. However, in tumor cells harbouring HRD, the resulting DSBs accumulate and cannot be accurately repaired, ultimately triggering cell death. This selective vulnerability allows PARP inhibitors to preferentially kill HRD cancer cells while sparing non-cancerous cells and tissues with functional HR.^{17,176} (Figure 2)

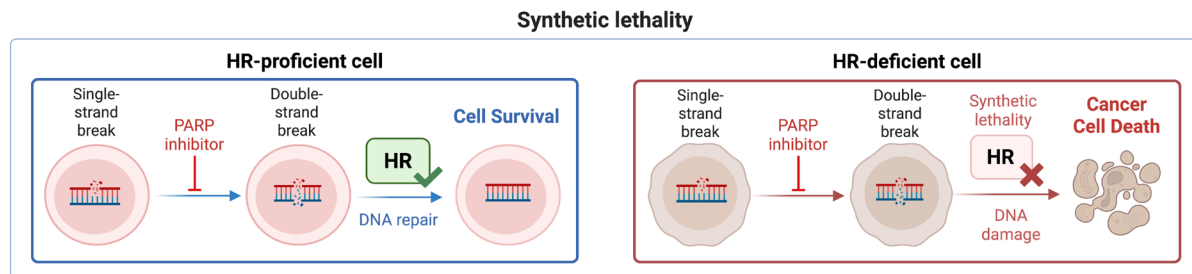


Figure 2: Synthetic lethality between PARP inhibition and HR deficiency. PARP inhibitors generate replication-associated DNA damage that is resolved by HR in proficient cells, allowing survival. In HR-deficient cancer cells, this damage persists unrepaired, triggering synthetic lethality and selective cancer cell death. Figure created with BioRender.

Although the synthetic lethal interaction was discovered in germline *BRCA*-mutant cancers, it extends to deficiencies in other HR factors, a phenotype termed "*BRCAness*".^{218,219} PARP inhibitors have transformed treatment approaches for *BRCA*-mutant and "*BRCAness*" cancers. Four agents have received FDA approval in ovarian and breast cancer, predominantly for maintenance therapy following platinum-based chemotherapy: olaparib, rucaparib, niraparib, and talazoparib.²²⁰

Most synthetic lethal effects in cancer are complex and polygenic,²²¹ but also extend beyond PARP. The syndrome discovery approach continues to reveal new synthetic lethal targets. Werner syndrome (section 1.3.2) recently emerged as therapeutically relevant when *WRN* was identified as a synthetic lethality target in microsatellite instability-high (MSI-H) tumors. MSI-H tumors depend on *WRN* helicase to resolve aberrant DNA structures arising from expanded microsatellite repeats, and defects in *WRN* promote treatment sensitivity.²²²⁻²²⁴ This discovery, more than a century after Werner's original clinical description, demonstrates that syndrome investigations can yield unexpected therapeutic opportunities as technology advances and molecular understanding deepens.

Extending the treatment opportunity of synthetic lethality to patients without pathogenic *BRCA* mutations requires reliable methods to identify HRD and "*BRCAness*".

1.7.2 Detecting homologous recombination deficiency in the clinic

Multiple approaches have been developed to detect HRD beyond germline *BRCA1/2* testing. Genomic scarring metrics that integrate LOH, TAI, and LST quantify the permanent genomic scars left by prolonged HRD.²²⁵⁻²²⁷

1. Introduction

Algorithmic approaches combine mutational signatures and rearrangement patterns to infer HRD from whole-genome sequencing (WGS), identifying *BRCA*-deficient tumors with 98.7 % sensitivity in discovery cohorts²²⁸ and *BRCAness* in 15 % of colorectal cancers²²⁹.

Functional assays measuring RAD51 foci formation provide readouts for HR capacity,²³⁰ while emerging technologies such as OGM enable detection of large structural variants missed by short-read sequencing.^{178,231} However, the predictive validity of these biomarkers for therapeutic response varies. Integrating multiple biomarker modalities is the current approach to improving HRD detection and expanding the eligible patient population. However, even patients who initially respond to PARP inhibition frequently develop resistance.^{232,233}

1.7.3 Resistance mechanisms limiting PARP inhibitor efficacy

The most common mechanism involves secondary reversion mutations in *BRCA* genes that restore the open reading frame and protein function. A meta-analysis identified such reversions in 26 % of resistant cases.²³² Additionally, loss of *53BP1* was shown to restore DNA end resection in *BRCA1*-deficient cells by removing barriers to DSB repair pathway choice and HR.²³³ Understanding these mechanisms informs development of combination strategies and alternative therapeutic approaches that exploit DNA repair defects through distinct mechanisms.

1.7.4 Genome instability and immune checkpoint inhibition

Beyond PARP inhibition, DNA repair defects create additional therapeutic opportunities through their effects on tumor immunogenicity, including innate immune activation through cytosolic DNA sensing²³⁴ and neoantigen presentation²³⁵.

The increased mutational burden resulting from DNA repair defects can generate neoantigens that can elicit anti-tumor immune responses. Antigens are presented to T-cells of the adaptive immune system via major histocompatibility complex (MHC) molecules.²³⁶ Neoantigens are novel peptides arising from somatic mutations in cancer cells. They are processed and displayed on MHC class I molecules, enabling T-cell recognition of tumor cells as "non-self".²³⁵ Increased neoantigen presentation from high mutation rates in cancer cells can enhance tumor immunogenicity by providing additional targets for T-cell-mediated cytotoxicity.²³⁷

However, tumors employ multiple strategies to evade immune surveillance. Downregulation of MHC class I expression through epigenetic silencing or degradation impairs antigen presentation and can prevent T-cell recognition.^{238,239} Additionally, tumors exploit immune checkpoint pathways, especially involving the programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) axis.

1. Introduction

PD-1 is an inhibitory receptor expressed on activated T cells, and engagement by its ligand PD-L1, frequently upregulated on tumor cells, delivers co-inhibitory signals that promote T-cell exhaustion.^{240,241} Blocking this interaction with anti-PD-1 or anti-PD-L1 antibodies can reactivate exhausted T-cells and restore anti-tumor immunity.^{240,242}

Tumors with high microsatellite instability (MSI-H) or MMR deficiency show elevated response rates to PD-1/PD-L1 inhibitors, compared to MMR-proficient tumors.^{237,242,243} Similarly, tumors with elevated tumor mutational burden (TMB ≥ 10 mutations/Mb) benefit from immunotherapy across multiple cancer types, establishing TMB as an emerging pan-cancer biomarker.^{244,245} Validating such biomarkers and testing combination strategies requires preclinical models that recapitulate the complexity of human tumors.

1.7.5 The KP mouse model for preclinical studies of lung cancer

Mouse models provide physiologically relevant model systems for this purpose, preserving the complex interactions between tumor cells, stromal components, and immune surveillance mechanisms that profoundly influence therapeutic response, unlike cell culture or xenograft systems.^{246,247}

The KP mouse model (*Kras*^{LSL-G12D/wt}; *Trp53*^{fl/fl}) exemplifies a widely adopted system for testing DDR-linked interventions in KRAS-driven non-small cell lung cancer (NSCLC).²⁴⁸ Mice develop tumors upon intratracheal instillation of Cre-expressing adenovirus, which induces lung-specific activation of oncogenic *Kras*^{G12D} (K) and deletion of the tumor suppressor *Trp53* (P).²⁴⁸ KRAS functions as an important molecular switch in cell signaling, cycling between active GTP-bound and inactive GDP-bound states to regulate the RAF/MEK/ERK cascade that controls cell proliferation and survival.^{249,250} Oncogenic mutations at the G12 residue, including G12D, G12V, and G12C, impair intrinsic GTPase activity and confer resistance to GTPase-activating proteins, thereby maintaining KRAS in its constitutively active state²⁵¹ and promoting malignant transformation in approximately 95 % of pancreatic cancers²⁴⁹ and approximately 30 % of lung adenocarcinoma²⁵².

Given that approximately 39-42 % of human KRAS-driven lung adenocarcinomas harbor TP53 loss-of-function mutations, the KP model provides a clinically relevant system for a substantial patient population with aggressive NSCLC.^{246,253-255} KP mice have proven valuable for evaluating therapeutic interventions, including models incorporating additional mutations to investigate cooperating driver mutations and DDR-targeted combination strategies.^{246,247,256} Importantly, all GEMM research adheres to the 3R framework (replacement, reduction, refinement), ensuring appropriate animal welfare standards.²⁵⁷ GEMM studies enable variant characterization and evaluation of therapeutic vulnerabilities for preclinical validation.²¹ *In vivo* studies with GEMMs are central to this dissertation.

1.8 Dissertation Architecture

Rare syndromes involving genome instability can illuminate regulatory mechanisms that, when compromised in cancer, may reveal therapeutic vulnerabilities. This cumulative dissertation follows a progression across four manuscripts to which I made substantial contributions as co-first or contributing author within multidisciplinary teams spanning clinical genetics, molecular biology, chemical biology, and preclinical modeling. Together, my work traces a path from clinical phenotype characterization through molecular mechanism discovery to preclinical therapeutic evaluation.

First, we characterized *CRIP*T syndrome as a Rothmund-Thomson-like progeroid disorder driven by premature cellular senescence rather than defective DNA repair, thereby expanding our understanding of mechanisms underlying progeroid phenotypes and illustrating the necessity of mechanistic syndrome characterization (**Manuscript I**). Second, we validated optical genome mapping as a clinical diagnostic approach for structural variant detection in a pediatric cohort with cancer predisposition. Application of this technology revealed a *de novo* duplication of 17p13.3 affecting *RPA1* in a young cancer patient whose fibroblasts exhibited DNA damage sensitivity, illustrating the diagnostic potential of combining structural variant detection with short-variant sequencing (**Manuscript II**). Third, discovery of the *UBQLN1* deficiency syndrome led us to identify a previously unrecognized role of UBQLN1 in regulating HR, with potential implications for cancer biomarker development and personalized therapy via synthetic lethality. This is the primary work of this dissertation, encompassing syndrome discovery using sequencing, biochemical and cell-biological mechanism characterization in patient-derived fibroblasts, immortalized cell lines, and an autochthonous mouse model, and subsequent preclinical evaluation in mouse models of lung cancer for potential therapeutic application (**Manuscript III**). Fourth, in collaboration with medicinal chemists and structural biologists who developed a first-in-class covalent PDE δ inhibitor targeting KRAS membrane trafficking, we conducted preclinical evaluation using the KP mouse model established in the lab during our UBQLN1 studies. This work demonstrated compound efficacy *in vitro* and *in vivo*, disrupting oncogenic signaling through a target-directed approach complementary to syndrome discovery and characterization (**Manuscript IV**).

Together, these studies represent two research strategies (**Figure 3**): syndrome discovery, where characterization can reveal mechanisms targetable through synthetic lethality, and target-directed therapeutic development validated in mouse models. The mechanistic insights and preclinical data provide a basis for further clinical development.

1. Introduction

Thesis Architecture: Overview of Manuscripts

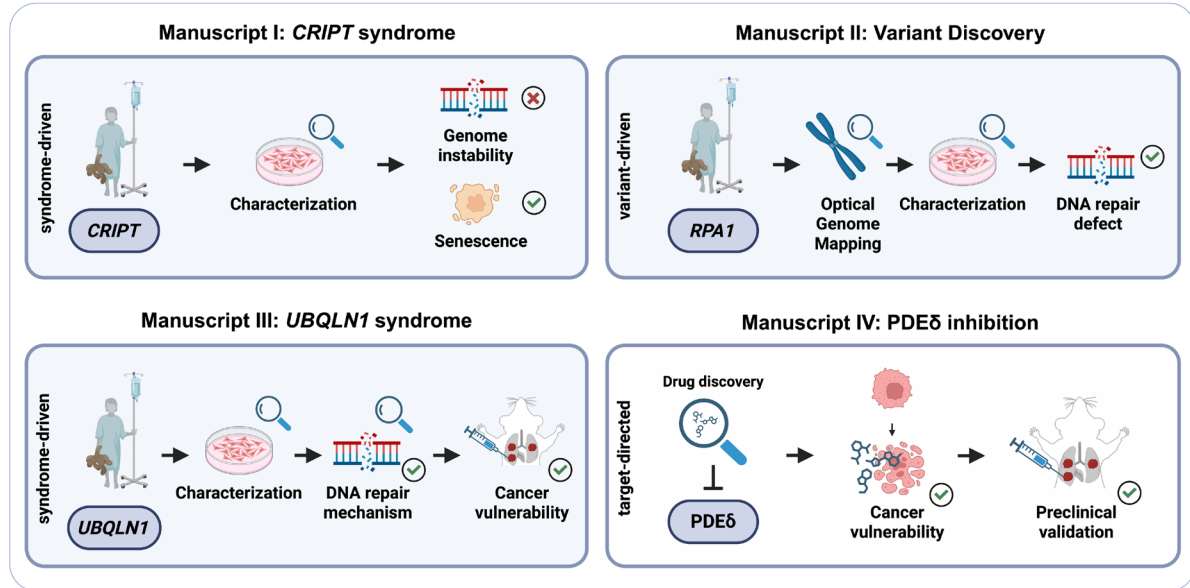


Figure 3: Thesis architecture and manuscript overview. The four manuscripts progress from clinical observation to therapeutic application. Manuscripts I-III employ a syndrome discovery approach: *CRIP1* syndrome (I) reveals that features of *CRIP1* deficiency arise through cellular senescence rather than canonical DNA repair defects; optical genome mapping is validated for structural variant detection, demonstrated by discovery of a *de novo* duplication encompassing *RPA1* in a patient with DNA damage sensitivity (II); and characterization of *UBQLN1* deficiency (III) uncovers a novel role for *UBQLN1* in HR regulation with actionable cancer vulnerabilities. Manuscript IV complements this discovery trajectory with target-directed preclinical evaluation of a first-in-class covalent PDE δ inhibitor against KRAS-driven cancers. Figure created with BioRender.

2. Results: Publications and Manuscripts

2.1 Manuscript I: Biallelic Variants in *CRIP1* Cause a Rothmund-Thomson-like Syndrome with Increased Cellular Senescence

Genetics in Medicine, July 2023

PMID: 37013901

doi: 10.1016/j.gim.2023.100836

Luisa Averdunk, [Maxim A Huetzen](#), Daniel Moreno-Andrés, Reinhard Kalb, Shane McKee, Tzung-Chien Hsieh, Annette Seibt, Marten Schouwink, Seema Lalani, Eissa Ali Fageih, Theresa Brunet, Peter Boor, Kornelia Neveling, Alexander Hoischen, Barbara Hildebrandt, Elisabeth Graf, Linchao Lu, Weidong Jin, Joerg Schaper, Jamal A Omer, Tanguy Demaret, Nicole Fleischer, Detlev Schindler, Peter Krawitz, Ertan Mayatepek, Dagmar Wieczorek, Lisa L Wang, Wolfram Antonin, Ron D Jachimowicz, Verena von Felbert, Felix Distelmaier

2.1.1 Contributions

As second author on this publication, I established a functional characterization workflow to assess DNA damage sensitivity in patient-derived fibroblasts. My contributions encompassed experimental design and execution, data analysis and visualization, and co-writing the original draft with L.A.

Particularly, I established and optimized live-cell-imaging-based viability assays using the IncuCyte system for high-throughput characterization of genotoxic stress responses. This work included cultivating patient-derived fibroblasts, validating *CRIP1* protein expression by immunoblotting, developing the staining and imaging strategy with multiple implementation screens, dosage evaluation experiments, orthogonal viability screens using the Cell-Titer-Glo assay, and expanding the DNA damage agent panel to include potassium bromate alongside ionizing radiation, mitomycin C, hydroxyurea, and etoposide (**I-Figure 4L-N**, implementation screens and CTG assays not shown). I analysed the resulting data and generated associated figures and visualizations.

During manuscript revision, I collaborated with L.A. to design siRNA knockdown experiments addressing reviewer feedback. I established the experimental approach for *CRIP1* downregulation, which was subsequently performed in the collaborator's laboratory (**I-Figures 3D-F**). I coordinated closely with L.A. and F.D. at Heinrich-Heine-University Düsseldorf, who led overall project coordination and patient characterization.

I contributed to the original manuscript by writing the results and discussion sections regarding DNA damage sensitivity experiments. I participated in drafting responses to reviewer comments, results interpretation, and manuscript finalization.

2. Results – Manuscript I

This functional characterization established that *CRIPT* deficiency causes increased cellular senescence rather than overt DNA repair defects, demonstrating shared molecular mechanisms with *RECQL4* deficiency and expanding the genetic heterogeneity of Rothmund-Thomson-like syndromes.

2.1.2 Peer-reviewed publication

The published manuscript PDF is attached in this section. The publisher allows the reuse of this Published Article in the dissertation. (<https://www.elsevier.com/about/policies-and-standards/copyright/permissions>)

Original page numbers were removed for formatting reasons.



ARTICLE

Biallelic variants in *CRIPT* cause a Rothmund-Thomson-like syndrome with increased cellular senescence



ARTICLE INFO

Article history:

Received 25 May 2022

Received in revised form

25 March 2023

Accepted 28 March 2023

Available online 31 March 2023

Keywords:

Aging

DNA damage and repair

Mitotic errors

Rothmund-Thomson syndrome

Senescence

ABSTRACT

Purpose: Rothmund-Thomson syndrome (RTS) is characterized by poikiloderma, sparse hair, small stature, skeletal defects, cancer, and cataracts, resembling features of premature aging. *RECQL4* and *ANAPC1* are the 2 known disease genes associated with RTS in >70% of cases. We describe RTS-like features in 5 individuals with biallelic variants in *CRIPT* (OMIM 615789).

Methods: Two newly identified and 4 published individuals with *CRIPT* variants were systematically compared with those with RTS using clinical data, computational analysis of photographs, histologic analysis of skin, and cellular studies on fibroblasts.

Results: All *CRIPT* individuals fulfilled the diagnostic criteria for RTS and additionally had neurodevelopmental delay and seizures. Using computational gestalt analysis, *CRIPT* individuals showed greatest facial similarity with individuals with RTS. Skin biopsies revealed a high expression of senescence markers (p53/p16/p21) and the senescence-associated β -galactosidase activity was elevated in *CRIPT*-deficient fibroblasts. *RECQL4*- and *CRIPT*-deficient fibroblasts showed an unremarkable mitotic progression and unremarkable number of mitotic errors and no or only mild sensitivity to genotoxic stress by ionizing radiation, mitomycin C, hydroxyurea, etoposide, and potassium bromate.

Conclusion: *CRIPT* causes an RTS-like syndrome associated with neurodevelopmental delay and epilepsy. At the cellular level, *RECQL4*- and *CRIPT*-deficient cells display increased senescence, suggesting shared molecular mechanisms leading to the clinical phenotypes.

© 2023 by American College of Medical Genetics and Genomics. Published by Elsevier Inc.

Introduction

Rothmund-Thomson syndrome (RTS) is a rare autosomal recessive disorder, which is characterized by clinical signs of premature aging and increased risk of cancer. The classical skin presentation is poikiloderma consisting of chronic, reticulated areas of depigmentation and hyperpigmentation, dilated blood vessels, and punctate atrophy giving a mottled

appearance.¹ Additional features of RTS include sparse hair, dystrophic teeth and nails, short stature, skeletal abnormalities including osteopenia, and juvenile cataracts.²

More than 300 individuals with RTS have been reported in the literature since its first description in 1868.^{3,4} Two forms of RTS can be distinguished. The characteristic feature of RTS type 1 is cataracts, and the typical hallmark of RTS type 2 is an increased susceptibility to cancer,

*Correspondence and requests for materials should be addressed to Luisa Averdunk, Department of General Pediatrics, Neonatology and Pediatric Cardiology, University Children's Hospital, Heinrich-Heine-University, Moorenstraße 5, 40225 Düsseldorf, Germany. Email address: luisasusan.averdunk@med.uni-duesseldorf.de OR Felix Distelmaier, Department of General Pediatrics, Neonatology and Pediatric Cardiology, University Children's Hospital, Heinrich-Heine-University, Moorenstraße 5, 40225 Düsseldorf, Germany. Email address: felix.distelmaier@med.uni-duesseldorf.de

A full list of authors and affiliations appears at the end of the paper.

doi: <https://doi.org/10.1016/j.gim.2023.100836>

1098-3600/© 2023 by American College of Medical Genetics and Genomics. Published by Elsevier Inc.

Key Messages

1. *CRIPT* causes a Rothmund-Thomson-like syndrome, which is associated with developmental delay.
2. Biallelic variants in *CRIPT* cause increased cellular senescence, which is a shared cellular phenotype of *CRIPT* and *RECQL4* deficiencies.
3. In fibroblasts derived from individuals with pathogenic variants in *CRIPT*, mitotic progression and mitotic arrest induction are not compromised, and the incidence of mitotic errors is not significantly increased.
4. Fibroblasts from *CRIPT* and *RECQL4* individuals show no or only very mild sensitivity to ionizing radiation, mitomycin C, hydroxyurea, etoposide, and potassium bromate compared with controls.

particularly osteosarcoma.² Neurodevelopmental delay of variable degree has been reported in RTS, but it has not been formally studied.⁵⁻⁹ The diagnosis of RTS can be made on clinical grounds based on the characteristic poikiloderma and other typical features of RTS (small stature, osteosarcoma or skin cancer, cataracts, and sparse hair).² Numerical and structural chromosomal aberrations have been observed in lymphocytes and fibroblasts from RTS individuals.^{2,10,11}

In 1999, biallelic variants in *RECQL4* were identified in a subset of RTS type 2 cases.¹² Although the genetic cause of RTS type 1 remained unknown for a long time, biallelic variants in *ANAPC1*, including intronic splice variants, were recently identified explaining a proportion of RTS type 1.¹³ *RECQL4* codes for the ATP-dependent RECQ-like DNA helicase type 4 (RECQL4) and is involved in DNA repair, DNA replication, telomeric maintenance, normal chromosome alignment in mitosis, and senescence.¹⁴⁻¹⁸ *RECQL4* contains a conserved DNA-unwinding domain, but it has only weak helicase activity.¹⁹ *ANAPC1* codes for the APC1 subunit of the multisubunit anaphase-promoting complex or cyclosome (APC/C). APC/C is an E3 ubiquitin ligase that controls cell cycle progression by proteasome-dependent degradation of cell cycle regulators.²⁰ The genetic cause in approximately 30% of RTS cases remains unknown, suggesting that not all RTS-associated genes have been identified so far.²¹

We report 2 unrelated individuals who fulfilled the diagnostic criteria of RTS but lacked pathogenic variants in *RECQL4* and *ANAPC1*. However, we identified pathogenic biallelic variants in *CRIPT* by exome sequencing. Biallelic variants in *CRIPT* were recently associated with primordial dwarfism and developmental delay in 4 families, but a link to RTS has not been drawn so far.²²⁻²⁴ *CRIPT* codes for a short, 101 amino acid cysteine-rich PDZ-binding protein that specifically binds to microtubules and to the post synaptic density protein, *Drosophila* disc large tumor suppressor and zonula occludens-1-(PDZ) domain 3 of the postsynaptic density protein 95 (PDS95). *CRIPT* has mainly been studied

in the context of synaptic formation; however, a functional role for *CRIPT* outside of the brain is unknown.^{25,26} Here, we systematically compare *CRIPT*-associated clinical phenotypes with RTS and identify *CRIPT* as a novel disease gene associated with an RTS-like phenotype. To delineate the driving and shared molecular mechanisms underlying RTS, cellular senescence, mitotic progression, and DNA repair were studied in fibroblasts from *RECQL4*- and *CRIPT*-deficient individuals. We show that *CRIPT* and *RECQL4* both play a functional role in preventing cellular senescence, whereas the mechanism that drives senescence remains unsolved.

Materials and Methods

Patient recruitment and clinical evaluation

Two individuals with typical features of RTS (#1 and #2) underwent exome sequencing. From the 3 individuals (#3-5) with biallelic *CRIPT* variants (*CRIPT* individuals) from the literature, follow-up clinical data and updated photographs were provided.^{22,23} Skin biopsies of #1 and #3 were performed and used for cell culture of fibroblasts and immunohistochemistry. Similarities among facial photographs were quantified using GestaltMatcher.²⁷ Frontal photographs from the 5 *CRIPT* individuals were compared with available, open-access photographs of published RTS cases (*RECQL4*), RecQ family-related syndromes, and syndromes characterized by poikiloderma. References of photographs are accessible in GestaltMatcher Database (<https://db.gestaltmatcher.org>). Methods of exome sequencing, electron microscopy of hair, and diagnostic skin histology are provided in the [Supplemental Methods](#).

Experimental methods

Fresh skin biopsies for the generation of fibroblasts cultures were available from *CRIPT* individuals #1, #2, and #3, and from control individuals (infants and adolescents).²⁸ The fibroblasts of #3 divided very slowly, and the number of cells was sufficient for live-cell imaging of mitotic progression but not for assays of senescence or genome stability. Fibroblasts from RTS individuals with pathogenic variants in *RECQL4* (*RECQL4* individuals) were obtained from Coriell Institute (NJ): *RECQL4* #1, AG05013: NM_004260.4:c.2492_2493del p.(His831Argfs*52), NM_004260.4:c.2059-1G>T, compound heterozygous; *RECQL4* #2, AG18371: NM_004260.4:c.1483+27_1483+37del, homozygous; and *RECQL4* #3, AG17524A: NM_004260.4:c.1391-1G>A NM_004260.4:c.2492_2493del p.(His831Argfs*52), compound heterozygous. The neonatal control fibroblast line was obtained from Lonza (Walkersville). The other control fibroblasts were generated by skin biopsies from healthy individuals. Fibroblasts with pathogenic variants in *ANAPC1* were not available. Cellular β -galactosidase activity was quantified in fibroblasts

from #1 and #2 and after downregulation of *CRIPT* expression by small interfering RNA (siRNA) in control cells using the CellEvent Senescence Green Flow Cytometry Kit (Thermo Fisher). Lymphocytes from whole blood samples of #1 were analyzed by optical genome mapping to search for somatic structural variants (Bionano Genomics). Mitotic progression of fibroblasts was analyzed after DNA staining in a Ti2 Eclipse (Nikon) equipped with an LED light engine SpectraX (Lumecor, Beaverton) and a Plan-Apochromat 20× NA 0.75 objective and environmental control system UNO-T-H-CO2 (Okolab). Fluorescence multiposition imaging was done every 3 minutes for 24 hours using the Elements software (Nikon). The dwell times and the images of mitotic progression were extracted using the machine learning CecogAnalyzer 1.5.2.²⁹ Mitotic arrest was induced with 100 ng/mL nocodazole treatment for 30 minutes. To study the sensitivity of patient-derived cell lines to genotoxic stress, cell viability was quantified at high resolution and high sensitivity using IncuCyte-S3 Live-Cell-Imaging system (Essen Bioscience). After treatment of cells with different genotoxic agents, viable and dead cells were stained and tracked for 96 hours. A cell death score was calculated as the dead cell over live-cell signal, allowing for normalization of different growth kinetics. Comprehensive description of methods, including siRNA downregulation, optical mapping, cytoplasmic and nuclear extraction of *CRIPT* protein, and mitotic progression are provided in the [Supplemental Methods](#).

Statistical analysis

Differences of β -galactosidase activity was compared using student's *t*-test, and live-cell imaging-based mitotic timing and cell viability was analyzed with one-way analysis of variance.

Results

Clinical characteristics

Individual #1 is a 3-year-old Moroccan female born in Germany who presented at the age of 6 months with a cutaneous rash starting on the face and spreading to the hands and legs, sparing the trunk, and developing into reticulated hypo- and hyperpigmentation with punctate atrophy resembling poikiloderma ([Figure 1B](#)).

Individual #2 is an 8-year-old Romanian male born in Northern Ireland by cesarean delivery at 28 weeks of gestation because of intrauterine growth retardation. Starting at 4 weeks of age, he had a mottled, reticulated rash affecting the limbs and abdomen spreading to the hands and scalp. In both individuals, edematous swelling of the limbs and palmoplantar hyperkeratotic lesions accompanied the skin rash. Histologic examination of skin biopsies from both individuals revealed atrophic epidermis, inflammatory

infiltration, pigment incontinence, and dilated blood vessels confirming the diagnosis poikiloderma ([Figure 1F](#)).¹ Additional histologic findings were fragmented elastic fibers and increased mucinous deposits ([Supplemental Figure 1](#)).

Both individuals had sparse hair, including eyebrows and eyelashes ([Figure 1B](#) and [C](#)). The children showed failure to thrive ([Figure 1G](#)), and full skeletal survey indicated osteopenia and skeletal abnormalities ([Figure 1H](#)). Individual #2 was diagnosed with bilateral central cataract at the age of 8 years. Both children had muscular hypotonia and moderate motor and profound language delay. Head magnetic resonance imaging showed reduced brain volume. Individual #2 had microcephaly ([Figure 1I](#)). Both had a prominent forehead with frontal bossing, a broad, depressed nasal root, and telecanthal folds.

In summary, the clinical features included poikiloderma, sparse hair, short stature, skeletal anomalies, developmental delay, and facial dysmorphic features. Because the diagnostic criteria of RTS were fulfilled, RTS was diagnosed on clinical grounds ([Table 1](#)).²

Identification of homozygous variants in *CRIPT* in 2 individuals with RTS

There were no pathogenic variants in *RECQL4* or *ANAPC1* identified by exome sequencing, and protein expression of APC1 and RECQL4 was normal in fibroblasts from individuals #1 and #2 ([Figure 2B](#)). By Sanger sequencing of individual #1, we additionally did not detect the recurrent deep intronic c.2705–198C>T variant that had been identified in *ANAPC1* ([Supplemental Figure 2A](#)).¹³

Instead, in both individuals, we identified homozygous variants in the *CRIPT* gene ([Figure 1J](#)). In 4 families (#3–6), pathogenic *CRIPT* variants were reported in association with abnormal skeletal and neurologic development and abnormal skin ([Table 1](#)).^{22–24} The term “short stature with microcephaly and distinctive facies” was suggested (OMIM 615789).^{22,23}

Individual #1 harbors the homozygous variant c.132del (NM_014171.4), which results in the same frameshift p.(Ala45Glnfs*86) that has been reported in individual #3 (ACMG class 5).²³ The missense variant c.227G>A, p.(Cys76Tyr) (NM_014171.4) of #2 is unreported (highly conserved among different species, including mammals, zebrafish, chicken, frog, and African claw frog; Combined Annotation Dependent Depletion score 31, <https://cadd.gs.washington.edu/>). The variant reported by Leduc et al.²² (#5) is also a missense variant substituting one of the 8 cysteine residues in 101-aa *CRIPT* ([Figure 1J](#)). The cysteine residues have recently been suggested to play a crucial role in redox modulation during disassembly of mitotic spindle.^{22,30} The variants have been confirmed by Sanger sequencing in the probands' parents ([Supplemental Figure 2B](#)). By western blot of *CRIPT* protein in patient-derived fibroblast cell lysates, *CRIPT* was only detectable in control and *RECQL4* individuals but not in *CRIPT*

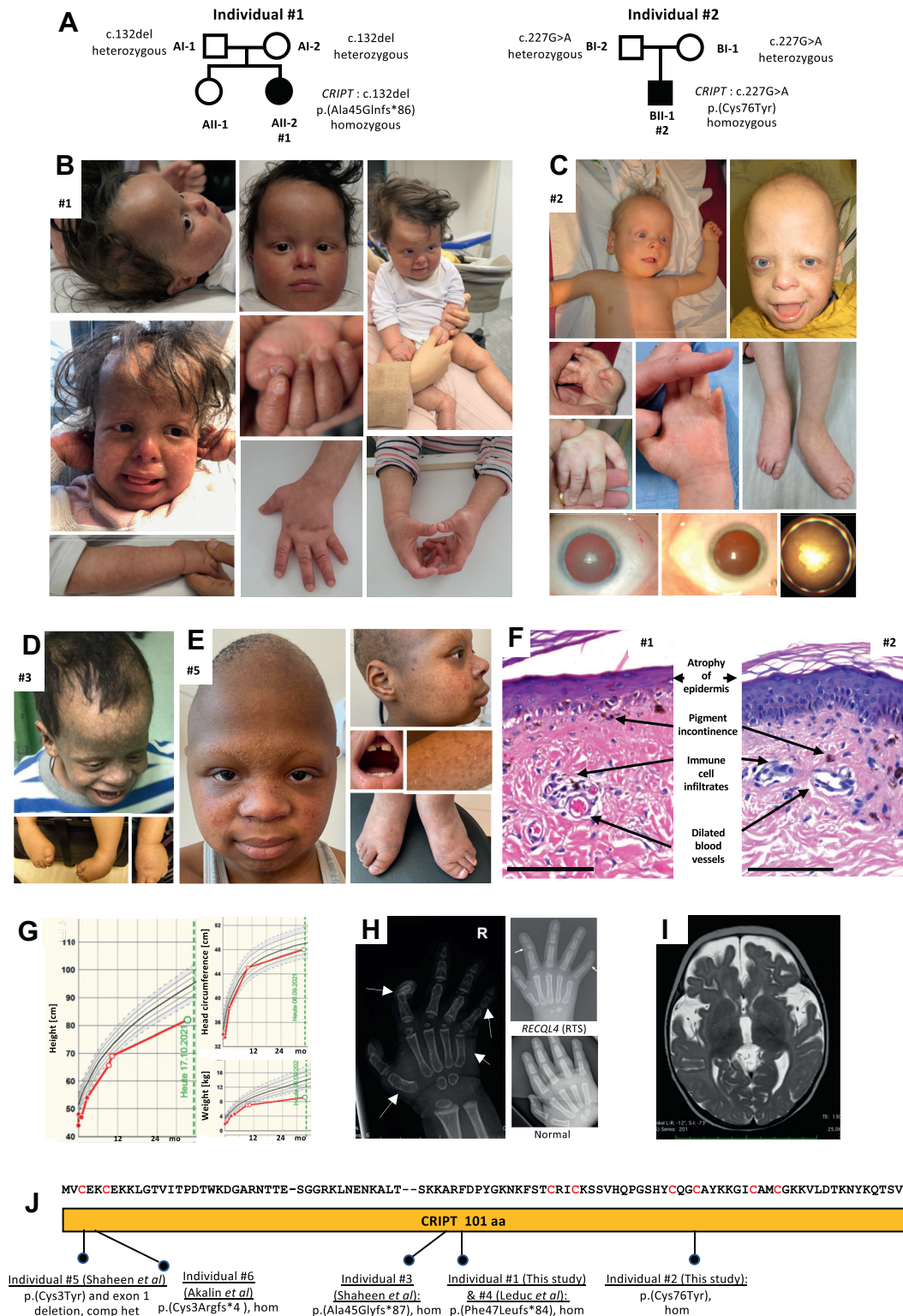


Figure 1 Genetic, clinical and imaging findings in individuals with biallelic variants in *CRIP1*. A. Family pedigrees of the *CRIP1* individual #1 (Moroccan) and #2 (Romanian) both harboring homozygous pathogenic variants in *CRIP1* (NM_014171.4.). B. Photographs of individual #1 showing characteristic frontal bossing, depressed nasal root, telecanthus, reticulated pigmentation of the skin (1 year), progressive hair loss with absent eye lashes and eyebrows, edematous swelling of extremities, muscular hypotonia, and dystrophic nails. Proximal placed and short thumbs. Difficulties to grasp with thumb and finger (first row: 1 year, second row: 2 years, hands: 4 years). C. Photographs of individual #2 showing sparse hair and progressive hair loss, reticulated rash of cheeks, dystrophic nails, and proximally placed thumb and edematous swelling, talipes equinus and nuclear cataract of both eyes (cataract of left eye only visible under the operating microscope). D, E. Photographs of individuals #3 and #5 at their follow-up examinations (unpublished photos of published cases)^{22,23} #3 at

individuals (Figure 3B). Despite the clinical features resembling RTS in our subjects, a link between *CRIPT* and RTS has not been previously established.

Clinical comparison of *CRIPT* individuals with RTS

Clinical updates on *CRIPT* individuals #3-5 were obtained, and clinical features of RTS types 1 and 2 were systematically compared with the 6 *CRIPT* individuals (Table 1).²²⁻²⁴ Individual #4 had died at the age of 70 days because of pulmonary infection. All *CRIPT* individuals who survived presented with reticulated, hypopigmented skin changes (5 of 5) compatible with poikiloderma, the hallmark of RTS (Figure 1B-E). All individuals had sparse hair and progressive hair loss, resulting in an almost complete absence of eyebrows and eyelashes in #1 and #2 (6 of 6). Additional features were dysplastic nails (2 of 5, #1 and #5) and delayed eruption of abnormal teeth (3 of 5, #1, #2, #5, and #6).¹ All *CRIPT* individuals had short stature (6 of 6). RTS often presents with skeletal anomalies, including osteoporosis, abnormal metaphyseal trabeculation, brachymesophalangy, first metacarpal or thumb hypoplasia, or hypoplastic terminal phalanges.³¹ Skeletal survey of *CRIPT* individuals revealed osteopenia with abnormal metaphyseal striation (6 of 6), brachymesophalangy (3 of 6), first metacarpal hypoplasia (1 of 6), hypoplastic terminal phalanges (5 of 6), flexible talipes equinus or pes planus (6 of 6), and scoliosis (6 of 6). The typical feature distinguishing RTS type 1 from RTS type 2 are juvenile-onset cataracts.¹³ Individual #2 developed bilateral cataracts at the age of 8 years, consistent with RTS type 1 individuals. Of 6 *CRIPT* individuals, 5 had myopia and 4 of 6 were suspected to have optic atrophy. RTS type 2 is associated with an increased risk of neoplasm, but none of the *CRIPT* individuals had a history of cancer so far.² Although RTS has rarely been associated with reduced brain volume or microcephaly, most RTS individuals are reported to develop normally or to only have mild speech delay.⁵⁻⁷ All *CRIPT* individuals presented with moderate motor delay and clearly impaired language development (Supplemental Table 1: skills and development of #1 and #2). The oldest individual #5 was able to speak short sentences and read 3 to 4 letter words at the age of 11 years. All *CRIPT* individuals had proportionate microcephaly (5 of 6) or reduced brain volumes on magnetic resonance imaging (Figure 1J). Although epilepsy is not common in RTS, 4 of 6 *CRIPT* individuals developed epilepsy (difficult to control in #1 and #2; Supplemental Table 2). Of 5 individuals, 2 had postprandial

emesis and dysphagia improving with age. All individuals were affected by recurrent upper respiratory tract infections, and in RTS, recurrent infections have been reported although rare.³² A laboratory workup of immunodeficiency revealed only mildly reduced titer of IgG in *CRIPT* individual #1. Additional hematologic findings included anisopoikilocytosis (2 of 6), microcytic anemia (2 of 6), and thrombocytopenia (1 of 6). RTS clinically overlaps with progeroid syndromes, including the 2 RecQ family-related syndromes (Bloom syndrome and Werner syndrome) and poikiloderma-associated syndromes (eg, Kindler syndrome and Fanconi anemia). We systematically compared the *CRIPT* individuals and RTS syndrome with these differential diagnoses, but none of the *CRIPT* individuals were more appropriately classified as any of these differential diagnoses than as RTS (Supplemental Table 3).¹ All *CRIPT* individuals fulfilled RTS diagnostic criteria and were additionally affected by neurodevelopmental delay, seizures, and microcephaly.

Sanger sequencing of *CRIPT* in 10 *RECQL4*-negative RTS individuals from the RTS registry

We searched for pathogenic variants in *CRIPT* in all ($N = 10$) *RECQL4*-negative RTS type 1 individuals from the RTS registry (Baylor College of Medicine, Houston, TX). Four of these individuals had mild developmental delay, and 6 had reportedly unremarkable neurologic development. Pathogenic variants in *CRIPT* were not detected in either of these 2 groups of individuals, which supports the notion that *CRIPT* is not a common disease gene among RTS individuals with unremarkable neurologic development.

Loss of cuticle in hair from *CRIPT* and *RECQL4* individuals

RECQL4 and *CRIPT* individuals have brittle and sparse hair. The healthy hair surface consists of several layers of overlapping cells, termed cuticle. Electron microscopy of the hair surface of *CRIPT* individuals revealed a partial (#2) to complete (#1) loss of the cuticle that was similar to that of the hair from 2 *RECQL4* individuals #1 and #2 (Figure 2A). Besides, the hair from #1 and #2 and *RECQL4* #1 showed fragility of the hair surface after bending stress. In contrast, the hair surface from young and old healthy controls showed intact cuticle layers.

the age of 11 years with reticulated hypo- and hyperpigmentation and sparse hair, edematous swelling, and talipes equinus, and #5 at the age of 10.5 years with reticulated rash on the cheeks, vesicles and hypo- and hyperpigmentation, and talipes equinus. F. Skin of #1 showing typical histopathologic findings of poikiloderma (H&E, 20X). Scale bars = 50 μ m. G. Growth charts illustrating failure to thrive and short stature (dashed line illustrates third and 97th percentiles) of #1. H. Radiographic images of the hand of individual #1 showing osteopenia, brachymesophalangy third to fifth digits, metacarpal hypoplasia first and fifth and bowing of second and fifth digits (top middle panel), and hypoplastic terminal phalanges, in comparison to x-ray images of unaffected (bottom middle panel) and *RECQL4*/RTS (right panel) individuals³¹ and a healthy control individual. I. MRI of the head showing reduced brain volume in *CRIPT* individual #1. J. Schematic illustration of *CRIPT*, a cysteine-rich PDZ domain-binding protein (cysteines in red). Pathogenic variants indicated. *MRI*, magnetic resonance imaging; *RTS*, Rothmund-Thomson syndrome.

Table 1 Clinical characteristics of *ANAPC1*, *RECQL4*, and *CRIP1* individuals

Feature	RTS						Individual #6 (Akalin et al. ²⁴)
	RTS Type 1 <i>ANAPC1</i>	RTS Type 2 <i>RECQL4</i>	Individual #1 (New)	Individual #2 (New)	Individual #3 (Shaheen et al. ²³)	Individual #4 (Shaheen et al. ²³)	Individual #5 (Leduc et al. ²⁵)
Disease gene							
Variants			<i>CRIP1</i> c.132del p.(Ala45Glyfs*82), homozygous	<i>CRIP1</i> c.227G>A, p.(Cys76Tyr), homozygous	<i>CRIP1</i> c.133_134insGG, p.(Ala45Glyfs*82), homozygous	<i>CRIP1</i> c.141del p.(Phe47Leufs*84), homozygous	<i>CRIP1</i> c.86>A p.(Cys3Tyr), 1,331 bp del exon 1, compound- heterozygous
Origin, sex			Morocco, female 4 y	Romania, male 8 y	Saudi Arabia, male 11 y	Saudi Arabia, male 70 d	African American, female 10.5 y
Age at last follow-up							Turkey, male 4 y
RTS—major criteria							
Facial rash	+	+	+	+	+	n/r	+
Poikiloderma	+	+	+	+	+	n/r	n/r
RTS—minor criteria (at least 2)							
Sparse hair	+	+	+	+	n/r	+	+
Short stature	+	+	+	+	+	+	+
Neoplasia	—	+	— ^a	— ^a	— ^a	— ^a	—1
Cataracts	+	—	—	+	—	—	—
Other clinical features							
Skeletal anomalies ^{78,79}							
Osteopenia/metaphyseal striations	+	—	+	+	+	+	+
Brachydactyly/Brachymeso- phalangy	II + V	n/r	+	n/r	+	n/r	+
Metacarpal hypoplasia	I	n/r	III + IV + V (plus mild bowing)	n/r	n/r	n/r	n/r
Hypoplastic terminal phalanges	+	n/r	I + V (no nail spikes)	+	+	+	+
Feet anomalies	—	—	Skewfoot	Pes planus	Pes planus	Skewfoot	Pes planus
Syndactyly of toes 4/5	—	—	+	+	+	+	+
Trunk anomalies			Scoliosis	Scoliosis + platyspondyly	Scoliosis + platyspondyly	Scoliosis 11 pairs of ribs	Scoliosis 11 pairs of ribs
Aplastic radius, ulna, thumb	Common	—	Proximally placed thumb	—	—	—	—
Frontal bossing	+	+	+	+	+	+	+
Dystrophic nails	+	+	+	—	n/r	n/r	+
Abnormal teeth			+	+	n/r	n/r	+
Microcephaly	Occasionally	10%	—	+	+	+	+
Developmental delay	10%	10%	+	+	+	+	+

(continued)

Table 1 Continued

RTS								
Feature	RTS Type		Individual #1 (New)	Individual #2 (New)	Individual #3 (Shaheen et al ²³)	Individual #4 (Shaheen et al ²³)	Individual #5 (Leduc et al ²²)	Individual #6 (Akalin et al ²⁴)
	Type 1	Type 2						
Motor development	Usually normal		Independent sitting at 15 mo, walking at 30 mo	Independent sitting at 12 mo, walking at 36 mo	Can run and jump, not able to ride bicycle	—	Independent sitting at 13 mo, walking at 23 mo	Independent sitting 30 mo, walking 4 y
Speech development	Mild delay		Single words, no sentences, understands simple commands	Single words, no sentences, understands simple commands	Single words, no sentences, understands simple commands	—	Speaks in short sentences, follows simple commands, reads 3-and 4-letter words	Significant delay
Gastrointestinal disturbance	Some		+ (dysphagia)	—	+ (mild dysphagia)	—	—	—
Recurrent chest infections	Rarely Recurrent (Kubota et al ³²)		Yes	Yes	Yes	Yes, lethal pneumonia	Yes	Yes, hospitalized twice
Anemia			Macrocytic	n/r	n/r	Anisopoikilocytosis	Aniso-poikilocytosis	Yes, additionally thrombocytopenia
Additional features primarily in CRPT individuals								
Seizures (age of onset)	—	—	+ (1 y) Well controlled	+ (3 y) Control difficult	—	+ (2 mo)	+ (3 y) Well controlled	— +
Myopia	—	—	+	+	+	—	+	+
Retina	—	—	Normal	Normal	Bilateral optic atrophy macular dystrophy	Bilateral pale optic discs	Hypopigmented mottling retina	Retinal pigmentation defect

^aNo history of cancer at last follow-up.

Variants refer to the transcript NM_014171.4 (*CRPT*).

+, present; –, not present; n/r, not reported; RTS, Rothmund-Thomson syndrome.

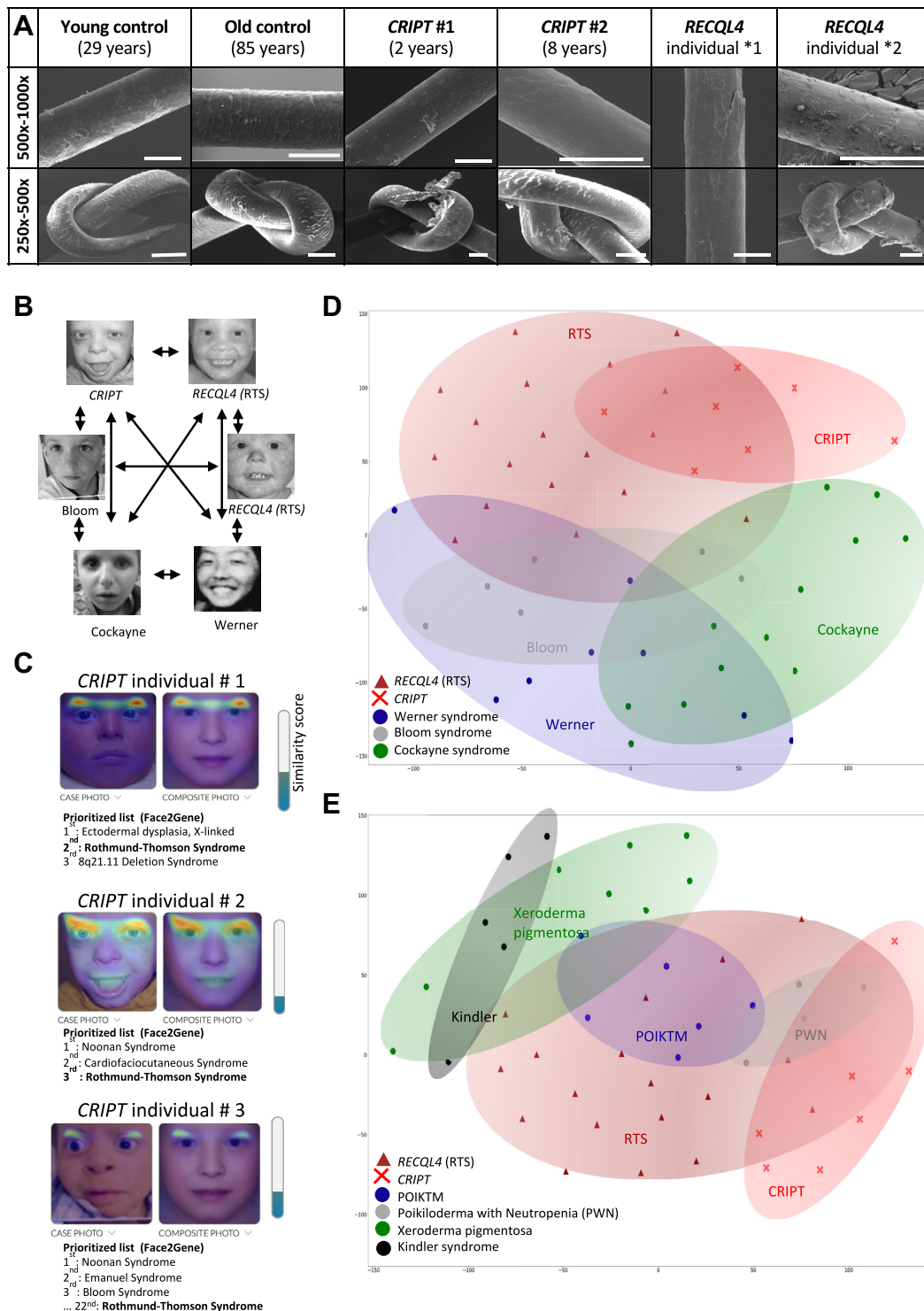


Figure 2 Comparison of hair ultrastructure and facial profile of individuals with *CRIPT* deficiency and differential diagnoses.

A. Electron microscopy showing the normal hair cuticle (overlapping layers of dead cells forming the hair surface) from young (29 years) and old (85 years) healthy controls (left panels) in comparison with (complete #1 or partial #2) loss of the cuticle in the hair from *CRIPT* and *RECQL4* individuals. Easy breaking of the hair shaft or surface after bending stress (knots). Scale bars = 50 μ m. B. Illustration of pairwise computational comparison of facial morphology using the DeepGestalt analysis framework (Face2Gene, FDNA Inc)³³. C. Heatmaps of frontal images of *CRIPT* #1, #2, and #3 highlighting the facial regions (left side) with greatest overlaps with the merged, composite image RTS (*RECQL4*) individuals (right side) with prioritized lists of differential diagnoses based on the analysis of frontal images for *CRIPT* #1, #2, and #3. D. Analysis of the morphologic similarities of published photographs using GestaltMatcher. The 2D visualization illustrates the morphologic distances between facial images of *CRIPT* individuals, and individuals with pathogenic variants in *RECQL4* (RTS) and related syndromes—RecQ family—related syndromes (Bloom syndrome and Werner syndrome), and the progeroid Cockayne syndrome. E. The 2D visualization illustrates the morphologic distance of *CRIPT* and *RECQL4* individuals and other syndromes that are characterized by poikiloderma. *POIKTM*, hereditary fibrosing poikiloderma with tendon contractures, myopathy, and pulmonary fibrosis; *PWN*, poikiloderma with neutropenia; *RTS*, Rothmund-Thomson syndrome.

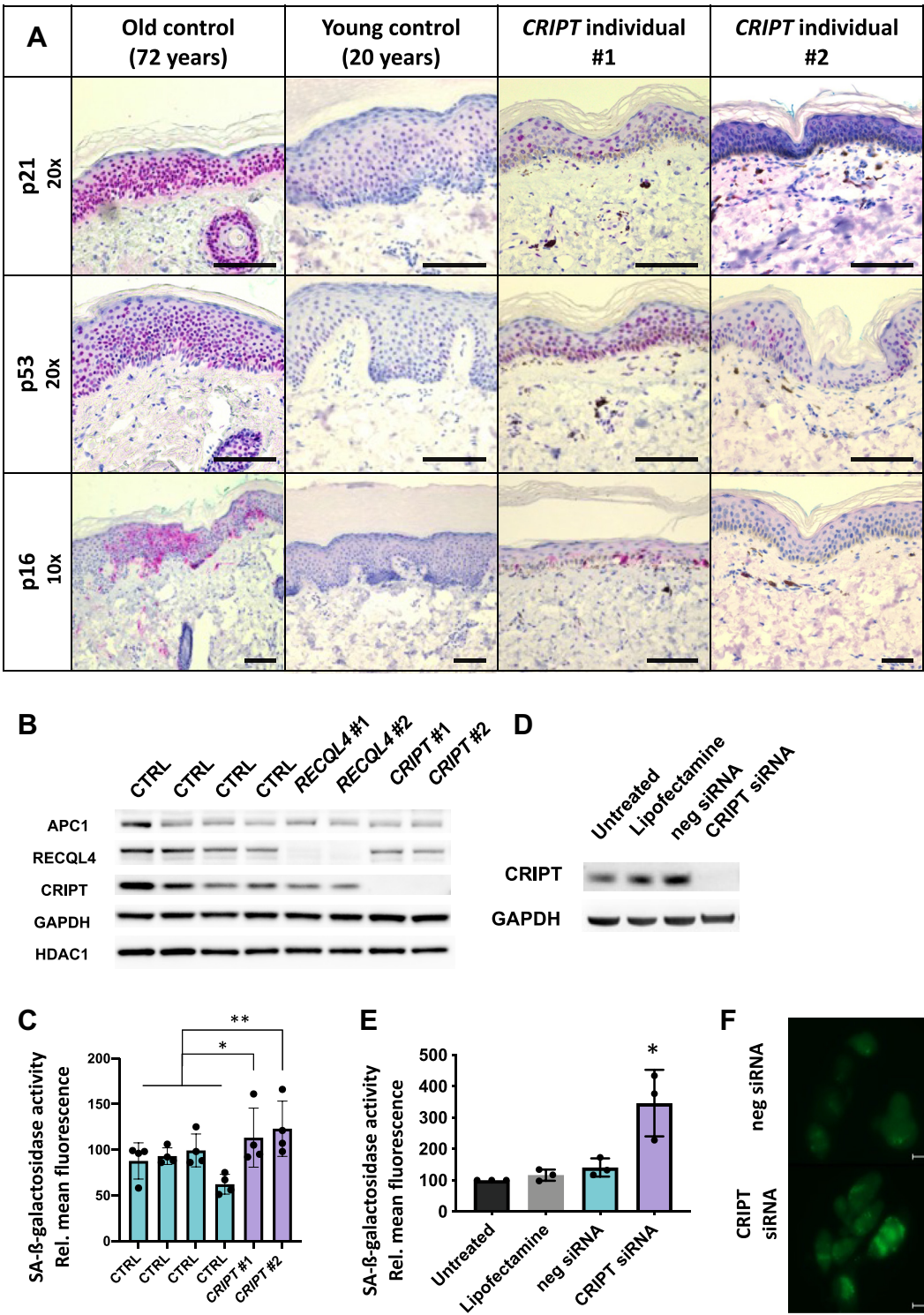


Figure 3 Senescence markers in skin biopsies from *CRIPT*-deficient individuals and in *CRIPT*-deficient cultured fibroblasts. A. Immunohistochemical staining of p21, p53, and p16 (senescence markers). Expression of the p21, p53, and p16 in the skin of a 72-year-old control individual and *CRIPT* individual #1, and expression of p53 and minor expression of p21 in *CRIPT* individual #2, but not in the 20-year-old individual. Scale bars = 50 μ m. B. Western blot of APC1, RECQL4, and *CRIPT* from fibroblast cell lysates of *CRIPT* individuals #1 and #2 and RECQL4 cell lines #1 and #2. HDAC1 was used as a loading control for nuclear proteins. C. Senescence-associated-(SA-) β -galactosidase activity in fibroblasts from controls and from *CRIPT* individuals #1 + #2. D. Immunoblotting of *CRIPT* after small interfering RNA-mediated *CRIPT* downregulation in control fibroblasts. E, F. SA- β -galactosidase activity in fibroblasts after downregulation of *CRIPT* expression on day 14. Scale bars = 10 μ m. All experiments were performed 3 times. Mean $\pm$ SD. APC1, anaphase-promoting complex subunit 1; CTRL, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HDAC1, Hda1 histone deacetylase complex. * $P < .05$; ** $P < .01$.

Computational comparison of photographs

RTS is characterized by a distinct facial phenotype, which includes frontal bossing, depressed nasal root, and epicanthal folds.¹ All *CRIPT* individuals show these craniofacial features. However, the assessment of facial features is biased by the clinician's experience. Therefore, the facial appearance of *CRIPT* individuals ranging from #1 to #5 was compared with individuals with other syndromic genetic diagnoses using the unbiased, computational DeepGestalt analysis (Face2Gene and FDNA) (Figure 2B).³³ Based on photographs of #1 and #2, RTS was listed among the top 3 differential diagnoses, indicating a significant morphologic similarity (Figure 2C). For the photograph of #3 and #6, RTS was ranked as top 22 and top 9, respectively; for #4 and #5, RTS was not in the prioritized list of differential diagnoses, which might be attributed to a reduced performance of DeepGestalt for very young age or African background.

The morphologic similarity between *CRIPT* and *RECQL4* individuals and related differential diagnoses was quantified by GestaltMatcher.²⁷ The photographs of *CRIPT* individuals showed a strong overlap with *RECQL4* individuals but no overlap with the RecQ family-related Bloom, Werner, or Cockayne syndromes (Figure 2D).

Facial photographs of *CRIPT* individuals showed the highest similarity to images of *RECQL4* individuals when compared with other “poikiloderma syndromes” (Figure 2E). Interestingly, photographs of *RECQL4* and *CRIPT* individuals overlapped with hereditary fibrosing poikiloderma (POIKTM) with tendon contractures, myopathy, and pulmonary fibrosis and poikiloderma with neutropenia syndrome.

Increased senescence in skin and in *CRIPT*-deficient fibroblasts

CRIPT individuals display the typical hallmarks of aging. Aged tissues accumulate senescent cells with a permanently arrested cell cycle.³⁴ Previous studies in skin and cartilage tissues of *Recql4*-deficient mice and in human fibroblasts after *RECQL4* downregulation demonstrated exuberant senescence.³⁵⁻³⁷ Cell cycle arrest in senescence is induced by activation of p53 and p21 and maintained by p16.³⁸

To analyze if increased senescence could contribute to aging symptoms in *CRIPT* deficiency, we stained skin samples from *CRIPT* individual #1 and #2 and from 20- and 72-year-old healthy controls for p53, p16, and p21. The skin of the younger control was negative for senescence markers (Figure 3A). In contrast, keratinocytes in the skin of the *CRIPT* children #1 and #2 showed strong expression of p53. In the 2-year-old *CRIPT* individual #1, there was additionally strong expression of p21 and p16, similar to the older control. The activity of senescence-associated β -galactosidase (SA- β -galactosidase) in fibroblasts, a marker of senescent cells, was quantified by

FACS. The SA- β -galactosidase activity was significantly higher in patient-derived *CRIPT*-deficient fibroblasts from individuals #1 and #2 compared with that of control (Figure 3C). The extent of senescence in cultured cells depends on the age and passage number and can be influenced by selective pressure. To better control these factors, we downregulated *CRIPT* expression by siRNA in wild-type fibroblasts (Figure 3D). *CRIPT* downregulation caused a significant 2.5-fold upregulation of SA- β -galactosidase activity when compared with negative control siRNA (Figure 3E and F). In line with the increased senescence observed in *RECQL4*-deficient tissues, *CRIPT* deficiency also induces cellular senescence.

Karyotypes and mitotic progression are unchanged in patient-derived cells

The underlying mechanism driving cellular senescence in *CRIPT*- and *RECQL4*-deficient cells is unknown. Senescence can be induced by mitotic, genotoxic, or oxidative stress.

In up to 6% of RTS cases, low-level mosaicism of numerical or structural chromosomal aberrations, most frequently of chromosomes 8, 7, or 2, were observed in lymphocytes and fibroblasts, suggesting a defect in chromosomal segregation.^{2,10,11} In addition, in cells derived from a viable mouse model of RTS, up to 25% of cells exhibited defective sister chromatid cohesion with precocious segregation of sister chromatids.³⁹ Karyotypes of lymphocytes (#1 and #2) and fibroblasts (#1) from *CRIPT* individuals did not indicate increased rates of chromosomal aberrations, precocious segregation of sister chromatids, or sister chromatid exchanges (>100 cells evaluated, Figure 4A and H; Supplemental Figure 3G). For the detection of low-grade mosaicism, interphase fluorescence in situ hybridization of chromosomes 2, 7, 8, 11, and 17 was performed but no isochromosomes were detected (Figure 4B). To search for somatic structural variants (SVs) and copy number variants, fibroblasts and lymphocytes of individual #1 were additionally analyzed with optical genome mapping (Bionano Genomics). Rare germline SVs present in both tissues with variant allele fractions were filtered out. Using stringent filter settings, we did not detect somatic SVs or copy number variants with variant allele fractions ranging from >5% to 10% (single-cell SVs cannot be excluded, Figure 4C).

RECQL4 is a microtubule-associated protein and localizes to the mitotic spindle.^{18,39} *RECQL4* downregulation in HeLa cells delayed mitotic progression and induced chromosome misalignment, but these aberrations were not observed in patient-derived *RECQL4*-deficient fibroblasts.¹⁸ *CRIPT* also binds to microtubules and was recently suggested to be crucial for redox-dependent microtubule depolymerization at spindle ends during anaphase.^{26,30} In this publication, it was shown that *CRIPT* knockout and cysteine substitution of *CRIPT* in HeLa cells caused either

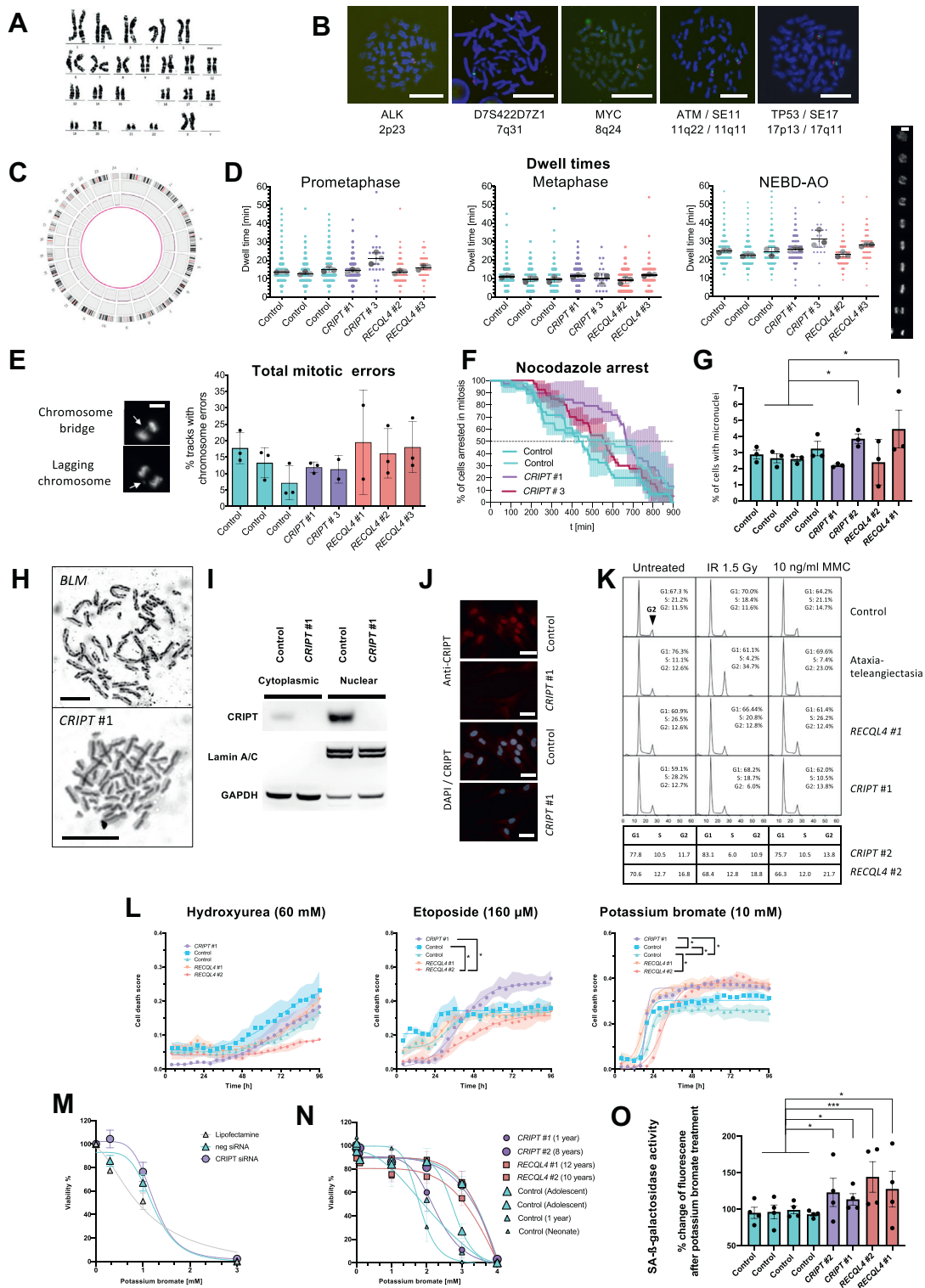


Figure 4 Analysis of chromosomal aberrations, mitotic progression, mitotic errors and sensitivity to chemotoxic stress in CRIPT- and RECQL4-deficient cells. A. Representative karyograms of cultured fibroblasts from CRIPT individual #1 showing no numerical or structural aberrations B. No mosaicism of numerical or structural aberrations detectable by inter- and metaphase-FISH (>1000 cells) using different probes. Representative metaphases are shown. C. Circos plot of somatic SVs and CNVs of CRIPT individual #1 analyzed by optical genome mapping (Bionano Genomics; minimal detectable variant allele fraction 5%-10%). After filtering rare SVs that were present in both tissues with variant allele fractions close to 50% to 100% (suggestive of germline SVs), no somatic SVs or CNVs were detected D. Mitotic progression was measured by live-cell imaging and quantified with the machine learning-algorithm CecogAnalyzer.²⁹ Superplots of mitotic timing of prometaphase, metaphase, and the time from NEBD to anaphase onset in cultured cells from healthy controls and from CRIPT and RECQL4 individuals: The thick gray dots represent the means of the populations of each independent experiment. The colored small dots

mitotic arrest or multipolar spindles and/or chromosome alignment with long-lasting spindle remnants. To test if CRIPT patient-derived fibroblasts show a similar phenotype with mitotic arrest, we analyzed mitotic progression and mitotic errors in patient-derived CRIPT- and RECQL4-deficient cells by live-cell imaging. Interestingly, prometaphase to anaphase progression did not significantly differ between control and CRIPT- or RECQL4-deficient and control cells (Figure 4D, *P* values of one-way analysis of variance: Supplemental Table 6). In RECQL4- and CRIPT-deficient cells, incidence of lagging chromosomes, misaligned chromosomes, or chromosomal bridges was not consistently increased when compared with control cells (Figure 4E; Supplemental Figure 3B-D). In addition, the proportion of dividing cells with tilted spindle axes indicative of spindle microtubule defects was not different from that of controls (Supplemental Figure 3E).

Because a defective spindle assembly checkpoint (SAC) can cause low levels of chromosome segregation errors, we also intended to check the integrity of the SAC.⁴⁰ Challenging cells with nocodazole, a drug that disturbs the microtubule polymerization, results in prometaphase arrest via SAC activation. The duration of this arrest serves as a readout for SAC integrity. After nocodazole challenge, the duration of arrest was not reduced in CRIPT-deficient fibroblasts, indicating an intact SAC (Figure 4F).

Micronuclei can result from chromosome malsegregation, for example, arising from mitotic spindle or kinetochore defects, or from acentric chromosome fragments generated by unrepaired DNA breaks. In patient-derived RECQL4-deficient fibroblasts, an increased number of micronuclei was reported.¹⁸ We quantified the number of micronuclei in CRIPT #1 and #2 and RECQL4 #1 and #2 patient-derived fibroblasts and only found inconsistently increased numbers of micronuclei in fibroblasts from CRIPT #2 (3.8%) and RECQL4 #1 (4.4%) when compared with that of control cells (2.8%) (Figure 4G). However, because of

the high variability between cell lines, no clear conclusions can be drawn.

Nuclear localization of CRIPT and sensitivity to genotoxic stress

CRIPT was originally described as a cytoplasmic, microtubule-binding protein that localizes to synapses in neurons.^{25,26} Recently, it was shown that during interphase CRIPT is localized in the nucleus.³⁰ By immunofluorescence and immunoblotting of cytoplasmic and nuclear fractions, we could confirm that CRIPT is expressed in both cytoplasmic and, more pronounced, in nuclear compartments in wild-type fibroblasts (Figure 4I and J).

Based on initial findings of RECQL4 localizing to nuclear foci, RECQL4 was suggested to be involved in DNA repair.⁴¹ Later studies demonstrated that RECQL4 acts in DNA replication, homologous recombination, nonhomologous end joining, base excision repair, and telomere maintenance.¹⁴⁻¹⁷ However, studies with primary cell lines derived from Recql4-deficient mice and RTS individuals yielded conflicting results regarding sensitivity to genotoxic agents. In fibroblasts from 10 RTS individuals, only agents interfering with DNA replication (eg, hydroxyurea) impaired growth in colony survival assays, whereas cells showed only minor sensitivity to DNA damaging agents (eg, cisplatin).⁴²

We analyzed the sensitivity of patient-derived fibroblasts to ionizing radiation (IR; 1.5 Gy) and mitomycin C using a diagnostic pipeline for screening for ataxia-telangiectasia, ligase-IV-syndrome, and Fanconi anemia.^{43,44} The sensitivity of fibroblasts from CRIPT #1 and #2 and RECQL4 #1 and #2 to IR and mitomycin C was in the range of control cells, only showing a trend toward a higher shift from S to G1 phase after IR (Figure 4K). These findings are in line with the unremarkable sensitivity of Recql4 knockout mouse embryonic fibroblasts to IR.⁴⁵

represent the time for each cell. No differences in dwell timing were observed. *P* values of ordinary one-way ANOVA are reported in Supplemental Table 6. E. Numbers of mitotic cell trajectories with chromosomal errors, including misaligned, lagging chromosomes, and chromosome bridges. Fisher's exact test was used to compare the pooled percentages and showed no differences between CRIPT-, RECQL4-deficient, and control cells. F. After nocodazole (100 ng/mL) treatment for 30 minutes, the arrest time of CRIPT-deficient fibroblasts is not reduced when compared with control cells. D-F show the results of 3 pooled experiments. Mean $\pm$ SD. G. The percentage of cells with micronuclei was inconsistently increased in fibroblasts from individuals CRIPT #2 and RECQL4 #1. H. Differential staining of sister chromatids in chromosomes from patient-derived lymphocytes (BS: Bloom Syndrome, CRIPT #1). The number of sister chromatid exchanges has been assessed by light microscopy. I. Western blot after extraction of cytoplasmic and nuclear (laminin A/C) fractions from fibroblasts. J. Immunofluorescent labeling of CRIPT (red) showing the presence of CRIPT in the cytoplasm and in the nucleus (DAPI, blue). K. Cell cycle distribution of primary fibroblasts (control, ataxia-telangiectasia, RECQL4 #1 and #2, CRIPT #1 and #2) after ionizing radiation 1.5 Gy or exposure to mitomycin C (MMC). G2: G2/m phase of the cell cycle. L. IncuCyte live-cell imaging of cell viability of patient-derived fibroblasts upon challenge with hydroxyurea, etoposide, and potassium bromate. One-way-ANOVA, **P* < .05. M, N Cell viability of fibroblasts analyzed by CellTiterGlo Assay (Promega) (M) after siRNA targeting of CRIPT expression and (N) in patient-derived fibroblasts after potassium bromate challenge for 72 hours measured, means of relative change of viability of 3 independent experiments. O. Relative change of senescence-associated β -galactosidase activity after treatment of patient-derived fibroblasts with low-dose potassium bromate (10 μ M) for 5 days, quantified by flow cytometry. Mean $\pm$ SD. Scale bars = 10 μ m. ANOVA, analysis of variance; BLM, BLM gene; CNV, copy number variant; FISH, fluorescence in situ hybridization; MMC, mitomycin C; NEBD, nuclear envelope breakdown; neg siRNA, negative control small interfering RNA; SV, structural variant; RTS, Rothmund-Thomson syndrome.

We next screened the sensitivity to hydroxyurea, etoposide, and potassium bromate by live-cell imaging of cell viability. Hydroxyurea interferes with DNA replication. In contrast to a previous report, *RECQL4*- and *CRIPT*-deficient fibroblasts did not show an increased susceptibility to hydroxyurea compared with those in controls (Figure 4L).⁴² The topoisomerase II inhibitor etoposide induces double-strand breaks. Although fibroblasts from *CRIPT* individual #1 showed a slightly increased sensitivity to etoposide, the overall sensitivity was in the range of controls (Figure 4L). Potassium bromate oxidizes DNA generating 7,8-dihydro-8-oxoguanine; 7,8-dihydro-8-oxoguanine is repaired by base excision repair, and *RECQL4* knockout was reported to impair base excision repair.⁴⁶ The fibroblasts from individuals *CRIPT* #1 and *RECQL4* #1 and #2 showed a mildly increased sensitivity to potassium bromate (10 mM) compared with control (Figure 4L). To further explore a potential sensitivity to potassium bromate, we downregulated *CRIPT* expression in wild-type fibroblasts. Although targeting *CRIPT* expression by siRNA caused a significant decrease in the number of proliferating cells, the sensitivity to potassium bromate, or alternatively oxidative stress by hydrogen peroxide, was unaffected by *CRIPT* downregulation (Figure 4M; Supplemental Figure 4). Likewise, patient-derived *CRIPT*- or *RECQL4*-deficient fibroblasts were not more sensitive to different doses of potassium bromate than controls. As expected, however, we noted a trend toward a higher sensitivity to potassium bromate in fibroblasts with shorter doubling times (Supplemental Figure 3A; Figure 4N). Because this was not systematically assessed, further studies would be required to prove this association. To investigate if sublethal doses of potassium bromate might trigger cellular senescence, we challenged patient-derived fibroblasts with a low concentration of potassium bromate (10 μ M) for 5 days. Although the SA- β -galactosidase activity remained unchanged in control fibroblasts, *CRIPT*- and *RECQL4*-deficient fibroblasts responded with mild upregulation of SA- β -galactosidase activity upon low concentration potassium bromate challenge (Figure 4O).

In conclusion, though low-level genotoxic stress might induce cellular senescence in *CRIPT*- and *RECQL4*-deficient fibroblast, the overall sensitivity to lethal doses of genotoxic stress is in the range of control cells.

Discussion

RTS is characterized by poikiloderma, sparse hair, small stature, susceptibility to cancer, and cataracts, features usually found in the aging population. Genetically, RTS is a heterogeneous disease with *RECQL4* and *ANAPC1* as the established disease-causing genes. However, in up to 30% of RTS individuals, no causative variants are detected in either *RECQL4* or *ANAPC1*, suggesting other, not yet identified disease genes.

We initially report 2 individuals who fulfill the diagnostic RTS criteria and lack pathogenic variants in *RECQL4* or *ANAPC1*, but who both have biallelic variants in the *CRIPT* gene. Biallelic variants in *CRIPT* have been reported in 4 individuals in the literature, accounting for a multisystemic disorder, including short stature, skin rash, skeletal, and neurodevelopmental anomalies.²²⁻²⁴ Our systematic evaluation of the clinical, histologic, and radiologic features showed that all 6 *CRIPT* individuals matched very well to the clinical spectrum of RTS. In a computational approach, facial images of *CRIPT* individuals showed the highest similarity to RTS individuals when compared with other premature aging and poikiloderma-associated syndromes. Given the phenotypic overlap, we propose that, in addition to *RECQL4* and *ANAPC1*, *CRIPT* causes an RTS-like syndrome.

One possible explanation why the published *CRIPT* cases had not yet been linked to RTS is that most ectodermal features in RTS develop over time starting at the age of 3 to 6 months and that published *CRIPT* individuals had not yet manifested the full dermatologic picture, as exemplified by follow-up data on individuals #3 and #5 (Figure 1D and E).^{22,23}

All *CRIPT* individuals presented with mild motor and more severe speech developmental delay, 5 of 6 *CRIPT* individuals had microcephaly proportional to body size, and 4 of 6 had well-controlled or refractory epilepsy. Although cognition has not formally been assessed in RTS, *RECQL4* individuals from the RTS registry are reported to have mild speech delay and minor delays in meeting developmental milestones.¹³ In addition, cases of non-*RECQL4*-RTS individuals with more profound neurodevelopmental delay have been reported.⁵⁻⁹ Mechanistically, the neurodevelopmental phenotype of *CRIPT* deficiency might be related to the known function of *CRIPT* in dendritic and synaptic plasticity and memory formation.^{30,45}

POIKTM and poikiloderma with neutropenia (PN) are 2 poikiloderma syndromes that have been published as distinct diseases. Although the facial profiles of POIKTM and PN overlap with RTS, these 2 diseases have features (PN: no sparse hair and no skeletal defect; POIKTMP: psoriasis-like lesions, blisters, almost obligatory internal organ involvement, myopathy, and contractures) that clearly distinguish them from RTS. In view of the neurodevelopmental phenotype as apparently outstanding feature of *CRIPT* individuals, it is debatable if the *CRIPT*-associated RTS-like syndrome should rather be classified as a distinct RTS-like disease entity or as a novel subtype of RTS (eg, type 3). Cataracts and the absence of cancer are the typical features of RTS type 1. Additionally, developmental delay has been observed in 2 of 10 *ANAPC1* RTS type 1 individuals.^{30,46} *CRIPT* individual #2 developed bilateral cataracts at the age of 8 years; none of the *CRIPT* individuals had cancer to date and all have developmental delay. In view of this overlap, the clinical phenotype of *CRIPT* deficiency appears to be closer to RTS type 1 than 2. However, long-time clinical follow-up is warranted to

systematically assess the risk of cancer and cataracts and neurologic development and to compare the frequency of these features in *CRIPT*, *ANAPC1*, and *RECQL4* individuals.

Although *CRIPT* was primarily studied in the context of neuronal connectivity and recently mitotic spindle disassembly, *RECQL4* has been implicated in many cellular processes, including DNA repair, cellular senescence, and cell cycle progression, but the crucial mechanism that accounts for the multisystemic and aging-related findings in RTS is under debate.^{30,47} The so far unrecognized link between *CRIPT* and RTS opens avenues to delineate further the molecular pathology of RTS symptoms. In the experimental part of this study, we searched for shared cellular pathways of *CRIPT*- and *RECQL4*-deficient cells, focusing on cellular senescence, mitotic progression, and sensitivity to genotoxic stress.

Similar to *RECQL4* deficiency, we found that *CRIPT* deficiency induces a senescence phenotype as demonstrated by high levels of p16, p21, and p53 expression in skin biopsies and increased SA- β -galactosidase activity in patient-derived fibroblasts and upon *CRIPT* downregulation. Senescent cells change their metabolic profile into proinflammatory secretory cells and this senescence-associated secretory inflammatory phenotype might explain the immune cell infiltration and dilated blood vessels observed in skin from *CRIPT* individuals and other poikiloderma disorders.⁴⁸ Overriding senescence of dermal hair matrix or papilla cells might cause a loss of the hair cuticle cells that we observed by electron microscopy in *CRIPT* and *RECQL4* individuals.

Senescence can occur in response to different kinds of stress, including genotoxic and oxidative damage and mitotic errors. Although structural and numerical chromosomal aberrations were reported in subsets of RTS individuals (eg, in 3 of 17 published individuals), we did not detect any chromosomal aberrations in *CRIPT* patient-derived cells.² Fibroblasts from *RECQL4* or *CRIPT* individuals progressed normally through mitosis, and the mitotic defects were not overrepresented compared with those from controls. In summary, in patient-derived cells with disease-associated *RECQL4* and *CRIPT* variants, there were no quantifiable mitotic defects. In a previous study, the number of misaligned chromosomes increased after *RECQL4* downregulation in HeLa cells, but it was normal in *RECQL4* patient-derived cells.¹⁸ Similarly, the divergent results for *CRIPT* with regard to mitotic defects might be likely because of differences between patient-derived fibroblasts and immortalized cells, for example, cancer cells but do not exclude a functional role of *CRIPT* or *RECQL4* in mitosis.

Cellular senescence could also be triggered by DNA repair defects and genomic instability. The overall sensitivity of *RECQL4* and *CRIPT* patient-derived cells and fibroblasts after siRNA downregulation of *CRIPT* expression to different types of genotoxic stress was in the range of control cells.

In conclusion, we identified *CRIPT* as a novel RTS-like syndrome causing gene with neurologic impairment and further expand the genetic heterogeneity of the RTS spectrum of disorders. We searched for shared cellular defects as the drivers for overlapping RTS phenotypes and narrowed down that *CRIPT* and *RECQL4* deficiency are both associated with (1) increased cellular senescence and (2) only mild sensitivity to chemotoxic agents, (3) while the numbers of mitotic errors was not significantly different from controls. Of note, only *CRIPT*- and *RECQL4*-deficient patient-derived fibroblasts were available for this study, and future studies elucidating the functional impact in *APC1*-deficient cells are warranted. Thus, the question regarding which precise mechanism drives senescence remains unanswered at the current stage.

Our findings will open the door for further studies exploring the role of *CRIPT*, *RECQL4*, and also *ANAPC1* in preventing cellular senescence and the potential of senolytic drugs as a therapeutic approach. “Splitter and lumpers debates” are decisive in the field of syndromic diseases. Formal neurologic assessment of RTS individuals and long-term clinical follow-up of additional *CRIPT* individuals will add clarification to the debate whether the *CRIPT*-associated RTS-like syndrome should rather be split as a separate disease entity or rather should be classified as a novel subtype of RTS.

Data Availability

Data and materials that are not provided in this article because of space limitations will be supplied upon request for purposes of replicating procedures and results and follow-up research studies. Please send your request to luisa.aver@gmail.com or felix.distelmaier@med.uni-duesseldorf.de.

Acknowledgments

We thank the individuals and their families for participating in our research study. We acknowledge the FACS and Imaging Core Facility at the Max Planck Institute for Biology of Ageing for providing technical support, and the Bioinformatics Core Facility at the Max Planck Institute for Biology of Ageing for providing data analysis support. We thank the colleagues from the Department of Translational Genomics of King Faisal Specialist Hospital and Research Centre for sharing the fibroblast cell line of *CRIPT* individual #3. We also thank Dr. Xi Luo (Department of Molecular and Human Genetics at Baylor College of Medicine) who annotated the sequence variants of the published *RECQL4* cell lines in compliance with Human Genome Variation Society recommendations. Maxim A. Huetzen is a member of the Cologne Graduate School of Ageing Research.

Funding

L.A. was supported by the Elterninitiative Kinderkrebsklinik e.V. F.D. was supported by a grant of the German Research Foundation/Deutsche Forschungsgemeinschaft (DI 1731/2-2) and by a grant from the “Elterninitiative Kinderkrebsklinik e.V.” (Düsseldorf; #701900167). P.B. was supported by the German Research Foundation/DFG, Project-IDs 454024652 and 445703531), the European Research Council (Consolidator Grant No 101001791), and the Federal Ministry of Education and Research (STOP-FSGS-01GM1901A). R.K. and D.S. were supported by the Federal Ministry of Education and Research (ADDRESS - 01GM1909B).

Author Information

Conceptualization: L.A., F.D.; Data Curation (clinical data): L.A., S.M., E.A.F., S.L., P.B., J.A.O.; Formal Analysis (clinical data): L.A., S.M., E.A.F., S.L., T.D., T.-C.H., J.A.O., T.B., J.S., D.W., L.L.W., F.D.; Funding Acquisition: P.K., E.M., W.A., R.D.J., V.v.F., F.D.; Investigation (functional data): L.A., M.A.H., D.M.-A., A.S., K.N., A.H., P.B., B.H., W.A., R.D.J., V.v.F., M.S., E.G., L.L., W.J., D.S., R.K.; Methodology: L.A., D.M.-A., T.-C.H., K.N., P.B., P.K., W.A., R.D.J., D.S., R.K., F.D.; Project Administration: L.A., F.D.; Resources: P.K., E.M., W.A., R.D.J., V.v.F., F.D.; Software: D.M.-A., A.H., N.F., P.K., R.D.J.; Supervision: W.A., R.D.J., L.L.W., F.D.; Validation: L.L.W., F.D.; Visualization: L.A., M.A.H., D.M.-A., T.-C.H., A.S., B.H., N.F., V.v.F.; Writing-original draft: L.A., M.A.H.; Writing-review and editing: L.A., D.M.-A., T.B., J.S., T.D., L.L.W., W.A., E.M., D.S., D.W., F.D.

Ethics Declaration

The study was performed according to the Declaration of Helsinki and was approved by the institutional ethical review board (Heinrich-Heine-University, Düsseldorf, vote 2021-1340-LA/FD). The legal guardians provided written informed consent to participate in the study and gave their explicit permission for the publication of photographs. The authors attest that the research included in this report was conducted in a manner consistent with the principles of research ethics, such as those described in the Declaration of Helsinki and/or the Belmont Report. In particular, this research was conducted with the voluntary, informed consent of any research participants, free of coercion or coercive circumstances, and received institutional review board or research ethics committee approval consistent with the principles of research ethics and the legal requirements of the lead authors' jurisdiction(s).

Conflict of Interest

The authors declare no conflicts of interest.

Additional Information

The online version of this article (<https://doi.org/10.1016/j.gim.2023.100836>) contains supplementary material, which is available to authorized users.

