

Elucidating the role of Musashi RNA-binding proteins in
tubular epithelial cells and acute kidney injury

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III. Abbreviations

% (v/v)	Volume concentration
% (w/v)	Mass concentration
ACTA/ACTN1	Actinin alpha 1
AKI	Acute kidney injury
ALYREF	Aly/REF export factor
ANXA2	Annexin A2
aPC	anticoagulant Protease activated protein C
APS	Ammonium persulfate
ASO	Antisense oligonucleotide
ATF6	Activating transcription factor 6
ATL	thin ascending limb of the loop of Henle
ATM	Ataxia telangiectasia mutated
ATP	Adenosine 5'-triphosphate
BLMH	Bleomycin hydrolase
bp	base pair
BSA	Bovine serum albumin
CAA	Chloroacetamide
CaCl ₂	Calcium chloride
CCD	Cortical collecting duct
CCT3	Chaperonin containing TCP1 subunit 3
CDS	Coding sequence
cDNA	complementary DNA
CHRAc	Chromatin-accessibility complex
circRNA	Circular RNA
CIRP	Cold-inducible RNA-binding protein
CKD	Chronic kidney disease
CLIP	Crosslinking and immunoprecipitation
CMV	Cytomegalovirus
CNS	Central nervous system
CNT	Connecting tubules
CO ₂	Carbon dioxide
CTAL	Cortical thick ascending limb of the loop of Henle
CTNNA1	Catenin alpha 1
CTNNB1	Catenin beta 1
DAMPs	Damage-associated molecular patterns
DCT	Distal convoluted tubule
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTL1	short descending limb of the loop of Henle
DTL2	long descending limb of the loop of Henle in the outer medulla
DTL3	long descending limb of the loop of Henle in the inner medulla
DTT	Dithiothreitol
DYNC1H1	Dynein cytoplasmic 1 heavy chain 1
e.g.	exempli gratia
ECL	Enhanced chemiluminescence
eCLIP	enhanced CLIP
EDTA	Ethylenediaminetetraacetic acid
EF1a	Elongation factor 1-alpha
EGFP	enhanced green fluorescent protein
EIF	Eukaryotic translation initiation factor
ER	endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FXR1	FMR1 autosomal homolog 1
GO	Gene ontology
H+L	heavy and light chain
H1F0	H1.0 linker histone

H ₂ O ₂	Hydrogen peroxide
HCl	Hydrogen chloride
HEBS	HEPES-buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia-inducible transcription factor
HIST1H2B	H2B clustered histone
HIST2HA	histone variants H2A clustered histone
HITS-CLIP	High-throughput sequencing of RNA isolated by CLIP
HRP	Horseradish peroxidase
HSP90AB1	Heat shock protein 90 alpha family class B member 1
HSR	Heat shock response
HuR	Human antigen R
iCLIP	Individual-nucleotide resolution UV CLIP
IF	Immunofluorescence
iHEK	inducible HEK
IMCD	Inner medullary collecting duct
IP	Immunoprecipitation
IRE1	Inositol-requiring enzyme 1
IRI	Ischemia reperfusion injury
Katna1	Katanin catalytic subunit A1
kb	Kilobase
kDa	Kilodalton
kgBW	Kilogramm bodyweight
LAD1	Ladinin 1
LB	Lysogeny broth
LLPS	Liquid-liquid phase separation
LMNB1	Lamin B1
lncRNA	long non-coding RNA
LSD	least significant difference
LTL	Lotus tetragonolobus lectin
MAPK	Mitogen-activated protein kinase
Mff	Mitochondrial fission factor
MgCl ₂	Magnesium chloride
MgSO ₄	Magnesium sulphate heptahydrate
mIMCD	murine inner medullary collecting duct
miRNA	micro RNA
MLKL	Mixed-lineage kinase domain-like protein
mRNA	messenger RNA
mRNPs	mRNA ribonucleoprotein complexes
MSI1	Musashi-1
MSI2	Musashi-2
MTAL	medullary thick ascending limb of the loop of Henle
MYH14	Myosin heavy chain 14
Na ₂ HPO ₄	Sodium hydrogen phosphate
Na ₃ VO ₄	Sodium orthovanadate
Na ₄ P ₂ O ₇	Tetrasodium pyrophosphate
NaCl	Sodium chloride
NADP	Nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluoride
NaOH	Sodium hydroxide
ncRNA	non-coding RNA
NDS	Normal donkey serum
Nec-1s	Necrostatin-1 stable
Neo	neomycin
NES	Nestin
NLRP3	NLR family pyrin domain containing 3
no PQ	unstimulated conditions
Numb	NUMB endocytic adaptor protein
OMCD	outer medullary collecting duct
p53	Tumor protein P53
PAA	Polyacrylamide

Paraquat (PQ)	Methyl viologen dichloride hydrate
PAR-CLIP	Photoactivatable ribonucleoside enhanced CLIP
PAS	Periodic acid–schiff
PBS	Phosphate buffered saline
PBs	Processing bodies
PCBP2	Poly(RC) binding protein 2
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
PERK1	Proline-rich receptor-like protein kinase
PFA	Paraformaldehyde
PGK	Phosphoglycerate kinase I
PHD	Prolyl hydroxylases
PI3K	Phosphatidylinositol 3-kinase
piRNA	Piwi-interacting RNA
PKS	Protein kinase
PMSF	Phenylmethylsulfonyl fluoride
PNK	Polynucleotide kinase
POLR2A	RNA polymerase II subunit A
PRRs	Pattern recognition receptors
PRPF	Pre-mRNA processing factor
PTS1	proximal convoluted tubule
PTS2	proximal straight tubule in cortical-medullary rays
PTS3	proximal straight tubule in outer stripe of outer medulla
Pum2	Pumilio 2
PVDF	Polyvinylidene fluoride
qPCR	quantitative PCR
Ran	RAN, member RAS oncogene family
RASL2-9	RAS-like, family 2, locus 9
RBD	RNA-binding domain
RBP	RNA-binding protein
RBPome	RNA-binding proteome
RIC	RNA interactome capture
RIPA	Radioimmunoprecipitation assay
RIPK	Receptor-interacting serine/threonine protein kinase
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RNPs	Ribonucleoprotein complexes
ROS	Reactive oxygen species
RP	Ribosomal Protein
rpm	revolutions per minute
RRM	RNA recognition motif
rRNA	ribosomal RNA
RT	Reverse transcriptase / Room temperature
scaRNA	small cajal body-specific RNA
SCIN	Scinderin
SCL25A3	Solute carrier family 25 member 3
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
seCLIP	single-end enhanced CLIP
SEM	Standard error of the mean
SG	Stress granule
siRNA	small interfering RNA
SMI	size-matched input
snoRNA	small nucleolar RNA
SNRPD1	Small nuclear ribonucleoprotein D1 polypeptide
SOC	Super optimal broth with catabolite repression medium
SP3	Single-pot, solid-phase-enhanced sample preparation
SPTAN1	Spectrin alpha, non-erythrocytic 1
STING	Stimulator of interferon genes
TAE	Tris-acetate-EDTA
TALEN	Transcription activator-like effector nuclease

TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
THP	Tamm horsefall protein
TJP	Tight junction protein
TMOD3	Tropomodulin 3
TPM	Transcripts per million
Tris-HCL	Tris hydrochloride
tRNA	transfer RNA
UAG	uracil,adenine,guanine
UTR	Untranslated region
Wac	WW domain containing adaptor with coiled-coil
WT	Wild type
Ybx-1	Y-box binding protein 1
γH2AX	phosphorylated histone H2A variant H2AX

IV. Abstract

RNA-binding proteins (RBPs) are essential mediators of post-transcriptional gene regulation and interaction of RBPs with target RNAs fundamentally influences every step of RNA metabolism. Consequently, dysregulation and malfunctioning of several RBPs is implicated in various human diseases including cancer, muscular atrophies, neurological disorders and kidney diseases. Recently, RBPs *e.g.* Human antigen R or cold-inducible RNA-binding protein have been linked to acute kidney injury (AKI), a serious complication strongly associated with increased mortality in hospitalized patients. Previous data of our group identified the RBP Musashi-1 (MSI1) as highly upregulated in a mouse model of AKI. MSI1 and its family member Musashi-2 (MSI2) are evolutionarily conserved RBPs, which have been studied extensively for their role in governing stem cell maintenance in the blood and nervous system. Unsurprisingly, both Musashi proteins are dysregulated in multiple forms of cancer, implicated in several neurodegenerative diseases and play a role in viral replication in the human body. The role of Musashi proteins in general kidney physiology and AKI however remain elusive. This work shows that MSI1, but not MSI2, is highly expressed in necrotic tubules at the cortico-medullary border in kidneys after ischemia reperfusion injury. Moreover, both Musashi proteins closely interact with a network of stress granule proteins, and oxidative stress causes robust localization of MSI1 and MSI2 in stress granules in tubular epithelial cells. In line with their function as RBPs, MSI1 and MSI2 bind to a common set of proteins involved in mRNA metabolism, translation and complex formation in tubular epithelial cells. While MSI1 interacts with 417 proteins in tubular epithelial cells, the interactome of MSI2 only consists of 296 proteins. Within this study RNA interactors of both Musashi proteins in tubular epithelial cells were characterized and it was shown that MSI1 and MSI2 bind to a high number of RNA binding sites (17922/17399), as well as similar target regions (3'UTRs) and binding motifs (UAG). Moreover, MSI1 (5264 identified RNA targets) and MSI2 (5318 RNA targets) interact with 4207 common transcripts in tubular epithelial cells. Induction of oxidative stress altered binding site and binding region preferences of both Musashi proteins and influenced in particular the composition of the MSI1 RNA interactome in tubular epithelial cells. These findings implicate that both Musashi proteins engage in similar ribonucleoprotein complexes in tubular epithelial cells. Cellular stress induces distinctive alterations to the RNA interactome, especially of MSI1, while the protein interactome remained unaffected. Examining the relevance of individual RNA target interactions and translating these observations into mouse models will help deciphering the role of MSI1 and MSI2 in AKI. Moreover, unraveling pathophysiological mechanisms in the emerging field of RNA-protein interactions holds the promise to uncover innovative therapeutic targets, which are urgently awaited for AKI.

