Mono-ADP-ribosylation Signaling in DNA Damage: New Tools and Biological Insights

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List of abbreviations

γH2AX	Phosphorylated form of **H2A** histone family member **X**
53BP1	p**53**-**B**inding **P**rotein **1**
ADP	**A**denosine **d**i**p**hosphate
ADPr	**ADP**-**r**ibosylation
ALC1	**A**mplified in **L**iver **C**ancer 1
AP	**Ap**urinic/apyrimidinic
APLF	**A**prataxin-**P**NK-**L**ike **F**actor
ARH	**A**DP-**R**ibosyl**h**ydrolase
ART	**A**DP-**R**ibosyl**t**ransferase
ATM	**A**taxia **T**elangiectasia **M**utated
ATP	**A**denosine **t**riphos**p**hate
ATR	**A**taxia **T**elangiectasia and **R**ad3 related
BRCA	**Br**east **Ca**ncer Susceptibility Protein
BRCA1	**Br**east **Ca**ncer type **1** Susceptibility Protein
BRCT	**BR**CA1 **C** **T**erminus
CHD2	**C**hromodomain **H**elicase **D**NA Binding Protein 2
CHD3	**C**hromodomain **H**elicase **D**NA Binding Protein 3
CHD4	**C**hromodomain **H**elicase **D**NA Binding Protein 4
CHD7	**C**hromodomain **H**elicase **D**NA Binding Protein 7
CHFR	**Ch**eckpoint With **F**orkhead And **R**ing Finger Domains
DDR	**D**NA **D**amage **R**esponse
DMSO	**D**i**m**ethyl **s**ulf**o**xide
DNA	**D**eoxyribo**n**ucleic **a**cid
DSB	**D**ouble-**s**trand **b**reak
DTX3L	**D**el**tex** **3** **L**ike, E3 Ubiquitin Ligase
ELISA	**E**nzyme-**l**inked **i**mmuno**s**orbent **a**ssay
FDA	**F**ood and **D**rug **A**dministration
FEN1	**F**lap **E**ndo**n**uclease 1
H1	**H**istone **1**
H2A	**H**istone **2A**
H2AX	**H2A** histone family member **X**
H2B	**H**istone **2B**
H_2O_2	Hydrogen Peroxide
H3	**H**istone **3**
H4	**H**istone **4**
HD domain	**H**istidine-aspartate **domain**
HPF1	**H**istone **P**ARylation **F**actor **1**
HR	**H**omologous **r**ecombination
HRP	**H**orseradish **P**eroxidase

IF	**I**mmuno**f**luorescence
MACROD1	**Macrod**omain-Containing Protein **1**
MACROD2	**Macrod**omain-Containing Protein **2**
MDC1	**M**ediator of **D**NA Damage **C**heckpoint Protein **1**
MMS	**M**ethyl**m**ethane **s**ulphonate
MRE11	**M**eiotic **Re**combination **11** Homolog A
MS	**M**ass-**S**pectrometry
NAD	**N**icotinamide **a**denine **d**inucleotide
NAM	**N**icotin**am**ide
NBS1	**N**ijmegen **b**reakage **s**yndrome **1**
NER	**N**ucleotide **E**xcision **R**epair
NH_2OH	Hydroxylamine
NHEJ	**N**on-**h**omologous **e**nd **j**oining
NMR	**N**uclear **M**agnetic **R**esonance
PARG	**P**oly-(**A**DP-**R**ibose) **G**lycohydrolase
PARP	**P**oly-(**A**DP-**R**ibose) **P**olymerase
PBZ	**P**oly(ADP-ribose)-**b**inding **z**inc-finger
POL	DNA **P**olymerase
PTM	**P**ost-**t**ranslational **m**odification
RNA	**R**ibo**n**ucleic **a**cid
RNF114	**R**i**n**g **F**inger Protein **114**
RNF146	**R**i**n**g **F**inger Protein **146**
RNF168	**R**i**n**g **F**inger Protein **168**
RNF8	**R**i**n**g **F**inger Protein **8**
SDS	**S**odium **d**odecyl **s**ulphate
SNM1A	**D**NA **c**ross-**l**ink **re**pair **1A** (**DCLRE1A**)
TARG1	**T**erminal **A**DP-**R**ibose Protein **G**lycohydrolase 1
UV	**U**ltra**v**iolet
WGR	Tryptophan-Glycine-Arginine domain
WWE	Tryptophan-tryptophan-glutamate domain
XPC	**X**eroderma **P**igmentosum Group **C** Protein
XRCC1	**X**-ray **R**epair **C**ross-**C**omplementing Protein **1**

// Summary

The repair of DNA damage is a key process to maintain the integrity of the genome and prevent dysregulation resulting in diseased states, such as cancer. To ensure efficient repair, this process is carried out by dedicated DNA repair pathways. The signaling cascade orchestrated to promote DNA repair relies not only on recruitment dynamics of specific repair factors, but also on the enzymatic synthesis of several post-translational modifications (PTMs). ADP-ribosylation (ADPr) is one of the most common PTMs in DNA repair, synthesized by the poly(ADP-ribosyl)polymerases PARP1 and PARP2. The biological and clinical relevance of this PTM is underscored by the therapeutic use of four FDA-approved PARP1/2 inhibitors against cancer. However, the chemical and structural complexity of ADPr resulted in a dearth of research tools, which severely limits our ability to study it.

In this thesis, we address this key issue by the development of new research tools and methodologies for the study of ADPr. We then leverage these tools to investigate the regulation and functional outcome of this PTM in the DNA damage response.

In the first study, we describe the development of a phospho-guided, chemoenzymatic strategy for the preparation of ADP-ribosylated peptides. We apply these peptides to develop a repertoire of new recombinant antibodies against several forms of ADPr, including site-specific and broad-specificity antibodies against mono-ADPr. We use these antibodies to discover that mono-ADPr is prevalent upon DNA damage, and describe its dependence on the hydrolases ARH3 and PARG.

In the second study, we further improve the versatility and sensitivity of our antibodies by applying affinity maturation and the SpyTag/SpyCatcher technology. We leverage these reagents to discover that, in DNA damage, ADPr forms a two-phase signaling pathway characterized by a rapid but transient poly-ADPr signal followed by a delayed, long-lived mono-ADPr signal. We find that persistent mono-ADPr on histones recruits proteins to the site of DNA damage, including RNF114, an E3 ubiquitin ligase. We investigate the functional consequence of RNF114 to discover that it acts as a mono-ADPr effector protein to modulate the DNA damage response and telomere maintenance.

Lastly, in an unpublished study, we turn our attention to aspartate- and glutamate-linked mono-ADPr. We find that, due to its high chemical lability, routine detection methods are unsuitable for the analysis of this PTM. This leads to a dramatic underestimation of its abundance in cellular processes. Here, we develop a method that reveals DNA damage-induced mono-ADPr on aspartate and glutamate residues by PARP1. Unexpectedly, we find that PARG, a poly-ADPr hydrolase that is thought to be inactive on mono-ADPr, can remove aspartate- and glutamate-linked mono-ADPr.

Chapter 1: Introduction

An overview of the DNA damage repair responses

The integrity of the human genome is constantly threatened by endogenous and exogenous mutagens, resulting in an estimated 10^5 lesions per day[1]. Damaged DNA can compromise vital processes such as DNA replication and transcription, and, if left unrepaired, can result in mutations that threaten the integrity of the genetic information.

The importance of this process is underscored by established and emerging evidence that repair of DNA damage is a central process in human health, from cancer[2], to ageing [3]. Indeed, germline mutations in genes coding for DNA repair proteins give rise to a heterogenous group of human diseases characterized by developmental defects, increased cancer incidence, and accelerated ageing phenotypes[4].

The large number of potential DNA-damaging agents and size of the genome makes the task of monitoring DNA and correctly repairing most lesions a staggeringly complex task. Nonetheless, the fact that the vast majority of cells throughout our lifetime do not become cancerous and manage to propagate and survive for decades of human lifespan, is a testament to the cell's incredible ability to manage DNA repair and its consequences in an efficient manner. At the heart of a cellular response to DNA damage is an orchestrated cascade of proteins and post-translational modifications (PTMs) that must coordinate in space and time to carry out repair.

The cellular response to DNA damage is not a single all-purpose pathway, but is rather a series of distinct, albeit interrelated, pathways each tailored to a set of possible lesions and cell cycle state. Nonetheless, at their core most if not all DNA repair pathways are signal transduction pathways with common elements. Firstly, they must have sensors, proteins dedicated to the recognition of aberrant DNA sequence or structure, such as strand breaks or modified bases. For example, to detect the entire repertoire of small chemical adducts that do not elicit helix distortions, such as alkylation by the cancer drug methyl methane sulfonate (MMS), human cells employ at least 11 different DNA glycosylases, each of which is responsible for the detection and excision of one or few kinds of modified bases[5].

By contrast, bulky lesions that generates helix distortions, such as 6-4 photoproducts and cyclopyrimidine dimers from UV radiation, can be sensed by stalled RNA polymerase II[6], or through recognition of the distorted, ssDNA “bubble” by XPC[7].
Secondly, dedicated enzymes remove one or more bases, followed by re-synthesis and ligation of the missing DNA sequence, usually templated on the complementary strand or on the homologous chromosome. In practice, these common steps can take place by different mechanisms, even within related pathways, or can be shared across different repair pathways. For example, after formation of the abasic site by DNA glycosylases and subsequent cleavage by apurinic/apyrimidinic (AP) endonuclease, repair can proceed via direct synthesis and ligation of the missing base by DNA polymerase ß (DNA pol ß) and DNA ligase I or III; alternatively, DNA pol δ/ε can synthesize multiple bases generating an overhang, which is cleaved by FEN1 and the resulting gap ligated by DNA ligase I[5]. In addition to this basic framework of effectors, a number of auxiliary proteins and PTMs performs additional roles from the local unfolding of the DNA strand to large-scale chromatin remodeling, signal amplification, recruitment of relevant proteins in a coordinated manner, scaffolding, pathway choice, integration of additional cellular signals, and signal propagation to other aspects of cellular function such as cell cycle.

Post-translational modifications in the DNA damage response

PTMs rapidly and reversibly control protein function and localization by fundamentally altering its chemical characteristics. In doing so, PTMs dramatically expand the functional repertoire of the proteome and provide a means to rapidly respond to many different cellular cues. Because of their utility and versatility, they play a role in virtually any biological process and therefore are central in our understanding of human physiology.
Within the context of DNA damage there is a large and expanding list of PTMs that have been found to regulate DDR signaling[8], with some the most prominently studied modifications being phosphorylation, namely on Serine 139 of the histone variant H2AX (γH2AX) mediated by ATM and ATR, ubiquitination of histones by the E3 ubiquitin ligases RNF8 and RNF168, and ADP-ribosylation (ADPr) mediated by PARP1 and PARP2.

Generally, these PTMs mark damaged regions to amplify the initial damage recognition signal and recruit other factors to the damage site. For example γH2AX spreads up to megabases away from the DNA lesion[9] and through MDC1 it act as a signal for the recruitment of several DNA repair factors, such as NBS1, 53BP1, and BRCA1[10,11]. ADPr is another important PTM that plays a crucial role in many DNA repair pathways, from NER to HR and NHEJ. Four FDA-approved PARP1 inhibitors are used in the clinic as cancer therapeutics. Therefore, the study of this PTM has important implications for human physiology and health.

In the coming sections we will provide a detailed overview of the molecular mechanisms leading to ADPr formation and removal, as well as the roles it plays in regulating the DDR.

ADP-ribosylation in the DNA damage response

ADPr is generated through the use of nicotinamide adenine dinucleotide (NAD) to modify target substrates with ADP-ribose **(Fig. 1)**.

Chemically and structurally, ADPr is a complex PTM that exhibits a wide variety in its structure and acceptor sites. It is made up by two phosphate groups with negative charge at physiological pH, two ribose moieties, and an adenine ring capable of hydrogen bonding and hydrophobic interactions. It can be synthetized on several nucleophilic amino acids, most notably serine (Ser), aspartate (Asp), glutamate (Glu), arginine (Arg), cysteine (Cys), tyrosine (Tyr), and threonine (Thr); as well as nucleic acids, such as DNA and RNA[12]. A single ADPr unit is termed mono-ADPr and, depending on the enzyme, it can be further extended to produce polymeric ADPr termed poly-ADPr. Poly-ADPr can be highly heterogeneous in the number of units, up to 200 in length[13], and can also contain branching sites[14]. The potential to modify several different amino acid residues, in both monomeric and polymeric form makes ADPr conceptually similar to other highly complex PTMs, such as glycosylation and ubiquitination. Historically, these PTMs have been proven difficult to characterize, and advances in our methods and tools used to analyze them holds enormous potential for new biological discoveries[15,16].