Authors

Luisa Averdunk^{1,*} , Maxim A. Huetzen^{2,3,4,5}, Daniel Moreno-Andrés⁶, Reinhard Kalb⁷, Shane McKee⁸, Tzung-Chien Hsieh⁹, Annette Seibt¹, Marten Schouwink¹, Seema Lalani¹⁰, Eissa Ali Faqih¹¹, Theresa Brunet^{12,13}, Peter Boor¹⁴, Kornelia Neveling¹⁵, Alexander Hoischen¹⁵, Barbara Hildebrandt¹⁶, Elisabeth Graf¹², Linchao Lu¹⁷, Weidong Jin¹⁷, Joerg Schaper¹⁸, Jamal A. Omer¹⁹, Tanguy Demaret²⁰, Nicole Fleischer²¹, Detlev Schindler⁷, Peter Krawitz⁹, Ertan Mayatepek¹, Dagmar Wiczorek¹⁶, Lisa L. Wang¹⁷, Wolfram Antonin⁶, Ron D. Jachimowicz^{2,3,4,5}, Verena von Felbert²², Felix Distelmaier^{1,*}

Affiliations

¹Department of General Pediatrics, Neonatology and Pediatric Cardiology, Medical Faculty, University Hospital Düsseldorf, Heinrich-Heine-University, Düsseldorf, Germany; ²Max Planck Research Group Mechanisms of DNA Repair, Max Planck Institute for Biology of Ageing, Cologne, Germany; ³Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne and Düsseldorf, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany; ⁴Cologne Excellence Cluster on Cellular Stress Response in Aging-Associated Diseases, University of Cologne, Cologne, Germany; ⁵Center for Molecular Medicine Cologne, University of Cologne, Cologne, Germany; ⁶Institute of Biochemistry and Molecular Cell Biology, Medical School, RWTH Aachen University, Aachen, Germany; ⁷Institute for Human Genetics, Biocenter, University of Würzburg, Würzburg, Germany; ⁸Northern Ireland Regional Genetics Service, Belfast City Hospital, Belfast HSC Trust, Belfast, United Kingdom; ⁹Institute of Genomic Statistics and Bioinformatics, University of Bonn, Bonn, Germany; ¹⁰Department of Molecular Genetics, Baylor College of Medicine, Houston, TX; ¹¹Division of Medical Genetics, Children's Specialized Hospital, King Fahad Medical City, Riyadh, Saudi Arabia; ¹²Technical University of Munich,

School of Medicine, Institute of Human Genetics, Munich, Germany; ¹³Institute of Neurogenomics, Helmholtz Zentrum München, Munich, Germany; ¹⁴Institute of Pathology and Electron Microscopy Facility, Medical Faculty, RWTH Aachen University, Aachen, Germany; ¹⁵Department of Human Genetics, Radboud University Medical Center, Nijmegen, The Netherlands; ¹⁶Institute of Human Genetics, University Hospital Düsseldorf, Heinrich-Heine-Universität, Düsseldorf, Germany; ¹⁷Division of Hematology/Oncology, Department of Pediatrics, Texas Children's Cancer Center, Baylor College of Medicine, Houston, TX; ¹⁸Center of Rare Diseases, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany; ¹⁹Department of General Pediatrics, Children's Specialized Hospital, King Fahad Medical City, Riyadh, Saudi Arabia; ²⁰Centre de Génétique Humaine, Institut de Pathologie et Génétique, Gosselies, Belgium; ²¹FDNA Inc., Boston, MA; ²²Department of Dermatology and Allergy, Medical Faculty, RWTH Aachen University, Aachen, Germany

References

- Vennos EM, Collins M, James WD. Rothmund-Thomson syndrome: review of the world literature. *J Am Acad Dermatol*. 1992;27(5Pt1):750-762. [http://doi.org/10.1016/0190-9622\(92\)70249-f](http://doi.org/10.1016/0190-9622(92)70249-f)
- Wang LL, Levy ML, Lewis RA, et al. Clinical manifestations in a cohort of 41 Rothmund-Thomson syndrome patients. *Am J Med Genet*. 2001;102(1):11-17. [http://doi.org/10.1002/1096-8628\(20010722\)102:1<11::aid-ajmg1413>3.0.co;2-a](http://doi.org/10.1002/1096-8628(20010722)102:1<11::aid-ajmg1413>3.0.co;2-a)
- Rothmund A. Ueber Cataracten in Verbindung mit einer eigenthümlichen Hautdegeneration. *Graefes Archiv für Ophthalmologie*. 1868;14(1):159-182. <http://doi.org/10.1007/BF02720945>
- Thomson MS. An hitherto undescribed familial disease. *Br J Dermatol*. 1923;35(12):455-462. <http://doi.org/10.1111/j.1365-2133.1923.tb09077.x>
- Gelaw B, Ali S, Becker J. Rothmund-Thomson syndrome, Klippel-Feil syndrome, and osteosarcoma. *Skeletal Radiol*. 2004;33(10):613-615. <http://doi.org/10.1007/s00256-004-0798-2>
- Hall JG, Pagon RA, Wilson KM. Rothmund-Thomson syndrome with severe dwarfism. *Am J Dis Child*. 1980;134(2):165-169. <http://doi.org/10.1001/archpedi.1980.02130140039013>
- Kassner EG, Qazi QH, Haller JO. Rothmund-Thomson syndrome (poikiloderma congenitale) associated with mental retardation, growth disturbance, and skeletal features. *Skeletal Radiol*. 1977;2(2):99-103. <http://doi.org/10.1007/BF00360989>
- Kraus BS, Gottlieb MA, Meliton HR. The dentition in Rothmund's syndrome. *J Am Dent Assoc*. 1970;81(4):895-915. <http://doi.org/10.14219/jada.archive.1970.0328>
- Nathanson M, Dandine M, Gaudelus J, Mousset S, Lasry D, Perelman R. Rothmund-Thompson syndrome with glaucoma. Endocrine study. *Sem Hop*. 1983;59(48):3379-3384.
- Miozzo M, Castorina P, Riva P, et al. Chromosomal instability in fibroblasts and mesenchymal tumors from 2 sibs with Rothmund-Thomson syndrome. *Int J Cancer*. 1998;77(4):504-510. [http://doi.org/10.1002/\(sici\)1097-0215\(19980812\)77:4<504::aid-ijc5>3.0.co;2-y](http://doi.org/10.1002/(sici)1097-0215(19980812)77:4<504::aid-ijc5>3.0.co;2-y)
- Lindor NM, Devries EMG, Michels VV, et al. Rothmund-Thomson syndrome in siblings: evidence for acquired in vivo mosaicism. *Clin Genet*. 1996;49(3):124-129. <http://doi.org/10.1111/j.1399-0004.1996.tb03270.x>
- Kitao S, Shimamoto A, Goto M, et al. Mutations in RECQL4 cause a subset of cases of Rothmund-Thomson syndrome. *Nat Genet*. 1999;22(1):82-84. <http://doi.org/10.1038/8788>
- Ajeawung NF, Nguyen TTM, Lu L, et al. Mutations in ANAPC1, encoding a scaffold subunit of the anaphase-promoting complex, cause Rothmund-Thomson syndrome type 1. *Am J Hum Genet*. 2019;105(3):625-630. <http://doi.org/10.1016/j.ajhg.2019.06.011>
- Sangrithi MN, Bernal JA, Madine M, et al. Initiation of DNA replication requires the RECQL4 protein mutated in Rothmund-Thomson syndrome. *Cell*. 2005;121(6):887-898. <http://doi.org/10.1016/j.cell.2005.05.015>
- Kumata Y, Tada S, Yamanada Y, et al. Possible involvement of RecQL4 in the repair of double-strand DNA breaks in *Xenopus* egg extracts. *Biochim Biophys Acta*. 2007;1773(4):556-564. <http://doi.org/10.1016/j.bbamcr.2007.01.005>
- Lu H, Shamanna RA, Keijzers G, et al. RECQL4 promotes DNA end resection in repair of DNA double-strand breaks. *Cell Rep*. 2016;16(1):161-173. <http://doi.org/10.1016/j.celrep.2016.05.079>
- Ghosh AK, Rossi ML, Singh DK, et al. RECQL4, the protein mutated in Rothmund-Thomson syndrome, functions in telomere maintenance. *J Biol Chem*. 2012;287(1):196-209. <http://doi.org/10.1074/jbc.M111.295063>
- Yokoyama H, Moreno-Andres D, Astrinidis SA, et al. Chromosome alignment maintenance requires the MAP RECQL4, mutated in the Rothmund-Thomson syndrome. *Life Sci Alliance*. 2019;2(1):e201800120. <http://doi.org/10.26508/lsa.201800120>
- Xu X, Liu Y. Dual DNA unwinding activities of the Rothmund-Thomson syndrome protein, RECQ4. *EMBO J*. 2009;28(5):568-577. <http://doi.org/10.1038/emboj.2009.13>
- Shirayama M, Tóth A, Gálová M, Nasmyth K. APC(Cdc20) promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5. *Nature*. 1999;402(6758):203-207. <http://doi.org/10.1038/46080>
- Larizza L, Roversi G, Volpi L. Rothmund-Thomson syndrome. *Orphanet J Rare Dis*. 2010;5:2. <http://doi.org/10.1186/1750-1172-5-2>
- Leduc MS, Niu Z, Bi W, et al. CRIPT exonic deletion and a novel missense mutation in a female with short stature, dysmorphic features, microcephaly, and pigmentary abnormalities. *Am J Med Genet A*. 2016;170(8):2206-2211. <http://doi.org/10.1002/ajmg.a.37780>
- Shaheen R, Faqeih E, Ansari S, et al. Genomic analysis of primordial dwarfism reveals novel disease genes. *Genome Res*. 2014;24(2):291-299. <http://doi.org/10.1101/gr.160572.113>
- Akalın A, Şimşek-Kiper PÖ, Taşkıran EZ, Karaosmanoğlu B, Utine GE, Boduroğlu K. A novel biallelic CRIPT variant in a patient with short stature, microcephaly, and distinctive facial features. *Am J Med Genet A*. 2023;191(4):1119-1127. <http://doi.org/10.1002/ajmg.a.63120>
- Niethammer M, Valtchanoff JG, Kapoor TM, et al. CRIPT, a novel postsynaptic protein that binds to the third PDZ domain of PSD-95/SAP90. *Neuron*. 1998;20(4):693-707. [http://doi.org/10.1016/s0896-6273\(00\)81009-0](http://doi.org/10.1016/s0896-6273(00)81009-0)
- Passafaro M, Sala C, Niethammer M, Sheng M. Microtubule binding by CRIPT and its potential role in the synaptic clustering of PSD-95. *Nat Neurosci*. 1999;2(12):1063-1069. <http://doi.org/10.1038/15990>
- Hsieh TC, Bar-Haim A, Moosa S, et al. GestaltMatcher facilitates rare disease matching using facial phenotype descriptors. *Nat Genet*. 2022;54(3):349-357. <http://doi.org/10.1038/s41588-021-01010-x>
- Villegas J, McPhaul M. Establishment and culture of human skin fibroblasts. *Curr Protoc Mol Biol*. 2005;Chapter (28):Unit 28.3. <https://doi.org/10.1002/0471142727.mb2803s71>
- Held M, Schmitz MHA, Fischer B, et al. CellCognition: time-resolved phenotype annotation in high-throughput live cell imaging. *Nat Methods*. 2010;7(9):747-754. <http://doi.org/10.1038/nmeth.1486>
- Xu K, Wang C, Keinänen K, Li H, Cai C. Mitotic spindle disassembly in human cells relies on CRIPT having hierarchical redox signals. *J Cell Sci*. 2022;135(18):jcs259657. <http://doi.org/10.1242/jcs.259657>
- Mehollin-Ray AR, Kozinetz CA, Schlesinger AE, Guillerman RP, Wang LL. Radiographic abnormalities in Rothmund-Thomson syndrome and genotype-phenotype correlation with RECQL4 mutation status. *AJR Am J Roentgenol*. 2008;191(2):W62-W66. <http://doi.org/10.2214/AJR.07.3619>

32. Kubota M, Yasunaga M, Hashimoto H, et al. IgG4 deficiency with Rothmund-Thomson syndrome: a case report. *Eur J Pediatr*. 1993; 152(5):406-408. <http://doi.org/10.1007/BF01955898>
33. Gurovich Y, Hanani Y, Bar O, et al. Identifying facial phenotypes of genetic disorders using deep learning. *Nat Med*. 2019;25(1):60-64. <http://doi.org/10.1038/s41591-018-0279-0>
34. Lombard DB, Chua KF, Mostoslavsky R, Franco S, Gostissa M, Alt FW. DNA repair, genome stability, and aging. *Cell*. 2005;120(4):497-512. <http://doi.org/10.1016/j.cell.2005.01.028>
35. Lu H, Fang EF, Sykora P, et al. Senescence induced by RECQL4 dysfunction contributes to Rothmund-Thomson syndrome features in mice. *Cell Death Dis*. 2014;5(5):e1226. <http://doi.org/10.1038/cddis.2014.168>
36. Lu L, Harutyunyan K, Jin W, et al. RECQL4 regulates p53 function in vivo during skeletogenesis. *J Bone Miner Res*. 2015;30(6):1077-1089. <http://doi.org/10.1002/jbmr.2436>
37. Davis T, Tivey HSE, Brook AJC, Grimstead JW, Rokicki MJ, Kipling D. Activation of p38 MAP kinase and stress signalling in fibroblasts from the progeroid Rothmund-Thomson syndrome. *Age (Dordr)*. 2013;35(5):1767-1783. <http://doi.org/10.1007/s11357-012-9476-9>
38. Stein GH, Drullinger LF, Soulard A, Dulić V. Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. *Mol Cell Biol*. 1999;19(3):2109-2117. <http://doi.org/10.1128/MCB.19.3.2109>
39. Mann MB, Hodges CA, Barnes E, Vogel H, Hassold TJ, Luo G. Defective sister-chromatid cohesion, aneuploidy and cancer predisposition in a mouse model of type II Rothmund-Thomson syndrome. *Hum Mol Genet*. 2005;14(6):813-825. <http://doi.org/10.1093/hmg/ddi075>
40. Weaver BAA, Cleveland DW. Decoding the links between mitosis, cancer, and chemotherapy: the mitotic checkpoint, adaptation, and cell death. *Cancer Cell*. 2005;8(1):7-12. <http://doi.org/10.1016/j.ccr.2005.06.011>
41. Petkovic M, Dietschy T, Freire R, Jiao R, Stagljar I. The human Rothmund-Thomson syndrome gene product, RECQL4, localizes to distinct nuclear foci that coincide with proteins involved in the maintenance of genome stability. *J Cell Sci*. 2005;118(18):4261-4269. <http://doi.org/10.1242/jcs.02556>
42. Jin W, Liu H, Zhang Y, Otta SK, Plon SE, Wang LL. Sensitivity of RECQL4-deficient fibroblasts from Rothmund-Thomson syndrome patients to genotoxic agents. *Hum Genet*. 2008;123(6):643-653. <http://doi.org/10.1007/s00439-008-0518-4>
43. Schindler D, Friedl R, Gavrovic I, et al. Applications of cell cycle testing in fanconi anemia. In: *Fanconi Anemia*. Karger; 2007:110-130. <http://doi.org/10.1159/000102552>
44. Beamish H, Lavin MF. Radiosensitivity in Ataxia-telangiectasia: anomalies in radiation-induced cell cycle delay. *Int J Radiat Biol*. 1994;65(2):175-184. <http://doi.org/10.1080/09553009414550211>
45. Hoki Y, Araki R, Fujimori A, et al. Growth retardation and skin abnormalities of the Recql4-deficient mouse. *Hum Mol Genet*. 2003;12(18):2293-2299. <http://doi.org/10.1093/hmg/ddg254>
46. Duan S, Han X, Akbari M, Croteau DL, Rasmussen LJ, Bohr VA. Interaction between RECQL4 and OGG1 promotes repair of oxidative base lesion 8-oxoG and is regulated by SIRT1 deacetylase. *Nucleic Acids Res*. 2020;48(12):6530-6546. <http://doi.org/10.1093/nar/gkaa392>
47. Lu L, Jin W, Wang LL. Aging in Rothmund-Thomson syndrome and related RECQL4 genetic disorders. *Ageing Res Rev*. 2017;33:30-35. <http://doi.org/10.1016/j.arr.2016.06.002>
48. Young ARJ, Narita M. SASP reflects senescence. *EMBO Rep*. 2009;10(3):228-230. <http://doi.org/10.1038/embor.2009.22>

2.2 Manuscript II: Optical Genome Mapping Identifies Structural Variants in Potentially New Cancer Predisposition Candidate Genes in Pediatric Cancer Patients

International Journal of Cancer, September 2023

PMID: 37776287

doi: 10.1002/ijc.34721

Rabea Wagener*, Danielle Brandes*, Marie Jung, Maxim A Huetzen, Anke K Bergmann, Stephanie Panier, Daniel Picard, Ute Fischer, Ron D Jachimowicz, Arndt Borkhardt, Triantafyllia Brozou

2.2.1 Contributions

With third authorship on this publication, I performed functional characterization to validate the pathogenic significance of the 17p13.3 duplication encompassing *RPA1* identified through optical genome mapping.

I assessed RPA1 protein expression by immunoblotting, measured telomere length using the TeloTAGGG Southern blot-based assay, and evaluated cellular sensitivity to the genotoxic agents hydroxyurea and cisplatin through CellTiter-Glo luminescence-based cell viability assays (**II-Figure S5; section 2.2.3**). These experiments demonstrated that the *RPA1* duplication conferred cellular hypersensitivity to replication stress and DNA crosslinking, providing mechanistic support for pathogenicity.

I co-wrote the manuscript sections describing RPA1 functional characterization and coordinated with the partner research group at Heinrich-Heine-University Düsseldorf, who led patient recruitment, clinical characterization, and optical genome mapping analysis.

This work adds to the growing body of evidence supporting optical genome mapping as a clinically relevant approach for detecting pathogenic structural variants in cancer predisposition genes that escape conventional sequencing methods. The functional characterization provided essential evidence linking *RPA1* duplication to cellular DNA damage hypersensitivity, underscoring the importance of functional validation in interpreting novel structural variants and contributing to the growing understanding of *RPA1* dosage sensitivity in genome maintenance and cancer predisposition.

2.2.2 Peer-reviewed publication

The published manuscript PDF is attached in this section. Under the Wiley Copyright Transfer Agreement, authors retain the right to include their article in a dissertation. (<https://authors.wiley.com/author-resources/Journal-Authors/licensing/licensing-info-faqs.html>)

Original page numbers were removed for formatting reasons.

SHORT REPORT

Innovative Tools and Methods

Optical genome mapping identifies structural variants in potentially new cancer predisposition candidate genes in pediatric cancer patients

Rabea Wagener^{1,2}  | Danielle Brandes¹ | Marie Jung¹ | Maxim A. Huetzen³ | Anke K. Bergmann⁴ | Stephanie Panier^{3,5} | Daniel Picard¹ | Ute Fischer^{1,2} | Ron D. Jachimowicz^{3,6,7,8} | Arndt Borkhardt^{1,2}   | Triantafyllia Brozou¹

¹Department of Pediatric Oncology, Hematology and Clinical Immunology, Medical Faculty, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

²German Cancer Consortium (DKTK), partner site Essen/Düsseldorf, Düsseldorf, Germany

³Max Planck Research Group Mechanisms of DNA Repair, Max Planck Institute for Biology of Ageing, Cologne, Germany

⁴Institute of Human Genetics, Hannover Medical School (MHH), Hannover, Germany

⁵Institute for Genome Stability in Aging and Disease, Medical Faculty, University of Cologne, Cologne, Germany

⁶Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne and Düsseldorf, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany

⁷Cologne Excellence Cluster on Cellular Stress Responses in Ageing-Associated Diseases, University of Cologne, Cologne, Germany

⁸Center for Molecular Medicine Cologne, University of Cologne, Cologne, Germany

Correspondence

Arndt Borkhardt, Department of Pediatric Oncology, Hematology and Clinical Immunology, Medical Faculty, Moorenstr. 5, 40225 Düsseldorf, Germany.
 Email: arndt.borkhardt@med.uni-duesseldorf.de

Funding information

Deutsche Forschungsgemeinschaft, Grant/Award Number: 495318549; Deutsche Krebshilfe; Forschungskommission of the Medical Faculty of the Heinrich Heine University Düsseldorf, Grant/Award Number: 2020-28; Forschungskommission of the Medical Faculty of the Heinrich Heine University of Düsseldorf; German José Carreras leukemia foundation, Grant/Award Numbers: DJCLS 07 R/2019, DJCLS 18R/202, DJCLS 21 R/2019; Katharina Hardt foundation; Löwenstern e.V.

Abstract

Genetic predisposition is one of the major risk factors for pediatric cancer, with ~10% of children being carriers of a predisposing germline alteration. It is likely that this is the tip of the iceberg and many children are underdiagnosed, as most of the analysis focuses on single or short nucleotide variants, not considering the full spectrum of DNA alterations. Hence, we applied optical genome mapping (OGM) to our cohort of 34 pediatric cancer patients to perform an unbiased germline screening and analyze the frequency of structural variants (SVs) and their impact on cancer predisposition. All children were clinically highly suspicious for germline alterations (concomitant conditions or congenital anomalies, positive family cancer history, particular cancer type, synchronous or metachronous tumors), but whole exome sequencing (WES) had failed to detect pathogenic variants in cancer predisposing genes. OGM detected a median of 49 rare SVs (range 27–149) per patient. By analysis of

Abbreviations: aCGH, array comparative genome hybridization; BCP-ALL, B-cell-precursor acute lymphoblastic leukemia; CPG, cancer predisposition gene; CPS, cancer predisposition syndrome; DSBs, double strand breaks; GoF, gain of function; HMW, high molecular weight; HU, hydroxyurea; indels, insertions and deletions; OGM, optical genome mapping; PBMC, peripheral blood mononuclear cells; PFBMT6, telomere-related pulmonary fibrosis and/or bone marrow failure-6 syndrome; SNVs, single nucleotide variants; STS, short telomere syndrome; SVs, structural variants; WES, whole exome sequencing.

Rabea Wagener and Danielle Brandes have contributed equally to this work and shared first authorship.

Arndt Borkhardt and Triantafyllia Brozou have contributed equally to this work and shared last authorship.

This is an open access article under the terms of the [Creative Commons Attribution](https://creativecommons.org/licenses/by/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2023 The Authors. *International Journal of Cancer* published by John Wiley & Sons Ltd on behalf of UICC.

18 patient-parent trios, we identified three de novo SVs. Moreover, we discovered a likely pathogenic deletion of exon 3 in the known cancer predisposition gene *BRCA2*, and identified a duplication in *RPA1*, which might represent a new cancer predisposition gene. We conclude that optical genome mapping is a suitable tool for detecting potentially predisposing SVs in addition to WES in pediatric cancer patients.

KEYWORDS

cancer predisposition, optical genome mapping, pediatric cancer, structural variants

What's new?

Around 10% of patients with pediatric cancer have been found to harbor a cancer predisposing germline alteration. However, the entire spectrum of genetic alterations has yet to be considered. Here, the authors analyzed 34 pediatric patients with a suspected underlying cancer predisposition syndrome by using the new optical genome mapping technique. They identified de novo or inherited genetic structural variants in *BRCA2* and in the potential novel cancer predisposition gene *RPA1*. The study demonstrates that optical genome mapping is a suitable tool for detecting potentially predisposing structural variants in addition to whole exome sequencing in pediatric cancer patients.

1 | INTRODUCTION

In recent years, several studies have shown that ~10% of pediatric cancer patients harbor a cancer predisposing germline alteration.¹⁻⁵ However, most of these studies focused on single nucleotide variants (SNVs) and small insertions and deletions (indels) ≤ 20 bp. In all of these studies, the whole exome sequencing (WES) analysis was mainly concentrated on a predefined gene panel of known cancer predisposition genes (CPG). Therefore, there is some question as to whether the remaining ~90% of patients carry any predisposing germline alteration, in particular as not the entire spectrum of genetic alterations was considered. In line with this, no causative genetic alteration was detectable in ~80% of pediatric cancer patients with clinical signs indicative of an underlying cancer predisposition syndrome (CPS).^{4,6} Although SNVs outnumber other types of DNA sequence variants by 7:1, structural variants (SVs) are 30-fold more prone to alter the gene expression. Moreover, 34% of all disease-causing variants are larger than SNVs, with 1/3 being >20 bp in size.⁷ Based on previous studies, 1.5% of adult cancer patients harbor a cancer predisposing SV.⁸ By contrast, the frequency seems to be more variable in pediatric cancer patients, with reports of 0.4%-3.2% of patients harboring a pathogenic germline SV.^{1,5} These studies are mostly based on short-read next generation sequencing approaches that have limitations with respect to accurate alignment in challenging genomic regions, which are prone to breakpoints causing SVs.⁹ Hence, the exact prevalence of SVs in the germline of cancer patients might be substantially underestimated. Recent proof-of-principle studies have shown that optical genome mapping (OGM) is a new method of reliably detecting constitutional SVs that can be used as an alternative to karyotyping, fluorescence in situ hybridization and DNA microarrays.^{10,11}

We here report on the applicability of OGM as a complementary instrument to the existing sequencing methods for unbiased germline screening of pediatric cancer patients regarding cancer predisposing SVs.

2 | MATERIALS AND METHODS

2.1 | Patient cohort

We included 34 patients of our germline study and, if available, their parents.¹² Those patients were positive for clinical criteria suggesting the presence of an underlying CPS,^{13,14} but lacking pathogenic cancer predisposing variants in WES.⁴ Refer to [Supplementary Material and Methods](#) for a detailed description of the cohort.

2.2 | Optical genome mapping

2.2.1 | Extraction and labeling of high-molecular weight DNA

We extracted high-molecular weight (HMW) DNA peripheral blood or bone marrow mononuclear cells from remission or, if available, from fibroblasts of the children and peripheral blood of the parents. Sufficient material was available from 18 child-parent trios, six child-parent duos and 10 child-singletons. The HMW DNA (750ng) was labeled, loaded on a Saphyr G2.3 chip and molecules were imaged by the Saphyr instrument (Gen 2, Bionano Genomics). Refer to Table S1 for an overview of the run statistics and quality metrics and [Supplementary Material and Methods](#) for a detailed description.

2.2.2 | De novo assembly and variant calling

The de novo assemblies against the human reference genome GRCh38, structural variant calling and annotation were performed using Bionano Solve (version 3.5.1, Bionano Genomics). The Bionano Access software (version 1.5.2, Bionano Genomics)

was used to visualize the data. For OGM data from the patient and one or both parents, we performed “Dual” or “Trio” variant annotation, respectively, to differentiate de novo and transmitted SVs. Refer to the [Supplementary Material and Methods](#) for a detailed description of the performed analysis and SV filtering.

2.3 | Variant annotation and interpretation

The final list of SVs was manually annotated using the UCSC genome browser tracks to identify whether an SV targets a gene (NCBI RefSeq, Ensembl: MANE Project [v1.0], GENCODE V39) and whether the SV region overlaps with a common SV ($\geq 1\%$ of the population) using the NCBI dbVAR Curated Common SVs database.¹⁵ We also checked whether the SVs overlap with known regulatory regions using GeneHancer (Double Elite).¹⁶ We annotated the overlap of an SV with a CPG using a published list.⁴ Genes targeted by an SV that potentially hits a coding region were explored with regard to clinical relevance for the cancerogenesis of the respective patient.

2.4 | Validation and functional testing

We performed validation experiments using DNA microarray, PCR-based assays and/or RNA sequencing for selected SVs and performed functional testing on P28 fibroblasts and age/gender-matched controls. Refer to the [Supplementary Material and Methods](#) for a detailed description of the performed analysis.

3 | RESULTS AND DISCUSSION

3.1 | Description of patient cohort

We analyzed 34 pediatric cancer patients with a median age of 5.4 years (range 0–18.5) at initial tumor diagnosis and a male to female ratio of 1.6:1 (Table S2). All patients harbored clinical signs indicative of an underlying predisposition such as congenital anomalies, concomitant conditions, multiple tumors or excessive toxicity to the chemotherapy (Figure 1A). Fifty-five percent of the patients harbored congenital anomalies, mainly of a mild form and not corresponding to a specific syndrome. Thirty-eight percent of the patients harbored positive family histories of pediatric and/or adult cancer. Nine patients were diagnosed with types of cancer strongly associated with genetic germline predisposition, such as sonic hedgehog-activated medulloblastoma. Moreover, three patients harbored more than two tumors (synchronous or metachronous) or showed excessive toxicity after chemotherapy.

3.2 | Identification of structural variants using optical genome mapping

We generated an average of 422.5 Gb of data per sample resulting in average $106\times$ effective coverage of the de novo assemblies

(Table S1). SV calling yielded a median of 6455 (range 5696–6685) SVs per sample. Applying filter steps to exclude artifacts and to detect rare SVs, yielded 1831 SVs, with a median of 49 (range 27–149) SVs per patient. Deletions and insertions were the most frequent SV types (median of 30 [range 15–93] and 18 [range 10–53], respectively; Figure 1B, Table S3). 95.6% of unbalanced SVs (considering deletions, duplications and insertions that can also constitute duplications) had a length of less than 50 kbp (Figure 1C). The majority of SVs were either intergenic (55%) or were located in an intron (19%) (Table S3). However, 26% of the SVs potentially affected coding sequences of one or more genes.

Due to our unique OGM dataset of 18 child-parent trios, we were able to identify de novo SVs (two deletions and one insertion) in 17% (3/18) of the children, affecting the *DDX10* or *FLG1* gene or an intergenic locus, respectively (Figure 1D, Table S4). This is in line with estimates based on WES published by Kloosterman et al, who reported de novo SVs >20 bp or >500 bp in 16% or 8% of children, respectively.¹⁷

3.3 | Structural variants targeting known cancer predisposition genes

In our list of high-confidence SVs, we checked whether they targeted known CPGs.⁴ Twenty-one SVs overlapped with a CPG, of which five potentially affected a coding region (Table S5).

Patients P6 and P10 both carried the same 1.4 kbp deletion in 17p13.1, potentially targeting the *TP53* gene (Figure S1A,B). Further analysis revealed that the deletion maps 5.5 kbp upstream of the *TP53* gene, overlapping with a described common deletion (nssv16197110) (Figure S1C,D).

After literature mining, we classified a deletion in *BRCA2*, which occurred in P26 as likely pathogenic (Table 1, Figure 2A). A maternally transmitted 4.8 kbp deletion was detected that overlapped with the exons 2–9 of the *BRCA2* gene. Performing PCR-based Sanger sequencing, we could confirm the presence of a 4.7 kbp deletion in chr13:32318724–32 323 440 bp, leading to deletion of the third exon of the *BRCA2* gene (*BRCA2* ^{Δ ex3}, ENST00000380152) (Figure 2B). Variants leading to *BRCA2* exon 3 deletions have been reported in individuals with breast and ovarian cancer.^{18,19} Although this deletion does not alter the reading frame, a part of the highly conserved N-terminal domain is lost that is important for the interaction with PALB2²⁰ and DNA repair. Next, we performed a semiquantitative PCR to check whether both the *BRCA2* ^{Δ ex3} and *BRCA2*^{wt} transcripts were expressed. While material was available from the child and the father, unfortunately, no material was available of the mother or the initially diagnosed leukemia of the patient to be examined for down-stream analysis. Interestingly, the child exhibited a higher expression of *BRCA2* ^{Δ ex3} than *BRCA2*^{wt} compared to the father and the control samples with predominant expression of *BRCA2*^{wt} transcript (Figure 2C). There has been some debate about the pathogenic effect of exon 3 deletion, as the *BRCA2* ^{Δ ex3} transcript is constitutively expressed in tissue samples from apparently healthy individuals. However, Muller et al reported an allelic imbalance in favor of the

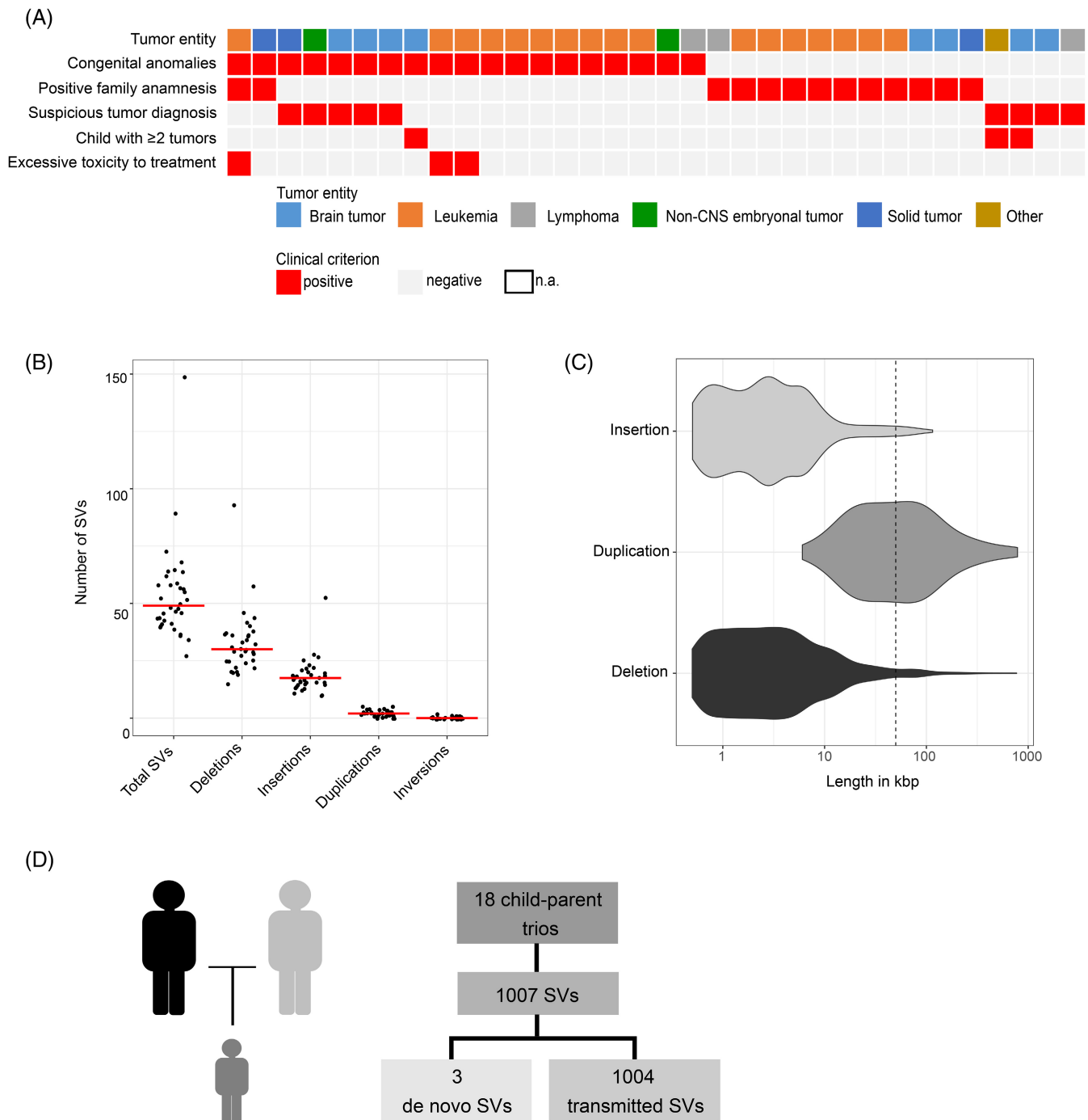


FIGURE 1 Structural variants detected by OGM in 34 pediatric patients suspicious for an underlying cancer predisposition syndrome. (A) Summary of patients' tumor diagnoses and status of clinical criteria. Each line represents a clinical feature and each column represents a patient. (B) Overview of structural variants identified using OGM. The dot plot represents the number of germline SVs per type, with every dot representing a patient. The red horizontal lines represent the median. (C) The violin plot depicts the length distribution of the identified SVs. The dashed line represents the 50 kbp threshold that is a lower limit of copy number variant length detectable by conventional DNA array-based methods. (D) Analysis of 18 child-parent trios using OGM revealed that 3/1007 detected SVs were de novo variants.

BRCA2 ^{Δ ex3} mutant allele¹⁸ and Caputo et al proposed that only variants producing transcripts lacking exon 3 should be classified as pathogenic.¹⁹ In line with this, we observed an allelic imbalance toward the *BRCA2* ^{Δ ex3} transcript in our patient. The maternally transmitted

deletion segregated within the cancer history in the maternal lineage. The grandmother (carrier status unknown) was diagnosed with breast cancer at the age of 32, and the patient developed an *ETV6::RUNX1* rearranged BCP-ALL at the age of 12, whereas the patients' mother

TABLE 1 Structural variants and affected genes potentially playing a role in cancer predisposition of two pediatric patients.

Case	P26	P28
Tumor type	BCP-ALL	BCP-ALL
Subtype	<i>ETV6::RUNX1</i>	<i>KMT2A::AFF1</i>
Phenotype	Congenital cardiac anomaly (HP:0001627)	Arched eyebrows (HP:0002553)
Genomic region (hg38)	chr13:32 315 668-32 331 479 bp	chr17:1 747 307-1 899 562 bp
SV type	Deletion	Duplication
Size	4.8 kbp	166 kbp
Affected genes of interest	<i>BRCA2</i>	<i>RPA1</i>
Overlap common dbVar	None	None
Validation	WES, Sanger, RT-qPCR	WES, SNP-array, qPCR
Functional testing	n.a.	Telomere length, DNA damage
Interpretation	Likely pathogenic	Likely pathogenic

Note: Overlap common dbVAR: Indicating whether the region in which the SV was observed overlaps with a known copy number alteration using the common dbVar database (>1% in the population) as reference.

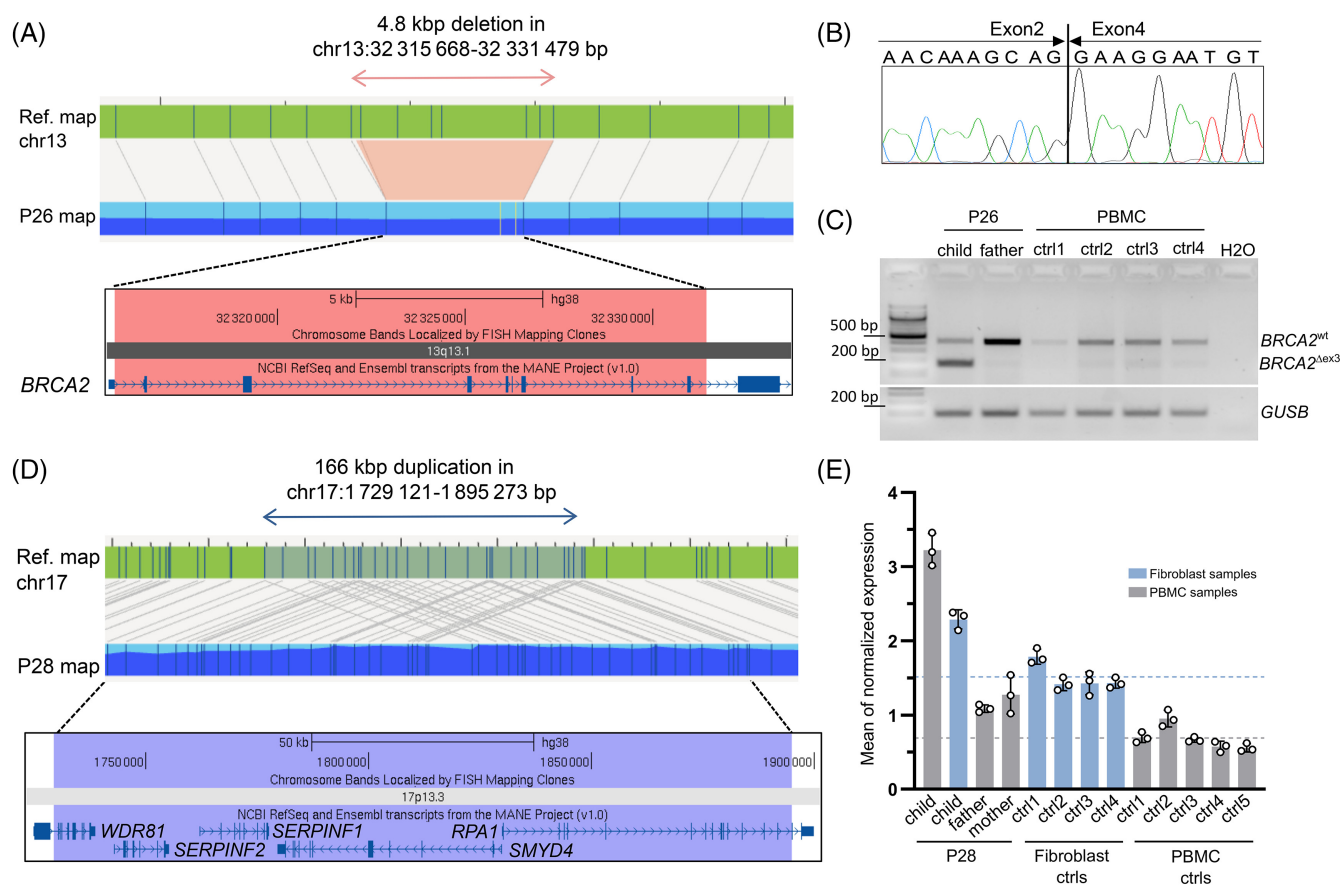


FIGURE 2 Structural variants affecting the known CPG *BRCA2* and the potential candidate CPG *RPA1* in two pediatric cancer patients. (A) Germline deletion detected in the cancer predisposition gene *BRCA2* in P26. Genome maps (green bar above reference and blue bar below patient) indicating the presence of a 4.8 kbp deletion in 13q13.1. Lower part of the figure depicts the location of the deleted region, highlighted in red, which is located within the *BRCA2* gene. (B) Sanger sequencing of the mRNA of the patient confirms the deletion of exon 3 in *BRCA2* (ENST00000380152). (C) Upper panel: Semiquantitative PCR of *BRCA2*^{wt} and *BRCA2*^{Δex3} transcript RNA extracted from the PBMCs of P26 carrying the 4.8 kbp deletion in comparison to the father (P26_father) and four control samples (PBMC ctrl 1-4). Lower panel: *GUSB* was used as control. (D) Genome map of duplicated region affecting the *RPA1* gene locus (17p13.3) in P28 (green bar represents reference map and blue bars the patient map) and the location within the genome using UCSC genome browser (duplication highlighted in blue). (E) Analysis of *RPA1* mRNA expression levels reveals higher expression in PBMCs and fibroblasts of P28 in comparison to the parents and controls. The controls were separated based on tissue of origin, including fibroblasts (blue) and PBMC (gray) control samples. The dashed lines represent the mean expression levels of the fibroblast controls (blue) and PBMC controls (gray).

had not yet developed any cancer at the age of 40. Although, *BRCA2* alterations have not been described to increase the susceptibility to ALL as yet, the internal *BRCA2* deletion might be a possible risk factor.

3.4 | Identification of novel candidate cancer predisposition genes

98.8% of all SVs identified by OGM in our study did not overlap with a known CPG. Performing a comprehensive literature search and further analysis, we identified three interesting SVs potentially targeting novel CPGs.

In patient P16, a girl who synchronously developed a medulloblastoma and an astrocytoma at the age of 3, we identified a maternally transmitted 180 kbp tandem duplication in 8p11.22, verified by SNP array and coverage-based WES data (Figure S2A-C). The duplicated region encompasses the *ADAM9* and *ADAM32* genes, which belong to the “disintegrin and metalloprotease” (ADAM) family. *ADAM9* is overexpressed in glioblastoma, where it promotes invasiveness and proliferation.^{21,22} To test the effect on *ADAM9* and *ADAM32* expression or if the duplication leads to a potential fusion transcript, we performed whole transcriptome analysis of the patient and the noncarrier father. We did not detect an *ADAM32::ADAM9* fusion transcript or altered expression of both genes in the affected child (Figure S2D). In addition, we identified compound heterozygous SVs in the *RAP1B* gene, in P10, a boy with B-ALL. The patient carried a maternally transmitted 9.3 kbp insertion and a paternally transmitted 10.8 kbp deletion, both affecting regulatory regions of *RAP1B* gene locus (Figure S3A,B). *RAP1B* encodes for a protein of the RAS family of small GTP binding proteins with mitogenic and oncogenic properties.^{23,24} Activating missense variants in *RAP1B* have been described in three patients with syndromic thrombocytopenia and multiple malformations, including microcephaly and learning difficulties.^{25,26} In line with these findings, P10 had mild global developmental delay and a growth disorder in addition to his B-ALL. However, the analysis of *RAP1B* mRNA expression in the PBMCs of the patient revealed no altered expression in comparison to the father or the controls (Figure S3C).

Conclusively, additional experiments including corresponding tumor analysis are required to clarify whether the identified germline SVs affecting *ADAM9* and *RAP1B* have a functional influence on the development of the patient's tumors.

The most interesting SV was a 166 kbp duplication in 17p13.3 in P28, an infant diagnosed with *KMT2A*-rearranged B-cell-precursor acute lymphoblastic leukemia (BCP-ALL) at the age of 4 months, who relapsed 6 months after initial diagnosis (Table 1, Figure 2D). The duplication was present in the germline and the corresponding tumor of P28 (Figure S4A). We verified the copy number gain in the child using coverage-based information from WES and could show that this gain was not present in the WES data of the parents, indicating that the SV occurred de novo (Figure S4B). Using SNP array, we could show that the duplication is located in a region of copy number neutral loss of heterozygosity in 17p13.3-p13.1 (Figure S4C). Due to the

consanguinity of the parents (first-degree cousins), 11.2% of the patient's autosomal genome is homozygous (Figure S4D). The duplication affected five genes, including *RPA1*, which has essential functions in DNA replication, repair, and recombination.²⁷ It is associated with autosomal dominant inherited telomere-related pulmonary fibrosis and/or bone marrow failure-6 syndrome (PFBMT6, MIM:619767). The PFBMT6 syndrome belongs to the short telomere syndromes (STS) with variable symptoms, genetic heterogeneity and variable expressivity. Analysis of the *RPA1* mRNA level in PBMCs and fibroblasts of the child revealed an increased expression in comparison to parents and controls, which points toward the duplication having a gain-of-function (GoF) effect (Figure 2E). This is in line with reports by Sharma et al, who described GoF *RPA1* missense variants in patients with short telomeres and varying clinical features of STS.²⁸ We further assessed *RPA1* protein expression in the patient's fibroblasts in comparison to two age/gender-matched fibroblast samples from children without *RPA1* alterations, which revealed similar *RPA1* expression (Figure S5A). We screened for a potential telomeropathy by performing TeloTAGGG Assay on fibroblasts of P28 carrying the *RPA1* duplication in comparison to age/gender-matched control fibroblasts (Figure S5B,C). However, we even detected a mild increase of mean telomere restriction fragment length in the *RPA1* duplicated cells compared to the controls (P28 = 13.3 kb vs Ctrl1 = 10.4 kb; Ctrl2 = 11.6 kb, respectively).

Additionally, it has been reported that *RPA1* duplication induces elevated sensitivity to DNA damage.²⁹ Therefore, we also checked for an underlying DNA repair defect in P28 by performing cell viability assays to analyze sensitivity to genotoxic agents (Figure S5D,E). We used the chemotherapeutic agent cisplatin, inducing DNA cross-linking and DNA double-strand breaks (DSBs), and the ribonucleotide reductase inhibitor hydroxyurea (HU) that depletes nucleotides and induces replication stress leading to subsequent DSBs. Fibroblasts of P28 carrying the *RPA1* duplication exhibited a significant increase in sensitivity to cisplatin and HU compared to wild-type age/gender-matched controls. Thus, in line with the published findings,²⁹ P28 fibroblasts with *RPA1* duplication showed elevated sensitivity to DNA damage. The extent to which the described genotype might be associated with the origin of the *KMT2A*+ infant ALL and if the patient, who remains very young, may develop shortened telomeres needs to be monitored in the future.

4 | CONCLUSION

By applying OGM, we comprehensively characterized the SV landscape of 34 pediatric cancer patients who were clinically highly suspicious for an underlying CPS and had a nonconclusive WES result. We identified three de novo SVs among 18 child-parent trios and two hot germline alterations affecting the known CPG *BRCA2* and the potential new CPG candidate *RPA1* in two respective leukemia patients.

Our study highlights the suitability of OGM in a clinical setting to detect potentially predisposing SVs in suspicious pediatric cases. However, there remains the necessity for applying additional

sequencing-based methods to determine the exact breakpoint boundaries when interpreting OGM reported SVs. We expect that extended germline screens, improvement of the OGM reference database and integration of the telomere-to-telomere reference genome will enhance SV detection and interpretation of disease-causing events. Further studies will likely use a combination of OGM and long-read sequencing technologies to overcome the current limitations of OGM resolution (<500 bp) to cover the full spectrum of genetic predisposition in children with cancer.

AUTHOR CONTRIBUTIONS

Rabea Wagener organized the project and wrote the paper. Rabea Wagener, Danielle Brandes, Marie Jung and Triantafyllia Brozou analyzed and interpreted the data. Danielle Brandes and Marie Jung extracted the DNA and performed the analysis. Triantafyllia Brozou recruited patients, provided clinical information, obtained informed consent and revised the manuscript. Maxim A. Huetzen, Stephanie Panier and Ron D. Jachimowicz performed functional analysis of RPA1. Daniel Picard analyzed RNA-sequencing data. Anke K. Bergmann provided cytogenetic and molecular genetic data. Ute Fischer and Arndt Borkhardt discussed the data and outlined the project. All authors contributed to and approved the final manuscript. The work reported in the paper has been performed by the authors, unless clearly specified in the text.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. Daniel Scholtysik and Ms. Silke Furlan for their excellent technical assistance. Computational support and infrastructure was provided by the Centre for Information and Media Technology (ZIM) at the University of Düsseldorf (Germany). Maxim A. Huetzen is member of the Cologne Graduate School of Ageing Research. The graphical abstract was created with the help of Biorender. Open Access funding enabled and organized by Projekt DEAL.

FUNDING INFORMATION

This work was supported by the Katharina Hardt foundation and Löwenstern e.V. Rabea Wagener acknowledges the financial support of the Forschungskommission of the Medical Faculty of the Heinrich Heine University Düsseldorf (2020-28). Ute Fischer (DJCLS 18R/2021 and DJCLS 21 R/2019) and Arndt Borkhardt (DJCLS 07 R/2019) were funded by the German José Carreras leukemia foundation. Ute Fischer has been funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation—Projektnummer 495318549) and the German Cancer Aid (Priority Programme ‘Cancer Prevention—Graduate School [CPGS]’).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of the study are available from the corresponding author upon request.

ETHICS STATEMENT

The study was approved by the ethics committee of Heinrich Heine University, Düsseldorf, Germany (ethics vote number 4886 R and study registration number 2014112933).

ORCID

Rabea Wagener  <https://orcid.org/0000-0002-5394-0907>

Arndt Borkhardt  <https://orcid.org/0000-0002-6121-4737>

TWITTER

Arndt Borkhardt  [Borkhardt_Lab](https://twitter.com/Borkhardt_Lab)

REFERENCES

1. Zhang J, Walsh MF, Wu G, et al. Germline mutations in predisposition genes in pediatric cancer. *N Engl J Med*. 2015;373(24):2336–2346. doi:[10.1056/NEJMoa1508054](https://doi.org/10.1056/NEJMoa1508054)
2. Diets IJ, Waanders E, Ligtenberg MJ, et al. High yield of pathogenic germline mutations causative or likely causative of the cancer phenotype in selected children with cancer. *Clin Cancer Res*. 2018;24(7):1594–1603. doi:[10.1158/1078-0432.Ccr-17-1725](https://doi.org/10.1158/1078-0432.Ccr-17-1725)
3. Gröbner SN, Worst BC, Weischenfeldt J, et al. The landscape of genomic alterations across childhood cancers. *Nature*. 2018;555(7696):321–327. doi:[10.1038/nature25480](https://doi.org/10.1038/nature25480)
4. Wagener R, Taeubner J, Walter C, et al. Comprehensive germline-genomic and clinical profiling in 160 unselected children and adolescents with cancer. *Eur J Hum Genet*. 2021;29(8):1301–1311.
5. Wong M, Mayoh C, Lau LMS, et al. Whole genome, transcriptome and methylome profiling enhances actionable target discovery in high-risk pediatric cancer. *Nat Med*. 2020;26(11):1742–1753. doi:[10.1038/s41591-020-1072-4](https://doi.org/10.1038/s41591-020-1072-4)
6. Byrjalsen A, Hansen TVO, Stoltze UK, et al. Nationwide germline whole genome sequencing of 198 consecutive pediatric cancer patients reveals a high incidence of cancer prone syndromes. *PLoS Genet*. 2020;16(12):e1009231. doi:[10.1371/journal.pgen.1009231](https://doi.org/10.1371/journal.pgen.1009231)
7. Eichler EE. Genetic variation, comparative genomics, and the diagnosis of disease. *N Engl J Med*. 2019;381(1):64–74. doi:[10.1056/NEJMr1809315](https://doi.org/10.1056/NEJMr1809315)
8. Whitworth J, Smith PS, Martin JE, et al. Comprehensive cancer-predisposition gene testing in an adult multiple primary tumor series shows a broad range of deleterious variants and atypical tumor phenotypes. *Am J Hum Genet*. 2018;103(1):3–18. doi:[10.1016/j.ajhg.2018.04.013](https://doi.org/10.1016/j.ajhg.2018.04.013)
9. Mahmoud M, Gobet N, Cruz-Dávalos DI, Mounier N, Dessimoz C, Sedlazeck FJ. Structural variant calling: the long and the short of it. *Genome Biol*. 2019;20(1):246. doi:[10.1186/s13059-019-1828-7](https://doi.org/10.1186/s13059-019-1828-7)
10. Dremsek P, Schwarz T, Weil B, Malashka A, Laccone F, Neesen J. Optical genome mapping in routine human genetic diagnostics—its advantages and limitations. *Genes*. 2021;12(12):1958. doi:[10.3390/genes12121958](https://doi.org/10.3390/genes12121958)
11. Mantere T, Neveling K, Pebrel-Richard C, et al. Optical genome mapping enables constitutional chromosomal aberration detection. *Am J Hum Genet*. 2021;108(8):1409–1422. doi:[10.1016/j.ajhg.2021.05.012](https://doi.org/10.1016/j.ajhg.2021.05.012)
12. Brozou T, Taeubner J, Velleuer E, et al. Genetic predisposition in children with cancer—affected families' acceptance of Trio-WES. *Eur J Pediatr*. 2018;177(1):53–60.
13. Jongmans MC, Loeffen JL, Waanders E, et al. Recognition of genetic predisposition in pediatric cancer patients: an easy-to-use selection tool. *Eur J Med Genet*. 2016;59(3):116–125. doi:[10.1016/j.ejmg.2016.01.008](https://doi.org/10.1016/j.ejmg.2016.01.008)
14. Ripperger T, Bielack SS, Borkhardt A, et al. Childhood cancer predisposition syndromes—a concise review and recommendations by the

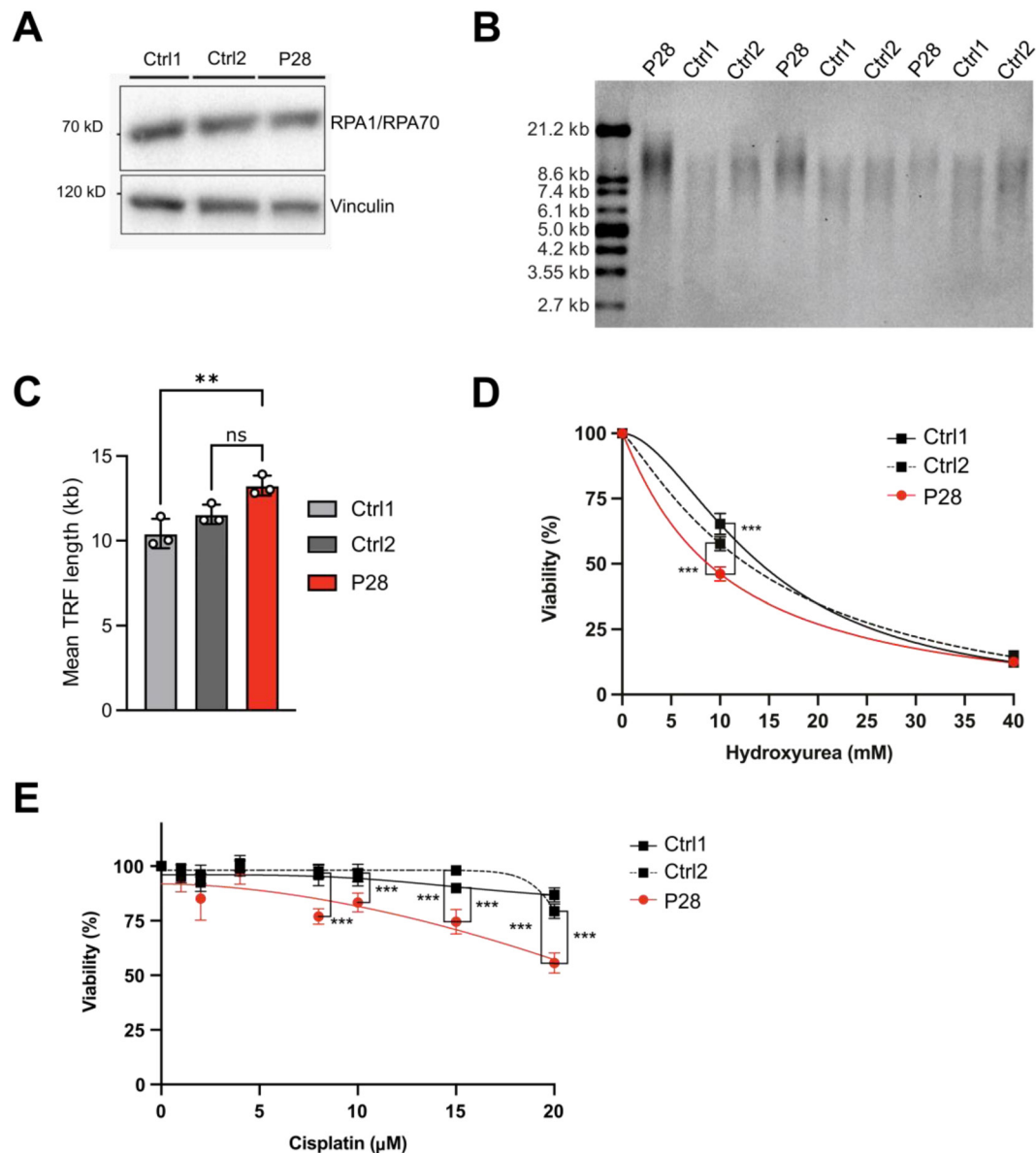
- cancer predisposition working Group of the Society for pediatric oncology and hematology. *Am J Med Genet A*. 2017;173(4):1017-1037.
15. Lappalainen I, Lopez J, Skipper L, et al. DbVar and DGVA: public archives for genomic structural variation. *Nucleic Acids Res*. 2013;41-(Database issue):D936-D941. doi:[10.1093/nar/gks1213](https://doi.org/10.1093/nar/gks1213)
 16. Fishilevich S, Nudel R, Rappaport N, et al. GeneHancer: genome-wide integration of enhancers and target genes in GeneCards. *Database*. 2017;2017:bax028. doi:[10.1093/database/bax028](https://doi.org/10.1093/database/bax028)
 17. Kloosterman WP, Francioli LC, Hormozdiari F, et al. Characteristics of de novo structural changes in the human genome. *Genome Res*. 2015; 25(6):792-801. doi:[10.1101/gr.185041.114](https://doi.org/10.1101/gr.185041.114)
 18. Muller D, Rouleau E, Schultz I, et al. An entire exon 3 germ-line rearrangement in the BRCA2 gene: pathogenic relevance of exon 3 deletion in breast cancer predisposition. *BMC Med Genet*. 2011;12:121. doi:[10.1186/1471-2350-12-121](https://doi.org/10.1186/1471-2350-12-121)
 19. Caputo SM, Léone M, Damiola F, et al. Full in-frame exon 3 skipping of BRCA2 confers high risk of breast and/or ovarian cancer. *Oncotarget*. 2018;9(25):17334-17348. doi:[10.18632/oncotarget.24671](https://doi.org/10.18632/oncotarget.24671)
 20. Xia B, Sheng Q, Nakanishi K, et al. Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. *Mol Cell*. 2006;22(6): 719-729. doi:[10.1016/j.molcel.2006.05.022](https://doi.org/10.1016/j.molcel.2006.05.022)
 21. Ji T, Zhang X, Li W. MicroRNA-543 inhibits proliferation, invasion and induces apoptosis of glioblastoma cells by directly targeting ADAM9. *Mol Med Rep*. 2017;16(5):6419-6427. doi:[10.3892/mmr.2017.7332](https://doi.org/10.3892/mmr.2017.7332)
 22. Fan X, Wang Y, Zhang C, et al. ADAM9 expression is associate with glioma tumor grade and histological type, and acts as a prognostic factor in lower-grade gliomas. *Int J Mol Sci*. 2016;17(9):1276. doi:[10.3390/ijms17091276](https://doi.org/10.3390/ijms17091276)
 23. Altschuler DL, Ribeiro-Neto F. Mitogenic and oncogenic properties of the small G protein Rap1b. *Proc Natl Acad Sci U S A*. 1998;95(13): 7475-7479. doi:[10.1073/pnas.95.13.7475](https://doi.org/10.1073/pnas.95.13.7475)
 24. Lou L, Urbani J, Ribeiro-Neto F, Altschuler DL. cAMP inhibition of Akt is mediated by activated and phosphorylated Rap1b. *J Biol Chem*. 2002;277(36):32799-32806. doi:[10.1074/jbc.M201491200](https://doi.org/10.1074/jbc.M201491200)
 25. Niemann JH, Du C, Morlot S, et al. De novo missense variants in the RAP1B gene identified in two patients with syndromic thrombocytopenia. *Clin Genet*. 2020;98(4):374-378. doi:[10.1111/cge.13807](https://doi.org/10.1111/cge.13807)
 26. Miller D, Saeed A, Nelson AC, Bower M, Aggarwal A. Third reported patient with RAP1B-related syndromic thrombocytopenia and novel clinical findings. *Am J Med Genet A*. 2022;188(9):2808-2814. doi:[10.1002/ajmg.a.62760](https://doi.org/10.1002/ajmg.a.62760)
 27. Wold MS. Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. *Annu Rev Biochem*. 1997;66:61-92. doi:[10.1146/annurev.biochem.66.1.61](https://doi.org/10.1146/annurev.biochem.66.1.61)
 28. Sharma R, Sahoo SS, Honda M, et al. Gain-of-function mutations in RPA1 cause a syndrome with short telomeres and somatic genetic rescue. *Blood*. 2022;139(7):1039-1051. doi:[10.1182/blood.2021011980](https://doi.org/10.1182/blood.2021011980)
 29. Outwin E, Carpenter G, Bi W, Withers MA, Lupski JR, O'Driscoll M. Increased RPA1 gene dosage affects genomic stability potentially contributing to 17p13.3 duplication syndrome. *PLoS Genet*. 2011;7(8): e1002247. doi:[10.1371/journal.pgen.1002247](https://doi.org/10.1371/journal.pgen.1002247)

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Wagener R, Brandes D, Jung M, et al. Optical genome mapping identifies structural variants in potentially new cancer predisposition candidate genes in pediatric cancer patients. *Int J Cancer*. 2024;154(4):607-614. doi:[10.1002/ijc.34721](https://doi.org/10.1002/ijc.34721)

2.2.3 Relevant supplementary figure (II-Figure S5)



Supplementary Figure 5: Functional validation of cells of patient P28 with focal duplication of 17p13.3, including *RPA1* gene locus. Cells show comparable RPA1 protein expression, a mild increase of telomere length and higher sensitivity to DNA damage and replication stress, compared to age/gender-matched controls. **A)** Immunoblot detecting RPA1 protein expression in dermal fibroblasts of P28 and age/gender-matched fibroblast controls (ctrl1 and ctrl2) from children without RPA1 alterations. **B)** TeloTAGGG assay: Southern blot showing telomere fragment lengths of dermal fibroblasts of P28 and age/gender-matched fibroblast controls (ctrl1 and ctrl2) from children without RPA1 alterations. Telomere length is calibrated against a DIG-labelled molecular weight marker. Biological triplicates are shown. **C)** Quantification of the representative TeloTAGGG Southern blot. Chemiluminescence signal peaks of the telomere restriction fragments (TRF) were defined as mean TRF length. Biological triplicates were analyzed. p-values were calculated using One-Way-ANOVA. Ns $p > 0.05$ and $**p < 0.01$. **D)** Cell-Titer-Glo viability assay: Replication stress was induced through nucleotide depletion by hydroxyurea treatment. A representative of two biological replicates is shown. Every biological replicate contained four technical replicates. **E)** Cell-Titer-Glo viability assay: DNA damage was induced by cisplatin treatment. One of two biological replicates is shown. Every biological replicate contained four technical replicates. p-values were calculated using Two-Way-ANOVA with multiple comparisons. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

2.3 Manuscript III: UBQLN1-mediated Repression of Homologous Recombination is an Actionable Target for Cancer Therapy

Manuscript under review at *Molecular Cell*

SSRN preprint: <http://dx.doi.org/10.2139/ssrn.6502243>

Maxim A. Huetzen*, Orsolya Leidecker*, Ann-Sophie Hopff*, Pavlína Višková Gajarská*, Maria-Teodora Pandrea, Rosa Camarillo, Maria del Carmen Dominguez-Perez, Jonas Goergens, Claus M. Graef, Paul J. Bröckelmann, Alana S. T. Stuedle, Jessica Wirtz, Emile van Weert, Anne Kruessmann, Neha Patwardhan, Leonie Müller, Saindhabi Ghosh, Moritz Reese, Simon E. Tröder, Lucia Torres Fernandez, Haylie Murray, Kelly K. Chong, Sebastian Klein, Jérôme Basquin, Filippo Beleggia, Andrea Mesaros, F. Thomas Wunderlich, Stephanie Panier, Alexander Quaas, Yosef Shiloh, Dave S. B. Hoon, Tim Hucho, Johannes Brägelmann, Martin Peifer, Hatip Aydin, Davut Pehlivan, James R. Lupski, Reinhard Büttner, Melanie Thelen, Pablo Huertas, Tobias Marschall, Arndt Borkhardt, Maria Cartolano, Thorsten Hoppe, Matias A. Bustos, Jörg Isensee, Dagmar Wiczorek, H. Christian Reinhardt[#] and Ron D. Jachimowicz[#]

2.3.1 Contributions

As listed first shared-first author, I led core aspects of this study, substantially contributing to study design, experimental execution, data analysis, and manuscript preparation.

I conducted high-content imaging (HCI) microscopy of syndrome mouse model-derived murine embryonic fibroblasts for cell viability and γ H2AX foci, and isogenic cell lines for RAD51 and MRE11 foci quantification, with automated imaging by J.I. (**III-Figure 1I-L, 2B, 3A, 3E, 4C, 4E, S2A-B, S3C, S4F-G, S5C-D, S6A-B**), demonstrating that *UBQLN1* loss impairs RAD51 foci maintenance, while *UBQLN1* overexpression suppresses both RAD51 and MRE11 foci formation. I performed Sanger sequencing to validate the *UBQLN1* variant in syndrome patients and the corresponding mouse model (**III-Figure 1B, 1H**), WES of patient fibroblasts (**III-Figure S1C** with S.K.), and RNA-seq expression analysis of patient fibroblasts together with F.B. (**III-Figure S1D**).

I established, managed, validated, and phenotypically characterized the *Ubpq1n1^{c.377delC}* (**III-Figure 1H-L, S3A-D**), the *Rosa26^{LSL.HA-Ubpq1n1};Actb^{Cre/wt}* (**III-Figure 5E-H, S7M**), the *Kras^{LSL.G12D/wt};Trp53^{fl/fl}* (KP), and the *Rosa26^{LSL.HA-Ubpq1n1};Kras^{LSL.G12D/wt};Trp53^{fl/fl}* (KPU1) mouse strains (**III-Figure 6, 7, S8A-Q, S9A, S9E**). The KP strain was established together with J.G. These experiments established that the syndrome mouse model recapitulates human *UBQLN1* deficiency including seizure susceptibility, and that constitutive *Ubpq1n1* overexpression promotes spontaneous lung adenocarcinoma and accelerates tumor progression in autochthonous murine NSCLC. I developed the 3D computed tomography analysis workflow for tumor burden quantification, with image acquisition by A.S.T.S., J.W., J.G., and me, and analysis performed with A.S.T.S. and A.M. (**III-Figure 6C-D, 7B, 7D, S9A, S9E**).

2. Results – Manuscript III

I planned and executed *in vivo* treatment experiments with olaparib and anti-PD1, with injections performed together with J.G. and the Comparative Biology core facility (**III-Figure 7A-D**), demonstrating enhanced sensitivity of *UBQLN1*-overexpressing tumors to PARP inhibition and immune checkpoint blockade. I performed histopathological analysis of mouse tumor models (**III-Figure 5H, 6B, S7M**) and colony survival assays in human lung cancer cell lines (**III-Figure S9B-D**), showing that high *UBQLN1* expression correlates with olaparib sensitivity *in vitro*.

I generated samples and designed the experimental framework for KP/KPU1 genomics and transcriptomics, with bioinformatic analysis by M.C., L.T.F., and C.M.G. (**III-Figure 6E-H, S8E-Q**), revealing increased tumor mutational burden, elevated Indel frequency, and genomic scars consistent with homologous recombination deficiency. I coordinated the TCGA data analysis collaboration with K.K.C. and M.A.B., providing scientific input on analysis design (**III-Figure 5A-D, S7A-L**), which established the clinical association between high *UBQLN1* expression and reduced overall survival.

I contributed to Western blot validation in syndrome patient cells, performed by O.L. (**III-Figure 1C**) and HCI microscopy of patient-derived fibroblasts with O.L. (**III-Figure 1D-G, S1E-F, S2**), demonstrating hypersensitivity to genotoxic agents and persistent DNA damage. I contributed to establishment of microirradiation and laser line recruitment assays with O.L., P.V.G., and E.v.W. (**III-Figure 2A, 2C**). Further, I acquired DR-GFP reporter experiments with P.V.G. and J.G. (**III-Figures 2D, 4A, S4H**), confirming that both *UBQLN1* loss and overexpression impair HR efficiency. Following up on my HCI RAD51 foci data, I performed cycloheximide chase experiments together with A.S.H. and E.v.W. (**III-Figure 3B-D, S5B**), demonstrating accelerated turnover of chromatin-bound RAD51, which was reversed by proteasome inhibition. Additionally, I contributed to the experimental design for recombinant protein work by M.-T.P. (**III-Figure 3H, S5F**), revealing that *UBQLN1* preferentially binds to non-ubiquitinated RAD51.

Further, I contributed to experimental planning of Turbo-ID proximity labeling by J.G. (**III-Figure 4B**), immunoprecipitation experiments by P.V.G., A.S.H., and H.M. (**III-Figure 3F, 3G, 3I, 3J, 4D, S5E**), and the cell viability rescue experiment by O.L. (**III-Figure S4A-D**). Of note, earlier versions of III-Figure 1A,B,C,H and III-Figure S1D were included in my master's thesis and have been reformatted and adapted for the present manuscript.

Beyond experimental work, I significantly contributed to intellectual development of this work, conceptualizing the RAD51-shielding hypothesis and coordinating experiments.

2. Results – Manuscript III

Further, I organized collaborative efforts across international research groups in the USA, Israel, Turkey, Germany, and Spain. I curated and analyzed final manuscript data, optimized experimental and analytical methods throughout the project, and finalized all figures for publication. I contributed to writing the original manuscript, prepared supplementary materials and methods, and integrated feedback from all co-authors during internal revision.

This work established UBQLN1 as a critical regulator of HR through a protein shielding mechanism, linking *UBQLN1* deficiency syndrome to impaired RAD51 stabilization while identifying *UBQLN1* overexpression as a therapeutically exploitable vulnerability in lung cancer. The comprehensive characterization from clinical phenotype through molecular mechanism to preclinical therapeutic validation demonstrates the translational potential of syndrome discovery.

2.3.2 Manuscript status

The manuscript was submitted to *Molecular Cell (Cell Press)* on January 6, 2026, and has been under review since March 31, 2026.

It is available as a preprint: <http://dx.doi.org/10.2139/ssrn.6502243>

2.3.3 Manuscript (under review)

The manuscript presented here was adapted for inclusion in this dissertation. The syndrome discovery figure and description were moved to the beginning of the results section, whereas it is placed at the end of the submitted manuscript.

Supplementary figures (III-Figure S1-9) and methods are included in **section 12.1.3**.

UBQLN1-Mediated Repression of Homologous Recombination is an Actionable Target for Cancer Therapy

Maxim A. Huetzen^{1,2,3,4,*}, Orsolya Leidecker^{1,2,3,4,5,*}, Ann-Sophie Hopff^{1,2,3,4,*}, Pavlína Víšková Gajarská^{1,2,3,4,*}, Maria-Teodora Pandrea^{3,4,5}, Rosa Camarillo⁶, Maria del Carmen Dominguez-Perez⁶, Jonas Goergens^{1,2,3,4}, Claus M. Graef^{1,2,3,4}, Paul J. Bröckelmann^{1,2,3,4}, Alana S. T. Stüdle^{1,2,3,4}, Jessica Wirtz^{1,2,3,4}, Emile van Weert^{1,2,3,4}, Anne Kruessmann^{1,2,3,4}, Neha Patwardhan^{1,2,3,4}, Leonie Müller^{3,4,5}, Saindhabi Ghosh^{1,2,3,4}, Moritz Reese^{1,2,3,4}, Simon E. Tröder^{3,7}, Lucia Torres Fernandez⁴, Haylie Murray^{1,2,3,4}, Kelly K. Chong⁸, Sebastian Klein⁹, Jérôme Basquin¹⁰, Filippo Beleggia^{2,11}, Andrea Mesaros¹, F. Thomas Wunderlich¹², Stephanie Panier^{1,3}, Alexander Quaas¹³, Yosef Shiloh¹⁴, Dave S. B. Hoon⁸, Tim Hucho¹⁵, Johannes Brägelmann^{4,11}, Martin Peifer^{4,11}, Hatip Aydin¹⁶, Davut Pehlivan¹⁷, James R. Lupski¹⁸, Reinhard Büttner¹³, Melanie Thelen^{1,2,3,4}, Pablo Huertas⁶, Tobias Marschall¹⁹, Arndt Borkhardt²⁰, Maria Cartolano⁴, Thorsten Hoppe^{3,4,5}, Matias A. Bustos⁸, Jörg Isensee¹⁵, Dagmar Wiczorek²¹, H. Christian Reinhardt^{9,§} and Ron D. Jachimowicz^{1,2,3,4,§,#}

¹Max Planck Institute for Biology of Ageing, Cologne, Germany;

²Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf (CIO ABCD), University of Cologne, Germany;

³Cologne Excellence Cluster on Cellular Stress Response in Aging-Associated Diseases, University of Cologne, Germany;

⁴Center for Molecular Medicine Cologne, University of Cologne, Germany;

⁵Institute for Genetics, University of Cologne, Germany;

⁶Centro Andaluz de Biología Molecular y Medicina Regenerativa-CABIMER, University of Sevilla, Spain;

⁷*in vivo* Research Facility, Faculty of Medicine and University Hospital Cologne, University of Cologne, Germany;

⁸Department of Translational Molecular Medicine, Saint John's Cancer Institute (SJCI), Providence Saint John's Health Center (SJHC), Santa Monica, California, USA;

⁹University Hospital Essen, Dept. of Hematology and Stem Cell Transplantation, West German Cancer Center, Essen, Germany;

¹⁰Max Planck Institute for Biochemistry, Martinsried, Germany;

¹¹Department of Translational Genomics, Faculty of Medicine and University Hospital Cologne, University of Cologne, Germany;

¹²Max Planck Institute for Metabolism Research, Germany;

¹³Institute of Pathology, University Hospital Cologne, Cologne, Germany; Medical Faculty, University of Cologne, Germany;

¹⁴David and Inez Myers Laboratory for Cancer Genetics, School of Medicine, Tel Aviv University, Tel Aviv, Israel;

¹⁵Department of Anesthesiology and Intensive Care Medicine, Translational Pain Research, Faculty of Medicine and University Hospital of Cologne, University of Cologne, Germany;

¹⁶Zeynep Kamil Children's and Maternity Hospital, Istanbul, Turkey;

¹⁷Section of Pediatric Neurology and Developmental Neuroscience, Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA;

¹⁸Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA;

¹⁹Institute of Medical Statistics, Heinrich-Heine-University, Düsseldorf, Germany;

²⁰Department of Paediatric Oncology, Haematology and Immunology, Heinrich-Heine-University, Düsseldorf, Germany;

²¹Institute of Human Genetics, Medical Faculty and University Hospital Düsseldorf, Heinrich-Heine University, Düsseldorf, Germany

*These authors contributed equally; §These authors contributed equally; #correspondence: rjachimowicz@age.mpg.de

Abstract

Maintaining DNA damage response (DDR) protein homeostasis is vital for genome integrity. This requires coordinated crosstalk between repair factors, such as RAD51 and MRE11, and the ubiquitin-proteasome system. Here, we identified a new node in the DDR network – ubiquilin-1 (UBQLN1), a member of the ubiquilin protein family that act as shuttles to transport ubiquitinated substrates to the proteasome. We observed that the loss of *UBQLN1* exposes RAD51 to ubiquitination and degradation, thus functionally repressing homologous recombination (HR). Mechanistically, we showed that UBQLN1 exhibits a non-canonical function whereby it stabilizes RAD51 localized to the sites of DNA damage by preventing its ubiquitination. Intriguingly, overexpression of *UBQLN1* repressed HR-mediated DSB repair by engaging ubiquitinated MRE11, shifting repair towards mutagenic single-strand annealing. We also noted frequent overexpression of *UBQLN1* in human tumors, and its association with an aggressive phenotype. Using a *Ubqln1* overexpression allele, we demonstrated an oncogenic role of *Ubqln1* in lung adenocarcinoma resulting in increased tumor mutational burden, *in vivo*. Lastly, we showed that *Ubqln1* overexpression causes lung adenocarcinomas to sensitize to PARP1 inhibition and immune-checkpoint blockade *in vivo*. We also identified human patients with a deleterious mutation in *UBQLN1* that results in a phenotype consistent with the role of UBQLN1 in HR. Taken together, our study of UBQLN1 reveals that this ubiquilin constitutes an important node in the DDR network, in part by inducing ubiquitin-independent RAD51 stabilization. Furthermore, *UBQLN1* overexpression can serve as a predictor of clinical sensitivity to PARP1 inhibition and immune-checkpoint blockade, which has important implications for cancer patient treatment.

Introduction

DNA damage repair, which ensures genomic stability and integrity, and proteasome-mediated protein degradation, which regulates protein homeostasis, are highly integrated and connected cellular processes^{1,2}. Their accurate coordination is essential for maintaining healthy cellular physiology, and dysregulation in one often affects the other, causing genomic instability and disease (e.g. cancer). The intersection between DNA damage repair and the ubiquitin-proteasome system (UPS)³ is primarily mediated via ubiquitination of proteins involved in DNA repair. In general, the DNA damage response (DDR) is triggered by the recruitment of the MRN (MRE11, RAD50, and NBS1) complex to the sites of DNA double-strand breaks (DSBs)⁴. The MRN complex then brings additional repair factors (e.g. BRCA1) to the site of damage and initiates DSB repair via either canonical non-homologous end-joining (c-NHEJ) or homologous recombination (HR)⁵. Importantly, in addition to an orchestrated

recruitment of DDR factors to DSBs, DDR execution and repair pathway choice also depends on ubiquitination and regulated turnover of repair factors mediated by the UPS³.

Although typically viewed through the lens of ubiquitination (involving the E1-E2-E3 enzyme cascade that catalyzes ubiquitination of substrate proteins), and the proteasome (a large macromolecular assembly that catalyzes proteolysis of ubiquitinated substrates), the UPS is significantly more complex and involves numerous other proteins and points of regulation. One such example are ubiquilins (UBQLNs), a small family of five proteins (UBQLN1 through 4, and UBQLNL) that shuttle ubiquitinated substrates to the proteasome, thus facilitating degradation⁶. UBQLNs share high levels of sequence similarity and a general domain organization involving: (1) two domains that connect these proteins to the UPS, the N-terminal Ubiquitin (Ub)-like domain that binds to the proteasome, and the C-terminal Ub-associated (UBA) domain that binds to ubiquitin;

and (2) two low complexity STI1-like regions in the middle that facilitate interactions with a wide range of binding partners⁶. As mentioned, UBQLNs are involved in the UPS and other protein quality control processes (e.g. ER-associated protein degradation), and loss of UBQLNs results in accumulation of misfolded proteins, protein mislocalization and proteotoxicity⁷⁻¹⁰. In addition, UBQLNs are starting to be recognized as important modulators of cancer^{11,12}, although their roles in tumorigenesis and cancer progression remain mostly unknown. In this context, we previously showed that UBQLN4 plays an important role in the cellular capacity to respond to genotoxic stress, and that elevated *UBQLN4* expression correlates with poor overall survival (OS) in a wide range of cancer entities¹³. Mechanistically, we showed that *UBQLN4* overexpression represses HR and enhances NHEJ pathway use by functionally crippling HR through enhanced MRE11 degradation¹³. These discoveries were the first indication that UBQLNs serve to connect DSB repair pathways with the UPS. Furthermore, they indicate the possibility that cancers upregulate expression of UBQLNs as a way of minimizing effects of genotoxic agents by upregulating mutagenic, yet efficient NHEJ pathway usage. However, whether ubiquilins beyond UBQLN4 play a role in these processes remains unknown.

To explore potential roles of other ubiquilins in DDR, we focused on UBQLN1, a primary ubiquilin that is involved in a wide range of cellular processes, including autophagy¹⁴, mitochondrial protein clearance^{8,15}, and amyloid precursor protein processing in Alzheimer's disease^{7,16,17}. Despite being homologous to UBQLN4, the role of UBQLN1 in regulating the DDR has not been reported before. We now identify a homozygous *UBQLN1* p.Asn126MetfsTer24 germline mutation that leads to loss of UBQLN1 and is causative for a novel genome instability syndrome which we term *UBQLN1* deficiency syndrome. Affected patients shared features that resemble the UBQLN4 deficiency syndrome and other genome instability

syndromes¹³. We discovered that loss of *UBQLN1* exposes RAD51 to ubiquitination and subsequent proteasomal degradation, thus impairing HR-mediated DSB repair. We demonstrated that UBQLN1 binds to RAD51 and protects this protein from ubiquitination, which represents a previously unrecognized function for a ubiquilin in the DDR. We also observed that, when overexpressed, UBQLN1 interacts with MRE11 to repress HR, which drives genomic instability and promotes cancer progression. Mechanistically, our data indicate that *UBQLN1* overexpression shifts repair towards mutagenic alternative repair pathways that require longer resection, such as single-strand annealing (SSA). We found that *UBQLN1* overexpression is associated with poor prognosis in various cancer entities. More specifically, we showed that *UBQLN1* functions as an oncogene in lung adenocarcinoma (LUAD). Furthermore, we demonstrated that *UBQLN1* overexpression is associated with increased sensitivity to PARP1 inhibitors and immune-checkpoint blockade therapy.

Results

A deleterious UBQLN1 mutation is causative for a novel genome instability syndrome

Through the outpatient genetics department of the Zeynep Kamil Hospital in Turkey, we consulted a consanguineous family with three children, of which two, a female and a male (A-II-1, A-II-4), displayed phenotypic features of known genome instability syndromes, such as microcephaly, intellectual disability, and facial dysmorphisms (**Fig. 1A, Suppl. Fig. 1A,B**). A detailed clinical description of the patients is provided in **Suppl. Figure 1B** and the human subjects section within the supplementary methods. Whole exome sequencing of both affected individuals revealed a deleterious homozygous c.377delA mutation of the *UBQLN1* gene creating a premature stop codon c.377delA (p.Asn126MetfsTer24).

2. Results – Manuscript III

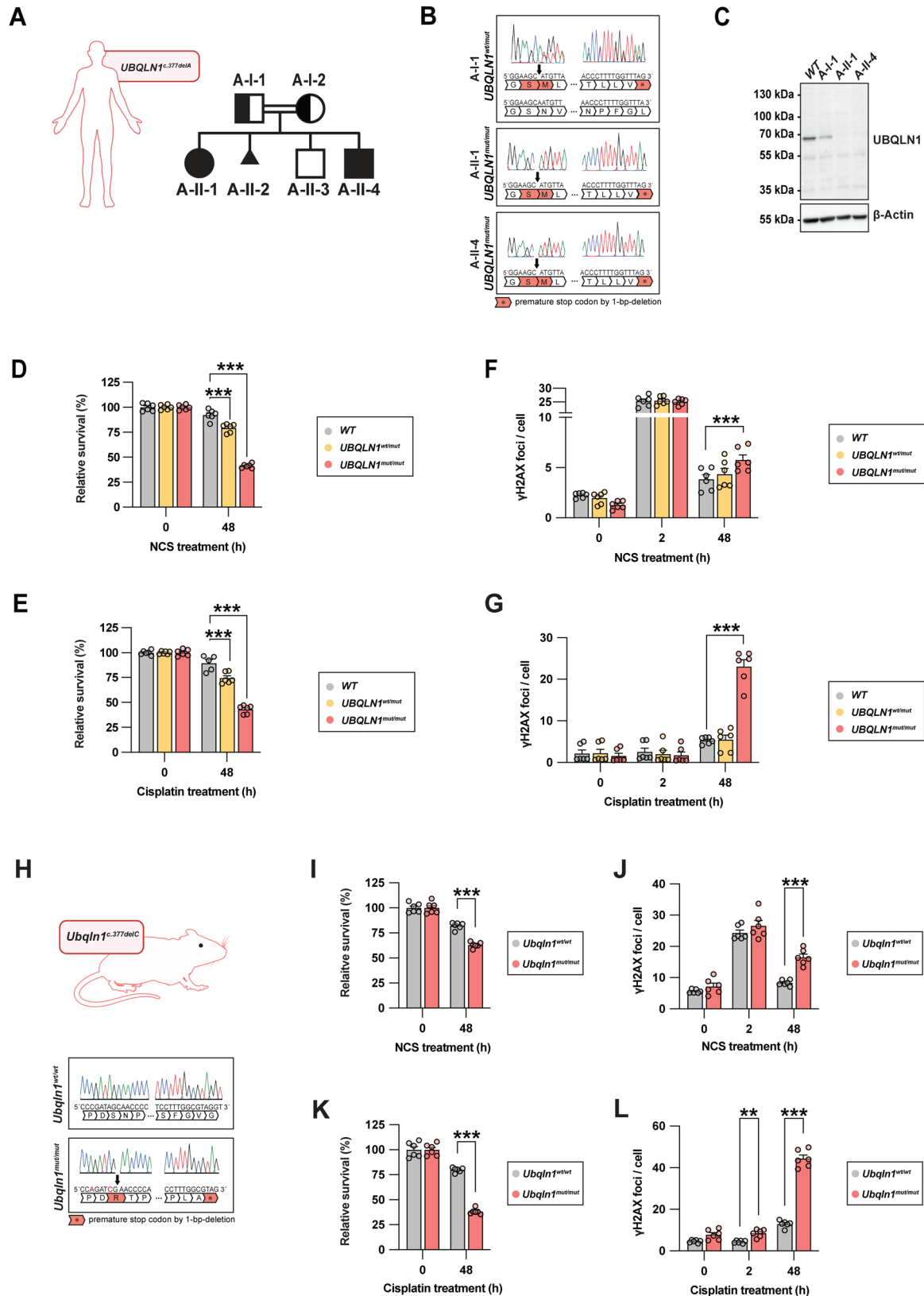


Figure 1. A deleterious *UBQLN1* mutation is causative for a novel genome instability syndrome. **A)** Pedigree of consanguineous family with autosomal recessive *UBQLN1*-deficiency syndrome. **B)** Sanger sequencing of fibroblast gDNA from A-II-1, A-II-4, and a heterozygous parent (A-I-1). The arrow indicates the *c.377delA* frameshift mutation. **C)** Immunoblot detecting *UBQLN1* in the human dermal fibroblasts (HDFs) of *UBQLN1* deficiency syndrome patients and heterozygous carrier. **D)** Cell viability of HDFs treated with NCS (500 ng/ml) for 48h. **E)** Cell viability of HDFs treated with cisplatin (20 μM) for 48h. **F)** Nuclear γH2AX foci in HDFs treated with NCS (500 ng/ml) at 2 and 48h. **G)** Nuclear γH2AX foci in HDFs treated with cisplatin (20 μM) at 2 and 48h. →

2. Results – Manuscript III

H) Sanger sequencing of gDNA from wildtype and *Ubqln1*^{c.377delC} MEFs. The arrow indicates the c.377delC frameshift mutation. **I)** Cell viability of MEFs treated with NCS (500 ng/ml) for 48h. **J)** Nuclear γH2AX foci in MEFs treated with NCS (500 ng/ml) at 2 and 48h. **K)** Cell viability of MEFs treated with cisplatin (20 μM) for 48h. **L)** Nuclear γH2AX foci in MEFs treated with cisplatin (20 μM). **Statistics:** Error bars represent SEM of 6 biological replicates. Two-way ANOVA (D, E, I, K) or two-way ANOVA with Bonferroni post hoc test (F, G, J, L). Each datapoint represents the mean of 3000 analyzed nuclei. *p < 0.05, **p < 0.01, ***p < 0.001.