1. Introduction

1.1 Acute kidney injury

1.1.1 Incidence, causes and treatment options

Acute kidney injury (AKI) is characterized by a rapid loss of kidney function occurring within hours to days. AKI leads to a decline in urine output and the accumulation of waste products *e.g.* creatinine in the blood, making serum creatinine concentrations and urine volume the two main parameters to diagnose and classify AKI ¹. Worldwide, AKI has a prevalence of approximately 21% in hospitalized patients ². With an incidence of more than 50%, critically ill patients in intensive care units are at higher risk of developing AKI ³. AKI is associated with an increased risk for chronic kidney disease (CKD) ⁴⁻⁶, cardiovascular morbidity ⁷⁻⁹, kidney failure and death ^{2,10,11}. AKI therapy focuses on preventing, identifying and treating the underlying cause and managing symptoms *e.g.* fluid status / blood pressure control or kidney replacement therapy ¹². Targeted approaches to intervene with AKI development and its progression to chronic kidney disease are still missing. In order to create novel and innovative treatment options, it is necessary to unravel the pathophysiological mechanisms underlying AKI.

1.1.2 Susceptibility of the kidney to AKI

1.1.2.1 Kidney structure and function

The kidney is a highly complex organ, responsible for waste excretion and regulation of whole-body homeostasis *e.g.* through controlling acid-base balance, extracellular fluid volume and blood pressure ¹³. Anatomically, the kidney is structured into cortex, medulla and renal pelvis, and nephrons represent the functional and structural units of the kidney (Figure 1). Blood is initially filtered by the glomeruli located in the kidney cortex. This ultrafiltrate is transported into the proximal tubules where ions, water and nutrients are reabsorbed into the blood stream. The descending limb of the loop of Henle is responsible for water reabsorption. The filtrate gets further concentrated through sodium and chloride ion reabsorption in the ascending limb of the loop of Henle. Different ions are finally reabsorbed in the distal convoluted tubules and the filtrate is processed to urine and excreted through the collecting duct segment.

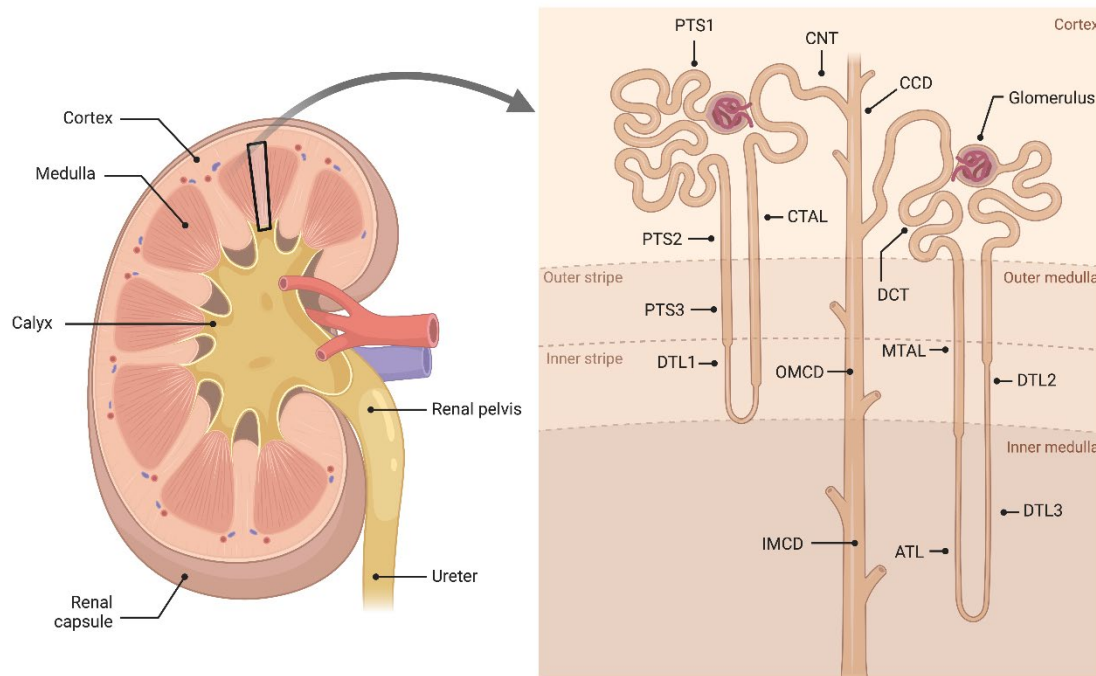


Figure 1. Schematic representation of kidney and nephron structure

The kidney consists of cortex, medulla and renal pelvis (left). Nephrons represent the functional and structural units of the kidney (right). The nephron is structured into proximal tubules (initial segment of the proximal convoluted tubule (PTS1), proximal straight tubule in cortico-medullary rays (PTS2), last segment of the proximal straight tubule in outer stripe of outer medulla (PTS3)), descending limb of the loop of Henle (short descending limb of the loop of Henle (DTL1), long descending limb of the loop of Henle in the outer medulla (DTL2), long descending limb of the loop of Henle in the inner medulla (DTL3)), ascending limb of the loop of Henle (thin ascending limb of the loop of Henle (ATL), medullary thick ascending limb of the loop of Henle (MTAL), cortical thick ascending limb of the loop of Henle (CTAL)), distal convoluted tubules (DCT), collecting duct segment (connecting tubule (CNT), cortical collecting duct (CCD), outer medullary collecting duct (OMCD), and inner medullary collecting duct (IMCD)). Created with BioRender.com (IO253JEK0G).

1.1.2.2 Distinct nephron segments are particularly susceptible to hypoxic injury

AKI is a heterogeneous and complex disease. The pathophysiology varies according to the type of insult and the affected part of the kidney. While some factors e.g. rhabdomyolysis primarily affect tubules in the outer stripe of the outer medulla ¹⁴, other complications like volume depletion mostly damage epithelial cells in the inner medulla ¹⁵. Except for nephrotoxic drugs, which directly injure tubular epithelial cells, the majority of AKI-inducing events lead to hypoperfusion and subsequently to the development of hypoxia in the kidney ¹⁶. Two compartments, the S3 proximal tubules and the thick ascending limb (TAL) of the loop of Henle, are particularly susceptible to ischemic injury ¹⁷. Anatomically, S3 proximal tubules and TAL are localized in the outer medulla, which is the segment with the lowest estimated oxygen pressure in the kidney ¹⁸. Functionally, the proximal tubule is responsible for reabsorption of nutrients into the blood stream and excretion of waste products into the filtrate, two processes showing the highest energy demand within the kidney ¹⁹. Likewise, the loop of Henle possesses high transporter activity and participates in urine concentration and salt excretion. Under

normoxic conditions, tubular epithelial cells cover their energy demand via oxidative phosphorylation (proximal tubules) or anaerobic glycolysis (distal tubules) ¹⁷. Hypoxia induces an imbalance between oxygen / energy supply and demand in the kidney ¹⁶. This deficiency renders tubules with a general low oxygen supply and high energy demand, like the S3 proximal tubules and TAL, vulnerable to acute tubular injury.

1.1.3 Acute tubular injury as the central hallmark of AKI

1.1.3.1 Studying AKI pathophysiology in experimental models

While kidney organoids and zebrafish models provide efficient tools for high throughput drug screening, our understanding of pathophysiological mechanisms in AKI has primarily been gained from observations made in rodent and large animal models ²⁰. Research on AKI in rodents focuses largely on models of ischemia reperfusion injury (IRI), nephrotoxic drugs and sepsis. The ultimate consequence of all types of insults is the development of acute tubular injury ¹⁵. Common pathophysiological mechanisms, which lead to the onset of acute tubular injury, are inflammation ^{21–23}, endothelial injury ^{24–26} and activation of tubular epithelial stress response pathways ^{27,28}. Although most insight into pathophysiological mechanisms was obtained through IRI models ^{29,30}, many insults leading to AKI share at least one of the hallmarks converging into acute tubular injury. As this work focuses on tubular epithelial cells, understanding activated stress response pathways within these cells is of fundamental importance to decipher the pathophysiology of AKI.

1.1.3.2 Stress response of tubular epithelial cells

1.1.3.2.1 Endoplasmic reticulum stress

Different insults *e.g.* ischemia-reperfusion injury (IRI) and nephrotoxic drugs are known to induce endoplasmic reticulum (ER) stress in tubular epithelial cells ³¹. Accumulation of unfolded and misfolded proteins in the ER triggers diverse unfolded protein response pathways. In order to maintain protein homeostasis PERK1 (Proline-rich receptor-like protein kinase), IRE1 (Inositol-requiring enzyme 1), and ATF6 (activating transcription factor 6) signaling is activated to cope with short-term cellular stress in tubular epithelial cells after damage ³². However, sustained ER stress activates the transcriptional regulator CCAAT/enhancer-binding protein, which triggers apoptosis of endothelial and tubular epithelial cells in AKI ³³. Finally, unresolved ER stress is linked to the development of CKD after AKI ^{34,35}.

1.1.3.2.2 Mitochondrial dysfunction

One pathophysiological mechanism activated by many causes of AKI is mitochondrial dysfunction^{36,37}. Sepsis and IRI have been shown to induce major structural abnormalities in mitochondria including swelling and fragmentation^{38,39}. Disruption of mitochondrial integrity is accompanied by the release of pro-inflammatory molecules e.g. cytochrome c³⁸ and mitochondrial DNA⁴⁰. The following impairment of mitochondrial oxidative metabolism leads to reduced ATP production and the accumulation of non-esterified fatty acids and ROS, which trigger inflammation and tubular cell death^{41–43}.

1.1.3.2.3 DNA damage response

Cisplatin and IRI are known to induce DNA damage and DNA damage response^{44,45}. Upon kidney injury, the DNA damage sensors Ataxia telangiectasia mutated (ATM) and Rad-3 related are activated⁴⁶. Increased levels of the checkpoint kinase 2 lead to activation of p53 and induce G2/M cell cycle arrest of tubular epithelial cells⁴⁷. Importantly, unresolved G2/M cell cycle arrest triggers maladaptive tubular epithelial repair, senescence and kidney fibrosis^{48,49}.