Notably, ADPr is involved in many biological processes beyond DNA damage. For the purpose of this thesis we will focus on the function and regulation of ADPr in DNA damage, and we point the reader to the following recent reviews for a broader overview of this topic[17-20].

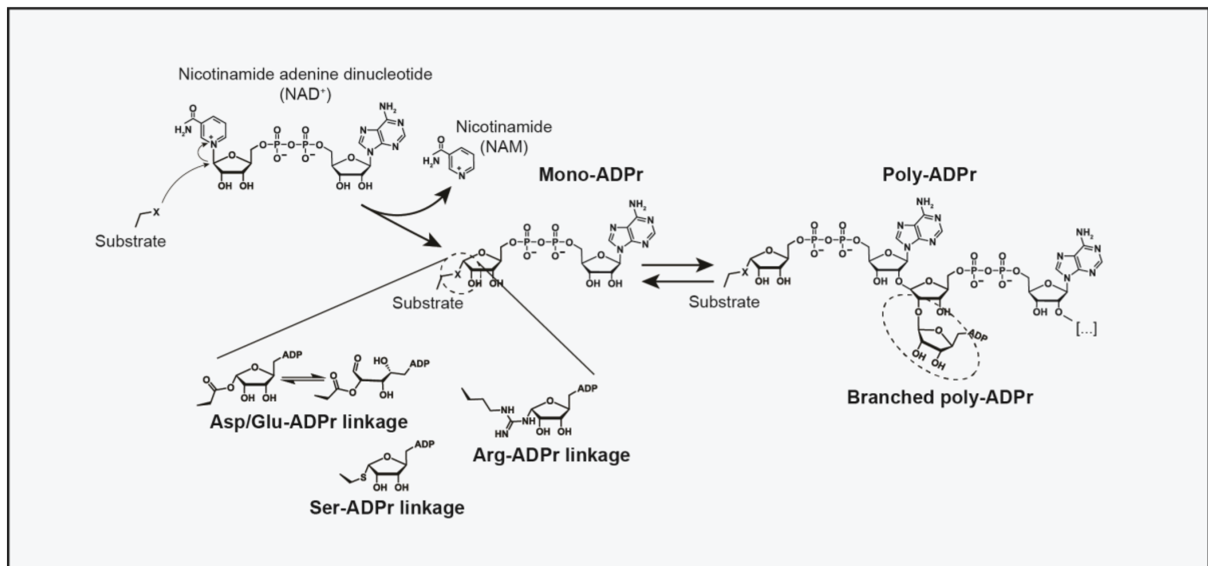

Figure 1. A chemical and structural overview of the ADPr alphabet

Simplified schematic of the ADP-ribosylation reaction. The nucleophilic attack of a substrate (target protein or nucleic acid) to NAD^+ results in covalent bonding of the ADP-ribose moiety and associated release of nicotinamide (NAM). The reaction can occur on several amino acid substrates (e.g. aspartate/glutamate, arginine, serine) resulting in different linkage types. Following the addition of the initial ADP-ribose to the substrate (mono-ADPr), the ADP-ribosylation reaction can be repeated with the hydroxyl groups of the initial ADP-ribose for linear or branched chain elongation to form poly-ADPr.

Molecular overview of ADPribosylation signaling

Writers

In humans there are two main enzyme families capable of synthetizing ADPr: the poly-(ADP-ribose)polymerases (PARP) family, characterized by the H-Y-(E/D/Q) catalytic triad; and the ART cholera toxin-like (ARTC) family, with R-(S/T)-E catalytic triad.

The human PARP family includes 17 members, although one member, PARP13, may be catalytically inactive. PARP3, PARP4, PARP6, PARP8, PARP7, PARP9, PARP10, PARP12, PARP14, PARP15, and PARP16 catalyze exclusively mono-ADPr, while PARP1, PARP2, PARP5a, and PARP5b can catalyze poly-ADPr. As a whole, the PARP family members are involved in many biological pathways, ranging from regulation of the immune response, stress response, and gene transcription[20,21].

Of these, PARP1, PARP2, PARP3 are canonically involved in the DDR. PARP1 and PARP2 share many sequence and structural features, and are consequently activated and regulated by similar signals. While individual mouse knockouts of PARP1 and PARP2 are viable, PARP1PARP2 double KO are embryonically lethal[22]. PARP1 synthesizes most of the ADPr signal detected upon DNA damage, with minor contributions from PARP2[23,24], however PARP2 might be responsible for more poly-ADPr branching sites[25].

PARP1 is a large 100 kDa protein comprising six domains connected by flexible linkers (**Figure 2A)**: three zinc finger domains (Zn1, Zn2, Zn3), a BRCT domain, a WGR (named after the conserved residues Trp-Gly-Arg) domain, and a catalytic domain (HD+ART). In the free, basal state these domains are highly flexible and act independently in a "beads on a string" model[26]. PARP1's dynamic and modular architecture is a feature which, as we will see, underlies many of PARP1 properties.

The zinc fingers and WGR domains are responsible for sensing and binding damaged DNA. Through these domains, PARP1 can recognize a disparate number of lesions including single- or double-strand breaks[27-29] and stalled replication forks[30]. PARP1 also binds to intact DNA with high affinity and moves along the DNA fiber with the coordinated action of the zinc fingers, WGR, and BRCT domains[31-33].

PARP1 is one of the most abundant nuclear enzymes and when active can consume large amounts of NAD^+ to synthesize poly-ADPr. Excessive PARP1 activation has been linked to depletion of NAD^+ pools and other metabolic disruptions[34], which can ultimately lead to cell death[35,36]. This presents an interesting biochemical problem.

Most PARP1 molecules should remain catalytically inactive while interacting with DNA to scan the genome, then rapidly switch to a catalytically competent state once a DNA lesion is encountered. Structural and biochemical studies revealed important insights into how this regulation is achieved. The catalytic domain, composed of the ADP-ribosyltransferase (ART) domain, also contains a helical domain (HD). In the native position, the HD acts as an autoinhibitory domain by preventing NAD^+ binding in the ART pocket[37,38]. While the HD domain itself does not contact the DNA break[27], PARP1 bound to damaged DNA induces a structural change which creates an HD-binding platform on the WGR domain, displacing the HD from the active site and enabling NAD^+ entry and catalytic activity[39,40]. In agreement with this model, deletion of the HD results in a hyperactive version of PARP1[37].

In analogy with other PTMs, knowledge of which amino acids are targeted for ADPr is essential to understand its function and regulation, and since its inception the ADPr field has devoted large efforts in attempting to understand the amino acid specificity of PARPs.

Initial biochemical studies suggested that, upon activation by DNA lesions, PARP1 can modify the acidic residues Aspartate and Glutamate[41,42]. The development of several sophisticated enrichment strategies that enabled mass spectrometry-based identification of ADPr peptides seemingly identified hundreds of PARP1-mediated Aspartate and Glutamate sites upon DNA damage[43-45]. However, the subsequent development of a mass-spectrometry approach for unbiased identification of ADPr sites revealed serine as an additional target residue for PARP1/2-mediated ADP-ribosylation in DNA damage[46]. Follow-up studies and bioinformatic re-analysis of previous mass-spec data showed that Ser-ADPr is the main form of ADPr in DNA damage[47,48]. This observation was in apparent contradiction with biochemical data, showing that PARP1 can synthesize *bona fide* aspartate and glutamate ADPr, but cannot produce Ser-ADPr. This inconsistency was resolved with the discovery that HPF1, a PARP1-interacting protein[49] changes the amino acid specificity of PARP1 from aspartate and glutamate residues to serine[50]. Accordingly, HPF1 depletion largely abolishes Ser-ADPr in cells[50]. Following a flurry of studies identifying thousands of Ser-ADPr sites upon DNA damage[47,51-55], a now commonly-held belief in the field is that Ser-ADPr is not only the most abundant but might also be the only relevant form upon DNA damage.

How does HPF1 enable PARP1 to perform Ser-ADPr and how is this interaction regulated during DNA damage? **Figure 2B** summarizes our knowledge in a simplified schematic. Biochemical and structural work revealed that upon formation of the PARP1/HPF1 complex, HPF1 is brough in close spatial proximity to the PARP1 active site and provides an additional key catalytic residue, HPF1E284, required for Ser-ADPr catalysis[56-58]. HPF1 also exhibits a negatively charged patch which might promote the interaction of the PARP1/HPF1 complex with target substrates, which typically contain a positively charged lysine residue[56]. Additionally, in *in vitro* reactions, increasing HPF1 concentration shortens and limits the formation of poly-ADPr[49,59,60], in line with structural observations suggesting that the presence of HPF1 in close vicinity of PARP1's active site might be sterically incompatible with poly-ADPr[56]. Overall, the PARP1/HPF1 complex has a radically different catalytic output than PARP1 alone. In this light, HPF1 is not simply an interaction partner of PARP1, but rather a key catalytic player that toggles between different signaling outcomes. Puzzlingly, PARP1 is about twenty fold more abundant than HPF1 in cells[61], yet the abundance of Ser-ADPr indicates that HPF1 must regulate PARP1 very efficiently and sub-stoichiometrically. Several key observations provide pieces to this puzzle. Firstly, biochemical and structural work revealed that the HD subdomain in inactive PARP1 dramatically impairs the formation of the PARP1-HPF1 interaction. Upon DNA binding, the PARP1 structural rearrangements that pry apart the HD from the catalytic site allow for the formation of the PARP1-HPF1 complex[56,62]. Through this mechanism, the interaction of HPF1 is biased towards PARP1 molecules that have encountered a DNA lesion, therefore increasing the number of catalytically-competent HPF1/PARP1 complexes. Secondly, the interaction of HPF1 with PARP1 is transient and dynamic, allowing HPF1 to rapidly sample multiple PARP1 molecules[59]. Lastly, in cells, PARP1 is rapidly recruited to the lesion site but also leaves early, while HPF1 concentration remains elevated much longer[63]. Therefore, the PARP1/HPF1 ratio in the local damage milieu might be much higher than in the whole nucleus. By contrast to PARP1 and PARP2, the role of PARP3 is less understood. It has been shown to participate in the DNA damage response, particularly with DNA double strand breaks[64,65]. In *in vitro* reactions, PARP3 is activated by DNA breaks with 5'-phosphorylated DNA[66] to catalyze mono-ADPr on aspartate and glutamate[67] and, indeed, it does not interact with HPF1[49].

Erasers

ADPr is a reversible modification with a rapid turnover. The chemical and structural diversity of ADPr is reflected in the variety of enzymes capable of removing this modification. ADPr hydrolysis is carried out by seven known hydrolases, belonging to either to the macrodomain family (PARG, TARG1, MACROD1, MACROD2) or to the ARH family (ARH1, ARH2, ARH3). Each of these hydrolases exhibits different substrate specificities and catalytic efficiency, which impairs different biological roles **(Figure 2C)**. Of these, biochemical in vitro studies have indicated that MACROD1, MACROD2, and TARG1 act on the ester bond of modified aspartates and glutamates[68]. PARG degrades poly-ADPr chains by hydrolysis of the ribose-ribose ether bond[69,70]. While PARG possesses both exo- and endo-glycohydrolase activity, removing either the terminal ADPr unit or within the poly-ADPr chain to release longer fragments, the former is more efficient[71,72]. The consensus in the field is that human PARG is not capable of removing the last protein-linked mono-ADPr unit[67,70,71], therefore PARG action results in conversion of poly-ADPr to mono-ADPr. ARH1 is active on arginine residues[73], while ARH2 is considered to be inactive. ARH3 can hydrolyze the ether bond of Ser-ADPr as well as poly-ADPr chains, albeit less efficiently[74-76]. ARH3 remains to date the only known human enzyme capable of reversing serine-linked ADPr[76] although, interestingly, in *Drosophila melanogaster*, which lacks an ARH3 homologue, PARG is capable of hydrolysing the Ser-ADPr linkage[77]. PARG and ARH3 are both recruited to sites of DNA damage[78-80] and participate in the DNA damage response by coordinating the removal of poly- and mono-ADPr. Depletion of ARH3 results in Ser-ADPr accumulation even in the absence of DNA damage[81-83], which impairs transcription[81]. The detectable levels of Ser-ADPr that occur upon ARH3 depletion in the absence of genotoxic stress indicate that even under physiological condition this modification is constantly produced and removed. One proposed source of physiological Ser-ADPr are unligated Okazaki fragments generated by a failure of DNA polymerase δ (POLδ) to reach the downstream Okazaki fragment, which activate PARP1[84-86]. In humans, loss-of-function mutations in ARH3 lead to childhood-onset neurodegeneration with ataxia and seizures[87-90].