The unaffected parents were heterozygous carriers, thus pinpointing this deleterious germline *UBQLN1* mutation as the likely cause of an autosomal recessive condition, which we refer to as the *UBQLN1* deficiency syndrome. The pathogenic c.377delA *UBQLN1* mutation was also confirmed by dideoxynucleotide sequencing (**Fig. 1B**). It is important to note that the *UBQLN1* c.377delA mutation was the only shared homozygous aberration between both affected individuals born to related parents predicted to be truncating (**Suppl. Fig. 1C**). Analysis of human dermal fibroblasts (HDFs) of the two patients (*A-II-1*, *A-II-4*) demonstrated loss of *UBQLN1* expression and reduced mRNA levels, compared to the heterozygous father (*A-I-1*) (**Fig. 1C**) and an unaffected healthy brother (*A-II-3*) (**Suppl. Fig. 1D**), which suggests that nonsense-mediated decay may contribute to the reduced *UBQLN1* protein levels. We next assessed the sensitivity of HDFs obtained from *UBQLN1*^{mut/mut} patients towards a series of genotoxic agents with distinct molecular mechanisms of action. Compared to *WT* and *UBQLN1*^{mut/wt} cells, *UBQLN1*^{mut/mut} cells displayed a significantly increased sensitivity towards a radiomimetic drug neocarzinostatin (NCS), an interstrand DNA crosslinking agent cisplatin, and a topoisomerase I inhibitor camptothecin (**Fig. 1D,E**, **Suppl. Fig. 1E**). To further substantiate this observation, we performed immunofluorescence experiments staining for the DSB marker γH2AX in cell cultures either exposed to NCS, cisplatin, or left untreated as control. These experiments demonstrated significantly delayed clearance of γH2AX foci in *UBQLN1*^{mut/mut} HDFs following NCS and cisplatin treatment throughout the 48h time course (**Fig. 1F,G**, **Suppl. Fig. 2A,B**). Similarly, *UBQLN1*^{mut/mut} cells displayed significantly increased accumulation of γH2AX foci following camptothecin exposure (**Suppl. Fig. 1F**). To unequivocally determine the pathogenic role of

the c.377delA frameshift mutation in the *UBQLN1* deficiency syndrome, we deployed CRISPR/Cas9 genome editing to generate a mouse model featuring the corresponding c.377delC frameshift mutation in the germline. We confirmed the homozygous presence of the *Ubqln1*^{c.377delC} mutation in murine embryonic fibroblasts (MEFs) by sequencing (**Fig. 1H**).

Of note, *Ubqln1*^{c.377delC/c.377delC} mice were born significantly below the expected Mendelian ratio (**Suppl. Fig. 3A**). In line with the data in human cells, *UBQLN1* protein levels were completely abolished in *Ubqln1*^{c.377delC/c.377delC} MEFs (**Suppl. Fig. 3B**). Exactly as demonstrated for the human HDFs, *Ubqln1*^{c.377delC/c.377delC} MEFs displayed significantly increased sensitivity to NCS and cisplatin, as well as altered γH2AX kinetics compared to *Ubqln1*^{wt/wt} controls (**Fig. 1I-L**, **Suppl. Fig. 3C**). Strikingly, and fully in line with the observation in human patients, we observed an increased occurrence of seizures specifically in *Ubqln1*^{c.377delC/c.377delC} mice (**Suppl. Fig. 3D**). Altogether, we documented a new genome instability syndrome caused by a deleterious homozygous *UBQLN1* mutation termed *UBQLN1* deficiency syndrome.

UBQLN1* increases homologous recombination via *RAD51

To establish a potential role within the DDR network, we initially assessed the recruitment of *UBQLN1* to sites of genotoxic damage. We observed dynamic recruitment of GFP-*UBQLN1* to laser-induced DNA damage in U2OS cells (**Fig. 2A**). To further probe the mechanistic role of *UBQLN1* within the DDR network, we performed high-content imaging (HCI) in *UBQLN1*-deficient cells using *UBQLN1* knockout (KO) U2OS cells. These cells demonstrated delayed γH2AX clearance after exposure to the DNA damaging agent neocarzinostatin (NCS), compared to *UBQLN1*-proficient controls, indicating decreased

capacity to repair damaged DNA (Suppl. Fig. 4A,B). Consistent with a role of UBQLN1 in DNA repair, *UBQLN1* KO cells exhibited a significantly increased sensitivity to NCS in cell viability assays, compared to control cells, which could be rescued upon re-expression of ectopic UBQLN1 (Suppl. Fig. 4C,D). Furthermore, *UBQLN1*-deficient cells showed significantly fewer RAD51 nuclear foci at 48h, compared to wildtype controls (Fig. 2B, Suppl. Fig. 4E-G), specifically in S- and G₂-M cell cycle phases (Suppl. Fig. 4G), which suggests decrease in ongoing HR. Intriguingly, these differences did not emerge until later, as *UBQLN1* KO and WT cells behaved identically at early time points (0-24h) (Fig. 2B). This suggests that the initial RAD51 recruitment to damaged chromatin is unaffected in *UBQLN1*-deficient cells. In agreement with this possibility,

GFP-RAD51 recruitment following microlaser-induced DNA damage was not affected in *UBQLN1*-deficient cells for up to 20 min (Fig. 2C). As RAD51 constitutes the central recombinase mediating HR-driven DSB repair, we next employed the DR-GFP reporter assay to functionally assess HR efficiency in *UBQLN1*-depleted or -proficient cells (Suppl. Fig. 4H). We used depletion of *CTIP* (involved in DNA end resection during HR), and *UBQLN4*, a ubiquitin that prevents HR¹³, as controls of decreased and increased HR pathway activity, respectively. These experiments revealed significantly reduced HR activity in *UBQLN1*-depleted cells, compared to control cells (Fig. 2D). Taken together, our results indicate that UBQLN1 increases HR pathway activity by affecting the number of RAD51 nuclear foci upon genotoxic damage.

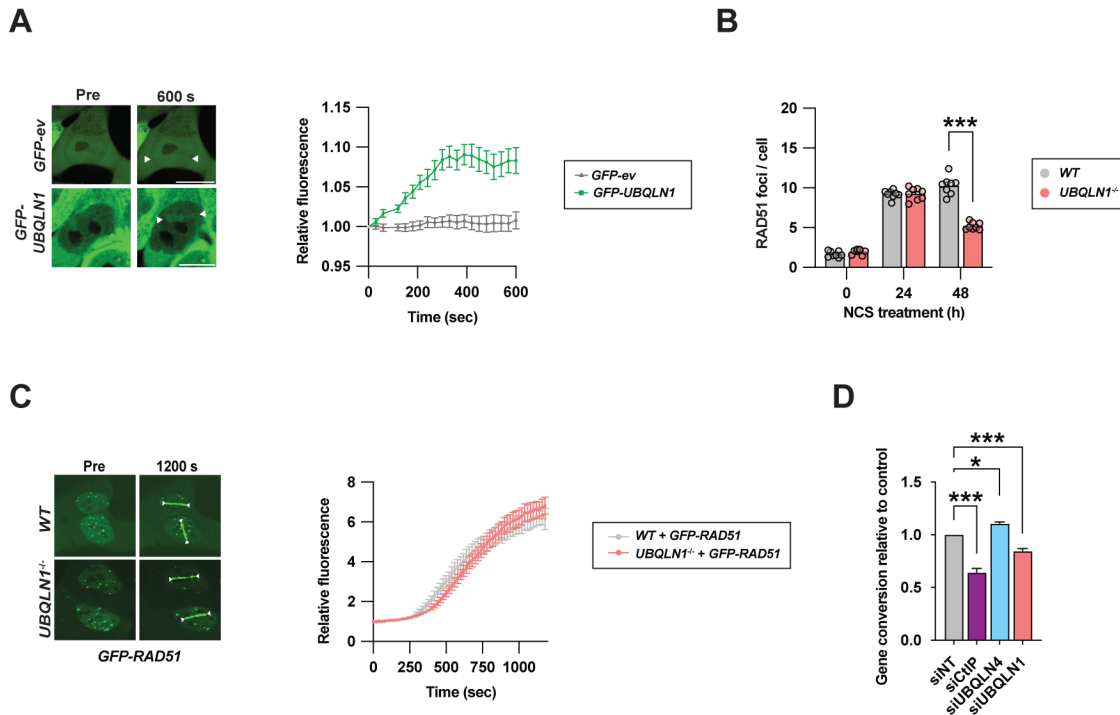


Figure 2. *UBQLN1* deletion leads to a DDR defect by decreasing the number of RAD51 foci.

A) Recruitment of *GFP-UBQLN1* and *GFP-ev* to 405 nm laser-induced damage over 10 min. Each data point represents the average of 10 cells. Representative images; scale bar, 15 μ m. **B)** Nuclear RAD51 foci in *WT* and *UBQLN1*^{-/-} U2OS cells treated with NCS (250 ng/ml) for 24 and 48h. Each datapoint represents the mean of 3000 analyzed nuclei. **C)** GFP-RAD51 recruitment kinetics to laser-induced damage over 20 min. Each data point represents the average of 10 cells. **D)** DR-GFP reporter assay for HR. Cells were transfected with the indicated siRNAs and transduced with BFP-tagged I-SceI. siCTIP served as positive control. **Statistics:** Error bars represent SEM. (A, C) n = 4 independent experiments; (B) n = 8 replicate wells, each represents the mean of 3000 analyzed nuclei, two-way ANOVA with Bonferroni post hoc test; (D) n = 3 biological replicates (3 technical replicates each), one-way ANOVA with Geisser-Greenhouse correction. *p < 0.05, **p < 0.01, ***p < 0.001.

UBQLN1 stabilizes RAD51 by preventing its ubiquitination

To test the possibility that UBQLN1 protects RAD51 from degradation at the site of genotoxic damage, we exposed cells to NCS, then treated them with a proteasome inhibitor (MG132) and quantified nuclear RAD51 foci using HCl. We observed that proteasome inhibition results in significant stabilization of DNA-associated RAD51 in both WT and *UBQLN1* KO cells (**Fig. 3A**); effectively, inhibiting the proteasome completely rescues the *UBQLN1* KO phenotype, suggesting that UBQLN1 is involved in regulating RAD51 proteasome-mediated degradation. We examined the relationship between UBQLN1 and RAD51 protein homeostasis by inhibiting protein synthesis via cycloheximide (CHX) chase experiments in cells that were released from a double thymidine block-mediated G₁/S arrest following an NCS-mediated genotoxic insult (**Suppl. Fig. 5A**). We detected a precipitous drop in RAD51 protein levels in the chromatin fraction of *UBQLN1* KO cells (**Fig. 3B,C**), whereas RAD51 levels in whole cell lysates did not change significantly (**Fig. 3B,C**). These effects were reversed by MG132 treatment (**Fig. 3D, Suppl. Fig. 5B**). Taken together, these results indicate that loss of UBQLN1 results in rapid depletion of chromatin-bound RAD51 levels due to proteasome-mediated degradation.

Next, we tested whether UBQLN1 stabilizes RAD51 by preventing its ubiquitination or by interfering with proteasomal degradation of ubiquitinated RAD51. We examined ubiquitination levels of RAD51 by deploying antibody co-staining experiments. These experiments revealed increased ubiquitin/RAD51 co-staining in *UBQLN1*-KO cells compared to WT controls at 24h (**Fig. 3E, Suppl. Fig. 5C,D**), indicating that loss of *UBQLN1* leads to increased levels of RAD51 ubiquitination. Furthermore, we examined polyubiquitination status of RAD51 using immunoprecipitation in HIS-Ubiquitin (HIS-Ub)

transfected cells and identified increased RAD51 K48-ubiquitination upon loss of *UBQLN1* (**Fig. 3F**). This was also confirmed by the reverse approach, by pulling on HIS-Ub (**Suppl. Fig. 5E**).

Lastly, to provide biochemical support that UBQLN1 engages RAD51 directly and prevents its ubiquitination, we tested whether the two proteins form a complex and whether the binding is RAD51 ubiquitination-dependent. We performed pulldown experiments from HEK293 Flp-In *UBQLN1*^{-/-} expressing GFP-UBQLN1 cells treated with NCS. In these cells we observed co-immunoprecipitation of UBQLN1 and RAD51, suggesting direct binding (**Fig. 3G**). We also used recombinant FLAG-UBQLN1-HIS and RAD51, which was either pre-ubiquitinated or left untreated. Our results showed that UBQLN1 binds exclusively to unmodified RAD51, and not Ub-RAD51 (**Fig. 3H, Suppl. Fig. 5F**). To identify the domain of UBQLN1 responsible for RAD51 interaction, we generated GFP-tagged UBQLN1 constructs containing a nuclear localization sequence (NLS) with a deletion of UBL, STI1-I, STI1-I.2/II.1, STI1-II, or UBA domain (**Fig. 3I**). Synchronized HEK293 Flp-In *UBQLN1*^{-/-} cells were transfected with GFP-UBQLN1-WT-NLS or GFP-UBQLN1-NLS deletion mutant constructs. We performed co-immunoprecipitation experiments after inducing DNA damage for 3h using NCS. These experiments revealed that deletion of the UBQLN1 STI1-II domain resulted in loss of interaction between UBQLN1 and RAD51 (**Fig. 3J**), whereas deletion of the UBL- or UBA-domain, which facilitate the proteasomal shuttle function of UBQLN1, had no effect (**Fig. 3J**). These findings indicate that the RAD51 stabilizing function of UBQLN1 may be mediated by the UBQLN1 STI1-II domain.

Collectively, our data support a model in which direct binding of UBQLN1 to RAD51 following DNA damage prevents RAD51 ubiquitination and subsequent proteasomal degradation, thus promoting HR-mediated DSB repair.

2. Results – Manuscript III

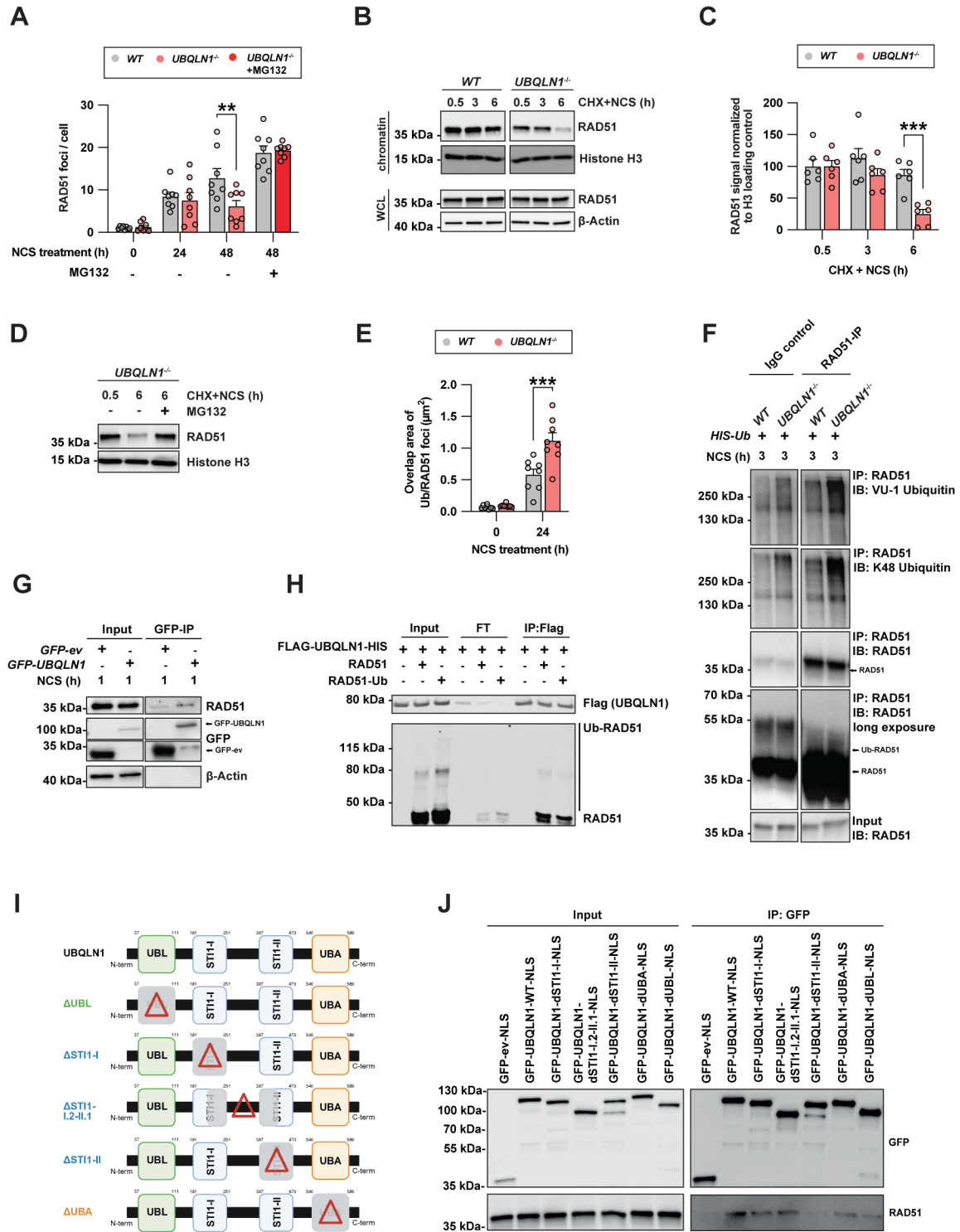


Figure 3. UBQLN1 stabilizes RAD51 by preventing its ubiquitination.

A) Nuclear RAD51 foci in *WT* and *UBQLN1*^{-/-} U2OS cells treated with NCS (250 ng/ml) for 24 and 48h, with or without MG132 pre-treatment (500 nM, 1h). Each datapoint represents the mean of 3000 analyzed nuclei. **B)** Cycloheximide chase assay. Synchronized U2OS cells were treated with CHX (500 μg/ml) and NCS (250 ng/ml). Chromatin fractions were probed for RAD51 at the indicated time points. H3 served as loading control. **C)** Quantification of (B). Chromatin-fraction RAD51 was normalized to H3; the 0.5h time point was set to 100%. **D)** Cycloheximide chase as in (B), with MG132 pre-treatment (2.5 μM, 30 min) in the indicated sample. **E)** Nuclear RAD51 and nuclear FK2 (ubiquitin) foci overlap in U2OS cells treated with NCS (250 ng/ml) for 24h. **F)** K48-ubiquitination of RAD51 in HEK293 Flp-In *WT* and *UBQLN1*^{-/-} cells transfected with HIS-Ubiquitin and treated with NCS (100 ng/ml, 3h). **G)** Immunoprecipitation of doxycycline-induced *GFP-UBQLN1* was probed for endogenous RAD51 in *UBQLN1*^{-/-} cells. *GFP-ev* served as control. NCS (100 ng/ml, 1h). **H)** Recombinant FLAG-UBQLN1-HIS was incubated with RAD51 exposed to *in vitro* ubiquitination machinery, immunoprecipitated via FLAG-tag, and probed for RAD51 interaction. **I)** Schematic of UBQLN1 domain architecture and deletion mutants. →

J) Immunoprecipitation of *GFP-UBQLN1* full-length and domain deletion mutants was probed for endogenous RAD51 in synchronized *UBQLN1*^{-/-} cells treated with NCS (500 ng/ml, 3h). **Statistics:** Error bars represent SEM. (A, E) n = 8 replicate wells, each represents the mean of 3000 analyzed nuclei, two-way ANOVA with Bonferroni post hoc test; (C) n = 3 independent fractionations (2 immunoblots each), two-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.

***UBQLN1* overexpression represses HR-mediated DSB repair**

To further probe the role of *UBQLN1* in regulating RAD51 and DSB repair pathway choice, we performed overexpression experiments. To this end, we examined how *UBQLN1* overexpression affects HR in U2OS cells as measured by gene conversion rate. We observed that *UBQLN1* overexpression causes an HR defect that was comparable to what we observed in *UBQLN1*-depleted cells following DSB induction (**Fig. 4A**). To identify all factors involved in mediating these effects, we assessed the *UBQLN1* interactome. We performed a Turbo-ID mass-spectrometry (MS) experiment following a 1h NCS exposure, employing exogenously expressed *UBQLN1* fused to Turbo-ID Ligase expressed in HEK293 cells. The recovered *UBQLN1* proximome contained a number of proteins directly involved in the DDR network (e.g. CHK1, RNF168, and CLSPN), and particularly in the HR pathway, such as BRCA1, NBS1, and MRE11 (**Fig. 4B**).

The detection of proteasomal subunits PSMD1 and PSMD4 in conjunction with MRE11, an HR factor that initiates DNA end-resection by processing DSBs, led us to ask whether *UBQLN1* overexpression represses HR activity by facilitating proteasomal degradation of HR pathway components – similar to what we had observed for *UBQLN4*¹³. To approach this question, we employed HCI to detect nuclear MRE11 foci upon genotoxic damage. Compatible with a role in mediating proteasomal degradation of MRE11, *UBQLN1* overexpression was associated with significantly reduced abundance of MRE11 foci following NCS exposure compared to empty-vector (EV) control (**Fig. 4C, Suppl. Fig. 6A**). These data

suggest that *UBQLN1* overexpression prevents effective execution of HR by reducing the abundance of MRE11 at the site of genotoxic damage. To probe whether *UBQLN1* engages MRE11 at the site of damage, and whether *UBQLN1* ubiquitin-associated domain is involved in mediating these interactions, we used co-immunoprecipitation experiments employing *GFP-UBQLN1-NLS* and *GFP-UBQLN1-dUBA-NLS* constructs. We observed that intact *UBQLN1* co-immunoprecipitated MRE11, whereas the UBA-deletion mutant failed to do so (**Fig. 4D**). Given that UBAs recognize and bind ubiquitin, our results suggest that *UBQLN1* engages ubiquitinated MRE11, which is in contrast to what we observed for RAD51 (**Fig. 3J**). This opens a possibility that *UBQLN1* regulates RAD51 and MRE11 activity via distinct mechanisms.

Moreover, we also observed significantly reduced RAD51 foci levels in *UBQLN1* overexpressing cells compared to control cells following NCS exposure (**Fig. 4E, Suppl. Fig. 6B**). As our data suggested that *UBQLN1* overexpression represses HR likely due to interfering with MRE11-dependent DNA end-resection, we tested whether resection processivity was affected. For that purpose, we employed the SMART assay that measures the length of resected DNA at the level of individual fibers¹⁸. Intriguingly, *UBQLN1* overexpression leads to a significant increase of resection track length (**Fig. 4F**). In this scenario, we further hypothesized that alternative repair pathways that require longer resection, such as single-strand annealing (SSA), would be stimulated as resection reaches outside the homologous stretch of DNA. Indeed, when using the SA-GFP reporter to measure SSA¹⁹, we observed that *UBQLN1* overexpression causes an increased usage of SSA (**Fig. 4G**).

2. Results – Manuscript III

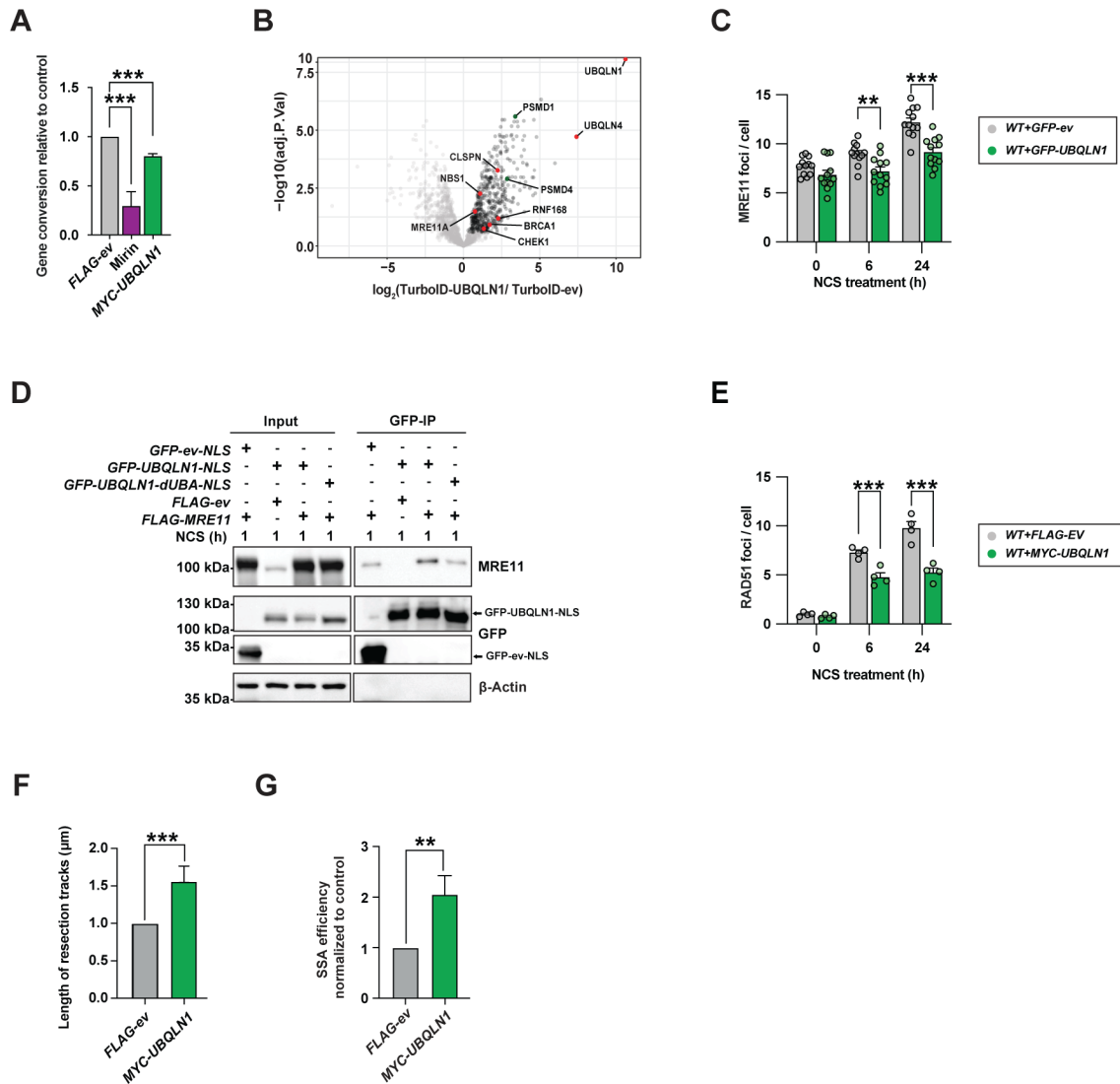


Figure 4. UBQLN1 overexpression represses HR-mediated DSB repair.

A) DR-GFP reporter assay. Cells expressing *FLAG-ev* or *MYC-UBQLN1* were transduced with BFP-tagged I-SceI. Mirin served as positive control. **B)** TurbID proximity proteomics of the UBQLN1 interactome. HEK293 cells expressing *TurbID-ev* or *TurbID-UBQLN1* were treated with NCS (500 ng/ml, 1h) and biotinylated for 10 min. DNA repair proteins (red) and proteasomal components (green) are highlighted. **C)** Nuclear MRE11 foci in doxycycline-induced U2OS Flp-In cells treated with NCS (250 ng/ml) for 6 and 24h. **D)** Immunoprecipitation of GFP-UBQLN1 and GFP-UBQLN1-dUBA domain deletion mutant was probed for FLAG-MRE11. Cells were treated with NCS (100 ng/ml, 1h). **E)** Nuclear RAD51 foci in doxycycline-induced U2OS Flp-In cells treated with NCS (250 ng/ml) for 6 and 24h. **F)** DNA end resection by SMART assay. Mean ssDNA track length was quantified in U2OS cells expressing the indicated constructs. **G)** SSA reporter assay. Cells expressing *FLAG-ev* or *MYC-UBQLN1* were assessed for SSA pathway usage. **Statistics:** Error bars represent SEM. (A) $n = 3$ biological replicates (3 technical replicates each), one-way ANOVA with Geisser-Greenhouse correction; (B) $n = 3$ biological replicates; (C) $n = 12$ replicate wells, each represents the mean of 3000 analyzed nuclei; (E) $n = 4$ replicate wells, each represents the mean of 3000 analyzed nuclei; (C, E) two-way ANOVA with Bonferroni post hoc test. (F) $n = 4$ biological replicates (800 measured tracks per replicate), normalized to *FLAG-ev*, Student's t-test; (G) $n = 3$ biological replicates, normalized to *FLAG-ev*, Student's t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Taken together, our results show that overexpression of *UBQLN1* produces an HR defect by significantly reducing abundance of MRE11 and RAD51 at sites of DNA damage and leads to the compensatory increase of the SSA pathway. Although it remains to be tested in future studies,

our evidence points to the possibility that *UBQLN1* regulates MRE11 and RAD51 via distinct mechanisms, involving ubiquitination-dependent and ubiquitination-independent binding, respectively.

***UBQLN1* overexpression is associated with reduced overall survival in lung adenocarcinoma**

Functional repression of the HR pathway is a common feature of human cancer²⁰. Given that *UBQLN1* overexpression resulted in substantially impaired HR activity, we examined a possible association between increased *UBQLN1* transcription and cancer by analyzing publicly available TCGA datasets. We found that *UBQLN1* mRNA expression is upregulated in various solid and hematological malignancies (Suppl. Fig. 7A). Next, we stratified patients according to *UBQLN1* mRNA levels, noting that patients with high *UBQLN1* expression levels (upper quartile) displayed significantly reduced disease-free

survival (DFS) and overall survival (OS) in a series of cancer types, including lung adenocarcinoma (LUAD) (Suppl. Fig. 7B-I).

Additionally, Confident Deconvolution For All Cell Subsets (CODEFACS)-mediated²¹ deconvolution confirmed that *UBQLN1* overexpression drives the DFS and OS difference specifically in the cancer cell fraction in LUAD cases (Fig. 5A-C, Suppl. Fig. 7J,K,L). Consistent with a more aggressive disease course, genomes of *UBQLN1* overexpressing LUADs displayed increased levels of tumor mutational burden (TMB) than tumors with low *UBQLN1* expression (Fig. 5D). We also observed significantly reduced OS in mice that constitutively overexpress *Ubqln1* (*Rosa26^{LSL}.HA-Ubqln1/wt*; *Actb^{Cre/wt}*), compared to wildtype animals (Fig. 5E-F).

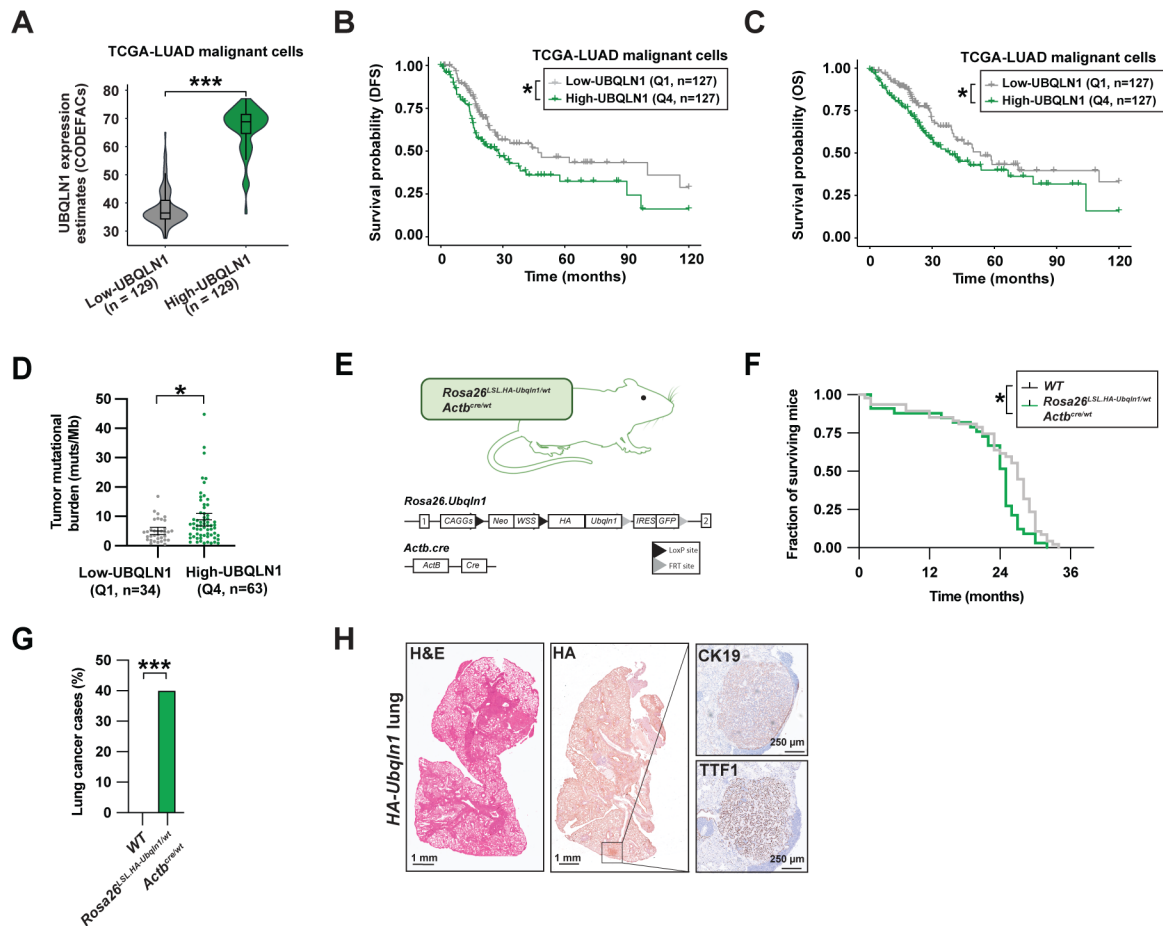


Figure 5. *UBQLN1* overexpression is associated with reduced overall survival in lung adenocarcinoma.

A) *UBQLN1* expression in malignant cells after CODEFACS deconvolution of TCGA-LUAD data. Two patients per group were excluded due to missing survival data. **B)** Disease-free survival for patients with low (n=127) vs. high (n=127) *UBQLN1*-expressing malignant cells. **C)** Overall survival for patients with low (n=127) vs. high (n=127) *UBQLN1*-expressing malignant cells. **D)** Comparison of tumor mutational burden between low-*UBQLN1* (Q1, n=34) and high-*UBQLN1* (Q4, n=63) patients. Patients with mutations in central DNA repair factors were excluded (2 in Q1: RAD50 or MRE11/BRCA2 variants). Error bars represent 95% CI. →

2. Results – Manuscript III

E) Schematic of *Rosa26^{LSL}HA-Ubqln1/wt;Actb^{Cre}/wt* mouse model for constitutive *Ubqln1* overexpression. **F)** Overall survival of *Ubqln1*-overexpressing mice (n=33) vs. controls (n=47). **G)** Cancer monitoring in aged mice. 40% of *Rosa26^{LSL}HA-Ubqln1/wt;Actb^{Cre}/wt* mice (8/20) developed lung cancer vs. 0% in wildtype controls (0/30). **H)** Immunohistochemistry staining (IHC) of *Ubqln1*-overexpressing mouse lungs stained for H&E, HA, TTF1, and CK19, confirming lung adenocarcinoma. Analyzed by a board-certified pathologist. **Statistics:** (B, C, F) Mantel-Cox test; (D) Wilcoxon test; (G) Student's t-test. *p < 0.05, **p < 0.01, ***p < 0.001.

Strikingly, 40% of these animals developed LUADs with a long latency (median OS 25 months), compared to none of the C57BL/6 *WT* mice (**Fig. 5G-H, Suppl. Fig. 7M**). Presence of LUAD and *Ubqln1* overexpression were confirmed using immunohistochemistry, with Thyroid Transcription Factor-1 (TTF1) and Cytokeratin 19 (CK19) as markers for LUAD and HA as marker for *HA-Ubqln1* (**Fig. 5H**). Altogether, these data strongly suggest that *UBQLN1* overexpression acts as a functional oncogene in LUAD carcinogenesis.

***UBQLN1* overexpression increases mutational burden, downregulates genes involved in HR and upregulates antigen processing and presentation genes**

To validate the cancer-driving function of *UBQLN1* overexpression in the background of a relevant autochthonous LUAD model, we employed the *Kras^{LSL}G12D/wt;Trp53^{fl/fl};Rosa26^{wt/wt}* mouse model²² and generated *Kras^{LSL}G12D/wt;Trp53^{fl/fl};Rosa26^{LSL}HA-Ubqln1/wt* (KPU1) mice. In these mice, expression of the *Ubqln1* transgene is prevented by a loxP-STOP-loxP (LSL) cassette in the absence of Cre recombinase (**Suppl. Fig. 8A-C**). Cre-mediated deletion of the LSL cassette and subsequent expression of *HA-Ubqln1* was verified in MEFs (**Suppl. Fig. 8D**).

We induced LUAD in KP and KPU1 mice by intratracheal Adeno-CMV-Cre instillation. While KP mice displayed a median OS of 117 days following Cre instillation (comparable to previously published survival data using this model²³), KPU1 mice exhibited significantly reduced OS of 91 days (22%

reduction) post instillation (**Fig. 6A**). Both serial μ CT assessment of tumor-bearing mice 11 weeks post Adeno-CMV-Cre instillation, as well as histological analysis showed that total tumor volume in KPU1 mice was significantly higher than that observed in KP control mice (**Fig. 6B-D**). In addition to our human TCGA dataset analysis, molecular characterization of the murine tumors revealed an increased number of single nucleotide variants (SNVs) and insertions/deletions (InDels) in KPU1 tumors, compared to KP controls (**Fig. 6E,F**). Furthermore, KPU1 tumors displayed an increase in an insertion/deletion mutational signature linked to DNA damage and repair processes, particularly NHEJ (*i.e.* ID8), and a mutational signature primarily characterized by 5 base pair insertions (*i.e.* ID19)²⁴ (**Fig. 6G, Suppl. Fig. 8E-P**), both in agreement with the observed reduction in HR activity in *UBQLN1* overexpression setting.

Gene-set enrichment analysis revealed significant downregulation of the gene-set *homologous recombination* in KPU1 tumors (**Fig. 6H**), concordant with reduced RAD51 and MRE11 foci (**Fig. 4C, E**) and impaired gene conversion in the DR-GFP assay (**Fig. 4A**). Given the increased TMB in KPU1 tumors, we further performed *in silico* neoepitope prediction analysis, which revealed an increased frequency of likely MHC-presented neoepitopes in KPU1 compared to KP controls (**Suppl. Fig. 8Q**). Altogether, these data indicate that increased *UBQLN1* expression in human and murine LUAD is associated with increased TMB, enhanced tumorigenesis, and features consistent with HR pathway repression.

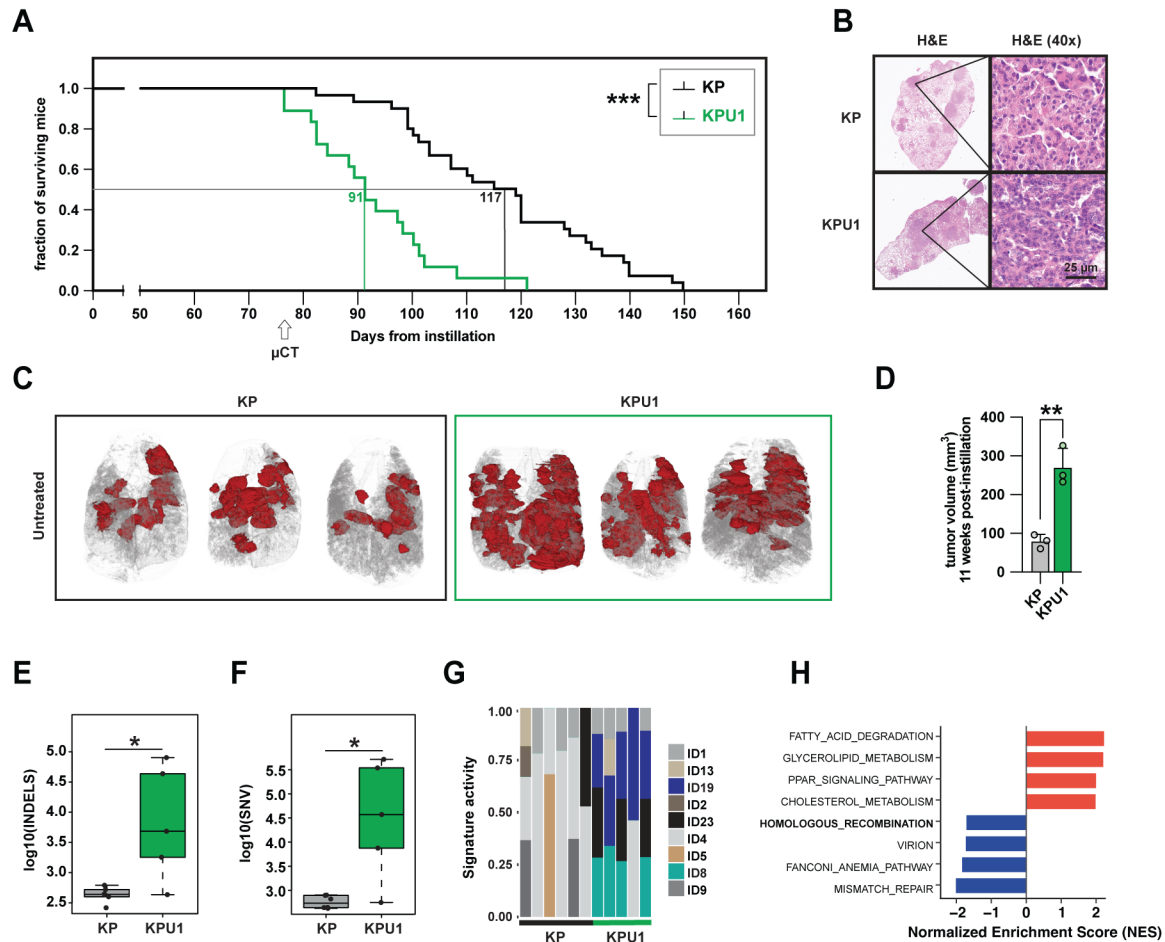


Figure 6. *UBQLN1* overexpression increases tumor mutational burden, downregulates HR and upregulates antigen processing and presentation in LUAD.

A) Overall survival of KP (n=29) and KPU1 (n=14) mice after bronchial AdenoCre instillation. Arrow indicates μ CT scan at 11 weeks. **B)** Representative H&E stainings of KP and KPU1 lungs, analyzed by a board-certified pathologist. **C)** Representative 3D rendering of μ CT tumor burden at 11 weeks post-instillation. **D)** Tumor volume quantification from μ CT at 11 weeks (3 mice per genotype, selected for representative survival). **E)** INDEL counts ($\log_{10}$) from whole-genome sequencing (WGS) of KP and KPU1 tumors. **F)** SNV counts from WGS of KP and KPU1 tumors. **G)** Genomic scar signatures in KP (n=6) and KPU1 (n=5) tumors. ID8 is associated with NHEJ; ID19 with 5 bp insertions²⁴. **H)** Gene set enrichment analysis (GSEA) of KPU1 vs. KP tumors showing NES for significantly enriched KEGG cancer-related pathways ($p < 0.05$).

Statistics: (A) Mantel-Cox test; (D) one-way ANOVA; (E, F) two-sided Wilcoxon test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

***UBQLN1* overexpression in lung adenocarcinoma provides distinct vulnerabilities to PARP1 and PD1 inhibition**

We next asked whether the HR defect in *UBQLN1* overexpression scenarios and the increased frequency of neoepitope presentation in these tumors might offer a window for molecularly rationalized therapeutic intervention. As HR deficiency has previously been identified as a strong predictor for sensitivity to PARP1 inhibitors^{23,25}, we exposed cohorts of tumor-bearing

(μ CT-verified) KP and KPU1 mice to the PARP1 inhibitor olaparib (50 mg/kg i.p., q.d.) and recorded OS as the endpoint of this study. PARP1 inhibitor treatment did not extend the survival of KP animals; however, we observed a statistically significant OS gain in KPU1 animals, illustrating an actionable HR defect brought about by *UBQLN1* overexpression (**Fig. 7A**). Furthermore, comparative analyses of total tumor volume at 11 weeks post-instillation showed limited, KP-like growth of KPU1 tumors upon PARP-inhibitor treatment, in line with the

2. Results – Manuscript III

improved OS (**Fig. 7B**, **Suppl. Fig. 9A**). To further validate olaparib sensitivity as a function of *UBQLN1* overexpression in the human setting, we analyzed a panel of six human LUAD cell lines. The NCIH1623, A427, and NCIH1651 cell lines, which harbored high *UBQLN1* expression levels (**Suppl. Fig. 9B**), displayed robust olaparib sensitivity, whereas Calu3, HCC461, and NCIH1793 cell lines, with low *UBQLN1* expression (**Suppl. Fig. 9B**), were resistant to olaparib (**Suppl. Fig. 9C, D**). In summary, these *in vitro* and *in vivo* data indicate that *UBQLN1* overexpression is associated with PARPi sensitivity in LUAD. Building on the increased TMB and frequency of predicted neoepitope presentation of KPU1 tumors, we next asked whether immune-checkpoint blockade (ICB) might display differential therapeutic efficacy in these tumors, compared to KP controls. Upon μ CT-

verified tumor manifestation, mice were treated with an anti-PD1 antibody (RMP1-14, 10mg/kg, i.p., 2qwk) and assessed for tumor volume and OS. ICB did not affect OS of KP animals (**Fig. 7C**). In contrast, ICB significantly increased OS of KPU1 tumor bearing animals compared to untreated controls (**Fig. 7C**). In line with these survival data, ICB treatment resulted in reduced tumor growth in KPU1 tumors compared to controls (**Fig. 7D**, **Suppl. Fig. 9E**). Altogether, these experiments provide a rationale for the use of *UBQLN1* as a clinical biomarker for stratifying LUAD patients. Importantly, based on our results we argue that LUAD patients that overexpress *UBQLN1* may benefit from treatment with PARP1 inhibitors or ICB therapy.

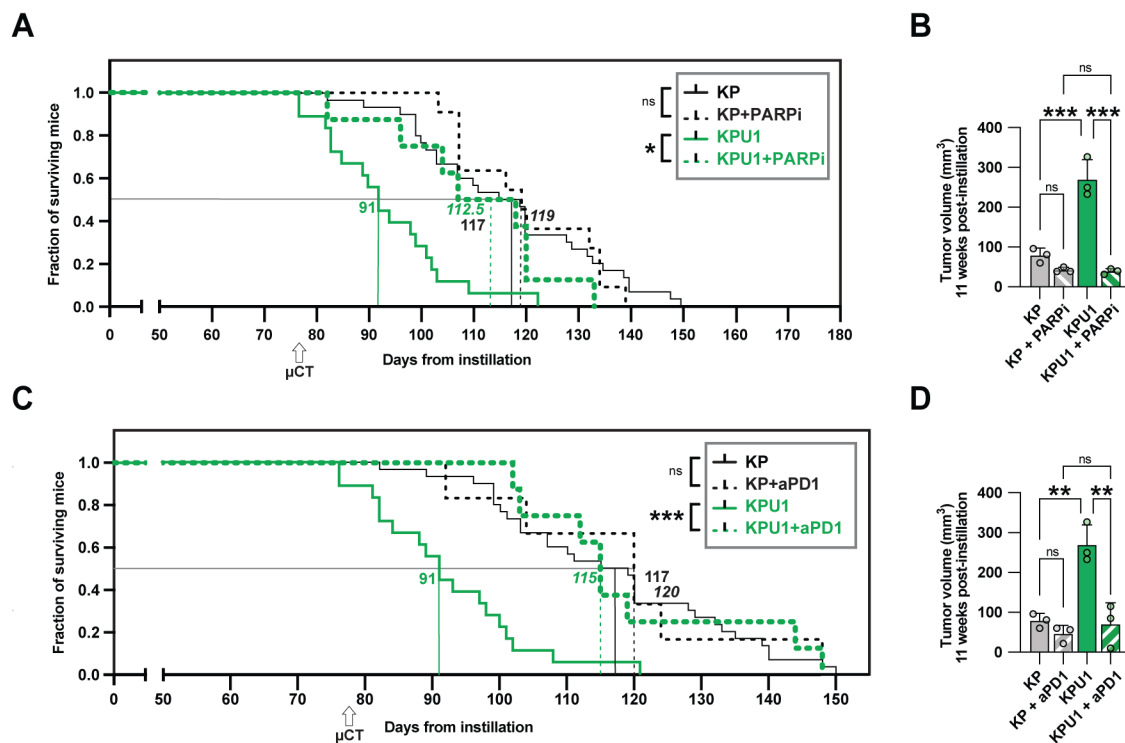


Figure 7. *UBQLN1* overexpression in lung adenocarcinoma is associated with distinct vulnerabilities to PARP1 and PD1 inhibition.

A) Overall survival of KP and KPU1 mice treated with Olaparib (50 mg/kg daily). KP n=29; KPU1 n=14; KP+PARPi n=11; KPU1+PARPi n=8. Arrow indicates μ CT scan. Untreated data correspond to Fig. 6A. **B)** Tumor volume from μ CT at 11 weeks (3 mice per group). Untreated data correspond to Fig. 6D. See Suppl. Fig. 9A for 3D images. **C)** Overall survival of KP and KPU1 mice treated with α PD1 (RMP1-14, 10 mg/kg bi-weekly). KP n=29; KPU1 n=14; KP+ α PD1 n=5; KPU1+ α PD1 n=5. Arrow indicates μ CT scan. Untreated data correspond to Fig. 6A. **D)** Tumor volume from μ CT at 11 weeks (3 mice per group). Untreated data correspond to Fig. 6D. See Suppl. Fig. 9E for 3D images.

Statistics: (A, C) Mantel-Cox test; (B, D) one-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.

Discussion

Here, we describe a role for the proteasomal shuttle factor UBQLN1 in regulating the HR process. We show that the *loss of UBQLN1* results in increased ubiquitination of the central HR mediator RAD51, leading to ubiquitination-dependent RAD51 proteasomal degradation and defective HR. We observed that *UBQLN1 overexpression* was also associated with an HR defect; however, our results indicate that the effect of overexpression likely involves an enhanced degradation of the HR initiating factor MRE11. Collectively, these results highlight the complex relationship between activity and degradation of multiple proteins involved in different steps of the HR pathway, and suggest that UBQLN1 regulates DSB repair pathway choice by controlling RAD51 and MRE11 levels. Ubiquitination of DDR-related factors is recognized as both a trigger for signaling cascades and a proteolytic signal that regulates the recruitment and removal of these proteins²⁶. Our previous studies, as well as those from other groups, have indicated that the primary function of ubiquilins involves facilitating the proteasomal turnover of ubiquitinated client proteins^{8,13}.

However, UBQLN1 can also play a stabilizing role as a chaperone that protects client proteins from ubiquitination and degradation^{6,27-30}. For example, UBQLN1 stabilizes an anti-apoptotic protein BCL-B to promote cell survival, a growth receptor IGF1R to regulate metabolic pathways, a membrane repair protein ESYT2 to maintain ER-membrane contact sites, γ -secretase components PSEN1/2 to support amyloid precursor protein processing in Alzheimer's disease, and the neuronal receptor GABAA-R to prevent epilepsy^{6,27-30}. Thus, UBQLN1 is emerging as an important factor that balances both aspects of proteostasis (stabilization vs. proteolysis) in numerous cellular processes, including the DDR. We specifically show that UBQLN1 exhibits its non-canonical function by binding RAD51 localized to sites of DNA DSBs in a

ubiquitin-independent manner and prevents its ubiquitination. This is an unprecedented function for ubiquilins, and an unprecedented mode of RAD51 stabilization. Thus, loss of UBQLN1 facilitates RAD51 ubiquitination and ultimately results in increased proteasomal degradation of RAD51 over time, and decrease in HR. This is in contrast to our previous work on UBQLN4¹³, where we showed that loss of UBQLN4 promotes HR pathway usage. Functional differences between UBQLN1 and UBQLN4 are also evident in distinct phenotypes of *UBQLN1 deficiency syndrome* and *UBQLN4 deficiency syndrome* patients. For example, although both the *UBQLN1-* and *UBQLN4 deficiency syndrome* patients exhibit failure to thrive, intellectual disability and facial dysmorphisms, seizures were observed exclusively in *UBQLN1 deficiency syndrome* patients, whereas ataxia was restricted to *UBQLN4 deficiency syndrome* patients.

In terms of translational and clinical implications of our work, we note that *UBQLN1* is overexpressed in a plethora of different cancer types, such as LUAD, ovarian- and breast cancer. This suggests that *UBQLN1* overexpression, and possibly the associated defect in genome maintenance, might constitute selected events in cancer³¹⁻³³. Our mechanistic studies lend support to a genome destabilizing and thus potentially selected effect of *UBQLN1* overexpression in cancer. Intriguingly, overexpression of *Ubqln1* led to the spontaneous formation of lung tumors in mice, indicating that *Ubqln1* bears oncogenic potential. Our experiments revealed that UBQLN1 engages with the HR initiating factor MRE11 through its Ub-binding UBA domain. This suggests a role of UBQLN1 in proteasomal degradation of MRE11, very similar to what we previously observed with *UBQLN4* overexpression¹³, potentially implying that overexpressed UBQLN1 hijacks natural UBQLN4 substrates. In line with this, we observe a significantly reduced abundance of MRE11 foci in *UBQLN1*-overexpressing cells following NCS exposure. Moreover, functional experiments using

2. Results – Manuscript III

DR-GFP assays and RAD51 foci quantification showed that *UBQLN1* overexpression drives a prominent HR defect.

Additionally, we found that human LUAD patients with *UBQLN1* overexpression and KPU1 mice, which overexpress *Ubqln1*, displayed a significantly reduced OS. KPU1 mice developed tumors harboring mutational signatures associated with HR deficiency and increased NHEJ pathway usage. We also showed that these mutational signatures represent a targetable vulnerability, as evidenced by sensitivity of *UBQLN1* overexpressing tumors to PARP1 inhibition in KPU1 mice. In addition, these tumors were also highly sensitive to ICB likely as a result of substantially increased TMB and frequency of predicted neoepitope presentation. The elevated neoantigen load increases tumor recognition by the immune system, rendering these cancers particularly sensitive to ICB³⁴⁻³⁶. In summary, our data unravel the mechanistic underpinnings of *UBQLN1* as a key node in the DDR network. Furthermore, our discovery that *UBQLN1* enhances a cell's capacity to repair DNA damage by binding to and protecting RAD51 from ubiquitination adds another dimension to our understanding of ubiquilins, as well as the intersection between two fundamental cellular processes, *i.e.* DNA repair and proteasomal degradation. Lastly, although the mechanism by which *UBQLN1* overexpression causes LUAD remains to be determined, our study nominates *UBQLN1* overexpression as a potential biomarker for PARP1 inhibitor and ICB therapy sensitivity, which has important implications for patient stratification and clinical decision making.

Acknowledgements

We are grateful to our patients and their family. We thank Hannah Goldfarb-Wittkopf and Matthew Arnett for excellent technical assistance. We thank Jeremy Stark for sharing the DR-GFP reporter cell line and Jeroen Essers for sharing the GFP-RAD51 plasmid. This work was tremendously supported by the Max Planck Biology for Ageing Core Facilities: All analyses using microscopy and FACS techniques were performed in the FACS&Imaging Core Facility. TurboID experiments and mass spectrometry analysis were performed in the Proteomics Core Facility. The μ CT analyses were performed in the Phenotyping Core Facility with support of Andrea Mesaros and Martin Purrio. Mouse line generation, mouse husbandry, and treatment studies were performed in the Comparative Biology Core Facility and supported by Frederik Bøddeker, Patrick Wollek, Jerome Henn, Sabrina Seyfarth, Silvia Bøddeker, and Bettina Bertalan. We thank Branko Zevnik and the *in vivo* Research Facility at CECAD for assistance in generating the *Ubqln1^{c.377delC}* mouse line. We further thank Alexandra Florin and Wiebke Jeske from the Pathology department of the University Hospital Cologne for support with immunohistochemistry staining. We thank Helen Dauben and Edoardo Longarini for technical assistance with laser line microscopy. We also acknowledge scientific advice and editorial services of Milka Kostic (Life Science Editors).

Author Contributions

H.C.R. and R.D.J. designed and R.D.J. supervised the study. M.A.H., O.L., A-S.H., P.V.G., R.D.J. designed the experiments. M.A.H., O.L., A-S.H., P.V.G., M.T-P., R.C., J.G., A.S.T.S., J.W., E.v.W., A.K., N.P., L.M., H.M., J.I. performed the experiments. M.A.H., O.L., A-S.H., P.V.G., M.-T.P., J.G., C.M.G., P.J.B, A.S.T.S., J.W., E.v.W., N.P., S.G., M.R., S.E.T., L.T.F., S.K., Je.B., F.B., A.M., F.T.W., S.P., A.Q., Y.S., D.S.B.H., T.Ho., Jo.B., M.P., H.A., D.P., J.R.L., R.B., M.T., P.H., T.M., A.B., M.C., T.Hu., K.K.C., M.A.B., J.I., D.W., H.C.R., R.D.J. performed data analysis. M.A.H., O.L., A-S.H., P.V.G., A.S.T.S., J.I., A.M., R.D.J. generated the figures. H.C.R. and R.D.J. wrote the manuscript. All authors read, revised, and approved the submitted version of the manuscript. M.A.H. adapted the manuscript for his dissertation.

References

- Schwertman, P., Bekker-Jensen, S., and Mairland, N. (2016). Regulation of DNA double-strand break repair by ubiquitin and ubiquitin-like modifiers. *Nat Rev Mol Cell Biol* 17, 379-394. 10.1038/nrm.2016.58.
- Jackson, S.P., and Durocher, D. (2013). Regulation of DNA damage responses by ubiquitin and SUMO. *Mol Cell* 49, 795-807. 10.1016/j.molcel.2013.01.017.
- Brinkmann, K., Schell, M., Hoppe, T., and Kashkar, H. (2015). Regulation of the DNA damage response by ubiquitin conjugation. *Front Genet* 6, 98. 10.3389/fgene.2015.00098.
- Shiloh, Y., and Ziv, Y. (2013). The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Nat Rev Mol Cell Biol* 14, 197-210. 10.1038/nrm3546.
- Dietlein, F., Thelen, L., and Reinhardt, H.C. (2014). Cancer-specific defects in DNA repair pathways as targets for personalized therapeutic approaches. *Trends Genet* 30, 326-339. 10.1016/j.tig.2014.06.003.
- Zheng, T., Yang, Y., and Castaneda, C.A. (2020). Structure, dynamics and functions of UBQLNs: at the crossroads of protein quality control machinery. *Biochem J* 477, 3471-3497. 10.1042/BCJ20190497.
- Stieren, E.S., El Ayadi, A., Xiao, Y., Siller, E., Landsverk, M.L., Oberhauser, A.F., Barral, J.M., and Boehning, D. (2011). Ubiquitin-1 is a molecular chaperone for the amyloid precursor protein. *J Biol Chem* 286, 35689-35698. 10.1074/jbc.M111.243147.
- Itakura, E., Zavodszky, E., Shao, S., Wohlever, M.L., Keenan, R.J., and Hegde, R.S. (2016). Ubiquitins Chaperone and Triage Mitochondrial Membrane Proteins for Degradation. *Mol Cell* 63, 21-33. 10.1016/j.molcel.2016.05.020.
- Chuang, K.H., Liang, F., Higgins, R., and Wang, Y. (2016). Ubiquitin/Dsk2 promotes inclusion body formation and vacuole (lysosome)-mediated disposal of mutated huntingtin. *Mol Biol Cell* 27, 2025-2036. 10.1091/mbc.E16-01-0026.
- Yang, H., Yue, H.W., He, W.T., Hong, J.Y., Jiang, L.L., and Hu, H.Y. (2018). PolyQ-expanded huntingtin and ataxin-3 sequester ubiquitin adaptors hHR23B and UBQLN2 into aggregates via conjugated ubiquitin. *FASEB J* 32, 2923-2933. 10.1096/fj.201700801RR.
- Tang, X., Liang, Y., Sun, G., He, Q., Qu, H., and Gao, P. (2021). UBQLN4 is activated by C/EBPbeta and exerts oncogenic effects on colorectal cancer via the Wnt/beta-catenin signaling pathway. *Cell Death Discov* 7, 398. 10.1038/s41420-021-00795-4.
- Shah, P.P., Lockwood, W.W., Saurabh, K., Kurlawala, Z., Shannon, S.P., Waigel, S., Zacharias, W., and Beverly, L.J. (2015). Ubiquitin1 represses migration and epithelial-to-mesenchymal transition of human non-small cell lung cancer cells. *Oncogene* 34, 1709-1717. 10.1038/onc.2014.97.
- Jachimowicz, R.D., Beleggia, F., Isensee, J., Velpula, B.B., Goergens, J., Bustos, M.A., Doll, M.A., Shenoy, A., ChecaRodriguez, C., Wiederstein, J.L., et al. (2019). UBQLN4 Represses Homologous Recombination and Is Overexpressed in Aggressive Tumors. *Cell* 176, 505-519 e522. 10.1016/j.cell.2018.11.024.
- Senturk, M., Lin, G., Zuo, Z., Mao, D., Watson, E., Mikos, A.G., and Bellen, H.J. (2019). Ubiquitins regulate autophagic flux through mTOR signalling and lysosomal acidification. *Nat Cell Biol* 21, 384-396. 10.1038/s41556-019-0281-x.
- Whiteley, A.M., Prado, M.A., Peng, I., Abbas, A.R., Haley, B., Paulo, J.A., Reichelt, M., Katakam, A., Sagolla, M., Modrusan, Z., et al. (2017). Ubiquitin1 promotes antigen-receptor mediated proliferation by eliminating mislocalized mitochondrial proteins. *Elife* 6. 10.7554/eLife.26435.
- Hiltunen, M., Lu, A., Thomas, A.V., Romano, D.M., Kim, M., Jones, P.B., Xie, Z., Kounnas, M.Z., Wagner, S.L., Berezovska, O., et al. (2006). Ubiquitin 1 modulates amyloid precursor protein trafficking and Abeta secretion. *J Biol Chem* 281, 32240-32253. 10.1074/jbc.M603106200.
- El Ayadi, A., Stieren, E.S., Barral, J.M., and Boehning, D. (2012). Ubiquitin-1 regulates amyloid precursor protein maturation and degradation by stimulating K63-linked polyubiquitination of lysine 688. *Proc Natl Acad Sci U S A* 109, 13416-13421. 10.1073/pnas.1206786109.
- Cruz-Garcia, A., Lopez-Saavedra, A., and Huertas, P. (2014). BRCA1 accelerates CtIP-mediated DNA-end resection. *Cell Rep* 9, 451-459. 10.1016/j.celrep.2014.08.076.
- Bennardo, N., Cheng, A., Huang, N., and Stark, J.M. (2008). Alternative-NHEJ is a mechanistically distinct pathway of mammalian chromosome break repair. *PLoS Genet* 4, e1000110. 10.1371/journal.pgen.1000110.
- Jachimowicz, R.D., Goergens, J., and Reinhardt, H.C. (2019). DNA double-strand break repair pathway choice - from basic

2. Results – Manuscript III

- biology to clinical exploitation. *Cell Cycle* 18, 1423-1434. 10.1080/15384101.2019.1618542.
21. Wang, K., Patkar, S., Lee, J.S., Gertz, E.M., Robinson, W., Schischlik, F., Crawford, D.R., Schaffer, A.A., and Rupp, E. (2022). Deconvolving Clinically Relevant Cellular Immune Cross-talk from Bulk Gene Expression Using CODEFACS and LIRICS Stratifies Patients with Melanoma to Anti-PD-1 Therapy. *Cancer Discov* 12, 1088-1105. 10.1158/21598290.CD-21-0887.
22. DuPage, M., Dooley, A.L., and Jacks, T. (2009). Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nat Protoc* 4, 1064-1072. 10.1038/nprot.2009.95.
23. Schmitt, A., Knittel, G., Welcker, D., Yang, T.P., George, J., Nowak, M., Leeser, U., Buttnar, R., Perner, S., Peifer, M., and Reinhardt, H.C. (2017). ATM Deficiency Is Associated with Sensitivity to PARP1- and ATR Inhibitors in Lung Adenocarcinoma. *Cancer Res* 77, 3040-3056. 10.1158/0008-5472.CAN-16-3398.
24. Jin, H., Gulhan, D.C., Geiger, B., Ben-Isvy, D., Geng, D., Ljungstrom, V., and Park, P.J. (2024). Accurate and sensitive mutational signature analysis with MuSiCal. *Nat Genet* 56, 541-552. 10.1038/s41588-024-01659-0.
25. Farmer, H., McCabe, N., Lord, C.J., Tutt, A.N., Johnson, D.A., Richardson, T.B., Santarosa, M., Dillon, K.J., Hickson, I., Knights, C., et al. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 434, 917-921. 10.1038/nature03445.
26. Kouranti, I., and Peyroche, A. (2012). Protein degradation in DNA damage response. *Semin Cell Dev Biol* 23, 538-545. 10.1016/j.semcdb.2012.02.004.
27. Kurlawala, Z., Dunaway, R., Shah, P.P., Gosney, J.A., Siskind, L.J., Ceresa, B.P., and Beverly, L.J. (2017). Regulation of insulin-like growth factor receptors by Ubiquilin1. *Biochem J* 474, 4105-4118. 10.1042/BCJ20170620.
28. Kurlawala, Z., Shah, P.P., Shah, C., and Beverly, L.J. (2017). The ST1 and UBA Domains of UBQLN1 Are Critical Determinants of Substrate Interaction and Proteostasis. *J Cell Biochem* 118, 2261-2270. 10.1002/jcb.25880.
29. Beverly, L.J., Lockwood, W.W., Shah, P.P., Erdjument-Bromage, H., and Varmus, H. (2012). Ubiquitination, localization, and stability of an anti-apoptotic BCL2-like protein, BCL2L10/BCLb, are regulated by Ubiquilin1. *Proc Natl Acad Sci U S A* 109, E119-126. 10.1073/pnas.1119167109.
30. Viswanathan, J., Haapasalo, A., Bottcher, C., Miettinen, R., Kurkinen, K.M., Lu, A., Thomas, A., Maynard, C.J., Romano, D., Hyman, B.T., et al. (2011). Alzheimer's disease-associated ubiquilin-1 regulates presenilin-1 accumulation and aggregate formation. *Traffic* 12, 330-348. 10.1111/j.1600-0854.2010.01149.x.
31. Kobayashi, Y., Bustos, M.A., Shoji, Y., Jachimowicz, R.D., Shiloh, Y., and Hoon, D.S.B. (2022). Genomic Amplification of UBQLN4 Is a Prognostic and Treatment Resistance Factor. *Cells* 11, 10.3390/cells11203311.
32. Feng, X., Cao, A., Qin, T., Zhang, Q., Fan, S., Wang, B., Song, B., Yu, X., and Li, L. (2021). Abnormally elevated ubiquilin-1 expression in breast cancer regulates metastasis and stemness via AKT signaling. *Oncol Rep* 46, 10.3892/or.2021.8187.
33. Bao, J., Jiang, X., Zhu, X., Dai, G., Dou, R., Liu, X., Sheng, H., Liang, Z., and Yu, H. (2018). Clinical significance of ubiquilin 1 in gastric cancer. *Medicine (Baltimore)* 97, e9701. 10.1097/MD.00000000000009701.
34. Huang, A.C., and Zappasodi, R. (2022). A decade of checkpoint blockade immunotherapy in melanoma: understanding the molecular basis for immune sensitivity and resistance. *Nat Immunol* 23, 660-670. 10.1038/s41590-022-01141-1.
35. Snyder, A., Makarov, V., Merghoub, T., Yuan, J., Zaretsky, J.M., Desrichard, A., Walsh, L.A., Postow, M.A., Wong, P., Ho, T.S., et al. (2014). Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N Engl J Med* 371, 2189-2199. 10.1056/NEJMoa1406498.
36. Muquith, M., Espinoza, M., Elliott, A., Xiu, J., Seeber, A., El-Deiry, W., Antonarakis, E.S., Graff, S.L., Hall, M.J., Borghaei, H., et al. (2024). Tissue-specific thresholds of mutation burden associated with anti-PD-1/L1 therapy benefit and prognosis in microsatellite-stable cancers. *Nat Cancer* 5, 1121-1129. 10.1038/s43018-024-00752-x.
37. Balaji, V., Muller, L., Lorenz, R., Kevei, E., Zhang, W.H., Santiago, U., Gebauer, J., Llamas, E., Vilchez, D., Camacho, C.J., et al. (2022). A dimer-monomer switch controls CHIP-dependent substrate ubiquitylation and processing. *Mol Cell* 82, 3239-3254 e3211. 10.1016/j.molcel.2022.08.003.
38. Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25, 1754-1760. 10.1093/bioinformatics/btp324.
39. Patro, R., Duggal, G., Love, M.I., Irizarry, R.A., and Kingsford, C. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nat Methods* 14, 417-419. 10.1038/nmeth.4197.
40. Yu, A.M., and McVey, M. (2010). Synthesis-dependent microhomology-mediated end joining accounts for multiple types of repair junctions. *Nucleic Acids Res* 38, 5706-5717. 10.1093/nar/gkq379.
41. Muller, L., Kutzner, C.E., Balaji, V., and Hoppe, T. (2021). In Vitro Analysis of E3 Ubiquitin Ligase Function. *J Vis Exp*. 10.3791/62393.
42. Lopez-Saavedra, A., Gomez-Cabello, D., Dominguez-Sanchez, M.S., Mejias-Navarro, F., Fernandez-Avila, M.J., Dinant, C., Martinez-Macias, M.I., Bartek, J., and Huertas, P. (2016). A genome-wide screening uncovers the role of CCAR2 as an antagonist of DNA end resection. *Nat Commun* 7, 12364. 10.1038/ncomms12364.
43. Karaca, E., Harel, T., Pehlivan, D., Jhangiani, S.N., Gambin, T., Coban Akdemir, Z., Gonzaga-Jauregui, C., Erdin, S., Bayram, Y., Campbell, I.M., et al. (2015). Genes that Affect Brain Structure and Function Identified by Rare Variant Analyses of Mendelian Neurologic Disease. *Neuron* 88, 499-513. 10.1016/j.neuron.2015.09.048.
44. Li, C., Zhi, D., Wang, K., and Liu, X. (2022). MetaRNN: differentiating rare pathogenic and rare benign missense SNVs and InDels using deep learning. *Genome Med* 14, 115. 10.1186/s13073-022-01120-z.

45. Vivian, J., Rao, A.A., Nothhaft, F.A., Ketchum, C., Armstrong, J., Novak, A., Pfeil, J., Narkizian, J., Deran, A.D., Musselman-Brown, A., et al. (2017). Toil enables reproducible, open source, big biomedical data analyses. *Nat Biotechnol* 35, 314-316. 10.1038/nbt.3772.
46. Liu, J., Lichtenberg, T., Hoadley, K.A., Poisson, L.M., Lazar, A.J., Cherniack, A.D., Kovatich, A.J., Benz, C.C., Levine, D.A., Lee, A.V., et al. (2018). An Integrated TCGA Pan-Cancer Clinical Data Resource to Drive High-Quality Survival Outcome Analytics. *Cell* 173, 400-416 e411. 10.1016/j.cell.2018.02.052.
47. Kim, N., Kim, H.K., Lee, K., Hong, Y., Cho, J.H., Choi, J.W., Lee, J.I., Suh, Y.L., Ku, B.M., Eum, H.H., et al. (2020). Single-cell RNA sequencing demonstrates the molecular and cellular reprogramming of metastatic lung adenocarcinoma. *Nat Commun* 11, 2285. 10.1038/s41467-020-16164-1.
48. Troder, S.E., Ebert, L.K., Butt, L., Assenmacher, S., Schermer, B., and Zevnik, B. (2018). An optimized electroporation approach for efficient CRISPR/Cas9 genome editing in murine zygotes. *PLoS One* 13, e0196891. 10.1371/journal.pone.0196891.
49. Cristescu, R., Mogg, R., Ayers, M., Albright, A., Murphy, E., Yearley, J., Sher, X., Liu, X.Q., Lu, H., Nebozhyn, M., et al. (2018). Pan-tumor genomic biomarkers for PD-1 checkpoint blockade-based immunotherapy. *Science* 362. 10.1126/science.aar3593.
50. Hirsch, F.R., McElhinny, A., Stanforth, D., Ranger-Moore, J., Jansson, M., Kulangara, K., Richardson, W., Towne, P., Hanks, D., Vennapusa, B., et al. (2017). PD-L1 Immunohistochemistry Assays for Lung Cancer: Results from Phase 1 of the Blueprint PD-L1 IHC Assay Comparison Project. *J Thorac Oncol* 12, 208-222. 10.1016/j.jtho.2016.11.2228.
51. Di Tommaso, P., Chatzou, M., Floden, E.W., Barja, P.P., Palumbo, E., and Notredame, C. (2017). Nextflow enables reproducible computational workflows. *Nat Biotechnol* 35, 316-319. 10.1038/nbt.3820.
52. George, J., Maas, L., Abedpour, N., Cartolano, M., Kaiser, L., Fischer, R.N., Scheel, A.H., Weber, J.P., Hellmich, M., Bosco, G., et al. (2024). Evolutionary trajectories of small cell lung cancer under therapy. *Nature* 627, 880-889. 10.1038/s41586-024-07177-7.

2.4 Manuscript IV: Targeting a Glutamic Acid in PDE δ with Fluoromethyl-Aryl Electrophiles Impairs KRAS Signaling

Journal of Medicinal Chemistry, January 2026

PMID: 41499451

doi: <https://doi.org/10.1021/acs.jmedchem.5c02082>

Ruirui Zhang*, Maxim A. Huetzen*, Aylin Binici*, Pablo Martín-Gago*, Raphael Gasper, Elena Rudashevskaya, Jie Liu, Chinta Nagaraju, Elena S. Reckzeh, Alana S.T. Stuedle, Ann-Sophie Hopff, Andrea Mesaros, Anke Unger, Melanie Thelen, Petra Janning, H. Christian Reinhardt, Slava Ziegler, Ron D. Jachimowicz[#], Herbert Waldmann[#]

2.4.1 Contributions

As shared first-author, I led the preclinical *in vivo* investigation that established Deltafluorine's therapeutic efficacy in the autochthonous KP lung cancer model, contributing substantially to experimental design, execution, data analysis, and manuscript preparation.

I designed and executed all mouse experiments, including tumor induction via intratracheal Adeno-CMV-Cre virus instillation, daily Deltafluorine treatment administration with support of the Comparative Biology Core Facility, and longitudinal μ CT imaging for tumor monitoring together with A.S.T.S. (**IV-Figure 6**). I developed the 3D computed tomography analysis workflow for tumor burden quantification with A.S.T.S. and A.M., applying computer-aided volumetry methods to reconstruct μ CT scans and measure tumor volumes with high precision. I generated associated figures and visualizations.

During revision, I performed additional validation experiments, generating immunoblotting data together with A.S.H., which confirmed proteomics findings on downstream MAPK and mTOR pathway suppression (**IV-Figure 4c**, **IV-Figure S6**, **S10**). I coordinated collaborative efforts with researchers at the MPI for Molecular Physiology in Dortmund for compound synthesis, structural biology, and proteomics, and with the Comparative Biology Core Facility and Phenotyping Core Facility at the MPI for Biology of Ageing for mouse experiments and imaging infrastructure. R.-R.Z. integrated project components, led the chemistry and proteomics studies, and wrote the first draft. I prepared and edited the manuscript collaboratively with P.M.G., A.B., S.Z., R.D.J., and H.W., contributing substantially to sections describing preclinical mouse data, therapeutic efficacy, and translational implications.

The quantitative 3D imaging methodology developed here can be applied to future therapeutic evaluations in genetically engineered mouse models of aggressive cancers. Overall, this work established that covalent PDE δ inhibition with Deltafluorine produces significant tumor burden reduction in an aggressive autochthonous lung adenocarcinoma mouse model.

2.4.2 Peer-reviewed publication

The published manuscript PDF is attached in this section. Original page numbers and an advertisement on the last page of the references were removed for formatting reasons. The publisher allows the reuse of this *Published Article* in the dissertation. (<https://pubs.acs.org/page/authors/sharing.htm#reuse-theses>)

Targeting a Glutamic Acid in PDE δ with Fluoromethyl-Aryl Electrophiles Impairs K-Ras Signaling

Ruirui Zhang,^{††} Maxim A. Huetzen,^{††} Aylin Binici,^{††} Pablo Martín-Gago,^{††} Raphael Gasper, Elena Rudashevskaya, Jie Liu, Chinta Nagaraju, Elena S. Reckzeh, Alana S. T. Stuedle, Ann-Sophie Hopff, Andrea Mesaros, Anke Unger, Melanie Thelen, Petra Janning, H. Christian Reinhardt, Slava Ziegler, Ron D. Jachimowicz,* and Herbert Waldmann*



Cite This: *J. Med. Chem.* 2026, 69, 964–981



Read Online

ACCESS |



Metrics & More

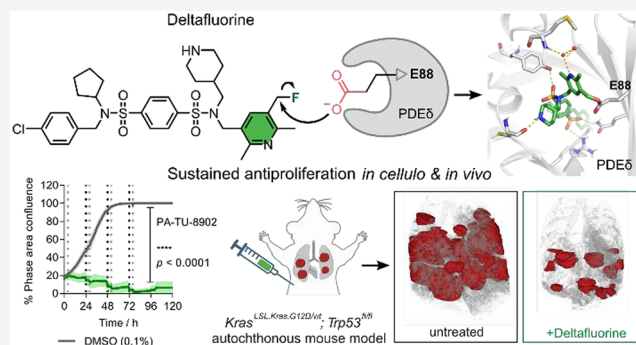


Article Recommendations



Supporting Information

ABSTRACT: For targeted covalent modification at low-reactivity carboxylates with biocompatible electrophiles, new approaches are in high demand. Engineering of the HaloTag protein facilitates such a covalent reaction between chloroalkanes and an aspartate residue. We demonstrate that conversely, engineering stable ligands can also enable covalent targeting of an acid residue in a protein binding site. Using the chaperone PDE δ , which shuttles lipidated oncoproteins and thereby mediates their signaling activity, we show that equipping noncovalent inhibitors with a benzyl fluoride-based electrophile leads to covalent modification of a specific glutamate p.E88 in the ligand binding site. The best inhibitor, Deltafluorine, embodies a 3-fluoromethyl-pyridyl group and is stable to nucleophiles like glutathione, phosphate, acetate, and citrate. In cells, Deltafluorine combines noncovalent and covalent reactivity to demonstrate distinct cellular profiles and inhibits signaling through the MAP-kinase and Akt-mTOR pathways. In an autochthonous mouse model of highly aggressive Kras^{G12D}-driven lung adenocarcinoma, Deltafluorine treatment significantly reduces tumor volume.



INTRODUCTION

Small molecules equipped with electrophilic reactive groups are invaluable tools for the study of protein function and hold significant therapeutic potential.^{1–3} Covalent targeting offers a reactivity-driven strategy that is particularly effective for the selective modification of specific residues.^{1–3} Among nucleophilic amino acid residues suitable for covalent strategies, carboxylates (Glu, E and Asp, D) are especially notable, as these residues are abundant in the proteome (approximately 12%). They are essential for numerous biochemical processes, functionally diverse, and often display heightened or aberrant activity in key drug targets,^{4–6} which makes them appealing sites for targeted covalent ligand discovery.^{7,8} However, due to their low intrinsic nucleophilicity, these residues in general are targeted with reactive electrophiles, which may have limited stability in physiologically relevant media and be prone to also covalently react with more nucleophilic amino acids, in particular Cys and Lys, such that Glu and Asp residues have thus far been targeted covalently in relatively few instances.^{5,6,9–19} Such undesired off-target reactivity and limited stability might be overcome if carboxylates in binding sites could be targeted with low reactivity electrophiles, and new approaches to achieve this goal are in high demand.

We recently developed a covalent glutamic acid targeting strategy utilizing biocompatible and selective warheads inspired by the HaloTag technology.²⁰ The HaloTag system employs a covalent conjugation reaction between ligands with a reactive chloroalkane linker and aspartic acid D106 in the HaloTag protein, forming a stable ester construct (Figure 1a).²¹ In parallel, covalent reactions were achieved in lipoprotein binding chaperone phosphodiesterase of retinal rod delta subunit (PDE δ) with alkyl bromides derived from reversible inhibitors to target its binding site glutamate p.E88.²⁰ In contrast to the HaloTag system, however, we observed that the corresponding alkyl chlorides derived from the same reversible PDE δ inhibitor failed to react with p.E88 in PDE δ .²⁰ The reactivity of alkyl chlorides for covalency in the HaloTag system is achieved through generations of protein engineering, during which key amino acid residues were selected for site-specific mutagenesis

Received: July 24, 2025

Revised: December 15, 2025

Accepted: December 19, 2025

Published: January 7, 2026



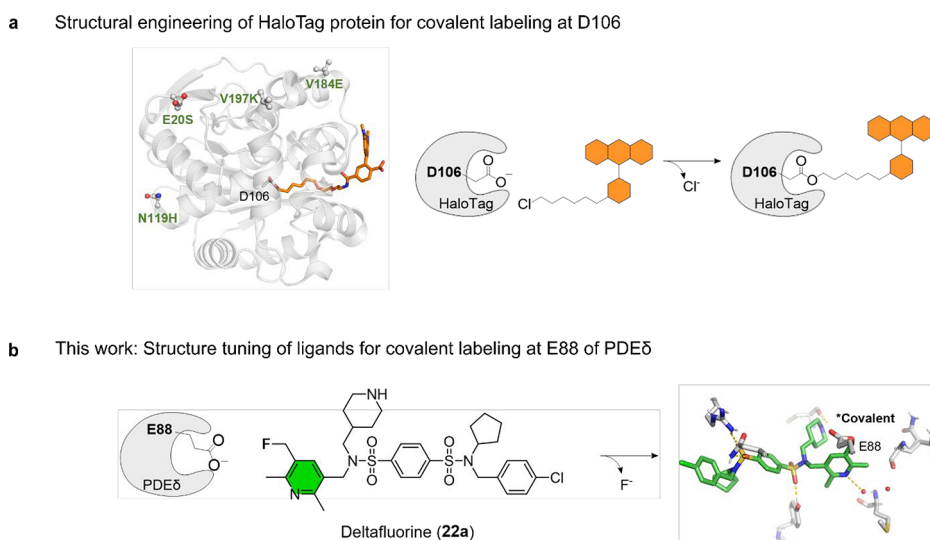


Figure 1. Concept for structural tuning to enable covalent targeting of carboxylate residues. (a) Structural engineering of HaloTag protein to optimize covalent binding between chloroalkane ligands and aspartate D106. Crystal structure of a TMR ligand bound to HaloTag protein (PDB code: 6Y7A) is shown.⁵² Beneficial mutation sites (green labels, specifically E20S, N119H, V184E, and V197 K mutations) that improve labeling speed are highlighted with ball-and-stick representations of side chains.²⁵ (b) This work: structural tuning of small molecule ligands to achieve covalency with a pyridylmethyl fluoride-based warhead. Crystal structure of the covalent adduct formed from Deltafluorine (22a) and PDEδ (this work; PDB code: 9RP7) is shown with key interactions in the PDEδ prenyl binding pocket (hydrogen bond as yellow dotted line, red spheres as bound water molecules). Structural development of Deltafluorine (22a) was guided by cocrystal structures of previously developed inhibitors with PDEδ (PDB codes: 4JVF, SE80, SML3, SNAL, and 9HMD,^{19,20,33–35} see Figure S1 for details).

to optimize the binding site and disposition of D106 for labeling kinetics and duration (Figure 1a).^{21–25} We reasoned that, alternatively, the precise position and orientation of the warhead toward the selected residue for covalency would also be possible from a classical ligand-directed TCI approach. We envisioned that with structural fine-tuning of small molecule ligands, targeted covalent binding can be achieved with innately low-reactivity warheads, such as alkyl fluoride-based electrophiles, which may offer reduced unspecific activity for covalent compounds (Figure 1b).²⁶

The intrinsic reactivity of fluoride motifs to nucleophiles was expected to be low, with poor leaving group properties of fluoride and the inherent stability of the C–F bond.^{27,28} However, in a few instances, fluoroalkyl groups have been employed to covalently target cysteines in cells.^{29,30} Additionally, the reactivity of alkyl halides in the α -position to carbonyl groups can be significantly enhanced, enabling α -fluoromethyl ketones to be employed as protease or kinase probes with demonstrated reactivity toward cysteines, and typically catalytic serines and threonines.^{12,31,32} The enhancement of the halide reactivity also applies to the benzylic position, albeit to a lesser extent. We therefore focused on covalent ligands incorporating benzyl fluoride-based warheads to achieve targeted covalency at p.E88 in the PDEδ model system. For compound design, we drew from insight gained during the development of potent reversible inhibitors, as well as two covalent inhibitor chemotypes previously reported (Figure S1).^{20,33–35} More reactive benzyl chloride-based counterparts were also considered for comparison during structure–activity analysis and refinement.

The lipoprotein binding chaperone PDEδ binds the prenylated termini of its lipidated cargos, including GTPases such as Ras, Rab, Rheb, and Rho proteins.³⁶ PDEδ establishes the dynamic intracellular localization of these cargos, for instance, the Ras^{33–35} and Rheb proteins,³⁷ and thereby plays an essential role in the regulation of their membrane localization and proper function. For inhibitor development targeting PDEδ,

we^{33–35} and others (the Abankwa group,^{38,39} the Ismail group,⁴⁰ and the Sheng group,^{41–44}) have reported several potent (K_D values in the low nanomolar and picomolar range), reversible inhibitors of PDEδ,^{33–35} but their efficacy is intrinsically limited by the counteracting Arl2/3 GTPases, which upon allosteric binding stabilize an ‘open’ form of PDEδ such that even high-affinity ligands have an increased off-rate and are released from the lipoprotein binding site.³⁷ PDEδ degraders that can act substoichiometrically circumvented the requirement for permanent binding PDEδ and showed improved antitumor activity as proof-of-concept for the design strategy.^{45–48} On the other hand, covalent-acting Deltasonamide-derived inhibitor bearing an isoxazolium warhead¹⁹ and Deltazinone-derived DeltaTag bearing an alkyl bromide warhead²⁰ overcame ligand release by Arl2, yet their physiological and PK/PD stability largely limited further *in vivo* validation of antitumor activity.