1.1.3.2.4 Cell death

Severe and sustained injury leads to tubular epithelial cell death. Apoptosis, necroptosis and ferroptosis are the main cell death pathways involved in AKI⁵⁰. Apoptosis was one of the first cell death pathways connected to AKI⁵¹ and it was shown that apoptosis contributes to tubular epithelial cell death and kidney dysfunction *in vivo*^{52,53}. Recent evidence suggests that regulated necrosis pathways majorly contribute to AKI pathophysiology^{54,55}. While apoptotic cells maintain membrane integrity when being removed by macrophages, regulated necrosis pathways e.g. necroptosis and ferroptosis, are characterized by release of damage-associated patterns (DAMPs) and a pro-inflammatory immune response, which typically aggravates acute tubular injury⁵⁶. Necroptosis relies on activated receptor-interacting serine/threonine protein kinase 3 (RIPK3) which phosphorylates its effector protein mixed-lineage kinase domain-like protein (MLKL). Necroptosis is a highly inflammatory type of cell death, as activated MLKL disrupts the cellular plasma membrane and releases a plethora of intracellular DAMPs⁵⁷. Together with ferroptosis, necroptosis is considered a major contributor to tubular epithelial cell death observed in AKI^{58,59}. Ferroptosis is an iron- and ROS-dependent programmed cell death with a unique signaling cascade relying on lipid peroxidation⁶⁰. Many studies report ferroptosis being the more significant form of cell death in AKI^{54,61,62}, but as ferroptosis and necroptosis are interconnected and compensatory, more research needs to decipher the contribution of both pathways to AKI pathogenesis^{50,59}.

1.1.3.3 Tubular epithelial repair and regeneration

The kidney has the capacity to repair and regenerate injured tubules ⁶³. Damaged tissue is predominantly replaced by surviving tubular epithelial cells, which undergo epigenetic reprogramming in order to enable transcriptional activation of dedifferentiation programs ^{64–66}. Dedifferentiated tubular epithelial cells rapidly proliferate to replace dead cells and reconstitute the tubular epithelium ⁶⁷. Of note, several studies report on the existence of a fixed population of renal progenitor cells with the capacity to regenerate tubular epithelial cells ^{68,69}. Despite the tremendous potential of the tubular epithelium for regeneration, it is meanwhile accepted that any injury comes with the consequence of increased susceptibility in the future.

1.1.4 Renoprotective strategies

Up until now, there is no dedicated therapy available to treat AKI as a disease entity, and targeted preventive measures are largely missing. Although many renoprotective strategies demonstrated promising results in experimental set ups, a successful implementation of these procedures into the clinic is lacking.

1.1.4.1 Targeting inflammation

Sustained inflammation is one of the hallmarks of AKI ^{21–23}. Promising approaches to target kidney inflammation after injury include blockage of stimulator of interferon genes (STING), which significantly reduced inflammation and ameliorated cisplatin-induced AKI in mice ⁷⁰. Inhibition of the complement system by administration of the C1-inhibitor led to a marked reduction of inflammation in mice after IRI ⁷¹. Treatment with an antibody targeting Toll-like receptor 2 significantly reduced neutrophil infiltration and kidney damage after IRI in mice ⁷² and is currently evaluated in clinical trials to prevent AKI in patients with kidney transplantation ⁷³.

1.1.4.2 Cell death inhibition

Inhibition of necroptosis and ferroptosis emerged as potent concepts to not only interfere with renal tubular cell death but also necrosis-induced inflammation in experimental AKI ^{74,75}. One of the most efficient inhibitors of necroptosis and ferroptosis, Necrostatin-1, significantly alleviated inflammation, cell death and overall renal damage in multiple models of AKI ^{55,58,76}. Although these two pathways are interconnected, suppression of either one of them was also shown to efficiently attenuate renal dysfunction upon acute tubular injury. Blocking necroptosis through direct or indirect RIPK1 inhibition ^{77,78} protected from AKI to a similar extend as observed when ferroptosis was inhibited *in vivo* ^{79–81}.

1.1.4.3 Mitochondrial protection

A multitude of novel strategies target mitochondrial dysfunction in AKI ³⁶. Many approaches target mitochondrial integrity *e.g.* by inhibiting fragmentation or enhancing biogenesis, restoring mitochondrial homeostasis and rescuing kidney function in experimental AKI ^{38,82,83}. Multiple other treatments aim to reconstitute mitochondrial metabolism and increase mitochondrial antioxidant defense to reduce ROS production and oxidative stress in AKI ^{84–86}. As an example, stimulating nicotinamide adenine dinucleotide biosynthesis and enhancing mitochondrial function was reported to induce renoprotection in animal models of AKI ^{84,87}.

1.1.4.4 Hypoxic adaptation

Many insults that cause AKI share the pathological development of hypoxia in the kidney ¹⁶. Under physiological conditions cells adapt to hypoxia via stabilization of hypoxia-inducible factor (HIF) which induces expression of genes involved in many cellular survival pathways ⁸⁸. While stabilization of HIFs through hypoxic preconditioning prior to injury was shown to potently protect from developing AKI in experimental models ^{89,90}, conflicting data exist on the effectiveness of remote ischemic preconditioning to protect from AKI in humans ^{91,92}. Prolyl hydroxylases (PHD) are the enzymes responsible for oxygen-dependent hydroxylation and degradation of HIFs under normoxic conditions ⁸⁸. Administration of inhibitors of PHDs ameliorated kidney damage after cisplatin treatment ⁹³ and IRI ⁸⁹ *in vivo* and is being evaluated in clinical trials to reduce AKI in patients after cardiac surgery (Clinical- Trials.gov Identifier: NCT05010460).

1.1.4.5 Stimulation of autophagy

Autophagy is activated upon AKI and protects from tubular injury through maintaining cellular integrity ^{94–96}. Autophagy is regulated by various cellular stress stimuli including nutrient/energy-sensing pathways *e.g.* phosphatidylinositol 3-kinase, AMP-activated protein kinase and sirtuin signaling, but also through cellular stress responses *e.g.* ROS, hypoxia or ER stress ⁹⁷. Activation of autophagy in AKI mediates cellular repair and homeostasis via *e.g.* removal of damaged organelles and protein aggregates ⁹⁸. Consequently, enhancing autophagy in AKI is a promising approach to promote repair and regeneration after injury and prohibit acute tubular injury and cell death ⁹⁹. In this context, many studies showed that stimulation of autophagy significantly protects against kidney damage in different models of AKI ^{96,100,101}.

1.1.4.6 Dietary interventions

Short-term caloric restriction (reduction to 10-40% of normal calorie intake), represents a dietary intervention which efficiently protects from AKI in several experimental models ^{102–104}. Interestingly, caloric restriction conveys renoprotection in AKI through activation of many pro-survival mechanisms described before *e.g.* reduction of oxidative stress and increased mitochondrial function and autophagy ^{105–107}. Other dietary interventions *e.g.* a fasting mimicking diet and a diet with restricted sulfur-containing amino acids, significantly protected from kidney damage in mice with IRI ¹⁰⁸. Likewise, a non-dairy diet with a restricted amount of sulfur-containing amino acids is being evaluated in patients with cardiac surgery in a clinical trial (Clinical- Trials.gov Identifier: NCT03715868). Importantly, renoprotective dietary interventions trigger cellular survival and stress resistance through several shared mechanisms including adaptation of oxidative and hydrogen sulfide (H₂S)-dependent cysteine catabolism ¹⁰⁸. The identification of common mechanisms between different dietary interventions emphasizes that understanding underlying molecular processes behind all renoprotective strategies is key to developing new therapy options for AKI.

1.2 RNA-binding proteins

RNA-binding proteins (RBPs) are one of the key players of post-transcriptional regulation of gene expression. By binding to their targets or contributing to the formation of macromolecular ribonucleoprotein complexes, they are involved in almost every step of RNA metabolism – from processing and maturation up to storage and degradation of target RNA molecules – and function. Only in recent years, the development of innovative technologies made it possible to start unraveling the various interactions of proteins with RNAs and their relevance in human diseases.

1.2.1 Characterization of the RNA-binding proteome

The first approaches used to define the total RNA-protein interactome, were made by applying techniques *e.g.* RNA interactome capture (RIC) ^{109–112} or RBDmap ¹¹³ in various human cell culture models. An integrative analysis of these published data sets assigned the human cellular RNA-binding proteome (RBPome) a size of 4257 proteins (<https://rbpbase.shiny.embl.de/>) ^{114,115}. Whilst half of the proteins contained in the human RBPome bind to RNAs through classical RNA-binding domains (RBDs), such as an RNA recognition motif (RRM), a DEAD box helicase domain or a K homology domain, the remaining RBPs astonishingly lack classical RBDs ¹¹⁴ and are termed “enigmRBPs”. EnigmRBPs comprise proteins, which have additional roles in cellular biology, apart from RNA binding - *e.g.* metabolic enzymes - extending our knowledge towards the functional versatility within the

RBPome¹¹⁶. Moreover, a lot of the non-classical RNA-binding regions contain intrinsically disordered regions - protein domains of low sequence complexity and highly variable structure - which among others play a key role in the formation of biomolecular condensates through liquid-liquid phase separation (LLPS)¹¹⁷. As LLPS is emerging as an important mechanism regarding the formation of membraneless organelles like the nucleolus, cajal bodies and stress granules (SGs), some of the novel RBPs could have pivotal roles in the compartmentalization, organization and stress response of the cell¹¹⁸. Importantly, global changes in the expression of RBPs are detected during cellular differentiation processes^{119,120} and differential binding of RBPs to their RNA targets is induced upon exposure to distinct cellular stressors^{121–123}.