Approximately one-third of patients die of neurogenic cardiac arrest and, in a mouse model of this disease, treatment with PARP1 inhibitors reduced myocardial dysfunction[91]. Interestingly, a homozygous mutation in TARG1 also leads to a neurodegenerative disease[92]. Similarly to ARH3, TARG1 is also recruited to sites of DNA damage[92,93], depletion of TARG1 with PARG inhibition leads to accumulation of poly-ADPr and induces replication stress and DSB formation[94]. Overall, these results suggest that ADPr formation and removal is a careful balancing act that can become toxic if tipped towards unconstrained synthesis. This toxicity may underlie some, if not all of the observed disorders in patients with mutations in ARH3 and TARG1 genes, although this remains to be elucidated. Interestingly, in *D. melanogaster*, loss-of-function mutations in PARG also lead to neurodegeneration[95], and to altered phenotypes in fly models of other neurodegenerative diseases. In this light, it is tempting to speculate that mono-ADPr, rather than poly-ADPr, might underly toxicity.

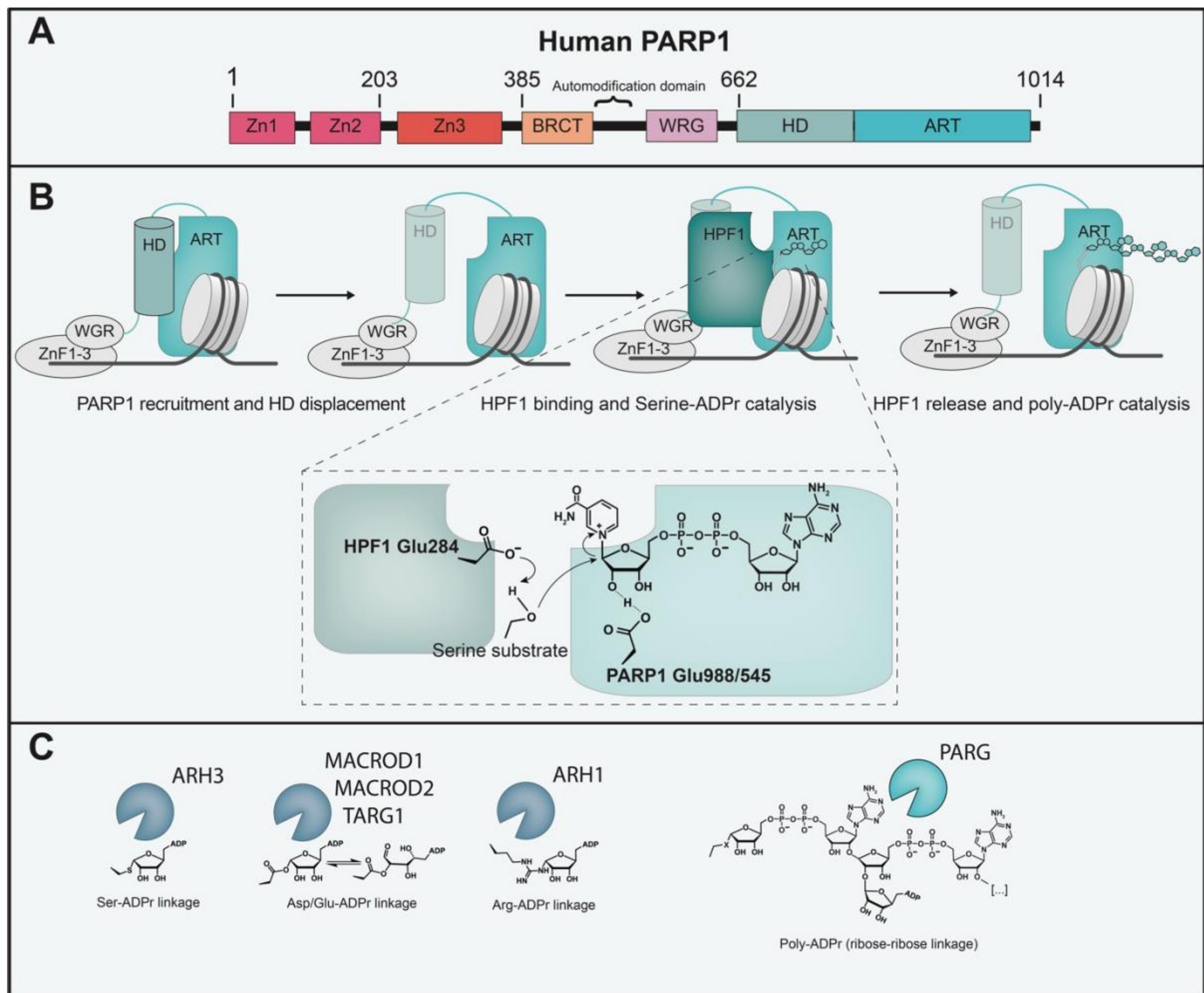

Figure 2. Main catalytic players in ADPr signaling

(A) Domain composition of human PARP1. The “automodification domain” contains the acceptor sites for auto-ADPribosylation. **(B)** Simplified schematic of the steps leading to PARP1/HPF1-mediated Ser-ADPr: The zinc finger 1–3 (Zn1–3) and WGR domains of PARP1 detect and bind to single-strand and double-strand breaks, inducing a structural rearrangement that displaces the HD subdomain from the active site of PARP1 and enables HPF1 binding. HPF1 binding brings the E284 residue of HPF1 close to the catalytic site of PARP1, enabling Ser-ADPr synthesis. Upon HPF1 release, PARP1 can elongate mono-ADPr to form poly-ADPr. Image adapted from Longarini and Matic, DNA Repair, 2022. **(C)** The main human ADPr hydrolases and the associated ADPr-protein bond target.

Roles of ADP-ribosylation signaling

The main roles of ADPr in DNA damage are modulation of intermolecular interactions to recruit or displace proteins from sites of DNA damage, and regulation of the chromatin structure **(Figure 3A)**. Many of these roles are accomplished via direct interaction with ADPr-binding domains on effector proteins, which will be reviewed below **(Figure 3B)**.

Modulation of intermolecular interactions

One of the main roles of PARP1 signaling in DNA damage is to modulate the recruitment or the release of proteins from damaged chromatin. The diversity of possible ADPr structures is reflected in the large number of different protein domains, modules, and motifs that can interact with ADPr, arguably the most out of any known PTM[96]. These structures exhibit different preferences in the kind of ADPr configuration they recognize, and therefore further contribute to signaling by translating this PTM into different biological effects **(Figure 3A)**. In the context of the DNA damage response, many repair factors harbor ADPr-binding domains. Here, we will provide a brief overview of the main known binding domains that are relevant in DNA damage, with particular emphasis to data linking ADPr binding to defined cellular outcomes.

Macrodomain

The macrodomain is one of the most studied ADPr-binding domain. We previously mentioned the macrodomain in the context of ADPr hydrolysis, however the genome also encodes for catalytically-inactive versions of this domain which serve as ADPr readers. ALC1 is a chromatin remodeler that contains a catalytically-inactive macrodomain and an Snf2-like ATPase domain[97]. ALC1 is recruited to sites of damage by PARP1-mediated ADPr through its macrodomain[97,98], although its recruitment is not dramatically affected by loss of HPF1[49], indicating that this process is not serine-specific. Under baseline conditions, the macrodomain inhibits its ATP hydrolysis required for chromatin remodeling. However, binding to ADPr causes a conformational shift that releases autoinhibition and converts the ATPase domain into a catalytically competent state[99-101]. Biochemically, ALC1 shows a preference towards short oligomers of ADPr ribose, particularly trimers[60,101,102], and this activation is not linkage specific, occurring with similar efficiency with glutamate-linked or serine-linked histone ADPr[103], in line with the recruitment data. ADPr-mediated recruitment and activation of ALC1 directly induce its chromatin relaxation activity, which, as we will see in more

detail in the **chromatin remodeling** section, directly contributes to DNA repair. Therefore, ALC1 is one of the best understood models on how ADPr binding affects downstream functions.

Among the PARPs, PARP9, PARP13, and PARP14 are known to contain a macrodomain. In this regard, PARP14 is particularly interesting because it harbors three tandem macrodomains, one of which acts as an eraser towards Glu-ADPr[104,105], while the other two are catalytically inactive and bind mono-ADPr[106,107]. PARP14 was shown to regulate DNA repair and the DNA stress response[108-110]. The identification of PARP14 target sites through a chemical genetics approach[111] suggested that PARP14 can modify over 100 proteins, predominantly on glutamate and aspartate residues[112]. Notably, how PARP14 catalytic activities could mechanistically affect DNA damage signaling is still largely unknown.

PARP9 forms a stable complex with DTX3L[113], an E3 ubiquitin ligase that can ubiquitylate lysine residues on proteins, as well as ADP-ribose itself[114], forming a composite substrate-ADPribose-ubiquitin signal. PARP9 has also been reported to recruit to DNA lesion in a PARP1-dependent manner, via its two tandem macrodomains[115,116]. Loss of PARP9 in cells is associated with defects in NHEJ repair pathway and 53BP1 signaling[116,117].

PBZ domain

The poly(ADP-ribose)-binding zinc-finger (PBZ) domain is characterized by a C2H2 zinc-finger which binds poly-ADPr[118,119]. In humans, APLF, CHFR, and SNM1A contain a PBZ domain and are associated with DNA repair pathways[118]. Of these, the PBZ in APLF is the most studied from a structural point of view, with NMR and crystal structures[119,120]. APLF participates in the DNA damage response as a histone chaperone[121,122] and, may also have AP endonuclease activity[123,124]. CHFR is also recruited to DNA damage sites via the PBZ domains, although its role in the DDR is less understood, with reports suggesting that it might ubiquitinate target substrates, including PARP1 and histones[125,126].

WWE domain

The WWE domain family is canonically considered a poly-ADPr binding domain. The most studied is the WWE domain of RNF146 which, in this protein, binds poly-ADPr via the ribose-ribose linkage that characterizes poly-ADPr chains[127]. RNF146 is an E3 ubiqutin ligase that ubiquitinates its targets in a poly-ADPr-dependent manner. In analogy to ALC1, this enzyme is not catalytically active until binding to its cognate

ADPr signal induces a conformational change that creates a functional enzyme[128]. While RNF146 is more commonly studied in the context of the Wnt signaling pathway[129,130], it might also function in the DNA damage response by targeting other repair factors, such as PARP1 and XRCC1, for ubiquitin-dependent degradation[131]. Notably, members of the WWE family have low degree of sequence homology[127], and therefore different WWE-containing proteins may exhibit different specificities. For example, PARP13 contains two tandem WWE domains which, by contrast to the WWE or RNF146, form a combined binding site to engage multiple units of ADPr starting from the terminal unit of a poly-ADPr chain[132].

Beyond protein recruitment, ADPr can also induce the displacement of proteins from the damage site. This effect is most striking and emblematic on PARP1 itself. Upon automodification, PARP1 rapidly dissociates from nucleosomes[62]. Interestingly, this effect seems to be specifically Ser-ADPr dependent, considering that, in cells, preventing PARP1 automodification on serine residues, either with HPF1 depletion or with alanine mutants of the target residues, results in persistent accumulation of PARP1 at the damage site[133]. The timely release of PARP1 from the damage site is likely important to allow for the downstream DNA repair factors to access the lesion site, and to prevent PARP1 hyperactivation.