Herein, we present the development and evaluation of benzyl fluoride-based ligands to achieve targeted modification at p.E88 in PDEδ. We demonstrate that ligand-focused structural fine-tuning may enable covalent modification of a low reactivity nucleophile with an inherently unreactive electrophile. The best fluoro-substituted ligand, termed Deltafluorine, achieves slow yet sustained covalent labeling of PDEδ (>85%) *in vitro* over a 7 day incubation at physiologically relevant conditions and induces a marked difference in phenotypic cellular profiles when compared to its nonfluorine-containing reversible counterpart. We also demonstrate that Deltafluorine impacts signal transduction through the MAPK family- and PI3K-Akt-mTOR signaling cascades, where PDEδ cargos such as the GTPases Ras and Rheb play a central role.^{35,36,49–51} Moreover, application of Deltafluorine in KRAS-dependent human and murine lung cancer models, both *in cellulo* and *in vivo*, proves antiproliferative activity of the inhibitor. These findings highlight the potential of fluoromethyl-substituted aryl systems for targeted covalent labeling of a specific carboxylate residue in proteins. With the observed promising biological effects of

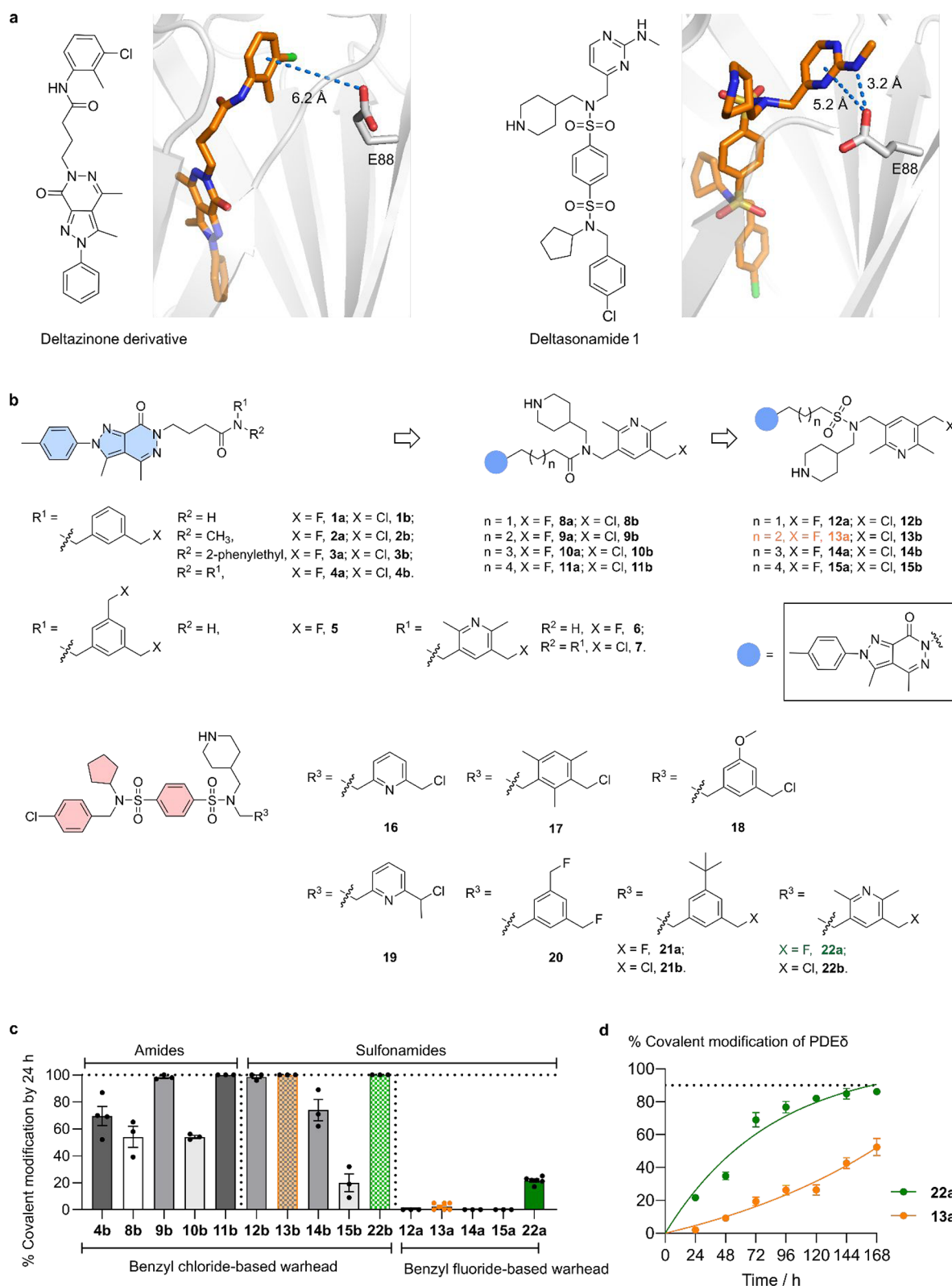


Figure 2. Structural development of halide electrophiles for covalent modification of PDE δ . (a) Cocrystal structures of Deltazinone derivative (PDB code: 5E80)³³ and Deltasonamide 1 (PDB code: 5ML3)³⁴ with PDE δ , highlighting the distance of the distal aryl ring to its glutamic acid residue p.E88. (b) Structures of Deltazinone-based and Deltasonamide-based compounds. IC₅₀ values of PDE δ binding for 13a and 22a were determined to be 336 ± 106 nM and 27 ± 13 nM, respectively, by a competitive fluorescence polarization assay; their K_D values were further characterized with isothermal titration calorimetry (ITC) to be 667 ± 20 nM for 13a and 148 ± 9 nM for 22a, see Figure S3 for details. (c) Covalent modification [%] of representative compounds at 24 h as determined by MALDI mass spectrometry. PDE δ (20 μ M) was incubated with compounds (60 μ M, 0.6% DMSO) in HEPES buffer (20 mM HEPES, 150 mM NaCl, pH = 7.5) at 37 $^{\circ}$ C for 24 h before analysis by MALDI. Percentages of covalent adduct formation were estimated by the relative intensity of the respective peaks in MALDI spectra. Data are presented as mean $\pm$ standard error of the mean

Figure 2. continued

($n = 3$, except $n = 6$ for **13a** and **22a**). (d) Kinetics of covalent modification of PDE δ (20 μ M) by **13a** and **22a** (60 μ M). Data are presented as mean $\pm$ s.e.m. ($n = 6$).

deltafluorine, it could also offer an exciting new therapeutic strategy for cancer treatment.

RESULTS

Structural Development of Benzyl Halide Electrophiles for Covalent Targeting of a Glutamic Acid

To explore the possibility of covalently targeting PDE δ at its glutamic acid residue p.E88 with benzyl halide electrophiles, we examined the cocrystal structures of the reversible inhibitors Deltazinone derivative and Deltasonamide 1 and found that the distance of the common aryl moiety at the distal end of the structures to the p.E88 residue in the protein is approximately 3–6 Å, which suggested the suitability to attach a one-carbon unit reactive group (Figure 2a). Therefore, we structurally modified Deltazinone and Deltasonamide at the position proximal to p.E88 with benzyl halide electrophiles, yielding structures **1–22** (Figure 2b), and screened for covalent modification of PDE δ by matrix-assisted laser desorption/ionization (MALDI) intact protein mass spectrometry. In this screen, the degree of covalent modification was estimated from the relative intensity of the signals recorded for the formed adducts (Figure 2c, see Figure S2 for details). For structural development based on Deltazinone 1, initial investigations (**1–7**, Figure 2b) found relatively low labeling efficiency (Figure S2). While we observed a moderate extent of modification with the reactive benzyl chloride warheads (at most $70 \pm 7\%$ modification with **4b** by 24 h at 37 °C, Figure 2c), all benzyl fluoride-based warheads failed to label PDE δ under the same conditions. To improve the binding affinity of Deltazinone-based structures, we incorporated a piperidine ring into the side chain of the amide, which is found in both the more potent Deltarasin ($K_D = 38 \pm 16$ nM) and Deltasonamide 1 ($K_D = 203 \pm 31$ pM) and forms a hydrogen bond with the carbonyl group of Cys56 (CS6) of PDE δ (Figure S1).^{34,35} This fragment-based hybrid design strategy³³ was successfully employed in the development of Deltazinone-based DeltaTag in the covalent modification of PDE δ (Figure S1).²⁰ However, although pyridylmethyl-chloride-containing amides **8–11** displayed covalent labeling efficiency up to 100% after 24 h, we could not observe any reactivity of the fluoride counterparts (Figures 2c and S2). For unsymmetrically substituted amides, we consistently observed rotamers due to slow rotation around the amide bond, which might limit the extent of covalent modification. Bioisosteric replacement of amides by sulfonamides⁵⁴ overcame this limitation, as previously exemplified in the development of DeltaTag in covalent labeling of PDE δ .²⁰ Consistent with the bioisosteric approach yielding sulfonamides **12–15**, we further improved labeling efficiency to 100% by 30 min with the pyridylmethyl chloride warhead in **13b** (Figures 2c and S2) and interestingly observed some very limited labeling of $2 \pm 1\%$ with the fluoride counterpart in **13a** at 24 h (Figure 2c).

In parallel, structural modifications of the Deltasonamide structure similarly yielded a series of compounds (**16–18**, **21–22b**) that achieved fast labeling of PDE δ in vitro with reactive benzyl chloride-based warheads (100% by 24 h, Figures 2c and S2) and some modification with the fluoride counterpart (**22a**, $22 \pm 1\%$ by 24 h, Figures 2c and S2). Among compounds

bearing benzyl fluoride-based warheads, the compound with the highest labeling efficiency was **22a**, which achieved a maximum modification of $86 \pm 2\%$ after 7 days (with an approximate second-order rate constant $k_{\text{inact}}/K_I = 5.4 \text{ M}^{-1} \text{ s}^{-1}$, Figure S2c), while the Deltazinone-based counterpart **13a** was limited to $52 \pm 5\%$ labeling over this extended time frame (Figure 2d).

Chloroalkane and chloromethyl-substituted aryl ring-based warheads are known to covalently label cysteine residues in proteins.^{12,55,56} We therefore investigated whether compounds **9b**, **11b**, and **13b**, which all showed rapid and full labeling of PDE δ by 24 h (Figures 2c and 3a), indeed modified p.E88 in its binding pocket instead of other reactive residues, including cysteines. We analyzed covalent adducts formed from PDE δ and these compounds with mass spectrometric evaluation of the peptides formed following a Glu-C digestion and identified the peptide sequence QKVQKVYFKGQCLEE with the exact mass, which corresponds to p.E88-modified adduct peptide (amino acids 78–89 of PDE δ , Figure 3b). The results indicated that **9b** and **13b**, both with four methylene units between the pyrazolopyridazinone fragment and the amide or sulfonamide, covalently modified PDE δ at a glutamate (either at p.E88 or at p.E89) with a higher probability for modification at p.E88 (Figure 3b). We also searched in parallel for other potential nucleophilic sites for covalent modifications (for example, other Glu, Asp, Cys, Thr, Ser, Tyr, Lys, and His residues) and did not observe other modified peptides (Table S1), suggesting that the selectivity of labeling the weakly nucleophilic glutamic acid p.E88 as compared to other more reactive and/or more accessible residues of PDE δ was conferred by the relatively high affinity of the core Deltazinone scaffold (Deltazinone 1: $K_D = 8 \pm 4$ nM) to its binding pocket. It is noteworthy that for amide structure **11b** with six methylene units between the pyrazolopyridazinone fragment and the amide, we identified other possible modification sites, consistently at p.E114/S115 in addition to p.E88/E89 (Figure 3b and Table S1). While **9b** and **11b** demonstrated similar kinetics of modifying PDE δ over 24 h in vitro (Figure 3a), consistent with the results for the sulfonamides series **12–15**, the linker length of four methylene units was the most favorable. As with **9b**, the labeling kinetics represents selective modification at p.E88, while with **11b**, the comparable kinetics reflects heterogeneous modification at different nucleophilic residues, especially as p.E114 and p.S115 are located in proximity at the entrance of the PDE δ pocket and are surface exposed. This result reinstates the significance of structural optimization of the core scaffold for correct positioning and orientation of the electrophilic warhead toward the desired amino acid residue to control reaction specificity.

Although **13b** exhibited favorable reactivity toward glutamate p.E88 in PDE δ , its instability in aqueous media and high reactivity with GSH (Figure S4) led us to discontinue further investigation. In contrast, in vitro stability and GSH reactivity tests demonstrated that the alkyl fluoride-based warhead in **22a** was highly stable and nonreactive in the presence of different nucleophiles such as glutathione, phosphate, acetate, and citrate in aqueous environments (Figure S4). The stability of the warhead and its demonstrated reactivity toward glutamate p.E88 in PDE δ prompted us to investigate **22a** further. We analyzed the cocrystal structure of the adduct formed from the treatment

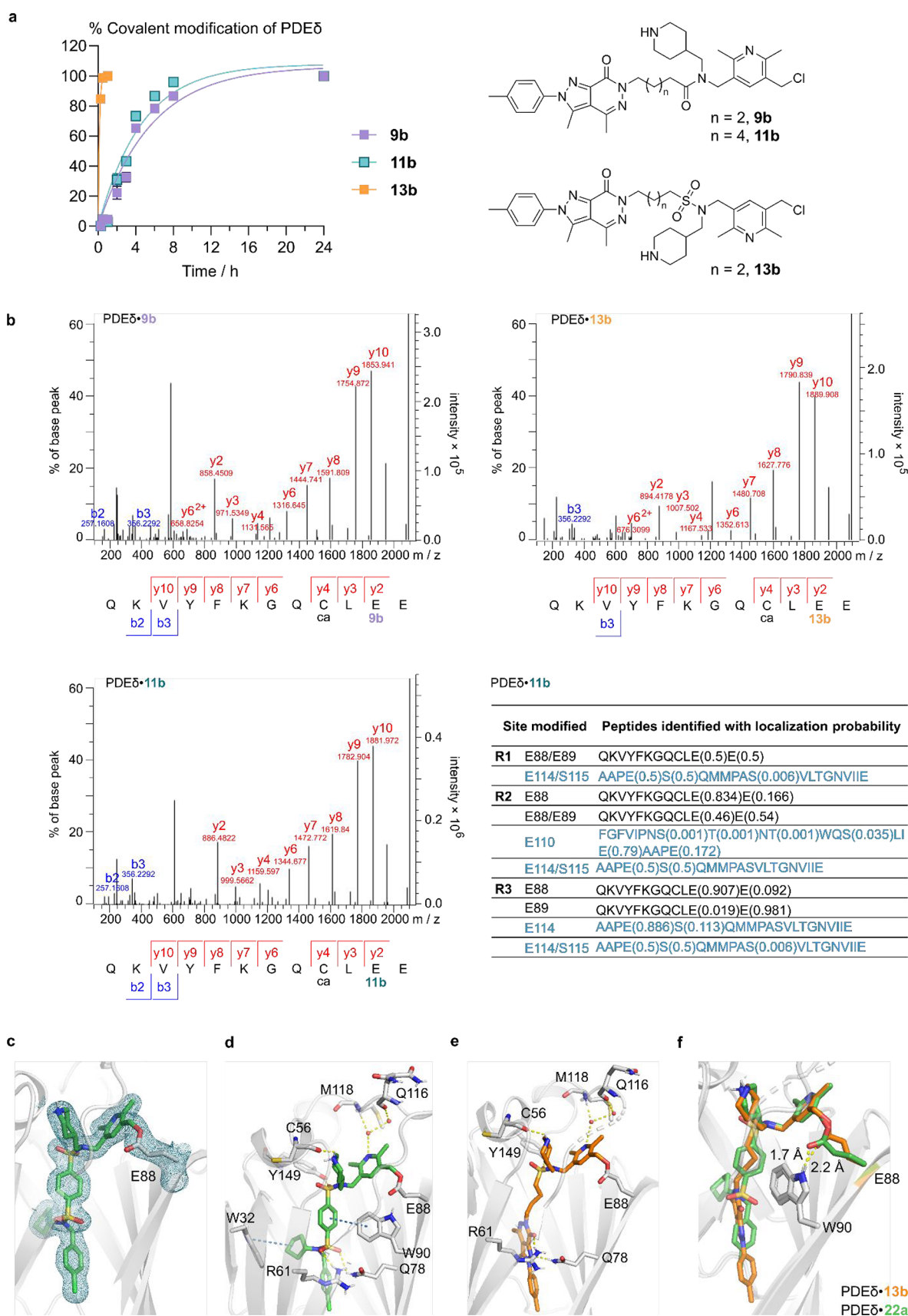
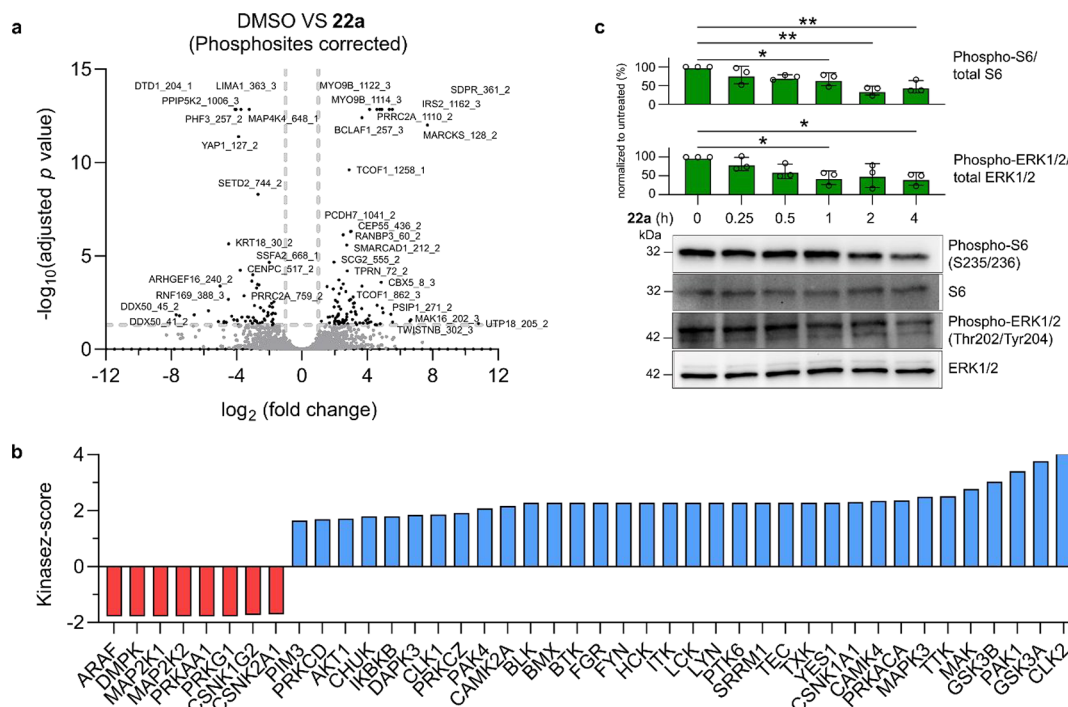


Figure 3. Structural basis for selectivity of covalent modification at p.E88 and reactivity of benzyl fluoride-based warheads. (a) Kinetics of covalent modification of PDE δ by 9b, 11b, and 13b. PDE δ (20 μ M) was incubated with compounds (60 μ M, 0.6% DMSO) in HEPES buffer (20 mM HEPES, 150 mM NaCl, pH = 7.5) at 37 °C before analysis by MALDI. Percentages of covalent adduct formation were estimated by the relative intensity of the respective peaks in MALDI spectra. Data are presented as mean $\pm$ s.e.m. (n = 3). (b) Representative LC–MS/MS spectra of compound 9b-, 11b-, or

13b-modified PDE δ peptides (amino acid 78–89), showing the modification site localization at p.E88. The sequence and detected fragments of the peptide are shown. Sequence-matched peaks are labeled in red and blue. Identified modifications at the amino acids are indicated below the peptide sequence—“ca” stands for carbamidomethylation of cysteine. Other **11b**-modified adduct peptides are shown in the table with the peptide sequence and localization probability. (c) $2F_o - F_c$ map for ligand **22a** and p.E88 in cyan mesh ($\sigma = 1.0$). (d,e) Key bonding interactions of ligand **22a** (green) and **13b** (orange) to PDE δ , respectively. Hydrogen bonds are shown as yellow dotted line; aromatic- π interactions as blue dotted line. Bound water molecules are represented as red spheres. (f) Superimposition of X-ray cocrystal structures of covalent adducts, PDE δ -**22a** (1.9 Å, PDB code: 9RP7) and PDE δ -**13b** (1.8 Å, PDB code: 9RP6). See [Table S5](#) for details of X-ray Crystallography Data Collection and Refinement Statistics.



Trp32 (W32) and Trp90 (W90) to **22a** (Figure 3d) provide enhanced stabilization, which might explain the observation of enhanced apparent binding affinity of **22a** ($K_D = 148 \pm 9$ nM for **22a** and $K_D = 667 \pm 20$ nM for **13a**, Figure S3). Overlay of the crystal structures indicated that the ligand from **13b** with the Deltazinone scaffold is located considerably deeper in the prenyl binding pocket of PDE δ as compared to **22a**. Additionally, the ester bond formed between ligand **13b** and the p.E88 residue is oriented at a different angle relative to the ester bond in covalent adduct PDE δ -**22a** (Figure 3f). The carbonyl group of p.E88 forms a hydrogen bond with the proximal Trp90 (W90), which was determined as 1.7 and 2.2 Å, respectively, in the covalent PDE δ -**22a** and PDE δ -**13a** adducts (Figure 3f). The significant difference in binding affinity, relative position of the ligands in the binding pocket of PDE δ , and the different orientation of the p.E88 side chain might provide an explanation why **22a** exhibited approximately twice the labeling rate of PDE δ compared to **13a** in vitro. The much higher binding affinity stabilized **22a** in the PDE δ binding pocket, and the higher

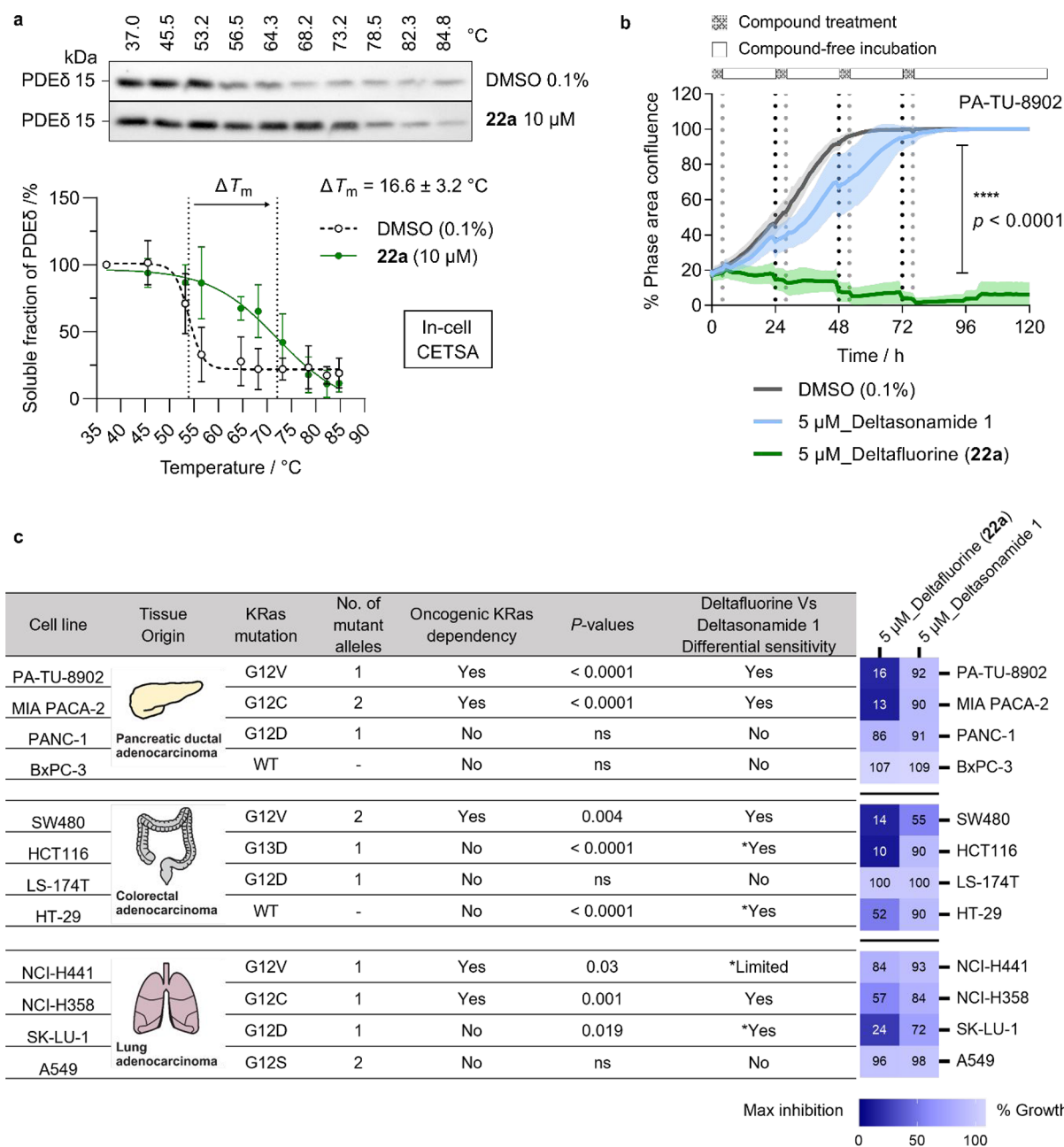


Figure 5. Profiling of Deltafluorine (**22a**) in KRAS mutant cell lines. (a) In-cell CETSA. Jurkat cells were treated with the compound (**22a**) at 10 μ M or vehicle (0.1% DMSO) for 2 h under live-cell settings before cell lysis and heat treatment. Intensities of the PDE δ immunoblot bands were analyzed by Image Lab and normalized to the first band of each condition. Data are presented as mean $\pm$ standard deviation, representative of three biological replicates ($n = 3$, see Figure S11 for details). (b) Scheme and representative cellular growth curves of the wash-out assay in PA-TU-8902 cells. Time-points for compound addition are marked with black dotted lines and compound removal by washing with fresh culture medium are marked with gray dotted lines. Data are presented as mean $\pm$ standard deviation, representative of three biological replicates ($n = 3$). (c) Overview of growth inhibition in the wash-out assay for the selected cell panel. Cell growth was determined by area under the curve integration at 120 h after the first dose of compound treatment monitored by real-time cell analysis of percent phase area confluence, normalized to DMSO control. Mean values of relative growth were plotted for the heat map, representative of three biological replicates ($n = 3$). Unpaired t -tests were carried out between Deltafluorine (**22a**) and Deltasonamide 1 treatment conditions with two tailed p -values denoting differential sensitivity of Deltafluorine (**22a**) in each cell line. Exceptions to the general correlation between differential sensitivity of Deltafluorine (**22a**) in comparison to Deltasonamide 1 and KRAS dependency are marked with * in the table.

positioning of **22a** (Figure 3f) and the closer distance of the aryl moiety to p.E88 (approximately 3 Å, Figure 2a) could orient the pyridylmethyl fluoride warhead in close proximity to the carboxylate residue with a high degree of overlap in their molecular orbitals, forcing a covalent reaction. In contrast, **13a** binds to PDE δ with a much weaker binding affinity, and the

deeper and lower position of the Deltazinone scaffold in the binding pocket left the same warhead more distant from p.E88. The more reactive pyridylmethyl chloride in **13b**, with chloride being a much better leaving group, could forge a covalent modification with an altered orientation of p.E88 away from its interaction with W90 to accommodate a bond formation with

adjusted angles, but we hypothesized that the probability of the same reaction could be much lower with the fluoride counterpart in **13a**. This observation highlights the potential for structural fine-tuning of small-molecule ligands to achieve targeted covalent labeling of a glutamic acid, even with an apparently inert benzyl fluoride-based warhead. We combined two parallel structural investigations based on Deltazinone and Deltasonamide derivatives and collectively chose to advance **22a** further to biological characterization. The compound was termed Deltafluorine to highlight its unique fluorine-containing structure.

Deltafluorine Induces Unique Cellular Profiles in KRAS-Dependent Cancer Cell Lines

In the development of the reversible PDE δ inhibitor Deltasonamide 1, a correlation had been demonstrated between the inhibition of the cellular interaction of PDE δ and its client protein KRAS and the antiproliferative activity of Deltasonamide 1 in a panel of KRAS-dependent human cancer cell lines.³⁴ The strongest antiproliferative activity of Deltasonamide 1 and PDE δ inhibitors Deltarasin and Deltazinone 1, was consistently observed in KRAS-dependent pancreatic ductal adenocarcinoma PA-TU-8902 cells.^{33–35} By analogy, we investigated the dynamic biological effects of Deltafluorine through the global proteome (Figure S5a–d) and phosphoproteome analysis in PA-TU-8902 cells (Figures 4c,d, and S5e,f). Upon treatment with Deltafluorine (**22a**) at 5 μ M for 2 h in a live-cell setting, we identified 17 out of 3694 (0.46%) proteins differentially regulated (adjusted p -value ≤ 0.05 , $\log_2$ fold change ≥ 1 or ≤ -1 , paired t -statistics-based FDR correction, Figure S5a). We observed the most significant and pronounced downregulation in PTB domain-containing engulfment adapter protein 1 (GULP1, $\log_2$ fold change = -2.09 , adjusted p -value = 1.62×10^{-13}), which plays a role in endosomal trafficking and lipid transport, and syntaxin-10 (STX10, $\log_2$ fold change = -1.82 , adjusted p -value = 1.62×10^{-13}), a Golgi apparatus associated membrane protein involved in intracellular protein transport. Additionally, we noted modulation in HLA-C ($\log_2$ fold change = 3.97 , adjusted p -value = 1.62×10^{-13}), Golgin subfamily A member 1 (GOLGA1, $\log_2$ fold change = 1.75 , adjusted p -value = 0.03) and WASH complex subunit 2C (FAM21C, $\log_2$ fold change = -2.03 , adjusted p -value = 0.04), all of which are involved in the endosomal pathway and membrane trafficking of protein cargos. When analyzing these hits for clustering or functional relatedness, we performed a Reactome pathway overrepresentation analysis of the significant hits,⁵⁷ which revealed notable regulation of pathways related to vesicle-mediated transport across the extended Golgi-Endoplasmic Reticulum (ER) membrane system (see Figure S5b–d for overview and Table S2 for details), besides general interferon-mediated signal translation pathways (see Figure S5e,f and Table S2 for details). The PDE δ lipoprotein chaperone binds to the prenylated termini of several GTPases, including Ras^{33–35} and Rheb³⁷ family members, influencing their intracellular trafficking between membrane compartments. This regulation of membrane localization and signal transduction may provide a link to the observed biological activity of Deltafluorine.

To gain further insight into the molecular basis downstream of PDE δ -Ras and PDE δ -Rheb inhibition, we employed Phospho-Analyst to analyze the phosphoproteome monitored in parallel with the global proteome upon Deltafluorine treatment.⁵⁸ With background correction to the global proteome, we identified 161 out of 8062 (2%) phosphosites significantly changed with 5 μ M

Deltafluorine (**22a**) treatment for 2 h (adjusted p -value ≤ 0.05 , $\log_2$ fold change ≥ 1 or ≤ -1 , paired t -statistics-based FDR correction, Figure 4a). From kinase-substrate enrichment analysis (KSEA) of the significant changes in phosphorylation,^{59–61} we observed significant regulation of kinases related to the MAPK family signaling cascades (PhosphoSitePlus data set with NetworKIN cutoff = 1, substrate count cutoff = 2, $p \leq 0.05$, Figure 4b). In particular, we noted strong downregulation of Ras-mediated A-Raf kinase (encoded by ARAF) and dual specificity mitogen-activated protein kinases 1 and 2 (MAP2K1 and 2), as well as the Ras homologue gene family member A (RhoA)-implicated cGMP-dependent protein kinase 1 (PRKG1), all of which presented with a kinase z-score of -1.78 (Figure 4b). Although we observed a higher number of upregulated compared to downregulated kinases, pathway analysis of the upregulated kinases suggested that their increased activity was likely due to secondary effects of intracellular signaling (Table S3). Further input of the significantly suppressed kinases into Reactome for pathway overrepresentation analysis⁵⁷ additionally confirmed the marked downregulation of oncogenic MAPK signaling (number of reactions found = 33, p -value = 2.86×10^{-5} , see Figure S5e,f and Table S4 for details) upon Deltafluorine (**22a**) treatment. Voronoi visualization of the Reactome pathway analysis also revealed that, in addition to Ras-mediated MAPK signaling, there was considerable suppression in the Rheb-mediated mTOR pathway (number of reactions found = 5, p -value = 0.03 , see Figure S5e,f and Table S4 for details), which is also connected to Ras signaling.

Consistently for Deltafluorine, the downregulation of the Ras pathway downstream of MAPK and the related PI3K-Akt-mTOR signaling pathway was evident from phosphoproteome analysis. Additionally, we performed immunoblot analysis examining phosphorylation of downstream targets of the MAPK and PI3K pathways following a time-course of exposure of PA-TU-8902 cells to Deltafluorine (Figure 4c). Our results demonstrate that Deltafluorine treatment leads to decreased phosphorylation of ERK1/2 and S6 dephosphorylation between 1 and 4 h. These timed dependencies are in line with our proteomic analysis (Figure 4a,b), and thus provide additional support on the activity of Deltafluorine on suppression of Ras-related signaling.

Therefore, we further examined the antiproliferative activity of Deltafluorine along with Deltasonamide 1 in a panel of human cancer cell lines, selected for their varied KRAS dependency,^{62–64} mutational status, and diverse tissue origin (Figure 5). We first demonstrated target engagement of Deltafluorine with PDE δ by means of a cellular thermal shift assay (CETSA) showing significant thermal stabilization of PDE δ upon compound treatment (in-cell CETSA with **22a** treatment at 10 μ M for 2 h, melting temperature shift $\Delta T_m = 16.6 \pm 3.2$ °C, paired t -test $*p = 0.01$, Figure 5a). Morphological cellular profiling using the Cell Painting assay (CPA)⁶⁵ revealed that for both Deltafluorine and Deltasonamide 1, cytotoxicity was observed for concentrations above 5 μ M upon 20 h treatment, and that at nontoxic doses, both compounds showed high similarity to the previously identified lysosomotropic profile (Figure S7a).^{66,67} The lysosomotropic activity and cytotoxicity of Deltafluorine and Deltasonamide 1 could mask the specific cellular effects resulting from ligand–target interactions, for in single-dose treatment, we did not observe significant changes in their cellular potency (72 h IC₅₀ of Deltafluorine = 3.1 ± 0.1 μ M versus IC₅₀ of Deltasonamide 1 = 3.8 ± 0.1 μ M in PA-TU-8902

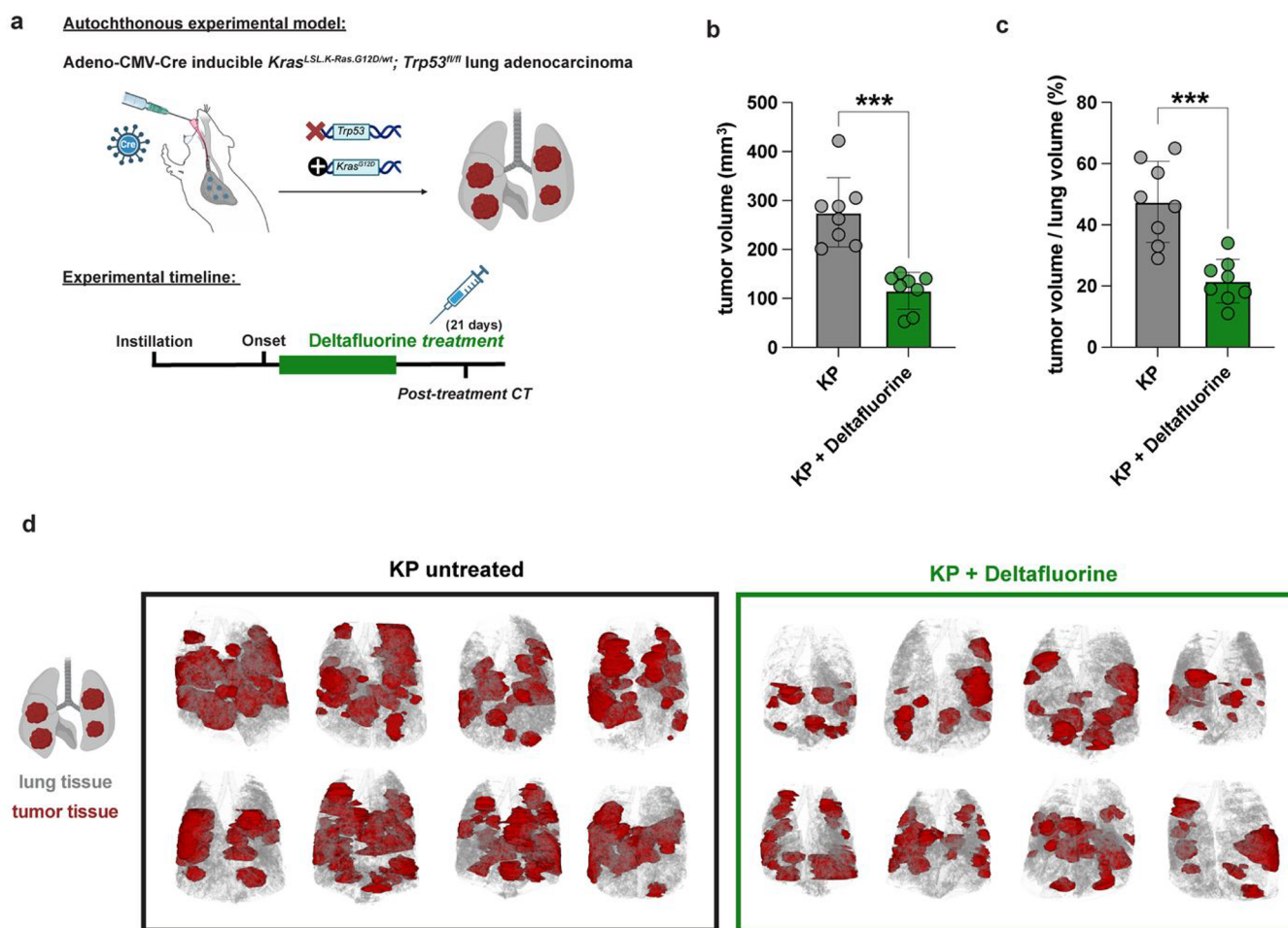


Figure 6. In vivo study of Deltafluorine (22a) in $Kras^{LSL;G12D/wt}; Trp53^{fl/fl}$ (KP) mouse model. (a) Experimental setup. (b) Tumor volumes were measured in post-treatment μ CT scans of lungs from eight mice per group. Unpaired t -test *** p < 0.001. (c) Tumor volumes from (b) were normalized to total lung volume of the respective lung, resulting in percentage of tumor-occupied lung volume. Unpaired t -test *** p < 0.001. (d) 3D renderings of analyzed lung μ CT scans. Gray represents lung tissue, white air, and red tumor tissue.

cells, Figure S7b). Therefore, we investigated their antiproliferative effects by a wash-out experiment with a sequential treatment regimen mimicking therapeutic dosing and noted that the superiority in cellular activity of Deltafluorine over Deltasonamide 1 was only evident under the wash-out conditions (Figures 5b and S7c). By measuring cell confluency over time with real-time live-cell imaging by Incucyte, we observed a marked difference in cellular potency of Deltafluorine and Deltasonamide 1 with a general trend of correlation with KRAS dependency (Figure 5c, see Figure S8 for details). In-cell target engagement for the short-term treatment regime employed in the wash-out experiments was supported by the observation that growth impairment of HAP1 PDE δ knockout cells by Deltafluorine (22a) was less pronounced compared to HAP1 wild type cells at concentrations 5–10 μ M for 4 h treatment (paired t -test, two-tailed **** p -value < 0.0001, Figure S7d). Strong inhibitory activity of Deltafluorine (22a) was observed in KRAS-dependent pancreatic ductal adenocarcinoma cell lines PA-TU-8902 and MIA PaCa-2 cells with an average growth inhibition of $84 \pm 2\%$ and $87 \pm 4\%$, respectively, upon repeated dosing and washing at 5 μ M concentration (4 h treatment +20 h wash-out regimen) over 4 days. The reversible nonfluoride-containing PDE δ inhibitor Deltasonamide 1 failed to retain the antiproliferative activity under the same washing conditions (unpaired t -test **** p < 0.0001, Figure 5b,c). The

differential sensitivity to the treatment regimen was also observed in KRAS-dependent SW480 (colorectal) and NCI-H358 (lung) cells, while the difference diminished in KRAS-independent PANC-1 (pancreatic ductal), LS-174T (colorectal), A549 (lung), and KRAS wild-type-expressing BxPC-3 (pancreatic) and HT-29 (colorectal) cells (Figure 5c). Deltafluorine was slightly more active in the aforementioned KRAS mutant and dependent cell lines in comparison to the HRAS mutant Hs 578T cell line, with an approximately 2-fold difference in their IC₅₀ values, while no difference in IC₅₀ was observed to the BRAF mutant HT-29 cells (Figure S8). Exceptions to the correlation between differential sensitivity and KRAS dependency were noted in HCT116 (colorectal), NCI-H441 (lung), and SK-LU-1 (lung) cells (Figure 5c). HCT116 and SK-LU-1 cells were classified as KRAS independent, but their degree of Ras dependency was at the borderline of cutoff^{62–64} (Figure 5c, $90 \pm 3\%$ growth inhibition by 22a vs $10 \pm 7\%$ by Deltasonamide 1 at 5 μ M in HCT-116, unpaired t -test **** p < 0.0001; $76 \pm 5\%$ growth inhibition by 22a vs $28 \pm 21\%$ by Deltasonamide 1 at 5 μ M in SK-LU-1, unpaired t -test * p = 0.02). For KRAS-dependent NCI-H441 cells, there was no substantial difference in the cellular response observed between the pair (Figure 5c, $16 \pm 4\%$ growth inhibition by 22a vs $7 \pm 2\%$ by Deltasonamide 1 at 5 μ M, unpaired t -test p = 0.03). This discrepancy may be related to the

abundance and balance level of Ras isoforms, for NCI-H441 cells exhibit elevated levels of KRAS4a, while PDE δ is only capable of translocating the other splice variant KRAS4b between membrane compartments.^{68,69}

Deltafluorine Demonstrates In Vivo Single-Agent Therapeutic Efficacy in a Kras-Driven Mouse Model of Lung Adenocarcinoma

Given the demonstrated general correlation between in cellulo investigation of Deltafluorine and cell line KRAS dependency, as well as the modulation of Ras downstream signaling of the MAPK and of the PI3K-mTOR pathway, we further investigated Deltafluorine in vivo. First, the Deltafluorine dosage was evaluated in healthy wild-type mice to assess pharmacokinetics and tolerability. Intraperitoneal administration showed the highest bioavailability (Figure S9a–c). A 21 day repeat dose study in wild-type animals 15 mg/kg q.d. i.p. was generally well tolerated with only transient signs of discomfort and a maximum mean body weight loss of 10% (Figure S9d). With Deltafluorine being a tool compound and not yet a fully pharmacologically optimized drug candidate, we selected the highest tolerated dose for further experiments. For a proof-of-concept study, we utilized an autochthonous, conditional Kras^{LSL.G12D/wt};Trp53^{fl/fl} (KP) mouse model.^{70,71} In this mouse model for highly aggressive lung adenocarcinoma, we induced tumor formation by intratracheal Adeno-CMV-Cre instillation, leading to Kras^{G12D} activation and Trp53 deletion (Figure 6a), and monitored tumor onset via μ CT imaging. Once mice developed measurable tumor lesions (pretreatment CT), we randomized mice 1:1 to either Deltafluorine, 15 mg/kg i.p., q.d. for 21 days, or left them untreated. Of note, all mice included in this study had comparable cancer onset and tumor burden before treatment start, as assessed by μ CT imaging (Figure S10). All KP lung cancer mice received a post-treatment μ CT scan within 6 weeks of treatment initiation. For accurate tumor volume measurements, we reconstructed the μ CT scans from the post-treatment time point and measured the volume of detectable lung tumors with 3D image modeling. This sensitive computer-aided method of tumor volumetry calculation revealed that the average tumor load after 3 week Deltafluorine treatment was significantly reduced by 42% compared to the untreated KP control group (Figure 6b,d). We further normalized tumor load with the corresponding lung volume of each animal to account for variability in the lung sizes of tested mice. Strikingly, while tumors occupied 47.5% of the lung volume in untreated KP mice, Deltafluorine-treated KP-tumor-bearing animals exhibited only 21.6% tumor burden (Figure 6c,d). Although future investigations are needed to confirm that the activity of Deltafluorine in cellulo translates to its in vivo effect, the current data demonstrated the clear effectiveness of short-term Deltafluorine treatment in reducing tumor growth in this highly aggressive lung adenocarcinoma in vivo model.

DISCUSSION

KRAS-mutated cancers were considered undruggable for a long time. The FDA-approved non-small cell lung cancer drugs, sotorasib⁷² and adagrasib,⁷³ along with the more recently discovered MK-1084,⁷⁴ covalently target the acquired cysteine in the K-Ras^{G12C} mutation, thereby locking K-Ras in a signaling-incompetent state and effectively inhibiting its activity. Furthermore, the recently reported first-in-class small molecule inhibitor BBO-8520 engages KRAS^{G12C} in both active and inactive conformations.⁷⁵ In contrast, covalent inhibition of K-

Ras^{G12D}, the most prevalent single variant in K-Ras-driven tumors, has remained elusive due to the more challenging carboxylate-targeting chemistry. Isolated cases of covalent inhibitors for K-Ras^{G12D} have been reported—Yu et al. have reported a dual K-Ras^{G12D/G12C} inhibitor bearing an epoxide warhead;⁵ Shokat et al. have employed a malolactone-based warhead for mutant-selective targeting of K-Ras^{G12D};⁶ and RMC-9805, which bears a trisubstituted aziridine warhead, has also been investigated in preclinical models of K-Ras^{G12D} cancers.⁹ We were hence interested in the expansion of carboxylate-targeting chemistry for covalent inhibitor design and exemplified the utility of a fluoromethyl-substituted aryl warhead with a PDE δ inhibitor Deltafluorine targeting its binding site, glutamate p.E88. For PDE δ as a lipoprotein chaperone involved in membrane localization and hence proper signaling of GTPases, including Ras,^{33–35} targeting PDE δ may also offer an alternative strategy to treat K-Ras-driven cancers, evidenced from the clear effectiveness of Deltafluorine demonstrated in the in vivo Kras^{LSL.G12D/wt};Trp53^{fl/fl} (KP) mouse model of lung adenocarcinoma.

From structure-based analysis of reversible PDE δ inhibitors Deltazinone and Deltasonamide, we explored the possibility of benzyl fluorides as covalent warheads to achieve targeted inhibition of a glutamate residue p.E88 in PDE δ 's binding pocket. Being otherwise stable and unreactive toward other nucleophiles, the specific reactivity of Deltafluorine to PDE δ 's p.E88, might be attributed to its high binding affinity and optimal positioning within the binding pocket, with favorable ligand disposition ensuring close proximity between the warhead and p.E88 to facilitate the reaction. In comparison, the Deltazinone counterpart, with approximately 10-fold lower binding affinity and more distant targeting of p.E88 in PDE δ (~3 Å further), could only achieve ~50% covalent inhibition under the same conditions. The Deltasonamide-derived Deltafluorine, with a pyridylmethyl fluoride-based warhead, covalently bound to PDE δ at p.E88 with >85% modification efficiency in a sustained rate over a 7 day course in vitro. We approximated its apparent second order rate constant, k_{inact}/K_I to be 5.4 M⁻¹ s⁻¹, which is admittedly extremely slow as compared to many TCIs with reported k_{inact}/K_I in the range of 10⁵–10⁷ M⁻¹ s⁻¹.⁷⁶ For PDE δ 's catalytic role in translocating membrane-associated GTPases,³⁷ a high level of target inhibition would be required for any observed cellular activity.³³ There seemed to be an apparent discordance between the very slow covalent reactivity observed in vitro and the distinct cellular phenotype under the wash-out assays with 4 h incubation for each dose. This could in part be explained by cellular PDE δ concentration in the nanomolar range³⁸ coupled with treatment in the micromolar range, leading to a pseudo-first reaction kinetic. We also reasoned that PDE δ 's slow cellular turnover (synthesis half-life = 113 h; degradation half-life = 40 h)⁷⁷ compensated for the weak covalent reactivity of Deltafluorine and that its covalency-driven effect could be accumulated to a substantial amount for biological effects with repeated dosing and sustained exposure. Therefore, using a wash-out assay mimicking a therapeutic dosing regimen, we observed a distinct phenotypic difference between Deltafluorine and the reversible nonfluoride-containing counterpart Deltasonamide 1 in suppressing the proliferation of KRAS-dependent cell lines. Statistical analysis of the dynamic changes in the proteome revealed significant regulation in the endosomal-related membrane trafficking of protein cargos, with notable suppression of KRAS-related MAPK- and PI3K-Akt-mTOR signaling

pathways. These in cellulo observations upon Deltafluorine interference were consistent with PDE δ inhibition^{20,33–35} and aligned with PDE δ 's role as a lipoprotein chaperone regulating membrane localization of GTPases, including Ras.^{33–35} The link between a unique phenotypic change with Deltafluorine treatment in cells and its covalent mode of action, however, remains unclear, which is most likely due to a mixed effect of noncovalent and covalent inhibitions to PDE δ .

To evaluate Deltafluorine in a clinically relevant in vivo context, we employed the autochthonous Kras^{LSL.G12D/wt};Trp53^{fl/fl} (KP) mouse model of lung adenocarcinoma. Lung cancer remains the leading cause of cancer-related mortality,⁷⁸ and KRAS mutations are found in approximately 32% of cases,⁷⁹ rendering this mouse strain a highly relevant model system to test the effectiveness of Deltafluorine in cancer. Importantly, this mouse model represents extraordinarily aggressive lung adenocarcinoma with a mean survival of only 120 days, and the strain has remained incurable since its implementation over 15 years ago. Prior publications in the autochthonous KP model tested the effect of chemotherapy. Cisplatin was shown to yield only marginal tumor control,⁸⁰ while paclitaxel–carboplatin was ineffective relative to untreated controls.⁸¹ This well-documented chemoresistance reflects the distinctive KP tumor biology, establishing a high yet appropriate bar for proof-of-concept evaluations aligned with clinical need and translational development in this very aggressive lung tumor model, in which both KRAS and TRP53 are altered. From a clinical point of view, this is exactly the patient population with the biggest need for novel therapeutic approaches. Consistently, published trajectories for untreated cohorts and for standard chemotherapies in KP mirror the behavior observed in our study and experience with the model.^{81,82}

Remarkably, we observed a marked reduction in tumor growth following a short-term Deltafluorine treatment. This robust in vivo effect, together with minimal observed side-effects, indicates the compound's therapeutic potential. We therefore presented a generally interesting covalent mode of action with a fluoromethyl-substituted aryl ring system reacting with a glutamate in a target protein binding site. Notably, with elaborate downstream biological characterizations both in cellulo and in vivo, the best compound, Deltafluorine, bearing a fluoromethyl-pyridyl ring, also demonstrates promising therapeutic potentials, for example, for targeted cancer treatment approaches.

MATERIALS AND METHODS

Materials and Equipment

Materials, reagents, and equipment are listed in [Supporting Information](#), Section 4—Material List.

Compound Synthesis and Analysis

Details related to compound synthesis and analysis can be found in [Supporting Information](#), Section 3—Chemical Synthesis. All compounds investigated were isolated and purified and were $\geq 95\%$ pure by HPLC analysis.

Protein Purification

All proteins were expressed in *Escherichia coli* strain Rosetta (BL21DE3). Competent cells were transformed with the respective pET plasmids and inoculated on a cell plate with TB medium supplemented with 100 $\mu\text{g/mL}$ ampicillin and 30 $\mu\text{g/mL}$ chloramphenicol for overnight incubation at 37 °C. A single colony was used to inoculate TB medium supplemented with 100 $\mu\text{g/mL}$ ampicillin and incubated at 37 °C with simultaneous shaking overnight. The next day, 5 L of TB medium was inoculated with 50 mL of the Rosetta suspension

and incubated at 37 °C until an OD ~ 1.0 . Cells were induced at OD ~ 1.0 with 100 μM isopropyl β -D-1-thiogalactopyranoside (IPTG) and incubated at 20 °C for 7 h. Cells were harvested and lysed in lysis buffer (30 mM Tris–HCl, pH = 7.5, 150 mM NaCl and 1 mM β -mercaptoethanol, 1 mM PMSF, i.e., phenylmethylsulfonyl fluoride) with sonication on ice. Supernatant of histidine-tagged protein was collected by centrifugation at 13,000g and 10 °C for 35 min and subsequently loaded onto a Ni-NTA column (QIAGEN) and eluted with elution buffer (30 mM Tris–HCl, pH = 7.5, 150 mM NaCl, 1 mM dithiothreitol (DTE), and 250 mM imidazole), followed by gel filtration on a Superdex G75 S26/60 column using elution buffer without imidazole. Protein purity was checked by SDS-PAGE.

MALDI Mass Spectrometry

PDE δ (20 μM) was incubated with compounds (60 μM) in HEPES buffer (20 mM HEPES, 150 mM NaCl, pH = 7.5) with 0.6% DMSO at 37 °C for the specified time (2, 4, 6, 8, 24 h, and subsequently every 24 h up to 7 days). The solution was briefly centrifuged before a sample was taken at the designated time for analysis by MALDI. A saturated solution of sinapinic acid (SA) in EtOH was used as matrix A, and it was added on an MTP 384 ground steel target plate (Bruker) and dried in air. A saturated solution of SA in 30/70 acetonitrile (ACN)/H₂O with 0.1% TFA was used as matrix B. One microliter portion of the sample was mixed with 2 μL of matrix B, and 1 μL of this mixture was placed on top of matrix A on the MTP 384 ground steel target plate and dried in air. Mass spectra were obtained over the m/z range 15,000–25,000 using a Bruker UltrafleXtreme XIAL DI-TOF/TOF mass spectrometer. Percentages of covalent adduct formation were estimated by the relative intensities of the respective peaks in MALDI spectra.

Fluorescence Polarization Assay

Binding to PDE δ was validated and quantified by means of a direct displacement assay employing a fluorescence labeled analogue of the HMG-CoA reductase inhibitor atorvastatin (FA probe), which has previously been shown to also bind to PDE δ .⁸³ Reported K_D of FA probe = 7.1 ± 4 nM and experimentally validated K_D of FA probe = 10.6 ± 2.3 nM. The K_D and IC_{50} values against PDE δ were determined by competitive fluorescence polarization assay, adapted from the method we described before.³⁵ IC_{50} values were generated and fitted with GraphPad Prism 9.2 (GraphPad software, USA) using a four-parameter variable slope nonlinear regression curve fit. To serially diluted solutions of compound in PBS buffer (containing 0.05% Chaps, 1% DMSO) in a black, nonbinding round-bottom 384-well plate (Corning #4514) (10 $\mu\text{L}/\text{well}$) was added an equal volume of premixed solution of PDE δ (80 nM) and FA probe (48 nM) in PBS buffer (containing 0.05% Chaps, 1% DMSO) so that the final concentration of compound was adjusted to the range of 0–500 nM, PDE δ to 40 nM, FA probe to 24 nM and DMSO to 1% for all conditions. The sealed plates were centrifuged, shaken (600 rpm) overnight at room temperature, and briefly centrifuged again before measurement of fluorescence polarization values (excitation wavelength at 485 nm and emission wavelength at 535 nm) by a plate reader at 25 °C (Tecan SPARK).

Isothermal Titration Calorimetry

ITC was performed using the MicroCal PEAQ-ITC system (Malvern) at 25 °C. Purified PDE δ was buffer-exchanged into PBS buffer containing 1 mM TCEP using a 3K Amicon Ultra centrifugal filter (Millipore). The buffer exchange was repeated ten times to ensure complete removal of interfering components. The final protein sample was diluted in the same phosphate buffer prior to titration. 300 μM protein in the buffer was loaded into the syringe, while 30 μM 22a or 40 μM 13a in the buffer was loaded to the cell. All samples were adjusted to 25 °C and degassed before loading. Titrations were performed at 25 °C with one injection of 0.4 μL , followed by 18 injections of 2 μL . Experiments were performed with three biological replicates ($n = 3$). Data were analyzed using the MicroCal PEAQ-ITC Analysis software and were plotted using GraphPad Prism 9.0 (GraphPad, USA).

Compound Stability in Aqueous Buffers and in the Presence of GSH

Compound (1 mM) was incubated with the respective buffers (HEPES 20 mM, 150 mM NaCl, pH = 7.5; 0.2 M sodium acetate buffer, pH = 5.6; 0.2 M sodium citrate buffer, pH = 6.2; 0.2 M potassium phosphate buffer, pH = 7.4, and 1 mM EDTA, with or without 10 mM GSH), with 1% DMSO adjusted for all conditions. The resulting solutions were incubated with shaking (600 rpm) at 37 °C and protected from light. At the respective time points (0, 2, 4, 6, 8, 24, 30, 48, 56, and up to 72 h), a sample was taken and diluted in acetonitrile and analyzed by HPLC-MS (Agilent Technologies 1290 Infinity, 6150 Quadrupole LC/MS). Percent (%) compound remaining in the solution was estimated by the ratio of areas under the curve.

Mass Spectrometry Analysis of Covalent Peptide Adducts of PDE δ after Glu-C Digestion

Covalent adducts of PDE δ or unbound vehicle DMSO-treated PDE δ (1.5 μ g/sample) were denatured, reduced, alkylated, and digested by Glu-C and desalted before analysis by mass spectrometry. For denaturation, 4 μ L of each sample was added to 18 μ L of 8 M guanidine hydrochloride (cas 50-01-1, Carl Roth, #0037.1) solution (to a final 6.5 M) and boiled for 15 min at 95 °C. For reduction, the solution was then cooled and added to 0.5 μ L of 50 mM dithiothreitol (DTT, cas 3483-12-3, Gerbu Biotechnik, #1008-100g) solution (to a final 1 mM DTT) and incubated for 20 min at 60 °C. Subsequently, for alkylation, 2.5 μ L of 50 mM 2-chloroacetamide (CAA, cas 79-07-2, Sigma-Aldrich, #22790) was added to each sample (to a final 5 mM CAA) and incubated for 30 min at room temperature with protection from light. For each sample, 225 μ L of 20 mM ammonium bicarbonate (cas 1066-33-7, Sigma-Aldrich, #A6141–500g) solution was then added to dilute the concentration of guanidine hydrochloride to less than 0.8 M. For digestion, 1.5 μ L of Glu-C (0.05 μ g/ μ L, Promega V1651) was added to each sample (1:20 enzyme to protein, w/w) and incubated overnight at 37 °C, 400 rpm with protection from light. On the next day, the reaction was quenched by adding 5 μ L of 10% trifluoroacetic acid (TFA, cas 76-05-1, Sigma-Aldrich, #302031), and samples were desalted by stage tip purification with C18 extraction disks (Empore high performance extraction disks, 47 mm, 3 M Bioanalytical Technologies #2215). Each stage tip (2 layers of C18 disks) was activated by 100 μ L of methanol, washed once by 100 μ L of buffer B (containing 0.1% formic acid, 80% acetonitrile in water), followed by twice washing with 100 μ L of buffer A (containing 0.1% formic acid in water) prior to sample loading. Loaded stage tip was further washed once with 100 μ L of buffer A, and sample was eluted with 20 μ L of buffer B, with centrifugation at 4000 rpm at room temperature for 5 min and then dried by SpeedVac at 30 °C. All details related to nanoHPLC-MS/MS analysis and data evaluation can be found in [Supporting Information—Methods section](#).

X-ray Crystallography

Compound **13b** was cocrystallized with PDE δ by incubating 2 mM of small molecule (10:1 compound to protein) with 200 μ M of PDE δ in HEPES buffer (HEPES 20 mM, 150 mM NaCl, pH = 7.5), with final 1% DMSO, at 37 °C, 600 rpm for 30 min (when complete covalent modification was verified by MALDI-TOF mass spectrometry measurement), followed by washing and concentration with Amicon 3k filters (Millipore, UFC5003BK) in protein buffer (containing 30 mM Tris–HCl, 150 mM NaCl, 1 mM β -mercaptoethanol, pH = 7.5) to approximately 20 g/L.

Compound **22a** was cocrystallized with PDE δ by incubating 2 mM of small molecule (4:1 compound to protein) with 500 μ M of PDE δ in TRIS buffer (25 mM TRIS–HCl, pH 7.5, 150 mM NaCl, and 3 mM DTE), with final 2.5% DMSO, at 37 °C, 400 rpm, until complete covalent modification was verified by MALDI-TOF mass spectrometry measurement. Precipitates were removed via centrifugation at 20,000 g and 4 °C for 5 min, followed by washing and concentration with Amicon 3k filters (Millipore, UFC5003BK) in protein buffer (containing 30 mM Tris–HCl, 150 mM NaCl, 1 mM β -mercaptoethanol, pH = 7.5) to approximately 20 g/L.

0.1 μ L portion of each protein solution was mixed with 0.1 μ L of precipitant solution in a sitting-drop setup (MRC 3-drop plates, Jena

Bioscience, UK for PDE δ -**13b** and iQ plates, SPT Labtech for PDE δ -**22a**), and crystals were obtained from a self-made crystal optimization plate (0.1 M NaOAc, pH = 4.71, 5.6% w/v PEG4000, 30% v/v glycerol) for PDE δ -**13b** and from a Qiagen Classics suite (20% (w/v) PEG3350 and 0.2 M potassium formate) for PDE δ -**22a**, harvested after 10–12 days incubation at 20 °C and flash frozen in liquid nitrogen with cryoprotectant solution containing the mother liquor components in addition to 25% glycerol or 20% PEG400.

A data set of PDE δ -**13b** was taken at the European Synchrotron Radiation Facility (ESRF) with beamline ID 30B. A data set of PDE δ -**22a** was obtained from the Synchrotron X-ray diffraction data using the X10SA beamline at the Swiss Light Source. Both data sets were analyzed and scaled using XDS and XSCALE.⁸⁴ The structures were solved using PHASER⁸⁵ within the PHENIX software suite,⁸⁶ with the structure of 5E80 and 5ML3, respectively, chain A serving as the model for molecular replacement. Refinement was conducted using COOT⁸⁷ for manual refinement and phenix.refine.⁸⁸ Figures were created with Pymol (Version 2.5.4, Schrödinger, LLC). Topology files for both ligands were generated using AceDRG⁸⁹ within the CCP4 suite,⁹⁰ based on their SMILES strings. Restraints for the geometry of the covalent bonds were manually created by modifying the parameter file for phenix.refine. The PDB ID code, data collection, and refinement statistics are presented in [Table S5](#). The crystal structures of PDE δ -**13b** and PDE δ -**22a** were deposited in the Protein Data Bank (PDB) with accession numbers 9RP6 and 9RP7.

Cell Lines and Cell Culture

All mammalian cells were cultured and maintained in a sterile environment with a humidified atmosphere at 37 °C and 5% CO₂. Jurkat (ACC282, RRID: CVCL_0065), PA-TU-8902 (ACC179, RRID: CVCL_1845), BxPC3 cells (ACC760, RRID: CVCL_0186), HCT116 (ACC581, RRID: CVCL_0291), LS-174T (ACC759, RRID: CVCL_1384), and A549 (ACC107, RRID: CVCL_0023) were purchased from DSMZ GmbH (Germany). MIA PaCa-2 (CRM-CRL-1420, RRID: CVCL_0428), PANC-1 (ATCC-CRL-1469, RRID: CVCL_0480), SW480 (CCL-228, RRID: CVCL_0546), HT-29 (ATCC-HTB-38, RRID: CVCL_0320), and NCI-H441 (ATCC-CRM-HTB-174, RRID: CVCL_1561) were obtained from ATCC (USA). NCI-H358 (ATCC-CRL-5807, RRID: CVCL_1559) cells were purchased from LGC Standards (Germany). U2OS (CLS-300364, RRID: CVCL_0042) and SK-LU-1 (CLS-300335, RRID: CVCL_0629) cells were obtained from CLS Cell Lines Service GmbH (Germany). HAP1 wild type (Horizon #C631, RRID: CVCL_Y019) and HAP1 PDE6D knockout (Horizon #HZGHC006484c003, RRID: CVCL_XR47) cells were purchased from Horizon (Horizon Discovery, UK). Hs 578T (RRID: CVCL_0332) cells were obtained from the NIH/NCI-DTP. PA-TU-8902, MIA PaCa-2, PANC-1, HCT116, A549, U2OS, and Hs 578T cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, P04-03550, PAN Biotech) supplemented with 10% FBS (Gibco, #10270-106), 1% nonessential amino acids (NEAA, P08-32100, PAN Biotech), and 1 mM sodium pyruvate (P04-43100, PAN Biotech). Jurkat, BxPC3, SW480, LS-174T, NCI-441, and NCI-H358 cells were cultured in RPMI-1640 medium (P04-18047, PAN Biotech) with 10% of FBS (Gibco, #10270-106) and 1% NEAA (P08-32100, PAN Biotech). HT-29 cells were cultured in MyCoy's 5A (P04-05500, PAN Biotech) and SK-LU-1 in MEM Eagle (P04-08500, PAN Biotech) supplemented with 10% FBS, respectively. HAP1 wild type and PDE6D knockout cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, P04-20350, PAN Biotech) supplemented with 10% FBS. Mycoplasma tests with the MycoAlert Mycoplasma Detection Kit (Lonza, #LT07-318) were carried out on a regular basis and confirmed that cells were free of contaminations at all times.

Fluorescence Lifetime Imaging Microscopy

Fluorescence lifetime images were acquired by using a confocal laser-scanning microscope (Leica SP8). For the detection of the donor mCitrine, the sample was excited with a supercontinuum White Light Laser (WLL) with a notch line filter at 470 nm at a 40 MHz repetition frequency. Fluorescence signals were collected through an oil immersion objective and spectrally filtered using a narrow-band

emission filter and detected with a photon-counting HyD detector from 519–541 nm. Images were analyzed in real-time on a global scale using the in-built Leica Application Suite X (LAS X) for FLIM, with lifetime calculated with fitting to the in-built model of monoexponential reconvolution.

Global Proteome and Phosphoproteome Analysis

PA-TU-8902 cells (8×10^6 cells/dish) were seeded in two 15 cm dishes and incubated in a humidified atmosphere at 37 °C and 5% CO₂ overnight. Cells were then treated with 5 μ M of compound **22a** or DMSO, with 0.1% DMSO adjusted for both conditions in fresh medium for 2 h incubated in a humidified atmosphere at 37 °C and 5% CO₂. All details related to subsequent sample preparation, nanoHPLC–MS/MS analysis, and data evaluation can be found in the [Supporting Information](#)—Methods section.

CETSA in Intact Cells (In-Cell CETSA)

Jurkat cells (adjusted to 9×10^6 cells/flask) were seeded in two T75 tissue culture flasks and treated with 10 μ M compound or vehicle (DMSO), with 0.1% DMSO adjusted, for 15 min at 37 °C. Cells were collected and resuspended in cold PBS and further washed three times in cold PBS. The sample from each treatment condition was distributed equally into ten tubes and subjected to heating at different temperatures in the MasterCycler EpGradient S (Eppendorf SE, DE). Afterward, the NP40 alternative was added to a final concentration of 0.4% (v/v), and cells were lysed by five consecutive freeze/thaw cycles. Soluble fractions were separated from denatured proteins by ultracentrifugation at 100,000 g and 4 °C for 25 min (Beckman Coulter Optima MAX-XP, with TLA-120.1 rotor). Supernatants were transferred to new tubes, and equal volumes of each sample were loaded on a self-made 15% Tris-glycine SDS-polyacrylamide gel and subjected to immunoblot analysis with gel electrophoresis in Tris-glycine SDS running buffer (25 mM Tris, 0.2 M glycine, 0.1% SDS) at 90 V for 15 min stacking, followed by 120 V until sufficient separation was visualized by the PageRuler prestained protein ladder. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, #88518, 0.45 μ m) using a wet-tank blotting system (Bio-Rad) with precooled transfer buffer (25 mM Tris, 0.2 M glycine, 10% methanol) at 100 V for 30 min. Membranes were blocked with Intercept Blocking Buffer (LI-COR Biosciences, #927-70001) for 1 h at room temperature, incubated with the primary antibody anti-PDE δ (Invitrogen, PA5-22008, RRID: AB_11154288, 1:500) overnight at 4 °C in blocking buffer, then washed with PBS-T (PBS with 0.1% Tween-20) five times and incubated with IRDye 800CW-conjugated secondary antibody (LI-COR Biosciences, #926-32210, RRID: AB_621842, 1:5000) in blocking buffer for 1 h at room temperature with protection from light. After washing with PBS-T five times, secondary antibody-incubated membranes were imaged with the ChemiDocMP Imaging System (BIO-RAD Laboratories) directly with IRDye800CW channel for visualization of bands. Relative band intensities were quantified using Image Lab (BIO-RAD) and normalized to the intensities of the bands at 37 °C.

SDS-PAGE and Immunoblotting

PA-TU-8902 cells (8×10^6 cells/dish) were seeded in two 15 cm dishes and incubated in a humidified atmosphere at 37 °C and 5% CO₂ overnight. Cells were then treated with 5 μ M of compound **22a** for the indicated time points. Cells were washed and harvested by scraping. Cells were lysed by resuspension in 100 μ L of sodium dodecyl sulfate (SDS) lysis buffer and sonicated in the Bioruptor (Diagenode Inc.). SDS-lysed samples were heated at 95 °C for 10 min and quantified via the DC assay. Samples were diluted in Laemmli buffer, heated at 95 °C for 10 min, and loaded on an SDS polyacrylamide gel (5% stacking gel, 10% separating gel). The Mini-PROTEAN Tetra Cell immunoblotting system (BioRad Laboratories, Inc.) was used for electrophoresis and transfer. Gels were transferred onto a PVDF membrane (Merck Millipore) via Trans-Blot Turbo Semi Dry Blotting at 25 V for 7 min. Membranes were washed in 1x TBS-T and blocked in 5% BSA in TBS-T for 1 h. For immunostaining, the membranes were incubated overnight with primary antibodies diluted in 5% BSA at 4 °C (Anti-ERK1/2, CST9107, 1:1000; Anti-ERK1/2, Abcam36991, 1:1000;

Anti-pS6, CST4856, 1:1000; Anti-S6, CST2317, 1:100). After three washes in 1x TBS-T for 10 min each, the membranes were incubated with Horseradish Peroxidase (HRP) conjugated secondary antibody diluted in 5% BSA at RT for 1 h, following another three washes. ECL Prime Blotting Detection Reagent (Merck Millipore) was used for protein detection using the ChemiDoc Imaging System (BioRad Laboratories Inc.). Signal intensities from SDS-PAGE bands were quantified by using FIJI software. Square ROI were defined around each band, and background correction was performed by subtracting the mean intensity of an equivalent ROI positioned above each band. For phosphorylated proteins, band intensities were normalized to their corresponding total protein levels. **22a** treatment data were presented in percentages relative to the untreated control lane (set as 100%).

Real-Time Live-Cell Analysis by Incucyte

Real-time live-cell analysis monitoring cell growth was performed with an IncuCyte ZOOM and Incucyte S3 live-cell analysis instrument (Sartorius AG, Germany). For each adherent cell line, 5×10^3 to 1.2×10^4 cells were seeded in each well of the 96-well plates in 100 μ L of cell culture medium and incubated overnight in a humidified incubator at 37 °C with 5% CO₂ before replacement of fresh medium and addition of respective compounds, with 0.5% DMSO adjusted for all conditions. Plates were inserted into the Incucyte S3 live-cell analysis instrument and incubated in a humidified incubator at 37 °C with 5% CO₂. Images of cells (2 images/well) were taken with the phase channel every 2 h until full confluency was reached in the DMSO controls. Images were analyzed with the basic analyzer inbuilt in the Incucyte Zoom (2018A) and IncuCyte S3 (2019B, Rev2) analysis software with a confluence mask calculating the percent phase area confluence of cells as an indicator of cell growth. To display dose–response curves, percent phase area confluence at 72 h of treatment was normalized to the DMSO control, plotted, and fitted with GraphPad Prism 9.2 (GraphPad software, USA) using a four-parameter variable slope nonlinear regression curve fit to calculate cellular IC₅₀ values. For calculation of growth rate, percent phase area confluence was plotted against time for each respective condition in GraphPad Prism, and the area below the curve was integrated for 96 h after administration of the first dose of drugs and normalized to the DMSO control.

Kras^{LSL.G12D/wt};Trp^{53fl/fl} (KP) Mouse Model Study

All details related to the in vivo mouse model study can be found in the [Supporting Information](#)—Methods section. The mouse experiments were licensed by the State Agency for Food and Consumer Protection (LAVE) under license 81-02.04.2020.A281. Breeding of mouse lines was permitted by the State Agency for Food and Consumer Protection (LAVE) under licenses 81-02.04.2019.A009 and 2024-225.

■ ASSOCIATED CONTENT

Data Availability Statement

All unique/stable materials and reagents generated in this study are available from the lead contact with a completed material transfer agreement. The crystal structures of covalent PDE δ adducts modified by compounds **13b** and **22a** (Deltafluorine) were deposited in the Protein Data Bank (PDB) with the accession numbers 9RP6 and 9RP7.

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.5c02082>.

Structures of selected PDE δ inhibitors and their cocrystal structures with PDE δ describing the same figure; supplementing design strategy of compound development; structural development of benzyl halide electrophiles; binding affinities of **13a** and **22a** to PDE δ ; stability of **13b** and **22a** in aqueous buffers and in the presence of GSH; global proteome profiling of **22a**; phosphoproteome profiling of **22a**; quantified immunoblots of phosphorylation of ERK1/2 and S6 in PA-TU-8902

cells; cellular profiling of Deltafluorine (22a), including cell painting, single-dose treatment in PA-TU-8902 cells, and HAP1 wildtype versus PDE δ knockout cells; cellular dose–response curves of Deltafluorine 22a; PK/PD studies of Deltafluorine 22a; pretreatment μ CT data; uncropped immunoblots of CETSA; mass spectrometry analysis of covalent peptide adducts of PDE δ ; Reactome pathway analysis of global proteome upon 22a treatment; Reactome pathway analysis of upregulated kinases upon 22a treatment; Reactome pathway analysis of down-regulated kinases upon 22a treatment; X-ray crystallography data collection and refinement statistics; detailed methods for mass spectrometry analysis of covalent peptide adducts of PDE δ after Glu-C digestion, global proteome and phosphoproteome analysis, and Kras^{LSL.G12D/wt};Trp^{S38/fl} (KP) Mouse Model Study; detailed description, methods, and analysis of compound synthesis; material list for this study (PDF)

SMILES and activity data of all investigated compounds (CSV)

AUTHOR INFORMATION

Corresponding Authors

Ron D. Jachimowicz – Max Planck Research Group Mechanisms of DNA Repair, Max Planck Institute for Biology of Ageing, Cologne 50931, Germany; Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf (CIO ABCD), Cologne Excellence Cluster on Cellular Stress Response in Aging-Associated Diseases, and Center for Molecular Medicine Cologne, University of Cologne, Cologne 50931, Germany; orcid.org/0000-0001-9522-7061; Email: rjachimowicz@age.mpg.de

Herbert Waldmann – Department of Chemical Biology, Max Planck Institute of Molecular Physiology, Dortmund 44227, Germany; Faculty of Chemistry and Chemical Biology, Technical University Dortmund, Dortmund 44221, Germany; orcid.org/0000-0002-9606-7247; Email: Herbert.waldmann@mpi-dortmund.mpg.de

Authors

Ruirui Zhang – Department of Chemical Biology, Max Planck Institute of Molecular Physiology, Dortmund 44227, Germany; orcid.org/0000-0001-9342-2613

Maxim A. Huetzen – Max Planck Research Group Mechanisms of DNA Repair, Max Planck Institute for Biology of Ageing, Cologne 50931, Germany; Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf (CIO ABCD), Cologne Excellence Cluster on Cellular Stress Response in Aging-Associated Diseases, and Center for Molecular Medicine Cologne, University of Cologne, Cologne 50931, Germany; orcid.org/0009-0008-2217-813X

Aylin Binici – Department of Chemical Biology, Max Planck Institute of Molecular Physiology, Dortmund 44227, Germany; Faculty of Chemistry and Chemical Biology, Technical University Dortmund, Dortmund 44221, Germany

Pablo Martín-Gago – Department of Chemical Biology, Max Planck Institute of Molecular Physiology, Dortmund 44227, Germany

Raphael Gasper – Crystallography and Biophysics Facility, Max Planck Institute of Molecular Physiology, Dortmund 44227, Germany

Elena Rudashevskaya – Department of Chemical Biology, Max Planck Institute of Molecular Physiology, Dortmund 44227, Germany

Jie Liu – Department of Chemical Biology, Max Planck Institute of Molecular Physiology, Dortmund 44227, Germany

Chinta Nagaraju – Department of Chemical Biology, Max Planck Institute of Molecular Physiology, Dortmund 44227, Germany

Elena S. Reckzeh – Department of Chemical Biology, Max Planck Institute of Molecular Physiology, Dortmund 44227, Germany

Alana S. T. Stuedle – Max Planck Research Group Mechanisms of DNA Repair, Max Planck Institute for Biology of Ageing, Cologne 50931, Germany; Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf (CIO ABCD), Cologne Excellence Cluster on Cellular Stress Response in Aging-Associated Diseases, and Center for Molecular Medicine Cologne, University of Cologne, Cologne 50931, Germany

Ann-Sophie Hopff – Max Planck Research Group Mechanisms of DNA Repair, Max Planck Institute for Biology of Ageing, Cologne 50931, Germany; Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf (CIO ABCD), Cologne Excellence Cluster on Cellular Stress Response in Aging-Associated Diseases, and Center for Molecular Medicine Cologne, University of Cologne, Cologne 50931, Germany; orcid.org/0000-0002-0042-4941

Andrea Mesaros – Phenotyping Core Facility, Max Planck Institute for Biology of Ageing, Cologne 50931, Germany; orcid.org/0000-0003-2394-1412

Anke Unger – Lead Discovery Center GmbH, Dortmund D-44227, Germany

Melanie Thelen – Max Planck Research Group Mechanisms of DNA Repair, Max Planck Institute for Biology of Ageing, Cologne 50931, Germany; Department I of Internal Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf (CIO ABCD), Cologne Excellence Cluster on Cellular Stress Response in Aging-Associated Diseases, and Center for Molecular Medicine Cologne, University of Cologne, Cologne 50931, Germany; orcid.org/0000-0003-0707-5921

Petra Janning – Department of Chemical Biology, Max Planck Institute of Molecular Physiology, Dortmund 44227, Germany

H. Christian Reinhardt – Department of Hematology and Stem Cell Transplantation, University Hospital Essen, West German Cancer Center, German Cancer Consortium Partner Site Essen, Center for Molecular Biotechnology, University of Duisburg-Essen, Essen 45147, Germany

Slava Ziegler – Department of Chemical Biology, Max Planck Institute of Molecular Physiology, Dortmund 44227, Germany; orcid.org/0000-0003-4398-7741

Complete contact information is available at:
<https://pubs.acs.org/10.1021/acs.jmedchem.5c02082>

Author Contributions

^{††}R.-R.Z., M.A.H., A.B., and P.M.G. contributed equally to this work. H.W., S.Z., H.C.R., and R.D.J. supervised the research. R.-R.Z., P.M.G., J.L., and C.N. designed the compound library and performed organic synthesis of the compounds. R.-R.Z., P.M.G., A.B., J.L., E.S.R., and E.R. performed and analyzed biophysical and biological experiments. R.G. solved the crystal structures;

R.-R.Z., A.B., and R.G. analyzed the crystallographic data. P.J. supervised the proteomics experiments; R.-R.Z., E.R., and A.B. performed the proteomics experiments; and R.-R.Z., E.R., A.B., and P.J. analyzed the proteomics data. M.A.H. and A.S.H. performed immunoblot validation of proteomics data. H.C.R. supervised the PK/PD studies. A.U. performed and analyzed P.K./P.D. experiments. R.D.J. supervised the animal model study. M.A.H. performed the in vivo experiments; M.A.H., A.S.T.S., A.M., A.S.H., M.T. and R.D.J. analyzed the in vivo study data. R.-R.Z. integrated project parts and wrote the first draft and R.-R.Z., M.A.H., P.M.G., A.B., S.Z., R.D.J., and H.W. prepared and edited the manuscript. All authors discussed the results and commented on the manuscript.

Funding

This work was funded by the Max Planck Society (to H.W. and R.D.J.), the German Research Foundation (grant nos. 496650118, 5504 FOR JA 2439/5-1 to R.D.J., JA2439/4-1 to R.D.J., grant 455784452 as part of CRC1530), with project funding for H.C.R. (A01) and R.D.J. (C02), grant no. 413326622 as part of CRC1399 to H.C.R., grant no. 424228829 as part of CRC1430 to H.C.R. (A09), the German Ministry of Education and Research (BMBF e:Med Consortium InCa, grant 01ZX1901 and 01ZX2201A to H.C.R.), the Ministry for Culture and Science North-Rhine-Westphalia (NW21–062A CANTAR to H.C.R. and R.D.J.), the Behrens-Weise-Foundation (Grant for the Improvement of Human Health to R.D.J.), the Center for Molecular Medicine Cologne (Project A10 to R.D.J.), the German Cancer Aid 1117240 to H.C.R., 70113041 to H.C.R., Cancer Aid excellence Program to H.C.R., and preclinical drug discovery program TACTIC to H.C.R. M.A.H. is a member of the Cologne Graduate School of Aging Research. Open access funded by Max Planck Society.

Notes

The authors declare the following competing financial interest(s): H.C.R. received consulting and lecture fees from Roche, Novartis, Takeda, Janssen, Lilly, Abbvie, AstraZeneca, Vertex, and Merck. H.C.R. received research funding from AstraZeneca and Gilead Pharmaceuticals. H.C.R. is a co-founder of CDL Therapeutics GmbH. R.D.J. received consulting and lecture fees from Beigene, AstraZeneca and Janssen. The remaining authors declare no competing interest.

ACKNOWLEDGMENTS

Research at the Max Planck Institute of Molecular Physiology and the Max Planck Institute for Biology of Ageing was supported by the Max Planck Society. We acknowledge the Swiss Light Source (SLS) and European Synchrotron Radiation Facility (ESRF) for the provision of synchrotron radiation facilities, and we would like to thank the local beamline support scientists for assistance and support in using SLS beamline X10SA and ESRF beamline ID 30B. We acknowledge the compound management and screening center (COMAS) in Dortmund for performing high-throughput cell painting screening. We thank Andreas Brockmeyer for mass spectrometry measurements, Christine Nowak for protein and plasmid purification, and Sasikala Thavam, Jens Warmers, Beate Schölermann, Anna Sophie Sickau, and Anne Krüßmann for technical support. Mouse husbandry and treatment studies were performed in the Comparative Biology Core Facility of the Max Planck Institute for Biology of Ageing and we acknowledge Frederik Bøddeker, Patrick Wollek, Jerome Henn, Sabrina Seyfarth, Silvia Bøddeker, and Bettina Bertalan. The μ CT

analyses were performed with support of the Phenotyping Core Facility of the Max Planck Institute for Biology of Ageing. Graphics in the TOC and Figure 6 were designed with BioRender by M.A.H. with a publication license. Huetzen, M. (2025) <https://BioRender.com/o1ha4l>.

ABBREVIATIONS

Akt, protein kinase B; Arl, ADP ribosylation factor-like GTPase; HLA-C, human leukocyte antigen-C; KP, Kras^{LSL.G12D/wt}; Trp53^{fl/fl}; mTOR, mammalian target of rapamycin; PDE δ , phosphodiesterase (6) delta; PTB, phosphotyrosine-binding domain; Rab, Ras-related protein; Raf, rapidly accelerated fibrosarcoma protein; Rheb, Ras homologue enriched in brain; Rho, Ras homologue; S6, ribosomal protein S6; TCI, targeted covalent inhibitor; TMR, tetramethylrhodamine; Trp53, transformation-related protein 53; μ CT, micro-computed tomography

REFERENCES

- (1) Boike, L.; Henning, N. J.; Nomura, D. K. Advances in covalent drug discovery. *Nat. Rev. Drug Discovery* **2022**, 21 (12), 881–898.
- (2) Abdeldayem, A.; Raouf, Y. S.; Constantinescu, S. N.; Moriggl, R.; Gunning, P. T. Advances in covalent kinase inhibitors. *Chem. Soc. Rev.* **2020**, 49 (9), 2617–2687.
- (3) Singh, J.; Petter, R. C.; Baillie, T. A.; Whitty, A. The resurgence of covalent drugs. *Nat. Rev. Drug Discovery* **2011**, 10 (4), 307–317.
- (4) Eder, J.; Hommel, U.; Cumin, F.; Martoglio, B.; Gerhartz, B. Aspartic Proteases in Drug Discovery. *Curr. Pharm. Des.* **2007**, 13 (3), 271–285.
- (5) Yu, Z.; He, X.; Wang, R.; Xu, X.; Zhang, Z.; Ding, K.; Zhang, Z.-M.; Tan, Y.; Li, Z. Simultaneous Covalent Modification of K-Ras(G12D) and K-Ras(G12C) with Tunable Oxirane Electrophiles. *J. Am. Chem. Soc.* **2023**, 145 (37), 20403–20411.
- (6) Zheng, Q.; Zhang, Z.; Guiley, K. Z.; Shokat, K. M. Strain-release alkylation of Asp12 enables mutant selective targeting of K-Ras-G12D. *Nat. Chem. Biol.* **2024**, 20 (9), 1114–1122.
- (7) Brosnan, J. T.; Brosnan, M. E. Glutamate: a truly functional amino acid. *Amino Acids* **2013**, 45 (3), 413–418.
- (8) Lin, J.; Pozharski, E.; Wilson, M. A. Short Carboxylic Acid–Carboxylate Hydrogen Bonds Can Have Fully Localized Protons. *Biochemistry* **2017**, 56 (2), 391–402.
- (9) Jiang, L.; Menard, M.; Weller, C.; Wang, Z.; Burnett, L.; Aronchik, I.; Steele, S.; Flagella, M.; Zhao, R.; Evans, J. W. W.; Chin, S.; Chou, K.-J.; Mu, Y.; Longhi, M.; McDowell, L.; Knox, J. E.; Gill, A.; Smith, J. A.; Singh, M.; Quintana, E.; Jiang, J. Abstract S26: RMC-9805, a first-in-class, mutant-selective, covalent and oral KRASG12D(ON) inhibitor that induces apoptosis and drives tumor regression in preclinical models of KRASG12D cancers. *Cancer Res.* **2023**, 83 (7_Supplement), 526.
- (10) Jung, Y.; Noda, N.; Takaya, J.; Abo, M.; Toh, K.; Tajiri, K.; Cui, C.; Zhou, L.; Sato, S.-i.; Uesugi, M. Discovery of Non-Cysteine-Targeting Covalent Inhibitors by Activity-Based Proteomic Screening with a Cysteine-Reactive Probe. *ACS Chem. Biol.* **2022**, 17 (2), 340–347.
- (11) Hillebrand, L.; Liang, X. J.; Serafim, R. A. M.; Gehringer, M. Emerging and Re-emerging Warheads for Targeted Covalent Inhibitors: An Update. *J. Med. Chem.* **2024**, 67 (10), 7668–7758.
- (12) Gehringer, M.; Laufer, S. A. Emerging and Re-Emerging Warheads for Targeted Covalent Inhibitors: Applications in Medicinal Chemistry and Chemical Biology. *J. Med. Chem.* **2019**, 62 (12), 5673–5724.
- (13) Zhang, H.; Liu, H.; Blankenship, R. E.; Gross, M. L. Isotope-Encoded Carboxyl Group Footprinting for Mass Spectrometry-Based Protein Conformational Studies. *J. Am. Soc. Mass Spectrom.* **2016**, 27 (1), 178–181.