1.2.2 Interaction of RBPs and messenger RNAs

RBPs recognize RNAs through their specific sequence and/or structure and can interact with messenger RNA (mRNA), as well as non-coding RNA (ncRNA). Due to similarities in structure and sequence shared by many RNA molecules, most RBPs are reported to bind to more than one class of RNA and therefore often participate in the regulation of a row of processes^{124–126}. Importantly, in line with their vital role in translation, RBPs which bind to mRNA, represent one of the most abundant classes of RBPs in eukaryotic cells and the dynamic assembly and disassembly of mRNA ribonucleoprotein complexes (mRNPs) regulate the fate and function of mRNAs at every post-transcriptional stage¹²⁷. RBPs are crucial for nuclear pre-mRNA processing, including 5' capping, splicing, chemical base modification and 3' polyadenylation, in part executed through large and complex RNP machineries like the spliceosome (Figure 2 a)¹²⁸. Mature mRNAs are packed into mRNP export complexes and transported into the cytoplasm (Figure 2 b)¹²⁹, where translation through ribosomes is tightly controlled by the binding of RBPs to defined motifs and structures in the mRNA *e.g.* the 5'cap, the 5' untranslated region (5'UTR), the open reading frame, the 3'UTR and the poly(A) tail¹³⁰. Beyond governing all aspects of the translational process – from initiation, rate and efficiency up to termination (Figure 2 c)¹³¹, RBPs regulate mRNA stability (Figure 2 d)¹³² and control mRNA decay (Figure 2 e). As mentioned before, aside from their crucial function in pre-mRNA processing and protein translation, mRNA interacting proteins transport and shuttle mRNAs between different cytoplasmic locations *e.g.* between ribosomes and SGs or processing bodies (PBs). Thereby they enable not only the correct storage of mRNAs but also a highly dynamic subcellular organization which is needed to counteract cellular stress and environmental challenges (Figure 2 f)¹³³.

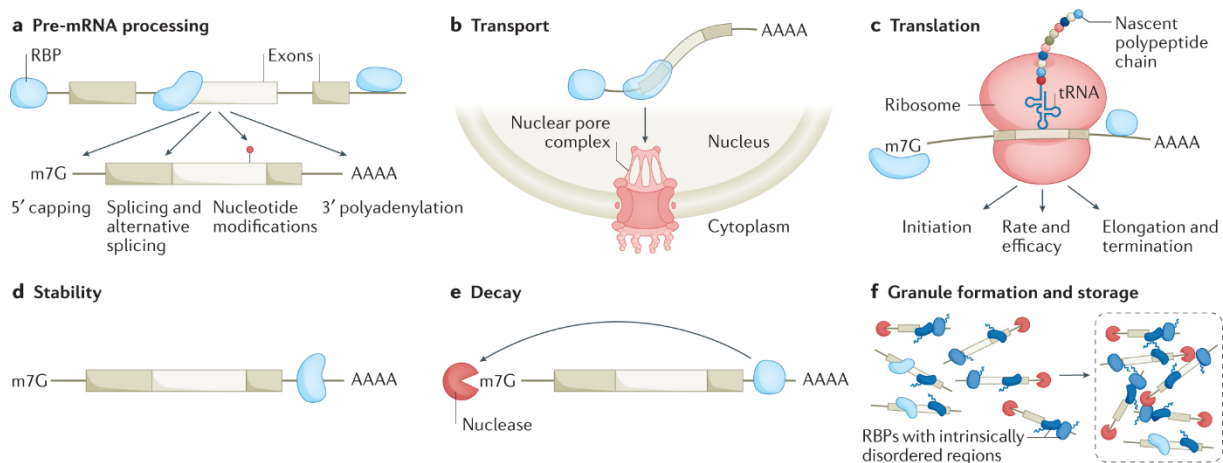


Figure 2. Interaction of RBPs with mRNA

Interactions with RBPs regulate the fate and function of mRNAs at every level. **a|** RBPs are crucial for the processing of pre-mRNAs regarding 5'capping, splicing/alternative splicing, chemical base modifications and 3' polyadenylation. **b|** The dynamic assembly and disassembly into packed mRNA ribonucleoprotein complexes (mRNPs) enables mRNA transport e.g. from the nucleus into the cytoplasm. **c|** Here, protein synthesis is tightly controlled by RBPs which regulate initiation, rate and efficacy as well as elongation and termination of translation. **d|** Moreover, RBPs influence mRNA stability, **e|** determine mode and time of mRNA decay **e|** or participate in the formation of membraneless organelles e.g. SGs, in order to store all types of RNAs (including both mRNA and ncRNA). Figure origin: Seufert, L., Benzing, T., Ignarski, M. & Müller, R. U. RNA-binding proteins and their role in kidney disease. *Nat. Rev. Nephrol.* 2021 183 18, 153–170 (2021).

1.2.3 Interaction of RBPs and non-coding RNAs (ncRNAs)

While a lot of research focusses on mRNA-RBP interactions, the interplay of RBPs with the ncRNA world is just recently gaining ground. Early methods e.g. RIC or RBDmap used oligo(dT) pulldowns for the purification of crosslinked RNP complexes and therefore primarily captured polyadenylated and highly abundant RNAs e.g. most mRNAs, leaving non-polyadenylated and low-expressed RNAs such as several groups of ncRNAs behind. Innovative techniques like the phenol toluol extraction ¹³⁴, orthogonal organic phase separation ¹³⁵ and protein-crosslinked RNA extraction ¹³⁶ make use of the unique physicochemical properties of crosslinked RNA-protein complexes to enrich and isolate RNPs containing all RNA species, in the interface between an organic and aqueous phase.

Similar to mRNA-RBP interactions, binding of RBPs to ncRNAs fulfils tasks, which are central to RNA biogenesis and function, such as various pre-RNA processing steps, chemical base modification and transport of target ncRNAs to different cellular compartments (Figure 3 a-d). Depending on the type of bound ncRNA, RBPs can be distinguished into translation-related and gene-regulatory non-coding RBPs, with each group undertaking specialized cellular roles in addition to the common features described before ¹³⁷. Translation-related ncRNA RBPs include RBPs which interact with ncRNAs e.g. small nuclear RNAs (snRNAs), small nucleolar and small cajal body-specific RNAs (snoRNAs and scaRNAs), as well as ribosomal RNAs

(rRNAs) and transfer RNAs (tRNAs). Interaction with these ncRNAs enables important cellular processes e.g. splicing¹³⁸, RNA modification¹³⁹ and translation^{140,141}.

A more indirect, but not less important impact on protein synthesis is mediated through the group of gene-regulatory non-coding RBPs, including RBPs, which bind to small silencing RNAs e.g. micro RNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs), as well as RBPs which bind to the heterogeneous group of long non-coding RNAs (lncRNAs). While miRNAs, siRNAs and piRNAs mediate post-transcriptional gene silencing via RNA interference (Figure 3 e+f)¹⁴², circRNAs are primarily involved in miRNA sponging (Figure 3 g)¹⁴³ and lncRNA-RBP complexes execute many gene-regulatory processes (Figure 3 h)¹⁴⁴.

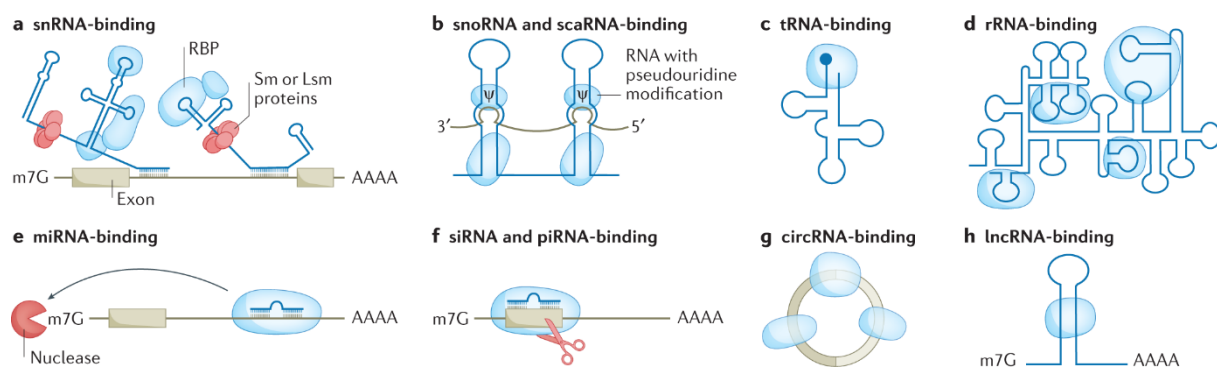


Figure 3. Interaction of RBPs with ncRNA

Similar to the interaction of RBPs with mRNAs, RBPs binding to non-coding RNAs (ncRNAs) affect general RNA biology e.g. ncRNA processing, transport, storage and nucleotide modification. Depending on the type of ncRNA bound, the cellular roles of these interactions can be divided into two large groups: translation-related processes (a-d) or gene-regulatory functions (e and f). Translation-related functions primarily include interactions of RBPs with **a**| small nuclear RNAs (snRNAs), **b**| small nucleolar and small Cajal body-specific RNAs (snoRNAs and scaRNAs), **c**| ribosomal RNAs (rRNAs) and **d**| transfer RNAs (tRNAs) and are therefore crucial for splicing, nucleotide modification, ribosome biogenesis and delivery of amino acids to the translating ribosome. RBPs (primarily of the Argonaute family) which bind to **e**| micro RNAs (miRNAs), **f**| short interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs), mediate post-transcriptional gene- and epigenetic silencing and are thus involved in gene-regulatory processes. **g**| Interaction of circRNA with RBPs influences protein functionality, scaffolding, degradation and recruitment to distinct cellular compartments. The interaction of RBPs with **h**| long non-coding RNAs (lncRNAs) enables multiple cellular processes including regulation of gene expression on the transcriptional level, alternative splicing, genome stability & organization and mRNA stability & translational regulation. However, RBP-lncRNA interactions may also act as scaffolds in large RNP organelles e.g. SGs, in order to store all types of RNAs (including both mRNA and ncRNA)¹⁴⁵. Figure origin: Seufert, L., Benzing, T., Ignarski, M. & Müller, R. U. RNA-binding proteins and their role in kidney disease. *Nat. Rev. Nephrol.* 2021 183 18, 153–170 (2021).

1.2.4 RBPs in AKI

In contrast to protein-protein interactions, RNA-protein interactions remained elusive for a long time, partly due to technical difficulties hampering their specific enrichment. The revolution in RNA sequencing technologies on the one hand, paired with innovative techniques to study RNA-protein interactions paved the way to entirely new opportunities towards a global analysis of the RBPome in health and disease ^{110,136,146}. The pivotal role of RBPs in cellular biology rests on the plethora of target RNAs they bind to and hence the multitude of processes they are involved in. Consequently, dysregulation and malfunction of various RBPs are associated with human diseases e.g. muscular atrophies, neurological disorders like amyotrophic lateral sclerosis ¹⁴⁷, cancer ¹⁴⁸ and kidney disease ¹⁴⁹.