This is the mechanism exploited by the current class of PARP1/2 inhibitors used in the clinic for cancer therapy. Initially developed on the basis of a synthetic lethality interaction with BRCA1 mutant tumors[134,135], our current understanding of the mechanism of action of these inhibitors is that cancer toxicity arises through a "PARP trapping" mechanism[136]. By preventing PARP1 automodification, these inhibitors prevent the efficient release and therefore "trap" PARP1/2 at the damage site. A separate class of inhibitors trap PARP1 via an allosteric mechanism that directly increases the affinity of PARP1 to damaged DNA[137], although so far no inhibitor in this class is used therapeutically. Notably, the notion of "trapped" PARP1 is vague and still poorly understood. This term was initially used to describe an enrichment of PARP1 at chromatin upon PARP inhibition[138]. More recently, live-cell imaging showed that, upon PARP1 inhibition, PARP1 molecules may not be physically stalled at chromatin but can still freely exchange between DNA-bound and soluble states, albeit with increased dwell time on chromatin, depending on the type of inhibitor[139,140].

Chromatin remodeling

The DNA damage response takes place within the context of a highly organized and dynamic chromatin structure. The nucleosome structure itself poses significant constraints to the DNA repair machinery[141,142]. For the coordinated action of the DNA repair machinery to occur, pre-existing proteins must be displaced and the DNA needs to be made accessible. To accomplish this task, the chromatin structure is remodeled in a sequence of events that has been dubbed the “access-repair-restore” model[143,144]. Under this conceptualized framework, chromatin is first transiently relaxed, either by nucleosome mobilization or disruption. This enables the repair machinery to access the lesion site unimpeded. Then, after the repair is complete, chromatin recondenses and the original organization is restored. Indeed, this model is supported by empirical data showing chromatin relaxation followed by recompaction after a DNA damage trigger[145,146]. Upon laser microirradiation-induced DNA damage, early chromatin decompaction is triggered by PARP1-mediated ADPr[146,147] and this unfolding facilitates the recruitment of proteins to the damage site[148]. How does ADPr accomplish this task? First, PARP1-dependent ADPr mediates the recruitment of ATP-dependent chromatin remodelers to the lesion site. As previously mentioned, ALC1 and APLF are directly recruited and activated by ADPr to remodel chromatin at the site of DNA damage. Accordingly, loss of ALC1 impairs chromatin relaxation[148]. By early recruitment to sites of damage, ALC1 induces an initial, rapid chromatin relaxation, which increases DNA accessibility and promotes the accessibility of other chromatin remodelers, such as CHD3, CHD4, and CHD7[148].

Interestingly, depletion of ATP impairs but does not fully abolish PARP1-dependent chromatin relaxation[63,146]. Indeed, in one of the earliest studies performed after the discovery of ADPr, PARP1 could induce chromatin relaxation independently of ATP.

Recent biochemical experiments showed that Ser-ADPribosylation of nucleosomes induces a chance in their compaction state[149] and ADP-ribosylated H1 linker histones impair compaction of chromatin arrays[150]. These experiments suggest that ADPr might also influence chromatin structure via direct biophysical effects, for example by electrostatic repulsion of the highly negatively charged ADPr molecule to DNA. Emerging evidence shows that, in cells, ADPr specifically on histone serine residues, over other targets, is important for the chromatin relaxation process. Loss of HPF1 – and therefore of Ser-ADPr – severely impairs relaxation, but does not fully abolish it

and the observed relaxation depends on histone Ser-ADPr specifically, rather than PARP1 automodification[63]. Taken together, these results are consistent with a model in which ADPr induces early DNA damage through three complementary mechanisms, direct recruitment of ALC1, indirect recruitment of additional chromatin remodelers, and biophysical effects on the nucleosome structure. In agreement with the "access-repair-restore" framework, the PARP1-dependent chromatin relaxation enables the recruitment of the DNA-repair machinery[63]. Consequently, preventing chromatin relaxation, for example by depletion of ATP-dependent chromatin remodelers, results in defects in DNA repair[97,146,151,152].

Following relaxation and repair, chromatin needs to be restored to its original status. This process is likely crucial to ensure that the intended processes, such as transcription, can resume appropriately. Compared to relaxation, we know relatively little on how this is achieved.

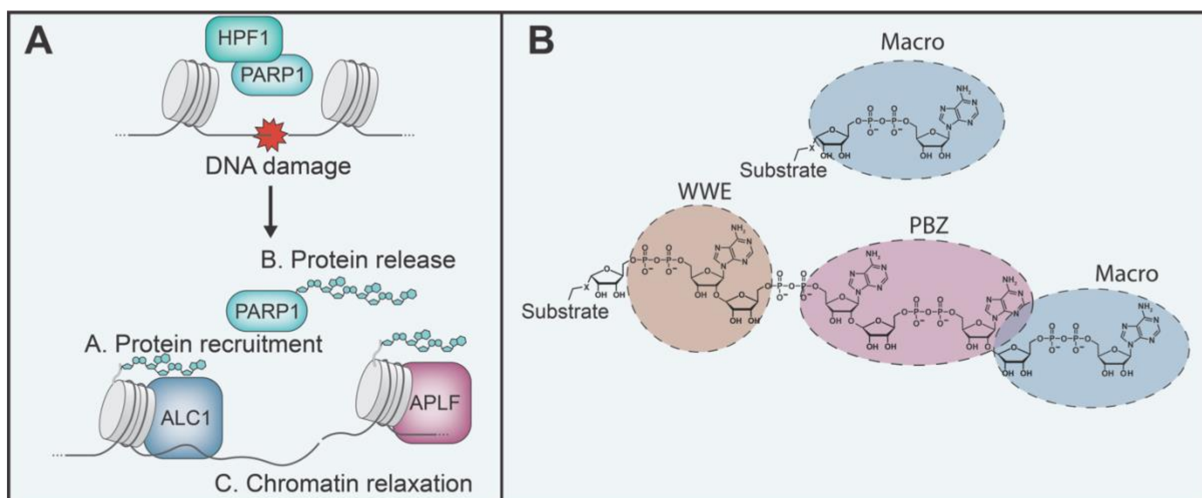

Figure 3. Main ADPr-mediated outcomes in the DNA damage response

(A) DNA breaks trigger the recruitment of PARP1 and HPF1 to sites of damage to catalyze Ser-ADPr. This results in the recruitment of other DNA damage repair factors (e.g. XRCC1, CHD4); release of target proteins (e.g. PARP1); and chromatin relaxation, partly mediated by ALC1 and other chromatin remodelers. **(B)** Many ADPr effector proteins have well-defined fold that recognize specific feature of the ADPr monomer or polymer. For example, the PAR-binding zinc finger (PBZ), WWE domain, and macrodomain. The dotted circle represents the binding preference for each domain based on structural data. For example, macrodomain preferentially recognize mono-ADPr or the terminal ADPr unit in poly-ADPr. Notably, the specific preference of these domains depends on their specific sequence and protein context. For example, the macrodomain in ALC1 preferentially recognizes three ADPr units in a chain (oligo-ADPr).

Tools to study ADPribosylation

Mass spectrometry-based detection

Mass spectrometry (MS)-based proteomics has emerged as a powerful tool for the analysis of protein PTMs. Nonetheless, the highly charged, labile, and heterogenous nature of this PTM make its identification challenging. For instance, long poly-ADPr units are not amenable to analysis and need to be hydrolyzed to a single, recognizable unit[43,44]. Additionally, the peptide-ADPr linkage is often labile. In practice, during peptide fragmentation, loss of the modifier from the peptide can hamper the identification of the modified site(s). The development and application of techniques that preserve the peptide-ADPr linkage during fragmentation and allow for the unambiguous assignment of the modification site was instrumental in our ability to identify and assign ADPr sites[48], and led to the identification of serine-linked ADPr[46].

Antibody-based detection

The study of widespread PTMs such as acetylation and phosphorylation have benefited tremendously by development of site-specific and broad-specificity antibodies. Much of these efforts began more than four decades ago and, since then, these antibodies have constituted a foundational tool in our efforts to understand how these PTMs function and their role in health and disease. For example, the first antibodies against phosphorylation were broad-specificity antibodies against phosphotyrosine developed in 1981[153]. Prior to these reagents, protein phosphorylation was visualized using laborious and hazardous radiolabeling with ^{32}P. Therefore, these antibodies greatly facilitated detection of phosphorylation events. Nonetheless, their utility was still limited by the inability to identify on which protein and residue the phosphorylation occurred. To circumvent this limitation, the development of site-specific antibodies, that recognize a given PTM only on a unique peptide sequence, was underway. In the DNA damage response, a key signaling event is phosphorylation of gamma-H2A.X on Serine 139. This modification was first identified in 1998[154] and shortly after a site-specific antibody was developed[155]. Since then, this antibody has been routinely used by thousands of laboratories worldwide as a marker of DNA damage. However, unlike phosphorylation, the study of ADPr has been hampered by a lack of research tools.

For most of its history, the ADPr field has largely relied on the 10H antibody, which recognizes long poly-ADPr chains but does not recognize any form of ADPr of less than 10-20 units in length[106]. This antibody has been instrumental in the field, but the inability to recognize other forms of ADPr represent a crucial blindspot. An important advance towards an improved ADPr toolbox came repurposing of naturally-occurring ADPr-binding domains into tools to detect ADPr[106]. That study developed antibody-like reagents against mono-ADPr, oligo-ADPr, and poly-ADPr. In particular, the WWE domain, a poly-ADPr binding domain from RNF146, has proven to be a complementary reagent to the 10H antibody and has also been used, tagged with a fluorescent protein, to track poly-ADPr in live cells[148,156]. Recently, the utility of the WWE reagent has been further expanded to track poly-ADPr *in vivo*[157]. Another domain, the Af1521 macrodomain from *Archaeoglobus fulgidus*, has been extensively used in this field, particularly as an enrichment strategy prior to mass spectrometry[45,106,158]. Af1521 exhibits high affinity towards ADPr, and has been recently improved further through random mutagenesis[54]. A limitation of this reagent is that it recognizes both mono- as well as poly-ADPr and therefore cannot be used to distinguish between these two signals. Despite these recent breakthroughs, the study of ADPr is constrained by a lack of detection methods to cover the full spectrum of ADPr's heterogeneity, for example by the lack of site-specific antibodies.

Chapter 2: An HPF1/PARP1-Based Chemical Biology Strategy for Exploring ADP-ribosylation

The publication can be found here:
Juan José Bonfiglio*, Orsolya Leidecker*, Helen Dauben*, **Edoardo José Longarini***, Thomas Colby, Pablo San Segundo-Acosta, Kathryn A. Perez, and Ivan Matic. "An HPF1/PARP1-based chemical biology strategy for exploring ADP-ribosylation." *Cell* 183, no. 4 (2020): 1086-1102.
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Chapter 3: Modular antibodies reveal DNA damage-induced mono-ADP-ribosylation as a second wave of PARP1 signaling

The publication can be found here:

Edoardo José Longarini, Helen Dauben, Carolina Locatelli, Anne R Wondisford, Rebecca Smith, Charlotte Muench, Andreas Kolvenbach, Michelle Lee Lynskey, Alexis Pope, Juan José Bonfiglio, Eva Pinto Jurado, Roberta Fajka-Boja, Thomas Colby, Marion Schuller, Ivan Ahel, Gyula Timinszky, Roderick J O'Sullivan, Sébastien Huet, Ivan Matic
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Author contributions:

Conceptualization of the project: I.M.; design of research: I.M., S.H., and E.J.L; design of phage-display strategy, selection and validation of the antibodies and the SpyTag formats: H.D., E.J.L., and J.J.B; proteomic analyses: E.J.L., J.J.B., and T.C; establishment of Bead-loading/fab system: H.D.; immunofluorescence and live-cell imaging experiments: E.J.L., H.D., C.L., R.S., E.P.J., C.M., and A.R.W.; generation of RNF114KO cell lines: A.K.; execution of telomere-related experiments: A.R.W., M.L.L., and R.J.O.; NHEJ-reporter assay: R.F.-B. and G.T.; production of recombinant proteins and experiments with ADP-ribosylated gDNA: M.S. and I.A.; immunoprecipitation and immunoblotting experiments: E.J.L., C.L., A.P., C.M., and H.D.; FCS experiments: S.H.; writing of the manuscript: I.M. and E.J.L. with input from all authors

Chapter 4: DNA Damage-Induced Asp/Glu mono-ADP-ribosylation by PARP1 and its Reversal by PARG

Unpublished manuscript

Author contributions:

Conceptualization of the project: I.M. and E.J.L.; design of research: I.M. and E.J.L; execution of experiments: E.J.L.; writing: E.J.L. with input from I.M.