- (14) Zhang, H.; Wen, J.; Huang, R. Y. C.; Blankenship, R. E.; Gross, M. L. Mass spectrometry-based carboxyl footprinting of proteins: Method evaluation. *Int. J. Mass Spectrom.* **2012**, *312*, 78–86.
- (15) Qiu, N.; Tan, H.; Pechalieu, D.; Abegg, D.; Fnu, D.; Powers, D. C.; Adibekian, A. Proteome-Wide Covalent Targeting of Acidic Residues with Tunable N-Aryl Aziridines. *J. Am. Chem. Soc.* **2025**, *147* (20), 17517–17528.
- (16) Ma, N.; Hu, J.; Zhang, Z.-M.; Liu, W.; Huang, M.; Fan, Y.; Yin, X.; Wang, J.; Ding, K.; Ye, W.; Li, Z. 2H-Azirine-Based Reagents for Chemoselective Bioconjugation at Carboxyl Residues Inside Live Cells. *J. Am. Chem. Soc.* **2020**, *142* (13), 6051–6059.
- (17) Bach, K.; Beerken, B. L. H.; Zanon, P. R. A.; Hacker, S. M. Light-Activatable, 2,5-Disubstituted Tetrazoles for the Proteome-wide Profiling of Aspartates and Glutamates in Living Bacteria. *ACS Cent. Sci.* **2020**, *6* (4), 546–554.
- (18) Qian, Y.; Schürmann, M.; Janning, P.; Hedberg, C.; Waldmann, H. Activity-Based Proteome Profiling Probes Based on Woodward's Reagent K with Distinct Target Selectivity. *Angew. Chem., Int. Ed. Engl.* **2016**, *55* (27), 7766–7771.
- (19) Martín-Gago, P.; Fansa, E. K.; Winzker, M.; Murarka, S.; Janning, P.; Schultz-Fademrecht, C.; Baumann, M.; Wittinghofer, A.; Waldmann, H. Covalent Protein Labeling at Glutamic Acids. *Cell Chem. Biol.* **2017**, *24* (5), 589–597.
- (20) Waldmann, H.; Zhang, R.; Liu, J.; Gasper, R.; Janning, P. Covalent Modification of Glutamic Acid Inspired by HaloTag Technology. *ChemRxiv* **2025**.
- (21) Los, G. V.; Encell, L. P.; McDougall, M. G.; Hartzell, D. D.; Karassina, N.; Zimprich, C.; Wood, M. G.; Learish, R.; Ohana, R. F.; Urh, M.; Simpson, D.; Mendez, J.; Zimmerman, K.; Otto, P.; Vidugiris, G.; Zhu, J.; Darzins, A.; Klaubert, D. H.; Bulleit, R. F.; Wood, K. V. HaloTag: A Novel Protein Labeling Technology for Cell Imaging and Protein Analysis. *ACS Chem. Biol.* **2008**, *3* (6), 373–382.
- (22) Frei, M. S.; Tarnawski, M.; Roberti, M. J.; Koch, B.; Hiblot, J.; Johnsson, K. Engineered HaloTag variants for fluorescence lifetime multiplexing. *Nat. Methods* **2022**, *19* (1), 65–70.
- (23) England, C. G.; Luo, H.; Cai, W. HaloTag Technology: A Versatile Platform for Biomedical Applications. *Bioconjugate Chem.* **2015**, *26* (6), 975–986.
- (24) Cook, A.; Walterspiel, F.; Deo, C. HaloTag-Based Reporters for Fluorescence Imaging and Biosensing. *ChemBioChem* **2023**, *24* (12), No. e202300022.
- (25) Wilhelm, J.; Nickel, L.; Lin, Y.-H.; Hiblot, J.; Johnsson, K. Improving split-HaloTag through computational protein engineering. *Protein Sci.* **2025**, *34* (5), No. e70123.
- (26) Gillis, E. P.; Eastman, K. J.; Hill, M. D.; Donnelly, D. J.; Meanwell, N. A. Applications of Fluorine in Medicinal Chemistry. *J. Med. Chem.* **2015**, *58* (21), 8315–8359.
- (27) Blanksby, S. J.; Ellison, G. B. Bond Dissociation Energies of Organic Molecules. *Acc. Chem. Res.* **2003**, *36* (4), 255–263.
- (28) Parr, R. G.; Szentpály, L. v.; Liu, S. Electrophilicity Index. *J. Am. Chem. Soc.* **1999**, *121* (9), 1922–1924.
- (29) Eckstein, J. W.; Foster, P. G.; Finer-Moore, J.; Wataya, Y.; Santi, D. V. Mechanism-Based Inhibition of Thymidylate Synthase by 5-(Trifluoromethyl)-2'-deoxyuridine 5'-Monophosphate. *Biochemistry* **1994**, *33* (50), 15086–15094.
- (30) Grishin, N. V.; Osterman, A. L.; Brooks, H. B.; Phillips, M. A.; Goldsmith, E. J. X-ray Structure of Ornithine Decarboxylase from *Trypanosoma brucei*: The Native Structure and the Structure in Complex with α -Difluoromethylornithine. *Biochemistry* **1999**, *38* (46), 15174–15184.
- (31) Cohen, M. S.; Hadjivassiliou, H.; Taunton, J. A clickable inhibitor reveals context-dependent autoactivation of p90 RSK. *Nat. Chem. Biol.* **2007**, *3* (3), 156–160.
- (32) Powers, J. C.; Asgian, J. L.; Ekici, O. D.; James, K. E. Irreversible Inhibitors of Serine, Cysteine, and Threonine Proteases. *Chem. Rev. (Washington, DC, U.S.)* **2002**, *102* (12), 4639–4750.
- (33) Papke, B.; Murarka, S.; Vogel, H. A.; Martín-Gago, P.; Kovacevic, M.; Truxius, D. C.; Fansa, E. K.; Ismail, S.; Zimmermann, G.; Heinelt, K.; Schultz-Fademrecht, C.; Al Saabi, A.; Baumann, M.; Nussbaumer, P.; Wittinghofer, A.; Waldmann, H.; Bastiaens, P. I. H. Identification of pyrazolopyridazinones as PDE δ inhibitors. *Nat. Commun.* **2016**, *7* (1), 11360.
- (34) Martín-Gago, P.; Fansa, E. K.; Klein, C. H.; Murarka, S.; Janning, P.; Schürmann, M.; Metz, M.; Ismail, S.; Schultz-Fademrecht, C.; Baumann, M.; Bastiaens, P. I. H.; Wittinghofer, A.; Waldmann, H. A PDE δ -KRAS Inhibitor Chemotype with up to Seven H-Bonds and Picomolar Affinity that Prevents Efficient Inhibitor Release by Arl2. *Angew. Chem., Int. Ed. Engl.* **2017**, *56* (9), 2423–2428.
- (35) Zimmermann, G.; Papke, B.; Ismail, S.; Vartak, N.; Chandra, A.; Hoffmann, M.; Hahn, S. A.; Triola, G.; Wittinghofer, A.; Bastiaens, P. I. H.; Waldmann, H. Small molecule inhibition of the KRAS–PDE δ interaction impairs oncogenic KRAS signalling. *Nature* **2013**, *497* (7451), 638–642.
- (36) Küchler, P.; Zimmermann, G.; Winzker, M.; Janning, P.; Waldmann, H.; Ziegler, S. Identification of novel PDE δ interacting proteins. *Bioorg. Med. Chem.* **2018**, *26* (8), 1426–1434.
- (37) Ismail, S. A.; Chen, Y.-X.; Rusinova, A.; Chandra, A.; Bierbaum, M.; Gremer, L.; Triola, G.; Waldmann, H.; Bastiaens, P. I. H.; Wittinghofer, A. Arl2-GTP and Arl3-GTP regulate a GDI-like transport system for farnesylated cargo. *Nat. Chem. Biol.* **2011**, *7* (12), 942–949.
- (38) Kaya, P.; Schaffner-Reckinger, E.; Manoharan, G. b.; Vukic, V.; Kiriazis, A.; Ledda, M.; Burgos Renedo, M.; Pavic, K.; Gaigneaux, A.; Glaab, E.; Abankwa, D. K. An Improved PDE δ Inhibitor Combines with Sildenafil To Inhibit KRAS Mutant Cancer Cell Growth. *J. Med. Chem.* **2024**, *67* (11), 8569–8584.
- (39) Siddiqui, F. A.; Alam, C.; Rosenqvist, P.; Ora, M.; Sabt, A.; Manoharan, G. b.; Bindu, L.; Okutachi, S.; Catillon, M.; Taylor, T.; Abdelhafez, O. M.; Lönnberg, H.; Stephen, A. G.; Papageorgiou, A. C.; Virta, P.; Abankwa, D. PDE δ Inhibitors with a New Design Principle Selectively Block K-Ras Activity. *ACS Omega* **2020**, *5* (1), 832–842.
- (40) Yelland, T.; Garcia, E.; Parry, C.; Kowalczyk, D.; Wojnowska, M.; Gohlke, A.; Zalar, M.; Cameron, K.; Goodwin, G.; Yu, Q.; Zhu, P.-C.; ElMaghloob, Y.; Pugliese, A.; Archibald, L.; Jamieson, A.; Chen, Y. X.; McArthur, D.; Bower, J.; Ismail, S. Stabilization of the RAS:PDE δ Complex Is a Novel Strategy to Inhibit RAS Signaling. *J. Med. Chem.* **2022**, *65* (3), 1898–1914.
- (41) Chen, L.; Zhuang, C.; Lu, J.; Jiang, Y.; Sheng, C. Discovery of Novel KRAS–PDE δ Inhibitors by Fragment-Based Drug Design. *J. Med. Chem.* **2018**, *61* (6), 2604–2610.
- (42) Chen, L.; Zhang, J.; Wang, X.; Li, Y.; Zhou, L.; Lu, X.; Dong, G.; Sheng, C. Discovery of novel KRAS–PDE δ inhibitors with potent activity in patient-derived human pancreatic tumor xenograft models. *Acta Pharm. Sin. B* **2022**, *12* (1), 274–290.
- (43) Chen, D.; Chen, Y.; Lian, F.; Chen, L.; Li, Y.; Cao, D.; Wang, X.; Chen, L.; Li, J.; Meng, T.; Huang, M.; Geng, M.; Shen, J.; Zhang, N.; Xiong, B. Fragment-based drug discovery of triazole inhibitors to block PDE δ -RAS protein-protein interaction. *Eur. J. Med. Chem.* **2019**, *163*, 597–609.
- (44) Jiang, Y.; Zhuang, C.; Chen, L.; Lu, J.; Dong, G.; Miao, Z.; Zhang, W.; Li, J.; Sheng, C. Structural Biology-Inspired Discovery of Novel KRAS–PDE δ Inhibitors. *J. Med. Chem.* **2017**, *60* (22), 9400–9406.
- (45) Winzker, M.; Friese, A.; Koch, U.; Janning, P.; Ziegler, S.; Waldmann, H. Development of a PDE δ -Targeting PROTACs that Impair Lipid Metabolism. *Angew. Chem., Int. Ed. Engl.* **2020**, *59* (14), 5595–5601.
- (46) Bao, J.; Chen, Z.; Li, Y.; Chen, L.; Wang, W.; Sheng, C.; Dong, G. Discovery of Novel PDE δ Autophagic Degradators: A Case Study of Autophagy-Tethering Compound (ATTEC). *ACS Med. Chem. Lett.* **2024**, *15* (1), 29–35.
- (47) Cheng, J.; Li, Y.; Wang, X.; Dong, G.; Sheng, C. Discovery of Novel PDE δ Degradators for the Treatment of KRAS Mutant Colorectal Cancer. *J. Med. Chem.* **2020**, *63* (14), 7892–7905.
- (48) Chen, S.; Wang, W.; Chen, Z.; Li, R.; Wu, Z.; Dong, G.; Sheng, C. Peptide-Mediated Small Molecule Lysosome-Targeting Chimeras for Targeted Degradation of Membrane and Intracellular Proteins. *J. Med. Chem.* **2024**, *67* (17), 15807–15815.
- (49) Chandra, A.; Grecco, H. E.; Pisupati, V.; Perera, D.; Cassidy, L.; Skoulidis, F.; Ismail, S. A.; Hedberg, C.; Hanzal-Bayer, M.;

Venkitaraman, A. R.; Wittinghofer, A.; Bastiaens, P. I. H. The GDI-like solubilizing factor PDE δ sustains the spatial organization and signalling of Ras family proteins. *Nat. Cell Biol.* **2012**, *14* (2), 148–158.

(50) Schmick, M.; Kraemer, A.; Bastiaens, P. I. H. Ras moves to stay in place. *Trends Cell Biol.* **2015**, *25* (4), 190–197.

(51) Nancy, V.; Callebaut, I.; El Marjou, A.; de Gunzburg, J. The δ Subunit of Retinal Rod cGMP Phosphodiesterase Regulates the Membrane Association of Ras and Rap GTPases. *J. Biol. Chem.* **2002**, *277* (17), 15076–15084.

(52) Wilhelm, J.; Kühn, S.; Tarnawski, M.; Gotthard, G.; Tünnermann, J.; Tänzer, T.; Karpenko, J.; Mertes, N.; Xue, L.; Uhrig, U.; Reinstein, J.; Hiblot, J.; Johnsson, K. Kinetic and Structural Characterization of the Self-Labeling Protein Tags HaloTag7, SNAP-tag, and CLIP-tag. *Biochemistry* **2021**, *60* (33), 2560–2575.

(53) Erlanson, D. A.; Fesik, S. W.; Hubbard, R. E.; Jahnke, W.; Jhoti, H. Twenty years on: the impact of fragments on drug discovery. *Nat. Rev. Drug Discovery* **2016**, *15* (9), 605–619.

(54) Kumari, S.; Carmona, A. V.; Tiwari, A. K.; Trippier, P. C. Amide Bond Bioisosteres: Strategies, Synthesis, and Successes. *J. Med. Chem.* **2020**, *63* (21), 12290–12358.

(55) Wang, C.; Abegg, D.; Hoch, D. G.; Adibekian, A. Chemo-proteomics-Enabled Discovery of a Potent and Selective Inhibitor of the DNA Repair Protein MGMT. *Angew. Chem., Int. Ed. Engl.* **2016**, *55* (8), 2911–2915.

(56) Otrubova, K.; Cravatt, B. F.; Boger, D. L. Design, Synthesis, and Characterization of α -Ketoheterocycles That Additionally Target the Cytosolic Port Cys269 of Fatty Acid Amide Hydrolase. *J. Med. Chem.* **2014**, *57* (3), 1079–1089.

(57) Milacic, M.; Baers, D.; Conley, P.; Gong, C.; Gillespie, M.; Griss, J.; Haw, R.; Jassal, B.; Matthews, L.; May, B.; Petryszak, R.; Ragueneau, E.; Rothfels, K.; Sevilla, C.; Shamovsky, V.; Stephan, R.; Tiwari, K.; Varusai, T.; Weiser, J.; Wright, A.; Wu, G.; Stein, L.; Hermjakob, H.; D'Eustachio, P. The Reactome Pathway Knowledge-base 2024. *Nucleic Acids Res.* **2024**, *52* (D1), D672–D678.

(58) Zhang, H.; Steele, J. R.; Kahrood, H. V.; Lucas, D. D.; Shah, A. D.; Schittenhelm, R. B. Phospho-Analyst: An Interactive, Easy-to-Use Web Platform To Analyze Quantitative Phosphoproteomics Data. *J. Proteome Res.* **2023**, *22* (9), 2890–2899.

(59) Casado, P.; Rodriguez-Prados, J.-C.; Cosulich, S. C.; Guichard, S.; Vanhaesebroeck, B.; Joel, S.; Cutillas, P. R. Kinase-Substrate Enrichment Analysis Provides Insights into the Heterogeneity of Signaling Pathway Activation in Leukemia Cells. *Sci. Signaling* **2013**, *6* (268), rs6.

(60) Horn, H.; Schoof, E. M.; Kim, J.; Robin, X.; Miller, M. L.; Diella, F.; Palma, A.; Cesareni, G.; Jensen, L. J.; Linding, R. KinomeXplorer: an integrated platform for kinome biology studies. *Nat. Methods* **2014**, *11* (6), 603–604.

(61) Hornbeck, P. V.; Zhang, B.; Murray, B.; Kornhauser, J. M.; Latham, V.; Skrzypek, E. PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Res.* **2015**, *43* (D1), D512–D520.

(62) Singh, A.; Greninger, P.; Rhodes, D.; Koopman, L.; Violette, S.; Bardeesy, N.; Settleman, J. A Gene Expression Signature Associated with “K-Ras Addiction” Reveals Regulators of EMT and Tumor Cell Survival. *Cancer Cell* **2009**, *15* (6), 489–500.

(63) Luo, J.; Emanuele, M. J.; Li, D.; Creighton, C. J.; Schlabach, M. R.; Westbrook, T. F.; Wong, K.-K.; Elledge, S. J. A Genome-wide RNAi Screen Identifies Multiple Synthetic Lethal Interactions with the Ras Oncogene. *Cell* **2009**, *137* (5), 835–848.

(64) Scholl, C.; Fröhling, S.; Dunn, I. F.; Schinzel, A. C.; Barbie, D. A.; Kim, S. Y.; Silver, S. J.; Tamayo, P.; Wadlow, R. C.; Ramaswamy, S.; Döhner, K.; Bullinger, L.; Sandy, P.; Boehm, J. S.; Root, D. E.; Jacks, T.; Hahn, W. C.; Gilliland, D. G. Synthetic Lethal Interaction between Oncogenic KRAS Dependency and STK33 Suppression in Human Cancer Cells. *Cell* **2009**, *137* (5), 821–834.

(65) Ziegler, S.; Sievers, S.; Waldmann, H. Morphological profiling of small molecules. *Cell Chem. Biol.* **2021**, *28* (3), 300–319.

(66) Tandon, A.; Santura, A.; Waldmann, H.; Pahl, A.; Czodrowski, P. Identification of lysosomotropism using explainable machine learning

and morphological profiling cell painting data. *RSC Med. Chem.* **2024**, *15* (8), 2677–2691.

(67) Pahl, A.; Schölermann, B.; Lampe, P.; Rusch, M.; Dow, M.; Hedberg, C.; Nelson, A.; Sievers, S.; Waldmann, H.; Ziegler, S. Morphological subprofile analysis for bioactivity annotation of small molecules. *Cell Chem. Biol.* **2023**, *30* (7), 839–853.

(68) Hood, F. E.; Sahraoui, Y. M.; Jenkins, R. E.; Prior, I. A. Ras protein abundance correlates with Ras isoform mutation patterns in cancer. *Oncogene* **2023**, *42* (15), 1224–1232.

(69) Nuevo-Tapióles, C.; Philips, M. R. The role of KRAS splice variants in cancer biology. *Frontiers in cell and developmental biology* **2022**, *10*, 1033348.

(70) DuPage, M.; Dooley, A. L.; Jacks, T. Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nature protocols* **2009**, *4* (7), 1064–1072.

(71) Dietlein, F.; Kalb, B.; Jokic, M.; Noll, E. M.; Strong, A.; Tharun, L.; Ozretić, L.; Küntlinger, H.; Kambartel, K.; Randerath, W. J.; Jungst, C.; Schmitt, A.; Torgovnick, A.; Richters, A.; Rauh, D.; Siedek, F.; Persigehl, T.; Mauch, C.; Bartkova, J.; Bradley, A.; Sprick, M. R.; Trumpf, A.; Rad, R.; Saur, D.; Bartek, J.; Wolf, J.; Büttner, R.; Thomas, R. K.; Reinhardt, H. C. A Synergistic Interaction between Chk1- and MK2 Inhibitors in KRAS-Mutant Cancer. *Cell* **2015**, *162* (1), 146–159.

(72) Lanman, B. A.; Allen, J. R.; Allen, J. G.; Amegadzie, A. K.; Ashton, K. S.; Booker, S. K.; Chen, J. J.; Chen, N.; Frohn, M. J.; Goodman, G.; Kopecky, D. J.; Liu, L.; Lopez, P.; Low, J. D.; Ma, V.; Minatti, A. E.; Nguyen, T. T.; Nishimura, N.; Pickrell, A. J.; Reed, A. B.; Shin, Y.; Siegmund, A. C.; Tamayo, N. A.; Tegley, C. M.; Walton, M. C.; Wang, H.-L.; Wurz, R. P.; Xue, M.; Yang, K. C.; Achanta, P.; Bartberger, M. D.; Canon, J.; Hollis, L. S.; McCarter, J. D.; Mohr, C.; Rex, K.; Saiki, A. Y.; San Miguel, T.; Volak, L. P.; Wang, K. H.; Whittington, D. A.; Zech, S. G.; Lipford, J. R.; Cee, V. J. Discovery of a Covalent Inhibitor of KRASG12C (AMG 510) for the Treatment of Solid Tumors. *J. Med. Chem.* **2020**, *63* (1), 52–65.

(73) Fell, J. B.; Fischer, J. P.; Baer, B. R.; Blake, J. F.; Bouhana, K.; Briere, D. M.; Brown, K. D.; Burgess, L. E.; Burns, A. C.; Burkard, M. R.; Chiang, H.; Chicarelli, M. J.; Cook, A. W.; Gaudino, J. J.; Hallin, J.; Hanson, L.; Hartley, D. P.; Hicken, E. J.; Hingorani, G. P.; Hinklin, R. J.; Mejia, M. J.; Olson, P.; Otten, J. N.; Rhodes, S. P.; Rodriguez, M. E.; Savechenkov, P.; Smith, D. J.; Sudhakar, N.; Sullivan, F. X.; Tang, T. P.; Vigers, G. P.; Wollenberg, L.; Christensen, J. G.; Marx, M. A. Identification of the Clinical Development Candidate MRTX849, a Covalent KRASG12C Inhibitor for the Treatment of Cancer. *J. Med. Chem.* **2020**, *63* (13), 6679–6693.

(74) Ma, X.; Sloman, D. L.; Duggal, R.; Anderson, K. D.; Ballard, J. E.; Bharathan, I.; Brynczka, C.; Gathiaka, S.; Henderson, T. J.; Lyons, T. W.; Miller, R.; Munsell, E. V.; Orth, P.; Otte, R. D.; Palani, A.; Rankic, D. A.; Robinson, M. R.; Sather, A. C.; Solban, N.; Song, X. S.; Wen, X.; Xu, Z.; Yang, Y.; Yang, R.; Day, P. J.; Stoeck, A.; Bennett, D. J.; Han, Y. Discovery of MK-1084: An Orally Bioavailable and Low-Dose KRASG12C Inhibitor. *J. Med. Chem.* **2024**, *67*, 11024–11052.

(75) Maciag, A. E.; Stice, J. P.; Wang, B.; Sharma, A. K.; Chan, A. H.; Lin, K.; Singh, D.; Dyba, M.; Yang, Y.; Setoodeh, S.; Smith, B. P.; Ju, J. H.; Jeknic, S.; Rabara, D.; Zhang, Z.; Larsen, E. K.; Esposito, D.; Denson, J.-P.; Ranieri, M.; Meynardie, M.; Mehdizadeh, S.; Alexander, P. A.; Abreu Blanco, M.; Turner, D. M.; Xu, R.; Lightstone, F. C.; Wong, K.-K.; Stephen, A. G.; Wang, K.; Simanshu, D. K.; Sinkevicius, K. W.; Nissley, D. V.; Wallace, E.; McCormick, F.; Beltran, P. J. Discovery of BBO-8520, a First-In-Class Direct and Covalent Dual Inhibitor of GTP-Bound (ON) and GDP-Bound (OFF) KRASG12C. *Cancer Discovery* **2025**, *15* (3), 578–594.

(76) Schwartz, P. A.; Kuzmic, P.; Solowiej, J.; Bergqvist, S.; Bolanos, B.; Almaden, C.; Nagata, A.; Ryan, K.; Feng, J.; Dalvie, D.; Kath, J. C.; Xu, M.; Wani, R.; Murray, B. W. Covalent EGFR inhibitor analysis reveals importance of reversible interactions to potency and mechanisms of drug resistance. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111* (1), 173–178.

(77) Zecha, J.; Meng, C.; Zolz, D. P.; Samaras, P.; Wilhelm, M.; Kuster, B. Peptide Level Turnover Measurements Enable the Study of Proteoform Dynamics. *Mol. Cell. Proteomics* **2018**, *17* (5), 974–992.

- (78) Jemal, A.; Bray, F.; Center, M. M.; Ferlay, J.; Ward, E.; Forman, D. Global cancer statistics. *Ca-Cancer J. Clin.* **2011**, 61 (2), 69–90.
- (79) Clinical Lung Cancer Genome Project (CLCGP); Network Genomic Medicine (NGM). A Genomics-Based Classification of Human Lung Tumors. *Sci. Transl. Med.* **2013**, 5 (209), 209ra153.
- (80) Oliver, T. G.; Mercer, K. L.; Sayles, L. C.; Burke, J. R.; Mendus, D.; Lovejoy, K. S.; Cheng, M.-H.; Subramanian, A.; Mu, D.; Powers, S.; Crowley, D.; Bronson, R. T.; Whittaker, C. A.; Bhutkar, A.; Lippard, S. J.; Golub, T.; Thomale, J.; Jacks, T.; Sweet-Cordero, E. A. Chronic cisplatin treatment promotes enhanced damage repair and tumor progression in a mouse model of lung cancer. *Genes Dev.* **2010**, 24 (8), 837–852.
- (81) Pfirschke, C.; Engblom, C.; Rickelt, S.; Cortez-Retamozo, V.; Garriss, C.; Pucci, F.; Yamazaki, T.; Poirier-Colame, V.; Newton, A.; Redouane, Y.; Lin, Y.-J.; Wojtkiewicz, G.; Iwamoto, Y.; Mino-Kenudson, M.; Huynh, T. G.; Hynes, R. O.; Freeman, G. J.; Kroemer, G.; Zitvogel, L.; Weissleder, R.; Pittet, M. J. Immunogenic Chemotherapy Sensitizes Tumors to Checkpoint Blockade Therapy. *Immunity* **2016**, 44 (2), 343–354.
- (82) Schmitt, A.; Knittel, G.; Welcker, D.; Yang, T. P.; George, J.; Nowak, M.; Leiser, U.; Büttner, R.; Perner, S.; Peifer, M.; Reinhardt, H. C. ATM Deficiency Is Associated with Sensitivity to PARP1- and ATR Inhibitors in Lung Adenocarcinoma. *Cancer Res.* **2017**, 77 (11), 3040–3056.
- (83) Chidley, C.; Haruki, H.; Pedersen, M. G.; Muller, E.; Johnsson, K. A yeast-based screen reveals that sulfasalazine inhibits tetrahydrobiopterin biosynthesis. *Nat. Chem. Biol.* **2011**, 7 (6), 375–383.
- (84) Kabsch, W. XDS. *Acta Crystallogr., Sect. D: Struct. Biol.* **2010**, 66 (2), 125–132.
- (85) McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J. Phaser crystallographic software. *J. Appl. Crystallogr.* **2007**, 40 (4), 658–674.
- (86) Liebschner, D.; Afonine, P. V.; Baker, M. L.; Bunkóczi, G.; Chen, V. B.; Croll, T. I.; Hintze, B.; Hung, L. W.; Jain, S.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R. D.; Poon, B. K.; Prisant, M. G.; Read, R. J.; Richardson, J. S.; Richardson, D. C.; Sammito, M. D.; Sobolev, O. V.; Stockwell, D. H.; Terwilliger, T. C.; Urzhumtsev, A. G.; Videau, L. L.; Williams, C. J.; Adams, P. D. Macromolecular structure determination using X-rays, neutrons and electrons: recent developments in Phenix. *Acta Crystallogr., Sect. D: Struct. Biol.* **2019**, 75 (10), 861–877.
- (87) Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K. Features and development of Coot. *Acta Crystallogr., Sect. D: Struct. Biol.* **2010**, 66 (4), 486–501.
- (88) Afonine, P. V.; Grosse-Kunstleve, R. W.; Echols, N.; Headd, J. J.; Moriarty, N. W.; Mustyakimov, M.; Terwilliger, T. C.; Urzhumtsev, A.; Zwart, P. H.; Adams, P. D. Towards automated crystallographic structure refinement with phenix.refine. *Acta Crystallogr., Sect. D: Struct. Biol.* **2012**, 68 (4), 352–367.
- (89) Long, F.; Nicholls, R. A.; Emsley, P.; Graafland, S.; Merkys, A.; Vaitkus, A.; Murshudov, G. N. AceDRG: a stereochemical description generator for ligands. *Acta Crystallogr., Sect. D: Struct. Biol.* **2017**, 73 (2), 112–122.
- (90) Agirre, J.; Atanasova, M.; Bagdonas, H.; Ballard, C. B.; Baslé, A.; Beilstein-Edmands, J.; Borges, R. J.; Brown, D. G.; Burgos-Mármol, J. J.; Berrisford, J. M.; Bond, P. S.; Caballero, I.; Catapano, L.; Chojnowski, G.; Cook, A. G.; Cowtan, K. D.; Croll, T. I.; Debreczeni, J.; Devenish, N. E.; Dodson, E. J.; Drevon, T. R.; Emsley, P.; Evans, G.; Evans, P. R.; Fando, M.; Foadi, J.; Fuentes-Montero, L.; Garman, E. F.; Gerstel, M.; Gildea, R. J.; Hatti, K.; Hekkelman, M. L.; Heuser, P.; Hoh, S. W.; Hough, M. A.; Jenkins, H. T.; Jiménez, E.; Joosten, R. P.; Keegan, R. M.; Keep, N.; Krissinel, E. B.; Kolenko, P.; Kovalevskiy, O.; Lamzin, V. S.; Lawson, D. M.; Lebedev, A. A.; Leslie, A. G. W.; Lohkamp, B.; Long, F.; Malý, M.; McCoy, A. J.; McNicholas, S. J.; Medina, A.; Millán, C.; Murray, J. W.; Murshudov, G. N.; Nicholls, R. A.; Noble, M. E. M.; Oeffner, R.; Pannu, N. S.; Parkhurst, J. M.; Pearce, N.; Pereira, J.; Perrakis, A.; Powell, H. R.; Read, R. J.; Rigden, D. J.; Rochira, W.; Sammito, M.; Sánchez Rodríguez, F.; Sheldrick, G. M.; Shelley, K. L.; Simkovic, F.; Simpkin, A. J.; Skubak, P.; Sobolev, E.; Steiner, R. A.; Stevenson, K.; Tews, I.; Thomas, J. M. H.; Thorn, A.; Valls, J. T.; Uski,
- V.; Usón, I.; Vagin, A.; Velankar, S.; Vollmar, M.; Walden, H.; Waterman, D.; Wilson, K. S.; Winn, M. D.; Winter, G.; Wojdyr, M.; Yamashita, K. The CCP4 suite: integrative software for macromolecular crystallography. *Acta Crystallogr., Sect. D: Struct. Biol.* **2023**, 79 (6), 449–461.

3. Extended Discussion of Individual Manuscripts

The discussion sections of the manuscripts presented in this dissertation are subject to word limits that can constrain comprehensive literature analysis, mechanistic reasoning, and extensive discussion of limitations and future directions. This extended discussion chapter enables thorough literature contextualization, articulation of my own conclusions, and development of hypotheses that extend beyond the published interpretations and mark my own additional scientific contributions.

Further, I follow a two-part discussion structure. Chapters 3.1 through 3.4 address each manuscript individually, providing project-specific contextualization, mechanistic interpretation, and exploration of unanswered questions. Where the data and existing literature warrant, I extrapolate testable models to guide future directions. Chapter 4 then synthesizes the findings cumulatively, tracing how syndrome discovery informs therapeutic prediction, how gene dosage and protein homeostasis define boundaries of DNA repair, and how these insights might affect cancer treatment strategies.

3.1 Manuscript I: Characterization of the *CRIPT* Syndrome

3.1.1 Rationale and phenotypic overlap with Rothmund-Thomson syndrome

We investigated *CRIPT*-deficient fibroblasts to determine whether the phenotypic overlap with Rothmund-Thomson syndrome (RTS) reflects shared molecular mechanisms and found that biallelic *CRIPT* variants contribute to a RTS-like phenotype.²⁵⁸ In patient-derived fibroblasts, we observed elevated cellular senescence but no overt defects in the canonical genotoxic stress responses tested. This expands our understanding of the genetic heterogeneity of RTS and illustrates the challenge of distinguishing genome instability syndromes from phenotypically similar disorders.^{259,260} While the clinical overlap is detailed in the manuscript (**I-Figure 1B, 1C, 1F, 1H, 2A, 3A**), the key distinction is that the main RTS gene *RECQL4* possesses established roles in DNA replication and repair,^{174,261} while *CRIPT* has no previously established role in DNA metabolism and was instead primarily studied for its role in dendritic spine formation and synaptic plasticity.^{262-264,260} Given the phenotypic overlap with RTS, we first investigated whether *CRIPT*-deficient fibroblasts share the genotoxic sensitivity characteristic of genome instability syndromes.

Our systematic DNA damage testing revealed sensitivity in the range of wildtype and RTS controls to IR, mitomycin C, hydroxyurea, etoposide, and potassium bromate (**I-Figure 4K, 4L**), agents that probe DSB, ICLR, replication stress, and oxidative damage pathways, respectively, alongside an absence of chromosomal aberrations (**I-Figure 4A-C**).

This finding must be interpreted in the context of mixed literature for RTS and *RECQL4* deficiency itself, where genotoxic sensitivity has proven variable across studies depending on mutation spectrum.

Of note, severe truncating mutations in *RECQL4* that abolish multiple protein domains produce a more pronounced phenotype than missense variants concentrated in the helicase domain.^{265,266} Importantly, the loss of *CRIPT* expression in tested patient fibroblasts would not necessarily allow for such domain-specific compensatory effects. The absence of genotoxic sensitivity in *CRIPT*-deficient fibroblasts prompted investigation of alternative cellular mechanisms underlying the patient phenotype.²⁵⁸

3.1.2 Senescence as a primary phenotype and implications for *CRIPT* syndrome classification

We found that the cellular senescence observed in *CRIPT*-deficient fibroblasts (**I-Figure 3C, 3E, 3F**) may represent a primary phenotype independent of canonical DSBR defects.²⁵⁸

The link between cellular senescence and poikiloderma histopathology, discussed in the manuscript (**I-Figure 1F**) and literature^{267,268}, also raises questions about a connection between *CRIPT* deficiency and long-term cancer risk. Cellular senescence has opposing effects in tumorigenesis, where permanent proliferative arrest constitutes a potent tumor-suppressive barrier, while persistent senescent cells secrete SASP factors that can promote a tumor-permissive microenvironment over time.^{269,270} If intact DNA repair in *CRIPT*-deficient cells limits the acquisition of oncogenic mutations, this would predict a fundamentally different cancer risk profile compared to *RECQL4*-RTS, where SASP-driven microenvironmental changes may act synergistically with the DNA repair defects caused by *RECQL4* deficiency. No malignancies have been reported in *CRIPT*-deficient individuals,²⁵⁸ but the young and small patient group precludes conclusions about long-term cancer risk. Longitudinal follow-up will be essential to determine whether *CRIPT* deficiency confers elevated cancer prevalence comparable to *RECQL4*-RTS, which carries a well-established osteosarcoma risk.^{271,272}

However, the question of cancer risk is only one dimension of a more fundamental classification challenge for the *CRIPT* deficiency syndrome. Our findings raise the question of whether *CRIPT* deficiency should be overall classified within the RTS spectrum. *CRIPT* syndrome shares phenotypic hallmarks of genome instability syndromes, but lacks evidence for canonical DNA repair involvement (**I-Figure 4**). One interpretation is that cellular senescence, rather than DNA repair deficiency, may be the shared pathogenic mechanism across the genetically heterogeneous RTS spectrum.

This observation suggests that genome instability phenotypes may extend beyond canonical DNA damage defects to encompass elevated cellular senescence through DDR-independent pathways, with overlapping clinical consequences.

3.1.3 Convergence of Rothmund-Thomson syndrome-associated genes

Since publication, our findings have been incorporated into a comprehensive review of RTS genetics. Martins *et al.* cited our work and included *CRIPT* variants in their comparative RTS phenotype table alongside *RECQL4*, *ANAPC1*, and *DNA2*, discussing our senescence and genotoxicity data as potential evidence that *CRIPT* deficiency might produce RTS features through non-canonical mechanisms.²⁷³ The Online Mendelian Inheritance in Man (OMIM) database classified *CRIPT*-associated disease as RTS type 3 (MIM #615789), reflecting formal recognition of the phenotypic overlap. The expanding genetic spectrum of RTS, now encompassing *RECQL4*, *ANAPC1*, *DNA2*, and *CRIPT*, represents the molecular heterogeneity underlying a clinically coherent phenotype.^{273,274}

Of particular relevance to this classification, Hudson *et al.* demonstrated in 2025 that *DNA2* depletion causes cellular senescence through loss of DNA2-mediated fork processing that leads to uncontrolled replication and cell cycle exit.²⁷⁵ Similarly, Lu *et al.* showed that *RECQL4* dysfunction triggers premature senescence through accumulation of persistent DNA damage, with functional consequences including reduced blood cell counts in bone marrow and hair follicle senescence in an RTS mouse model.²⁷⁶ The convergence of *RECQL4*, and now *DNA2* and *CRIPT* deficiency on cellular senescence, each through apparently distinct upstream mechanisms, strengthens the hypothesis that senescence represents a unifying cellular phenotype across the RTS genetic heterogeneity spectrum, as proposed in our original study. This implies that senescence could be the common downstream effector across RTS subtypes regardless of the upstream genetic lesion. If so, targeting senescent cells by senolytics might be broadly applicable across the RTS spectrum rather than requiring gene-specific interventions.²⁷⁶⁻²⁷⁸ It also suggests that senescence-based cellular screening could serve as a functional readout for identifying additional RTS candidate genes.

3.1.4 Limitations

While the convergence of multiple RTS-associated genes on senescence supports our central hypothesis of *CRIPT* as a RTS variant, some methodological constraints should be considered. First, fibroblasts deficient in the additional RTS gene *ANAPC1* were unavailable, precluding a direct comparison with *ANAPC1*-RTS that could have clarified whether senescence is universal across RTS-spectrum disorders. Second, all cellular experiments were performed in patient-derived fibroblasts, which may not fully recapitulate the biology of other affected tissues.

Fibroblasts are a surrogate tissue, while the neurodevelopmental phenotype in *CRIPT* deficiency likely reflects neuronal functions that cannot be captured in fibroblasts. Similarly, poikiloderma involves keratinocytes and vascular endothelium alongside fibroblasts. Stem cell-derived neuronal models or keratinocyte cultures could provide more disease-relevant insights into *CRIPT* deficiency. Third, the limited number of patient-derived cell lines in cell viability experiments constrains statistical power and generalizability due to potential variability between cell lines. Fourth, while the combination of elevated *p53*, *p16*, and *p21* expression with increased SA- β -gal activity is consistent with premature cellular senescence, irreversible proliferative arrest and SASP cytokine secretion were not tested. Incorporating proliferation markers and SASP profiling would strengthen the senescence classification. The detected senescence in fibroblasts connects to the phenotype of *CRIPT*-deficient patients and, while further mechanistic work is required, demonstrates impact on RTS research and diagnosis.

3.1.5 Conclusions and future directions

Clinically, our findings expand the differential diagnosis of RTS in pediatric practice, particularly for individuals presenting with neurodevelopmental features atypical for classical *RECQL4*-RTS, and may inform prognosis, cancer surveillance, and genetic counseling. Mechanistically, the results highlight senescence as a shared endpoint that can arise through DNA damage-independent pathways, broadening the pathogenic spectrum of progeroid syndromes beyond the DDR spectrum, as laminopathies illustrate.¹⁶⁷ The consistent senescence phenotype across tested RTS subtypes provides a rational basis for preclinical investigation of senescence-targeting approaches.²⁷⁶⁻²⁷⁸ For syndrome discovery, computational gestalt analysis provided phenotypic evidence supporting pathogenicity and syndrome-associations of *CRIPT* variants before functional data were available (**I-Figure 2C-D**), demonstrating how computational phenotyping can initiate and guide variant interpretation in unsolved cases. With established genes explaining approximately 70 % of RTS cases,²⁷³ systematic application of such approaches could accelerate identification of additional candidate genes.

In summary, this work established *CRIPT* as a novel potential RTS-spectrum gene and identified cellular senescence as a shared phenotype arising independently of canonical DNA repair defects.²⁵⁸ This identification depended on sequencing-based discovery of the underlying genetic variants. While exome sequencing remains the clinical standard for such cases and was sufficient to detect *CRIPT* variants, it cannot reliably detect larger genomic alterations such as structural variants, leaving potential syndrome candidates unidentified and their mechanistic insights unexplored. The following chapter examines how optical genome mapping addresses this diagnostic gap.²⁷⁹

3.2 Manuscript II: Optical Genome Mapping Identifies Structural Variants

3.2.1 Rationale and study overview

Hereditary cancer predisposition extends beyond the single nucleotide variants and small indels detectable by whole-genome and whole-exome sequencing (WGS, WES), which dominate current diagnostic workflows.¹⁸² Structural variants (SV) represent an underexplored category of cancer predisposition, with emerging evidence suggesting they account for a relevant fraction of unexplained familial cancer risk.²⁸⁰ Short-read-based WGS and WES have limited sensitivity for detecting structural variants and balanced chromosomal rearrangements. Long-read sequencing technologies improve SV detection but remain costly for routine clinical screening. This limitation has historically constrained systematic investigation of this variant class. OGM offers a complementary approach that enables genome-wide SV detection at comparatively low cost and high throughput.²⁸¹⁻²⁸³ Applying OGM, we screened 34 pediatric cancer patients (**II-Figure 1A**) with suspected but unconfirmed hereditary predisposition and identified potential clinically relevant SVs in two cases (**II-Figure 1B-C**).²⁷⁹ Overall, *de novo* SVs were detected in 17 % (3/18) of analyzed trios (**II-Figure 1D**), which is consistent with population-based estimates from Kloosterman *et al.*, who reported *de novo* SVs at a rate of 0.16 per generation, approximately 16 % of individuals carrying at least one *de novo* SV, across 250 families.²⁸⁴

Several recent studies across different clinical contexts reinforce the complementary value of OGM and highlight the broader diagnostic gap created by reliance on any single detection method. Tang *et al.* demonstrated significant advantages of OGM through detection of additional aberrations in comparison to karyotyping, fluorescence *in situ* hybridization, and RT-PCR specifically in *BCR::ABL1* and found no additional diagnostic value beyond OGM.²⁸⁵ This finding adds to our observations with P28, where the *de novo* 166 kb duplication of 17p13.3 would have been likely overlooked by standard cytogenetic approaches. Budurlean *et al.* integrated OGM with WGS for somatic SV detection in 29 pediatric B-cell acute lymphoblastic leukemia (ALL) patients and reported only 11.6 % overlap between the two approaches.²⁸⁶ This demonstrates that OGM and WGS capture largely non-overlapping variant populations and suggests that both technologies may be required to achieve diagnostic completeness for germline cancer predisposition screening.

Larger studies have begun to quantify the contribution of SVs to cancer risk. Gillani *et al.* examined germline SVs in 1,765 pediatric solid tumor patients and estimated that rare SVs explain 1.1 % to 5.6 % of cancer liability.²⁸⁰

Together with earlier estimates that WES-based SNV/InDel screening identifies germline predisposition in 8.5 % of pediatric cancer patients,²⁸⁷ these data suggest that true predisposition prevalence is likely higher when the full SV spectrum is captured. Our own analysis with *de novo* SVs in 17 % of families reinforces that *de novo* structural changes may contribute to pediatric cancer risk independently of inherited alleles, a possibility that has not been systematically investigated.

For single nucleotide variants and small indels, the field has developed robust classification frameworks combining population frequency databases (gnomAD)²⁸⁸ and clinical variant databases (ClinVar)²⁸⁹. These integrated approaches enable classification of sequence variants encountered in clinical practice. However, SV interpretation remains largely case-by-case and dependent on gene context, with systematic frameworks for functional assessment being developed.²⁹⁰ Moving SVs from detection to interpretation requires development of systematic validation approaches including RNA-seq to assess gene expression and functional assays to determine phenotypes consistent with the suspected disease, as demonstrated by our work.²⁷⁹ It would be beneficial to introduce OGM as a screening standard alongside WES/WGS for variant detection. High-resolution SV detection technologies and variant characterization have the potential to expand the spectrum of known cancer predisposition variants, which ultimately would benefit clinical decision making.

3.2.2 Characterization of a *de novo* 17p13.3 duplication encompassing *RPA1*

This potential of OGM is demonstrated by our identification of a *de novo* 166 kb duplication of 17p13.3 in patient P28 (**II-Figure 2D**), an infant with *KMT2A*-rearranged leukemia, affecting five genes including *RPA1*.²⁷⁹ Among these, *RPA1* represents the strongest candidate for functional relevance, as the remaining four genes, *WDR81*, *SERPINF1*, *SERPINF2*, and *SMYD4*, lack established roles in genome maintenance. *SMYD4* has been implicated in tumor biology with context-dependent roles across cancer types,²⁹¹ but its functions are unrelated to DDR pathways and the observed phenotype. Germline *RPA* perturbations would be predicted to compromise genomic stability across several DNA damage response axes simultaneously due to RPA's essential role in DNA replication, recombination, and multiple repair pathways.²⁹² The duplication elevated *RPA1* mRNA expression (**II-Figure 2E**), yet protein levels remained comparable to controls (**II-Figure S5A; section 2.2.3**), suggesting that protein regulation and turnover could have partially buffered the dosage effect. If protein levels are maintained near normal, functional consequences could reflect altered expression dynamics during S phase or following DNA damage, or disrupted RPA complex stoichiometry, rather than constitutive overexpression. Germline *RPA1* perturbations have independently been linked to human disease. Sharma *et al.* identified heterozygous *RPA1* missense variants with increased ssDNA-binding affinity in patients with short telomere syndromes.²⁹³

Both observations establish *RPA1* as a dosage- and function-sensitive gene where modest germline changes can have clinical consequences.

However, in contrast to the telomere shortening observed in those patients, P28 fibroblasts showed mildly increased rather than shortened telomeres (**II-Figure S5B, S5C; section 2.2.3**), suggesting that distinct *RPA1* perturbation types may have different effects on telomere homeostasis. Further, tissue-specific differences between tested fibroblasts and hematopoietic cells, or age-dependent penetrance in an infant whose telomere phenotype may not yet have manifested, could explain the discrepancy between these observations.

The hypersensitivity of P28 fibroblasts to both cisplatin and hydroxyurea (**II-Figure S5D, S5E, section 2.2.3**), mechanistically distinct genotoxic stressors, points to a generalized defect in DNA damage tolerance rather than a pathway-specific impairment, consistent with the earlier observation by Outwin *et al.* that 17p13.3 duplication elevates DNA damage sensitivity.²⁹⁴ Because RPA coats ssDNA intermediates generated during both repair and replication, increased gene dosage may disrupt the stoichiometric balance within the RPA complex or with downstream effectors, impairing coordinated handoff to repair factors beyond saturating ssDNA binding capacity.²⁹²

KMT2A-rearranged infant ALL is characterized by chromosomal translocations arising *in utero*, potentially through error-prone DNA repair.²⁹⁵ While genome instability from germline *RPA1* perturbation could plausibly contribute to leukemogenesis risk, establishing such a link would require systematic germline sequencing of larger infant ALL cohorts, which was beyond the scope of this work.

3.2.3 Additional structural variants of uncertain significance

Functional validation of two additional SVs, a tandem *ADAM9/ADAM32* duplication in patient P16 (**II-Figure S2**) and compound heterozygous *RAP1B* regulatory variants in patient P10 (**II-Figure S3**), showed no altered gene expression or fusion transcripts in peripheral blood.²⁷⁹ These negative results in accessible tissue do not exclude pathogenicity, as these variants may exert tissue-specific or context-dependent effects not captured in peripheral blood samples. Alternatively, the variants might contribute to tumorigenesis through somatic second hits or clonal selection.

This highlights a broader limitation in SV interpretation, where negative results from functional validation in accessible tissues remain difficult to interpret, while still narrowing the hypothesis space and demonstrating the rigor of the validation process. These challenges are addressed further in the study's limitations.

3.2.4 Limitations

The *RPA1* finding derives from a single patient, precluding the classification of this specific SV as a recurrent cancer predisposition mutation, while serving as a precedent for the necessity of SV screening and functional characterization in the clinic.

The genomic context of patient P28 with parental consanguinity complicates phenotype attribution, as homozygous regions may harbor recessive variants contributing to the clinical presentation.

While we have argued that *RPA1* is the most plausible candidate among the five duplicated genes, definitive attribution would require functional characterization of each gene individually, which was beyond the scope of this study. Additionally, functional characterization of P28 was performed in patient-derived fibroblasts, which may not recapitulate the biology of hematopoietic cells where leukemogenesis occurred. Nevertheless, fibroblasts were sufficient to demonstrate DNA damage sensitivity, providing initial functional evidence linking the *RPA1* duplication to a cellular phenotype.

3.2.5 Conclusions and future directions

This work demonstrates that OGM captures clinically relevant SVs missed by standard sequencing approaches, reinforcing the limited overlap between OGM and WGS/WES approaches and supporting integration of OGM into germline cancer predisposition screening. The identification of a *de novo* duplication encompassing *RPA1* in an infant with *KMT2A*-rearranged leukemia shows how SV detection can implicate established DDR genes in cancer predisposition through variant classes that escape conventional diagnostics. This work suggests that the true prevalence of germline cancer predisposition may be underestimated when SV screening is omitted.

Our findings illustrate a gap between SV detection and clinical interpretation. OGM identified the 17p13.3 duplication, but determining its pathogenicity required functional characterization. Such multi-step validation is not yet standardized for SVs in the way that classification frameworks exist for SNVs and indels. Developing comparable SV interpretation guidelines remains an important unmet need. The central contribution of this work is not definitive proof that the 17p13.3 duplication causes cancer predisposition. Rather, it shows that integrating OGM into clinical sequencing pipelines, followed by functional characterization, could identify pathogenic variants that current workflows miss. This approach could be applicable to the substantial fraction of hereditary cancer cases that remain unexplained by current diagnostic standards.

3.3 Manuscript III: From Syndrome to DNA Repair Mechanism to Actionable Cancer Vulnerabilities: UBQLN1 in DNA Repair

3.3.1 *UBQLN1* deficiency syndrome discovery

Our discovery and characterization of the *UBQLN1* deficiency syndrome demonstrates how ultra-rare genome instability disorders can reveal previously unknown regulatory mechanisms of DNA repair.²⁹⁶ The two known affected individuals presented with hallmark features of genome instability, prompting systematic investigation that revealed genotoxic sensitivity, and delayed DSB repair (**III-Figure 1, S1**). The mechanism of this genotoxic sensitivity was examined in isogenic cell lines that demonstrated *UBQLN1* recruitment to DNA damage sites and impaired HR by premature degradation of RAD51 when *UBQLN1* was deleted (**III-Figure 2-3, S2-5**). Together, these findings support classification of *UBQLN1* deficiency as a DSBR disorder and identify *UBQLN1* as a regulatory factor governing HR through RAD51 protein homeostasis.²⁹⁶

Validation of DNA damage sensitivity in embryonic fibroblasts of the *Ubqln1*-deficient mouse model harboring the corresponding human mutation strengthens confidence in the causal relationship between *Ubqln1* loss and the observed phenotype (**III-Figure 1H-L, S1B, S3A-C**). Beyond cellular sensitivity, the mouse model recapitulated the seizure phenotype observed in patients, supporting physiological relevance of the mutation (**III-Figure S1B, S3D**). However, mice lacked major morphological characteristics of the *UBQLN1* deficiency syndrome patients, such as developmental delay. Mouse models of human genome instability syndromes frequently exhibit attenuated phenotypes, and species-specific differences must be considered, as documented for *Atm*-deficient mice introduced in section 1.5.4.^{192,193} Additionally, the recency of this syndrome discovery results in limited patient follow-up data, and longitudinal studies will be essential to characterize cancer predisposition, a feature expected based on the DNA repair defect but not documented in our young syndrome patients.

As introduced, Jachimowicz *et al.* published the *UBQLN4* deficiency syndrome in 2019.¹³⁶ Both paralogs, *UBQLN1* and *UBQLN4*, share close genetic and structural homology, which makes it tempting to compare the physiological consequence of each deficiency (**III-Figure S1B**). Both syndromes show developmental delay, intellectual disability, and facial dysmorphisms consistent with genome instability disorders. However, seizures uniquely characterize *UBQLN1* deficiency, while ataxia marks *UBQLN4* deficiency.¹³⁶ This divergence is consistent with the distinct role of *UBQLN1* in DSBR, which we discovered to be the protection of RAD51. The following sections examine and develop this molecular mechanism, and discuss future directions.

3.3.2 The "shielding" model of UBQLN1

Mechanistically, our data are consistent with a "shielding" model whereby UBQLN1 interaction protects RAD51 from ubiquitin-dependent proteasomal degradation (**Figure 4**).²⁹⁶ Proteasome inhibition with MG132 rescued RAD51 protein levels in the chromatin fraction (**III-Figure 3B-D, S5B**) and nuclear RAD51 foci formation (**III-Figure 3A**) in *UBQLN1*-deficient cells, demonstrating increased proteasome-dependent degradation of RAD51 in these cells. Further, *in vitro* pulldown experiments showed that UBQLN1 preferentially binds non-ubiquitinated RAD51 compared to pre-ubiquitinated RAD51 (**III-Figure 3H**), suggesting that UBQLN1 may compete with the ubiquitin-conjugating machinery rather than recognizing ubiquitinated substrates for delivery to the proteasome. This binding preference distinguishes RAD51 stabilization from UBQLN1's canonical function, shuttling ubiquitinated substrates for proteasomal degradation.¹⁵⁰ Additionally, loss of *UBQLN1* increased RAD51 polyubiquitination in immunoprecipitation experiments (**III-Figure 3F, S5F**). In line with this, further immunoprecipitation experiments indicated that UBQLN1 interacts with RAD51 via the UBQLN1 STI1-II domain (**III-Figure 3J**) and this type of binding has been shown to facilitate substrate stabilization, but not canonical degradation.¹⁵⁰

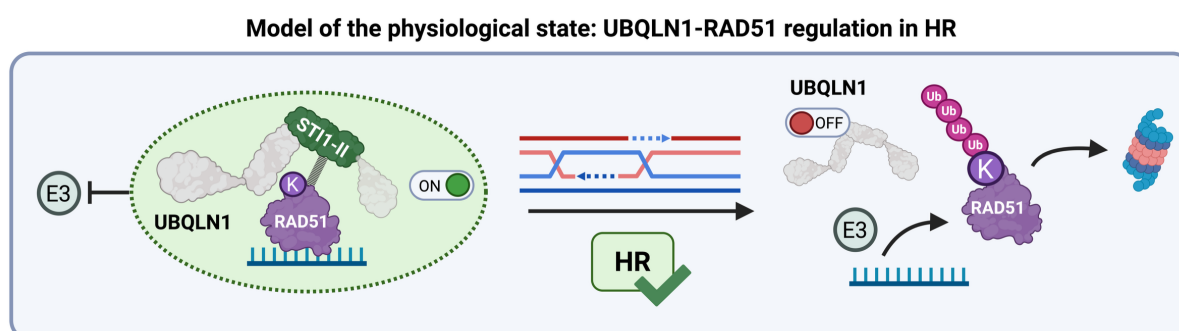


Figure 4: Proposed model of UBQLN1-mediated RAD51 regulation during HR. Under physiological conditions, UBQLN1 may bind chromatin-associated RAD51 via its STI1-II domain, potentially shielding cryptic lysine residues from E3 ligase access and thus preventing premature ubiquitination. This stabilizes RAD51 on resected ssDNA, enabling strand invasion and homology-directed repair. Following completion of strand invasion, UBQLN1 is proposed to disengage through an as-yet-uncharacterized mechanism, exposing RAD51 to polyubiquitination and proteasomal degradation, thereby permitting downstream HR. Figure created with BioRender.

Based on these findings, I hypothesize that RAD51 may possess at least one cryptic ubiquitination site that is shielded from E3 interaction and ubiquitination when UBQLN1 is bound. This would result in the shown RAD51 stabilization on chromatin and efficient HR. Upon *UBQLN1* loss, these sites would become accessible to E3 ligase activity prematurely, leading to K48-linked polyubiquitination and proteasomal targeting (**Figure 5**), as we observed (**III-Figure 3F**). This model would benefit from structural characterization of the UBQLN1-STI1-II binding interface on RAD51 and mass spectrometric comparison of RAD51 ubiquitination sites in *UBQLN1*-proficient versus *UBQLN1*-deficient contexts.

The identity of the E3 ligase that is presumably antagonized by UBQLN1 remains unknown. Numerous lysine residues have been identified as potential ubiquitination sites on RAD51, including K40, K57, K58, K64, K70, K73, K80, K107, K156, and K285.²⁹⁷⁻³⁰⁰ The E3 ligase RFWD3 was found to polyubiquitinate RAD51, involving residues K57, K58, K64, K107, and K156, promoting RAD51 chromatin extraction and proteasomal degradation.³⁰¹ Lysine-to-arginine mutation of all five residues largely abolished ubiquitination and caused persistent RAD51 chromatin retention.³⁰¹ Of note, RFWD3 activity requires ATR/ATM-dependent phosphorylation,³⁰¹ implicating it specifically in damage-dependent RAD51 regulation. RFWD3 is among the best-characterized RAD51 E3 ligases with defined target sites. It would be worthwhile to test whether mutation of the five identified residues would stabilize RAD51 upon *UBQLN1* loss. A deletion of a region encompassing these clustered candidate target lysines on RAD51 could be tested in immunoprecipitation and functional experiments for constituting the UBQLN1-STI1-II binding interface and subsequent protection of RAD51 from ubiquitination.

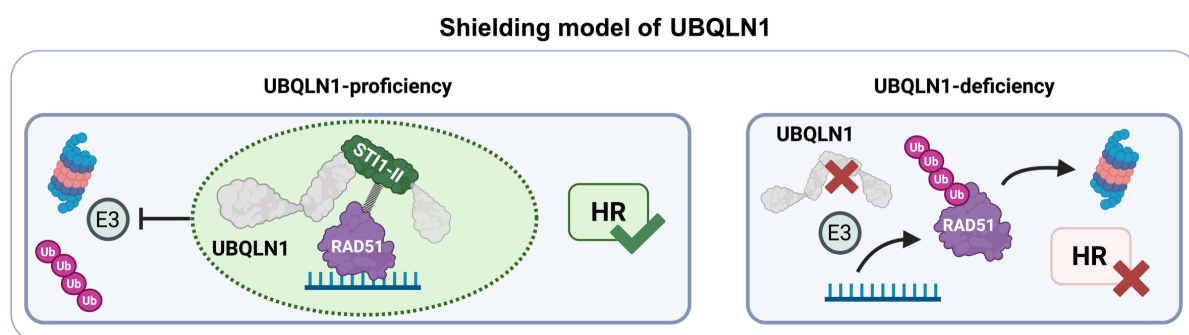


Figure 5: The "shielding" model of UBQLN1 in HR. In UBQLN1-proficient cells (left), UBQLN1 is proposed to shield RAD51 from E3 ligase-mediated polyubiquitination, maintaining chromatin-bound RAD51 levels sufficient for efficient HR. In *UBQLN1*-deficient cells, unprotected RAD51 is prematurely ubiquitinated and degraded by the proteasome, depleting the chromatin-bound RAD51 pool and impairing HR. Figure created with BioRender.

Of note, additional E3 ligases have been shown to regulate RAD51 in distinct contexts. For example, EMI1 orchestrates SCF complex assembly to promote cytoplasmic RAD51 degradation,^{302,303} a mechanism potentially regulating the overall cellular RAD51 pool beyond our focused observations at the chromatin. Further, FBH1 mediates RAD51 mono-ubiquitination at K58/K64, controlling localization rather than degradation.²⁹⁷ Recent work revealed that UHRF1 maintains RAD51 levels by targeting RFWD3 for proteasomal degradation.³⁰⁴ Our data suggest that UBQLN1 acts directly on RAD51, where it may function independently of or in competition with the UHRF1-RFWD3 axis. Epistasis experiments would address whether UBQLN1 competes with the E3 ligase targeting RAD51, presumably RFWD3, and if other RAD51 degradation pathways converge or operate in parallel. Until the relevant E3 ligase is identified, the E3-competition aspect of the "shielding" model remains a hypothetical framework.

Several additional mechanistic questions remain unresolved. The stoichiometry of UBQLN1:RAD51 binding is unknown. Furthermore, how UBQLN1 is recruited to and displaced from RAD51 to permit physiological ubiquitination and removal from ssDNA remains to be determined. UBQLN1 PTMs, competition with other RAD51-binding proteins, or conformational changes in RAD51 itself could potentially regulate this dissociation.

3.3.3 UBQLN1 within the multi-layered RAD51 regulatory network

Our identification of UBQLN1 as a RAD51 stabilizer adds to an increasingly complex regulatory network controlling RAD51 protein homeostasis.²⁹⁶ To place UBQLN1 within this network, it is useful to compare it with established regulators that operate through similar or opposing mechanisms.

Our finding that UBQLN1 stabilizes RAD51 through physical association (**III-Figure 3F-J, S5F-G**) parallels the mechanism described for DDB2, a DNA damage recognition factor that functions primarily in the NER pathway. DDB2 stabilizes RAD51 by direct binding, disrupting its ubiquitination and subsequent proteasomal degradation in triple-negative breast cancer cells.³⁰⁵ DDB2 depletion sensitized cells to PARP inhibitors by destabilizing RAD51 and impairing HR. Whether DDB2 and UBQLN1 function redundantly, in distinct cellular contexts, or in different pathways, suggested by the NER-specific function of DDB2, remains to be determined.

However, RAD51 stabilization extends beyond ubiquitin-proteasome regulation. For example, GRB2 stabilizes RAD51 on replication forks by inhibiting its ATPase activity rather than preventing degradation.³⁰⁶ RAD51 stabilization therefore involves mechanistically diverse processes with different functional outcomes.

While UBQLN1 stabilizes RAD51 protein levels on chromatin (**III-Figure 3A-D**), the FLIP-FIGNL1 complex could represent an opposing regulatory axis that actively removes RAD51 from chromatin. FLIP forms a stable complex with FIGNL1, and loss of either protein leads to persistent RAD51 foci and increased RAD51 chromatin association following DNA damage.³⁰⁷ FIGNL1 was proposed to promote RAD51 dissociation from DNA substrates.³⁰⁸ Given that either excessive or insufficient RAD51 chromatin association causes genomic instability, UBQLN1 and FLIP-FIGNL1 may function as counterbalancing regulators maintaining optimal RAD51 dynamics. Whether UBQLN1-mediated stabilization influences the pool of RAD51 available for FIGNL1-dependent removal, and whether UBQLN1 binding might protect RAD51 from FIGNL1 engagement, remain to be investigated.

How UBQLN1 is recruited to DSB sites remains unknown. Whether UBQLN1 relocates to damage foci through direct DNA damage signaling, co-recruitment with RAD51 or E3, or constitutive chromatin association will be important for understanding its spatiotemporal relationship with the regulators discussed above.

3.3.4 The "domain switch" theory of UBQLN1

Since RAD51 interacts with UBQLN1 via the STI1-II domain rather than the canonical UBA-UBL degradation axis, it is tempting to speculate that UBQLN1's function, protective or degrading, may be determined by the domain through which substrate engagement occurs (**III-Figure 3I-J**).²⁹⁶

Prior work supports UBQLN1-STI1 domains as mediators of ubiquitin-independent substrate recognition and protective interactions.¹⁵⁰ This is in line with our observation that UBQLN1 preferentially binds non-ubiquitinated RAD51 *in vitro* (**III-Figure 3H**). This preference remains to be validated *in cellulo* within the chromatin context. Structurally, STI1-like domains share high similarity to the co-chaperone Stress-inducible-phosphoprotein 1 (STI1). Each STI1 domain of UBQLN1 comprises five amphipathic alpha-helices arranged in a configuration that forms hydrophobic grooves for substrate binding.³⁰⁹ This architecture is well-suited for direct protein-protein interactions independent of substrate ubiquitination.

Critically, Kurlawala *et al.* showed that UBQLN1 recognizes and binds transmembrane proteins via STI domains, while UBA domain binding presumably blocks ubiquitin chain elongation and shields the substrate from proteasomal recognition.^{150,310} Whether our observed protection of RAD51 additionally requires the UBA domain for functional shielding upon RAD51 binding remains to be investigated. If so, this would place RAD51 within the same protective mode as the transmembrane protein stabilization mechanism, with STI1-mediated recognition coupled to UBA-mediated shielding.

Functional evidence across diverse substrates supports such a stabilizing role for UBQLN1. UBQLN1 has been shown to stabilize transmembrane proteins,^{150,311-313} and UBQLN1 has been shown to protect GABA_A-receptors from degradation in the murine brain, connecting *Ubqln1* deficiency to epilepsy.^{314,315} Indeed, we observed seizures in our *Ubqln1*-deficient mouse strain and the syndrome patients alongside cellular sensitivity to DNA damage. Very recently, UBQLN1 was shown to protect cells from ferroptotic cell death by stabilizing the antioxidant enzyme GPX4 via its STI1 domain.³¹⁶ These examples illustrate that the role of UBQLN1 as a stabilizing protein, rather than solely a degrading shuttle factor, is increasingly recognized.

The RAD51 protection mechanism may exemplify a broader principle governing UBQLN1 substrate selectivity. Under this model, substrate fate would be determined by the mode of substrate recognition: STI1-mediated ubiquitin-independent recognition, potentially coupled with UBA-mediated shielding, or UBA-mediated ubiquitin-dependent engagement coupled with UBL-mediated proteasome targeting and degradation.

The accessibility or activation of STI1-mediated versus ubiquitin-UBA-UBL-mediated substrate engagement may therefore represent a critical decision point in UBQLN1 function: a "domain switch" where the interaction mode determines UBQLN1's function and substrate fate (Figure 6).

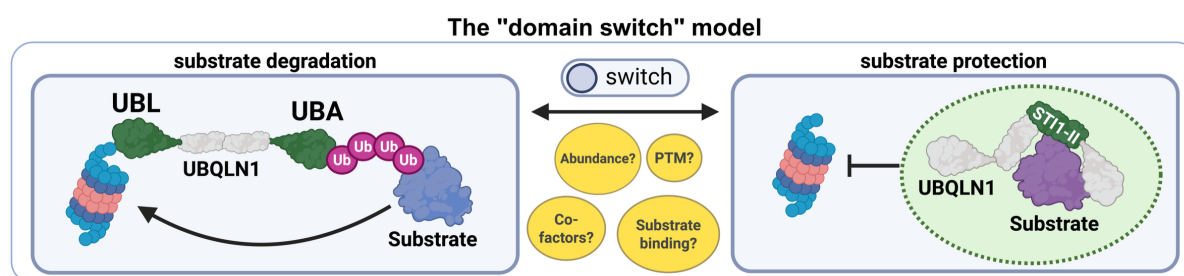


Figure 6: Proposed "domain-switch" theory of UBQLN1 function. UBQLN1 is hypothesized to toggle between two functional states. In its canonical degradation shuttle mode (left), the UBA domain recognizes polyubiquitinated substrates and the UBL domain delivers them to the proteasome, as observed for MRE11 in HR. In the protective mode (right), the STI1-II domain engages client proteins in a ubiquitin-independent manner, potentially shielding them from E3 ligase access and premature degradation, as observed for RAD51 in HR. The switch between these states may be regulated by post-translational modifications, protein abundance, cofactor binding, or substrate-induced conformational changes, though the mechanism remains uncharacterized. Figure created with BioRender.

The "domain switch" theory is a working hypothesis that organizes the current observations and generates testable predictions, rather than a proven mechanism. How UBQLN1 switches between degradation-promoting and stabilization-promoting mode remains unknown. PTM-induced conformational changes could alter STI1 accessibility for protective ubiquitin-independent substrate engagement. Alternatively, cofactor binding might actively redirect UBQLN1's function toward specific substrates. Resolving the mechanism of functional switching would benefit from structural approaches. However, no high-resolution structural data exist for full-length UBQLN1 and AlphaFold prediction models show very low confidence, compounded by the intrinsically disordered nature of the STI1 domains. Cryo-electron microscopy of UBQLN1 domain deletion mutants in substrate complexes could address this gap and may enable the separation-of-function experiments needed to test the "domain switch" theory.

3.3.5 The UBQLN1/UBQLN4 paradox of opposing HR phenotypes

If UBQLN1's functional outcome depends on the domain through which it engages its substrate, it is tempting to ask how structurally similar Ubiquilins with identical domain architecture achieve distinct and even opposing functional outcomes in the same pathway.

The case of UBQLN4 in HR provides a direct test of this question. UBQLN4 is phosphorylated by ATM following DNA damage and interacts with ubiquitinated MRE11 to promote proteasomal degradation of MRE11.¹³⁶ *UBQLN4* loss causes MRE11 chromatin retention and enhances HR activity, while *UBQLN4* overexpression increases MRE11 degradation, which represses DNA end resection, induces HRD, and sensitizes cells to PARP inhibitors.¹³⁶ The question arises why *UBQLN1* loss impairs HR (**III-Figure 2D**) while *UBQLN4* loss promotes it, but overexpression of both represses HR, despite both paralogs share approximately 70 % sequence similarity and identical domain architecture.³¹⁷

At physiological abundance, UBQLN4 regulates MRE11,¹³⁶ whereas UBQLN1 stabilizes RAD51,²⁹⁶ each enabling HR. Because these paralogs exert functionally opposing effects on their respective substrates, loss of *UBQLN1* or *UBQLN4* produces opposing HR outcomes. Importantly, our data showing that *UBQLN1* overexpression reduces MRE11 foci suggests potential substrate overlap at supraphysiological expression levels (**III-Figure 4C**). Overexpressed UBQLN1 may engage MRE11 through its UBA-UBL-dependent canonical degradation function, which shows broader substrate selection via UBA-mediated interaction (**III-Figure 4D**), rather than through direct STI1-II-mediated binding as observed with RAD51. This implies that substrate specificity could operate at physiological concentrations but may be overridden by expression level and domain function.

Sequence analysis revealed a potential structural basis for paralog-specific substrate recognition. While the STI1-II domains share high sequence similarity between UBQLN1 and UBQLN4, my comparative sequence analysis via UniProt³¹⁸ revealed that an intrinsically disordered region (IDR) immediately preceding the UBQLN1 STI1-II domain (Q327-V367) diverges substantially from other Ubiquilins and may function as a UBQLN1-specific selectivity filter. This IDR contains an SQ motif representing a potential ATM/ATR phosphorylation site.¹⁴⁴ Whether this site is phosphorylated following DNA damage and whether this IDR confers STI1-II selectivity for RAD51 are testable predictions.

Beyond substrate identity, the timing of substrate engagement may distinguish paralog function. UBQLN4 degrades MRE11 early in DSBR initiation, guiding repair toward NHEJ before committing to HR.¹³⁶ By contrast, we found that *UBQLN1* overexpression promotes MRE11 degradation, but also enhances long-range DNA end resection, and elevates SSA while suppressing HR (**III-Figure 4F-G**), suggesting timed effects on MRE11 after commitment to end resection. These observations suggest that *UBQLN1* overexpression might cause MRE11 degradation less efficiently or later than UBQLN4. A later- or less-depleted MRE11 pool could suffice for resection initiation but might prove inadequate for MRE11's downstream negative regulation role in limiting EXO1/DNA2-BLM-dependent resection.³¹⁹⁻³²¹

Incomplete or late MRE11 degradation may permit uncontrolled long-range resection, generating the observed extensive ssDNA tracts that expose distant homologous repeats and favor RAD52-mediated SSA over RAD51-dependent HR (**III-Figure 4F-G**). Testing this model would require assessing whether EXO1 or DNA2 depletion rescues the UBQLN1-induced SSA phenotype and whether chromatin fractionation reveals differential MRE11 clearance kinetics between *UBQLN1* and *UBQLN4* overexpression. Resolving the inter-paralog paradox, however, does not address a fundamental observation within UBQLN1 itself.

3.3.6 A dosage-dependent model of UBQLN1 function

Both *UBQLN1* deficiency and overexpression impair HR, albeit through distinct mechanisms (**III-Figure 3-4**). This observation suggests a dosage-dependent model with an optimal physiological range of UBQLN1. Such "Goldilocks" models, where both insufficient and excessive activity produce deleterious outcomes, have been observed for other signaling proteins. For instance, balanced ERK1/2 activation promotes cell proliferation and survival, while inadequate or excessive activity impairs these processes.³²² UBQLN1 may function optimally within a defined concentration range, with deviations in either direction disrupting HR.

The model describes three functional states. In *UBQLN1* deficiency, the protective RAD51 interaction is lost, leading to RAD51 destabilization and impaired strand invasion, a downstream HR defect. At physiological levels, UBQLN1 might preferentially engage RAD51 via its ST11-II domain as a substrate of potential higher affinity, maintaining RAD51 stability and enabling HR, while MRE11 levels remain unaffected. In *UBQLN1* overexpression, RAD51 binding sites might become saturated or excess UBQLN1 might be redirected and could engage MRE11 via its UBL-UBA-axis, promoting its degradation and impairing resection, an early and upstream HR defect. Thus, *UBQLN1* deficiency and overexpression both impair HR, but at different pathway steps, as deficiency compromises strand invasion, while overexpression compromises resection. (**Figure 7**)

An additional mechanistic layer potentially contributing to this dosage dependence may be UBQLN1 concentration-dependent oligomerization and phase separation, as Ubiquilins have been shown to form biomolecular condensates through self-association.³²³ Such condensate formation may reinforce the proposed "shielding" model, as a recent preprint demonstrated that ubiquilin condensates can restrict proteasomal access to substrates,³²⁴ and sequestration of RAD51 filaments within potential UBQLN1 condensates could similarly contribute to their protection. Conversely, at supraphysiological concentrations, excessive self-association could redirect excess UBQLN1 toward UBL-UBA-dependent degradation substrates such as MRE11.

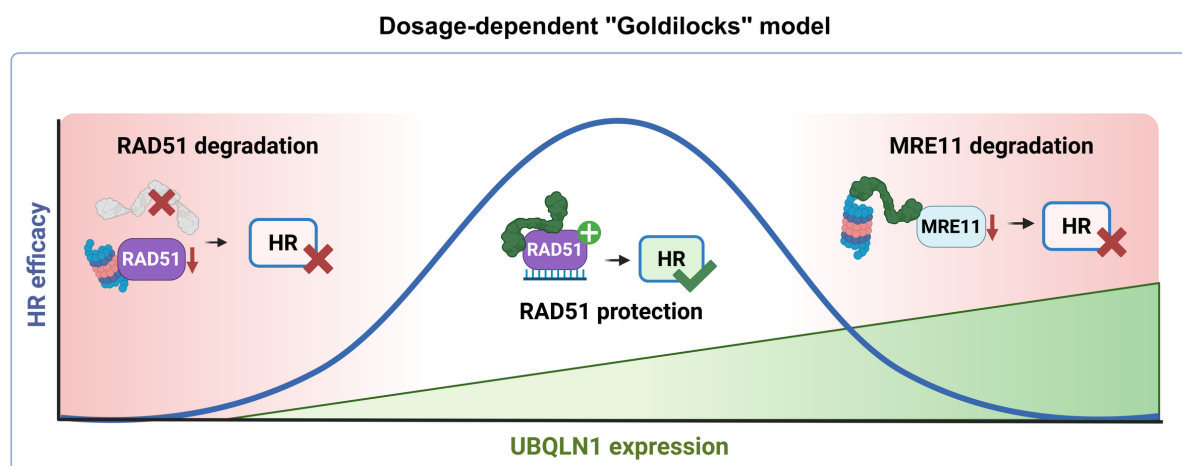


Figure 7: Dosage-dependent "Goldilocks" model of UBQLN1 in HR regulation. HR efficacy is proposed to follow a bell-shaped relationship with *UBQLN1* expression, describing three functional states. At *UBQLN1* deficiency (left), loss of the protective interaction leads to premature RAD51 ubiquitination and degradation, impairing strand invasion, a downstream HR defect. At physiological *UBQLN1* expression (center), RAD51 is stabilized on chromatin, enabling proficient HR. At supraphysiological *UBQLN1* levels, excess UBQLN1 is proposed to engage MRE11 via the UBA domain, promoting its degradation and impairing end resection, an upstream HR defect. Thus, both deficiency and overexpression compromise HR, but at distinct pathway steps. Figure created with BioRender.

The dosage-dependent behavior also aligns with the "domain switch" theory (section 3.3.4), where expression level might determine the predominant interaction mode. Quantitative determination of UBQLN1-RAD51 and UBQLN1-MRE11 binding affinities would directly test the impact of substrate affinity in the HR hierarchy. Additionally, titration experiments assessing HR efficiency across a range of *UBQLN1* expression levels using the DR-GFP reporter would establish whether an optimal concentration range exists and would define the boundaries of UBQLN1 functionality in HR.

3.3.7 UBQLN1 as a proteostasis regulator in genome maintenance

The preceding sections established UBQLN1 as a context-dependent regulator whose effect on HR depends on expression level, domain engagement, and substrate identity. The functional versatility of acting as a context-dependent stabilizer or degradation factor within the same pathway places UBQLN1 within a group of proteostasis regulators with "moonlighting" roles in genome maintenance. This parallels RAD23B, which stabilizes the NER damage recognition factor XPC through direct binding, independent of its UBL-UBA-dependent proteasomal shuttle function.³²⁵ That structurally related proteins independently evolved stabilization mechanisms suggests that proteostatic control of repair factor abundance might be a recurring regulatory principle.³²⁶

UBQLN1's proteostasis function extends beyond DSB repair to other genome maintenance pathways. Independent evidence for the degradation role of UBQLN1 in genome maintenance emerged from Zhou *et al.*, who demonstrated that UBQLN1 facilitates RPA1 removal from replication forks via its UBA domain.³²⁷

Inducing replication stress in *UBQLN1* knockdown cells resulted in persistent RPA1 foci, slowed replication fork progression, rapid telomere shortening, and impaired RAD51 loading onto ssDNA.³²⁷ In the context of replication stress, RAD51 protects stalled replication forks from nucleolytic degradation and facilitates fork reversal,³²⁸ independent of its function in HR.

Their findings both complement and contrast with our work. Both studies identified defective RAD51 chromatin association as a downstream consequence of *UBQLN1* deficiency, yet through distinct mechanisms. In the replication stress context of Zhou *et al.*, RAD51 fails to load because persistent RPA1 retention physically blocks ssDNA access, whereas in our DSB repair context, RAD51 loads normally but is subsequently destabilized and removed through proteasome-dependent degradation (**III-Figure 3A-F**). The domain of engagement further distinguishes the two interactions, as the *UBQLN1*-RPA1 interaction requires the UBA domain and results in substrate removal from ssDNA, whereas the *UBQLN1*-RAD51 interaction characterized in our work is mediated by the STI1-II domain (**III-Figure 3J**) and results in substrate stabilization on chromatin. These opposing outcomes, degradation versus protection, mediated through different domains of the same protein, provide independent support for the "domain switch" theory proposed in section 3.3.4.

Of note, I also examined the consequence of *UBQLN1* loss on RPA1 in context of screening the HR pathway where I ultimately detected the RAD51 phenotype using high-content screening microscopy (**Figure 8**) and did not observe the RPA1 retention phenotype reported by Zhou *et al.*, which used siRNA knockdown. This discrepancy may reflect methodological differences. I specifically induced DSBs through neocarzinostatin treatment, while Zhou *et al.* focused on replication stress through camptothecin and hydroxyurea treatment. The absence of an RPA1 retention phenotype in our *UBQLN1*-deficient knockout system (**Figure 8**), the normal initial recruitment of RAD51 to DSBs (**III-Figure 2B-C**), and the proteasome-dependent rescue of RAD51 on the chromatin by MG132 (**III-Figure 3D, S5B**), establish mechanistically separable functions.

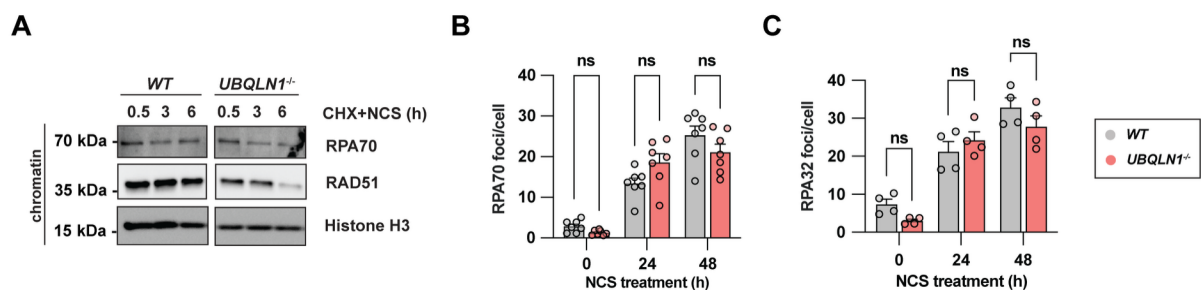


Figure 8: *UBQLN1* deficiency does not affect RPA1 in our system. A) Cycloheximide chase assay similar to **III-Figure 3B**. Chromatin fraction probed for RPA1 (RPA70) and RAD51 at the indicated time points. H3 served as loading control. Data were acquired in collaboration with A.S.H. **B-C)** High-content microscopy similar to **III-Figure 2B**. Nuclear RPA1 (RPA70) foci or RPA2 (RPA32) foci in WT and *UBQLN1*^{-/-} U2OS cells treated with NCS (250 ng/ml). Each datapoint represents the mean of 3000 analyzed nuclei. Methods correspond to Manuscript III (section 12.1.3).

If impaired RPA1 clearance were the primary defect, RAD51 would fail to load onto the chromatin rather than being destabilized after loading, and proteasome inhibition could not rescue chromatin-bound RAD51. Instead, we observed that RAD51 loaded normally but was subsequently destabilized through proteasome-dependent degradation, establishing these as distinct mechanisms – HR versus replication stress.

The consequences of disrupted proteostasis extend beyond impaired repair factor stabilization. *UBQLN1* deficiency has been connected to protein aggregation and mitochondrial dysfunction that cause telomeric and oxidative DNA damage.³²⁹ These findings suggest that *UBQLN1* loss simultaneously increases the endogenous DNA damage burden through mitochondrial ROS while compromising the HR capacity to repair such damage through RAD51 destabilization, as demonstrated in our work. Thus, *UBQLN1* loss may compromise genome maintenance through multiple mechanisms, elevated oxidative damage,³²⁹ impaired replication fork progression through RPA1 retention,³²⁷ and defective DSBR through RAD51 destabilization, each reflecting a different facet of *UBQLN1*'s proteostasis function, but all leading towards genome instability. Therefore, more damage coupled with less repair capacity would contribute to the pronounced genome instability phenotype observed in *UBQLN1* deficiency syndrome. This illustrates how proteostasis regulators can influence genome maintenance, but also would anticipate cancer predisposition in the young *UBQLN1* deficiency syndrome patients with progressing age, consistent with the delayed cancer onset observed in other genome instability syndromes.¹⁶⁴

While these mechanisms illustrate the extent of *UBQLN1*'s proteostasis functions, the DSBR-specific findings warrant closer examination of how proteostatic abundance control operates as a regulatory principle within the DNA damage response.

3.3.8 Proteostasis as a regulatory layer in DNA double-strand break repair

Controlling how much repair factor protein is available adds a distinct level of regulation to DSBR. Traditionally, DSBR regulation has been understood through ATM/ATR kinase signaling³³⁰ and competitive interactions such as 53BP1-BRCA1 antagonism in DSBR pathway choice³³¹. Unlike kinase signaling, which modulates protein activity and localization within existing protein pools, degradative ubiquitin signaling modulates local abundance of pathway components available for repair. That upstream factor depletion constrains downstream pathway output is a general principle, but *UBQLN1* represents a specific case where a single factor imposes such "ceiling effects" at multiple sequential HR steps through dosage-dependent substrate engagement.

In DSBR, data on UBL-UBA shuttle factors has been limited to UBQLN4, which mediates removal of ubiquitinated MRE11 from damaged chromatin to regulate DSB pathway choice.¹³⁶ Now, we add UBQLN1, which stabilizes RAD51 to enable HR.²⁹⁶

With UBQLN1, we demonstrate how a single proteostasis factor can set functional ceilings at sequential HR steps in a dosage-dependent manner. Under physiological conditions, UBQLN1 shields RAD51 from proteasomal degradation (section 3.3.2), maintaining RAD51 abundance at ssDNA at the level required for productive strand invasion. Loss of *UBQLN1* removes this protective stabilization, exposing RAD51 to E3 ligase-dependent degradation and lowering the "ceiling" below the threshold presumably required for efficient strand invasion.

The "ceiling effect" is most evident in *UBQLN1* overexpression, where MRE11 is depleted through proteasomal degradation, potentially lowering the "ceiling" on MRE11 availability below the level required for its role in limiting long-range end resection, which shifts pathway outcome from HR toward SSA and phenocopies HRD even when downstream repair machinery remains intact (section 3.3.5). Here, no amount of downstream signaling could compensate for the depleted upstream factor pool. The "ceiling" has been set by proteostatic degradation rather than kinase regulation. This dual function, maintaining RAD51 stabilization and, in overexpression, decreasing the MRE11 pool through degradation, positions UBQLN1 as a proteostasis factor whose dosage-dependent effects on sequential HR steps can shift DSBR pathway outcome through abundance control. Identifying additional proteostasis factors that regulate repair protein stability, and determining how their degradative and stabilizing activities are coordinated with canonical DDR signaling, represents an important direction for understanding the regulatory architecture of DSBR.

3.3.9 UBQLN1 dysregulation in cancer

If proteostatic control of repair factor abundance represents a regulatory layer governing DSBR pathway choice, then dysregulation of such control in cancer would be expected to have functional consequences for tumor biology and therapy response. *UBQLN1* dysregulation has been implicated across multiple cancer types, exhibiting context-dependent oncogenic or tumor-suppressive functions. In line with our observation of frequent *UBQLN1* overexpression in human tumors (**III-Figure 5A-C, S7A-L**), immunohistochemical studies have demonstrated *UBQLN1* upregulation in breast cancer³³², gastric cancer³³³, colorectal cancer³³⁴, and hepatocellular carcinoma³³⁵. *UBQLN1* overexpression has also been associated with activation of oncogenic signaling pathways, including AKT³³⁶ and ERK-c-Myc³³⁴, consistent with UBQLN1's broader proteostasis functions beyond DNA repair.^{150,337-339} Importantly, however, we observed UBQLN1 recruitment to DNA damage sites (**III-Figure 2B**) and interaction with RAD51 and MRE11 (**III-Figure 3G, S5G, 4D**), establishing DSBR regulation as a *bona fide* function of UBQLN1 distinct from its broader cellular roles.

Indeed, *Ubqln1* overexpression-induced HRD elevated mutational burden in our mouse experiments and in TCGA patient data (**III-Figure 4A, 5D, 6E-F, S8E-Q**).

While UBQLN1's diverse proteostasis functions may contribute additional phenotypic consequences to the tumor aggressiveness observed in the *Kras*^{LSL.G12D/wt}; *Trp53*^{fl/fl}; *Rosa26*^{LSL.HA-Ubqln1} (KPU1) mouse model (**III-Figure 6A-D**), this does not diminish the observation that *UBQLN1* overexpression drives cancer aggressiveness. This phenotype encompasses overall biological consequences of *UBQLN1* dysregulation present in the tested cell lines and lung tissue. In particular, the therapeutic sensitivities to PARP inhibition (**III-Figure 7**) are most directly explained as a functional consequence of the resulting HRD and DSB defect, rather than secondary proteostasis effects.