Multiple RBPs are suggested to play a role in AKI including Human antigen R (HuR), Y-box binding protein 1 (Ybx-1), cold-inducible RNA binding protein (CIRP) and Pumilio 2 (Pum2) (Figure 4) ¹⁴⁹. HuR expression was associated with a potential pro-survival function in AKI. HuR levels and cytoplasmic localization was increased upon IRI in the proximal tubules of rats ¹⁵⁰ and HuR was critical for Akt (Protein kinase B) activation¹⁵¹, autophagosome formation and survival of proximal tubular cells *in vitro* ¹⁵². Similarly, Ybx-1 was identified as a mediator of anticoagulant protease activated protein C (aPC)-dependent cytoprotection of the kidney upon IRI in mice ¹⁵³. In contrast, many studies connected elevated levels of CIRP with aggravated AKI in patients and experimental models. Increased secretion of CIRP after cardiac surgery was associated with the development of AKI in patients ¹⁵⁴ and mediated inflammation, oxidative stress and mitochondrial dysfunction in rats ^{155,156}. In line with these reports, inhibition of CIRP as well as knockout of *Cirp* in mice protected from kidney damage in experimental mouse models of AKI ^{157–159}. The RBP Pum2 was identified as a negative regulator of mitochondrial fission factor (Mff) expression in AKI ¹⁶⁰. *Mff* knockout in mice improved mitochondrial function and attenuated kidney damage upon IRI. Interestingly, Pum2 overexpression in primary proximal tubule cells, isolated after IRI, markedly reduced Mff levels, mitochondrial dysfunction and tubular cell injury, highlighting Pum2 as a potential therapeutic target in AKI.

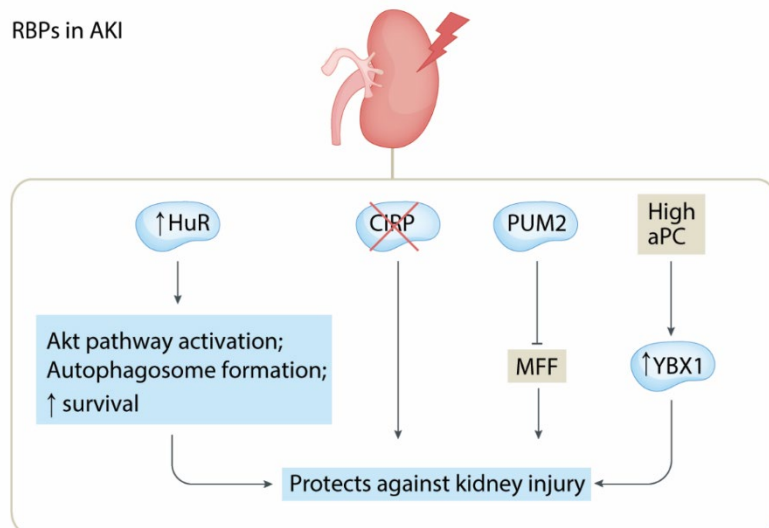


Figure 4. RBPs implicated in AKI

Implications of RBPs in AKI. Increased expression and cytoplasmic shuttling of Human antigen R (HuR) was detected upon IRI and was associated with activation of Akt (Protein kinase B) pathway, autophagosome formation and cellular survival in proximal tubule cells. Increased secretion of cold-inducible RNA-binding protein (CIRP) was linked to AKI in patients after cardiac surgery and reduced levels of CIRP protected from kidney damage in experimental AKI. As a negative regulator of mitochondrial fission factor (Mff), Pumilio 2 (Pum2) was shown to enhance mitochondrial function and protect from tubular injury. Y-box binding protein 1 (Ybx-1) was identified as downstream target of anticoagulant protease activated protein C (aPC) and mediated renoprotection after IRI in mice. Modified after: Seufert, L., Benzing, T., Ignarski, M. & Müller, R. U. RNA-binding proteins and their role in kidney disease. *Nat. Rev. Nephrol.* 2021 183 18, 153–170 (2021).

1.2.5 RBPs as therapeutic targets

As described before, RBPs are associated with multiple human pathologies including neurological disorders¹⁴⁷, cancer¹⁴⁸ and kidney disease¹⁴⁹. Development of RBP therapeutics has focused on posttranslational inhibition of RBP function on the one hand¹⁶¹ and interference with mRNA expression through RNA therapeutics on the other hand¹⁶². Especially high-throughput compound screening allowed for the identification of specific inhibitors e.g. natural compounds, small molecules or peptides which impede the function of RBPs¹⁶³. In this context, multiple small molecule inhibitors targeting HuR demonstrated anti-tumor properties in various cancer cells^{164–166}. Moreover, forced nuclear compartmentalization of Ybx-1, using small molecule inhibitors, was shown to ameliorate kidney inflammation and damage in a mouse model of kidney fibrosis¹⁶⁷. RNA therapeutics comprise antisense-based technologies e.g. double-stranded siRNAs and antisense oligonucleotides (ASOs), as well as modified mRNAs or RNA aptamers¹⁶². HuR expression was efficiently reduced by ASO administration in a mouse model of neuropathic pain¹⁶⁸ and by siRNA in mice with xenograft ovarian tumors¹⁶⁹. In summary RBPs represent promising targets for novel therapies of different human diseases based on their versatile functions and RNA targets.

1.3 Musashi RNA-binding proteins

The family of Musashi RNA-binding proteins is evolutionary conserved and was first described in *Drosophila melanogaster*, where Musashi was identified as an essential protein for asymmetric cell division of sensory organ precursor cells ¹⁷⁰. Mammalian cells express two homologous proteins – Musashi-1 (MSI1) and Musashi-2 (MSI2). MSI1 was extensively studied for its role in the central nervous system (CNS). Multiple reports found MSI1 to be highly expressed in undifferentiated neural stem and progenitor cells and MSI1 levels rapidly declined during neural differentiation. Interestingly, MSI1 was identified to contribute to proliferation and expansion of neural stem and progenitor cell populations through regulating their self-renewal capacity ^{171–176}. In this context, MSI1 was shown to bind to target mRNA e.g. *Numb* (NUMB Endocytic Adaptor Protein) and regulate post-translational gene expression ^{177,178}. Of note, MSI2 was found to be co-expressed with MSI1 in the CNS and the two are thought to act cooperatively ¹⁷⁹. Besides the nervous system, Musashi proteins were demonstrated to be important during spermatogenesis ^{180–182} and morphogenesis of photoreceptor neurons ^{183–185}. While MSI1 is primarily associated with the CNS, MSI2 is predominantly connected to hematopoietic stem cells. Similar to the role of MSI1 in neural stem cells, MSI2 was shown to regulate stem-cell maintenance and normal hematopoiesis via regulating asymmetric cell division of hematopoietic stem cells ^{186,187}. In summary, Musashi proteins are key post-transcriptional regulators, which are not only responsible for self-renewal of many germline- and somatic- stem cells ^{188,189}, but also play a role in development and maintenance in other tissues e.g. testis ^{180–182} or eye ^{183–185}.

1.3.1 Musashi-RNA interactions

Mouse MSI1 and MSI2 share 75% overall amino acid sequence similarity ¹⁷⁹ with two RNA recognition motifs (RRMs) at the N-terminal end governing the binding to RRM recognition sites at the 3' end of target mRNAs ^{176,190}. Due to the high sequence similarity of their RRMs (up to 91% between MSI1 and MSI2 ¹⁷⁹), the two Musashi proteins were found to bind to a largely, but not completely, overlapping population of target transcripts in many different cell types ^{184,191–198}. In recent years, various crosslinking and immunoprecipitation (CLIP) technologies have been developed to study the interaction of RBPs with RNAs ^{199,200}. Application of these CLIP technologies on different tissues and cell lines identified the UAG base triplet as predominant RNA binding motif and the 3' untranslated region (3'UTR) as preferred binding site of both Musashi proteins (Table 1).

On a molecular level the Musashi proteins are known to repress mRNA translation^{177,201–204} as well as stimulate translation through direct mRNA stabilization^{205,206} or enhanced polyadenylation of target transcripts^{207–211}. In addition, several studies report on a role of Musashi proteins in alternative splicing^{183,212} and their interference with miRNA biogenesis^{213,214}.

Table 1. Overview of different CLIP methods used to identify RNA targets of MSI1 and MSI2

Protein	Method	Cell type	Organism	Top two binding sites	Year
MSI1	HITS-CLIP	Intestinal epithelium	Mouse	3'UTR CDS	2015 ¹⁹¹
	iCLIP	U251 glioblastoma cell line	Human	3'UTR 5'UTR	2015 ¹⁹²
	eCLIP	Retina	Mouse	3'UTR introns	2022 ¹⁸⁴
	eCLIP	pediatric medulloblastoma cell line (SU_MB002) embryonic cerebellar neural stem cells (NSC201cb)	Human	3'UTR CDS	2022 ¹⁹⁶
MSI2	HITS-CLIP	K562 chronic myeloid leukemia cell line	Human	CDS 3'UTR	2014 ¹⁸⁶
	HITS-CLIP	Intestinal epithelium	Mouse	3'UTR CDS	2015 ¹⁹³
	HITS-CLIP	NB4 hematopoietic cell line	Human	3'UTR CDS	2016 ¹⁹⁷
	HITS-CLIP	Keratinocytes	Mouse	3'UTR ncRNA	2016 ¹⁹⁴
	PAR-CLIP	HEK293 cells	Human	3'UTR introns	2018 ¹⁹⁵
	iCLIP	K562 chronic myeloid leukemia cell line	Human	3'UTR introns	2022 ¹⁹⁸

Abbreviations: High-throughput sequencing of RNA isolated by CLIP (HITS-CLIP); individual-nucleotide resolution CLIP (iCLIP); enhanced CLIP (eCLIP); photoactivatable ribonucleoside-enhanced CLIP (PAR-CLIP); coding sequence (CDS)

1.3.2 Role of Musashi proteins in disease

MSI1 and MSI2 are overexpressed as oncoproteins in various types of cancer²¹⁵. In this context, MSI1 and MSI2 levels are not only prognostic for the outcome but drive disease progression and cancer aggressiveness e.g. in different types of leukemia^{187,202}, colorectal^{193,216} or pancreatic cancer²¹⁷. In addition to their oncogenic potential, MSI1 expression is

essential for replication of the zika virus ^{218,219}, MSI1 and MSI2 form toxic aggregates in Alzheimer's disease ^{220,221} and downregulation of *Msi1* is associated with kidney fibrosis ²²².