An immunoblotting method for mono-Asp/Glu-ADPr detection

Historically, glutamate and aspartate were considered the primary target residues for ADPr in the DDR. After our initial discovery of Ser-ADPr on histones, subsequent research from us and others established Ser-ADPr by the PARP1/HPF1 complex as a prevalent and functionally important PTM in DNA damage[47,51,56,63,83,133,159-161]. This has inspired a flurry of studies culminating with the notion that serine might be the only relevant amino acid target for ADPr in the DDR[55]. Indeed, in our own studies we were also unable to detect, by western blotting, mono-Asp/Glu-ADPr[160,161]. We were nonetheless intrigued by a clear detection of mono-ADPr in HPF1KO cells using immunofluorescence[161], and by a recent report suggesting that Asp/Glu-ADPr is a highly labile PTM, which could be lost under routine western blotting techniques[103]. This led us to consider the hypothesis that Asp/Glu-ADPr might be more widespread than currently thought, due to a lack of adequate detection methods

While the mono-ADPr antibody AbD43647 was generated an affinity-matured using Ser-ADPr peptides, it can also recognize other forms of mono-ADPr *in vitro*, including Asp/Glu-ADPr by PARP1[161]. Therefore, we hypothesized that when combined with a sample preparation protocol that preserves Asp/Glu-ADPr, the high sensitivity of the HRP-coupled antibody could be employed to probe the presence of mono-Asp/Glu-ADPr in cells. Towards this aim, we systematically tested the conditions which we reasoned would have the biggest effect on the lability of the Asp/Glu-ADPr linkage during sample preparation for immunoblotting: heat, DNA shearing, and pH. For this, we used HPF1 knockout (HPF1KO) cells in which lack of HPF1 prevents Ser-ADPr and would therefore unambiguously allow us to detect other forms of ADPr.

During routine sample preparation for immunoblotting, the samples are heated at high temperature to ensure protein denaturation and reduction. However, we reasoned that cell lysis under harsh denaturing conditions using high concentrations of SDS, a common reagent for solubilization of cells and tissue, would circumvent the requirement for this step. Strikingly, in HPF1KO cells, omission of sample boiling resulted in a dramatic increase in mono-ADPr signal **(Fig 1A)**. By contrast, in WT cells, the mono-ADPr signal was largely insensitive to boiling, consistent with the abundance of Ser-ADPr, which is stable at high temperatures[103]. We did not detect any noticeable difference in protein extraction and immunoblotting efficiency between the two conditions, as assessed by ponceau S staining and PARP1, GAPDH, and H3 staining. Encouraged by these results, we sought to further optimize the sample preparation

protocol by evaluating two other factors: DNA shearing to reduce the sample viscosity, and pH of the lysis buffer. We find that DNA shearing via sonication, a widespread technique for this purpose, results in a minor but significant loss of mono-ADPr signal in HPF1KO, a loss which is ameliorated by shearing DNA with recombinant benzonase **(Fig. 1B)**. Notably, sample sonication also resulted in a reduction in total PARP1, but not of the other proteins tested. Lastly, we find that pH of the lysis buffer (pH 7.2 – 7.9) has a negligible impact on mono-ADPr signal, albeit with short total sample processing time (< 1h) **(Fig. 1C)**.

When taken together, our refined sample processing steps enable the detection of mono-ADPr signals that would otherwise be undetectable **(Fig. 1D)**. Treatment of the HPF1KO cell lysate with hydroxylamine, which cleaves the Asp/Glu-ADPr bond but not Ser-ADPr[47], resulted in a complete loss of mono-ADPr signal **(Fig. 1D)**, leading us to reason that the detected signal might be mono-Asp/Glu-ADPr, in line with the biochemical activity of PARP1 in the absence of HPF1. Notably, at this stage we cannot rule out the contribution of other ADPr linkages that might be hydroxylamine and temperature sensitive, such as Arg-ADPr.

In a previous study, by leveraging the SpyTag/SpyCatcher platform to directly conjugate HRP to primary antibodies, we have dramatically increased the sensitivity of antibodies against mono-ADPr[161]. Here, we assessed the HRP-conjugated AbD43647 and its unconjugated IgG counterpart for detection of mono-Asp/Glu-ADPr. Despite 10-fold higher amounts for the IgG antibody, the HRP-coupled format resulted in vastly superior signal detection **(Fig. 1E)**. Lastly, we sought to assess whether room temperature incubation of the membrane, a common step during secondary antibody incubation, would affect mono-ADPr signal intensity. Indeed, prolonged room temperature incubation (3 h) resulted in a slight reduction in signal **(Fig. 1F)**. Therefore, the ability to circumvent this step via the use of HRP-conjugated primary antibodies represents another advantage of our modular format.

Overall, we developed a protocol that preserves the highly labile mono-Asp/Glu-ADPr, enabling detection of this elusive PTM. We used this method to show that this modification is prevalent in HPF1KO cells, where Ser-ADPr is absent. Compared to mono-Ser-ADPr, whose main biological substrates are PARP1 and histones[47], we find that mono-Asp/Glu-ADPr is distributed more uniformly across several targets **(Fig. 1A)**. Amongst those, we detect a prominent band between 100 and 150 kDa, corresponding to the observed migration of mono-ADP-ribosylated PARP1, leading us to hypothesize that mono-D/E-PARP1 might be one of the most abundant Asp/Glu-ADPr substrates. Additionally, while core histones are abundantly ADP-ribosylated on serine residues during the DNA damage, and on glutamate in other biological contexts[162], we did not observe histone Asp/Glu-ADPr under our conditions.

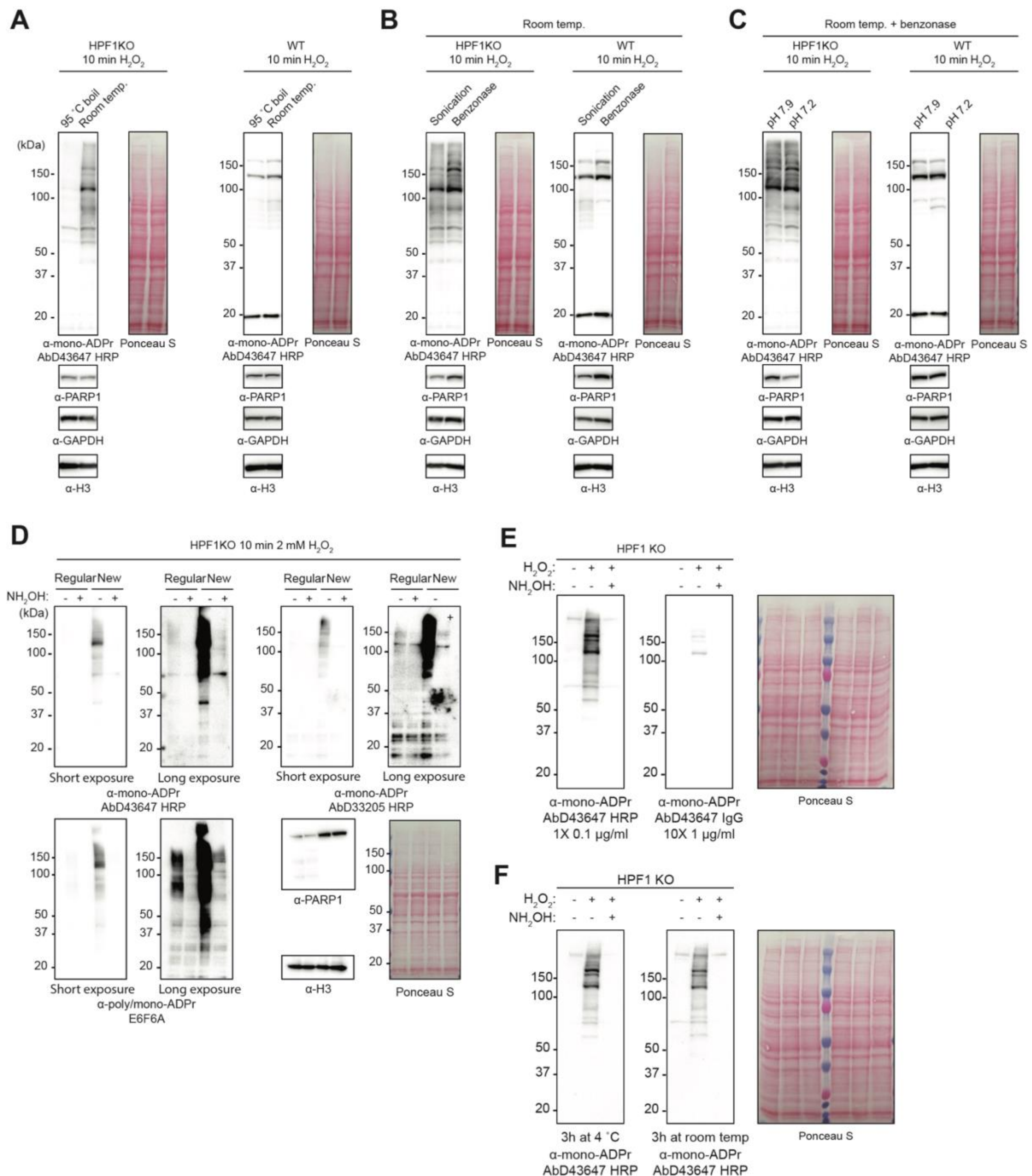

Figure 4. An immunoblotting method for mono-Asp/Glu-ADPr detection

(A) Immunoblotting images showing comparison between sample boiling (10 min at 95 ˚C) or room temperature. WT or HPF1KO U2OS cells were treated with 2 mM H_2O_2 for 10 min to induce DNA damage, harvested and either boiled for 10 min at 95 ˚C or kept at room temperature and analyzed by immunoblotting with the indicated antibodies.

(B) Immunoblotting analysis of DNA shearing by sonication or benzonase during the samples processing step. WT or HPF1KO U2OS cells were treated with 2 mM H_2O_2 for 10 min to induce DNA damage, harvested without boiling, then sonicated (10 cycles of 30 sec on/off on a bioruptor) or treated with benzonase (750 U benzonase per sample) and analyzed by immunoblotting with the indicated antibodies.

(legend continued on next page)

(C) Immunoblotting analysis of pH during the samples processing step. WT or HPF1KO U2OS cells were treated with 2 mM H_2O_2 for 10 min to induce DNA damage, harvested in lysis buffer at pH 7.9 or 2.7, without boiling, then treated with benzonase (750 U benzonase per sample) and analyzed by immunoblotting with the indicated antibodies. **(D)** Immunoblotting analysis comparing sample preparation workflows. HPF1KO U2OS cells were treated with 2 mM H_2O_2 for 10 min to induce DNA damage, then harvested according to the "regular" (processed by boiling and sonication at pH 7.9) or the "new" workflow (room temperature incubation with benzonase at pH 7.2). The lysed samples were split and treated or not with 1 M NH_2OH for 2 h at room temp. to remove Asp/Glu-ADPr. The samples were then analyzed by immunoblotting with the indicated antibodies. **(E)** Immunoblot images showing comparison between IgG and HRP-coupled formats of the AbD43647 anti-mono-ADPr antibody. HPF1KO U2OS cells were treated with 2 mM H_2O_2 for 10 min to induce DNA damage then harvested according to the "new" workflow and analyzed by immunoblotting with the indicated antibodies. Detection of mono-ADPr in WB is dramatically improved upon conversion of IgG to directly-coupled HRP antibody format. Note, 10-fold higher concentration of the IgG format was required to obtain signal in comparison to the HRP-coupled format. **(F)** Immunoblot images showing the effect of room temp. incubation of membranes on mono-Asp/Glu-ADPr signal. After overnight incubation at 4 ˚C with AbD43647 HRP, the membranes were washed, then further incubated for 3 h either at 4 ˚C or at room temp.