Conversely, physiological levels of UBQLN1 have been shown to exhibit tumor-suppressive properties in LUAD cell line models, where *UBQLN1* loss promoted epithelial-to-mesenchymal transition, migration, and metastasis through reciprocal regulation with ZEB1.³⁴⁰ Loss of *UBQLN1* also has been shown to increase MYC protein stability, a common lung cancer oncogene, driving cell proliferation and tumorigenesis *in vivo*, while increased *UBQLN1* expression counteracted *MYC* expression.³⁴¹ The consequence of *UBQLN1* overexpression might therefore be different in *MYC*-driven cancers compared to *KRAS*-driven cancers, the latter being the primary context examined in our study. However, our tumor-driver independent TCGA analysis across several cancer entities corroborated these dosage-dependent effects of *UBQLN1* overexpression (**III-Figure 5**).

The observation of tumor suppression at physiological levels versus promotion through overexpression likely reflects *UBQLN1*'s expression level-dependent effects on specific substrates, as proposed in the dosage-dependent "Goldilocks" model (section 3.3.6). Since I focused on *Ubqln1*-overexpression in the lung cancer mouse model, it remains unclear whether *Ubqln1* deficiency would produce comparable HRD-dependent effects on tumorigenesis and treatment vulnerability.

3.3.10 *UBQLN1* overexpression as an oncogenic driver

The constitutively *Ubqln1*-overexpressing model (*Rosa26*^{LSL.HA-Ubqln1}; *Actb*^{Cre/wt}) provides direct evidence that *Ubqln1* overexpression can promote oncogenesis (**III-Figure 5E-H**). Strikingly, 40 % of these mice spontaneously developed LUAD in older age, whereas no tumors were observed in wildtype controls. This suggests that *Ubqln1* overexpression is not merely a modulator of tumor aggressiveness, but potentially a candidate oncogene capable of initiating malignant transformation. Unlike the constitutional *UBQLN1* deficiency in syndrome patients, *UBQLN1* overexpression in cancer patients would represent a somatic, tumor-restricted event. The constitutive *Rosa26* model therefore represents a deliberate experimental extreme.

The KPU1 and *Rosa26^{LSL.HA-Ubqln1};Actb^{Cre/wt}* models address different aspects of *Ubqln1*-driven oncogenesis. In the KPU1 model, *Ubqln1* overexpression accelerated tumorigenesis in the context of established oncogenic drivers (*KRAS^{G12D}* activation, *Trp53* loss), demonstrating its role as a tumor promoter that enhances genomic instability in an already-initiated malignancy (**III-Figure 6A-D**). Of note, the multifocal disease pattern inherent to the autochthonous KP/KPU1 models recapitulates aggressive, advanced-stage LUAD,³⁴² making preclinical testing especially challenging and meaningful. The *Rosa26^{LSL.HA-Ubqln1};Actb^{Cre/wt}* model extends this by demonstrating that *Ubqln1* overexpression alone, without the cooperating mutations, is sufficient to drive tumorigenesis in the lung (**III-Figure 5G**). The long latency observed (median 25 months, **III-Figure 5F**) compared to KPU1 could reflect the time required for *Ubqln1*-induced HRD to generate secondary mutations in cancer driver genes together with age-related decline in repair capacity, progressively compromising genomic integrity until transforming alterations might emerge.^{7,13,14}

This would be in line with the established paradigm of HRD in cancer, where *BRCA* loss functions both as an initiating driver of hereditary breast-ovarian cancers and as an acquired vulnerability in sporadic tumors.³⁴³ If *Rosa26^{LSL.HA-Ubqln1};Actb^{Cre/wt}* lung tumors exhibit HRD genomic features and PARP inhibitor sensitivity compared to KPU1 tumors, then this would provide strong evidence that *Ubqln1* overexpression alone confers synthetic lethality independent of *Kras* or *Trp53* status.

3.3.11 Alternative repair pathway engagement in UBQLN1-dysregulated cells

The functional consequences of *UBQLN1* dysregulation in DSB repair extend beyond HRD and include compensatory engagement of alternative DSB repair pathways. Consistent with HR impairment at the resection step, *UBQLN1*-overexpressing cells exhibited elevated SSA in reporter assays (**III-Figure 4G**), suggesting compensatory pathway engagement driven by extensive dysregulated resection, as discussed in section 3.3.5. Additionally, *UBQLN1* overexpression generated genomic signatures of both NHEJ and MMEJ in murine KPU1 lung tumors (**III-Figure 6G**), suggesting a complex DSB repair dysregulation.

The choice among alternative pathways in HRD tumors may be cell cycle-dependent. In S/G₂ phase, HRD could be compensated through MMEJ or SSA depending on resection tract length and homology availability.³⁴⁴ The propagation of mutagenically resolved lesions through these error-prone pathways could translate into elevated damage burden entering G₁,³⁴⁵ potentially explaining the NHEJ signature observed in KPU1 mice.

These observations suggest therapeutic opportunities beyond PARP inhibition. Cells reliant on MMEJ might exhibit sensitivity to Pol θ inhibitors,³⁴⁶ while SSA dependence could confer vulnerability to RAD52 inhibition.³⁴⁷

Investigation of Pol θ or RAD52 inhibitors in combination with PARP inhibition in *UBQLN1* overexpressing models is warranted and would strengthen the clinical potential of *UBQLN1* overexpression as a novel potential biomarker for personalized cancer therapy.

3.3.12 *UBQLN1* as a candidate therapeutic biomarker

Our findings identify *UBQLN1* overexpression as a candidate biomarker for cancer aggressiveness that demonstrates sensitivity to PARP inhibitors and immune checkpoint blockade in preclinical models.²⁹⁶ Critically, *UBQLN1* overexpression could enforce an HRD state without mutations in canonical DNA repair genes, positioning *UBQLN1* as a dosage-dependent, expression-level driver of HRD. Of note, immunohistochemistry of elderly mice that overexpress *Ubqln1* constitutively showed no *UBQLN1* downregulation throughout the mouse lifespan (**III-Figure S7M**), further supporting its potential as a stable biomarker.

In the clinic, current HRD assessment relies on genomic scar-based methods that detect past HRD but may not reflect current repair status, while *BRCA1/2* mutation testing identifies only a subset of HRD tumors.²²⁵⁻²²⁷ *UBQLN1* overexpression could serve as a functional HRD indicator that could be independent of genomic scars and mutations in DNA repair genes. Tumors with high *UBQLN1* expression but negative genomic HRD scores might nonetheless respond to PARP inhibitors, a patient population currently missed by standard biomarkers. I propose that *UBQLN1*-dysregulated HRD represents another "functional HRD" or *BRCAness* mechanism requiring dedicated biomarker approaches, with the potential to expand the eligible patient population beyond those identifiable by current genomic assays.

Clinical validation requires establishing *UBQLN1* expression thresholds that predict treatment response. In a study using immunohistochemistry, high *UBQLN1* expression was detected in 54.4 % (68/125) of invasive breast cancer samples.³³² Staining positivity correlated with tumor size, lymph node metastasis, advanced disease stage, and poor prognosis,³³² demonstrating both the prevalence and potential clinical significance of *UBQLN1* as a biomarker for cancer aggressiveness. The poor prognosis observed in breast cancer parallels our findings in KPU1 lung cancer and TCGA data (**III-Figure 5,6**). To establish clinically relevant thresholds and validate patient stratification utility in NSCLC, *UBQLN1* immunohistochemistry scoring in clinically annotated tissue microarrays could be correlated with TMB, HRD signatures, and treatment sensitivities.

How *UBQLN1* is upregulated in cancer remains unclear. A recent study suggested that *UBQLN1* levels in hepatocellular carcinoma are mediated by the circularRNA SORE,³¹⁶ while the oncogenic microRNA MIR155 has been shown to negatively regulate *UBQLN1* expression. Carcinogen exposure was shown to repress MIR155, thereby upregulating *UBQLN1*.³⁴⁸

UBQLN1-driven cancer development and aggressiveness might be therefore linked to post-transcriptional regulation and environmental factors. Regardless of the upstream cause, the critical observation is that *UBQLN1* upregulation is sufficient to impair HR. Depending on the underlying cause of overexpression, this mechanism could escape detection by mutation-based, copy-number-based, and genomic scar-based HRD assays alike, as discussed. Understanding the source of *UBQLN1* upregulation would contribute to evaluating its potential as a diagnostic and therapeutic biomarker.

UBQLN1 may also serve as a serological marker for early detection. Serum autoantibodies against *UBQLN1* were significantly elevated in LUAD patients compared to healthy controls, alongside *UBQLN1* overexpression in tumor tissue.^{349,350} Such autoantibodies could enable minimally invasive patient identification before biopsy and potentially before the accumulation of genomic HRD scars.

Preclinical model systems, particularly GEMMs that recapitulate tumor-microenvironment interactions, are important for validating biomarker-therapy relationships. While the parental KP model exhibits low TMB and reduced immunogenicity, limiting its predictive value for immunotherapy studies,²⁵⁶ the KPU1 strain showed elevated TMB (**III-Figure 6E-F**), increased predicted neoantigen presentation (**III-Figure S8Q**), and sensitivity to immune checkpoint blockade (ICB) therapy (**III-Figure 7C-D**), demonstrating the impact of *Ubqln1* overexpression on immunogenicity in this aggressive mouse model. Our findings establish biological plausibility for *UBQLN1* as a biomarker candidate in cancer, also for immunotherapy.

Validation as a clinical biomarker requires prospective studies, ideally randomized controlled trials or biomarker-stratified cohorts, correlating *UBQLN1* expression levels with PARP inhibitor and ICB response rates to establish predictive thresholds and support clinical application.

3.3.13 Conclusions and future directions

This work identifies *UBQLN1* as a previously unrecognized regulator of HR through RAD51 protein homeostasis, discovered through the characterization of a novel genome instability syndrome. Methodological and interpretive limitations and experimental follow-up suggestions of individual findings were discussed in their respective sections above. The proposed "shielding" model, in which *UBQLN1* binding via its STI1-II domain protects RAD51 from ubiquitin-dependent proteasomal degradation, suggests a non-canonical function of *UBQLN1*. That RAD51 stabilization depends on the STI1-II domain rather than the canonical UBA-UBL degradation axis raises the possibility of a domain switch mechanism, whereby substrate fate – stabilization or degradation – may be determined by the mode of initial recognition.

The observation that both *UBQLN1* deficiency and overexpression appear to impair HR, albeit at different pathway steps, is consistent with a dosage-dependent model in which *UBQLN1* functions within an optimal concentration range. This dosage sensitivity may be mechanistically linked to the "domain switch" theory, where STI1-II-mediated RAD51 stabilization would predominate at physiological *UBQLN1* expression, whereas supraphysiological expression could increasingly engage the broader UBA-dependent substrate repertoire. The resulting model suggests that versatile shuttle factors may contribute to DSB repair pathway choice by controlling the availability of repair factors.

Translationally, *UBQLN1* overexpression drove tumorigenesis in autochthonous mouse models, induced HRD and elevated TMB, and conferred sensitivity to both PARP inhibition and immune checkpoint blockade in preclinical settings. As a dosage-dependent driver of HRD, *UBQLN1* overexpression could potentially identify patients who lack canonical *BRCA* and *BRCAness* mutations or genomic HRD scars but might still benefit from targeted therapies. The compensatory engagement of MMEJ and SSA observed in *UBQLN1*-overexpressing cells further suggests possible combinatorial vulnerability to Pol θ and RAD52 inhibitors, which could broaden therapeutic options. Clinical validation of *UBQLN1* expression thresholds in annotated tumor cohorts will be essential to determine whether these preclinical findings translate into viable patient stratification strategies.

While *UBQLN1* overexpression creates therapeutic vulnerability through HRD, complementary target-directed approaches can address oncogenic signaling and driver gene mutations directly. The following section discusses the development of Deltafluorine, a first-in-class covalent PDE δ inhibitor targeting KRAS signaling.

3.4 Manuscript IV: Targeting a Glutamic Acid in PDE δ with Fluoromethyl-Aryl Electrophiles Impairs KRAS Signaling

Deltafluorine achieves covalent modification of glutamate E88 in PDE δ through a fluoromethyl pyridyl electrophile (**IV-Figure 1b, 2, 3c-f**), demonstrating activity in *KRAS*-dependent cell lines and reducing tumor burden in the KP mouse model of lung adenocarcinoma (**IV-Figure 6**). Understanding the potential of Deltafluorine requires positioning in the context of recent advances in *KRAS* targeting strategies.

3.4.1 Advances in direct *KRAS*^{G12D} targeting

Direct targeting of *KRAS*^{G12D} has advanced considerably since the initiation of our work. MRTX1133, the first potent non-covalent *KRAS*^{G12D} inhibitor with picomolar affinity,^{351,352} is now in clinical trials (NCT05737706), and Kim *et al.* demonstrated that MRTX1133 allosterically

arrests the GTPase cycle³⁵³. HRS-4642, a high-affinity selective noncovalent inhibitor, has shown promising phase I antitumor activity, with Zhou *et al.* identifying through CRISPR screening that proteasome inhibition synergizes with KRAS^{G12D} targeting.³⁵⁴ Daraxonrasib, an RAS(ON) multi-selective inhibitor targeting active GTP-bound RAS, has received FDA breakthrough designation for KRAS^{G12X}-mutant pancreatic ductal adenocarcinoma in 2025 and is now in phase III evaluation.^{355,356}

These advances in direct KRAS targeting might appear to undermine the rationale for indirect targeting of KRAS membrane trafficking through PDE δ , yet several considerations suggest continued value. Resistance to direct KRAS inhibitors emerges rapidly through mechanisms including mTOR pathway activation, epithelial-to-mesenchymal transition, and KRAS amplification.³⁵⁷ Since single-agent resistance appears unavoidable, efforts have shifted toward rational combination strategies. Liaki *et al.* very recently demonstrated that simultaneous targeting of three independent KRAS signaling nodes, RAF1, EGFR, and STAT3, achieves regression of KRAS/TP53-driven pancreatic tumors without resistance emergence for over 200 days in a mouse model, whereas daraxonrasib monotherapy showed limited long-term durability.³⁵⁸ Their data confirmed that multi-node pathway blockade can prevent adaptive resistance by exceeding the tumor's compensatory capacity. PDE δ inhibition may offer combination opportunities with direct KRAS inhibitors. Additionally, PDE δ inhibitors provide chemical biology tools for dissecting KRAS trafficking biology regardless of their standalone therapeutic potential, which might provide new insights and future targeting opportunities.

3.4.2 Carboxylate-targeting chemistry in the context of emerging covalent inhibitors

The carboxylate targeting chemistry exemplified by Deltafluorine represents one of several emerging approaches to covalently engage aspartate and glutamate residues. Isolated examples of KRAS^{G12D}-directed covalent inhibitors have been reported, including epoxide-based compounds,³⁵⁹ malolactone warheads that engage both GDP- and GTP-bound states,³⁶⁰ and RMC-9805,³⁶¹ an orally bioavailable drug currently in clinical trials (NCT06040541). More recently, Shuai *et al.* designed tunable warheads for carboxylate targeting, demonstrating selectivity for carboxyl residues and antiproliferative activity in KRAS^{G12D}-dependent cells.³⁶² Deltafluorine's slow kinetics and slow cellular turnover may represent a stability advantage over more reactive warheads, though whether this translates to improved therapeutic efficacy has not yet been established.³⁶³

3.4.3 Therapeutic validity of PDEδ as a surrogate target

The therapeutic validity of PDEδ as a KRAS surrogate target has been directly challenged by very recent work that was published in 2025 upon acceptance of our Deltafluorine manuscript and warrants discussion. Gómez-Mulas *et al.* developed genetically encoded PDEδ inhibitors and concluded that PDEδ inhibition is not sufficient to effectively suppress KRAS membrane anchorage and mitogen-activated protein kinase (MAPK) activity.³⁶⁴ They found that inhibition of KRAS membrane anchorage and KRAS^{G12C}-dependent MAPK signaling was weak, with near-complete PDEδ knockdown in KRAS^{G12C}-overexpressing HEK cells yielding only approximately 20 % reduction in phospho-ERK levels.³⁶⁴ This may raise the possibility that even complete PDEδ inhibition could be insufficient to meaningfully suppress KRAS signaling in therapeutic contexts.

However, I observed substantially greater pathway suppression with Deltafluorine treatment, achieving approximately 50 % reduction in both phospho-ERK and phospho-S6 levels within 4 hours of exposure in KRAS^{G12V} PA-TU-8902 cells (**IV-Figure 4c**). Given the slow kinetics of Deltafluorine, the biological effects likely reflect a combination of reversible target occupancy and progressive covalent modification, with the relative contributions of each mechanism potentially varying across experimental timeframes and contexts. The experimental systems also differ in context, as Gómez-Mulas *et al.* relied on overexpressed KRAS^{G12C} in HEK cells, whereas I measured pathway suppression in PA-TU-8902 cells expressing endogenous KRAS^{G12V}, where PDEδ-dependent trafficking may account for a different fraction of total KRAS membrane delivery. Gómez-Mulas *et al.* themselves noted that PDEδ directs only an estimated 25-50 % of KRAS isoform 4B membrane trafficking, with redundant chaperones contributing to the remainder, though this estimate may vary across KRAS contexts.³⁶⁴ Even accounting for these differences, on-target PDEδ inhibition through the KRAS-MAPK axis alone is unlikely to account for the full extent of our observed phospho-ERK suppression. However, PDEδ clients extend beyond KRAS, and the simultaneous suppression of both phospho-ERK and phospho-S6 observed with Deltafluorine (**IV-Figure 4c**) suggests suppression of multiple PDEδ-dependent pathways, which was not examined by Gómez-Mulas and colleagues. While off-target contributions from modification of other carboxylate residues cannot be excluded, these observations suggest that evaluating PDEδ inhibition solely through KRAS-MAPK readouts may underestimate its therapeutic relevance. Our phosphoproteomic analysis provided orthogonal validation of these findings and strengthened confidence in the inhibitory effect observed with Deltafluorine (**IV-Figure 4a, 4b**).

3.4.4 Alternative mechanisms of antitumor efficacy

Our observation of phospho-S6 suppression with Deltafluorine is consistent with disruption of PDE δ -dependent RHEB trafficking. RHEB is a farnesylated PDE δ client and critical upstream activator of mTORC1.³⁶⁵

Armijo *et al.* demonstrated that PDE δ inhibitors may exert effects through the mTOR axis independently of KRAS.³⁶⁵ Dual suppression of both MAPK and mTOR pathways may therefore produce cooperative antitumor effects that neither pathway inhibition alone would achieve. Our global proteome analysis revealed regulation of proteins involved in vesicle-mediated transport across the Golgi-ER membrane system (**IV-Figure S5**), which aligns with PDE δ 's role as chaperone for prenylated cargo trafficking, and also suggests that Deltafluorine may influence membrane trafficking beyond direct PDE δ targeting. Distinguishing these mechanistic possibilities will require careful dissection that extends beyond the scope of the present work. Nonetheless, the correlation between enhanced signaling suppression and *in vivo* tumor reduction (**IV-Figure 4,6**) suggests that PDE δ inhibition with compounds exhibiting Deltafluorine's profile warrants continued investigation as a therapeutic strategy.

3.4.5 KRAS-induced DNA damage response rewiring

Recent work has begun to show how oncogenic KRAS rewires the DNA damage response, creating therapeutic vulnerabilities. Igarashi *et al.* demonstrated that *KRAS*^{G12V} expression triggers chromatin compaction and replication fork slowing that requires elevated ATR expression for cellular survival.³⁶⁶ Repriming generates ssDNA gaps at heterochromatin, contributing to genomic instability that may be further impaired by *TP53* loss.³⁶⁶ Smith *et al.* found that *KRAS*-mutated pancreatic ductal adenocarcinoma shows increased transcription replication conflicts compared to other solid tumors.³⁶⁷ Meng *et al.* identified BER factors as regulators of transcription replication conflicts in pancreatic cancer, with synergistic enhancement of DNA damage when BER and ATR inhibitors were combined.³⁶⁸ These findings suggest that *KRAS*-mutant cancers could exist in a state of chronic replication stress that, when combined with *Trp53* loss as in our KP model, might create synthetic lethal dependencies on DNA repair mechanisms.

3.4.6 Implications for combination therapy strategies

These DNA damage response alterations suggest specific therapeutic vulnerabilities that could be exploited in combination with PDE δ inhibitors. Oncogenic *RAS* increases replicative, metabolic, and oxidative stress while coopting adaptive responses to preserve survival, with the resulting rewiring of DDR pathways promoting resistance to chemotherapy and radiotherapy.³⁶⁹ Hoffman *et al.* demonstrated that *KRAS* mutant cancers rapidly escape MAPK-pathway-inhibitor induced quiescence through ATR-dependent stress responses.³⁷⁰

These observations raise the possibility that PDE δ inhibition, by disrupting KRAS membrane localization and potentially affecting the downstream signaling that drives replication stress adaptation, might synergize with ATR inhibitors or comparable DNA damage response modulators.

The combination of *Kras*^{G12D}-induced replication stress with *Trp53*-mediated checkpoint deficiency in the KP model creates a context where such vulnerabilities may be particularly pronounced given that p53 governs cell fate decisions following DNA damage.³⁷¹

3.4.7 Limitations

The contribution of PDE δ inhibition relative to potential alternative Deltafluorine targets to the observed antitumor effect remains incompletely resolved. While genetic rescue experiments in Hap1 cells (**IV-Figure S7d**) provided partial target validation, off-target modifications of other carboxylate residues cannot be excluded. *In vivo* evaluation was limited to the KP mouse model carrying *KRAS*^{G12D}, leaving uncertain whether efficacy extends to other *Kras*-mutant contexts or tumors retaining functional *Trp53*. Furthermore, pathway suppression was not validated in samples derived from the mouse experiments. Analysis of KP model-derived cell lines and comparison of untreated versus treated lung tissue would strengthen the mechanistic understanding of Deltafluorine activity *in vivo*. While relevant samples are available, these analyses were beyond the scope of the current manuscript and should be pursued if Deltafluorine advances through subsequent stages of preclinical development. Resistance mechanisms and combination strategies, particularly with direct KRAS inhibitors, also remain to be determined through future systematic evaluation.

3.4.8 Conclusions and future directions

In conclusion, Deltafluorine enters a rapidly evolving field of KRAS targeting strategies. The carboxylate targeting chemistry established in the manuscript (**IV-Figure 1b**, **IV-Figure 2**, **IV-Figure 3**), achieving covalent modification of glutamate with a fluoromethyl aryl warhead, expands the toolkit for engaging traditionally challenging targets and may inform development of inhibitors engaging other carboxylate-containing residues. Deltafluorine may find greatest utility as a chemical biology tool for studying prenyl protein trafficking and potentially as a combination partner with direct KRAS inhibitors or DDR modulators, rather than as a standalone therapeutic agent.³⁶³ The connections between KRAS signaling and DDR discussed here suggest avenues for potential combination strategies that warrant further investigation. Future work could focus on defining the mechanistic basis for observed antitumor effects through genetic approaches. Identifying synergistic combinations through systematic screening and characterizing resistance mechanisms would guide clinical translation strategies if Deltafluorine or related compounds advance toward therapeutic development.

4. Cumulative Discussion

The extended discussions in chapter 3 examined each study on its own terms. This chapter shifts to a cumulative perspective, integrating the four manuscripts to identify conceptual connections that span across individual publications.

4.1 From Syndrome Discovery to Therapeutic Vulnerabilities in Cancer

The core of this dissertation follows the traditional trajectory of DNA repair research, where mechanistic understanding revealed pathways now central to cancer therapeutics. Two paths from mechanistic insights to therapeutic relevance emerge from the four manuscripts. The first is syndrome-derived translation (**Manuscripts I-III**), where clinical phenotype prompted mechanistic investigation that revealed therapeutic predictions. The second path is target-directed development, where understanding of oncogenic pathway biology, independent of syndrome characterization, enabled the design of a novel inhibitor (**Manuscript IV**). Both trajectories require detailed mechanistic characterization before therapeutic potential becomes apparent, and both utilized the same KP mouse model for preclinical validation, showing how investment in preclinical systems can have impact across distinct projects (**Manuscripts III-IV**).

The *UBQLN1* deficiency syndrome illustrates the syndrome discovery approach. Clinical characterization of a genome instability phenotype led to the identification of *UBQLN1* as a HR regulator, which prompted investigation of the opposite expression extreme. This in turn established *UBQLN1* overexpression as a driver of functional HRD with sensitivity to PARP inhibition and immune checkpoint blockade (**Manuscript III**). This trajectory, from a consanguineous family to preclinical therapeutic validation, depends on patient identification, variant detection, and functional characterization rather than phenotypic resemblance alone.

Importantly, the syndrome discovery path shows that translation begins with variant detection, a significant bottleneck addressed by the OGM study (**Manuscript II**). The identification of a *de novo* duplication encompassing *RPA1* in an infant with *KMT2A*-rearranged leukemia shows that expanding variant detection beyond SNVs and indels can connect established DDR genes to defects in variant classes that escape conventional diagnostics.²⁷⁹ The analysis presented in Manuscript II suggests that the true prevalence of hereditary cancer and genome instability predisposition may be systematically underestimated, because structural variants affecting known and novel DDR genes could remain invisible to standard sequencing workflows.

4. Cumulative Discussion

Each undetected pathogenic variant could be a missed diagnosis and a missed opportunity for mechanistic investigation that can ultimately yield therapeutic predictions, as illustrated by the *UBQLN1* work (**Manuscripts II-III**). Without comprehensive combination of detection technologies, syndrome candidates might remain unidentified and the mechanistic insights they harbor unexplored.

The *CRIP*T syndrome illustrates the relevance of functional characterization within this trajectory (**Manuscript I**). Despite clinical overlap with established genome instability disorders, systematic genotoxicity testing revealed no canonical DDR defect, preventing misclassification as genome instability syndrome and redirecting mechanistic investigation toward senescence.²⁵⁸ The risk of misclassification has direct consequences for the discovery trajectory, because expanded variant detection (**Manuscript II**) will inevitably surface candidates whose clinical features suggest DDR involvement. Functional testing of the kind applied to patient fibroblasts across *CRIP*T, *RPA1*, and *UBQLN1* is therefore essential (**Manuscripts I-III**), as phenotypic resemblance to established DDR disorders does not imply shared molecular mechanisms.

While this approach depends on first detecting variants and then characterizing them functionally, Deltafluorine was developed through a different logic. Disruption of KRAS membrane trafficking through covalent PDE δ modification demonstrated antitumor activity in the KP mouse model through dual MAPK and mTOR suppression (**Manuscript IV**). This target-directed path proceeded from understanding of oncogenic pathway biology rather than from syndrome characterization, yet unites with the syndrome discovery path on the same preclinical model system.

Together, **Manuscripts I-IV** progress from variant detection through syndrome characterization, functional validation and mechanistic understanding, to potential therapeutic application.

4.2 Mechanistic Concepts and Therapeutic Perspectives

The preceding section described how the manuscripts connect as a progression from patient phenotype to therapeutic potential. Beyond this progression, broader principles emerge across manuscripts that were not fully apparent within any single study and inform therapeutic predictions. The observation that both *UBQLN1* deficiency and overexpression impair HR at different pathway steps establishes a "Goldilocks" principle for *UBQLN1* where only physiological expression supports efficient repair (section 3.3.6). This principle extends beyond *UBQLN1* within the work presented here.

4. Cumulative Discussion

The *RPA1* duplication identified through OGM is associated with genotoxic sensitivity through increased gene dosage, while *RPA1* haploinsufficiency has been reported to impair ATR-dependent checkpoint signaling and increase DNA damage sensitivity through a distinct mechanism associated with decreased dosage.^{294,372} Both cases suggest that DDR factor perturbation, in either direction, can compromise cellular homeostasis through distinct mechanisms (**Manuscripts II-III**). Together, these findings indicate that DDR factor abundance is tightly constrained. Perturbations exceeding these constraints, whether through germline variants, overexpression, or structural duplications, can compromise genome maintenance, each through distinct mechanisms.

This dosage sensitivity reflects a broader role of proteostasis in genome maintenance. UBQLN1 illustrates this most directly as a UBL-UBA shuttle factor that regulates RAD51 stability through its STI1-II domain, operating through a non-canonical protective mechanism distinct from its established degradation function.²⁹⁶ The "domain switch" theory (section 3.3.4) explains how UBQLN1 can both stabilize and degrade substrates, and the "ceiling effect" (section 3.3.8) illustrates that proteostasis-mediated abundance control can restrict repair capacity in a way that stabilization of downstream signaling cannot override. The context-dependent switching between stabilization and degradation distinguishes UBQLN1 from conventional shuttling-based DDR regulation and suggests that other UBL-UBA family members may similarly function as conditional DDR regulators.

Proteostasis encompasses not only protein degradation, but also protein trafficking and localization.³⁷³ This broader definition connects the UBQLN1 and PDE δ studies beyond the ubiquitin-proteasome axis. Deltafluorine disrupts PDE δ 's function as a chaperone that shuttles prenylated GTPases between membrane compartments, thereby regulating their intracellular localization and signaling competence.³⁶³ Protein homeostasis therefore affects cancer biology through multiple mechanisms, such as stabilizing or degrading repair factors, as shown for UBQLN1, and through controlling the intracellular localization of oncogenic proteins, as shown for PDE δ (**Manuscripts III-IV**). Investigating other proteostasis factors could therefore uncover new regulators with relevance to cancer therapy.

One such therapeutic opportunity is already apparent within the manuscripts presented here. The UBQLN1 and PDE δ studies, though mechanistically distinct, together suggest specific combination treatment strategies (**Manuscripts III-IV**). Oncogenic *KRAS* has been shown to trigger replication stress through chromatin compaction and replication fork slowing, creating dependency on ATR-mediated stress tolerance.³⁶⁶ When HR is attenuated by *UBQLN1* upregulation, this stress cannot be resolved with full fidelity, compounding genomic instability.

4. Cumulative Discussion

The *Trp53*-deficient context of the KP model is frequently observed in lung cancer patients,²⁵² removing checkpoint constraints governing cell fate decisions following DNA damage,³⁷¹ and rendering tumor cells reliant on DNA repair-based tolerance of replication stress.

These observations point toward specific combination strategies that require systematic evaluation. PDE δ inhibition combined with PARP inhibition could target mechanistically distinct vulnerabilities where both features co-occur. While *UBQLN1* overexpression would be expected to repress HR, creating vulnerability to PARP inhibition,²⁹⁶ PDE δ -mediated KRAS inhibition would primarily target tumor growth through disruption of oncogenic signaling.³⁶³ Preclinical studies have established that inhibition of the RAS/MAPK pathway synergizes with PARP inhibition across multiple *RAS*-mutant tumor lineages.³⁷⁴ Clinically, this concept is now being extended to direct KRAS-targeted agents with an ongoing phase I trial of adagrasib plus olaparib in advanced solid tumors (NCT06130254).³⁷⁵ Notably, this trial does not select for *UBQLN1*-overexpression, which according to our data²⁹⁶ would further potentiate PARP inhibitor sensitivity (**Manuscripts III-IV**). Whether *UBQLN1* overexpression coincides with *KRAS* mutations at sufficient frequency to define a clinically meaningful patient population awaits clarification. Considering that *KRAS* mutations are present in approximately 30 % of lung adenocarcinomas^{252,255} and that *UBQLN1* is significantly overexpressed in lung adenocarcinoma (**Manuscript III**), their co-occurrence at clinically relevant frequency appears plausible.

The functional independence of prenyl protein trafficking disruption and DNA repair targeting suggests non-overlapping mechanisms of action and, by extension, potentially distinct resistance mechanisms. This is relevant because resistance to single-agent KRAS inhibitors emerges rapidly through pathway rewiring.³⁵⁸ Of note, *UBQLN1* overexpression might be a disadvantage under *KRAS*-mutant-driven replication stress, as the increased damage burden enhances reliance on high-fidelity repair that *UBQLN1* overexpression compromises. However, the *Trp53*-deficient context common to *KRAS*-mutant cancers^{252,255} may resolve the apparent paradox of this co-occurrence by removing checkpoint constraints, as *Trp53* loss permits tumor cells to tolerate damage burden that would otherwise trigger apoptosis.³⁷¹ This defines the genetic context where the proposed combination rationale most directly applies – *KRAS*-mutant, *Trp53*-deficient tumors.

Further, *UBQLN1* overexpression does not simply impair HR but redirects repair toward error-prone alternative pathways.²⁹⁶ The increased engagement of MMEJ and SSA observed in *UBQLN1*-overexpressing cells (section 3.3.11) suggests additional therapeutic dependencies with vulnerability to Pol θ and RAD52 inhibition, respectively. These compensatory survival mechanisms for HRD make them candidates for combination with PARP inhibition in a multi-vulnerability targeting strategy.

5. Conclusion

Because these three potential targets act through separate mechanisms, combinations may achieve durable response even in the context of common PARP inhibitor resistance (section 1.7.3). However, such strategies would require further clinical development and careful toxicity evaluation.

Evaluating such strategies requires shared preclinical infrastructure. The KP model²⁴⁸ enabled assessment of both DDR-targeted (KPU1)²⁹⁶ and oncogene-targeted (Deltafluorine)³⁶³ approaches within the same *Kras*^{G12D}-driven, *Trp53*-deficient context (**Manuscripts III-IV**). The 3D-CT tumor measurement technique developed as part of this dissertation enabled whole-lung volumetric quantification across both studies, capturing the multifocal disease complexity inherent to autochthonous GEMMs.^{296,363} The *Ubqln1*-driven HRD together with *Kras*-mutant-induced replication stress in this shared model system provides a rational preclinical basis for the combination strategies proposed above, and a defined starting point for their systematic evaluation.

5. Conclusion

This dissertation presented four studies^{258,279,296,363} whose central contribution is the discovery of UBQLN1 as a novel dosage-sensitive regulator of HR through RAD51 stabilization. Investigation of the *UBQLN1* deficiency syndrome revealed a "shielding" model whereby UBQLN1 protects the recombinase from proteasomal degradation. Both *UBQLN1* deficiency and overexpression impair HR at different pathway steps, establishing a dosage sensitivity principle. Murine *Ubqln1*-overexpressing lung tumors exhibit HRD with elevated mutational burden and sensitivity to PARP inhibition and immune checkpoint blockade. *UBQLN1* overexpression represents a candidate functional biomarker that could expand eligibility for targeted therapies.²⁹⁶

The supporting manuscripts contribute distinct but related insights. Characterization of the *CRIP1* syndrome established that RTS-like features can arise through senescence rather than canonical DNA repair defects, broadening the recognized genetic heterogeneity of the RTS spectrum.²⁵⁸ Validation of OGM for structural variant detection demonstrated that clinically relevant variants escape conventional sequencing, highlighting the need for comprehensive detection technologies within the syndrome discovery trajectory.²⁷⁹ Deltafluorine established carboxylate-targeting covalent chemistry for PDE δ inhibition, achieving dual MAPK and mTOR suppression and antitumor activity in the KP model.³⁶³ The potential mechanistic complementarity with *Ubqln1*-driven HRD, where HRD creates PARP-associated synthetic lethality while KRAS inhibition targets the proliferative driver, suggests rational combination strategies acting on distinct cancer vulnerabilities within the same tumor context.

5. Conclusion

The combination strategies developed in chapter 4 remain untested predictions of this framework. On a mechanistic level, the findings generate testable hypotheses that UBQLN1 shielding might involve E3 antagonism, that *UBQLN1* expression may predict therapeutic sensitivity in clinical settings, and that similar proteostatic DDR regulators await discovery through systematic screening. More broadly, these findings show that controlled protein stability and turnover directly affect how cells repair DNA damage. While the contribution of proteostasis to DDR regulation is well established, UBQLN1 extends this framework by representing a proteasomal shuttle factor that protects a repair protein through non-degradative binding and whose dosage perturbation in either direction impairs HR through distinct mechanisms. Shuttle factors may therefore play a larger role in regulating DSB repair than currently recognized. Because their dysregulation arises from altered protein abundance, the resulting therapeutic vulnerabilities could be invisible to DNA-based tumor profiling and might require expression-level detection strategies.

How many additional proteostasis factors govern repair protein stability, and how their dysregulation shapes cancer vulnerabilities, are questions that future work can address by following the same approach of rare syndrome discovery, identification of mechanisms, and potential clinical translation (**Figure 9**). That a single consanguineous family led to the identification of a regulatory DNA repair mechanism with potential implications for precision oncology across common cancers suggests the answers are worth pursuing.

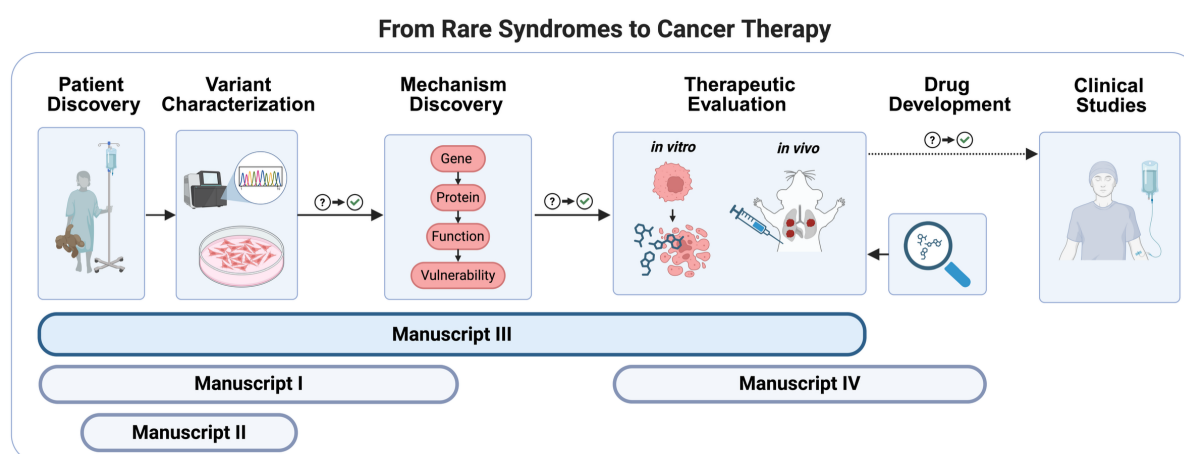


Figure 9: From rare syndromes to cancer therapy. The syndrome discovery approach (Manuscripts I-III, chapters 2.1-2.3) progresses from patient identification and variant characterization through mechanistic dissection of gene, protein, and pathway function to potential identification of cancer vulnerabilities and their preclinical evaluation *in vitro* and *in vivo*. Checkpoints between stages indicate that progression through the trajectory is dependent on positive findings. Only syndromes that reveal mechanistic insights and exploitable cancer vulnerabilities can advance to therapeutic evaluation. Manuscript III (*UBQLN1* syndrome, chapter 2.3) spans the full process from patient discovery to preclinical therapeutic evaluation. This approach is complemented by target-directed drug development and preclinical validation (Manuscript IV, chapter 2.4). Both trajectories can converge toward clinical studies (dashed arrow), dependent on subsequent safety, biomarker, and efficacy evaluation. The approach demonstrates how rare genome instability syndrome discovery can benefit cancer biology and therapy. Figure created with BioRender.

6. Publications and Manuscripts

6.1 Included in this Dissertation

Manuscript I – Peer-reviewed publication

Averdunk L, **Huetzen MA**, Moreno-Andrés D, *et al.* Biallelic variants in *CRIP1* cause a Rothmund-Thomson-like syndrome with increased cellular senescence. *Genet Med.* 2023;25(7):100836. doi:10.1016/j.gim.2023.100836

Manuscript II – Peer-reviewed publication

Wagener R, Brandes D*, Jung M*, **Huetzen MA**, *et al.* Optical genome mapping identifies structural variants in potentially new cancer predisposition candidate genes in pediatric cancer patients. *Int J Cancer.* 2024;154(4):607-614. doi:10.1002/ijc.34721

Manuscript III – Manuscript under review at *Molecular Cell (Cell Press)*; Preprint available

Huetzen MA*, Leidecker O*, Hopff A-S*, Višková Gajarská P*, *et al.* UBQLN1-mediated Repression of Homologous Recombination is an Actionable Target for Cancer Therapy. 2026. Preprint: <http://dx.doi.org/10.2139/ssrn.6502243>

Manuscript IV – Peer-reviewed publication

Zhang R*, **Huetzen MA***, Binici A*, Martín-Gago P*, *et al.* Targeting a Glutamic Acid in PDEδ with Fluoromethyl-Aryl Electrophiles Impairs K-Ras Signaling. *J. Med. Chem.* 2026; 69(2):964-981. doi:10.1021/acs.jmedchem.5c02082

6.2 Not Included in this Dissertation

Ratz L, Brambillasca C, Bartke L, **Huetzen MA**, *et al.* Combined inhibition of EZH2 and ATM is synthetic lethal in BRCA1-deficient breast cancer. *Breast Cancer Res.* 2022;24(1):41. doi:10.1186/s13058-022-01534-y

Sakthivelu V, Schmitt A, Odenthal F, ..., **Huetzen MA**, *et al.* Functional synapses between neurons and small cell lung cancer. *Nature.* 2025;646(8087):1243-1253. doi:10.1038/s41586-025-09434-9

7. Materials and Methods

7.1 Materials and Methods of Integrated Manuscripts

Materials and methods for individual manuscripts can be found in the original publications in chapter 2 (Results), or in the supplementary material for each publication in chapter 12. Experimental data are stored on the institute's file server.

7.2 Software Tools

Scientific figures were generated using GraphPad Prism and Adobe Illustrator.

Schematics were generated using BioRender (Figures 1-7, 9).

Gene/protein names in the list of abbreviations (chapter II) were annotated using the HUGO Gene Nomenclature Committee (genenames.org) resource.

Claude Opus 4.5 and 4.6 (Anthropic) with *PubMed* integration was used to support literature searches and to help improve the clarity and readability of the thesis text. All suggestions were critically reviewed, verified against primary sources, and implemented by the author.

The dissertation was written in *Microsoft Word*. *PaperPal* and *iThenticate* (access via the Max-Planck Society) were used for pre-submission plagiarism screening and no plagiarism was detected.

8. Eidesstattliche Erklärung

Removed for publication

Removed for publication

Removed for publication

10. Acknowledgements

Removed for publication

10. Acknowledgements

Removed for publication

11. References

1. Lindahl, T., and Barnes, D.E. (2000). Repair of endogenous DNA damage. *Cold Spring Harb Symp Quant Biol* 65, 127-133. 10.1101/sqb.2000.65.127.
2. Lombard, D.B., Chua, K.F., Mostoslavsky, R., Franco, S., Gostissa, M., and Alt, F.W. (2005). DNA repair, genome stability, and aging. *Cell* 120, 497-512. 10.1016/j.cell.2005.01.028.
3. Lindahl, T. (1993). Instability and decay of the primary structure of DNA. *Nature* 362, 709-715. 10.1038/362709a0.
4. Panier, S., Wang, S., and Schumacher, B. (2024). Genome Instability and DNA Repair in Somatic and Reproductive Aging. *Annu Rev Pathol* 19, 261-290. 10.1146/annurev-pathmechdis-051122-093128.
5. Roos, W.P., Thomas, A.D., and Kaina, B. (2016). DNA damage and the balance between survival and death in cancer biology. *Nat Rev Cancer* 16, 20-33. 10.1038/nrc.2015.2.
6. Gorbunova, V., Seluanov, A., Mao, Z., and Hine, C. (2007). Changes in DNA repair during aging. *Nucleic Acids Res* 35, 7466-7474. 10.1093/nar/gkm756.
7. Toiber, D., and Schumacher, B. (2026). Targeting Genome Stability to Mitigate Human Aging and Disease. *Annu Rev Pathol* 21, 213-238. 10.1146/annurev-pathmechdis-042624-105942.
8. López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. *Cell* 153, 1194-1217. 10.1016/j.cell.2013.05.039.
9. López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2023). Hallmarks of aging: An expanding universe. *Cell* 186, 243-278. 10.1016/j.cell.2022.11.001.
10. Moriwaki, S., Ray, S., Tarone, R.E., Kraemer, K.H., and Grossman, L. (1996). The effect of donor age on the processing of UV-damaged DNA by cultured human cells: reduced DNA repair capacity and increased DNA mutability. *Mutat Res* 364, 117-123. 10.1016/0921-8777(96)00029-8.
11. Rossi, M.L., Ghosh, A.K., and Bohr, V.A. (2010). Roles of Werner syndrome protein in protection of genome integrity. *DNA Repair (Amst)* 9, 331-344. 10.1016/j.dnarep.2009.12.011.
12. Siegel, R.L., Miller, K.D., and Jemal, A. (2018). Cancer statistics, 2018. *CA Cancer J Clin* 68, 7-30. 10.3322/caac.21442.
13. Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. *Cell* 100, 57-70. 10.1016/s0092-8674(00)81683-9.
14. Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646-674. 10.1016/j.cell.2011.02.013.
15. McGranahan, N., and Swanton, C. (2017). Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future. *Cell* 168, 613-628. 10.1016/j.cell.2017.01.018.
16. Negrini, S., Gorgoulis, V.G., and Halazonetis, T.D. (2010). Genomic instability--an evolving hallmark of cancer. *Nat Rev Mol Cell Biol* 11, 220-228. 10.1038/nrm2858.
17. Lord, C.J., and Ashworth, A. (2017). PARP inhibitors: Synthetic lethality in the clinic. *Science* 355, 1152-1158. 10.1126/science.aam7344.
18. Kim, Y.J., Kim, H.S., and Seo, Y.R. (2018). Genomic Approach to Understand the Association of DNA Repair with Longevity and Healthy Aging Using Genomic Databases of Oldest-Old Population. *Oxid Med Cell Longev* 2018, 2984730. 10.1155/2018/2984730.
19. Lee, J.W., Ratnakumar, K., Hung, K.F., Rokunoh, D., and Kawasumi, M. (2020). Deciphering UV-induced DNA Damage Responses to Prevent and Treat Skin Cancer. *Photochem Photobiol* 96, 478-499. 10.1111/php.13245.
20. D'Orazio, J., Jarrett, S., Amaro-Ortiz, A., and Scott, T. (2013). UV radiation and the skin. *Int J Mol Sci* 14, 12222-12248. 10.3390/ijms140612222.
21. Hoeijmakers, J.H. (2009). DNA damage, aging, and cancer. *N Engl J Med* 361, 1475-1485. 10.1056/NEJMr0804615.
22. Ward, J.F. (1988). DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability. *Prog Nucleic Acid Res Mol Biol* 35, 95-125. 10.1016/s0079-6603(08)60611-x.
23. Singh, R., Kaur, B., Kalina, I., Popov, T.A., Georgieva, T., Garte, S., Binkova, B., Sram, R.J., Taioli, E., and Farmer, P.B. (2007). Effects of environmental air pollution on endogenous oxidative DNA damage in humans. *Mutat Res* 620, 71-82. 10.1016/j.mrfmmm.2007.02.024.
24. Noda, A., Hirai, Y., Hamasaki, K., Mitani, H., Nakamura, N., and Kodama, Y. (2012). Unrepairable DNA double-strand breaks that are generated by ionising radiation determine the fate of normal human cells. *J Cell Sci* 125, 5280-5287. 10.1242/jcs.101006.
25. Yoshida, K., Gowers, K.H.C., Lee-Six, H., Chandrasekharan, D.P., Coorens, T., Maughan, E.F., Beal, K., Menzies, A., Millar, F.R., Anderson, E., et al. (2020). Tobacco smoking and somatic mutations in human bronchial epithelium. *Nature* 578, 266-272. 10.1038/s41586-020-1961-1.
26. Sugimura, T. (2000). Nutrition and dietary carcinogens. *Carcinogenesis* 21, 387-395. 10.1093/carcin/21.3.387.
27. Anand, U., Dey, A., Chandel, A.K.S., Sanyal, R., Mishra, A., Pandey, D.K., De Falco, V., Upadhyay, A., Kandimalla, R., Chaudhary, A., et al. (2023). Cancer chemotherapy and beyond: Current status, drug

11. References

- candidates, associated risks and progress in targeted therapeutics. *Genes Dis* 10, 1367-1401. 10.1016/j.gendis.2022.02.007.
28. Ames, B.N., and Gold, L.S. (1991). Endogenous mutagens and the causes of aging and cancer. *Mutat Res* 250, 3-16. 10.1016/0027-5107(91)90157-j.
29. Bielas, J.H., and Loeb, L.A. (2005). Mutator phenotype in cancer: timing and perspectives. *Environ Mol Mutagen* 45, 206-213. 10.1002/em.20111.
30. Berquist, B.R., and Wilson, D.M., 3rd (2012). Pathways for repairing and tolerating the spectrum of oxidative DNA lesions. *Cancer Lett* 327, 61-72. 10.1016/j.canlet.2012.02.001.
31. Jackson, S.P., and Bartek, J. (2009). The DNA-damage response in human biology and disease. *Nature* 461, 1071-1078. 10.1038/nature08467.
32. Chatterjee, N., and Walker, G.C. (2017). Mechanisms of DNA damage, repair, and mutagenesis. *Environ Mol Mutagen* 58, 235-263. 10.1002/em.22087.
33. Maslov, A.Y., and Vijg, J. (2009). Genome instability, cancer and aging. *Biochim Biophys Acta* 1790, 963-969. 10.1016/j.bbagen.2009.03.020.
34. Niedernhofer, L.J., Gurkar, A.U., Wang, Y., Vijg, J., Hoeijmakers, J.H.J., and Robbins, P.D. (2018). Nuclear Genomic Instability and Aging. *Annu Rev Biochem* 87, 295-322. 10.1146/annurev-biochem-062917-012239.
35. Jeppesen, D.K., Bohr, V.A., and Stevnsner, T. (2011). DNA repair deficiency in neurodegeneration. *Prog Neurobiol* 94, 166-200. 10.1016/j.pneurobio.2011.04.013.
36. Diderich, K., Alanazi, M., and Hoeijmakers, J.H. (2011). Premature aging and cancer in nucleotide excision repair-disorders. *DNA Repair (Amst)* 10, 772-780. 10.1016/j.dnarep.2011.04.025.
37. Hosfield, D.J., Daniels, D.S., Mol, C.D., Putnam, C.D., Parikh, S.S., and Tainer, J.A. (2001). DNA damage recognition and repair pathway coordination revealed by the structural biochemistry of DNA repair enzymes. *Prog Nucleic Acid Res Mol Biol* 68, 315-347. 10.1016/s0079-6603(01)68110-8.
38. Krokan, H.E., and Bjørås, M. (2013). Base excision repair. *Cold Spring Harb Perspect Biol* 5, a012583. 10.1101/cshperspect.a012583.
39. Dijk, M., Typas, D., Mullenders, L., and Pines, A. (2014). Insight in the multilevel regulation of NER. *Exp Cell Res* 329, 116-123. 10.1016/j.yexcr.2014.08.010.
40. Sameer, A.S., Nissar, S., and Fatima, K. (2014). Mismatch repair pathway: molecules, functions, and role in colorectal carcinogenesis. *Eur J Cancer Prev* 23, 246-257. 10.1097/cej.0000000000000019.
41. Nickoloff, J.A., Sharma, N., Taylor, L., Allen, S.J., and Hromas, R. (2021). The Safe Path at the Fork: Ensuring Replication-Associated DNA Double-Strand Breaks are Repaired by Homologous Recombination. *Front Genet* 12, 748033. 10.3389/fgene.2021.748033.
42. Klein Douwel, D., Boonen, R.A., Long, D.T., Szypowska, A.A., Räsche, M., Walter, J.C., and Knipscheer, P. (2014). XPF-ERCC1 acts in Unhooking DNA interstrand crosslinks in cooperation with FANCD2 and FANCP/SLX4. *Mol Cell* 54, 460-471. 10.1016/j.molcel.2014.03.015.
43. Daley, J.M., Kwon, Y., Niu, H., and Sung, P. (2013). Investigations of homologous recombination pathways and their regulation. *Yale J Biol Med* 86, 453-461.
44. Jacobs, A.L., and Schär, P. (2012). DNA glycosylases: in DNA repair and beyond. *Chromosoma* 121, 1-20. 10.1007/s00412-011-0347-4.
45. Petrusheva, I.O., Evdokimov, A.N., and Lavrik, O.I. (2014). Molecular mechanism of global genome nucleotide excision repair. *Acta Naturae* 6, 23-34.
46. Li, G.M. (2008). Mechanisms and functions of DNA mismatch repair. *Cell Res* 18, 85-98. 10.1038/cr.2007.115.
47. Abbotts, R., and Wilson, D.M., 3rd (2017). Coordination of DNA single strand break repair. *Free Radic Biol Med* 107, 228-244. 10.1016/j.freeradbiomed.2016.11.039.
48. Lavin, M.F., Kozlov, S., Gatei, M., and Kijas, A.W. (2015). ATM-Dependent Phosphorylation of All Three Members of the MRN Complex: From Sensor to Adaptor. *Biomolecules* 5, 2877-2902. 10.3390/biom5042877.
49. Niida, H., and Nakanishi, M. (2006). DNA damage checkpoints in mammals. *Mutagenesis* 21, 3-9. 10.1093/mutage/gei063.
50. Li, L., and Zou, L. (2005). Sensing, signaling, and responding to DNA damage: organization of the checkpoint pathways in mammalian cells. *J Cell Biochem* 94, 298-306. 10.1002/jcb.20355.
51. Iyer, D.R., and Rhind, N. (2017). The Intra-S Checkpoint Responses to DNA Damage. *Genes (Basel)* 8, 10.3390/genes8020074.
52. O'Connell, M.J., and Cimprich, K.A. (2005). G2 damage checkpoints: what is the turn-on? *J Cell Sci* 118, 1-6. 10.1242/jcs.01626.
53. Cann, K.L., and Dellaire, G. (2011). Heterochromatin and the DNA damage response: the need to relax. *Biochem Cell Biol* 89, 45-60. 10.1139/o10-113.
54. Podhorecka, M., Skladanowski, A., and Bozko, P. (2010). H2AX Phosphorylation: Its Role in DNA Damage Response and Cancer Therapy. *J Nucleic Acids* 2010, 10.4061/2010/920161.
55. Palomera-Sanchez, Z., and Zurita, M. (2011). Open, repair and close again: chromatin dynamics and the response to UV-induced DNA damage. *DNA Repair (Amst)* 10, 119-125. 10.1016/j.dnarep.2010.10.010.
56. Erol, A. (2011). Deciphering the intricate regulatory mechanisms for the cellular choice between cell repair, apoptosis or senescence in response to damaging signals. *Cell Signal* 23, 1076-1081. 10.1016/j.cellsig.2010.11.023.
57. Bartek, J., and Lukas, J. (2007). DNA damage checkpoints: from initiation to recovery or adaptation. *Curr Opin Cell Biol* 19, 238-245. 10.1016/j.ceb.2007.02.009.

11. References

58. Hinkal, G.W., Gatz, C.E., Parikh, N., and Donehower, L.A. (2009). Altered senescence, apoptosis, and DNA damage response in a mutant p53 model of accelerated aging. *Mech Ageing Dev* 130, 262-271. 10.1016/j.mad.2009.01.001.
59. Lopes-Paciencia, S., Saint-Germain, E., Rowell, M.C., Ruiz, A.F., Kalegari, P., and Ferbeyre, G. (2019). The senescence-associated secretory phenotype and its regulation. *Cytokine* 117, 15-22. 10.1016/j.cyto.2019.01.013.
60. Foo, M.X.R., Ong, P.F., and Dreesen, O. (2019). Premature aging syndromes: From patients to mechanism. *J Dermatol Sci* 96, 58-65. 10.1016/j.jdermsci.2019.10.003.
61. Shamanna, R.A., Croteau, D.L., Lee, J.H., and Bohr, V.A. (2017). Recent Advances in Understanding Werner Syndrome. *F1000Res* 6, 1779. 10.12688/f1000research.12110.1.
62. Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol* 35, 495-516. 10.1080/01926230701320337.
63. Pasparakis, M., and Vandenabeele, P. (2015). Necroptosis and its role in inflammation. *Nature* 517, 311-320. 10.1038/nature14191.
64. Stockwell, B.R., Friedmann Angeli, J.P., Bayir, H., Bush, A.I., Conrad, M., Dixon, S.J., Fulda, S., Gascón, S., Hatzios, S.K., Kagan, V.E., et al. (2017). Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell* 171, 273-285. 10.1016/j.cell.2017.09.021.
65. Mahaney, B.L., Meek, K., and Lees-Miller, S.P. (2009). Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining. *Biochem J* 417, 639-650. 10.1042/bj20080413.
66. Symington, L.S., and Gautier, J. (2011). Double-strand break end resection and repair pathway choice. *Annu Rev Genet* 45, 247-271. 10.1146/annurev-genet-110410-132435.
67. Mimitou, E.P., and Symington, L.S. (2009). DNA end resection: many nucleases make light work. *DNA Repair (Amst)* 8, 983-995. 10.1016/j.dnarep.2009.04.017.
68. Escribano-Díaz, C., Orthwein, A., Fradet-Turcotte, A., Xing, M., Young, J.T., Tkáč, J., Cook, M.A., Rosebrock, A.P., Munro, M., Canny, M.D., et al. (2013). A cell cycle-dependent regulatory circuit composed of 53BP1-RIF1 and BRCA1-CtIP controls DNA repair pathway choice. *Mol Cell* 49, 872-883. 10.1016/j.molcel.2013.01.001.
69. Lavin, M.F. (2007). ATM and the Mre11 complex combine to recognize and signal DNA double-strand breaks. *Oncogene* 26, 7749-7758. 10.1038/sj.onc.1210880.
70. Collins, P.L., Purman, C., Porter, S.I., Nganga, V., Saini, A., Hayer, K.E., Gurewitz, G.L., Sleckman, B.P., Bednarski, J.J., Bassing, C.H., and Oltz, E.M. (2020). DNA double-strand breaks induce H2Ax phosphorylation domains in a contact-dependent manner. *Nat Commun* 11, 3158. 10.1038/s41467-020-16926-x.
71. Collyer, J., and Rajan, D.S. (2024). Ataxia telangiectasia. *Semin Pediatr Neurol* 52, 101169. 10.1016/j.spen.2024.101169.
72. Viegas, J. (2025). Profile of Yosef Shiloh. *Proc Natl Acad Sci U S A* 122, e2426242122. 10.1073/pnas.2426242122.
73. Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D.A., Smith, S., Uziel, T., Sfez, S., et al. (1995). A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* 268, 1749-1753. 10.1126/science.7792600.
74. Thiriet, C., and Hayes, J.J. (2005). Chromatin in need of a fix: phosphorylation of H2AX connects chromatin to DNA repair. *Mol Cell* 18, 617-622. 10.1016/j.molcel.2005.05.008.
75. Cejka, P., and Symington, L.S. (2021). DNA End Resection: Mechanism and Control. *Annu Rev Genet* 55, 285-307. 10.1146/annurev-genet-071719-020312.
76. Chanut, P., Britton, S., Coates, J., Jackson, S.P., and Calsou, P. (2016). Coordinated nuclease activities counteract Ku at single-ended DNA double-strand breaks. *Nat Commun* 7, 12889. 10.1038/ncomms12889.
77. Lee, B.I., and Wilson, D.M., 3rd (1999). The RAD2 domain of human exonuclease 1 exhibits 5' to 3' exonuclease and flap structure-specific endonuclease activities. *J Biol Chem* 274, 37763-37769. 10.1074/jbc.274.53.37763.
78. Sertic, S., Quadri, R., Lazzaro, F., and Muzi-Falconi, M. (2020). EXO1: A tightly regulated nuclease. *DNA Repair (Amst)* 93, 102929. 10.1016/j.dnarep.2020.102929.
79. Sturzenegger, A., Burdova, K., Kanagaraj, R., Levikova, M., Pinto, C., Cejka, P., and Janscak, P. (2014). DNA2 cooperates with the WRN and BLM RecQ helicases to mediate long-range DNA end resection in human cells. *J Biol Chem* 289, 27314-27326. 10.1074/jbc.M114.578823.
80. Seppa, I.M., Ceppi, I., Tennakoon, M., Reginato, G., Jackson, J., Rouault, C.D., Agashe, S., Sviderskiy, V.O., Limbu, M., Lantelme, E., et al. (2025). MRN-CtIP, EXO1, and DNA2-WRN/BLM act bidirectionally to process DNA gaps in PARPi-treated cells without strand cleavage. *Genes Dev* 39, 582-602. 10.1101/gad.352421.124.
81. Wang, H., Shi, L.Z., Wong, C.C., Han, X., Hwang, P.Y., Truong, L.N., Zhu, Q., Shao, Z., Chen, D.J., Berns, M.W., et al. (2013). The interaction of CtIP and Nbs1 connects CDK and ATM to regulate HR-mediated double-strand break repair. *PLoS Genet* 9, e1003277. 10.1371/journal.pgen.1003277.
82. Huertas, P., and Jackson, S.P. (2009). Human CtIP mediates cell cycle control of DNA end resection and double strand break repair. *J Biol Chem* 284, 9558-9565. 10.1074/jbc.M808906200.
83. Buis, J., Stoneham, T., Spehalski, E., and Ferguson, D.O. (2012). Mre11 regulates CtIP-dependent double-strand break repair by interaction with CDK2. *Nat Struct Mol Biol* 19, 246-252. 10.1038/nsmb.2212.
84. Chen, H., Lisby, M., and Symington, L.S. (2013). RPA coordinates DNA end resection and prevents formation of DNA hairpins. *Mol Cell* 50, 589-600. 10.1016/j.molcel.2013.04.032.

11. References

85. Zhang, F., Fan, Q., Ren, K., and Andreassen, P.R. (2009). PALB2 functionally connects the breast cancer susceptibility proteins BRCA1 and BRCA2. *Mol Cancer Res* 7, 1110-1118. 10.1158/1541-7786.Mcr-09-0123.
86. Jensen, R.B., Carreira, A., and Kowalczykowski, S.C. (2010). Purified human BRCA2 stimulates RAD51-mediated recombination. *Nature* 467, 678-683. 10.1038/nature09399.
87. Joudeh, L., Appleby, R.E., Maman, J.D., and Pellegrini, L. (2025). Structural mechanism of strand exchange by the RAD51 filament. *Elife* 14. 10.7554/eLife.107114.
88. Dronkert, M.L., Beverloo, H.B., Johnson, R.D., Hoeijmakers, J.H., Jasin, M., and Kanaar, R. (2000). Mouse RAD54 affects DNA double-strand break repair and sister chromatid exchange. *Mol Cell Biol* 20, 3147-3156. 10.1128/mcb.20.9.3147-3156.2000.
89. Machida, S., Takaku, M., Ikura, M., Sun, J., Suzuki, H., Kobayashi, W., Kinomura, A., Osakabe, A., Tachiwana, H., Horikoshi, Y., et al. (2014). Nap1 stimulates homologous recombination by RAD51 and RAD54 in higher-ordered chromatin containing histone H1. *Sci Rep* 4, 4863. 10.1038/srep04863.
90. Kowalczykowski, S.C. (2015). An Overview of the Molecular Mechanisms of Recombinational DNA Repair. *Cold Spring Harb Perspect Biol* 7. 10.1101/cshperspect.a016410.
91. Sebesta, M., Burkovics, P., Juhasz, S., Zhang, S., Szabo, J.E., Lee, M.Y., Haracska, L., and Krejci, L. (2013). Role of PCNA and TLS polymerases in D-loop extension during homologous recombination in humans. *DNA Repair (Amst)* 12, 691-698. 10.1016/j.dnarep.2013.05.001.
92. West, S.C., Blanco, M.G., Chan, Y.W., Matos, J., Sarbajna, S., and Wyatt, H.D. (2015). Resolution of Recombination Intermediates: Mechanisms and Regulation. *Cold Spring Harb Symp Quant Biol* 80, 103-109. 10.1101/sqb.2015.80.027649.
93. Schwartz, E.K., Wright, W.D., Ehmsen, K.T., Evans, J.E., Stahlberg, H., and Heyer, W.D. (2012). Mus81-Mms4 functions as a single heterodimer to cleave nicked intermediates in recombinational DNA repair. *Mol Cell Biol* 32, 3065-3080. 10.1128/mcb.00547-12.
94. Ferguson, D.O., and Holloman, W.K. (1996). Recombinational repair of gaps in DNA is asymmetric in *Ustilago maydis* and can be explained by a migrating D-loop model. *Proc Natl Acad Sci U S A* 93, 5419-5424. 10.1073/pnas.93.11.5419.
95. Ira, G., Satory, D., and Haber, J.E. (2006). Conservative inheritance of newly synthesized DNA in double-strand break-induced gene conversion. *Mol Cell Biol* 26, 9424-9429. 10.1128/mcb.01654-06.
96. Johnson, R.D., and Jasin, M. (2000). Sister chromatid gene conversion is a prominent double-strand break repair pathway in mammalian cells. *Embo j* 19, 3398-3407. 10.1093/emboj/19.13.3398.
97. Hall, J.M., Lee, M.K., Newman, B., Morrow, J.E., Anderson, L.A., Huey, B., and King, M.C. (1990). Linkage of early-onset familial breast cancer to chromosome 17q21. *Science* 250, 1684-1689. 10.1126/science.2270482.
98. Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W., and et al. (1994). A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 266, 66-71. 10.1126/science.7545954.
99. Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C., and Micklem, G. (1995). Identification of the breast cancer susceptibility gene BRCA2. *Nature* 378, 789-792. 10.1038/378789a0.
100. Moynahan, M.E., Chiu, J.W., Koller, B.H., and Jasin, M. (1999). Brca1 controls homology-directed DNA repair. *Mol Cell* 4, 511-518. 10.1016/s1097-2765(00)80202-6.
101. Moynahan, M.E., Pierce, A.J., and Jasin, M. (2001). BRCA2 is required for homology-directed repair of chromosomal breaks. *Mol Cell* 7, 263-272. 10.1016/s1097-2765(01)00174-5.
102. Engel, C., and Fischer, C. (2015). Breast cancer risks and risk prediction models. *Breast Care (Basel)* 10, 7-12. 10.1159/000376600.
103. Moynahan, M.E. (2002). The cancer connection: BRCA1 and BRCA2 tumor suppression in mice and humans. *Oncogene* 21, 8994-9007. 10.1038/sj.onc.1206177.
104. Oshima, J. (2000). Comparative aspects of the Werner syndrome gene. *In Vivo* 14, 165-172.
105. Goto, M., Ishikawa, Y., Sugimoto, M., and Furuichi, Y. (2013). Werner syndrome: a changing pattern of clinical manifestations in Japan (1917~2008). *Biosci Trends* 7, 13-22.
106. Yu, C.E., Oshima, J., Fu, Y.H., Wijsman, E.M., Hisama, F., Alisch, R., Matthews, S., Nakura, J., Miki, T., Ouais, S., et al. (1996). Positional cloning of the Werner's syndrome gene. *Science* 272, 258-262. 10.1126/science.272.5259.258.
107. Hsu, T.Y., Hsu, L.N., Chen, S.Y., and Juang, B.T. (2021). MUT-7 Provides Molecular Insight into the Werner Syndrome Exonuclease. *Cells* 10. 10.3390/cells10123457.
108. Marabitti, V., Valenzisi, P., Lillo, G., Malacaria, E., Palermo, V., Pichierri, P., and Franchitto, A. (2022). R-Loop-Associated Genomic Instability and Implication of WRN and WRNIP1. *Int J Mol Sci* 23. 10.3390/ijms23031547.
109. Ellis, N.A., Groden, J., Ye, T.Z., Straughen, J., Lennon, D.J., Ciocci, S., Proytcheva, M., and German, J. (1995). The Bloom's syndrome gene product is homologous to RecQ helicases. *Cell* 83, 655-666. 10.1016/0092-8674(95)90105-1.
110. Ababou, M. (2021). Bloom syndrome and the underlying causes of genetic instability. *Mol Genet Metab* 133, 35-48. 10.1016/j.ymgme.2021.03.003.
111. Chaganti, R.S., Schonberg, S., and German, J. (1974). A manyfold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes. *Proc Natl Acad Sci U S A* 71, 4508-4512. 10.1073/pnas.71.11.4508.

11. References

112. Gupta, R., Somyajit, K., Narita, T., Maskey, E., Stanlie, A., Kremer, M., Typas, D., Lammers, M., Mailand, N., Nussenzweig, A., et al. (2018). DNA Repair Network Analysis Reveals Shieldin as a Key Regulator of NHEJ and PARP Inhibitor Sensitivity. *Cell* 173, 972-988.e923. 10.1016/j.cell.2018.03.050.
113. Mirman, Z., Lottersberger, F., Takai, H., Kibe, T., Gong, Y., Takai, K., Bianchi, A., Zimmermann, M., Durocher, D., and de Lange, T. (2018). 53BP1-RIF1-shieldin counteracts DSB resection through CST- and Pol α -dependent fill-in. *Nature* 560, 112-116. 10.1038/s41586-018-0324-7.
114. Walker, J.R., Corpina, R.A., and Goldberg, J. (2001). Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature* 412, 607-614. 10.1038/35088000.
115. Chaplin, A.K., Hardwick, S.W., Liang, S., Kefala Stavridi, A., Hnizda, A., Cooper, L.R., De Oliveira, T.M., Chirgadze, D.Y., and Blundell, T.L. (2021). Dimers of DNA-PK create a stage for DNA double-strand break repair. *Nat Struct Mol Biol* 28, 13-19. 10.1038/s41594-020-00517-x.
116. Radhakrishnan, S.K., and Lees-Miller, S.P. (2017). DNA requirements for interaction of the C-terminal region of Ku80 with the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). *DNA Repair (Amst)* 57, 17-28. 10.1016/j.dnarep.2017.06.001.
117. Cottarel, J., Frit, P., Bombarde, O., Salles, B., Négrel, A., Bernard, S., Jeggo, P.A., Lieber, M.R., Modesti, M., and Calsou, P. (2013). A noncatalytic function of the ligation complex during nonhomologous end joining. *J Cell Biol* 200, 173-186. 10.1083/jcb.201203128.
118. Lieber, M.R. (2008). The mechanism of human nonhomologous DNA end joining. *J Biol Chem* 283, 1-5. 10.1074/jbc.R700039200.
119. Bhargava, R., Onyango, D.O., and Stark, J.M. (2016). Regulation of Single-Strand Annealing and its Role in Genome Maintenance. *Trends Genet* 32, 566-575. 10.1016/j.tig.2016.06.007.
120. Ochs, F., Somyajit, K., Altmeyer, M., Rask, M.B., Lukas, J., and Lukas, C. (2016). 53BP1 fosters fidelity of homology-directed DNA repair. *Nat Struct Mol Biol* 23, 714-721. 10.1038/nsmb.3251.
121. Hanamshet, K., and Mazin, A.V. (2020). The function of RAD52 N-terminal domain is essential for viability of BRCA-deficient cells. *Nucleic Acids Res* 48, 12778-12791. 10.1093/nar/gkaa1145.
122. Blasiak, J. (2021). Single-Strand Annealing in Cancer. *Int J Mol Sci* 22. 10.3390/ijms22042167.
123. Seol, J.H., Shim, E.Y., and Lee, S.E. (2018). Microhomology-mediated end joining: Good, bad and ugly. *Mutat Res* 809, 81-87. 10.1016/j.mrfmmm.2017.07.002.
124. Truong, L.N., Li, Y., Shi, L.Z., Hwang, P.Y., He, J., Wang, H., Razavian, N., Berns, M.W., and Wu, X. (2013). Microhomology-mediated End Joining and Homologous Recombination share the initial end resection step to repair DNA double-strand breaks in mammalian cells. *Proc Natl Acad Sci U S A* 110, 7720-7725. 10.1073/pnas.1213431110.
125. Audebert, M., Salles, B., and Calsou, P. (2004). Involvement of poly(ADP-ribose) polymerase-1 and XRCC1/DNA ligase III in an alternative route for DNA double-strand breaks rejoining. *J Biol Chem* 279, 55117-55126. 10.1074/jbc.M404524200.
126. Wood, R.D., and Doublé, S. (2016). DNA polymerase θ (POLQ), double-strand break repair, and cancer. *DNA Repair (Amst)* 44, 22-32. 10.1016/j.dnarep.2016.05.003.
127. Ozdemir, A.Y., Rusanov, T., Kent, T., Siddique, L.A., and Pomerantz, R.T. (2018). Polymerase θ -helicase efficiently unwinds DNA and RNA-DNA hybrids. *J Biol Chem* 293, 5259-5269. 10.1074/jbc.RA117.000565.
128. Sharma, S., Javadekar, S.M., Pandey, M., Srivastava, M., Kumari, R., and Raghavan, S.C. (2015). Homology and enzymatic requirements of microhomology-dependent alternative end joining. *Cell Death Dis* 6, e1697. 10.1038/cddis.2015.58.
129. Ahrabi, S., Sarkar, S., Pfister, S.X., Pirovano, G., Higgins, G.S., Porter, A.C., and Humphrey, T.C. (2016). A role for human homologous recombination factors in suppressing microhomology-mediated end joining. *Nucleic Acids Res* 44, 5743-5757. 10.1093/nar/gkw326.
130. Tobin, L.A., Robert, C., Nagaria, P., Chumsri, S., Twaddell, W., Ioffe, O.B., Greco, G.E., Brodie, A.H., Tomkinson, A.E., and Rassool, F.V. (2012). Targeting abnormal DNA repair in therapy-resistant breast cancers. *Mol Cancer Res* 10, 96-107. 10.1158/1541-7786.Mcr-11-0255.
131. Kerzendorfer, C., and O'Driscoll, M. (2009). Human DNA damage response and repair deficiency syndromes: linking genomic instability and cell cycle checkpoint proficiency. *DNA Repair (Amst)* 8, 1139-1152. 10.1016/j.dnarep.2009.04.018.
132. Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer* 3, 155-168. 10.1038/nrc1011.
133. Vollebergh, M.A., Jonkers, J., and Linn, S.C. (2012). Genomic instability in breast and ovarian cancers: translation into clinical predictive biomarkers. *Cell Mol Life Sci* 69, 223-245. 10.1007/s00018-011-0809-0.
134. Setton, J., Hadi, K., Choo, Z.N., Kuchin, K.S., Tian, H., Da Cruz Paula, A., Rosiene, J., Selenica, P., Behr, J., Yao, X., et al. (2023). Long-molecule scars of backup DNA repair in BRCA1- and BRCA2-deficient cancers. *Nature* 621, 129-137. 10.1038/s41586-023-06461-2.
135. Zhang, T., Yang, H., Zhou, Z., Bai, Y., Wang, J., and Wang, W. (2022). Crosstalk between SUMOylation and ubiquitylation controls DNA end resection by maintaining MRE11 homeostasis on chromatin. *Nat Commun* 13, 5133. 10.1038/s41467-022-32920-x.
136. Jachimowicz, R.D., Beleggia, F., Isensee, J., Velpula, B.B., Goergens, J., Bustos, M.A., Doll, M.A., Shenoy, A., Checa-Rodriguez, C., Wiederstein, J.L., et al. (2019). UBQLN4 Represses Homologous Recombination and Is Overexpressed in Aggressive Tumors. *Cell* 176, 505-519.e522. 10.1016/j.cell.2018.11.024.
137. Garvin, A.J. (2019). Beyond reversal: ubiquitin and ubiquitin-like proteases and the orchestration of the DNA double strand break repair response. *Biochem Soc Trans* 47, 1881-1893. 10.1042/bst20190534.

11. References

138. Rezazadeh, S., Yang, D., Biashad, S.A., Firsanov, D., Takasugi, M., Gilbert, M., Tomblin, G., Bhanu, N.V., Garcia, B.A., Seluanov, A., and Gorbunova, V. (2020). SIRT6 mono-ADP ribosylates KDM2A to locally increase H3K36me2 at DNA damage sites to inhibit transcription and promote repair. *Aging (Albany NY)* 12, 11165-11184. 10.18632/aging.103567.
139. Markkanen, E., van Loon, B., Ferrari, E., Parsons, J.L., Dianov, G.L., and Hübscher, U. (2012). Regulation of oxidative DNA damage repair by DNA polymerase λ and MutYH by cross-talk of phosphorylation and ubiquitination. *Proc Natl Acad Sci U S A* 109, 437-442. 10.1073/pnas.1110449109.
140. Borsos, B.N., Majoros, H., and Pankotai, T. (2020). Ubiquitylation-Mediated Fine-Tuning of DNA Double-Strand Break Repair. *Cancers (Basel)* 12. 10.3390/cancers12061617.
141. Heyza, J.R., Mikhova, M., Bahl, A., Broadbent, D.G., and Schmidt, J.C. (2023). Systematic analysis of the molecular and biophysical properties of key DNA damage response factors. *Elife* 12. 10.7554/eLife.87086.
142. Traven, A., and Heierhorst, J. (2005). SQ/TQ cluster domains: concentrated ATM/ATR kinase phosphorylation site regions in DNA-damage-response proteins. *Bioessays* 27, 397-407. 10.1002/bies.20204.
143. Stucki, M., and Jackson, S.P. (2006). gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. *DNA Repair (Amst)* 5, 534-543. 10.1016/j.dnarep.2006.01.012.
144. Blackford, A.N., and Jackson, S.P. (2017). ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response. *Mol Cell* 66, 801-817. 10.1016/j.molcel.2017.05.015.
145. Cook, P.J., Ju, B.G., Telese, F., Wang, X., Glass, C.K., and Rosenfeld, M.G. (2009). Tyrosine dephosphorylation of H2AX modulates apoptosis and survival decisions. *Nature* 458, 591-596. 10.1038/nature07849.
146. Sekiguchi, M., and Matsushita, N. (2022). DNA Damage Response Regulation by Histone Ubiquitination. *Int J Mol Sci* 23. 10.3390/ijms23158187.
147. Nishi, R. (2017). Balancing act: To be, or not to be ubiquitylated. *Mutat Res* 803-805, 43-50. 10.1016/j.mrfmmm.2017.07.006.
148. Lange, S.M., Armstrong, L.A., and Kulathu, Y. (2022). Deubiquitinases: From mechanisms to their inhibition by small molecules. *Mol Cell* 82, 15-29. 10.1016/j.molcel.2021.10.027.
149. Komander, D., and Rape, M. (2012). The ubiquitin code. *Annu Rev Biochem* 81, 203-229. 10.1146/annurev-biochem-060310-170328.
150. Kurlawala, Z., Shah, P.P., Shah, C., and Beverly, L.J. (2017). The STI and UBA Domains of UBQLN1 Are Critical Determinants of Substrate Interaction and Proteostasis. *J Cell Biochem* 118, 2261-2270. 10.1002/jcb.25880.
151. Park, S.Y., and Kim, J.S. (2020). A short guide to histone deacetylases including recent progress on class II enzymes. *Exp Mol Med* 52, 204-212. 10.1038/s12276-020-0382-4.
152. Gursoy-Yuzugullu, O., House, N., and Price, B.D. (2016). Patching Broken DNA: Nucleosome Dynamics and the Repair of DNA Breaks. *J Mol Biol* 428, 1846-1860. 10.1016/j.jmb.2015.11.021.
153. Tuzon, C.T., Spektor, T., Kong, X., Congdon, L.M., Wu, S., Schotta, G., Yokomori, K., and Rice, J.C. (2014). Concerted activities of distinct H4K20 methyltransferases at DNA double-strand breaks regulate 53BP1 nucleation and NHEJ-directed repair. *Cell Rep* 8, 430-438. 10.1016/j.celrep.2014.06.013.
154. Pfister, S.X., Ahrabi, S., Zalmas, L.P., Sarkar, S., Aymard, F., Bachrati, C.Z., Helleday, T., Legube, G., La Thangue, N.B., Porter, A.C., and Humphrey, T.C. (2014). SETD2-dependent histone H3K36 trimethylation is required for homologous recombination repair and genome stability. *Cell Rep* 7, 2006-2018. 10.1016/j.celrep.2014.05.026.
155. Garvin, A.J., and Morris, J.R. (2017). SUMO, a small, but powerful, regulator of double-strand break repair. *Philos Trans R Soc Lond B Biol Sci* 372. 10.1098/rstb.2016.0281.
156. Morris, J.R., Boutell, C., Keppler, M., Densham, R., Weekes, D., Alamshah, A., Butler, L., Galanty, Y., Pangon, L., Kiuchi, T., et al. (2009). The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress. *Nature* 462, 886-890. 10.1038/nature08593.
157. Nosella, M.L., Kim, T.H., Huang, S.K., Harkness, R.W., Goncalves, M., Pan, A., Tereshchenko, M., Vahidi, S., Rubinstein, J.L., Lee, H.O., et al. (2024). Poly(ADP-ribosyl)ation enhances nucleosome dynamics and organizes DNA damage repair components within biomolecular condensates. *Mol Cell* 84, 429-446.e417. 10.1016/j.molcel.2023.12.019.
158. Pandey, N., and Black, B.E. (2021). Rapid Detection and Signaling of DNA Damage by PARP-1. *Trends Biochem Sci* 46, 744-757. 10.1016/j.tibs.2021.01.014.
159. Slade, D., Dunstan, M.S., Barkauskaite, E., Weston, R., Lafite, P., Dixon, N., Ahel, M., Leys, D., and Ahel, I. (2011). The structure and catalytic mechanism of a poly(ADP-ribose) glycohydrolase. *Nature* 477, 616-620. 10.1038/nature10404.
160. Dantuma, N.P., and van Attikum, H. (2016). Spatiotemporal regulation of posttranslational modifications in the DNA damage response. *Embo j* 35, 6-23. 10.15252/emboj.201592595.
161. Al-Hakim, A., Escribano-Diaz, C., Landry, M.C., O'Donnell, L., Panier, S., Szilard, R.K., and Durocher, D. (2010). The ubiquitous role of ubiquitin in the DNA damage response. *DNA Repair (Amst)* 9, 1229-1240. 10.1016/j.dnarep.2010.09.011.
162. Terabayashi, T., and Hanada, K. (2018). Genome instability syndromes caused by impaired DNA repair and aberrant DNA damage responses. *Cell Biol Toxicol* 34, 337-350. 10.1007/s10565-018-9429-x.
163. Weidenheim, K.M., Dickson, D.W., and Rapin, I. (2009). Neuropathology of Cockayne syndrome: Evidence for impaired development, premature aging, and neurodegeneration. *Mech Ageing Dev* 130, 619-636. 10.1016/j.mad.2009.07.006.

11. References

164. Taylor, A.M.R., Rothblum-Oviatt, C., Ellis, N.A., Hickson, I.D., Meyer, S., Crawford, T.O., Smogorzewska, A., Pietrucha, B., Weemaes, C., and Stewart, G.S. (2019). Chromosome instability syndromes. *Nat Rev Dis Primers* 5, 64. 10.1038/s41572-019-0113-0.
165. Freitas, A.A., and de Magalhães, J.P. (2011). A review and appraisal of the DNA damage theory of ageing. *Mutat Res* 728, 12-22. 10.1016/j.mrrev.2011.05.001.
166. Lu, H., and Davis, A.J. (2021). Human RecQ Helicases in DNA Double-Strand Break Repair. *Front Cell Dev Biol* 9, 640755. 10.3389/fcell.2021.640755.
167. Eriksson, M., Brown, W.T., Gordon, L.B., Glynn, M.W., Singer, J., Scott, L., Erdos, M.R., Robbins, C.M., Moses, T.Y., Berglund, P., et al. (2003). Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome. *Nature* 423, 293-298. 10.1038/nature01629.
168. Constantinescu, D., Csoka, A.B., Navara, C.S., and Schatten, G.P. (2010). Defective DSB repair correlates with abnormal nuclear morphology and is improved with FTI treatment in Hutchinson-Gilford progeria syndrome fibroblasts. *Exp Cell Res* 316, 2747-2759. 10.1016/j.yexcr.2010.05.015.
169. Lu, L., Jin, W., and Wang, L.L. (2017). Aging in Rothmund-Thomson syndrome and related RECQL4 genetic disorders. *Ageing Res Rev* 33, 30-35. 10.1016/j.arr.2016.06.002.
170. Larizza, L., Roversi, G., and Volpi, L. (2010). Rothmund-Thomson syndrome. *Orphanet J Rare Dis* 5, 2. 10.1186/1750-1172-5-2.
171. Kitao, S., Shimamoto, A., Goto, M., Miller, R.W., Smithson, W.A., Lindor, N.M., and Furuichi, Y. (1999). Mutations in RECQL4 cause a subset of cases of Rothmund-Thomson syndrome. *Nat Genet* 22, 82-84. 10.1038/8788.
172. Kitao, S., Lindor, N.M., Shiratori, M., Furuichi, Y., and Shimamoto, A. (1999). Rothmund-thomson syndrome responsible gene, RECQL4: genomic structure and products. *Genomics* 61, 268-276. 10.1006/geno.1999.5959.
173. Im, J.S., Ki, S.H., Farina, A., Jung, D.S., Hurwitz, J., and Lee, J.K. (2009). Assembly of the Cdc45-Mcm2-7-GINS complex in human cells requires the Ctf4/And-1, RecQL4, and Mcm10 proteins. *Proc Natl Acad Sci U S A* 106, 15628-15632. 10.1073/pnas.0908039106.
174. Lu, H., Shamanna, R.A., de Freitas, J.K., Okur, M., Khadka, P., Kulikowicz, T., Holland, P.P., Tian, J., Croteau, D.L., Davis, A.J., and Bohr, V.A. (2017). Cell cycle-dependent phosphorylation regulates RECQL4 pathway choice and ubiquitination in DNA double-strand break repair. *Nat Commun* 8, 2039. 10.1038/s41467-017-02146-3.
175. Balajee, A.S. (2021). Human RecQL4 as a Novel Molecular Target for Cancer Therapy. *Cytogenet Genome Res* 161, 305-327. 10.1159/000516568.
176. Farmer, H., McCabe, N., Lord, C.J., Tutt, A.N., Johnson, D.A., Richardson, T.B., Santarosa, M., Dillon, K.J., Hickson, I., Knights, C., et al. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 434, 917-921. 10.1038/nature03445.
177. Ng, S.B., Turner, E.H., Robertson, P.D., Flygare, S.D., Bigham, A.W., Lee, C., Shaffer, T., Wong, M., Bhattacharjee, A., Eichler, E.E., et al. (2009). Targeted capture and massively parallel sequencing of 12 human exomes. *Nature* 461, 272-276. 10.1038/nature08250.
178. Mantere, T., Neveling, K., Pebrel-Richard, C., Benoist, M., van der Zande, G., Kater-Baats, E., Baatout, I., van Beek, R., Yammine, T., Oorsprong, M., et al. (2021). Optical genome mapping enables constitutional chromosomal aberration detection. *Am J Hum Genet* 108, 1409-1422. 10.1016/j.ajhg.2021.05.012.
179. Levy, B., Baughn, L.B., Akkari, Y., Chartrand, S., LaBarge, B., Claxton, D., Lennon, P.A., Cujar, C., Kolhe, R., Kroeger, K., et al. (2023). Optical genome mapping in acute myeloid leukemia: a multicenter evaluation. *Blood Adv* 7, 1297-1307. 10.1182/bloodadvances.2022007583.
180. Cipriani, V., Vestito, L., Magavern, E.F., Jacobsen, J.O.B., Arno, G., Behr, E.R., Benson, K.A., Bertoli, M., Bockenhauer, D., Bowl, M.R., et al. (2025). Rare disease gene association discovery in the 100,000 Genomes Project. *Nature*. 10.1038/s41586-025-08623-w.
181. Slaba, K., Pokorna, P., Jugas, R., Palova, H., Prochazkova, D., Aulicka, S., Spanelova, K., Danhofer, P., Horak, O., Tuckova, J., et al. (2024). Diagnostic efficacy and clinical utility of whole-exome sequencing in Czech pediatric patients with rare and undiagnosed diseases. *Sci Rep* 14, 28780. 10.1038/s41598-024-79872-4.
182. Sullivan, J.A., Schoch, K., Spillmann, R.C., and Shashi, V. (2023). Exome/Genome Sequencing in Undiagnosed Syndromes. *Annu Rev Med* 74, 489-502. 10.1146/annurev-med-042921-110721.
183. Berwick, M., Satagopan, J.M., Ben-Porat, L., Carlson, A., Mah, K., Henry, R., Diotti, R., Milton, K., Pujara, K., Landers, T., et al. (2007). Genetic heterogeneity among Fanconi anemia heterozygotes and risk of cancer. *Cancer Res* 67, 9591-9596. 10.1158/0008-5472.Can-07-1501.
184. Gruber, S.B., Ellis, N.A., Scott, K.K., Almog, R., Kolachana, P., Bonner, J.D., Kirchhoff, T., Tomsho, L.P., Nafa, K., Pierce, H., et al. (2002). BLM heterozygosity and the risk of colorectal cancer. *Science* 297, 2013. 10.1126/science.1074399.
185. Thompson, E.R., Doyle, M.A., Ryland, G.L., Rowley, S.M., Choong, D.Y., Tothill, R.W., Thorne, H., Barnes, D.R., Li, J., Ellul, J., et al. (2012). Exome sequencing identifies rare deleterious mutations in DNA repair genes FANCC and BLM as potential breast cancer susceptibility alleles. *PLoS Genet* 8, e1002894. 10.1371/journal.pgen.1002894.
186. Han, W., Kim, K.Y., Yang, S.J., Noh, D.Y., Kang, D., and Kwack, K. (2012). SNP-SNP interactions between DNA repair genes were associated with breast cancer risk in a Korean population. *Cancer* 118, 594-602. 10.1002/cncr.26220.