1.3.3 Expression of Musashi proteins in the kidney

RNA sequencing of microdissected renal tubules from mouse kidneys revealed a low abundance of *Msi1* mRNA along the different nephron segments ²²³ (Figure 5 a). *Msi1* expression was only detected in distinct sections of the thin descending limb of the loop of Henle (DTL1, DTL2, DTL3), the ATL and the OMCD. In contrast, *Msi2* was expressed across all 14 nephron segments with the highest levels detected in medullary sections of the loop of Henle (MTAL, DTL1, DTL3, ATL) and the IMCD (Figure 5 b). Surprisingly, MSI2 protein levels in microdissected renal tubules from rat kidneys were highest in the proximal tubule segments PTS2 and PTS3 ²²⁴ (Figure 5 b), which depicted the lowest *Msi2* mRNA levels within the nephron (Figure 5 a). MSI1 protein was not detected in rat kidneys.

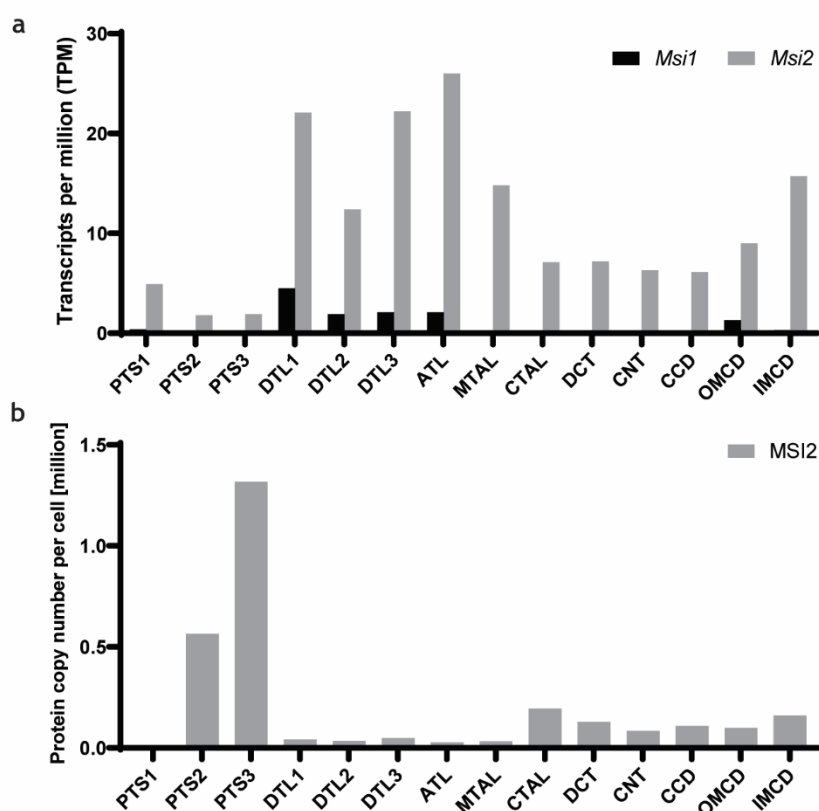


Figure 5. Expression of Musashi mRNA and protein across the renal tubule segments

a| mRNA expression of *Msi1* and *Msi2* along the kidney tubular system determined by RNA sequencing of microdissected mouse kidneys. While *Msi1* mRNA was exclusively measured in the loop of Henle and the OMCD, *Msi2* mRNA was quantified in all tubular segments and was highly expressed in medullary sections of the loop of Henle ²²³. Transcript expression levels were quantified as transcripts per million (TPM) and are plotted on the y-axis. *Msi1* (black) and *Msi2* (grey) transcript abundance across all 14 tubular segments is depicted on the x-axis. Data retrieved from: Chen, L., Chou, C.-L. & Knepper, M. A. A Comprehensive Map of mRNAs and Their Isoforms across All 14 Renal Tubule Segments of Mouse. J. Am. Soc. Nephrol. 32, 897–912 (2021) ²²³. *continued on next page*

b| Musashi protein expression along the kidney tubular system determined by mass-spectrometry of microdissected rat kidneys. MSI1 was not measured in the tubular segments of rat kidneys. MSI2 expression was detected to similar levels in CTAL, DCT and collecting duct of rat kidneys and highly abundant in PTS2 and PTS3 of the proximal tubules ²²⁴. Protein levels were quantified as copy number per cell and are plotted per million on the y-axis. Data retrieved from: Limbutara, K., Chou, C. L. & Knepper, M. A. Quantitative proteomics of all 14 renal tubule segments in rat. J. Am. Soc. Nephrol. 31, 1255–1266 (2020) ²²⁴.

1.3.4 Expression of Musashi proteins in AKI

Previously published data of our own group showed that in transcriptomes of cisplatin-damaged mouse kidneys, *Msi1* was one of the most highly upregulated RBPs compared to control mice ¹⁰⁴ (Figure 6). In contrast, expression of *Msi2* remained unchanged upon cisplatin-induced AKI in mice.

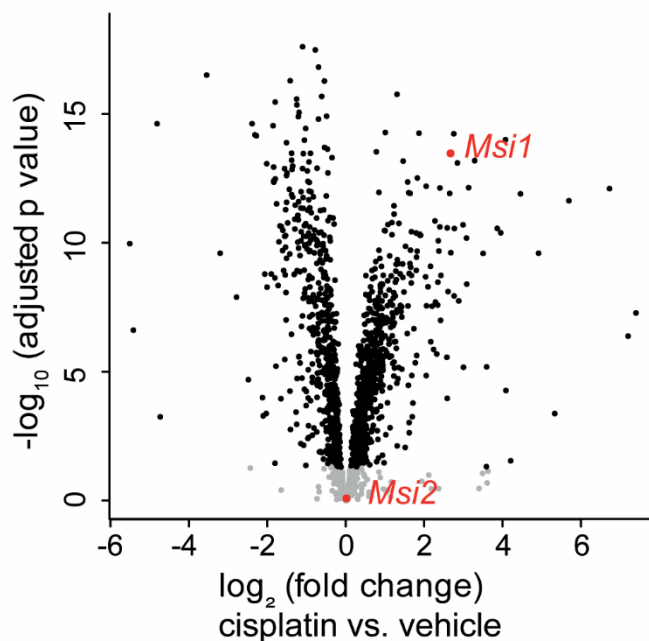


Figure 6. *Msi1* is one of the most upregulated genes in mouse kidneys after cisplatin treatment

While *Msi2* was not differentially regulated, *Msi1* was found to be one of the most upregulated genes in cisplatin-damaged kidneys compared to kidneys of vehicle-treated mice (Rank 34 from 1307 significant differentially regulated RBPs). Shown is a scatter plot depicting expression of *Msi1* and *Msi2* in transcriptomes of cisplatin damaged mouse kidneys compared to controls. Visualized are all differentially expressed genes encoding RBPs between cisplatin-treated and vehicle-treated mouse kidneys (transcriptomes) ¹⁰⁴. Plotted are the $-\log_{10}$ adjusted p values on the y-axis and the $\log_2$ fold change (cisplatin-treated kidneys vs. vehicle-treated kidneys) on the x-axis. Significantly regulated genes (student's t-test, $FDR < 0.05$) are illustrated in black, non-significant genes are depicted in grey. *Msi1* ($\log_2 FC = 2.67$; adj. p value = 3.38×10^{-14}) and *Msi2* ($\log_2 FC = 0.02$; adj. p value = 0.83) are colored in red. Data retrieved from: Späth, M. R. et al. The proteome microenvironment determines the protective effect of preconditioning in cisplatin-induced acute kidney injury. Kidney Int. 95, 333–349 (2019) ¹⁰⁴.

1.4 Thesis aims

Given the general role of RBPs in kidney disease ¹⁴⁹ and the implications for MSI1 in kidney fibrosis ²²², studying the role of MSI1 and MSI2 in the kidney and investigating their potential role in AKI will unravel new insights into AKI pathophysiology and highlight novel therapeutic targets.

The purpose of this work was to elucidate the role of MSI1 and MSI2 in AKI and tubular epithelial cells pursuing the following aims:

(1) Studying the impact of Musashi proteins on kidney function at baseline and in AKI

Our group has previously identified *Msi1*, but not *Msi2*, to be highly upregulated in cisplatin-damaged mouse kidneys ¹⁰⁴. As a first step towards deciphering the role of Musashi proteins within the kidney, expression of MSI1 and MSI2 at baseline and in experimental models of AKI was determined. In order to study the role of Musashi proteins in kidney physiology and their impact in AKI, knockout mouse models with conditional loss of MSI1 and MSI2 were generated, phenotyped and subjected to IRI.