DNA damage dependent mono-Asp/Glu-ADPr depends on PARP1

With our optimized detection method at hand, we investigated the nature of mono-Asp/Glu-ADPr. PARP1 is the predominant enzyme in the DNA damage response, and is responsible for the majority of mono- and poly- Ser-ADPr. However, there is emerging evidence that other PARPs, known to catalyze mono-Asp/Glu-ADPr, such as PARP3, PARP10, and PARP14 could also play a role in the DDR[163,164]. We therefore sought to evaluate which PARP is regulating this signal. Treatment of the cells with Olaparib, a PARP1/2 selective inhibitor, abolished mono-Asp/Glu-ADPr in HPF1KO cells **(Fig. 2A)**. Next, we sought to discern between PARP1- and PARP2-dependent ADPr. Treatment of the cells with AZD5305 and AZD9574, two inhibitors specific for PARP1 which do not cross-react with PARP2[165,166], showed dramatic reduction in signal, similar to Olaparib treatment **(Fig. 2B)**. We further validated these results with IF, which confirmed that mono-Asp/Glu-ADPr signal is abolished with PARP1-specific inhibitors **(Fig. 2C)**. Taken together, these results lead us to conclude that upon DNA damage PARP1 is the main writer of mono-Asp/Glu-ADPr.

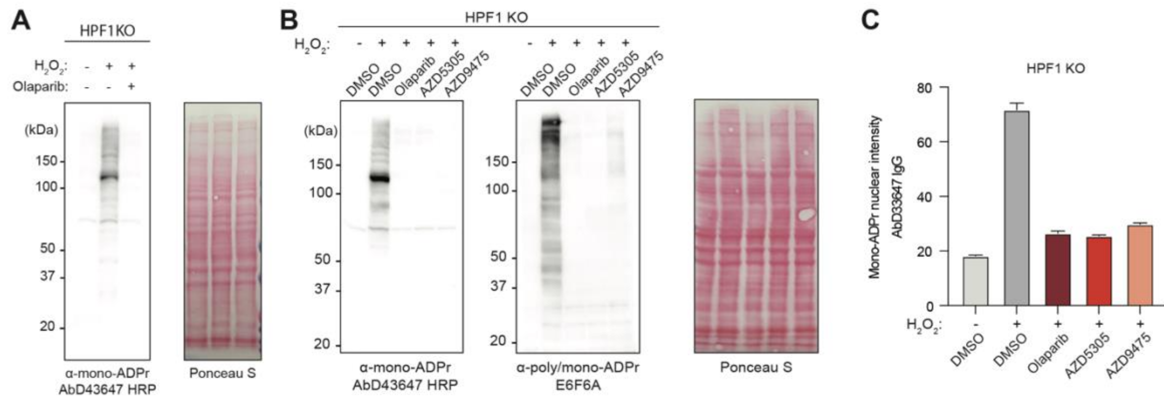

Figure 5. DNA damage dependent mono-Asp/Glu-ADPr depends on PARP1

(A) Immunoblotting images showing the effect of PARP1/2 inhibition by Olaparib on mono-Asp/Glu-ADPr signal. HPF1KO U2OS cells were pre-treated with either 1 μM Olaparib or DMSO (control) for 30 mins, followed by treatment with 2 mM H_2O_2 for 10 mins, where indicated, to induce DNA damage. Cells were harvested and analyzed by immunoblotting with the indicated antibodies. **(B)** Immunoblotting images showing the effect of PARP1-specific inhibition by AZD5305 and AZD9574 on mono-Asp/Glu-ADPr signal. HPF1KO U2OS cells were pre-treated with either 1 μM Olaparib, 20 nM AZD5305, 20 nM AZD9574, or DMSO (control) for 30 mins, followed by treatment with 2 mM H_2O_2 for 10 mins, where indicated, to induce DNA damage. Cells were harvested and analyzed by immunoblotting with the indicated antibodies. **(C)** Immunofluorescence analysis showing the effect of PARP1-specific inhibition by AZD5305 and AZD9574 on mono-Asp/Glu-ADPr signal, as in (B). HPF1KO U2OS cells were pre-treated with either 1 μM Olaparib, 20 nM AZD5305, 20 nM AZD9574, or DMSO (control) for 30 mins, followed by treatment with 2 mM H_2O_2 for 10 mins, where indicated, to induce DNA damage. Cells were fixed with Methanol and analyzed by immunofluorescence with the indicated antibodies.

Human PARG can hydrolyse mono-Asp/Glu-ADPr in cells

We sought an explanation for the prevalence of PARP1-dependent mono-Asp/Glu-ADPr. Using the anti-mono-ADPr antibodies we developed, which do not show poly-ADPr crossreactivity[160,161], we previously showed that mono-Ser-ADPr formation is partly dependent upon PARG-dependent degradation of poly-ADPr to mono-ADPr[160,161]. Therefore, we reasoned that mono-Asp/Glu-ADPr might also arise from a similar mechanism. Under this model, we expected a dramatic decrease in mono-Asp/Glu-ADPr upon chemical inhibition or genetic depletion of PARG.

To test this hypothesis, we treated HPF1KO cells with the PARG inhibitor PDD00017273 (PARGi)[167]. When compared to control, PARGi-treated HPF1KO cells showed higher levels of poly-ADPr upon DNA damage, as expected **(Fig. 3A)**. Accordingly, total PARP1 signal shows smearing across the membrane, rather than a distinct band, reflecting the high levels of heterogeneously poly-ADP-ribosylated PARP1.

Unexpectedly, however, PARGi also resulted in a dramatic increase in mono-Asp/Glu-ADPr, which was reversed by hydroxylamine treatment **(Fig. 3A)**.

Encouraged by a recent report showing that recombinant PARG can degrade mono-Asp/Glu-ADPr from modified peptides *in vitro*[103], these results led us to consider the hypothesis that PARG might remove mono-Asp/Glu-ADPr.

Next, we tested the effects of PARGi in WT cells **(Fig. 3B)**. Consistent with previous studies[160,161], PARGi resulted in a dramatic reduction of mono-Ser-ADPr, particularly on PARP1 and core histones, and effect that is accompanied by an increase in poly-Ser-ADPr **(Fig. 3B)**. However, similarly to HPF1KO data, PARGi resulted in an increase in mono-Asp/Glu-ADPr. Notably, poly-ADPr in WT cells is largely on serine residues and therefore preserved upon NH_2OH treatment, as indicated by the poly-ADPr specific antibody MABE1031. By contrast, the signal detected by AbD43647 is largely abolished upon NH_2OH treatment. Consequently, these results also confirm the mono-ADPr specificity of AbD43647 and that the signal we detect in HPF1KO cells and upon PARGi is not due to poly-ADPr cross-reactivity.

To confirm that the increase in mono-Asp/Glu-ADPr is not due to off-target effects of PARGi towards other Asp/Glu-ADPr hydrolyses, we sought to validate these results through genetic depletion of PARG. In PARG knockouts (PARGKO) we observe an increase in mono-Asp/Glu-ADPr, in agreement with PARGi data.

Importantly, PARGi in PARGKO cells did not result in a noticeable increase in mono-ADPr signal, validating the on-target activity of this inhibitor **(Fig. 3C)**. These observations where further validated by IF but, interestingly, hydroxylamine treatment did not fully abolish the mono-ADPr signal detected upon PARGi in HPF1KO cells **(Fig. 3D)**, suggesting that inhibition of PARG might also increase other forms of ADPr that are not detected by western blotting, such as the emerging DNA-ADPr[168,169].

Next, to confirm that the increase in mono-Asp/Glu-ADPr is directly due to PARG catalytic activity, as opposed to indirect effects of PARG inhibition or depletion, we characterized PARG biochemically. We took advantage of the PARP1 mutant E988Q which, in the absence of HPF1, generates mono-ADPr on aspartate and glutamate residues[170]. After PARP1E988Q automodification we then stopped the reaction with the PARP inhibitor Olaparib. Upon subsequent addition of PARG, we observed a time-dependent removal of mono-Asp/Glu-ADPr **(Fig. 3E)**. This effect becomes noticeable only after long incubation times, which might also explain why in earlier reports, using milder conditions, this phenomenon was not observed[67,70,71].

To characterize the activity of PARG more broadly, we automodified PARP1 WT using condition which generate poly-ADPr and no detectable mono-ADPr. Subsequent addition of PARG resulted, as expected, in rapid degradation of poly-ADPr which is converted to mono-ADPr. The rapid poly-ADPr hydrolysis is then followed by a slower and incomplete removal of mono-ADPr **(Fig. 3F)**, consistent with our previous results. Taken together, these data demonstrate that PARG is capable to hydrolyze the ester bond of Asp/Glu-ADPr, albeit with much lower efficiency than the ether bond in poly-ADPr. We were intrigued by this result, considering that PARG is canonically considered a poly-ADPr hydrolase that is unable to cleave the last mono-ADPr unit[67,70,71]. Despite the low *in vitro* activity of PARG towards mono-Asp/Glu-ADPr, this activity is prominent in cells, as indicated by the substantial increase in mono-Asp/Glu-ADPr upon PARG inhibition. Our results also demonstrate that, even in the presence of HPF1, mono-Asp/Glu-ADPr is constantly formed and readily hydrolyzed. By doing so, PARG controls the balance of mono-ADPr on Serine and Aspartate/Glutamate residues. PARG activity results concomitant mono-Ser-ADPr formation, via poly-ADPr degradation, and mono-Asp/Glu-ADPr removal. Conversely, inactivating PARG largely prevents the formation of mono-Ser-ADPr and steers the system towards increased mono-Asp/Glu-ADPr.

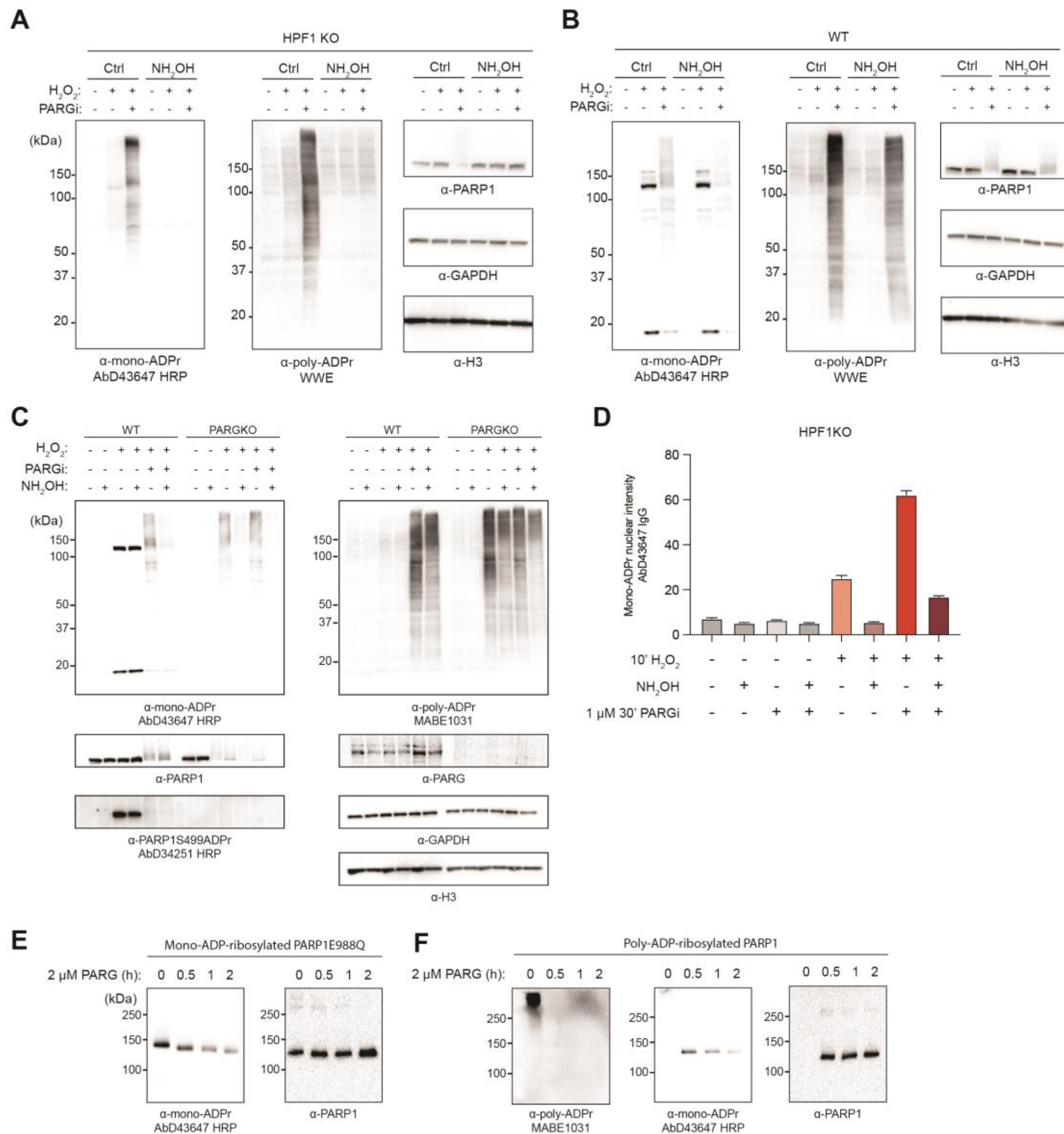

Figure 6. Human PARG can hydrolyze mono-Asp/Glu-ADPr in cells

(A) Immunoblotting images showing the effect of PARG inhibition by PDD00017273 (PARGi) on mono-Asp/Glu-ADPr signal. HPF1KO U2OS cells were pre-treated with either 1 μM PARGi or DMSO (control) for 30 mins, followed by treatment with 2 mM H_2O_2 for 10 mins, where indicated, to induce DNA damage. After harvesting, cells were lysed and treated or not with 1 M NH_2OH for 2 h to remove Asp/Glu-ADPr, then analyzed by immunoblotting with the indicated antibodies. **(B)** Immunoblotting images showing the effect of PARGi on mono-Asp/Glu-ADPr signal. WT U2OS cells were processed and analyzed as in (A). **(C)** Immunoblotting images showing the effect of PARG knockout and PARGi on mono-Asp/Glu-ADPr signal. WT and PARGKO U2OS cells were processed and analyzed as in (A). **(D)** Immunofluorescence analysis showing the effect of PARGi on mono-Asp/Glu-ADPr signal. Cells were treated as in (A), then fixed with methanol and analyzed by immunofluorescence with the indicated antibodies.