11. References

187. Ming-Shiean, H., Yu, J.C., Wang, H.W., Chen, S.T., Hsiung, C.N., Ding, S.L., Wu, P.E., Shen, C.Y., and Cheng, C.W. (2010). Synergistic effects of polymorphisms in DNA repair genes and endogenous estrogen exposure on female breast cancer risk. *Ann Surg Oncol* 17, 760-771. 10.1245/s10434-009-0802-0.
188. Vermeij, W.P., Hoeijmakers, J.H., and Pothof, J. (2016). Genome Integrity in Aging: Human Syndromes, Mouse Models, and Therapeutic Options. *Annu Rev Pharmacol Toxicol* 56, 427-445. 10.1146/annurev-pharmtox-010814-124316.
189. Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F., and Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153, 910-918. 10.1016/j.cell.2013.04.025.
190. Birling, M.C., Herault, Y., and Pavlovic, G. (2017). Modeling human disease in rodents by CRISPR/Cas9 genome editing. *Mamm Genome* 28, 291-301. 10.1007/s00335-017-9703-x.
191. Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J.N., Ried, T., Tagle, D., and Wynshaw-Boris, A. (1996). Atm-deficient mice: a paradigm of ataxia telangiectasia. *Cell* 86, 159-171. 10.1016/s0092-8674(00)80086-0.
192. Tal, E., Alfo, M., Zha, S., Barzilai, A., De Zeeuw, C.I., Ziv, Y., and Shiloh, Y. (2018). Inactive Atm abrogates DSB repair in mouse cerebellum more than does Atm loss, without causing a neurological phenotype. *DNA Repair (Amst)* 72, 10-17. 10.1016/j.dnarep.2018.10.001.
193. Chiesa, N., Barlow, C., Wynshaw-Boris, A., Strata, P., and Tempia, F. (2000). Atm-deficient mice Purkinje cells show age-dependent defects in calcium spike bursts and calcium currents. *Neuroscience* 96, 575-583. 10.1016/s0306-4522(99)00581-3.
194. Hakem, R., de la Pompa, J.L., and Mak, T.W. (1998). Developmental studies of Brca1 and Brca2 knock-out mice. *J Mammary Gland Biol Neoplasia* 3, 431-445. 10.1023/a:1018792200700.
195. Deng, C.X. (2002). Tumor formation in Brca1 conditional mutant mice. *Environ Mol Mutagen* 39, 171-177. 10.1002/em.10069.
196. Hanahan, D. (2026). Hallmarks of cancer-Then and now, and beyond. *Cell*. 10.1016/j.cell.2025.12.049.
197. Keuper, K., Wieland, A., Räschle, M., and Storchova, Z. (2021). Processes shaping cancer genomes - From mitotic defects to chromosomal rearrangements. *DNA Repair (Amst)* 107, 103207. 10.1016/j.dnarep.2021.103207.
198. Ceccaldi, R., Liu, J.C., Amunugama, R., Hajdu, I., Primack, B., Petalcorin, M.I., O'Connor, K.W., Konstantinopoulos, P.A., Elledge, S.J., Boulton, S.J., et al. (2015). Homologous-recombination-deficient tumours are dependent on Polθ-mediated repair. *Nature* 518, 258-262. 10.1038/nature14184.
199. Deriano, L., and Roth, D.B. (2013). Modernizing the nonhomologous end-joining repertoire: alternative and classical NHEJ share the stage. *Annu Rev Genet* 47, 433-455. 10.1146/annurev-genet-110711-155540.
200. Dagogo-Jack, I., and Shaw, A.T. (2018). Tumour heterogeneity and resistance to cancer therapies. *Nat Rev Clin Oncol* 15, 81-94. 10.1038/nrclinonc.2017.166.
201. Yurgelun, M.B., Kulke, M.H., Fuchs, C.S., Allen, B.A., Uno, H., Hornick, J.L., Ukaegbu, C.I., Brais, L.K., McNamara, P.G., Mayer, R.J., et al. (2017). Cancer Susceptibility Gene Mutations in Individuals With Colorectal Cancer. *J Clin Oncol* 35, 1086-1095. 10.1200/jco.2016.71.0012.
202. Ledermann, J.A., Drew, Y., and Kristeleit, R.S. (2016). Homologous recombination deficiency and ovarian cancer. *Eur J Cancer* 60, 49-58. 10.1016/j.ejca.2016.03.005.
203. Shao, C., Wan, J., Lam, F.C., Tang, H., Marley, A.R., Song, Y., Miller, C., Brown, M., Han, J., and Adeboyeje, G. (2022). A comprehensive literature review and meta-analysis of the prevalence of pan-cancer BRCA mutations, homologous recombination repair gene mutations, and homologous recombination deficiencies. *Environ Mol Mutagen* 63, 308-316. 10.1002/em.22505.
204. Riaz, N., Blecua, P., Lim, R.S., Shen, R., Higginson, D.S., Weinhold, N., Norton, L., Weigelt, B., Powell, S.N., and Reis-Filho, J.S. (2017). Pan-cancer analysis of bi-allelic alterations in homologous recombination DNA repair genes. *Nat Commun* 8, 857. 10.1038/s41467-017-00921-w.
205. Heeke, A.L., Pishvaian, M.J., Lynce, F., Xiu, J., Brody, J.R., Chen, W.J., Baker, T.M., Marshall, J.L., and Isaacs, C. (2018). Prevalence of Homologous Recombination-Related Gene Mutations Across Multiple Cancer Types. *JCO Precis Oncol* 2018. 10.1200/po.17.00286.
206. Nik-Zainal, S., Davies, H., Staaf, J., Ramakrishna, M., Glodzik, D., Zou, X., Martincorena, I., Alexandrov, L.B., Martin, S., Wedge, D.C., et al. (2016). Landscape of somatic mutations in 560 breast cancer whole-genome sequences. *Nature* 534, 47-54. 10.1038/nature17676.
207. Alexandrov, L.B., Nik-Zainal, S., Wedge, D.C., Aparicio, S.A., Behjati, S., Biankin, A.V., Bignell, G.R., Bolli, N., Borg, A., Børresen-Dale, A.L., et al. (2013). Signatures of mutational processes in human cancer. *Nature* 500, 415-421. 10.1038/nature12477.
208. von Wahlde, M.K., Timms, K.M., Chagpar, A., Wali, V.B., Jiang, T., Bossuyt, V., Saglam, O., Reid, J., Gutin, A., Neff, C., et al. (2017). Intratumor Heterogeneity of Homologous Recombination Deficiency in Primary Breast Cancer. *Clin Cancer Res* 23, 1193-1199. 10.1158/1078-0432.Ccr-16-0889.
209. Rempel, E., Kluck, K., Beck, S., Ourailidis, I., Kazdal, D., Neumann, O., Volckmar, A.L., Kirchner, M., Goldschmid, H., Pfarr, N., et al. (2022). Pan-cancer analysis of genomic scar patterns caused by homologous repair deficiency (HRD). *NPJ Precis Oncol* 6, 36. 10.1038/s41698-022-00276-6.
210. Golan, T., O'Kane, G.M., Denroche, R.E., Raites-Gurevich, M., Grant, R.C., Holter, S., Wang, Y., Zhang, A., Jang, G.H., Stossel, C., et al. (2021). Genomic Features and Classification of Homologous Recombination Deficient Pancreatic Ductal Adenocarcinoma. *Gastroenterology* 160, 2119-2132.e2119. 10.1053/j.gastro.2021.01.220.

11. References

211. Yndestad, S., Engebretsen, C., Herencia-Ropero, A., Nikolaienko, O., Vintermyr, O.K., Lillestøl, R.K., Minsaas, L., Leirvaag, B., Iversen, G.T., Gilje, B., et al. (2023). Homologous Recombination Deficiency Across Subtypes of Primary Breast Cancer. *JCO Precis Oncol* 7, e2300338. 10.1200/po.23.00338.
212. Faraoni, I., and Graziani, G. (2018). Role of BRCA Mutations in Cancer Treatment with Poly(ADP-ribose) Polymerase (PARP) Inhibitors. *Cancers (Basel)* 10. 10.3390/cancers10120487.
213. Feng, Z., Shao, D., Cai, Y., Bi, R., Ju, X., Chen, D., Song, C., Chen, X., Li, J., An, N., et al. (2023). Homologous recombination deficiency status predicts response to platinum-based chemotherapy in Chinese patients with high-grade serous ovarian carcinoma. *J Ovarian Res* 16, 53. 10.1186/s13048-023-01129-x.
214. Konecny, G.E., Chander, C., and Zhang, L. (2021). Integrated, Integral, and Exploratory Biomarkers in the Development of Poly(ADP-Ribose) Polymerase Inhibitors. *Cancer J* 27, 482-490. 10.1097/ppo.0000000000000564.
215. Bryant, H.E., Schultz, N., Thomas, H.D., Parker, K.M., Flower, D., Lopez, E., Kyle, S., Meuth, M., Curtin, N.J., and Helleday, T. (2005). Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 434, 913-917. 10.1038/nature03443.
216. Murai, J., Huang, S.Y., Das, B.B., Renaud, A., Zhang, Y., Doroshow, J.H., Ji, J., Takeda, S., and Pommier, Y. (2012). Trapping of PARP1 and PARP2 by Clinical PARP Inhibitors. *Cancer Res* 72, 5588-5599. 10.1158/0008-5472.Can-12-2753.
217. Petropoulos, M., Karamichali, A., Rossetti, G.G., Freudenmann, A., Iacovino, L.G., Dionellis, V.S., Sotiriou, S.K., and Halazonetis, T.D. (2024). Transcription-replication conflicts underlie sensitivity to PARP inhibitors. *Nature* 628, 433-441. 10.1038/s41586-024-07217-2.
218. Lord, C.J., and Ashworth, A. (2016). BRCAness revisited. *Nat Rev Cancer* 16, 110-120. 10.1038/nrc.2015.21.
219. Tutt, A., Tovey, H., Cheang, M.C.U., Kernaghan, S., Kilburn, L., Gazinska, P., Owen, J., Abraham, J., Barrett, S., Barrett-Lee, P., et al. (2018). Carboplatin in BRCA1/2-mutated and triple-negative breast cancer BRCAness subgroups: the TNT Trial. *Nat Med* 24, 628-637. 10.1038/s41591-018-0009-7.
220. Hennes, E.R., Dow-Hillgartner, E.N., Bergsbaken, J.J., and Piccolo, J.K. (2020). PARP-inhibitor potpourri: A comparative review of class safety, efficacy, and cost. *J Oncol Pharm Pract* 26, 718-729. 10.1177/1078155219895066.
221. Ryan, C.J., Devakumar, L.P.S., Pettitt, S.J., and Lord, C.J. (2023). Complex synthetic lethality in cancer. *Nat Genet* 55, 2039-2048. 10.1038/s41588-023-01557-x.
222. Chan, E.M., Shibue, T., McFarland, J.M., Gaeta, B., Ghandi, M., Dumont, N., Gonzalez, A., McPartlan, J.S., Li, T., Zhang, Y., et al. (2019). WRN helicase is a synthetic lethal target in microsatellite unstable cancers. *Nature* 568, 551-556. 10.1038/s41586-019-1102-x.
223. van Wietmarschen, N., Sridharan, S., Nathan, W.J., Tubbs, A., Chan, E.M., Callen, E., Wu, W., Belinky, F., Tripathi, V., Wong, N., et al. (2020). Repeat expansions confer WRN dependence in microsatellite-unstable cancers. *Nature* 586, 292-298. 10.1038/s41586-020-2769-8.
224. Picco, G., Cattaneo, C.M., van Vliet, E.J., Crisafulli, G., Rospo, G., Consonni, S., Vieira, S.F., Rodríguez, I.S., Cancelliere, C., Banerjee, R., et al. (2021). Werner Helicase Is a Synthetic-Lethal Vulnerability in Mismatch Repair-Deficient Colorectal Cancer Refractory to Targeted Therapies, Chemotherapy, and Immunotherapy. *Cancer Discov* 11, 1923-1937. 10.1158/2159-8290.Cd-20-1508.
225. Abkevich, V., Timms, K.M., Hennessy, B.T., Potter, J., Carey, M.S., Meyer, L.A., Smith-McCune, K., Broaddus, R., Lu, K.H., Chen, J., et al. (2012). Patterns of genomic loss of heterozygosity predict homologous recombination repair defects in epithelial ovarian cancer. *Br J Cancer* 107, 1776-1782. 10.1038/bjc.2012.451.
226. Birkbak, N.J., Wang, Z.C., Kim, J.Y., Eklund, A.C., Li, Q., Tian, R., Bowman-Colin, C., Li, Y., Greene-Colozzi, A., Iglehart, J.D., et al. (2012). Telomeric allelic imbalance indicates defective DNA repair and sensitivity to DNA-damaging agents. *Cancer Discov* 2, 366-375. 10.1158/2159-8290.Cd-11-0206.
227. Popova, T., Manié, E., Rieunier, G., Caux-Moncoutier, V., Tirapo, C., Dubois, T., Delattre, O., Sigal-Zafrani, B., Bollet, M., Longy, M., et al. (2012). Ploidy and large-scale genomic instability consistently identify basal-like breast carcinomas with BRCA1/2 inactivation. *Cancer Res* 72, 5454-5462. 10.1158/0008-5472.Can-12-1470.
228. Davies, H., Glodzik, D., Morganella, S., Yates, L.R., Staaf, J., Zou, X., Ramakrishna, M., Martin, S., Boyault, S., Sieuwerts, A.M., et al. (2017). HRDetect is a predictor of BRCA1 and BRCA2 deficiency based on mutational signatures. *Nat Med* 23, 517-525. 10.1038/nm.4292.
229. Corti, G., Buzo, K., Berrino, E., Miotto, M., Aquilano, M.C., Lentini, M., Bellomo, S.E., Lorenzato, A., Bartolini, A., Mauri, G., et al. (2024). Prediction of homologous recombination deficiency identifies colorectal tumors sensitive to PARP inhibition. *NPJ Precis Oncol* 8, 231. 10.1038/s41698-024-00706-7.
230. Naipal, K.A., Verkaik, N.S., Ameziame, N., van Deurzen, C.H., Ter Brugge, P., Meijers, M., Sieuwerts, A.M., Martens, J.W., O'Connor, M.J., Vrieling, H., et al. (2014). Functional ex vivo assay to select homologous recombination-deficient breast tumors for PARP inhibitor treatment. *Clin Cancer Res* 20, 4816-4826. 10.1158/1078-0432.Ccr-14-0571.
231. Iqbal, M.A., Broeckel, U., Levy, B., Skinner, S., Sahajpal, N.S., Rodriguez, V., Stence, A., Awayda, K., Scharer, G., Skinner, C., et al. (2023). Multisite Assessment of Optical Genome Mapping for Analysis of Structural Variants in Constitutional Postnatal Cases. *J Mol Diagn* 25, 175-188. 10.1016/j.jmoldx.2022.12.005.

11. References

232. Tobalina, L., Armenia, J., Irving, E., O'Connor, M.J., and Forment, J.V. (2021). A meta-analysis of reversion mutations in BRCA genes identifies signatures of DNA end-joining repair mechanisms driving therapy resistance. *Ann Oncol* 32, 103-112. 10.1016/j.annonc.2020.10.470.
233. Bunting, S.F., Callén, E., Wong, N., Chen, H.T., Polato, F., Gunn, A., Bothmer, A., Feldhahn, N., Fernandez-Capetillo, O., Cao, L., et al. (2010). 53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks. *Cell* 141, 243-254. 10.1016/j.cell.2010.03.012.
234. Shen, J., Zhao, W., Ju, Z., Wang, L., Peng, Y., Labrie, M., Yap, T.A., Mills, G.B., and Peng, G. (2019). PARPi Triggers the STING-Dependent Immune Response and Enhances the Therapeutic Efficacy of Immune Checkpoint Blockade Independent of BRCAness. *Cancer Res* 79, 311-319. 10.1158/0008-5472.Can-18-1003.
235. Schumacher, T.N., and Schreiber, R.D. (2015). Neoantigens in cancer immunotherapy. *Science* 348, 69-74. 10.1126/science.aaa4971.
236. Pishesha, N., Harmand, T.J., and Ploegh, H.L. (2022). A guide to antigen processing and presentation. *Nat Rev Immunol* 22, 751-764. 10.1038/s41577-022-00707-2.
237. Le, D.T., Durham, J.N., Smith, K.N., Wang, H., Bartlett, B.R., Aulakh, L.K., Lu, S., Kemberling, H., Wilt, C., Luber, B.S., et al. (2017). Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* 357, 409-413. 10.1126/science.aan6733.
238. Burr, M.L., Sparbier, C.E., Chan, K.L., Chan, Y.C., Kersbergen, A., Lam, E.Y.N., Azidis-Yates, E., Vassiliadis, D., Bell, C.C., Gilan, O., et al. (2019). An Evolutionarily Conserved Function of Polycomb Silences the MHC Class I Antigen Presentation Pathway and Enables Immune Evasion in Cancer. *Cancer Cell* 36, 385-401.e388. 10.1016/j.ccell.2019.08.008.
239. Yamamoto, K., Venida, A., Yano, J., Biancur, D.E., Kakiuchi, M., Gupta, S., Sohn, A.S.W., Mukhopadhyay, S., Lin, E.Y., Parker, S.J., et al. (2020). Autophagy promotes immune evasion of pancreatic cancer by degrading MHC-I. *Nature* 581, 100-105. 10.1038/s41586-020-2229-5.
240. Cha, J.H., Chan, L.C., Li, C.W., Hsu, J.L., and Hung, M.C. (2019). Mechanisms Controlling PD-L1 Expression in Cancer. *Mol Cell* 76, 359-370. 10.1016/j.molcel.2019.09.030.
241. Dolina, J.S., Van Braeckel-Budimir, N., Thomas, G.D., and Salek-Ardakani, S. (2021). CD8(+) T Cell Exhaustion in Cancer. *Front Immunol* 12, 715234. 10.3389/fimmu.2021.715234.
242. Le, D.T., Uram, J.N., Wang, H., Bartlett, B.R., Kemberling, H., Eyring, A.D., Skora, A.D., Luber, B.S., Azad, N.S., Laheru, D., et al. (2015). PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* 372, 2509-2520. 10.1056/NEJMoa1500596.
243. Overman, M.J., Lonardi, S., Wong, K.Y.M., Lenz, H.J., Gelsomino, F., Aglietta, M., Morse, M.A., Van Cutsem, E., McDermott, R., Hill, A., et al. (2018). Durable Clinical Benefit With Nivolumab Plus Ipilimumab in DNA Mismatch Repair-Deficient/Microsatellite Instability-High Metastatic Colorectal Cancer. *J Clin Oncol* 36, 773-779. 10.1200/jco.2017.76.9901.
244. Marabelle, A., Fakih, M., Lopez, J., Shah, M., Shapira-Frommer, R., Nakagawa, K., Chung, H.C., Kindler, H.L., Lopez-Martin, J.A., Miller, W.H., Jr., et al. (2020). Association of tumour mutational burden with outcomes in patients with advanced solid tumours treated with pembrolizumab: prospective biomarker analysis of the multicohort, open-label, phase 2 KEYNOTE-158 study. *Lancet Oncol* 21, 1353-1365. 10.1016/s1470-2045(20)30445-9.
245. Marcus, L., Fashoyin-Aje, L.A., Donoghue, M., Yuan, M., Rodriguez, L., Gallagher, P.S., Philip, R., Ghosh, S., Theoret, M.R., Beaver, J.A., et al. (2021). FDA Approval Summary: Pembrolizumab for the Treatment of Tumor Mutational Burden-High Solid Tumors. *Clin Cancer Res* 27, 4685-4689. 10.1158/1078-0432.Ccr-21-0327.
246. Schmitt, A., Knittel, G., Welcker, D., Yang, T.P., George, J., Nowak, M., Leiser, U., Büttner, R., Perner, S., Peifer, M., and Reinhardt, H.C. (2017). ATM Deficiency Is Associated with Sensitivity to PARP1- and ATR Inhibitors in Lung Adenocarcinoma. *Cancer Res* 77, 3040-3056. 10.1158/0008-5472.Can-16-3398.
247. Borchmann, S., Selenz, C., Lohmann, M., Ludwig, H., Gassa, A., Brägelmann, J., Lohneis, P., Meder, L., Mattlener, J., Breid, S., et al. (2022). Tripartite antigen-agnostic combination immunotherapy cures established poorly immunogenic tumors. *J Immunother Cancer* 10. 10.1136/jitc-2022-004781.
248. DuPage, M., Dooley, A.L., and Jacks, T. (2009). Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nat Protoc* 4, 1064-1072. 10.1038/nprot.2009.95.
249. Adamopoulos, C., Cave, D.D., and Papavassiliou, A.G. (2024). Inhibition of the RAF/MEK/ERK Signaling Cascade in Pancreatic Cancer: Recent Advances and Future Perspectives. *Int J Mol Sci* 25. 10.3390/ijms25031631.
250. Chen, Z., Chen, M., Fu, Y., and Zhang, J. (2023). The KRAS signaling pathway's impact on the characteristics of pancreatic cancer cells. *Pathol Res Pract* 248, 154603. 10.1016/j.prp.2023.154603.
251. Karnoub, A.E., and Weinberg, R.A. (2008). Ras oncogenes: split personalities. *Nat Rev Mol Cell Biol* 9, 517-531. 10.1038/nrm2438.
252. Ding, L., Getz, G., Wheeler, D.A., Mardis, E.R., McLellan, M.D., Cibulskis, K., Sougnez, C., Greulich, H., Muzny, D.M., Morgan, M.B., et al. (2008). Somatic mutations affect key pathways in lung adenocarcinoma. *Nature* 455, 1069-1075. 10.1038/nature07423.
253. Arbour, K.C., Jordan, E., Kim, H.R., Dienstag, J., Yu, H.A., Sanchez-Vega, F., Lito, P., Berger, M., Solit, D.B., Hellmann, M., et al. (2018). Effects of Co-occurring Genomic Alterations on Outcomes in Patients with KRAS-Mutant Non-Small Cell Lung Cancer. *Clin Cancer Res* 24, 334-340. 10.1158/1078-0432.Ccr-17-1841.
254. Scheffler, M., Ihle, M.A., Hein, R., Merkelbach-Bruse, S., Scheel, A.H., Siemanowski, J., Brägelmann, J., Kron, A., Abedpour, N., Ueckerth, F., et al. (2019). K-ras Mutation Subtypes in NSCLC and Associated

11. References

- Co-occurring Mutations in Other Oncogenic Pathways. *J Thorac Oncol* 14, 606-616. 10.1016/j.jtho.2018.12.013.
255. Skoulidis, F., Byers, L.A., Diao, L., Papadimitrakopoulou, V.A., Tong, P., Izzo, J., Behrens, C., Kadara, H., Parra, E.R., Canales, J.R., et al. (2015). Co-occurring genomic alterations define major subsets of KRAS-mutant lung adenocarcinoma with distinct biology, immune profiles, and therapeutic vulnerabilities. *Cancer Discov* 5, 860-877. 10.1158/2159-8290.CD-14-1236.
256. Zhu, M., Kim, J., Deng, Q., Ricciuti, B., Alessi, J.V., Eglenen-Polat, B., Bender, M.E., Huang, H.C., Kowash, R.R., Cuevas, I., et al. (2023). Loss of p53 and mutational heterogeneity drives immune resistance in an autochthonous mouse lung cancer model with high tumor mutational burden. *Cancer Cell* 41, 1731-1748.e1738. 10.1016/j.ccell.2023.09.006.
257. Richmond, J. (2002). Refinement, reduction, and replacement of animal use for regulatory testing: future improvements and implementation within the regulatory framework. *Ilar j* 43 Suppl, S63-68. 10.1093/ilar.43.suppl_1.s63.
258. Averdunk, L., Huetzen, M.A., Moreno-Andrés, D., Kalb, R., McKee, S., Hsieh, T.C., Seibt, A., Schouwink, M., Lalani, S., Fageih, E.A., et al. (2023). Biallelic variants in CRIPT cause a Rothmund-Thomson-like syndrome with increased cellular senescence. *Genet Med* 25, 100836. 10.1016/j.gim.2023.100836.
259. Leduc, M.S., Niu, Z., Bi, W., Zhu, W., Miloslavskaya, I., Chiang, T., Streff, H., Seavitt, J.R., Murray, S.A., Eng, C., et al. (2016). CRIPT exonic deletion and a novel missense mutation in a female with short stature, dysmorphic features, microcephaly, and pigmentary abnormalities. *Am J Med Genet A* 170, 2206-2211. 10.1002/ajmg.a.37780.
260. Akalin, A., Şimşek-Kiper, P., Taşkıran, E.Z., Karaosmanoğlu, B., Utine, G.E., and Boduroğlu, K. (2023). A novel biallelic CRIPT variant in a patient with short stature, microcephaly, and distinctive facial features. *Am J Med Genet A* 191, 1119-1127. 10.1002/ajmg.a.63120.
261. Lu, H., Shamanna, R.A., Keijzers, G., Anand, R., Rasmussen, L.J., Cejka, P., Croteau, D.L., and Bohr, V.A. (2016). RECQL4 Promotes DNA End Resection in Repair of DNA Double-Strand Breaks. *Cell Rep* 16, 161-173. 10.1016/j.celrep.2016.05.079.
262. Passafaro, M., Sala, C., Niethammer, M., and Sheng, M. (1999). Microtubule binding by CRIPT and its potential role in the synaptic clustering of PSD-95. *Nat Neurosci* 2, 1063-1069. 10.1038/15990.
263. Omelchenko, A., Menon, H., Donofrio, S.G., Kumar, G., Chapman, H.M., Roshal, J., Martinez-Montes, E.R., Wang, T.L., Spaller, M.R., and Firestein, B.L. (2020). Interaction Between CRIPT and PSD-95 Is Required for Proper Dendritic Arborization in Hippocampal Neurons. *Mol Neurobiol* 57, 2479-2493. 10.1007/s12035-020-01895-5.
264. Niethammer, M., Valtschanoff, J.G., Kapoor, T.M., Allison, D.W., Weinberg, R.J., Craig, A.M., and Sheng, M. (1998). CRIPT, a novel postsynaptic protein that binds to the third PDZ domain of PSD-95/SAP90. *Neuron* 20, 693-707. 10.1016/s0896-6273(00)81009-0.
265. Jin, W., Liu, H., Zhang, Y., Otta, S.K., Plon, S.E., and Wang, L.L. (2008). Sensitivity of RECQL4-deficient fibroblasts from Rothmund-Thomson syndrome patients to genotoxic agents. *Hum Genet* 123, 643-653. 10.1007/s00439-008-0518-4.
266. Cabral, R.E., Queille, S., Bodemer, C., de Prost, Y., Neto, J.B., Sarasin, A., and Daya-Grosjean, L. (2008). Identification of new RECQL4 mutations in Caucasian Rothmund-Thomson patients and analysis of sensitivity to a wide range of genotoxic agents. *Mutat Res* 643, 41-47. 10.1016/j.mrfmmm.2008.06.002.
267. Dańczak-Pazdrowska, A., Gornowicz-Porowska, J., Polańska, A., Krajka-Kuźniak, V., Stawny, M., Gostyńska, A., Rubiś, B., Nourredine, S., Ashiqueali, S., Schneider, A., et al. (2023). Cellular senescence in skin-related research: Targeted signaling pathways and naturally occurring therapeutic agents. *Aging Cell* 22, e13845. 10.1111/accel.13845.
268. Young, A.R., and Narita, M. (2009). SASP reflects senescence. *EMBO Rep* 10, 228-230. 10.1038/embor.2009.22.
269. Campisi, J. (2013). Aging, cellular senescence, and cancer. *Annu Rev Physiol* 75, 685-705. 10.1146/annurev-physiol-030212-183653.
270. Campisi, J. (2005). Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell* 120, 513-522. 10.1016/j.cell.2005.02.003.
271. Ho, S.K.L., Tong, G.P.Y., Leung, L.T., Cheng, S.S.W., Fu, E.C.H., Lo, I.F.M., Liu, A.P.Y., and Luk, H.M. (2025). RECQL4-related Rothmund-Thomson syndrome: A case series and literature review. *Cancer Genet* 292-293, 131-136. 10.1016/j.cancergen.2025.02.013.
272. Grossman, L.D., Baldino, S., Zelle, K., Balis, F., Bagatell, R., Kalish, J.M., and MacFarland, S.P. (2024). Atypical presentations of RECQL4-related syndromes. *Pediatr Blood Cancer* 71, e31315. 10.1002/pbc.31315.
273. Martins, D.J., Di Lazzaro Filho, R., Bertola, D.R., and Hoch, N.C. (2023). Rothmund-Thomson syndrome, a disorder far from solved. *Front Aging* 4, 1296409. 10.3389/fragi.2023.1296409.
274. Di Lazzaro Filho, R., Yamamoto, G.L., Silva, T.J., Rocha, L.A., Linnenkamp, B.D.W., Castro, M.A.A., Bartholdi, D., Schaller, A., Leeb, T., Kelmann, S., et al. (2023). Biallelic variants in DNA2 cause poikiloderma with congenital cataracts and severe growth failure reminiscent of Rothmund-Thomson syndrome. *J Med Genet* 60, 1127-1132. 10.1136/jmg-2022-109119.
275. Hudson, J.J.R., Appanah, R., Jones, D., Davidson, K., Budden, A.M., Vaitiankova, A., Chan, K.L., Caldecott, K.W., Carr, A.M., and Rass, U. (2025). DNA2 enables growth by restricting recombination-restarted replication. *Nature* 646, 992-1000. 10.1038/s41586-025-09470-5.

11. References

276. Lu, H., Fang, E.F., Sykora, P., Kulikowicz, T., Zhang, Y., Becker, K.G., Croteau, D.L., and Bohr, V.A. (2014). Senescence induced by RECQL4 dysfunction contributes to Rothmund-Thomson syndrome features in mice. *Cell Death Dis* 5, e1226. 10.1038/cddis.2014.168.
277. Baker, D.J., Wijshake, T., Tchkonja, T., LeBrasseur, N.K., Childs, B.G., van de Sluis, B., Kirkland, J.L., and van Deursen, J.M. (2011). Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* 479, 232-236. 10.1038/nature10600.
278. Yousefzadeh, M.J., Zhu, Y., McGowan, S.J., Angelini, L., Fuhrmann-Stroissnigg, H., Xu, M., Ling, Y.Y., Melos, K.I., Pirtskhalava, T., Inman, C.L., et al. (2018). Fisetin is a senotherapeutic that extends health and lifespan. *EBioMedicine* 36, 18-28. 10.1016/j.ebiom.2018.09.015.
279. Wagener, R., Brandes, D., Jung, M., Huetzen, M.A., Bergmann, A.K., Panier, S., Picard, D., Fischer, U., Jachimowicz, R.D., Borkhardt, A., and Brozou, T. (2024). Optical genome mapping identifies structural variants in potentially new cancer predisposition candidate genes in pediatric cancer patients. *Int J Cancer* 154, 607-614. 10.1002/ijc.34721.
280. Gillani, R., Collins, R.L., Crowdis, J., Garza, A., Jones, J.K., Walker, M., Sanchis-Juan, A., Whelan, C.W., Pierce-Hoffman, E., Talkowski, M.E., et al. (2025). Rare germline structural variants increase risk for pediatric solid tumors. *Science* 387, eadq0071. 10.1126/science.adq0071.
281. Tattini, L., D'Aurizio, R., and Magi, A. (2015). Detection of Genomic Structural Variants from Next-Generation Sequencing Data. *Front Bioeng Biotechnol* 3, 92. 10.3389/fbioe.2015.00092.
282. Demidov, G., Laurie, S., Torella, A., Piluso, G., Scala, M., Morleo, M., Nigro, V., Graessner, H., Banka, S., Lohmann, K., and Ossowski, S. (2024). Structural variant calling and clinical interpretation in 6224 unsolved rare disease exomes. *Eur J Hum Genet* 32, 998-1004. 10.1038/s41431-024-01637-4.
283. Sund, K.L., Liu, J., Lee, J., Garbe, J., Abdelhamed, Z., Maag, C., Hallinan, B., Wu, S.W., Sperry, E., Deshpande, A., et al. (2024). Long-read sequencing and optical genome mapping identify causative gene disruptions in noncoding sequence in two patients with neurologic disease and known chromosome abnormalities. *Am J Med Genet A* 194, e63818. 10.1002/ajmg.a.63818.
284. Kloosterman, W.P., Francioli, L.C., Hormozdiari, F., Marshall, T., Hehir-Kwa, J.Y., Abdellaoui, A., Lameijer, E.W., Moed, M.H., Koval, V., Renkens, I., et al. (2015). Characteristics of de novo structural changes in the human genome. *Genome Res* 25, 792-801. 10.1101/gr.185041.114.
285. Tang, Z., Wang, W., Toruner, G.A., Hu, S., Fang, H., Xu, J., You, M.J., Medeiros, L.J., Khoury, J.D., and Tang, G. (2024). Optical Genome Mapping for Detection of BCR::ABL1-Another Tool in Our Toolbox. *Genes (Basel)* 15. 10.3390/genes15111357.
286. Budurlean, L., Tukaramrao, D.B., Zhang, L., Dovat, S., and Broach, J. (2024). Integrating Optical Genome Mapping and Whole Genome Sequencing in Somatic Structural Variant Detection. *J Pers Med* 14. 10.3390/jpm14030291.
287. Zhang, J., Walsh, M.F., Wu, G., Edmonson, M.N., Gruber, T.A., Easton, J., Hedges, D., Ma, X., Zhou, X., Yergeau, D.A., et al. (2015). Germline Mutations in Predisposition Genes in Pediatric Cancer. *N Engl J Med* 373, 2336-2346. 10.1056/NEJMoa1508054.
288. Chen, S., Francioli, L.C., Goodrich, J.K., Collins, R.L., Kanai, M., Wang, Q., Alföldi, J., Watts, N.A., Vittal, C., Gauthier, L.D., et al. (2024). A genomic mutational constraint map using variation in 76,156 human genomes. *Nature* 625, 92-100. 10.1038/s41586-023-06045-0.
289. Landrum, M.J., Lee, J.M., Riley, G.R., Jang, W., Rubinstein, W.S., Church, D.M., and Maglott, D.R. (2014). ClinVar: public archive of relationships among sequence variation and human phenotype. *Nucleic Acids Res* 42, D980-985. 10.1093/nar/gkt1113.
290. Collins, R.L., Brand, H., Karczewski, K.J., Zhao, X., Alföldi, J., Francioli, L.C., Khera, A.V., Lowther, C., Gauthier, L.D., Wang, H., et al. (2020). A structural variation reference for medical and population genetics. *Nature* 581, 444-451. 10.1038/s41586-020-2287-8.
291. Hu, L., Zhu, Y.T., Qi, C., and Zhu, Y.J. (2009). Identification of Smdy4 as a potential tumor suppressor gene involved in breast cancer development. *Cancer Res* 69, 4067-4072. 10.1158/0008-5472.CAN-08-4097.
292. Sakaguchi, K., Ishibashi, T., Uchiyama, Y., and Iwabata, K. (2009). The multi-replication protein A (RPA) system--a new perspective. *Febs j* 276, 943-963. 10.1111/j.1742-4658.2008.06841.x.
293. Sharma, R., Sahoo, S.S., Honda, M., Granger, S.L., Goodings, C., Sanchez, L., Künstner, A., Busch, H., Beier, F., Pruett-Miller, S.M., et al. (2022). Gain-of-function mutations in RPA1 cause a syndrome with short telomeres and somatic genetic rescue. *Blood* 139, 1039-1051. 10.1182/blood.2021011980.
294. Outwin, E., Carpenter, G., Bi, W., Withers, M.A., Lupski, J.R., and O'Driscoll, M. (2011). Increased RPA1 gene dosage affects genomic stability potentially contributing to 17p13.3 duplication syndrome. *PLoS Genet* 7, e1002247. 10.1371/journal.pgen.1002247.
295. Bueno, C., Montes, R., Catalina, P., Rodríguez, R., and Menendez, P. (2011). Insights into the cellular origin and etiology of the infant pro-B acute lymphoblastic leukemia with MLL-AF4 rearrangement. *Leukemia* 25, 400-410. 10.1038/leu.2010.284.
296. (Under review) Huetzen, M.A., Leidecker, O., Hopff, A.-S., Víšková Gajarská, P., et al. (2026). UBQLN1-Mediated Repression of Homologous Recombination is an Actionable Target for Cancer Therapy, Manuscript under review at *Molecular Cell*. SSRN preprint: <http://dx.doi.org/10.2139/ssrn.6502243>
297. Chu, W.K., Payne, M.J., Beli, P., Hanada, K., Choudhary, C., and Hickson, I.D. (2015). FBH1 influences DNA replication fork stability and homologous recombination through ubiquitylation of RAD51. *Nat Commun* 6, 5931. 10.1038/ncomms6931.
298. Akimov, V., Barrio-Hernandez, I., Hansen, S.V.F., Hallenborg, P., Pedersen, A.K., Bekker-Jensen, D.B., Puglia, M., Christensen, S.D.K., Vanselow, J.T., Nielsen, M.M., et al. (2018). UbiSite approach for

11. References

- comprehensive mapping of lysine and N-terminal ubiquitination sites. *Nat Struct Mol Biol* 25, 631-640. 10.1038/s41594-018-0084-y.
299. Udeshi, N.D., Svinkina, T., Mertins, P., Kuhn, E., Mani, D.R., Qiao, J.W., and Carr, S.A. (2013). Refined preparation and use of anti-diglycine remnant (K- ϵ -GG) antibody enables routine quantification of 10,000s of ubiquitination sites in single proteomics experiments. *Mol Cell Proteomics* 12, 825-831. 10.1074/mcp.O112.027094.
300. Wagner, S.A., Beli, P., Weinert, B.T., Nielsen, M.L., Cox, J., Mann, M., and Choudhary, C. (2011). A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles. *Mol Cell Proteomics* 10, M111.013284. 10.1074/mcp.M111.013284.
301. Inano, S., Sato, K., Katsuki, Y., Kobayashi, W., Tanaka, H., Nakajima, K., Nakada, S., Miyoshi, H., Knies, K., Takaori-Kondo, A., et al. (2017). RFWD3-Mediated Ubiquitination Promotes Timely Removal of Both RPA and RAD51 from DNA Damage Sites to Facilitate Homologous Recombination. *Mol Cell* 66, 622-634.e628. 10.1016/j.molcel.2017.04.022.
302. Marzio, A., Puccini, J., Kwon, Y., Maverakis, N.K., Arbini, A., Sung, P., Bar-Sagi, D., and Pagano, M. (2019). The F-Box Domain-Dependent Activity of EMI1 Regulates PARPi Sensitivity in Triple-Negative Breast Cancers. *Mol Cell* 73, 224-237.e226. 10.1016/j.molcel.2018.11.003.
303. Kang, J.W., Zhan, Z., Ji, G., Sang, Y., Zhou, D., Li, Y., Feng, H., and Cheng, T. (2021). PUMA facilitates EMI1-promoted cytoplasmic Rad51 ubiquitination and inhibits DNA repair in stem and progenitor cells. *Signal Transduct Target Ther* 6, 129. 10.1038/s41392-021-00510-w.
304. Liu, G., Huang, K., Liu, S., Sheng, Z., and Zhang, P. (2025). UHRF1 Controls the Timing of RAD51 Removal During DNA Damage Repair Through Suppressing RFWD3. *Adv Sci (Weinh)* 12, e09901. 10.1002/advs.202509901.
305. Zhao, L., Si, C.S., Yu, Y., Lu, J.W., and Zhuang, Y. (2019). Depletion of DNA damage binding protein 2 sensitizes triple-negative breast cancer cells to poly ADP-ribose polymerase inhibition by destabilizing Rad51. *Cancer Sci* 110, 3543-3552. 10.1111/cas.14201.
306. Ye, Z., Xu, S., Shi, Y., Cheng, X., Zhang, Y., Roy, S., Namjoshi, S., Longo, M.A., Link, T.M., Schlacher, K., et al. (2024). GRB2 stabilizes RAD51 at reversed replication forks suppressing genomic instability and innate immunity against cancer. *Nat Commun* 15, 2132. 10.1038/s41467-024-46283-y.
307. Tischler, J.D., Tsuchida, H., Bosire, R., Oda, T.T., Park, A., and Adeyemi, R.O. (2024). FLIP(C1orf112)-FIGNL1 complex regulates RAD51 chromatin association to promote viability after replication stress. *Nat Commun* 15, 866. 10.1038/s41467-024-45139-9.
308. Matsuzaki, K., Shinohara, A., and Shinohara, M. (2024). Human AAA+ ATPase FIGNL1 suppresses RAD51-mediated ultra-fine bridge formation. *Nucleic Acids Res* 52, 5774-5791. 10.1093/nar/gkac263.
309. Fry, M.Y., Saladi, S.M., and Clemons, W.M., Jr. (2021). The STI1-domain is a flexible alpha-helical fold with a hydrophobic groove. *Protein Sci* 30, 882-898. 10.1002/pro.4049.
310. Kurlawala, Z. (2017). UBQLN1: a multi-domain protein with multiple functions. Doctoral Dissertation (University of Louisville).
311. Beverly, L.J., Lockwood, W.W., Shah, P.P., Erdjument-Bromage, H., and Varmus, H. (2012). Ubiquitination, localization, and stability of an anti-apoptotic BCL2-like protein, BCL2L10/BCLb, are regulated by Ubiquilin1. *Proc Natl Acad Sci U S A* 109, E119-126. 10.1073/pnas.1119167109.
312. Kurlawala, Z., Dunaway, R., Shah, P.P., Gosney, J.A., Siskind, L.J., Ceresa, B.P., and Beverly, L.J. (2017). Regulation of insulin-like growth factor receptors by Ubiquilin1. *Biochem J* 474, 4105-4118. 10.1042/bcj20170620.
313. Viswanathan, J., Haapasalo, A., Böttcher, C., Miettinen, R., Kurkinen, K.M., Lu, A., Thomas, A., Maynard, C.J., Romano, D., Hyman, B.T., et al. (2011). Alzheimer's disease-associated ubiquilin-1 regulates presenilin-1 accumulation and aggregate formation. *Traffic* 12, 330-348. 10.1111/j.1600-0854.2010.01149.x.
314. Kürten, T., Ihbe, N., Ueberbach, T., Distler, U., Sielaff, M., Tenzer, S., and Mittmann, T. (2022). GABA(A) Receptor-Stabilizing Protein Ubqln1 Affects Hyperexcitability and Epileptogenesis after Traumatic Brain Injury and in a Model of In Vitro Epilepsy in Mice. *Int J Mol Sci* 23. 10.3390/ijms23073902.
315. Zhang, Y., Li, Z., Gu, J., Zhang, Y., Wang, W., Shen, H., Chen, G., and Wang, X. (2015). Plic-1, a new target in repressing epileptic seizure by regulation of GABAAR function in patients and a rat model of epilepsy. *Clin Sci (Lond)* 129, 1207-1223. 10.1042/cs20150202.
316. Ji, L., Ruan, Y., Tong, M., Chen, T., Cai, J., Ye, Z., Cai, X., and Xu, J. (2025). circRNA-SORE/UBQLN1/GPX4 Mediates the Acquisition of Sorafenib Resistance in Hepatocellular Carcinoma Through Inhibition of Ferroptosis. *MedComm* (2020) 6, e70488. 10.1002/mco2.70488.
317. Lin, B.C., Higgins, N.R., Phung, T.H., and Monteiro, M.J. (2022). UBQLN proteins in health and disease with a focus on UBQLN2 in ALS/FTD. *Febs j* 289, 6132-6153. 10.1111/febs.16129.
318. UniProt, C. (2023). UniProt: the Universal Protein Knowledgebase in 2023. *Nucleic Acids Res* 51, D523-D531. 10.1093/nar/gkac1052.
319. Tomimatsu, N., Mukherjee, B., Deland, K., Kurimasa, A., Bolderson, E., Khanna, K.K., and Burma, S. (2012). Exo1 plays a major role in DNA end resection in humans and influences double-strand break repair and damage signaling decisions. *DNA Repair (Amst)* 11, 441-448. 10.1016/j.dnarep.2012.01.006.
320. Daley, J.M., Jimenez-Sainz, J., Wang, W., Miller, A.S., Xue, X., Nguyen, K.A., Jensen, R.B., and Sung, P. (2017). Enhancement of BLM-DNA2-Mediated Long-Range DNA End Resection by CtIP. *Cell Rep* 21, 324-332. 10.1016/j.celrep.2017.09.048.
321. Kijas, A.W., Lim, Y.C., Bolderson, E., Cerosaletti, K., Gatei, M., Jakob, B., Tobias, F., Taucher-Scholz, G., Gueven, N., Oakley, G., et al. (2015). ATM-dependent phosphorylation of MRE11 controls extent of

11. References

- resection during homology directed repair by signalling through Exonuclease 1. *Nucleic Acids Res* 43, 8352-8367. 10.1093/nar/gkv754.
322. Deschênes-Simard, X., Malleshaiah, M., and Ferbeyre, G. (2023). Extracellular Signal-Regulated Kinases: One Pathway, Multiple Fates. *Cancers (Basel)* 16. 10.3390/cancers16010095.
323. Gerson, J.E., Linton, H., Xing, J., Sutter, A.B., Kakos, F.S., Ryou, J., Liggans, N., Sharkey, L.M., Safren, N., Paulson, H.L., and Ivanova, M.I. (2021). Shared and divergent phase separation and aggregation properties of brain-expressed ubiquilins. *Sci Rep* 11, 287. 10.1038/s41598-020-78775-4.
324. (Preprint) Scheutzwow, A.N., Thanthirige, S., Siffer, G., Sorkin, A.D., and Wohlever, M.L. (2025). E3 ligase recruitment by UBQLN2 protects substrates from proteasomal degradation. *bioRxiv*. 10.1101/2024.07.04.602059.
325. Ng, J.M., Vermeulen, W., van der Horst, G.T., Bergink, S., Sugawara, K., Vrieling, H., and Hoeijmakers, J.H. (2003). A novel regulation mechanism of DNA repair by damage-induced and RAD23-dependent stabilization of xeroderma pigmentosum group C protein. *Genes Dev* 17, 1630-1645. 10.1101/gad.260003.
326. Grønbaek-Thygesen, M., Kampmeyer, C., Hofmann, K., and Hartmann-Petersen, R. (2023). The moonlighting of RAD23 in DNA repair and protein degradation. *Biochim Biophys Acta Gene Regul Mech* 1866, 194925. 10.1016/j.bbagr.2023.194925.
327. Zhou, H., Xie, C., Xie, Y., He, Y., Chen, Y., Zhang, C., Zhang, Y., Zhao, Y., and Liu, H. (2023). UBQLN1 deficiency mediates telomere shortening and IPF through interacting with RPA1. *PLoS Genet* 19, e1010856. 10.1371/journal.pgen.1010856.
328. Bhat, K.P., and Cortez, D. (2018). RPA and RAD51: fork reversal, fork protection, and genome stability. *Nat Struct Mol Biol* 25, 446-453. 10.1038/s41594-018-0075-z.
329. Zhao, S., Li, J., Duan, S., Liu, C., Wang, H., Lu, J., Zhao, N., Sheng, X., Wu, Y., Li, Y., et al. (2024). UBQLN1 links proteostasis and mitochondria function to telomere maintenance in human embryonic stem cells. *Stem Cell Res Ther* 15, 180. 10.1186/s13287-024-03789-y.
330. Burger, K., Ketley, R.F., and Gullerova, M. (2019). Beyond the Trinity of ATM, ATR, and DNA-PK: Multiple Kinases Shape the DNA Damage Response in Concert With RNA Metabolism. *Front Mol Biosci* 6, 61. 10.3389/fmolb.2019.00061.
331. Panier, S., and Boulton, S.J. (2014). Double-strand break repair: 53BP1 comes into focus. *Nat Rev Mol Cell Biol* 15, 7-18. 10.1038/nrm3719.
332. Wang, Y., Lu, J., Zhao, X., Feng, Y., Lv, S., Mu, Y., Wang, D., Fu, H., Chen, Y., and Li, Y. (2015). Prognostic significance of Ubiquilin1 expression in invasive breast cancer. *Cancer Biomark* 15, 635-643. 10.3233/cbm-150503.
333. Bao, J., Jiang, X., Zhu, X., Dai, G., Dou, R., Liu, X., Sheng, H., Liang, Z., and Yu, H. (2018). Clinical significance of ubiquilin 1 in gastric cancer. *Medicine (Baltimore)* 97, e9701. 10.1097/md.00000000000009701.
334. Ni, R., Jiang, J., Zhao, M., Huang, S., and Huang, C. (2023). Knockdown of UBQLN1 Functions as a Strategy to Inhibit CRC Progression through the ERK-c-Myc Pathway. *Cancers (Basel)* 15. 10.3390/cancers15123088.
335. Xu, J., Ji, L., Ruan, Y., Wan, Z., Lin, Z., Xia, S., Tao, L., Zheng, J., Cai, L., Wang, Y., et al. (2021). UBQLN1 mediates sorafenib resistance through regulating mitochondrial biogenesis and ROS homeostasis by targeting PGC1 β in hepatocellular carcinoma. *Signal Transduct Target Ther* 6, 190. 10.1038/s41392-021-00594-4.
336. Feng, X., Cao, A., Qin, T., Zhang, Q., Fan, S., Wang, B., Song, B., Yu, X., and Li, L. (2021). Abnormally elevated ubiquilin-1 expression in breast cancer regulates metastasis and stemness via AKT signaling. *Oncol Rep* 46. 10.3892/or.2021.8187.
337. Lim, P.J., Danner, R., Liang, J., Doong, H., Harman, C., Srinivasan, D., Rothenberg, C., Wang, H., Ye, Y., Fang, S., and Monteiro, M.J. (2009). Ubiquilin and p97/VCP bind erasin, forming a complex involved in ERAD. *J Cell Biol* 187, 201-217. 10.1083/jcb.200903024.
338. Liu, D., Huo, X.Y., Zhang, X., and Zhang, Z.R. (2025). The E3 ubiquitin ligase RNF126 facilitates quality control of unimported mitochondrial membrane proteins. *J Biol Chem* 301, 108403. 10.1016/j.jbc.2025.108403.
339. Safren, N., El Ayadi, A., Chang, L., Terrillion, C.E., Gould, T.D., Boehning, D.F., and Monteiro, M.J. (2014). Ubiquilin-1 overexpression increases the lifespan and delays accumulation of Huntingtin aggregates in the R6/2 mouse model of Huntington's disease. *PLoS One* 9, e87513. 10.1371/journal.pone.0087513.
340. Shah, P.P., Lockwood, W.W., Saurabh, K., Kurlawala, Z., Shannon, S.P., Waigel, S., Zacharias, W., and Beverly, L.J. (2015). Ubiquilin1 represses migration and epithelial-to-mesenchymal transition of human non-small cell lung cancer cells. *Oncogene* 34, 1709-1717. 10.1038/nc.2014.97.
341. Shah, P.P., and Beverly, L.J. (2023). UBQLN Family Members Regulate MYC in Lung Adenocarcinoma Cells. *Cancers (Basel)* 15. 10.3390/cancers15133389.
342. Herter-Sprie, G.S., Korideck, H., Christensen, C.L., Herter, J.M., Rhee, K., Berbeco, R.I., Bennett, D.G., Akbay, E.A., Kozono, D., Mak, R.H., et al. (2014). Image-guided radiotherapy platform using single nodule conditional lung cancer mouse models. *Nat Commun* 5, 5870. 10.1038/ncomms6870.
343. Le Page, C., Amuzu, S., Rahimi, K., Gottlieb, W., Ragoussis, J., and Tonin, P.N. (2021). Lessons learned from understanding chemotherapy resistance in epithelial tubo-ovarian carcinoma from BRCA1 and BRCA2 mutation carriers. *Semin Cancer Biol* 77, 110-126. 10.1016/j.semcancer.2020.08.005.
344. Larminat, F., Germanier, M., Papouli, E., and Defais, M. (2002). Deficiency in BRCA2 leads to increase in non-conservative homologous recombination. *Oncogene* 21, 5188-5192. 10.1038/sj.onc.1205659.

11. References

345. Harrigan, J.A., Belotserkovskaya, R., Coates, J., Dimitrova, D.S., Polo, S.E., Bradshaw, C.R., Fraser, P., and Jackson, S.P. (2011). Replication stress induces 53BP1-containing OPT domains in G1 cells. *J Cell Biol* 193, 97-108. 10.1083/jcb.201011083.
346. Patterson-Fortin, J., and D'Andrea, A.D. (2023). Targeting Polymerase Theta (POL θ) for Cancer Therapy. *Cancer Treat Res* 186, 285-298. 10.1007/978-3-031-30065-3_15.
347. Sullivan-Reed, K., Bolton-Gillespie, E., Dasgupta, Y., Langer, S., Siciliano, M., Nieborowska-Skorska, M., Hanamshet, K., Belyaeva, E.A., Bernhardt, A.J., Lee, J., et al. (2018). Simultaneous Targeting of PARP1 and RAD52 Triggers Dual Synthetic Lethality in BRCA-Deficient Tumor Cells. *Cell Rep* 23, 3127-3136. 10.1016/j.celrep.2018.05.034.
348. Yadav, S., Singh, N., Shah, P.P., Rowbotham, D.A., Malik, D., Srivastav, A., Shankar, J., Lam, W.L., Lockwood, W.W., and Beverly, L.J. (2017). MIR155 Regulation of Ubiquitin1 and Ubiquitin2: Implications in Cellular Protection and Tumorigenesis. *Neoplasia* 19, 321-332. 10.1016/j.neo.2017.02.001.
349. Chen, G., Wang, X., Yu, J., Varambally, S., Yu, J., Thomas, D.G., Lin, M.Y., Vishnu, P., Wang, Z., Wang, R., et al. (2007). Autoantibody profiles reveal ubiquitin 1 as a humoral immune response target in lung adenocarcinoma. *Cancer Res* 67, 3461-3467. 10.1158/0008-5472.Can-06-4475.
350. Wang, Y., Ouyang, S., Liu, M., Si, Q., Zhang, X., Zhang, X., Li, J., Wang, P., Ye, H., Shi, J., et al. (2024). Humoral immune response to tumor-associated antigen Ubiquitin 1 (UBQLN1) and its tumor-promoting potential in lung cancer. *BMC Cancer* 24, 283. 10.1186/s12885-024-12019-w.
351. Wang, X., Allen, S., Blake, J.F., Bowcut, V., Briere, D.M., Calinisan, A., Dahlke, J.R., Fell, J.B., Fischer, J.P., Gunn, R.J., et al. (2022). Identification of MRTX1133, a Noncovalent, Potent, and Selective KRAS(G12D) Inhibitor. *J Med Chem* 65, 3123-3133. 10.1021/acs.jmedchem.1c01688.
352. Hallin, J., Bowcut, V., Calinisan, A., Briere, D.M., Hargis, L., Engstrom, L.D., Laguer, J., Medwid, J., Vanderpool, D., Lifset, E., et al. (2022). Anti-tumor efficacy of a potent and selective non-covalent KRAS(G12D) inhibitor. *Nat Med* 28, 2171-2182. 10.1038/s41591-022-02007-7.
353. Kim, H.N., Gasmi-Seabrook, G.M.C., Uchida, A., Gebregiorgis, T., Marshall, C.B., and Ikura, M. (2025). Switch II Pocket Inhibitor Allosterically Freezes KRAS(G12D) Nucleotide-binding Site and Arrests the GTPase Cycle. *J Mol Biol* 437, 169162. 10.1016/j.jmb.2025.169162.
354. Zhou, C., Li, C., Luo, L., Li, X., Jia, K., He, N., Mao, S., Wang, W., Shao, C., Liu, X., et al. (2024). Anti-tumor efficacy of HRS-4642 and its potential combination with proteasome inhibition in KRAS G12D-mutant cancer. *Cancer Cell* 42, 1286-1300.e1288. 10.1016/j.ccell.2024.06.001.
355. Holderfield, M., Lee, B.J., Jiang, J., Tomlinson, A., Seamon, K.J., Mira, A., Patrucco, E., Goodhart, G., Dilly, J., Gindin, Y., et al. (2024). Concurrent inhibition of oncogenic and wild-type RAS-GTP for cancer therapy. *Nature* 629, 919-926. 10.1038/s41586-024-07205-6.
356. Cregg, J., Edwards, A.V., Chang, S., Lee, B.J., Knox, J.E., Tomlinson, A.C.A., Marquez, A., Liu, Y., Freilich, R., Aay, N., et al. (2025). Discovery of Daraxonrasib (RMC-6236), a Potent and Orally Bioavailable RAS(ON) Multi-selective, Noncovalent Tri-complex Inhibitor for the Treatment of Patients with Multiple RAS-Addicted Cancers. *J Med Chem* 68, 6064-6083. 10.1021/acs.jmedchem.4c02314.
357. Yin, G., Huang, J., Petela, J., Jiang, H., Zhang, Y., Gong, S., Wu, J., Liu, B., Shi, J., and Gao, Y. (2023). Targeting small GTPases: emerging grasps on previously untamable targets, pioneered by KRAS. *Signal Transduct Target Ther* 8, 212. 10.1038/s41392-023-01441-4.
358. Liaki, V., Barrambana, S., Kostopoulou, M., Lechuga, C.G., Zamorano-Dominguez, E., Acosta, D., Morales-Cacho, L., Álvarez, R., Sun, P., Rosas-Perez, B., et al. (2025). A targeted combination therapy achieves effective pancreatic cancer regression and prevents tumor resistance. *Proc Natl Acad Sci U S A* 122, e2523039122. 10.1073/pnas.2523039122.
359. Yu, Z., He, X., Wang, R., Xu, X., Zhang, Z., Ding, K., Zhang, Z.M., Tan, Y., and Li, Z. (2023). Simultaneous Covalent Modification of K-Ras(G12D) and K-Ras(G12C) with Tunable Oxirane Electrophiles. *J Am Chem Soc* 145, 20403-20411. 10.1021/jacs.3c05899.
360. Zheng, Q., Zhang, Z., Guiley, K.Z., and Shokat, K.M. (2024). Strain-release alkylation of Asp12 enables mutant selective targeting of K-Ras-G12D. *Nat Chem Biol* 20, 1114-1122. 10.1038/s41589-024-01565-w.
361. Weller, C., Burnett, G.L., Jiang, L., Chakraborty, S., Zhang, D., Vita, N.A., Dilly, J., Kim, E., Maldonado, B., Seamon, K., et al. (2025). A neomorphic protein interface catalyzes covalent inhibition of RAS(G12D) aspartic acid in tumors. *Science* 389, eads0239. 10.1126/science.ads0239.
362. Shuai, T., Zhang, R., Yuan, Z., Zhang, M., Fan, Z., Ruan, X., Qi, Z., Xie, W., Liu, P., Uesugi, M., et al. (2025). Expanding the Chemistry of Acyl Diazo Electrophile as a Tunable Warhead for Covalent Targeting of KRAS (G12D) Mutant. *J Med Chem* 68, 21427-21440. 10.1021/acs.jmedchem.5c01365.
363. Zhang, R., Huetzen, M.A., Binici, A., Martín-Gago, P., Gasper, R., Rudashevskaya, E., Liu, J., Nagaraju, C., Reckzeh, E.S., Stuedle, A.S.T., et al. (2026). Targeting a Glutamic Acid in PDE δ with Fluoromethyl-Aryl Electrophiles Impairs K-Ras Signaling. *J Med Chem* 69, 964-981. 10.1021/acs.jmedchem.5c02082.
364. Gómez-Mulas, A., Schaffner-Reckinger, E., Peeters, H., Chippalkatti, R., Dautbasic, A., Smith, M.J., Ismail, S., and Abankwa, D.K. (2025). Development of a Genetically Encoded and Potent PDE6D Inhibitor. *Chembiochem* 26, e202500739. 10.1002/cbic.202500739.
365. Armijo, M.E., Escalona, E., Peña, D., Farias, A., Morin, V., Baumann, M., Klebl, B.M., Pincheira, R., and Castro, A.F. (2022). Blocking the Farnesyl Pocket of PDE δ Reduces Rheb-Dependent mTORC1 Activation and Survival of Tsc2-Null Cells. *Front Pharmacol* 13, 912688. 10.3389/fphar.2022.912688.
366. Igarashi, T., Mazevet, M., Yasuhara, T., Yano, K., Mochizuki, A., Nishino, M., Yoshida, T., Yoshida, Y., Takamatsu, N., Yoshimi, A., et al. (2023). An ATR-PrimPol pathway confers tolerance to oncogenic KRAS-induced and heterochromatin-associated replication stress. *Nat Commun* 14, 4991. 10.1038/s41467-023-40578-2.

11. References

367. Smith, S.J., Meng, F., Lingeman, R.G., Li, C.M., Li, M., Boneh, G., Seppälä, T.T., Phan, T., Li, H., Burkhart, R.A., et al. (2025). Therapeutic Targeting of Oncogene-Induced Transcription-Replication Conflicts in Pancreatic Ductal Adenocarcinoma. *Gastroenterology* 169, 600-614.e611. 10.1053/j.gastro.2025.02.038.
368. Meng, F., Li, T., Singh, A.K., Wang, Y., Attiyeh, M., Kohram, F., Feng, Q., Li, Y.R., Shen, B., Williams, T., et al. (2024). Base-excision repair pathway regulates transcription-replication conflicts in pancreatic ductal adenocarcinoma. *Cell Rep* 43, 114820. 10.1016/j.celrep.2024.114820.
369. Cáceres-Gutiérrez, R.E., Alfaro-Mora, Y., Andonegui, M.A., Díaz-Chávez, J., and Herrera, L.A. (2022). The Influence of Oncogenic RAS on Chemotherapy and Radiotherapy Resistance Through DNA Repair Pathways. *Front Cell Dev Biol* 10, 751367. 10.3389/fcell.2022.751367.
370. Hoffman, T.E., Nangia, V., III, C.R., Passanisi, V.J., Armstrong, C., Yang, C., and Spencer, S.L. (2023). Multiple cancers escape from multiple MAPK pathway inhibitors and use DNA replication stress signaling to tolerate aberrant cell cycles. *Sci Signal* 16, eade8744. 10.1126/scisignal.ade8744.
371. Rizzotto, D., Englmaier, L., and Villunger, A. (2021). At a Crossroads to Cancer: How p53-Induced Cell Fate Decisions Secure Genome Integrity. *Int J Mol Sci* 22. 10.3390/ijms221910883.
372. O'Driscoll, M., Dobyns, W.B., van Hagen, J.M., and Jeggo, P.A. (2007). Cellular and clinical impact of haploinsufficiency for genes involved in ATR signaling. *Am J Hum Genet* 81, 77-86. 10.1086/518696.
373. Sala, A.J., Bott, L.C., and Morimoto, R.I. (2017). Shaping proteostasis at the cellular, tissue, and organismal level. *J Cell Biol* 216, 1231-1241. 10.1083/jcb.201612111.
374. Sun, C., Fang, Y., Yin, J., Chen, J., Ju, Z., Zhang, D., Chen, X., Vellano, C.P., Jeong, K.J., Ng, P.K., et al. (2017). Rational combination therapy with PARP and MEK inhibitors capitalizes on therapeutic liabilities in RAS mutant cancers. *Sci Transl Med* 9. 10.1126/scitranslmed.aal5148.
375. Miyashita, H., Kato, S., and Hong, D.S. (2024). KRAS G12C inhibitor combination therapies: current evidence and challenge. *Front Oncol* 14, 1380584. 10.3389/fonc.2024.1380584.

12. Appendix

12.1 Supplementary Data of Integrated Manuscripts

For the published Manuscripts I, II, and IV, supplementary data are accessible via the digital object identifiers listed below, which link to the publisher-hosted supplementary material. For **Manuscript III**, which is currently under review, the submitted supplementary figures referenced in chapter 2.3 (III-Figure S1-9), material and methods, and author declarations, are attached in full under **section 12.1.3**.

12.1.1 Biallelic Variants in *CRIP1* Cause a Rothmund-Thomson-like Syndrome with Increased Cellular Senescence

<https://doi.org/10.1016/j.gim.2023.100836>

12.1.2 Optical Genome Mapping Identifies Structural Variants in Potentially New Cancer Predisposition Candidate Genes in Pediatric Cancer Patients

<https://onlinelibrary.wiley.com/doi/10.1002/ijc.34721>

Supplementary Figure 5 (II-Figure S5) is shown in section 2.2.3, as it contains my primary experimental contribution to this manuscript.

12.1.3 UBQLN1-mediated Repression of Homologous Recombination is an Actionable Target for Cancer Therapy

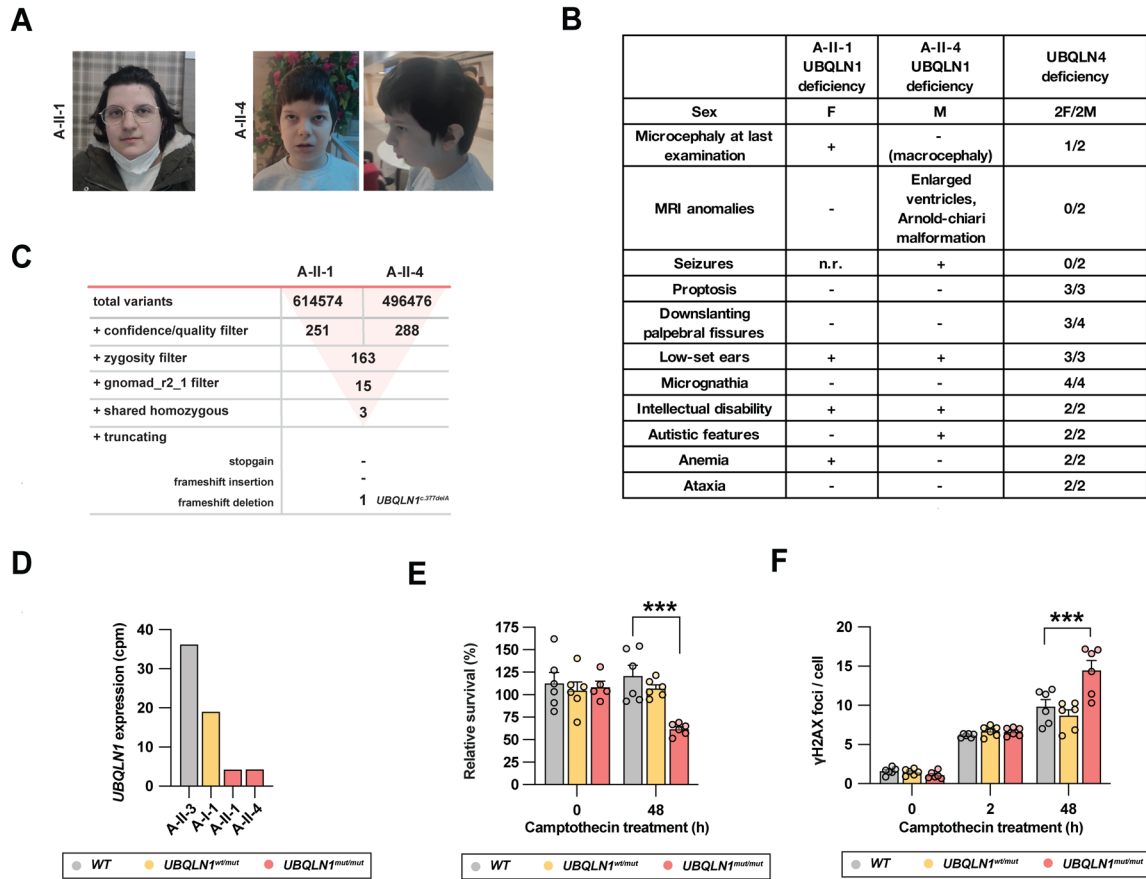
Supplementary figures, materials and methods, and declarations of the manuscript can be found on the following pages.

Supplementary:**UBQLN1-Mediated Repression of Homologous Recombination is an Actionable Target for Cancer Therapy****Table of Contents**

Supplementary Figure 1.	153
Supplementary Figure 2.	154
Supplementary Figure 3.	155
Supplementary Figure 4.	156
Supplementary Figure 5.	157
Supplementary Figure 6.	158
Supplementary Figure 7.	159
Supplementary Figure 8.	160
Supplementary Figure 9.	161
Material and Methods.	162
Material list	162
Cell Culture and Maintenance	165
Passaging Cells	165
Calculation of Seeding Density	165
Transient transfection of human cell lines	165
Generation of constitutively expressing Flp-In cell lines	165
Small interfering RNA (siRNA) mediated knockdown of cells	165
SDS-PAGE and Immunoblotting	166
Sample Preparation	166
SDS-PAGE and Immunoblotting	166
SDS-PAGE Signal Quantification	166
Cell cycle synchronization	166
Fixation with EtOH and propidium iodide staining for cell cycle analysis	166
Cycloheximide chase assays	166
Immunoprecipitation	166
Recombinant protein production	167
Co-immunoprecipitation of recombinant proteins	167
<i>In vitro</i> ubiquitylation reaction	167
Cell viability measurement	167
High-content Imaging	168
DSB repair pathway assays	168
DR-GFP	168

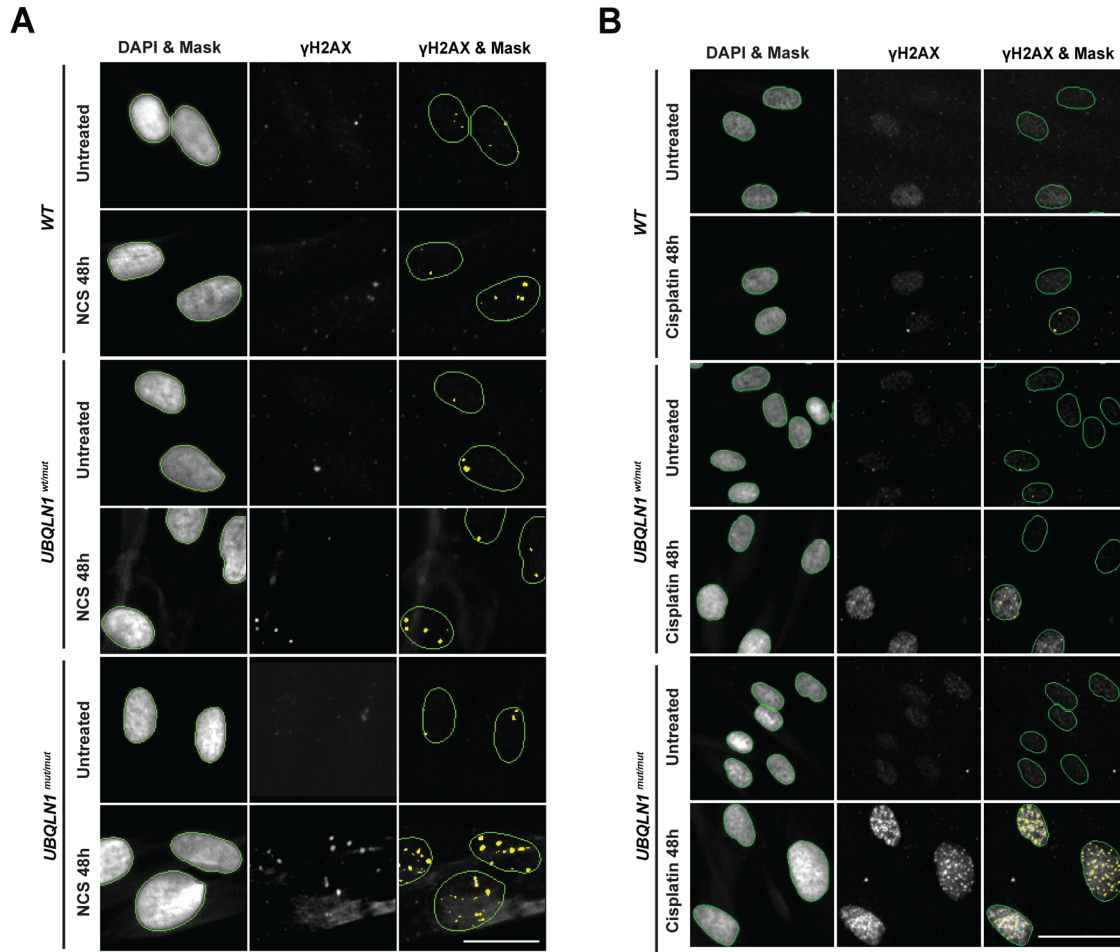
SA-GFP SSA reporter	168
Single Molecule Analysis of Resection Tracks	168
Laser microirradiation and imaging of cells.....	168
Patients.....	169
Patient A-II-1	169
Patient A-II-4	169
Sanger Sequencing	169
Whole-Exome-Sequencing Analysis of Patients.....	169
Patient fibroblasts RNA-Sequencing Analysis	169
TurboID proximity labelling assay and mass spectrometry analysis.....	169
The Cancer Genome Atlas (TCGA) / Genotype-Tissue Expression (GTEx) analysis	170
CODEFACs analyses	170
Licensing of animal experiments.....	170
Mouse Colony Statistics.....	170
C57BL/6J <i>Ubp1n1</i>^{c.377delC}	171
C57BL/6J <i>Rosa26</i>^{LSL.HA-Ubp1n1/wt}; <i>ACTB</i>^{Cre/wt}	171
C57BL/6J <i>K-Ras</i>^{LSL.G12D/wt}; <i>Trp53</i>^{fl/fl} and C57BL/6J <i>Rosa26</i>^{LSL.HA-Ubp1n1.IRES.GFP}; <i>K-Ras</i>^{LSL.G12D/wt}; <i>Trp53</i>^{fl/fl}	171
MEF Generation	171
Animal Housing	171
Lung Cancer Induction in autochthonous mouse models	171
In vivo Tumor Progression Monitoring with μ CT	171
In vivo Lung Cancer Treatment	171
3D CT Image Processing	172
RNA-Sequencing of KP/KPU1 lung tumors	172
Whole-Genome-Sequencing of KP/KPU1 lung tumors.....	172
Somatic mutation analysis of murine tumor WGS data	172
<i>In silico</i> prediction of mutant peptides based on WGS data	172
Colony Formation Assay	172
Histology analysis	173
Figures and Statistics	173
Data Availability.....	173
Declaration of Interest	173
Materials availability.....	173
Funding.....	173

Supplementary figures.



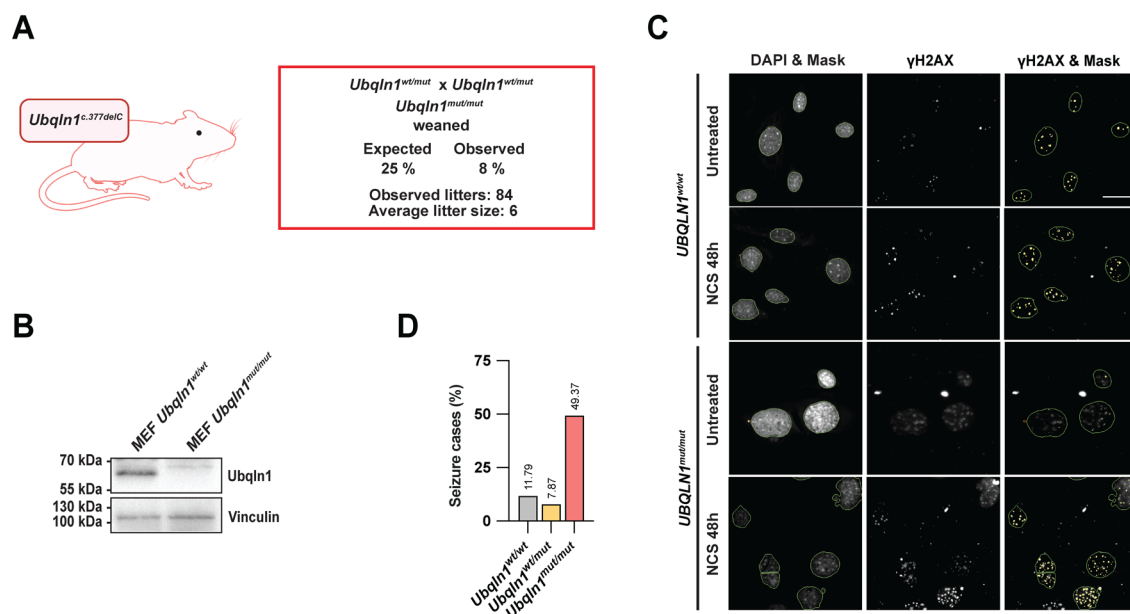
Supplementary Figure 1.

A) *UBQLN1* deficiency syndrome patients A-II-1 and A-II-4. **B)** Clinical evaluation of *UBQLN1* deficiency syndrome patients A-II-1 and A-II-4 compared to *UBQLN4* deficiency syndrome patients. (n.r. = not reported) **C)** Whole-exome-sequencing analysis of patient exomes to identify shared variants predicted to be truncating. All variants in the joint VCF were filtered for confidence and quality (minimizing false-positive calls, excluding common non-pathogenic variants, restricting on coding/splice regions), zygosity consistency (ensuring consistent inheritance), gnomad_r2_1 (excluding SNPs common in the population), shared variants (prioritizing disease-segregating variants), and protein impact (predicted truncating variants), defined as frameshift indels and stopgain, but not non-synonymous SNVs. *UBQLN1*^{c.377delA} was identified as the single shared homozygous mutation predicted to be truncating in both patients. **D)** *UBQLN1* expression analysis. RNA-Seq transcript counts of *UBQLN1* (ENST00000376395.9) obtained from 3' mRNAseq with Lexogen QuantSeq, Single-Read 1x50 bp, of patient fibroblasts. **E)** Cell viability assessment via DAPI immunofluorescence. Human dermal fibroblasts (HDFs) were treated with camptothecin (5 μ M) for 48h. Viability was assessed by detecting DAPI-positive nuclei with high-content imaging. Error bars represent the SEM of 6 biological replicates. Two-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **F)** Quantification of γ H2AX foci. HDFs were treated with camptothecin (5 μ M) and γ H2AX foci were counted 2 and 48h after treatment using high-content imaging. Error bars represent the SEM of 6 replicates. Two-way ANOVA with Bonferroni post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



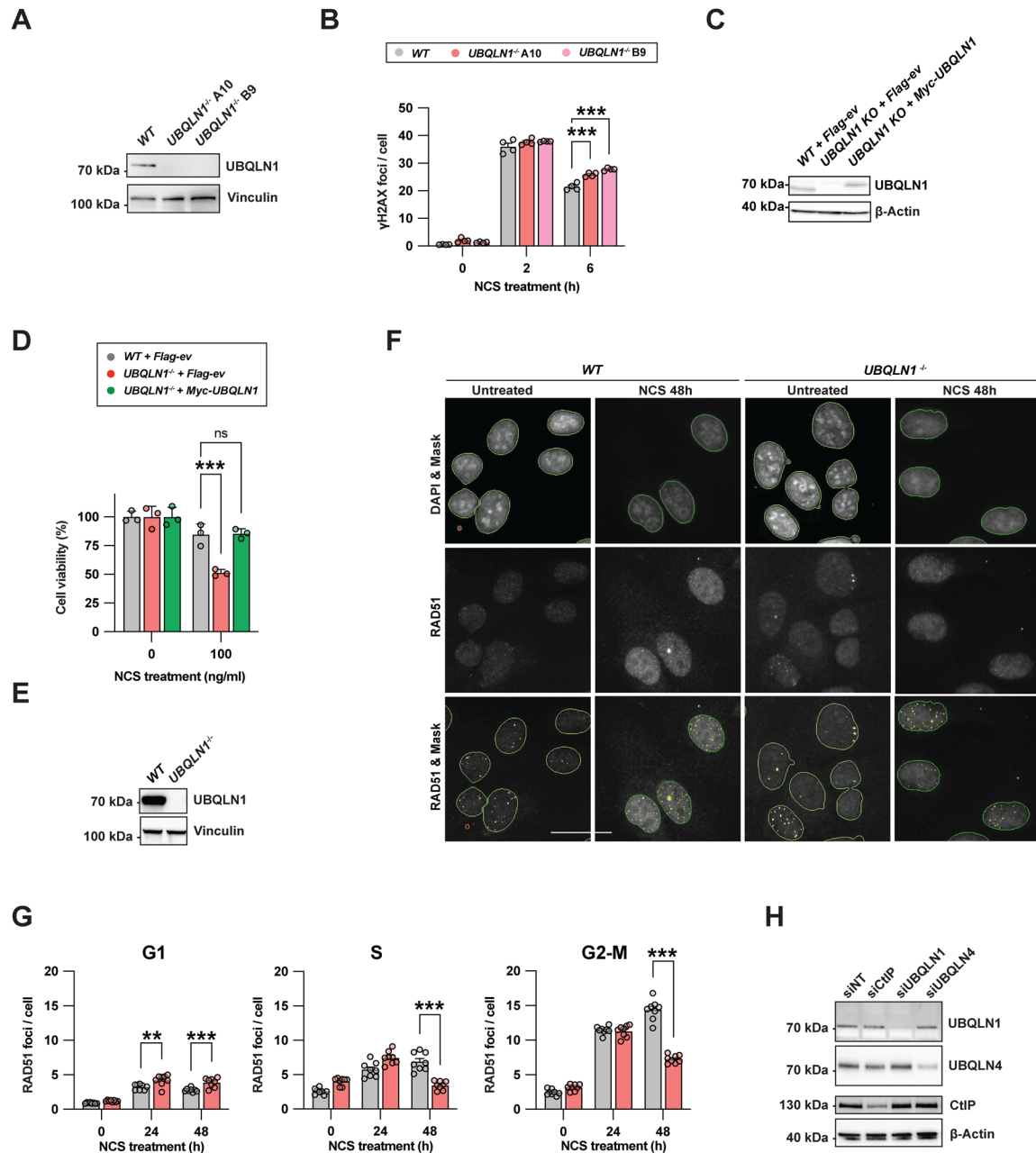
Supplementary Figure 2.

A) Representative images of γ H2AX foci 48h after neocarzinostatin (NCS, 500 ng/ml) or no treatment in human dermal fibroblasts (HDFs), using high-content imaging. DAPI served as a counterstain. Scale bar: 20 μ m. **B)** Representative images of γ H2AX foci 48h after cisplatin (20 μ M) or no treatment in HDFs, using high-content imaging. DAPI served as a counterstain. Scale bar: 30 μ m.



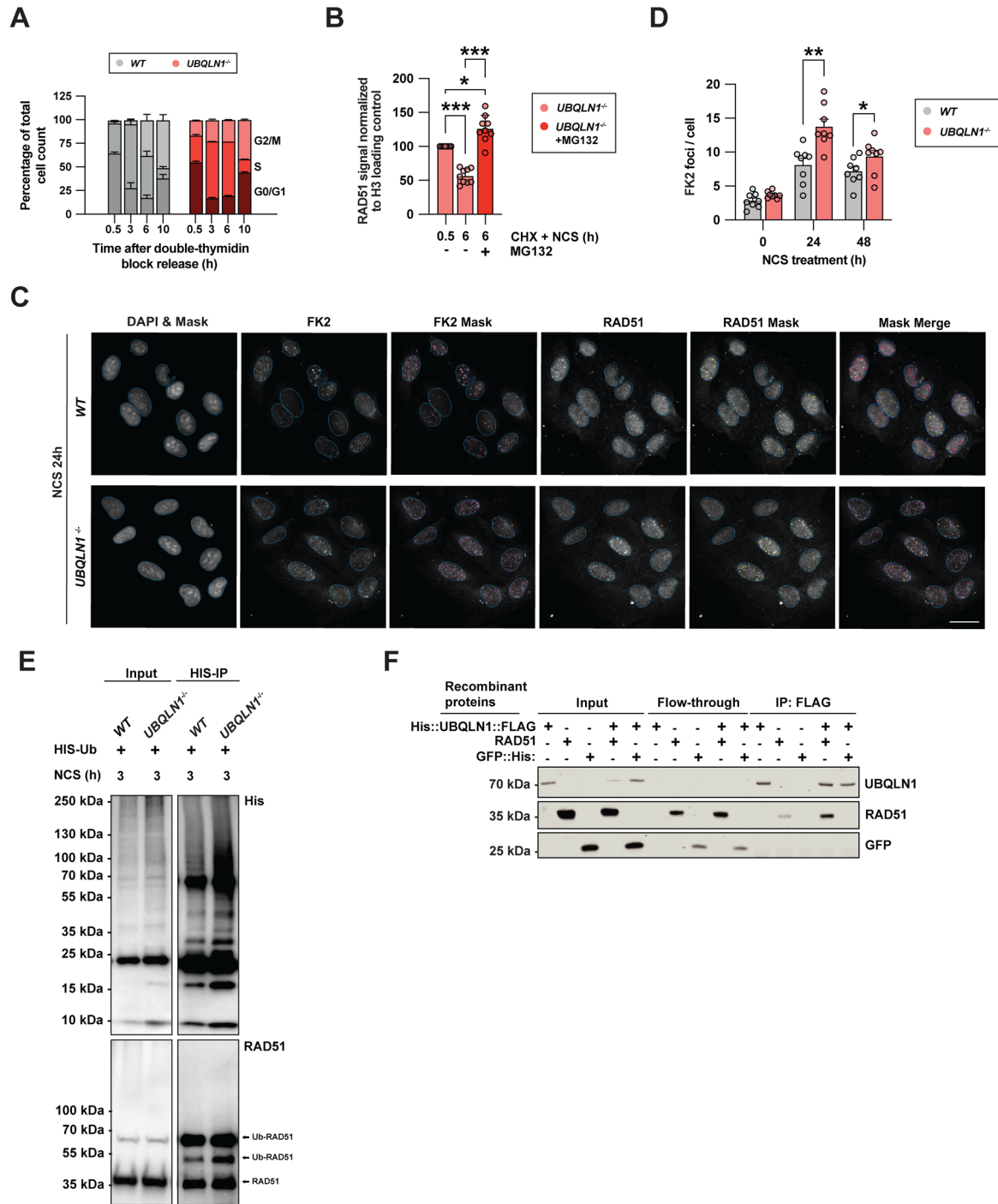
Supplementary Figure 3.