(2) Examining the molecular functions of Musashi proteins in tubular epithelial cells

Few data exist on the molecular function of Musashi proteins in cells of the kidney. To study MSI1 and MSI2 in tubular epithelial cells, transgenic cell lines were generated. The subcellular localization of Musashi proteins in response to diverse cellular stressors was examined using EGFP-tagged Musashi cell lines. Paraquat (PQ) stimulation was established as a useful tool to study certain AKI-associated pathomechanisms e.g. oxidative stress and ferroptotic cell death *in vitro*. To gain a deeper understanding of the function of MSI1 and MSI2 in kidney cells, the protein and RNA interactomes of both Musashi proteins were characterized using mass-spectrometry and single-end enhanced crosslinking and immunoprecipitation (seCLIP). Moreover, adaptations of the protein and RNA networks of MSI1 and MSI2 in response to oxidative stress were determined in order to investigate their role in oxidative stress response in tubular epithelial cells.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

Table 2. List of chemicals and reagents used within this work

Chemical / Reagent	Product no.	Provider
[gamma-P32] Adenosine 5'-triphosphate (ATP)	SRP-501	Hartmann Analytics
Acetic acid	7332.1	Carl Roth
Acetonitrile	701881	AppliChem
Agarose	A9539	Sigma-Aldrich
Ammonium persulfate (APS)	A0834	AppliChem
Ampicillin Sodium Salt	K029.2	Carl Roth
Bepanthen® Antiseptic Wound Cream	01578681	Bayer Vital GmbH
Betaine solution	B0300	Sigma-Aldrich
Betaisodona® solution	01931491	Mundipharma GmbH
Bovine Serum Albumin (BSA)	1066	Gerbu
Bromphenol Blue	A512	Carl Roth
Calcium chloride (CaCl ₂)	HN04.2	Carl Roth
Citric acid monohydrate	27490	Fluka
cOmplete™ EDTA-free Protease Inhibitor Cocktail	4693132001	Roche
Coumaric acid	C9008	Sigma-Aldrich
Dextran T 250	9233.1	Carl Roth
Dimethyl sulfoxide (DMSO) Hybri-Max	D2650	Sigma-Aldrich
Dithiothreitol (DTT)	6908.1	Carl Roth
DMEM/F12	D6421	Sigma-Aldrich
DNase/RNase-Free Distilled Water	15657708	Thermo Fisher Scientific
dNTP Mix (10 mM each)	R0191	Thermo Fisher Scientific
Dulbecco's Modified Eagle Medium (DMEM)	D6429	Sigma-Aldrich
Ethanol (99.8%)	9065.3	Carl Roth
Ethidium bromide solution	2218.1	Carl Roth
Ethylenediaminetetraacetic acid (EDTA)	15575020	Thermo Fisher Scientific
Fetal Bovine Serum (FBS)	S 0115	Biochrom AG
Formic Acid	94318	Fluka
Gel Loading Dye, Orange (6X)	B7022S	New England Biolabs
GeneRuler 1kb DNA Ladder	SM0311	Thermo Fisher Scientific
GeneRuler 50bp DNA Ladder	SM0372	Thermo Fisher Scientific
Glucose	G7021	Sigma-Aldrich
GlutaMAX	35050061	Thermo Fisher Scientific
Glycerole	3783	Carl Roth
Heparin Natrium solution	15782698	Braun
HEPES	H0887	Sigma-Aldrich
Histomount	HS-103	National Diagnostics

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Chemical / Reagent	Product no.	Provider
Hoechst 33342 Solution (20 mM)	62249	Thermo Fisher Scientific
Hydrogen chloride (HCl)	T134	Carl Roth
Hydrogen Peroxide (H ₂ O ₂) 30%	107209	Merck
Igepal CA-630	I8896	Sigma-Aldrich
Isopropanol	5752.3	Carl Roth
Ketaset	12467832	Zoetis
KH ₂ PO ₄	P5655	Sigma-Aldrich
LB Medium	X964.2	Carl Roth
LB-Agar	X965.1	Carl Roth
Lipofectamine 2000	11668019	Thermo Fisher Scientific
Lipofectamine RNAiMAX	13778075	Thermo Fisher Scientific
Luminol	9253	Fluka
Magnesium chloride (MgCl ₂)	1.05833.0250	Merck
Methanol	4627.5	Carl Roth
Methyl viologen dichloride hydrate (Paraquat)	856177-1G	Sigma-Aldrich
Meyer's hematoxylin solution	A0884	AppliChem
Magnesium sulphate heptahydrate (MgSO ₄)	P027.2	Carl Roth
N,N,N',N'-tetramethylethylenediamine (TEMED)	2367.3	Carl Roth
NEBuffer™ 3.1	B7203	New England Biolabs
Normal Donkey Serum (NDS)	017-000-121	Dianova
Nonidet P40	1694.0250	AppliChem
NuPAGE™ LDS Sample Buffer	NP0007	Thermo Fisher Scientific
NuPAGE™ MOPS SDS Running Buffer	NP0001	Thermo Fisher Scientific
NuPAGE™ Transfer Buffer	NP00061	Thermo Fisher Scientific
NuSieve® GTG Agarose	50084	Lonza
Opti-MEM	31985-047	Thermo Fisher Scientific
PageRuler Plus Protein Ladder	26620	Fermentas
Paraplast Plus	39502004	VWR
Paraformaldehyde (PFA)	P6148	Sigma-Aldrich
Penicillin-Streptomycin 10,000 U/mL (Pen-Strep)	15140-122	Thermo Fisher Scientific
Periodic acid (99%)	3257	Carl Roth
Phenylmethylsulfonyl fluoride (PMSF)	A0999	AppliChem
Phosphate buffered saline	15374875	Thermo Fisher Scientific
Polyacrylamide (PAA)	T802.1	Carl Roth
Potassium chloride	6781.1	Carl Roth
ProLong™ Gold Antifade Mountant	P36930	Thermo Fisher Scientific
Protease Inhibitor Cocktail III	539134-1ML	Merck
Puromycin	ant-pr-1	Invivogen
Rompun 2% (Xylazin)	01320422	Bayer Healthcare GmbH
ROTI®Block	A151.1	Carl Roth
Schiff's reagent	1.090.330.500	VWR
Sesame oil	S3547-250ML	Sigma-Aldrich
Sodium chloride (NaCl)	3957.1	Carl Roth
Sodium deoxycholate	D6750	Sigma-Aldrich
Sodium dodecyl sulfate (SDS) pellets	CN30.3	Carl Roth

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Chemical / Reagent	Product no.	Provider
Sodium dodecyl sulfate (SDS) powder	A2263	AppliChem
Sodium fluoride (NaF)	S1504	Sigma-Aldrich
Sodium hydrogen phosphate (Na_2HPO_4)	S9390	Sigma-Aldrich
Sodium hydroxide (NaOH)	A3910.1000	AppliChem
Sodium orthovanadate (Na_3VO_4)	S6508	Sigma-Aldrich
Tamoxifen	T5648	Sigma-Aldrich
TaqMan™ Universal PCR Master Mix	4304437	Thermo Fisher Scientific
Temgesic® Buprenorphin	PZN: 00345928	RB Pharmaceuticals Limited
Tetrasodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7$)	106591	Merck
TRI Reagent®	T3934-200ML	Sigma-Aldrich
Tris Hydrochloride (Tris-HCL)	9090.3	Carl Roth
TritonX-100	3051.2	Carl Roth
TriTrack DNA Loading Dye (6X)	R1161	Thermo Fisher Scientific
Trizma base	T1503	Sigma-Aldrich
Trypsin-EDTA Solution (1X), cell culture	T3924	Sigma-Aldrich
Tryptone	1010817	MP Biomedicals
Tween20	3472	Caesar & Loretz
VECTASTAIN® Elite® ABC-HRP Reagent	PK-7100	Vector laboratories
Xylene	371.5	Th. Geyer
Yeast Extract	2363.1	Carl Roth

2.1.2 Assays and Kits

Table 3. List of assays and kits used within this work

Assay / Kit	Product no.	Provider
Avidin/Biotin Blocking Kit	SP-2001	Vector laboratories
Dako Liquid DAB+ Substrate Chromogen System	K3468	Dako
Direct-zol RNA Miniprep Kit	R2052	Zymo Research
GeneJet Gel Extraction Kit	K0692	Thermo Fisher Scientific
GeneJet Plasmid Miniprep Kit	K0503	Thermo Fisher Scientific
High-Capacity cDNA Reverse Transcription Kit	4368814	Applied Biosystems
High-Performance GoTaq® G2 DNA Polymerase with Mg-Free Buffer System	M7801	Promega
PCR Mycoplasma Test Kit I/C	PK-CA91-1096	PromoKine
Q5® High-Fidelity DNA Polymerase	M0491L	New England Biolabs
QIAquick Gel Extraction Kit	28706X4	Qiagen
Qubit™ dsDNA HS-Assay-Kit	Q32854	Thermo Fisher Scientific
Qubit™ Protein Assay Kit	Q33211	Thermo Fisher Scientific
Qubit™ Broad Range (BR) Assay Kit	Q10211	Thermo Fisher Scientific
Quick-DNA Miniprep Plus Ki	D4069	Zymo Research
RNA Clean & Concentrator-5 (DNase not Included)	R1016	Zymo Research
SuperSignal West Femto Chemiluminescent Substrate	34095	Thermo Fisher Scientific

2.1.3 Buffers and solutions

Table 4. List of buffers and solutions used within this work

Buffer / Solution	Composition
Base solution (50x)	12.5 ml NaOH (5N) 1 ml EDTA (0.5 M) 36.5 ml ddH ₂ O
Cell Culture Medium (HEK293T)	10% (vol/vol) FBS in DMEM with GlutaMAX
Cell Culture Medium (mIMCD3)	10% (vol/vol) FBS 2 mM GlutaMAX 1 x Pen-Strep in DMEM/F12
Cell Culture Medium (mIMCD3, starvation)	2 mM GlutaMAX 1 x Pen-Strep in DMEM/F12
Cell Culture Medium (mIMCD3, selection)	10% (vol/vol) FBS 2 mM GlutaMAX 1 x Pen-Strep 1 µg/ml Puromycin in DMEM/F12
Citric acid buffer (10x)	87.4 mM Na ₃ C ₆ H ₅ O ₇ 12.6 mM citric acid monohydrate pH 6
Enhanced Chemiluminescence Solution (ECL)	100 mM Tris (pH 8.5) 1.25 mM Luminol 0.2 mM Coumaric acid 0.75% (vol/vol) H ₂ O ₂ pH 8.5
FACS buffer	2% (vol/vol) FBS in 1x PBS
Freezing medium	90% (vol/vol) FBS 10% (vol/vol) DMSO
HEBS (2x)	42 mM HEPES, Free Acid 274 mM NaCl 10 mM KCl 1.4 mM Na ₂ HPO ₄ Heptahydrate 15 mM Dextrose pH 7.09
High salt wash buffer	50 mM Tris-HCL pH 7.4 1 M NaCl 1% (vol/vol) Igepal CA-630 1 mM EDTA 0.1% (vol/vol) SDS 0.5% (wt/vol) Sodium deoxycholate