(legend continued on next page)

(E) Immunoblotting analysis of *in vitro* PARG activity. First, PARP1E988Q was automodified in the absence of HPF1 to produce mono-Asp/Glu-ADPr. After 30 mins the reaction was stopped with Olaparib and incubated with 2 μM PARG at room temperature for the indicated times before stopping the reaction with 1X loading buffer and immunoblotting with the indicated antibodies. Note, all samples were kept at room temperature for the same overall time, to control for time- and temperature-dependent chemical hydrolysis of Asp/Glu-ADPr. **(F)** Immunoblotting analysis of *in vitro* PARG activity. First, PARP1WT was automodified in the absence of HPF1 to produce poly-Asp/Glu-ADPr. After 30 mins the reaction was stopped with olaparib and incubated with 2 μM PARG at room temperature for the indicated times before stopping the reaction with 1X loading buffer and immunoblotting with the indicated antibodies. Note, all samples were kept at room temperature for the same overall time, to control for time- and temperature-dependent chemical hydrolysis of Asp/Glu-ADPr

Mono-Asp/Glu-ADPr is part of the first wave of PARP1 signaling

We then sought to evaluate the dynamics of mono-Asp/Glu-ADPr upon DNA damage. In a previous study we have shown that by contrast to poly-ADPr, which spikes early and is rapidly removed in response to DNA damage induction, mono-Ser-ADPr rises more gradually and remains elevated for longer[161]. To investigate the dynamics of mono-Asp/Glu-ADPr, we exposed cells to continuous H_2O_2 treatment before cell lysis at different time points. The cell lysates were processed with our newly developed immunoblotting protocol and, therefore, in WT cells the mono-ADPr antibody AbD43647 would detect both types of ADPr. To circumvent this limitation and compare the dynamics of Asp/Glu-ADPr to Ser-ADPr we leveraged the site-specific antibody against PARP1S499ADPr (AbD33251), which does not recognize Asp/Glu-ADPr[160]. By contrast to mono-Ser-ADPr, which peaks late (~ 20 mins), in agreement with our previous findings[161], mono-Asp/Glu-ADPr peaks early (~ 10 mins) and is then rapidly degraded **(Fig. 4A)**. These observations were further validated by IF **(Fig. 4B)**.

We then evaluated the dynamics of mono-Asp/Glu-ADPr in response to PARG inhibition. In agreement with our previous results, PARGi resulted in a dramatic increase in mono-Asp/Glu-ADPr, as well as poly-ADPr **(Fig. 4C)**. Interestingly, however, in contrast to poly-ADPr, which remained elevated after 40 minutes of treatment, we observed a decrease in mono-Asp/Glu-ADPr after the initial peak (~ 10-20 mins) **(Fig. 4C)**. Therefore, while in untreated cells poly- and mono-Asp/Glu-ADPr follow broadly the same dynamics upon DNA damage, treatment with PARGi results in their uncoupling. We reason this might be due, at least in part, to other hydrolases, such as TARG1 and MACROD1/2, that can eventually remove mono-Asp/Glu-ADPr.

Indeed, for mono-Ser-ADPr, depletion of ARH3 – the only known human mono-Ser-ADPr hydrolase – results in a monotonically increasing signal that remains elevated for up to 90 minutes, the maximum timepoint considered in previous experiments[160].

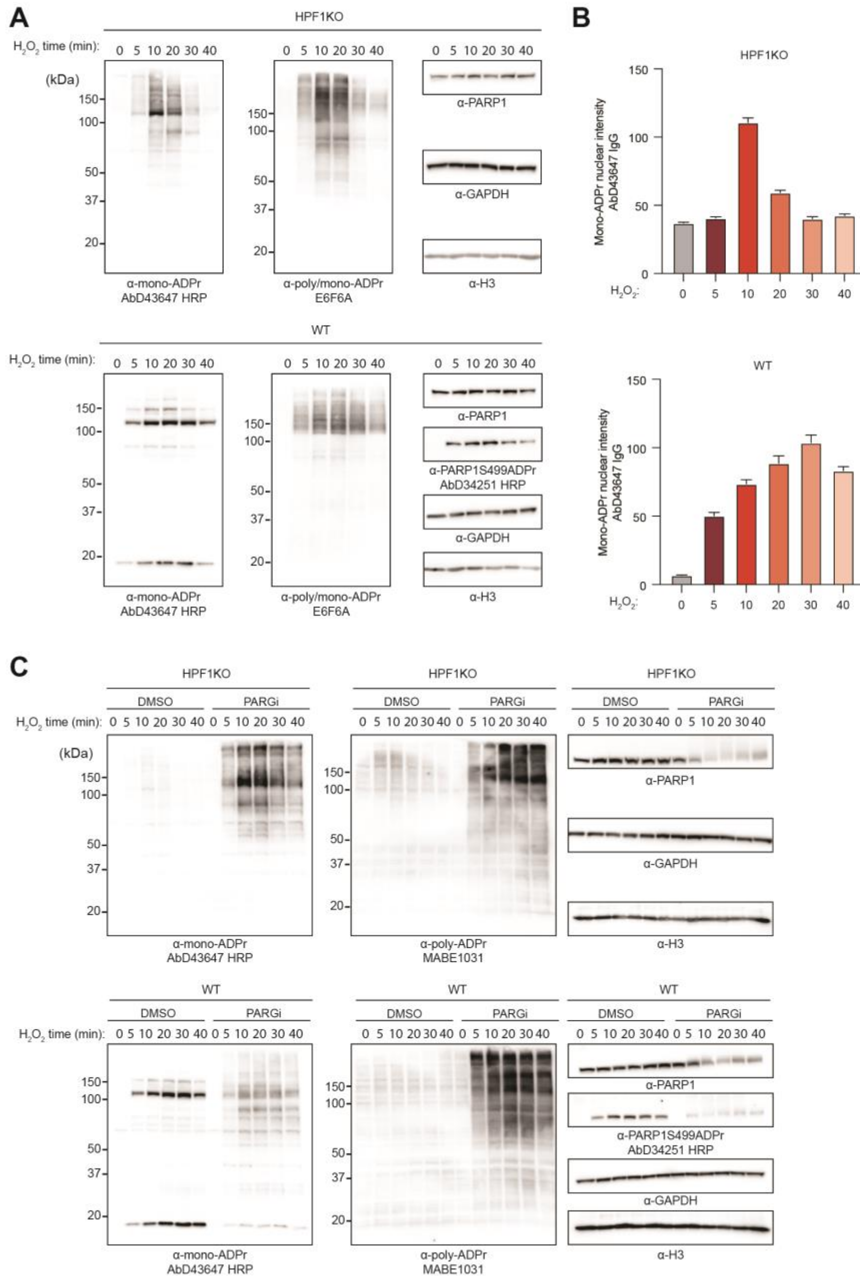

Figure 7. Mono-Asp/Glu-ADPr is part of the first wave of PARP1 signaling

(A) Immunoblotting images showing time-course of DNA damage treatment by H_2O_2. WT and HPF1KO U2OS cells were treated with 2 mM H_2O_2 for the indicated times to induce DNA damage. After harvesting, cells were lysed and analyzed by immunoblotting with the indicated antibodies. **(B)** Immunofluorescence analysis showing time-course of DNA damage treatment by H_2O_2. Cells were treated as in (A), then fixed with methanol and analyzed by immunofluorescence with the indicated antibodies. **(C)** Immunoblotting images showing time-course of DNA damage treatment by H_2O_2 upon PARGi. First, cells were pre-treated with 1 μM PARGi or DMSO (control) for 30 mins. Then, cells were treated with 2 mM H_2O_2 for the indicated times to induce DNA damage. After harvesting, cells were lysed and analyzed by immunoblotting with the indicated antibodies.

Materials and methods

Cell culture and drug treatments

U2OS cell lines were obtained, authenticated by *STR* profiling and confirmed *mycoplasma* free by ATCC cell line authentication services. Cells were routinely tested for mycoplasma contamination. HPF1KO U2OS cells were generously provided by Ivan Ahel (University of Oxford). PARGKO U2OS cells were generously provided by Roderick J. O'Sullivan (University of Pittsburgh). Each cell line was cultured in Glutamax-DMEM supplemented with 10% bovine serum and 100 U/ml penicillin/streptomycin at 37°C and 5% CO_2. To induce PARG inhibition, the cell medium was aspirated and replaced with 37 °C complete DMEM containing 2 μM PDD00017273 for 30 mins. To induce PARP inhibition, the cell medium was aspirated and replaced with 37 °C complete DMEM containing either 1 μM Olaparib, or 20 nM AZD5305, or 20 nM AZD9574 for 30 mins. To induce DNA damage, the cell medium was aspirated and replaced with 37 °C complete DMEM containing 2 mM H_2O_2 for the indicated times.

Immunoblotting

Regular sample preparation

U2OS cells (WT, HPF1KO, PARGKO) were treated as indicated in the figure legends, then lysed in SDS buffer (4% SDS; 50 mM HEPES, pH 7.9), boiled for 5 min at 95 ˚C, and sonicated for 10 cycles of 30 s on/off on a bioruptor (Diagenode) at 4 ˚C. The samples were then boiled for 5 min at 95 ˚C in 1x NuPAGE LDS sample buffer (Invitrogen) with DTT (Sigma), resolved on NuPAGE Novex 4-12% Bis-Tris gels (Invitrogen), and transferred onto nitrocellulose membranes (Amersham) using wet transfer at 110 V for 90 min. The membranes were blocked in 5% milk in PBS buffer with 0.1% Tween-20 for 1 h at room temperature and incubated overnight with primary antibody (0.1 μg/ml for AbD43647, 1:1000 for commercial antibodies)at 4 ˚C. For antibodies requiring secondary antibody, this was followed by a 1 h incubation at room temperature with peroxidase-conjugated secondary anti-mouse (Amersham, 1:8000) or anti-rabbit (Amersham, 1:8000).

Optimized sample preparation

U2OS cells (WT, HPF1KO, PARGKO) were treated as indicated in the figure legends, lysed in SDS buffer (4% SDS; 50 mM PIPES, pH 7.2), incubated for 5 minutes at room temperature with recombinant benzonase (smDNase, 750 U per sample). 1x NuPAGE LDS sample buffer (Invitrogen) with DTT (Sigma) was added to the samples, loaded and resolved on NuPAGE Novex 4-12% Bis-Tris gels (Invitrogen), and transferred onto nitrocellulose membranes (Amersham) using wet transfer at 110 V for 90 min. Ice-cold running and transfer buffer were used, and the tanks were kept on ice throughout. The membranes were then processed as described above.