A) Mouse breeding statistics of heterozygous intercrosses of the *Ubqln1^{c.377delC}* mouse strain. **B)** Immunoblot validation of *Ubqln1^{c.377delC}* murine embryonic fibroblasts (MEFs) depicting Ubqln1 expression levels. **C)** Representative images of γH2AX foci 48h after neocarzinostatin (NCS, 500 ng/ml) or no treatment in MEFs, using high-content imaging. DAPI served as a counterstain. Scale bar: 20 μm. **D)** Monitoring of seizure cases in the indicated mouse strains. Seizures were evaluated in daily observations by experienced animal caretakers. *Ubqln1^{wt/wt}* n=195; *Ubqln1^{wt/mut}* n=432; *Ubqln1^{mut/mut}* n=79.



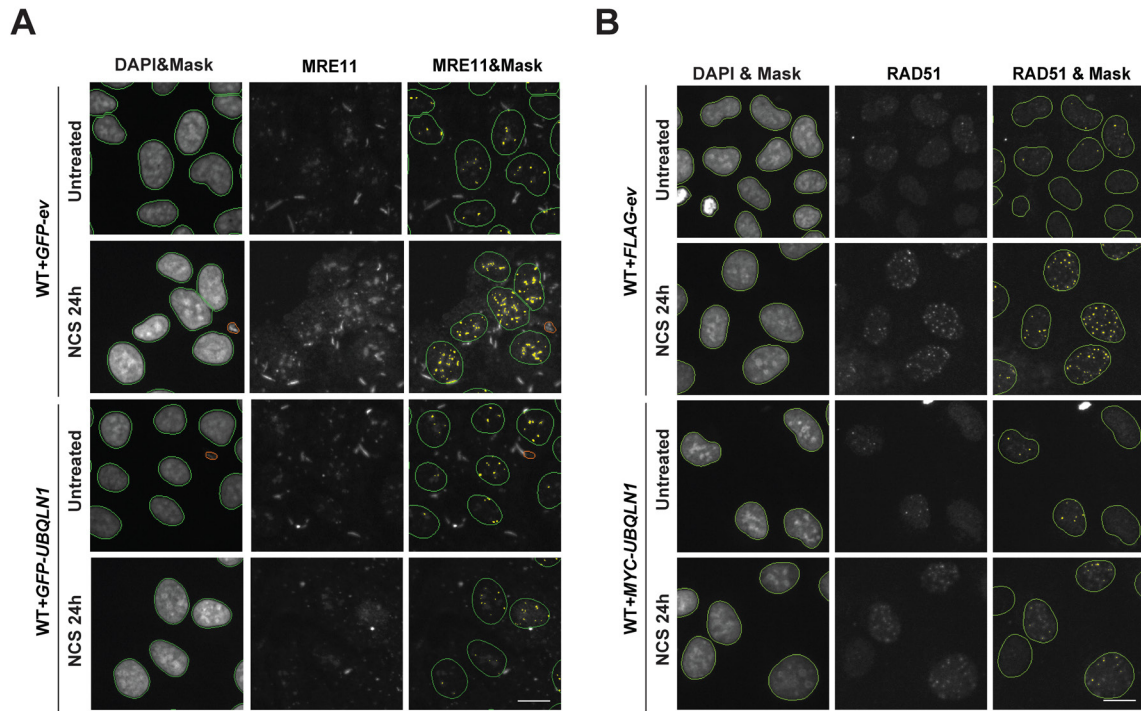
Supplementary Figure 4.

A) Immunoblot depicting UBQLN1 expression levels in the two *UBQLN1* CRISPR KO clones A10 and B9. **B)** Quantification of γ H2AX foci. U2OS CRISPR KO clones were treated with neocarzinostatin (NCS, 250 ng/ml) and γ H2AX foci were counted 2 and 6h after treatment using high-content imaging. Error bars represent the SEM of 4 replicates. Two-way ANOVA with Bonferroni post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **C)** Immunoblot depicting UBQLN1 expression levels in the HEK293 Flp-In *WT* and *UBQLN1*^{-/-} cells transiently transfected with *Flag-empty-vector* or *Myc-UBQLN1* as indicated. **D)** Cell viability rescue experiment of *UBQLN1* KO cells. HEK293 Flp-In *WT* and *UBQLN1*^{-/-} cells were transiently transfected with *Flag-empty-vector* or *Myc-UBQLN1* as indicated. Cells were treated with 100 ng/ml NCS and viability was measured 96h post-treatment using Cell-Titer-Glo reagent. Error bars represent the SD of 3 replicates. Two-way ANOVA with Bonferroni post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **E)** Immunoblot depicting UBQLN1 expression levels in U2OS *WT*, *UBQLN1*^{-/-} CRISPR KO cell lines. **F)** Representative images of RAD51 foci 48h after NCS (250 ng/ml) or no treatment in U2OS *WT*, *UBQLN1*^{-/-} cells, using high-content imaging. DAPI served as a counterstain. Scale bar: 20 μ m. **G)** Quantification of RAD51 foci from experiment Fig.2B with data gated for G1, S, and G2-M cell cycle stages via nuclear Hoechst staining intensity. Error bars represent the SEM of 8 replicates. Two-way ANOVA with Bonferroni post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **H)** Immunoblot depicting *UBQLN1*, *UBQLN4*, and *CtIP* expression levels in cells that were transfected with siRNAs against the respective targets and were used for DR-GFP reporter assay.



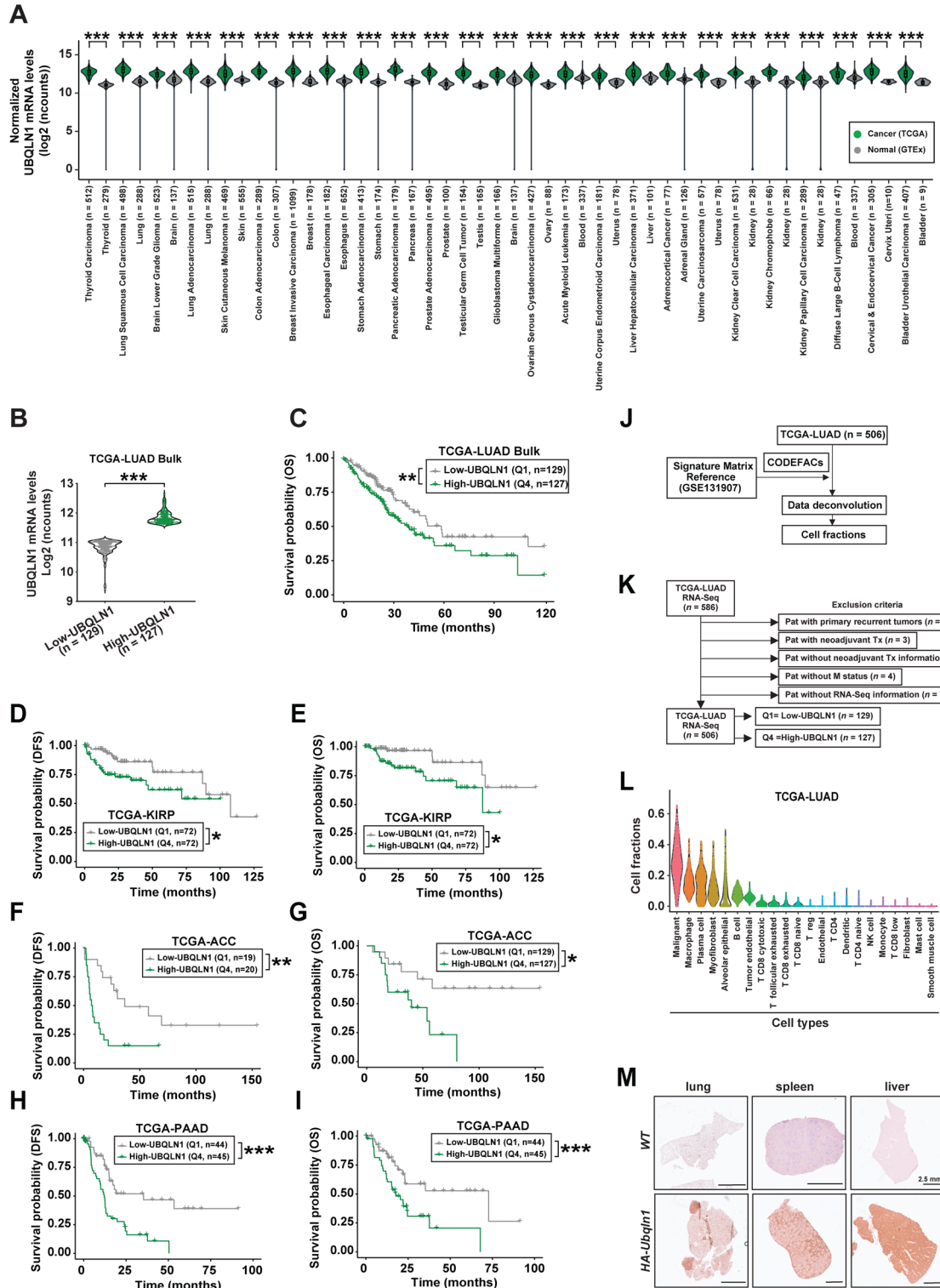
Supplementary Figure 5.

A) Quantification of cell cycle analysis performed on U2OS WT and *UBQLN1*^{-/-} cells. Cells were fixed in ethanol 0.5, 3, 6, and 10h after release from double-thymidine-block, stained with PI and subsequently analyzed via flow cytometry. Gating and data quantification was performed in FlowJo 10.10.0 software. **B)** Quantification of cycloheximide chase assay in Fig. 3D. Immunoblot bands of the chromatin fraction were quantified using FIJI and normalized to background and histone H3 loading control. Data represents 3 independent fractionations with 3 immunoblots each. Two-way ANOVA. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. **C)** Representative images of FK2 and RAD51 foci 24h after neocarzinostatin (NCS, 250 ng/ml) treatment in WT and *UBQLN1*^{-/-} cells using high-content imaging. DAPI served as a counterstain. Scale bar: 30 μm. **D)** Quantification of FK2 foci from overlap analysis in Fig. 3E. U2OS WT and *UBQLN1*^{-/-} cells were treated with NCS (250 ng/ml) and nuclear FK2 foci were quantified 24 and 48h after treatment using high-content imaging. Error bars represent the SEM of 8 replicate wells. Two-way ANOVA with Bonferroni post hoc test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. **E)** Co-immunoprecipitation of His-Ub with RAD51. HEK293 Flp-In WT and *UBQLN1*^{-/-} cell lines were transfected with 6 μg of His-Ubiquitin plasmid, synchronized via double-thymidine-block and treated with NCS (100 ng/ml) 3h before harvest. The amount of His-ubiquitinated protein and bound (ubiquitylated) endogenous RAD51 protein was detected via immunoblotting. **F)** Co-immunoprecipitation of recombinant UBQLN1 with RAD51. Recombinant FLAG-UBQLN1-HIS protein was incubated with recombinant RAD51 or GFP-HIS as a negative control. FLAG-UBQLN1-HIS and GFP-HIS were immunoprecipitated via FLAG-tag and probed for RAD51 interaction.



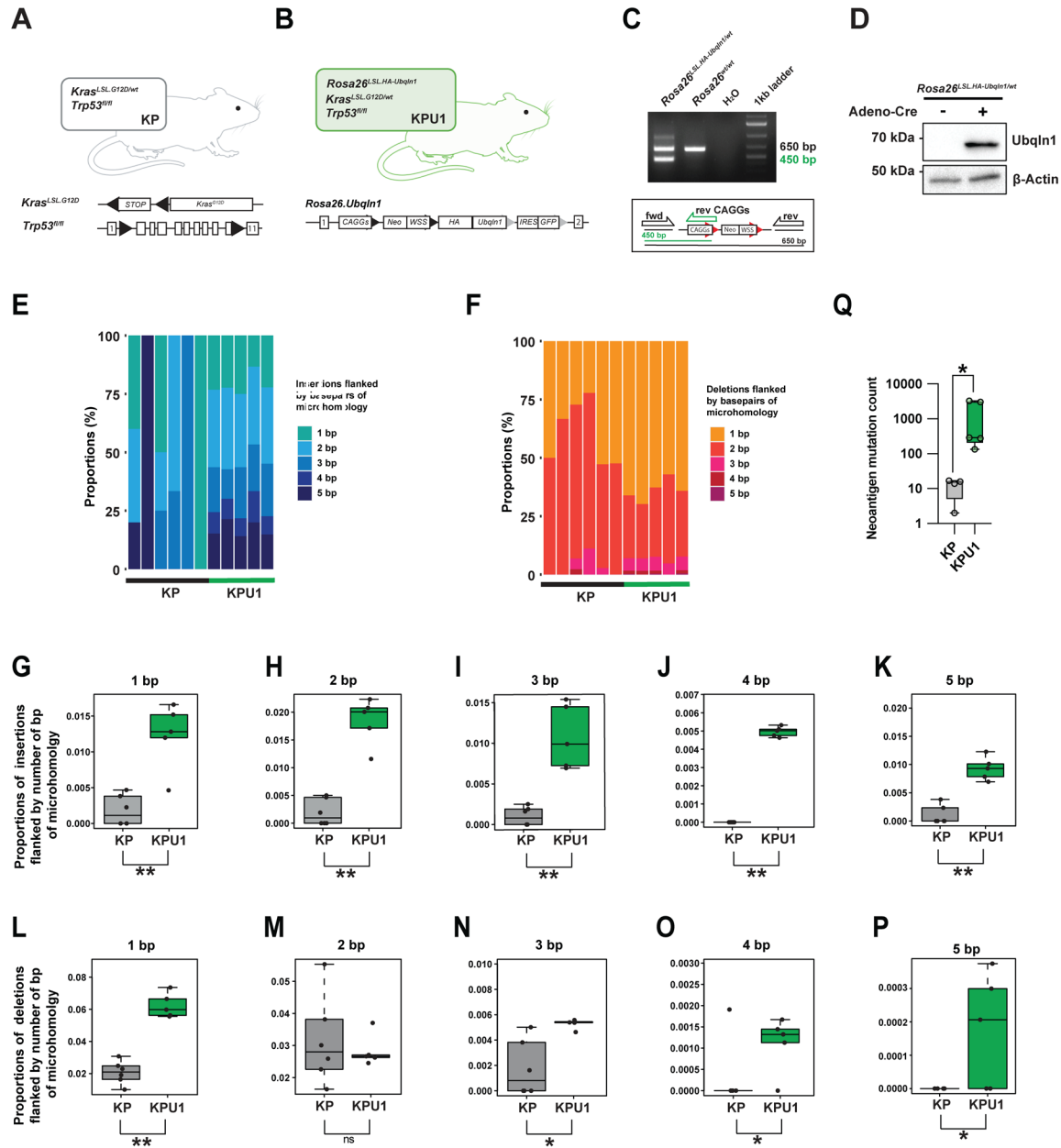
Supplementary Figure 6.

A) Representative images of MRE11 foci 24h after neocarzinostatin (NCS, 250 ng/ml) or no treatment in U2OS *WT* Flp-In +*GFP-ev*, and +*GFP-UBQLN1* cell lines that were induced with doxycycline (100 ng/ml) for 48h and imaged with high-content imaging. DAPI served as a counterstain. Scale bar: 20 μ m. **B)** Representative images of RAD51 foci 24h after NCS (250 ng/ml) or no treatment in U2OS Flp-In +*FLAG-ev*, and +*MYC-UBQLN1* cell lines that were induced with doxycycline (100 ng/ml) for 48h, using high-content imaging. DAPI served as a counterstain. Scale bar: 20 μ m.



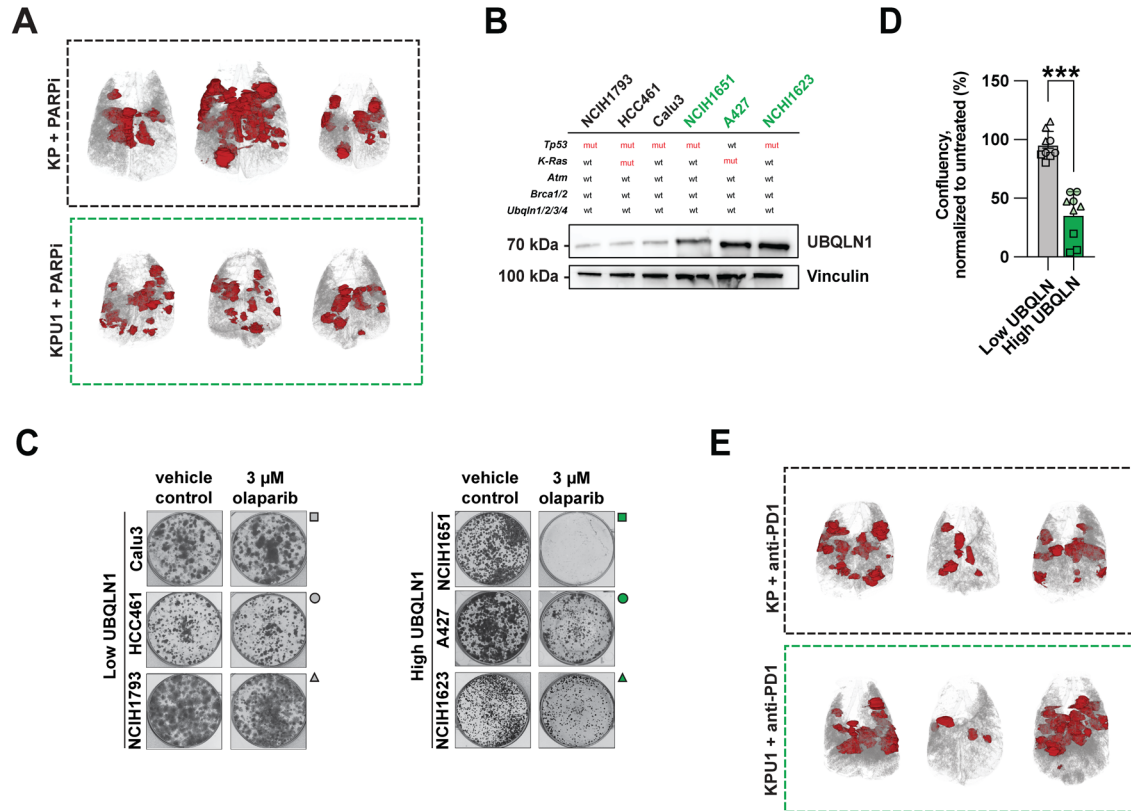
Supplementary Figure 7.

A) *UBQLN1* mRNA levels in different cancer entities (TCGA) were compared to their normal tissues (GTEx). **B)** TCGA lung adenocarcinoma bulk patient data was grouped by lower and higher quartile of *UBQLN1* expression. **C)** Overall survival of *UBQLN1* low (n=129) and high (n=127) expressing tumors. Mantel-Cox-Test. **D)** Disease-free survival of low or high *UBQLN1* expressing kidney renal papillary carcinoma (KIRP). Mantel-Cox-Test. **E)** Overall survival of low or high *UBQLN1* expressing kidney renal papillary carcinoma (KIRP). Mantel-Cox-Test. **F)** Disease-free survival of low or high *UBQLN1* expressing adenoid cystic carcinoma. Mantel-Cox-Test. **G)** Overall survival of low or high *UBQLN1* expressing adenoid cystic carcinoma. Mantel-Cox-Test. **H)** Disease-free survival of low or high *UBQLN1* expressing pancreatic adenocarcinoma (PAAD). Mantel-Cox-Test. **I)** Overall survival of low or high *UBQLN1* expressing pancreatic adenocarcinoma (PAAD). Mantel-Cox-Test. **J)** TCGA data stratification. Overview of the TCGA LUAD data selection and exclusion criteria. **K)** CODEFACs method. **L)** CODEFACs analysis of the TCGA LUAD dataset. Cell fractions were deconvoluted and used for downstream analysis (Fig. 5A-C). **M)** Immunohistochemistry validation. Lung, spleen, and liver obtained from *Rosa26^{LSL}HA-Ubqln1/wt*; *Actb^{cre/wt}* or wildtype mice were stained for *HA-Ubqln1* expression. Scale bar: 2.5 mm. **Statistics:** *p < 0.05, **p < 0.01, ***p < 0.001.



Supplementary Figure 8.

A) Genetic information on the KP mouse strain. **B)** Genetic information on the KPU1 mouse strain. **C)** Genotyping strategy for *Rosa26* insertion in KPU1 mice. **D)** Immunoblot depicting *Ubqln1* expression levels in murine embryonic fibroblast (MEF) lysates of *Rosa26^{LSL:HA-Ubqln1}* upon 24h of *in vitro* Adeno-CMV-Cre induction. **E)** Relative contribution of small insertions associated with flanking microhomologies in KP and KPU1 tumors. **F)** Relative contribution of small deletions associated with flanking microhomologies in KP and KPU1 tumors. **G-K)** Percentage of microhomology-associated insertions associated with breakpoint-induced microhomology from total insertions of KP and KPU1 whole-genome-sequencing data. Wilcoxon-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. **L-P)** Percentage of microhomology-associated deletions associated with breakpoint-induced microhomology from total deletions of KP and KPU1 whole-genome-sequencing data. Wilcoxon-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. **Q)** Neoepitope presentation analysis from whole-genome-sequencing data of KP and KPU1 mice. Student's *t*-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.



Supplementary Figure 9.

A) Representative 3D images. Volumetric tumor burden analysis for PARPi-treated KP and KPU1 mice. 3 mice per genotype and treatment group were selected by representative survival in the respective cohort. Tumors were mapped and interpolated from μ CT images 11 weeks post instillation using CTan/CTvox software. **B)** Immunoblot depicting *UBQLN1* expression in 6 human lung adenocarcinoma cell lines. Relevant mutational backgrounds are indicated according to public DepMap data. **C)** Colony formation assays of high or low *UBQLN1* expressing lung adenocarcinoma cell lines treated with Olaparib (3 μ M) or DMSO vehicle control for 14 days. Figure shows one representative image of 3 replicates. **D)** Quantification of the colony formation assays in Suppl. Fig. 9C. Triplicates were quantified using FIJI. Staining intensity was normalized against the background for measurement of well confluency. Treatment wells were normalized to vehicle control (100%). Normalized confluency in wells treated with Olaparib (3 μ M) is shown. Datapoints were assigned to the individual cell lines. Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **E)** Representative 3D images. Volumetric tumor burden analysis for aPD1-treated KP and KPU1 mice. 3 mice per genotype and treatment group were selected by representative survival in the respective cohort. Tumors were mapped and interpolated from μ CT images 11 weeks post instillation using CTan/CTvox software.

Material and Methods.

Material list

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-Phospho-Histone H2AX (Ser139) (IF 1:500)	Millipore	05-636
Goat AF488 anti-mouse	Invitrogen	A11001
Goat AF488 anti-rabbit IgG	Invitrogen	A11008
Goat AF647 anti-mouse	Invitrogen	A21235
Goat AF647 anti-rabbit	Invitrogen	A27040
Goat anti-mouse IgG peroxidase	EMD Millipore	12-349
Mouse anti-CtIP (WB 1:1000)	Santa Cruz Biotechnology	sc-271339
Mouse anti-FLAG M2 (WB 1:1000)	Sigma	F1804
Mouse anti-GFP (WB 1:1000)	Santa Cruz Biotechnology	sc-9996
Mouse anti-β-Actin (WB 1:10,000)	Sigma	A5316
Mouse anti-MRE11 (WB 1:1000)	Santa Cruz Biotechnology	sc-135992
Mouse anti-Ubiquitin (FK2) (IF 1:500, WB 1:1000)	Millipore	04-263
Mouse anti-Ubiquitin (P4D1) (WB 1:1000)	Cell Signaling Technology	3936
Rabbit anti-UBQLN1 (WB 1:1000)	Bethyl	BL-20647
Rabbit anti-UBQLN1 (WB 1:1000)	Cell Signaling Technology	14526
Rabbit anti-UBQLN1 (WB 1:1000)	Bio-Rad	VPA00453
Rabbit anti-UBQLN1 (WB 1:1000)	Bethyl	A305-896A-M
Rabbit anti-UBQLN4 (WB 1:1000)	Bethyl	BL2064
Rabbit anti-GFP (WB 1:1000)	Novus	NB600-308SS
Rabbit anti-HA-Tag (IHC: 1:500, WB 1:1000)	Cell Signaling Technology	3724
Rabbit anti-Histone H3 (WB 1:10,000)	Cell Signaling Technology	60932SF
Rabbit anti-IgG HRP	Merck	12-348
Rabbit anti-K48 (WB 1:1000)	Cell Signaling Technology	8081S
Rabbit anti-MRE11 (IF 1:500, WB 1:1000)	Cell Signaling Technology	4847
Rabbit anti-MYC-Tag (WB 1:1000)	Cell Signaling Technology	2276S
Rabbit anti-RAD51 (IF: 1:500, WB 1:1000)	Millipore	ABE257
Rabbit anti-RAP80 (D1T6Q) (WB 1:1000)	Cell Signaling Technology	14466
Rabbit anti-Vinculin (WB 1:10,000)	Abcam	ab129002
Bacterial and Virus Strains		
Ad5CMVCre	University of Iowa	Ad5CMVCre
<i>E. coli</i> stable competent (high efficiency)	New England Biolabs	C3040I
Chemicals, Peptides, and Recombinant Proteins		
Acrylamide	Carl Roth	3029.2
Agarose	Carl Roth	3810.3
Ammoniumpersulfate	Carl Roth	9592.5
Animal-Free Blocking Solution	Cell Signaling Technology	15019S
Anti-FLAG® M2 Magnetic Beads	Merck	M8823-1ML
ATP energy regenerating solution	Enzo Life Sciences	BML-EW9810-0100
Benzonase	Merck Millipore	103773
Biotin	Sigma-Aldrich	B4501
Blasticidin-S Hydrochlorid	ThermoFisher Scientific	10264913
Blotting paper	Millipore	CL66.1
Bovine Serum Albumin	Sigma-Aldrich	9048-46-8
2-chloroacetamide	Sigma-Aldrich	22790
CaCl ₂	Carl Roth	CN93.1
Camptothecin	Millipore	208925
CellTiter-Glo® Reagent	Promega	G7573
Cisplatin	Accord	N/A
Cre Recombinase, TAT-Cre (Tat-NLS-Cre, HTNC, HTNCre)	Excellgen	EG-1001
Crystal violet	ThermoFisher Scientific	11976931
Cycloheximide	Sigma-Aldrich	C7698-5G
Dharmafect transfection reagent	Horizon discovery	T-2001-02
DMSO	Carl Roth	A994.2
DNase I	ThermoFisher Scientific	EN0521
dNTP	ThermoFisher Scientific	R0181
DpnII	New England Biolabs	R0543S
Dulbecco's Modified Eagle Medium + GlutaMAX	ThermoFisher Scientific	2902879
Dulbecco's Phosphate Buffered Saline	ThermoFisher Scientific	2858855
DYKDDDDK Fab-Trap® Agarose beads	Chromotek	#ffa
DYNAL™ MyOne™ Dynabeads™ Streptavidin C1	ThermoFisher Scientific	65001
ECL Prime Blotting Detection Reagent	EMD Millipore	RPN2236
EDTA	ThermoFisher Scientific	AM9260G
EnVision FLEX Target Retrieval Solution, High pH	Agilent	K800421
EnVision FLEX Target Retrieval Solution, Low pH	Agilent	K800521
Eosin	Carl Roth	X883.2
Epredia SuperFrost Plus Histology Slides	ThermoFisher Scientific	Epredia™ J1800AMNZ
Ethanol	Carl Roth	0911.2
Ethanol 99,8% Rotipuran	Carl Roth	9065.4
Ethanol denatured 99,8%	Carl Roth	K928.4
Fetal bovine serum (FCS)	ThermoFisher Scientific	10270106

Supplementary Data – Manuscript III

G418	Sigma-Aldrich	G418-RO
GFP-Trap Magnetic Particles M-270	Chromotek	gtd-20
Glycerol	Carl Roth	3783.2
Glycin	Carl Roth	1979.4
GoTaq 2500U	Promega	M784B
GoTaq qPCR Master Mix	Promega	A6001
Haematoxylin	Carl Roth	3816.2
HEPES	Carl Roth	6763.2
Hoechst 34580	Merck	63493
Human ubiquitin	Boston Biochemicals	U-100H
Hydrogen Peroxide 30 %	Carl Roth	9681.4
2-Hydroxypropyl-β-cyclodextrin	Carbolution	CC20035
Hygromycin B	Carl Roth	1287.1
Igepal	Sigma-Aldrich	I8896
Imidazole	Sigma-Aldrich	56750
Isoflurane	Piramal critical care	66794-17
Ketamine	Serumwerk Bernburg AG	13690.00.00
Laemmli SDS sample buffer, non-reducing (4X)	ThermoFisher Scientific	J63615.AC
Lipofectamine 2000	ThermoFisher Scientific	11668027
Methanol	Sigma-Aldrich	32213
MG132	Selleckchem	S2619
MgCl ₂	Carl Roth	KK36.2
Mirin	Selleckchem	S8096
NaH ₂ PO ₄	Carl Roth	T879.2
N-Ethylmaleimide	Sigma-Aldrich	04259-5G
NaCl	Carl Roth	P029.4
Neocarzinostatin (NCS)	Sigma-Aldrich	N9162
Ni-NTA Magnetic Agarose Beads	Qiagen	36113
Normal goat serum	Sigma-Aldrich	G9023
NP40/Igepal	Sigma-Aldrich	I8896-50ml
Olaparib	MCE	HY-10162
Opti-MEM™ I Reduced Serum Medium	ThermoFisher Scientific	31985062
PageRuler Prestained Protein Ladder	ThermoFisher Scientific	26616
Paraformaldehyde	Carl Roth	0335.3
Penicillin/Streptomycin	ThermoFisher Scientific	15070063
Phosphatase inhibitor	Roche	4906837001
Polybrene	Sigma-Aldrich	TR-1003-G
Ponceau S Solution	Applchem	A2935
PR-619	Selleckchem	S7130
Propidium iodide	Invitrogen	P3566
Protein G Magnetic Beads	Cell Signaling Technology	70024S
Protease inhibitors (cOmplete, EDTA free)	Sigma-Aldrich	11873580001
Puromycin	Sigma-Aldrich	P8833
PVDF membrane	Merck	IPFL00010
QiAquick Gel Extraction Kit	Qiagen	28704
Recombinant E1 activating enzyme	Enzo Life Sciences	BML-UW-9610-0050
Recombinant E2 conjugating enzyme UBE2D2	Boston Biochemicals	E2-622
Recombinant Ubiquitin	Boston Biochemicals	U-100H
Recombinant RAD51	Abcam	ab63808
Recombinant HIS-RAD51	MCE	HY-P702777
Recombinant FLAG-UBQLN1-HIS	This paper	
Recombinant E3 CHIP	Balaji et al., 2022 ³⁷	
RMP1-14 (anti-PD1)	InVivoMAb	BE0146
RNAse A	ThermoFisher Scientific	EN0531
Roti-GelStain 1ml	Carl Roth	3865.1
RPMI1640 medium	ThermoFisher Scientific	21875091
SDS	Carl Roth	2326.2
SignalStain® Antibody Diluent	Cell Signaling Technology	8112S
SignalStain® Boost (HRP, Mouse)	Cell Signaling Technology	8125P
SignalStain® Boost (HRP, Rabbit)	Cell Signaling Technology	8114P
Sodium Azide	Merck	8223350100
Sucrose	Carl Roth	9286.1
TEMED	Carl Roth	2367.3
Thymidine	Sigma-Aldrich	T9250-5G
Trans-Blot Turbo 5X Transfer Buffer	Bio-Rad	10026938
Tris HCl	Carl Roth	9090.3
Triton X-100	Sigma-Aldrich	X100
Trypan blue	ThermoFisher Scientific	15250061
Trypsin	ThermoFisher Scientific	25200056
Tween20	Sigma-Aldrich	102717264
Ubiquitin conjugation reaction buffer	Enzo Life Sciences	BML-KW9885-0001
Urea	Merck	1.08487.1000
Xylazine	Serumwerk Bernburg AG	116565
Critical Commercial Assays		
DC assay kit	Bio-Rad	5000114
DNeasy Blood&Tissue Kit	Qiagen	69504
QiAquick Gel Extraction Kit	Qiagen	28704
RNeasy Mini Kit	Qiagen	74104
SignalStain® DAB Substrate Kit	Cell Signaling Technology	8059

Supplementary Data – Manuscript III

Subcellular Protein Fractionation Kit	ThermoFisher Scientific	78840
Trans-Blot Turbo RTA Mini 0.2 µm PVDF Transfer Kit	Bio-Rad	1704272
Experimental Models: Cell Lines		
U2OS	ATCC	HTB-96
A427	ATCC	
Calu3	ATCC	
HDF A-I-1	This paper	
HDF A-II-1	This paper	
HDF A-II-3	This paper	
HDF A-II-4	This paper	
HCC461	ATCC	
HEK293	ThermoFisher Scientific	R70007
NCIH1623	ATCC	
NCIH1651	ATCC	
NCIH1793	ATCC	
NHDF-c WT fibroblast	Sigma	C-12300
TRex 293 Flp-In UBQLN1 KO	Ubigen	
TRex 293 Flp-In	Panier Lab, Cologne	
TRex U2OS Flp-In	Ubigen	
TRex U2OS Flp-In UBQLN1 KO A10	Ubigen	
TRex U2OS Flp-In UBQLN1 KO B9	Ubigen	
U2OS DR-GFP	Stark Lab, Duarte	
Experimental Models: Mouse Lines		
<i>K-Ras</i> ^{LSL-G12D/wt ; Trp53^{fl/fl}}	DuPage et al., 2009 ²²	C57BL/6J
<i>Rosa</i> ^{LSL-HA-Ubqln1.IRES.GFP}	This paper	C57BL/6J
<i>Rosa26</i> ^{LSL-HA-Ubqln1.IRES.GFP ; K-Ras} ^{LSL-G12D/wt ; Trp53^{fl/fl}}	This paper	C57BL/6J
<i>Rosa26</i> ^{LSL-HA-Ubqln1.IRES.GFP ; Actb^{cre/wt}}	This paper	C57BL/6J
<i>Ubqln1</i> ^{c.377delC}	This paper	C57BL/6J
Oligonucleotides		
siRNA Non-Targeting Control Pool	Horizon	D-001810-10-20
siRNA RBBP8 (CtIP) human, SMARTpool	Horizon	L-0211376-00-0010
siRNA UBQLN1 human, SMARTpool	Horizon	L-012942-00-0020
siRNA UBQLN4 human, SMARTpool	Horizon	L-021178-01-0020
Recombinant DNA		
<i>3xHA-TurboID NLS</i>	This paper	N/A
<i>pcDNA3_3xHA-TurboID-Ubqln1_NLS</i>	This paper	N/A
<i>pcDNA3_FLAG_MRE11</i>	This paper	N/A
<i>pcDNA5_FRT_TO_eGFP_S-tag</i>	This paper	N/A
<i>pcDNA5_FRT_TO_eGFP_s-tag_UBQLN1_NLS</i>	This paper	N/A
<i>pcDNA5_FRT_TO_eGFP_s-tag_UBQLN1_dSTI1-I_NLS</i>	This paper	N/A
<i>pcDNA5_FRT_TO_eGFP_s-tag_UBQLN1_dSTI1-I.2-II.1_NLS</i>	This paper	N/A
<i>pcDNA5_FRT_TO_eGFP_s-tag_UBQLN1_dSTI1-II_NLS</i>	This paper	N/A
<i>pcDNA5_FRT_TO_eGFP_s-tag_UBQLN1_dUBA_NLS</i>	This paper	N/A
<i>pcDNA5_FRT_TO_eGFP_s-tag_UBQLN1_dUBL_NLS</i>	This paper	N/A
<i>pcDNA5_FRT_TO_MYC_UBQLN1</i>	This paper	N/A
<i>pcDNA5_FRT_TO_FLAG_EV</i>	This paper	N/A
<i>GFP-Rad51 in pEGFP-c1</i>	Essers Lab, Rotterdam	N/A
<i>pCVL.SFFV.Kozak.HA.NLS.SceOpt.IRES.BFP</i>	Addgene	#45574
<i>pgLAP1_GFP-UBQLN1</i>	This paper	N/A
<i>pMD2.G</i>	Addgene	#12259
<i>psPAX2</i>	Addgene	#12260
Software and Algorithms		
Adobe Illustrator	Adobe	
Adobe Photoshop CS4 (DNA fiber analysis)	Adobe	
Anaconda v2.0.4	Anaconda Inc.	
Benchling	Benchling	
BWA v0.7.17	Li and Durbin, 2009 ³⁸	
Cellomics software package (Colocalization V.4 Bioapplication)	Thermo Scientific	
Comet Score software	TriTek	
CTAn v.1.18	Bruker	
Cellomics HCS Studio	Version 6.6.3	Build 8568
ComplexHeatmap v2.22.0		
Cutadapt v3.4		
DAVID v2.1	Extron	
DESeq2 v1.48.0		
Docker	Docker Inc.	
Dupradar v1.28.0		
FASTQC v0.12.1		
FIJI v1.53c	FIJI	
GATK v3.7	Broad Institute	
ImageJ software with the PZfociEZ plugin	Wayne Rasband	
ImageLab	Bio-Rad	
MaxQuant v1.6.1.0	Cox et al., 2011	
Microsoft Office 365	Microsoft	
MultiQC v1.19		
mutSigExtractor		
NDP.view2 vU12388-02	Hamamatsu	
nextflow v23.10.1		

Supplementary Data – Manuscript III

nf-core/rnaseq pipeline v3.14.0		
NIS ELEMENTS	Nikon	
NRecon	Bruker	
Picard v2.26.0	Broad Institute	
PRISM	Graphpad	
PyRat	Scionics	
Python v3.6.5	Python Software Foundation	
R library BSgenome.Mmusculus.UCSC.mm10		
R v4.4.1	The R Foundation	
R Studio v2024.09	Posit Software, PBC	
data.table v1.16.0		
gridExtra v2.3		
tidyverse v2.0.0		
RSeQC v5.0.2		
Salmon v1.10.1	Patro et al., 2017 ³⁹	
SigProfilerAssignment v0.1.7		
SigProfilerMatrixGenerator v1.2.26		
Spyder v2.26.0	Spyder Website Contributors	
STAR v2.7.9a		
Trim Galore v0.6.7		
Tximport v 1.12.0		
Qualimap v2.3		
Other		
Glass Bottom dishes 35mm	MatTek	P35G-0-10-C
96-well solid white flat-bottom polystyrene TC-treated microplates	Corning	3917
Cell culture 96-well, clear flat-bottom microplates	Greiner	655090
Mini-PROTEAN TGX Stain-Free Gels 10%	Bio-Rad	4568036

Cell Culture and Maintenance

Human patient dermal fibroblasts (HDFs), U2OS, HEK293, and murine embryonic fibroblasts (MEFs) were grown in Dulbecco's Modified Eagle Medium with GlutaMAX (DMEM, ThermoFisher Scientific) containing 1 % Penicillin/Streptomycin (P/S, ThermoFisher Scientific) and 10 % fetal bovine serum (FCS, ThermoFisher Scientific). Lung adenocarcinoma cell lines were grown in Gibco Roswell Park Memorial Institute 1640 medium (RPMI 1640, ThermoFisher Scientific) containing 10 % FCS and 1 % P/S. Cells were grown at 37 °C and 5 % CO₂.

Passaging Cells

Cells were passaged by washing with 5 ml of 1x DPBS (ThermoFisher Scientific) and trypsinization with 1 ml of 0.25 % trypsin (ThermoFisher Scientific) at 37 °C. The appropriate volume of cell suspension was added to a new dish for maintenance or experiments.

Calculation of Seeding Density

Trypsinized cells in suspension were mixed 1:1 with 0.4 % Trypan blue (ThermoFisher Scientific). Samples were loaded into a cell counting Neubauer chamber slide and measured by an automated cell counter (CytoSMART, Corning Life Sciences).

Transient transfection of human cell lines

Cells were plated at a density of 6×10^6 cells in a 10 cm dish containing 10 ml of DMEM medium without P/S and incubated at 37 °C overnight. On the day of transfection, the indicated amount of DNA was diluted in 300 µl of Opti-MEM. In a separate tube, Lipofectamine 2000 was diluted in 300 µl of Opti-MEM to a 2:1 Lipofectamine/DNA ratio. After 5 min of incubation at RT, the diluted DNA was combined with the diluted Lipofectamine 2000 and incubated for 20 min at RT to allow the DNA-Lipofectamine 2000 complexes to form. DNA-Lipofectamine 2000 complex was added dropwise to the cells. The medium was replaced 5h after transfection and the cells were incubated at 37 °C for 48h. Alternatively, the respective DNA was diluted in 500 µl of 250 mM CaCl₂ and vortexed, while 500 µl of 2x HEPES-buffered saline (42 mM HEPES; 273.8 mM NaCl; 10 mM KCl; 1.76 mM Na₂HPO₄·H₂O; 0.2 % glucose) was added dropwise. The mixture was then added dropwise to the cells, medium was replaced 24h after transfection, and cells were incubated at 37 °C for 48h.

Generation of constitutively expressing Flp-In cell lines

The Invitrogen™ Flp-In™ system was used for the integration and constitutive expression of the gene of interest in mammalian cells. One day prior to transfection, HEK293 Flp-In or U2OS Flp-In cells were plated at a density of 5×10^6 cells in a 10 cm dish containing 10 ml DMEM medium without antibiotics and incubated overnight at 37 °C. On the day of transfection, 1 µg of expression plasmid containing an FRT site linked to the hygromycin and blasticidin resistance genes and 9 µg of Flp recombinase expression plasmid pOG44 were diluted in 300 µl of Opti-MEM. Cells were transfected as described above to allow Flp recombinase mediated DNA recombination at the FRT site in the cells. The cells were then incubated at 37 °C in 10 cm dishes and selected over the following days using 100 µg/ml hygromycin and 15 µg/ml blasticidin for colony expansion.

Small interfering RNA (siRNA) mediated knockdown of cells

Cells were seeded at a density of 1×10^6 in a 10 cm dish containing 10 ml of DMEM medium without antibiotics and incubated at 37 °C overnight. On the day of siRNA transfection, 1 µM of siRNA diluted in Opti-MEM was combined with 15 µl Dharmafect diluted in 235 µl of Opti-MEM and incubated for 25 min at RT. After incubation,

the siRNA-Dharmafect complex was added dropwise to the cells to allow siRNA mediated knockdown. The cells were then incubated at 37 °C in 10 cm dishes for 2 days until they were subjected to experiments.

SDS-PAGE and Immunoblotting

Sample Preparation

Cells were collected as described above. The cell suspension was centrifuged at 300 g for 5 min to obtain cell pellets. The cell pellets were washed thrice in 1x DPBS and lysed by resuspension in 50-100 µl sodium dodecyl sulfate (SDS) lysis buffer or RIPA-buffer, sonicated in the Bioruptor (Diagenode Inc.) using the following protocol: 30 sec ON, 30 sec OFF, 10 cycles at 4 °C; or additionally sonicated manually. SDS-lysed samples were heated at 95 °C for 10 min and quantified via DC assay.

SDS-PAGE and Immunoblotting

The obtained lysates were quantified using the DC assay (Bio-Rad Laboratories Inc.). Sample absorbance was quantified using the Tecan Spark 10M Luminescence Multi-Mode Microplate Reader (Tecan). Samples were diluted in Laemmli buffer, heated at 95 °C for 10 min, and loaded on an SDS polyacrylamide gel (5 % stacking gel, 10 % or 4-20 % gradient separating gels). The Mini-PROTEAN Tetra Cell immunoblotting system (Bio-Rad Laboratories Inc.) was used for gel casting, electrophoresis, and transfer. Upon system assembly, samples were loaded and electrophoretically separated at 80 V through the stacking gel and at 100 V through the separation gel, followed by either overnight wet transfer or Trans-Blot Turbo Semi Dry Blotting onto a PVDF membrane (Merck Millipore). Wet transfer was carried out at 100 mA for 18h. Semi-dry transfer was acquired at 25 V for 7 min. Membranes were washed in 1x TBS-T and blocked in 5 % BSA in TBS-T for 1h. For immunostaining, the membranes were incubated overnight with primary antibodies diluted in 5 % BSA at 4 °C. After three washes in 1x TBS-T for 10 min each, the membranes were incubated with Horseradish Peroxidase (HRP) conjugated secondary antibody diluted in 5 % BSA at RT for 1h, following another three washes. ECL Prime Blotting Detection Reagent (Merck Millipore) was used for protein detection using the ChemiDoc Imaging System (Bio-Rad Laboratories Inc.).

SDS-PAGE Signal Quantification

SDS-PAGE band signal intensity and background intensity were measured using square regions of interest in FIJI. Intensity values were normalized for the background signal in the same ROI size above the protein signal of interest.

Cell cycle synchronization

U2OS cells of indicated genotypes were plated at a density of 2×10^6 cells in a 10 cm dish containing 10 ml of DMEM medium, 1 % P/S and 10 % FCS and incubated at 37 °C overnight. Next day, 2 mM Thymidine was added onto the dishes and after 16h, cells were released from Thymidine by washing twice with DPBS and adding fresh DMEM medium containing 1 % P/S and 10 % FCS. After 8h of release, cells were again treated with 2 mM Thymidine and after 16h, cells were released again and harvested at the indicated time points either for a subsequent cycloheximide chase assay or fixed in ethanol for cell cycle analysis via flow cytometry.

Fixation with EtOH and propidium iodide staining for cell cycle analysis

Cells were first washed in 1x DPBS, trypsinized, and centrifuged at 300 g for 5 min at RT. The pellet was then thoroughly resuspended in 300 µl 1x DPBS and finally, 2.5 ml of 70 % ice-cold ethanol was added dropwise. The sample was then vortexed and stored at -20 °C for up to 1 week but at least 1h prior to staining. Fixed cells were washed twice in ice-cold 1x DPBS (5 ml and 1 ml), with the centrifugation steps at 17000 g for 5 min at 4 °C. After the second centrifugation, the sample was resuspended in 200-500 µl of 1x DPBS (according to the pellet size), 10 µl of propidium iodide (PI) and 0.5 µl of RNase A (ThermoFisher Scientific) were added per sample. Finally, all samples were incubated in the dark at 37 °C, shaking at 900 rpm, for approximately 40 min and subsequently taken for flow cytometry analysis using FACS CANTO II (BD Biosciences).

Cycloheximide chase assays

U2OS cells of indicated genotypes were synchronized via double-Thymidine-block as described above. Then, cells were treated with CHX and NCS at the indicated timepoints. For cellular fractionation, cells were chemically fractionated using the subcellular protein fractionation kit according to the manual (Thermo Fisher Scientific).

Immunoprecipitation

For GFP-Trap experiments, HEK293 cells were transfected with respective plasmids, synchronized when indicated, treated, and harvested. Cells were then incubated in lysis buffer (20 mM HEPES, pH 7.9, 300 mM NaCl, 2.5 mM MgCl₂, 0.5 % IGEPAL, 20 % glycerol, 10 µM PR-619, 10 mM N-ethylmaleimide, DNase I, phosphatase inhibitor tablets, and complete protease inhibitor cocktail) for 1h at 4 °C with rotation. The resulting cell lysates were pre-cleared at 15.000 g for 10 min at 4 °C and the concentration of samples was measured via DC assay. Equal amounts of protein were incubated with GFP-Trap magnetic particles at 4 °C for 1h. The resulting complexes were washed 5 times with wash buffer (10 mM HEPES, pH 7.9, 150 mM NaCl, 0.5 % IGEPAL, 0.5 mM EDTA, 10 µM PR-619, 10 mM N-ethylmaleimide, phosphatase inhibitor tablets, and protease inhibitor cocktail) and analyzed by SDS-PAGE. For endogenous RAD51-IP, HEK293 cells were transfected, treated, harvested and subsequently incubated with lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM MgCl₂, 1 % IGEPAL, 5 % glycerol, 10 µM PR-619, 10 mM N-ethylmaleimide, DNase I, phosphatase inhibitor tablets and complete protease inhibitor cocktail) for 1h at 4 °C with rotation. Lysates were then clarified by centrifuging at 13000 g for 10 min at 4 °C and protein concentration was measured via DC assay. Equal amounts of protein were incubated with RAD51 primary antibody or rabbit isotype control at 4 °C overnight with rotation. Next day, the immunocomplexes were

incubated with Protein G magnetic beads at 4 °C for 1h. Finally, samples were washed 5 times in wash buffer (20 mM HEPES, pH 7.9, 150 mM NaCl, 0.5 mM EDTA, 0.5 % IGEPAL, 10 % glycerol, 10 µM PR-619, 10 mM N-ethylmaleimide, phosphatase inhibitor tablets and complete protease inhibitor cocktail), eluted into Laemmli buffer via heating at 95 °C for 10 min and analyzed by SDS-PAGE.

For HIS-IP, HEK293 cells were transfected with 6 µg HIS-Ubiquitin plasmid and synchronized via double-Thymidine-block prior to harvest. Cells were then incubated in lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM Na₂HPO₄, 150 mM NaCl, 0.5 % IGEPAL, 10 mM imidazole, 8 M Urea, 10 µM PR-619, 10 mM N-ethylmaleimide, phosphatase inhibitor tablets, and protease inhibitor cocktail) for 1h at 4 °C with rotation and subsequently sonicated on ice with a manual probe. The resulting cell lysates were pre-cleared at 13000 g for 10 min at 4 °C and the concentration of samples was measured via DC assay. Equal amounts of protein were incubated with Ni-NTA magnetic agarose beads at 4 °C for 2h. The resulting complexes were washed 4 times with wash buffer 1 (same as lysis buffer but containing 20 mM imidazole), then washed once with wash buffer 2 (same as lysis buffer but containing 40 mM imidazole), and finally eluted into Laemmli buffer containing 200 mM imidazole and analyzed by SDS-PAGE.

Recombinant protein production

BL21 -CodonPlus®(DE3)-RIL competent cells (NEB, Cat# C2530H) were transformed with the *pTH_FLAG-UBQLN1-6xHis*, consisting of the pET-21b(+) expression vector backbone with *FLAG-UBQLN1* inserted in the multiple cloning site, according to the manufacturer's instructions. After transformation, a 50 mL starting culture was grown in LB medium supplemented with 1:1000 ampicillin at 37 °C/300 rpm overnight. The next day, two liters of LB-medium with 1:1000 ampicillin were inoculated with the starting culture at a 1:200 ratio. The expression culture was grown at 37 °C/300 rpm until an OD₆₀₀ of 0.4 – 0.8 was reached, measured by photometric analysis with the Thermo Genesis 30 (Thermo Scientific). To induce protein expression 0.5 mM IPTG was added, and protein expression was carried out at 18°C/300 rpm overnight. The next day, bacteria were harvested at 5000 rpm at 4 °C for 30 min. Then, 5 ml of lysis buffer (50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, 10 mM imidazole), supplemented with 1 mg/ml lysozyme and 2x proteinase inhibitor cocktail, was added per gram of wet pellet. The pellet was resuspended in lysis buffer and incubated on ice for 30 min. Subsequently, cells were lysed by sonication using the SONOPLUS (Bandelin Electronic GmbH & Co.KG) at 30 % amplitude with five repetitions of pulses set to 1 second ON/ 1 second OFF for 20 seconds, followed by a 20-seconds pause. Lysates were cleared by centrifugation at 10000 g at 4 °C for 1h and the supernatant was collected and stored on ice. For affinity purification, 500 µl settled resin of His-Pur™ His-Ni-NTA beads (Thermo Scientific) were transferred into a 5 mL polypropylene column (Qiagen) and washed with 3 column volumes of water, allowing the water to drain by gravity flow. To equilibrate the column, this step was repeated with 3 CVs of Ni-NTA wash buffer (50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, 20 mM imidazole). The cleared supernatant was then loaded onto the closed column, and the supernatant-bead suspension was incubated for 1h at 4 °C with gentle shaking. After incubation, the supernatant was allowed to drain from the column by gravity flow and the column was washed with 15 CVs of ice-cold Ni-NTA wash buffer. Elution was performed by adding 1 mL of Ni-NTA elution buffer (50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl, 250 mM imidazole) to the closed column, followed by 2 min incubation at room temperature. The eluate was collected by opening the column. This process was repeated seven times, and the collected elution fractions were analyzed for protein content by SDS-PAGE and Coomassie staining. Suitable fractions were combined and buffer-exchanged to storage buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM DTT) using PD10 columns (Cytiva, Cat#17085101), according to the manufacturer's instructions. Finally, 5 % (v/v) glycerol was added to the buffer-exchanged protein, and the protein was frozen in liquid nitrogen and stored at -80 °C until further use. ⁴¹

Co-immunoprecipitation of recombinant proteins

Purified *FLAG-UBQLN1-HIS* recombinant protein was incubated with equimolar amounts of recombinant RAD51 or purified HIS-GFP and diluted to 100 µl in dilution buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA). 10 µl were removed as input from each reaction before diluting to 500 µl in dilution buffer. Each reaction was incubated for 2h at 4 °C with end-to-end rotation. After 2h, 15 µl of DYKDDDDK Fab-Trap® Agarose beads were equilibrated in dilution buffer, added to each reaction, and incubated for 1h at 4 °C with end-to-end rotation. Beads were washed twice in wash buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.05 % IGEPAL). For the third wash, a high salt wash buffer (10 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.5 mM EDTA, 0.05 % IGEPAL) was used, and the supernatant was transferred to fresh tubes before centrifuging and discarding. Samples were eluted from the beads in 2x SDS sample buffer by heating at 95 °C for 5 min and analyzed by SDS-PAGE. Recombinant *FLAG-UBQLN1-HIS*, RAD51, and GFP-His were also incubated with the Fab-Trap® Agarose beads alone as controls.

In vitro ubiquitylation reaction

1 µM purified recombinant human CHIP along with equimolar concentration (unless stated otherwise) of recombinant HIS-RAD51 was mixed with E1 activating enzyme (100 nM), E2 conjugating enzyme (1 µM) (UBE2D2), human ubiquitin (50 µM), ATP energy regenerating solution, and ubiquitin conjugation reaction buffer. Samples were incubated at 37 °C for 2h, and reactions were terminated by adding EDTA to a final concentration of 20 mM.

Cell viability measurement

HEK293 cells were transfected with respective plasmids. Cells were plated into 96-well plates at 3500 cells/well. 24h later, cells were treated with 100 ng/ml NCS for 96h. After the incubation, room-temperature CellTiter-Glo® Reagent (Promega, USA) was added 1:1 to each well and the plates were incubated at room temperature for

10 min. Luminescence was measured with the Tecan Infinite M1000 Pro (Tecan, Männedorf, CHE) and normalized against control cells treated with vehicle solution.

High-content Imaging

High-content imaging (HCI) was performed on U2OS cells, MEFs, and HDFs using a Thermo Fisher Cellomics Cell Insight CX7 LZR Pro equipped with a 7-color laser light source. Images of 1104 × 1104 pixels (2x2 binned) were acquired with the 20x objective (Olympus microscope, NA = 0.45) and using the long-wave laser autofocus. The images were analyzed with the Cellomics HCS Studio software package version 6 using either the SpotDetectorV4.1 or ColocalizationV.4 bio-applications. Images of all channels were background corrected (3D surface fitting, value = 50). Hoechst 34580 stained cell nuclei were identified after uniform smoothing (value = 2), fixed thresholding (value = 500-1500 depending on cell model), and segmentation (value = 10-20 depending on cell model) using the following object selection parameters: size = 50–2000 μm^2 , ratio of perimeter squared to 4π area = 1–5, length-to-width ratio = 1–5, average intensity = 400–10000, total intensity: $2 \times 10^6 - 5 \times 10^7$. Foci of γH2AX , RAD51, and Ubiquitin were quantified within the nuclear region at another excitation wavelength (485 ± 20 nm or 650 ± 20 nm). Spot detection was performed after fixed thresholding (value = 500–800 depending on the staining intensity) using the spot detection method 'Box' (value = 3) in the SpotDetectorV4.1 bio-application or 'Spatial' (value = 2) in the ColocalizationV.4 bio-application. Object selection parameters for foci were size: 0.3–30 μm^2 , ratio of perimeter squared to 4π area: 1–5, length-to-width ratio: 1–5, average intensity: 500–15000, total intensity: $100 - 1 \times 10^6$. To determine the cell cycle stage, we analyzed the distribution of the total Hoechst 34580 intensity using a custom R script. In brief, four thresholds were adjusted manually to gate cells in 2N (G0/G1), 2-4N (S), and 4N (G2/M) stages, respectively. Cells in the G0/G1 phases possess a normal diploid DNA content (2N) whereas cells in G2/M contain exactly twice this amount (4N). As DNA is synthesized during S-phase, cells are found with a DNA content ranging between 2N and 4N. The gates were then applied to evaluate the mean number of foci in cells of the respective cell cycle stage. Single cell data were further processed and plotted using R packages. High content screening kinetic experiments were analyzed with R using ordinary two-way ANOVA. High-content imaging results are means $\pm$ standard deviation; n = 3-4 wells; > 2000 cells/well; Two-way ANOVA with Bonferroni's test. *p < 0.05; **p < 0.01; ***p < 0.001.

DSB repair pathway assays

DR-GFP

U2OS cells bearing a single copy integration of the DR-GFP were used to analyze HR capacity⁴². Cells were plated at 40 % confluency in 10 cm dishes. The next day, cells were transfected with the indicated siRNA or with the indicated plasmids. Medium was changed 5h after transfection and after 24h, cells were transduced with lentivirus containing IScel.IRES.BFP for an additional 24h. The following day, cells were then harvested and washed three times with DPBS and three times with DPBS containing 1 % BSA and stained with propidium iodide. Afterwards, BFP and GFP fluorescence were acquired using FACS with the FACS CANTO II (BD Biosciences, New Jersey, USA). For the gene conversion, the ratio of both BFP and GFP double-positive cells was normalized relative to siNT or empty vector controls to facilitate comparability between experiments.

SA-GFP SSA reporter

U2OS cells bearing a single copy integration of the reporter SA-GFP (SSA)¹⁹ were used to analyze SSA pathway usage. 4000 cells were plated in 96-well plates. One day after seeding, they were infected with a lentivirus harboring an I-SceI and labeled with Blue Fluorescent Protein (BFP) using an M.O.I (multiplicity of infection) of 5. Six hours after infection the same volume of fresh medium was added. Cells were grown during 48h, fixed with 4% paraformaldehyde, stained with Hoechst and washed with phosphate buffered saline (PBS) prior visualization with a fluorescent microscope for blue and green fluorescence. The repair frequency was calculated as the percentage of blue cells expressing Green Fluorescent Protein (GFP). To facilitate the comparison between experiments, this ratio was normalized with a control treated with dimethyl sulfoxide (DMSO). Data represent a minimum of three sets of duplicated experiments.

Single Molecule Analysis of Resection Tracks

Single Molecule Analysis of Resection Tracks (SMART) was performed as previously described¹⁸. For that purpose, U2-OS cells transfected with *FLAG-ev* or *MYC-UBQLN1* construct were cultured in the presence of 10 μM bromodeoxyuridine (BrdU) for 24h, irradiated with 10 Gy of IR, and harvested 1h later. Cells were embedded in low-melting agarose and in-gel DNA extraction followed. To stretch the DNA fibers, silanized coverslips were dipped into the DNA solution for 15 min and pulled out at constant speed (250 $\mu\text{m/s}$). Coverslips were baked for 2h at 60°C and incubated without denaturation with an anti-BrdU mouse monoclonal antibody. After washing with DPBS, coverslips were incubated with a secondary antibody. The coverslips were mounted with ProLong® Gold Antifade Reagent (Molecular Probes, Oregon, USA) and stored at -20°C. DNA fibers were observed using a Nikon NI-E microscope under a PLAN FLOUR40 × /0.75 PHL DLL objective. The images were recorded and processed using NIS ELEMENTS Nikon software. For each experiment, at least 200 DNA fibers were analyzed, and the length of DNA fibers was measured using Adobe Photoshop CS4 Extended version 11.0 (Adobe Systems Incorporated). Experiments were repeated independently four times. Statistical significance in these experiments was determined with the paired Student's t test.

Laser microirradiation and imaging of cells

U2OS or HEK293 cells expressing ectopic, GFP-tagged proteins were plated on glass bottom dishes (Ibidi) and pre-sensitized as indicated in the figure legends. The dishes were transferred into a microscope top-stage incubator

equipped with a control system for gas mixture and humidity. DNA damage was induced on an Olympus SpinSR confocal microscope (Olympus Corporation) using a 405 nm diode laser focused through an HC PL APO 63X, 1.4-numerical aperture oil immersion objective and microscope settings as indicated in the figure legends. Images were acquired using the same system. Signal quantification was performed in the CellSens imaging software (Olympus Corporation) and further processed in Microsoft Excel and GraphPad PRISM.

Patients

Written informed consent was obtained from the *UBQLN1-deficiency syndrome* family. The family was part of a cohort with neurogenetic disorders and homozygous loss of function variants in candidate genes⁴³. The study was performed according to the Declaration of Helsinki protocols. Ethical approval was obtained from Baylor College of Medicine under the protocol H-29697.

Patient A-II-1

The 19-year-old female individual (A-II-1) presented with intellectual disability and developmental delay (DD). She had frequent upper respiratory tract infections and a normal brain MRI. She displayed an early loss of teeth. The examination further revealed dolichocephaly and severe microcephaly with an occipital-frontal head circumference (OFC) of 49 cm (-5.8 SD). Facial dysmorphism consisted of low-set and simple ears, hypertelorism and a thin upper lip. Further, she had central hypotonia, strabismus, and a beginning of hypochromic anemia. Of note, the examining geneticist described no cancer or skin abnormalities.

Patient A-II-4

The male individual A-II-4 was examined at 10 years of age. He presented with ID, DD, and also with severe macrocephaly (OFC: 50 cm (+4.7 SD)). Facial dysmorphism consisted of a high forehead, deep-set eyes, hypertelorism, short nasal bridge with anteverted nares, a thin upper lip and low-set and posteriorly rotated ears. He showed autistic features with limited eye contact, and stereotypic movements of hands. He had epilepsy, frequent respiratory tract infections, and early loss of teeth. Brain MRI revealed bilaterally dilated ventricles and Arnold-Chiari malformation. He had no history of cancer and skin anomalies.

Sanger Sequencing

Sample DNA was extracted from fibroblasts using the DNeasy Blood&Tissue Kit (Qiagen). The sequence of interest was amplified by PCR. The band of the relevant PCR product size was excised and DNA was purified using the QIAquick Gel Extraction Kit (Qiagen). The DNA concentration was measured on a NanoDrop (ThermoFisher Scientific). 50 ng of DNA and 10 µM sequencing primer (human: GGCGCCAACTGATATGTAAG; mouse: TCACGGTGAAGACGCCTAAAGA) were mixed 1:1 in 10 µl and samples were submitted to Eurofins for Sanger Sequencing with the LightRun Protocol. The results were analyzed with the Benchling Alignment Tool.

Whole-Exome-Sequencing Analysis of Patients

Genomic DNA was extracted from patient dermal fibroblasts using the DNeasy Blood&Tissue Kit (Qiagen, Hilden). The sequencing was acquired at CeGaT GmbH using Twist Human Core Exome Plus kit (Twist Bioscience) for library preparation and NovaSeq6000 with 2x100 bp read length and 10 Gb output. Raw reads were aligned to the GRCh37 reference genome with BWA-MEM, and variants were called by GATK HaplotypeCaller in gVCF mode, then merged through joint genotyping and refined with Variant Quality Score Recalibration. The resulting variants were functionally annotated in ANNOVAR, incorporating RefSeq gene models, ExAC/gnomAD population frequencies, and in-silico impact scores from MetaRNN⁴⁴.

To isolate disease-relevant changes, nine sequential filters were applied. First, only variants with high technical confidence (genotype quality ≥ 99 and quality-by-depth > 10) were retained. Second, common polymorphisms were removed by restricting the ExAC_ALL allele frequency to below 1 %. Third, the analysis was limited to exonic and canonical splice-site positions. Fourth, only variants predicted not to be "tolerated" by MetaRNN were kept. Fifth, a segregation filter preserved variants present in every affected individual, and a sixth filter required that those variants show identical zygosity across the affected pair. Subsequently, any variant detected in the unaffected relatives with the same zygosity was discarded. Finally, we excluded variants with a frequency above 0.01 within gnomAD_r2_1, because of varying coverage within gnomAD populations and potential biases, we performed this step at the end. To this end, we extracted a total of 12 heterozygous variants (1 frameshift_deletion, 1 frameshift_insertion, 7 nonsynonymous_SNV and 3 stopgains), together with 3 homozygous alterations: 1 frameshift_deletion in *UBQLN1*^{c.377delA} predicted to be truncating; and 2 nonsynonymous_SNV.

Patient fibroblasts RNA-Sequencing Analysis

RNA was extracted from patient dermal fibroblasts using the RNeasy Mini Kit (Qiagen). 3' mRNAseq was acquired with Lexogen QuantSeq, Single-Read 1x50 bp. FPKM expression values were computed based on the RefSeq reference transcriptome. RNA copy numbers were collected from the alignments.

TurboID proximity labelling assay and mass spectrometry analysis

HEK293 cells were seeded to a target density of 70-90 % and transfected with pcDNA3 expression vectors encoding for TurboID or the *TurboID-UBQLN1* fusion constructs the next day. 48h after transfection, cells were treated for 10 min with 50 µM biotin. Subsequently, cells were harvested and snap-frozen in liquid nitrogen. Part of the sample was used to verify TurboID expression and biotinylation via immunoblot. After confirming both, pellets were lysed in TurboID lysis buffer supplemented with 2 % Triton-X 100 and sonicated using the Bioruptor Plus (Diagenode)

with 20 cycles, 30 sec pulses at 4 °C. Afterwards, sample volume was increased by adding 50 mM Tris-HCl pH 7.4 and sonicated for a further 10 cycles before clearing the lysate via centrifugation at 17000 g at 4 °C for 10 min. Equilibrated Dynabeads MyOne Streptavidin C1 (Invitrogen) were added to the cleared lysate and incubated at 4 °C overnight. Beads were washed three times each with wash buffer 1, wash buffer 2, wash buffer 3 and 50 mM Tris-HCl pH 7.4 and proteins were eluted in elution buffer while shaking at 1200 rpm at 24 °C for 1h. Overnight digestion was performed by adding 5 mM 2-chloroacetamide (CAA) and 5 ng/μl trypsin at 28 °C. Peptides were separated on a 40 cm, 75 μm internal diameter packed emitter column (Coann emitter from MS Wil, Poroshell EC C18 2.7micron medium from Agilent) using an EASY-nLC 1200 (Thermo Fisher Scientific). The column was maintained at 50 °C. Buffer A and B were 0.1 % formic acid in water and 0.1 % formic acid in 80 % acetonitrile respectively. Peptides were separated on a segmented gradient from 6% to 31% buffer B for 57 min and from 31 % to 44 % buffer B for 5 min at 300 nl/min. Eluting peptides were analyzed on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). Peptide precursor m/z measurements were carried out at 60000 resolution in the 350 to 1500 m/z range. The most intense precursors based on TOP Speed with charge state from 2 to 7 were selected for HCD fragmentation using 27 % normalized collision energy. The cycle time was set to 1 sec. The m/z values of the peptide fragments were measured at a resolution of 50000 using an AGC target of 2x10⁵ and 86 ms maximum injection time. Upon fragmentation, precursors were put on a dynamic exclusion list for 45 sec. The raw data were analyzed with MaxQuant version 1.6.1.0. Peptide fragmentation spectra were searched against a fasta database of Homo Sapiens (proteome ID UP000005640, reviewed and unreviewed, downloaded September of 2018 from UniProt). Methionine oxidation and protein N-terminal acetylation were set as variable modifications; cysteine carbamidomethylation was set as fixed modification. The digestion parameters were set to “specific” and “Trypsin/P.” The minimum number of peptides and razor peptides for protein identification was 1; the minimum number of unique peptides was 0. Protein identification was performed at a peptide spectrum matches and protein false discovery rate of 0.01. The “second peptide” option was on. Successful identifications were transferred between the different raw files using the “Match between runs” option. Label-free quantification (LFQ) was performed using an LFQ minimum ratio count of 2. LFQ intensities were filtered for at least 2 valid values in at least one group and imputed from a normal distribution with a width of 0.3 and down shift of 1.8. Differential expression analysis was performed using limma, version 3.34.9 in R, version 3.4.3.

The Cancer Genome Atlas (TCGA) / Genotype-Tissue Expression (GTEx) analysis

Both TCGA and GTEx RNA-Seq datasets raw counts were processed and normalized together using the DESeq2 pipeline from UCSC Xena (<https://xena.ucsc.edu/>), which used the UCSC Toil RNA-Seq recompute method⁴⁵. OS, DSS, and PFS data for TCGA patients were obtained from a previous study⁴⁶. The `surv_cutpoint` function from the R package `survminer` was utilized to stratify patients into high-*UBQLN1* (Q4) and low-*UBQLN1* (Q1) mRNA expression level using quartiles groups (Q1, Q2, Q3, and Q4).

The clinical information, RNA-Seq, and genomic for TCGA-LUAD datasets were obtained from cBioPortal for Cancer Genomics (<https://www.cbioportal.org>, accessed November 2024). Normalized counts data was obtained from the TCGA-LUAD (Firehose Legacy, n = 586). For survival analysis, patients from the TCGA-LUAD dataset were excluded from analysis: if the tumors were primary recurrent tumors (n = 2); if the patients received any neoadjuvant chemotherapy (NAC, n = 3); if there was no information about previous NAC treatment (n = 64) or M status (n = 4); or if there were no RNA-Seq data available (n = 7). The `surv_cutpoint` function from the R package `survminer` was utilized to stratify patients into high-*UBQLN1* (Q4) and low-*UBQLN1* (Q1) mRNA expression level using quartiles groups (Q1, Q2, Q3, and Q4). All statistical analyses and visualizations were conducted using R (version 4.3.2). Survival analyses, including the cutpoint determination and Kaplan-Meier plot generation, were performed using the `survminer` (version 0.4.9), `survival` (version 3.7.0) and `ggsurvfit` (version 1.0.0) packages.

For tumor mutational burden (TMB) analysis, TCGA patients were kept in the quartiles described above. Patients with available TMB data were included in the comparison. All groups were filtered for mutations in major DNA repair genes, which led to the exclusion of two patients in *UBQLN1* Q1 due to a mutation in *RAD50*, or *MRE11* and *BRCA2*, respectively.

CODEFACs analyses

The LUAD scRNA-Seq dataset used as reference signature matrix for cell type deconvolution is publicly available (GSE131907)⁴⁷. The reference signature matrices containing 22 cell types were applied to the TCGA-LUAD public datasets using CODEFACs²¹. A total of 506 LUAD patients with RNA-Seq data for FFPE tissue samples were imputed to estimate the cellular abundances and gene expression profiles in each cell type. Patients were stratified based on the median value of *UBQLN1* mRNA levels before deconvolution into high-*UBQLN1* and low-*UBQLN1* groups. OS and DFS were evaluated by using Kaplan-Meier methods and significant differences were assessed using the Log-rank test using the R package `survival` (3.5-7) and `survminer` (0.4.9).

Licensing of animal experiments

The mouse experiments were licensed by the State Agency for Nature, Environment and Consumer Protection (LANUV) under the licenses 81-02.04.2021.A097, 81-02.04.2019.A335, and 81-02.04.2020.A281. Breeding of mouse lines was permitted by the State Agency for Nature, Environment and Consumer Protection (LANUV) under the license VSG 81-02.04.2019.A009 and 2024-225.

Mouse Colony Statistics

The Python-based Relational Animal Tracking software (PyRat, Scionics Computer Innovation GmbH) was used for animal documentation. The colony data was exported, plotted, and analyzed using the GraphPad Prism9 software.

C57BL/6J *Ubqln1*^{c.377delC}

The *Ubqln1*^{c.377delC} mouse line was generated by the CECAD Transgenic Core Unit using EEZy-CRISPR/Cas9 genome editing⁴⁸. The deletion of cytosine 377 of the *Ubqln1* cDNA (*c.377delC*) was acquired with the crRNA ACCAGCGTGGGGTTGCTAT and the repair template ATTGTTATTTAATTATTTACACTAGGCCGCAAGATAATTCAGCTCAGCAAACAAATGCCCTGGAAGCACTGT GACCAGCTCACCAGCTCCAGATCGAACCCACGTCTGGTTCTGCTGCTAACAGCTCCTT to recapitulate the human *UBQLN1* patient mutation. The deletion was designed to induce a frameshift that results in a change of the amino acid sequence after residue D125 and a premature STOP codon at Aa140 of exon 3 (ENSMUST00000058735); p.Ser126ArgfsTer15. For genotyping, the primers *Ubqln1_c.377delC_1fwd* (ATGCTCAGTCATTTACAGGCAAAG) and *Ubqln1_c.377delC_1rev* (AACTGCTCTTGAGGAAGAGAAC) were used. The PCR product was used in RFLP analysis with a DpnII digestion for 2h. Genotyping resulted in a wildtype band of 324 bp for wildtype animals, two bands (114+209 bp) for animals that were homozygous for the deletion, or all three bands in case of heterozygous animals. Positive founder mice were backcrossed once to eliminate mosaicism and reduce the risk of potential unintended mutations. The F1 generation was used to generate homozygous animals. F6 animals were used for phenotyping experiments.

C57BL/6J *Rosa26*^{LSL.HA-Ubqln1/wt;ACTB^{Cre}/wt}

Animals with a conditional *Ubqln1* expression (*Rosa26*^{LSL.HA-Ubqln1.IRES.GFP}) were crossed to the *ACTB*^{Cre/wt} strain, harboring a random insertion of a human beta-actin promoter constitutively expressing nuclear localizing *Cre* for constitutive overexpression of *HA-Ubqln1.IRES.GFP*.

C57BL/6J *K-Ras*^{LSL.G12D/wt}; *Trp53*^{fl/fl} and C57BL/6J *Rosa26*^{LSL.HA-Ubqln1.IRES.GFP}; *K-Ras*^{LSL.G12D/wt}; *Trp53*^{fl/fl}

We employed the *K-ras*^{LSL.G12D/wt}; *Trp53*^{fl/fl} mouse line as described previously²². Animals with a conditional *Ubqln1* (*Rosa26*^{LSL.HA-Ubqln1.IRES.GFP}) were combined with the KP mouse line to obtain the *Rosa26*^{LSL.HA-Ubqln1.IRES.GFP}; *K-ras*^{LSL.G12D/wt}; *Trp53*^{fl/fl} (KPU1) C57BL/6J mouse line. The *Rosa26* insertion was genotyped using the primers *Rosa26_fwd* (GCAGGAAGCACTTGCTCTC), *Rosa26_CAGS_rev* (CGACAAGGCGTCTAGTTTATGTG), and *Rosa26_rev* (GATAAAGGCTGCAGAAGGAGC).

MEF Generation

A heterozygous breeding pair was mated and the female mouse was plug-checked daily until plug detection. At E13.5, the mother was culled by cervical dislocation and the embryos were extracted from the mother. Embryos were placed in 10 cm cell culture dishes containing 1x DPBS. Placental and other maternal tissues were removed. Embryos were placed in new 10 cm dishes and covered with 1x DPBS. Further, the embryos were decapitated and the liver was removed. Head and liver were collected for genotyping of the respective embryo. The remaining embryo tissue was placed in a new 10 cm dish. 400 µl of 10x Trypsin was added and the tissue was minced using a sterile razor blade. The plates were incubated at 37 °C for 45 min. The trypsin was quenched with 10 ml of DMEM containing 10 % FCS and 1 % P/S. The medium was pipetted up and down to break up the tissue further. MEFs were grown to confluency, usually after 24-48h. Cells were frozen at P3 and freshly thawed for experiments.

Animal Housing

In the animal husbandry of the MPI for Biology of Ageing, gamma-irradiated (25 kGy) laboratory animal feed was used and drinking water was sterile-filtered. The light cycle in the enclosure was 12h light/12h dark phase, the humidity was 50 % (+/- 5 %) and the room temperature was 22 °C (+/- 1 °C). The air exchange rate in the room was 8-fold. The animals were kept in individually ventilated cages (IVC).

Lung Cancer Induction in autochthonous mouse models

To induce lung tumors in KP and KPU1 mice, 8–15-week-old mice were anesthetized via intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Upon reaching adequate surgical tolerance, mice were administered 2.5×10⁷ PFU of replication-deficient adenovirus encoding Cre-recombinase (Adeno-CMV-Cre) intratracheally, and carefully monitored during recovery. Mice were scored for general and disease specific burden and dissection upon reaching heavy burden (score 20 on score sheet).

In vivo Tumor Progression Monitoring with µCT

Instilled mice underwent µCT scans to track tumor development using a Skyscan 1176 high-resolution µCT imaging system (Bruker). Mice were anesthetized with 3 Vol. % isoflurane through an isoflurane vaporizer (Dräger). Thoracic µCT images were acquired at 475 µA and 50 kV with a 35 µm resolution using a 0.5 mm aluminum filter. The images were reconstructed using NRecon software (Bruker) and analyzed in DataViewer software for presence of tumors (Bruker).

In vivo Lung Cancer Treatment

Mice were included into the survival monitoring upon identification of a tumor in µCT. Tumor bearing mice were randomly assigned to untreated and treated groups. Compounds were prepared by dissolving the PARP inhibitor Olaparib in dimethyl sulfoxide to 50 mg/ml, followed by dilution in a 10 % 2-hydroxy-propyl-beta-cyclodextrin/DPBS solution. Olaparib was administered daily at 50 mg/kg via intraperitoneal injection. The PD1 inhibitor RMP1-14⁵⁰ was diluted in DPBS to a concentration of 50 mg/ml and given bi-weekly at a dose of 10 mg/kg intraperitoneally. Mice were scored for general and disease specific burden and weighed weekly.

3D CT Image Processing

For 3D visualization of mouse lungs and tumors, individual tumors were identified and marked as ROIs within each animal's μ CT transverse image stack. Tumors were segmented using threshold values and interpolated in Bruker CTAn software, with 3D visualizations created in Bruker's CTVox software. The analyst was blinded to genotypes and treatments during tumor ROI identification.

RNA-Sequencing of KP/KPU1 lung tumors

RNA was extracted from snap frozen KP/KPU1 tumors using the RNeasy Mini Kit (Qiagen). 3'mRNA libraries were generated from 500ng total RNA using the Lexogen QuantSeq kit. After validation (TapeStation; Agilent Technologies) and quantification (Qubit, Thermo Fisher Scientific) pools of cDNA libraries were generated. The library pools were quantified using the KAPA Library Quantification kit and the 7900HT Sequence Detection System (Applied Biosystems) and subsequently sequenced on an Illumina NovaSeq 6000 sequencing instrument using a 1x100bp protocol. The resulting data were processed using the nf-core/rnaseq pipeline (version 3.14.0) executed with Nextflow (version 23.10.1), aligning reads to the mouse reference genome (GRCm39, Ensembl release 110) using STAR (version 2.7.9a)⁵¹. Quality assessment of raw reads was performed with FastQC (version 0.12.1), and adapter trimming was conducted using Trim Galore (version 0.6.7) incorporating Cutadapt (version 3.4). Transcript quantification was achieved with Salmon (version 1.10.1), and gene-level counts were generated using tximport (Bioconductor- version 1.12.0). Quality control metrics were compiled using RSeQC (version 5.0.2), Qualimap (version 2.3), and dupRadar (version 1.28.0). Duplicate reads were marked with Picard (version 3.0.0), and comprehensive quality metrics were summarized using MultiQC (version 1.19). Differential gene expression analysis was conducted using DESeq2 (version 1.48.0) within the R environment (version 4.3.2). Gene set enrichment analysis (GSEA) was done with clusterProfiler (version 4.16.0) using KEGG pathways retrieved with msigdb (version 24.1.0) package. *R26.Ubqln1* construct expression in KPU1 tumors was confirmed by detection of the 3' EGFP sequence expression.

Whole-Genome-Sequencing of KP/KPU1 lung tumors

Upon dissection of KP and KPU1 mice at humane endpoint according to animal scoring, lungs were extracted and left lung lobes were snap frozen in liquid nitrogen, while right lobes were fixed for histology analysis. For DNA extraction, tumors were separated from lung tissue on dry ice. For each lung, one tumor and one piece of visually healthy lung was acquired. DNA extraction was conducted with the DNeasy Blood&Tissue Kit. Concentrations were determined via NanoDrop and Qubit. DNA was submitted to CeGaT for whole-genome-sequencing. Library preparation was done with TruSeq DNA Nano (Illumina). Samples were sequenced using the WGS Flex pipeline (CeGaT GmbH) on NovaSeq 6000 with 2x151 bp read length and 30X coverage.

Somatic mutation analysis of murine tumor WGS data

Whole genome sequencing data were used to identify somatic mutations in the KP and KPU1 tumors. Raw sequencing reads were aligned to the mouse reference genome (GRCm38 / mm10) using the BWA-MEM algorithm (version 0.7.13-r1126). SNV and INDEL mutation calling was performed using our data processing workflow 'peiflyne'⁵². Briefly, our workflow controls for PCR duplicates, local sequencing depth, and forward/reverse read sequencing bias to call somatic alterations. Specifically, concordant read pairs were identified and annotated to the alignment file as potential PCR duplicates, thus excluded for further analysis. Somatic alterations were then considered if 1) a variant position in respect to the reference genome was observed in the tumor sample but not in the healthy sample; 2) the forward–reverse score was below 0.2 (forward–reverse score is 1.0 if 50 % of variant reads are found on the forward or reverse read, and 0 if all variant reads are on one orientation); 3) the sequence coverage at the genomic position of the variant was > 15x for SNV and > 5x for INDELS; 4) the allelic frequency of the variant was above background sequencing errors and potential cross library contamination of the samples.⁴⁰ Finally, to exclude possible germline mutations, somatic alterations were retained if unique across all tumors.

In silico prediction of mutant peptides based on WGS data

The exonic partitions of a whole exome sequencing experiment was used to generate a library of mutant short peptides using a custom script which was programmed in R and employing the following packages within R studio. The mutant peptide library included information for missense, in-frame and frameshift mutations which can potentially generate neoantigens.⁴⁹ For missense and in-frame mutations, mutant protein sequences were trimmed to short peptide sequences to contain the mutation in a centered position flanked by 3' and 5' short sequences of 13 residues. Frameshift mutant sequences were trimmed only in 3' direction while preserving the whole frameshift in the 5' end. This trimming strategy keeps the sequence information of all possible mutant peptides (8mers-14mers) which could be generated from each mutated sequence to bind the HLA-I peptide pocket.

Colony Formation Assay

Cells were plated on 6-well plates at a density of 1000 cells per well. Treatment of the cells started after 24h with 3 μ M Olaparib in 1 % DMSO or 1 % DMSO alone as vehicle control. The cells were fixed upon colony formation after 14 days. Cells were washed with 1x DPBS. Crystal violet solution (0.4 % crystal violet, 20 % methanol) was incubated for 10 min. The fixed plates were washed several times with ddH₂O. The plates were dried and images were captured and analyzed using the GelCount cell colony counter (Oxford Optronix).

Histology analysis

Mice with tumors were sacrificed upon reaching humane endpoint and tissues were fixed in 4% PFA for 24h. Formalin-fixed paraffin-embedded (FFPE) murine lung samples were cut into 3 µm-thick sections and mounted on slides. After staining with haematoxylin and eosin (H&E), the tumors were assessed by a board-certified pathologist to evaluate the relative tumor burden.

Figures and Statistics

Except where noted, figures were made with Adobe Illustrator (Adobe Systems, San José, CA, USA). BioRender was used for graphics (Biorender.com). Statistical analyses were performed with GraphPad Prism9 for MacOS. The type of statistical analyses, parameters and number of replicates are indicated for each experiment in the figure legends. For all tests, p-value significance was defined as follows: not significant (n.s.) Significant as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Data Availability

Mass spectrometry data was deposited to the ProteomeXchange Consortium via the PRIDE partner repository and is accessible via the ID PXD064336. High-content microscopy data will be deposited to the Mendeley repository. Sequencing data will be made accessible upon reasonable request due to ethical restrictions.

Declaration of Interest

H.C.R. received consulting and lecture fees from Roche, Novartis, Takeda, Janssen, Lilly, Abbvie, AstraZeneca, Vertex, and Merck. H.C.R. received research funding from AstraZeneca and Gilead Pharmaceuticals. H.C.R. is a co-founder of CDL Therapeutics GmbH. R.D.J received consulting and lecture fees from Beigene, AstraZeneca, and Janssen. D.P. provides consulting service for Ionis and Acadia Pharmaceuticals. The remaining authors declare no competing interest.

Materials availability

The research related to human use complied with all the relevant national regulations, institutional policies, is in accordance with the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board (approval no. 22-1293 University of Cologne, approval no. 2018-268_2 from Heinrich Heine University Düsseldorf, approval no. H-29697 from Baylor College of Medicine). All unique/stable materials and reagents generated in this study are available from the lead contact with a completed material transfer agreement.

Funding

This work was funded by the Max Planck Society (to R.D.J.), the German Research Foundation (grant no. 496650118, 5504 FOR JA 2439/5-1 TP05 to R.D.J., JA2439/4-1 to R.D.J., WI 1440/13-1 to D.W., MA 6907/6-1 to T.M., BO 1549/8-1 to A.B.; grant 455784452 as part of CRC1530, with project funding for H.C.R. (A01) and R.D.J. (C02), grant no. 413326622 as part of CRC1399 to H.C.R., F.B., J.B., and M.C. (A01), grant no. 424228829 as part of CRC1430 to H.C.R. (A09)), the German Ministry of Education and Research (BMBF e:Med Consortium InCa, grant 01ZX1901 and 01ZX2201A to H.C.R.), the Ministry for Culture and Science North-Rhine-Westphalia (NW21-062A CANTAR to H.C.R. and R.D.J.), the German Research Foundation under Germany's Excellence Strategy – EXC 2030 to 390661388 and FOR 5504 project number 496650118 (to T.H.), Dr. Miriam and Sheldon Adelson Medical Research Foundation (AMRF) to D.S.B.H. and Y.S., the Behrens-Weise-Foundation (Grant for the Improvement of Human Health to R.D.J.), the Center for Molecular Medicine Cologne (Project A10 to R.D.J.), the Center for Integrated Oncology Cologne (ABCD grant to R.D.J.), the EI foundation (to R.D.J.), the Hilde Kopp foundation (to R.D.J.), the German Cancer Aid (Post-doctoral stipend by the Mildred Scheel School of Oncology to O.L., 70113307 to J.B., 1117240 to H.C.R., 70113041 to H.C.R. and F.B., 70116707 to F.B., Cancer Aid excellence Program to H.C.R.) and the Else Kröner Forschungskolleg Clonal Evolution in Cancer (C.M.G.). M.A.H. and M.T.P. are members of the Cologne Graduate School of Ageing Research. This work was supported by the DFG Research Infrastructure West German Genome Center (project 407493903) as part of the Next Generation Sequencing Competence Network (project 423957469). NGS experiments were carried out at the production site Cologne (Cologne Center for Genomics). This work has been generated within the European Reference Network on Rare Congenital Malformations and Rare Intellectual Disability (ERN-ITHACA) [EU Framework Partnership Agreement ID: 3HP-HP-FPA ERN-01-2016/739516]. The results shown here are in part based upon data generated by the TCGA Research Network: <https://www.cancer.gov/tcga>.

12.1.4 Targeting a Glutamic Acid in PDE δ with Fluoromethyl-Aryl Electrophiles Impairs KRAS Signaling

<https://doi.org/10.1021/acs.jmedchem.5c02082>