Immunofluorescence

U2OS cells (WT, HPF1KO, PARGKO) were cultured on glass coverslips, treated as indicated, and fixed with ice-cold methanol for 20 min at -20 ˚C. The cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min, then blocked with 3% normal goat serum (Invitrogen) for 5 min. The coverslips were incubated with primary antibody for 1 h at room temperature, followed by 1 h incubation at room temperature with Alexa Fluor secondary antibodies (Invitrogen), then mounted with Prolong Diamond Antifade (ThermoScientific). Cells were imaged using a Leica SP8-DLS inverted laser-scanning confocal microscope using 63X objective. Image analysis and quantification was performed using the Fiji Software. Nuclei were identified based on DAPI, and used as a mask to measure the pixel intensity of other image channels.

Recombinant protein assays

PARP1E988Q and PARP1WT were automodified as previously described[160]. After stopping the reaction with 1 µM Olaparib, 40 nM PARP1 (E988Q or WT) was incubated with 2 µM recombinant PARG for the indicated times, before stopping the reaction with 1x NuPAGE LDS sample buffer and proceeding with immunoblotting as described above.

Chapter 5: Discussion

In this thesis work, we developed new tools and methodologies to study ADPribosylation, and applied them to investigate important biological questions.

In Bonfiglio et al., 2020 we have developed a chemoenzymatic method to generate Ser-ADPr peptides. This method relies on the enzymatic use of the PARP1/HPF1 Ser-ADPr writer complex to install ADPribosylation. A key aspect of our approach is the use of chemical phosphorylation of serine residues as a selective and reversible protecting group, directing the specificity of the complex only towards the intended serine target. Beyond Ser-ADPr peptides, we also achieved Tyr-ADPr and serine-phosphoribose peptides. Tyrosine has been identified as an alternative acceptor site for ADPr by the PARP1/HPF1 complex[159,171]. Whether this modification represents a functionally distinct signal than Ser-ADPr remains to be elucidated, but our peptides can provide a stepping stone towards this goal. Innovating upon our chemoenzymatic approach, other labs have used PARP1 alone to install poly-ADPr units on mono-ADPr substrates[60], and leveraged the catalytic domain of PARP14 for aspartate and glutamate ADPr synthesis[103].

Next, we used our newly produced peptides to generate the first site-specific antibodies, as well as broad specificity antibodies against mono-ADPr. When we applied these antibodies to the study of PARP1/HPF1 signaling, we made the unexpected discovered that mono-ADPr, and not poly-ADPr as previously thought, is the predominant form of ADPr in DNA damage. The prevalence of mono-ADPr was soon after validated by an independent lab[55]. In hindsight however, it is not surprising that this knowledge has remained hidden considering that, until recently, the only antibodies available against ADPr were limited to poly-ADPr. This represents a testament to the importance of challenging our assumptions on the systems under study, and to keep in mind the biases and blindspots introduced by our research tools. We expect that this study represents a catalyst for a further increase in the number and sophistication of antibodies and other tools for ADPr. We have proven that this pipeline can be used to generate novel antibodies with interesting properties but we are nonetheless far from the development ceiling, as our current toolbox only scratches the surface of the whole diversity of this PTM and, therefore, a continuous process of development and refinement is needed.

This is the aim we set for our next study, Longarini et al., 2023, in which we further improved our antibodies for greater affinity and sensitivity. We took advantage of the recombinant nature of these reagents, which directly allows for affinity maturation and application of the “molecular glue” SpyTag/SpyCatcher. With the former, we generated a new antibody against mono-ADPr, AbD43647, which displays ~ 20-fold higher affinity than the parental antibody, AbD33204. With the latter, we have a modular way of easily adding functional components to antibodies. For example, we have shown that direct conjugation of HRP via SpyTag/SpyCatcher results in a dramatic increase in antibody detection sensitivity in immunoblotting. Beyond antibodies, to further broaden the scope of our toolbox for ADPr, we described the use of the macrodomain domain of MACROD2 as a genetically-encoded probe that can be used to track mono-Ser-ADPr in live cells. With these reagents at hand, we investigated how poly- and mono-ADPr are dynamically regulated during the DNA damage response. We discovered that, by contrast to poly-ADPr, which increases rapidly after damage but is also promptly removed, mono-Ser-ADPr is a more enduring signal which raises more gradually but persists longer after the initial damage. The distinct temporal regulation of poly- and mono-Ser-ADPr further reinforced our model of mono-Ser-ADPr as a PTM that is linked but functionally distinct from poly-ADPr. Therefore, we reasoned that it might have a different role to play in the DDR. To tackle this hypothesis we used a combination of mass-spectrometry and live-cell confocal microscopy to discover readers of mono-ADPr. We found a set of proteins recruited by mono-ADPr, and were particularly intrigued by RNF114, an E3 ubiquitin ligase. We found that RNF114 preferentially binds mono-ADPr, and this interaction is dependent on a zinc finger domain. By contrast to poly-ADPr regulated proteins, which accumulate rapidly after damage induction, RNF114 is recruited in the second, mono-ADPr regulated phase of PARP1 signaling. In our efforts to test whether RNF114 shows a preference for mono- over poly-ADPr we developed methods that could selectively regulate, in live cells, the levels of mono-ADPr without affecting poly-ADPr, and viceversa. This is not a trivial undertaking, considering that mono- and poly-ADPr, in the DDR context, stem from the same enzyme and are intimately linked. We expect that these selective perturbations will be valuable in future studies aimed at deconvoluting the selective contributions of mono- and poly-ADPr in a given pathway.

It is interesting to note that since our discovery of RNF114 as an ADPr interacting protein in DNA damage, other recent publications have also suggested a link between ADPr and RNF114, even beyond DNA damage. PARP14 may regulate RNF114 ADP-ribosylation[104], and RNF114 might be involved in stabilizing PARP5a/b via diubiquitination[172]. To more precisely define the contribution of mono-ADPr to DNA damage repair, we found that loss of RNF114 sensitizes cells to DNA damaging agents, and show a defective 53BP1 signaling pathway, which we could rescue with reconstitution of WT RNF114 but not with the mono-ADPr-binding deficient mutant. In agreement with this data, cells depleted of RNF114 show a reduction in NHEJ efficiency.

While our work established a link between RNF114 recruitment by mono-ADPr and DNA repair, how RNF114 mechanistically contributes to DNA damage repair is still largely an open question. Given that RNF114 is an E3 ubiquitin ligase, and that ubiquitination plays a central role in the 53BP1 pathway, it is tempting to speculate that mono-ADPr recruitment of RNF114 may serve as a trigger to induce the ubiquitination of key target proteins, targeting them for degradation or stabilization. In this light, it is interesting to note that the connection between ADPribosylation and ubiquitination is a recurring theme. As we discussed in the **Roles of ADP-ribosylation signaling** section of the introduction, RNF146, CHFR, and DTX3L are ADPr effector proteins that link ADPr recognition to ubiquitination of target substrates. It suggests that to achieve a truly comprehensive understanding of the ADPr signaling pathway, we may need to consider the full spectrum of PTMs and how their interplay gives rise to specific cellular outcomes. This is a lofty goal, considering that we still have to uncover the full repertoire of ADPr signaling events, as our still recent discovery of mono-ADPr indicates.

Lastly, in an unpublished work-in-progress manuscript, we are working on characterizing mono-Asp/Glu-ADPr upon DNA damage. Towards this aim, we first explored western blotting conditions that could preserve this highly labile PTM. Combining an updated western blotting workflow with highly sensitive anti-mono antibodies, we observed abundant mono-Asp/Glu-ADPr in HPF1 depleted cells. Interestingly, upon PARG inhibition, we observed a dramatic increase in mono-Asp/Glu-ADPr sites. This was surprising because human PARG is canonically considered to be a hydrolase for poly-ADPr that is inactive towards the last mono-ADPr unit (see **Writers** section of the introduction for more details).

Under the current literature model, we would expect a decrease in mono-Asp/Glu-ADPr, reflecting an increase in poly-ADPr sites that are not hydrolysed. Indeed, Ser-ADPr is decreased upon PARGi, as expected from the lack of PARG-dependent degradation of poly-ADPr chains[160,161]. To explain this discrepancy, we reasoned that PARG could hydrolyse mono-Asp/Glu-ADPr. In this context, we were encouraged by a recent report showing that recombinant PARG is active towards mono-Asp/Glu-ADPr peptides *in vitro*. Indeed, using a recombinant PARP1 mutant that can only catalyze mono-ADPr (PARP1E988Q), we confirmed that PARG is capable of hydrolyzing Asp/Glu-ADPr *in vitro* and on a protein substrate, albeit with low efficiency. These results also represent one of the most striking testaments to the importance of reagents against mono-ADPr. PARG inhibition or depletion is known to dramatically increase poly-ADPr. Therefore, the signal detected with reagents that recognize both mono- and poly-ADPr simultaneously, such as the commonly used Af1521 and E6F6A reagents, could not be unambiguously assigned to either of these ADPr species and would make it impossible to discover a concomitant increase in mono-ADPr.

Additionally, we foresee that mono-ADPr selective reagents will be instrumental to determine the inhibition selectivity of future PARG inhibitors *in cells*. Unwanted cross-reactivity of PARG inhibitors towards other ADPr hydrolases, such as TARG1 and MACROD1/2, would result in increased mono-Asp/Glu-ADPr in PARG depleted cells, which can be detected with our mono-selective antibodies and Asp/Glu-ADPr detection methodology. This will be of practical relevance as there is an increasing interest in developing PARG inhibitors for cancer treatment[173].

Beyond the practical implications of our observation, it is interesting to note how proteins that have been under scrutiny for decades can still reveal surprising attributes. More broadly, the detection method we describe also opens up the possibility to study Asp/Glu-ADPr signaling in other biological contexts. Most PARPs are able to mono-ADP-ribosylate aspartate and glutamate residues *in vitro*, therefore this PTM might be involved in a wide range of biological and disease processes – ranging from the antiviral immune response to protein homeostasis and gene regulation[174].

For example PARP14, an enzyme that can catalyze aspartate and glutamate ADPr and is involved in the immune response and genome stability[163]. It is also emerging as a promising drug target due to its association with inflammatory diseases and several types of cancer, leading to the development of PARP14 inhibitors.

Recently, the Ahel group showed detection of PARP14-dependent mono-ADPribosylation using the mono-ADPr specific antibody that we developed[104]. Nonetheless, this was possible under non-physiological perturbations of the system by overexpression of PARP14. We foresee that our method would significantly enhance the detection of Asp/Glu-ADPr to enable its study under physiological conditions. Beyond basic research, this would also be useful for drug discovery and validation, as a way to show that a proposed inhibitor is active towards the intended target.

Nonetheless, more work is needed to confirm and refine our observations.

It will be important to unambiguously determine the nature of the mono-ADPr linkage we detect in HPF1KO cells and upon PARGi. The data presented so far strongly points in the direct of Asp/Glu-ADPr, although we cannot formally rule out other linkage types. Additionally, the detection of this form of ADPr in WT cells is severely limited, due to the strong mono-Ser-ADPr signal detected. We are currently working on developing a mass-spectrometry protocol for the proteome-wide identification of Asp/Glu-ADPr sites. Similarly to immunoblotting, several fundamental issues hinder the large-scale analysis of Asp/Glu-ADPr. Routine mass-spectrometry workflows involve harsh lysis conditions, and overnight trypsin digestion at 37 ˚C with slightly alkaline pH, conditions which would cause the chemical loss of most ADPr sites[103]. If successful, a method to circumvent these obstacles would set the stage for subsequent studies linking specific Asp/Glu-ADPr sites to biological outcomes.

Another major limitation to the study of Asp/Glu-ADPr with immunoblotting and immunofluorescence is the lack of reagents that can selectively recognize this linkage over other forms of ADPr. This makes the detection of Asp/Glu-ADPr in WT cells, where Ser-ADPr is abundant and strongly detected by our reagents, very challenging. Therefore, development of antibodies that are specific for Asp/Glu-ADPr would represent another major step forward.

Ultimately, it will be important to elucidate the biological role of mono-Asp/Glu-ADPr in DNA damage. The presence of several hydrolases capable of reversing mono-Asp/Glu-ADPr, namely TARG1, MACROD1/2, and now also PARG, is indicative of cellular fine-tuning over the levels of this PTM. Additionally, while loss of HPF1KO sensitizes cells to DNA damaging agents, loss of PARP1 is even more deleterious[49,63], which is suggestive of a role for Asp/Glu-ADPr in the DNA damage response.

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