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Buffer / Solution	Composition
IP buffer	20 mM Tris 1% (vol/vol) TritonX-100 50mM NaCl 15 mM Na ₄ P ₂ O ₇ 50 mM NaF 44 µg/µl PMSF 2 mM Na ₃ VO ₄ pH 7.5
Laemmli (5x)	62.5 mM Tris pH 6.8 10% (wt/vol) Glycerole 2% SDS 50 mM DTT Bromphenolblue
LICOR wash buffer (1x)	0.1% (vol/vol) Tween-20 in 1x PBS
Modified RIPA buffer	10 mM Tris, pH 8 1 mM EDTA 0.5 mM EGTA 1 % Triton-X100 0.1% Sodiumdeoxycholate 44 µg/µl PMSF 2 mM Na ₃ VO ₄
Neutralization solution (50x)	15.75 g Tris-HCl in 50 mL of H ₂ O
PBT-X	0.1 % Triton X-100 in PBS
PCR elution buffer	10 mM Tris-HCl pH 7.4 20 mM NaCl 0.1 mM EDTA
Phosphate Buffered Saline (PBS)	137 mM NaCl 2.7 mM KCl 10 mM Na ₂ HPO ₄ 2 mM KH ₂ PO ₄
PKS buffer	100 mM Tris-HCl pH 7.4 50 mM NaCl 10 mM EDTA 0.2% (vol/vol) SDS
PNK buffer	50 mM Tris-HCl pH 7.5 50 mM NaCl 10 mM MgCl ₂ 0.5% (wt/vol) NP-40

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Buffer / Solution	Composition
PNK lysis buffer	100 mM KCl 5 mM MgCl ₂ 10 mM Tris-HCl pH 7.5 0.5% (wt/vol) NP-40 1 mM DTT 1 x cOmplete™ EDTA-free Protease Inhibitor Cocktail
PNK wash buffer	500 mM NaCl 20 mM Tris-HCl pH 7.5 1 mM MgCl ₂ 0.5% (wt/vol) NP-40
PNK7 buffer (10x)	700 mM Tris-HCl pH 7.0 100 mM MgCl ₂
Protein wash buffer	30 mM Tris 300 mM NaCl 0.3% Tween20 pH 7.5
Quantilyse buffer	5 µM SDS 10 mM Tris-HCl pH 8.0 40 µg/ml Proteinase K
RLTW buffer	1 x RLT buffer 5% (wt/vol) Tween-20
RNA ligase buffer without DTT (10x)	500 mM Tris-HCl pH 7.4 100 mM MgCl ₂
Running buffer	25 mM Trizma base 192 mM Glycine 0.1% (wt/vol) SDS
seCLIP lysis buffer	50 mM Tris-HCL pH 7.4 100 mM NaCl 1% (vol/vol) Igepal CA-630 0.1% (vol/vol) SDS 0.5% (wt/vol) Sodium deoxycholate 1 x Protease Inhibitor Cocktail Set III, EDTA-Free
seCLIP wash buffer	20 mM Tris-HCL pH 7.4 10 mM MgCl ₂ 0.2% (vol/vol) Tween-20 5 mM NaCl
Separating gel buffer	750 mM Tris 10% (vol/vol) PAA 0.2% (wt/vol) SDS pH 8.8

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Buffer / Solution	Composition
SOC medium	2% (wt/vol) Tryptone 0.5% (wt/vol) Yeast Extract 8.6 mM NaCl 2.5 mM KCl 20 mM MgSO ₄ 20 mM Glucose
Solvent A	0.1% formic acid
Solvent B	0.1% formic acid in 80 % acetonitrile
SP3 buffer	5% (wt/vol) SDS in PBS
Stacking gel buffer	250 mM Tris 5% (vol/vol) PAA 0.2% (wt/vol) SDS pH 6.8
TAE buffer (1x)	40 mM Tris (pH 8.5) 20 mM Acetic Acid 1mM EDTA
Tris Buffered Saline (TBS) (20x)	300 mM Tris-HCl 92.5 mM Tris Base 3 M NaCl pH 7.6
Transfer buffer	25 mM Tris 188 mM Glycine 0.1% (wt/vol) SDS
Tris-EDTA buffer (10x)	100 mM Tris Base 12.7 mM EDTA 0.05% Tween-20 pH 9
TT elution buffer	10 mM Tris-HCl pH 7.4 0.1 mM EDTA 0.01% (vol/vol) Tween-20
Urea buffer	8 M Urea 50 mM TEAB 1 x Protease Inhibitor Cocktail Set III, EDTA-Free

2.1.4 Oligonucleotides

Table 5. List of oligonucleotides used for cloning

Name	Sequence 5' → 3'
mMsi1_nostop_NotI_rev	CGCGGGGGCGGCCCGCGTGGTACCCATTGGTGAAGGC TG
Msi1_MluI_fwd	CGCACGCGTATGGAGACTGACGCGCCCCAG

continued on next page

Name	Sequence 5'→ 3'
Msi1_NotI_rev	CGCGGGGGCGGCCGCTCAGTGGTACCCATTGGTGAAG GCTGT
Msi2_MluI_fwd	CGCACGCGTATGGAGGCAAATGGGAGCCCAGG
Msi2_NotI_rev	CGCGGGGGCGGCCGCTCAGTGGTATCCATTTGTAAAG GCCGTTGC
pcDNA6 (-mlu) fsp1	CGTGTACGGTGGGAGGTCTA
pcDNA6 (-mlu) rsp 1	AGGAAAGGACAGTGGGAGTG
R26_beforeHA_Talen_fp	GCGTGTTTTGGTTGGCGTAA
Typ_mTmG_tg_rp	TCAATGGGCGGGGGTCGTT
typ_ROSA_wt_fp	AAAGTCGCTCTGAGTTGTTATC
typ_ROSA_wt_rp	GATATGAAGTACTGGGCTCTT

Table 6. List of Taqman assays used for qPCR

Gene	Taqman assay ID	Provider
MSI1	Mm.PT.58.12220977	IDT
MSI2	Mm.PT.58.17674798	IDT
POLR2A	Mm.PT.39a.22214849	IDT

Table 7. List of oligonucleotides used for seCLIP

Name	Sequence 5'→ 3'
D501	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTT CCCTACACGACGCTCTTCCGATCT
D502	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTT CCCTACACGACGCTCTTCCGATCT
D503	AATGATACGGCGACCACCGAGATCTACACCCTATCCTACACTCTTT CCCTACACGACGCTCTTCCGATCT
D504	AATGATACGGCGACCACCGAGATCTACACGGCTCTGAACACTCTTT CCCTACACGACGCTCTTCCGATCT
D505	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTT CCCTACACGACGCTCTTCCGATCT
D506	AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTC CCTACACGACGCTCTTCCGATCT
D701	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCA GACGTGTGCTCTTCCGATC
D702	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTC AGACGTGTGCTCTTCCGATC
D703	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTC AGACGTGTGCTCTTCCGATC
D704	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCA GACGTGTGCTCTTCCGATC
D705	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCA GACGTGTGCTCTTCCGATC
D706	CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCA GACGTGTGCTCTTCCGATC
InvAR17	CAGACGTGTGCTCTTCCGA
InvRand3Tr3	5Phos/NNNNNNNNNAGATCGGAAGAGCGTCGTGT/3SpC3
InvRiL19	5Phos/rArGrArUrCrGrGrArArGrArCrArCrArCrUrC/3SpC3

Table 8. List of oligonucleotides used for mouse work

Name	Sequence 5'→ 3'
Beta-globin fp	TGCTCACACAGGATAGAGAGGGCAGG
Beta-globin rp	GGCTGTCCAAGTGATTCAGGCCATCG
Cre fp	GGACATGTTCAGGGATCGCCAGGCG
Cre rp	GCATAACCAGTGAAACAGCATTGCTG
Msi1 3F-2	CGGACTGGGAGAGGTTTCTT
Msi1 3R-2	AGCTCCCCTGATTCCTGGT
Msi1 ASO	5'BNA-A//iBNA-T//iBNA-A/TGATACAGGA/iBNAmeC//iBNA-G//3'BNA-G
Msi1 3loxP_fwd	CTTGCCTTGCAATTCGGCTT
Msi1_ex3_reverse1	GAAGTGGCCGAAGTATTCGC
Msi1_Neo_fwd	TCCTCGTGCTTTACGGTATCG
Msi1_PGK_rev	GCTTTTGAAGCGTGCGAGAATG
Msi2 F2	TCTCCTTGTTGCGCTCAGTA
Msi2 R2	GCTCGGCTGACAAAGAAAGT

2.1.5 Plasmids

Table 9. List of plasmids used within this work

Name	Vector	Provider
3xFlag.MSI1 pcDNA6	pcDNA6	Generated within this work
3xFlag.MSI2 pcDNA6	pcDNA6	Generated within this work
pDonor 3xFlag.MSI2 Rosa26	pDonor 3xFlag (Nterm) Rosa26	Generated within this work
pDonor EGFP.MSI1 Rosa26	pDonor GFP (Nterm) Rosa26	Generated within this work
pDonor EGFP.MSI2 Rosa26	pDonor GFP (Nterm) Rosa26	Generated within this work
pDonor MSI1.3xFlag Rosa26	pDonor 3xFlag (Cterm) Rosa26	Generated within this work
TALEN-mROSA26 ELD	TALEN-mROSA26 ELD	Addgene #60026
TALEN-mROSA26 KKR	TALEN-mROSA26 KKR	Addgene #60025

2.1.6 Cell lines

Table 10. List of cell lines used within this work

Name	Species	Parental cell line	Clone no.	Genotype
3xFlag.EGFP	mouse	mIMCD3#8	-	heterozygous
3xFlag.MSI2	mouse	mIMCD3#8	1.8	heterozygous
MSI1.3xFlag	mouse	mIMCD3#8	38.1	heterozygous
EGFP.MSI1	mouse	mIMCD3#8	37	heterozygous
EGFP.MSI2	mouse	mIMCD3#8	33	heterozygous
HEK293T	human	-	-	WT
mIMCD3#8	mouse	mIMCD3	8	WT

2.1.7 Live-cell imaging

Table 11. List of cell death inhibitors used for live-cell imaging

Reagent	Endconcentration	Company	Catalog number
Belnacasan (VX-765)	40 μ M	Selleckchem	S2228
CRID3	2.5 μ M	Tocris	5479
DIYO-1	150 nM	Biomol	ABD-17580
Emricasan	10 μ M	Biozol	SEL-S7775
Ferostatin-1	5 μ M	Sigma-Aldrich	SML0583-25MG
GSK-872	5 μ M	Sigma-Aldrich	HY-101872
Necrostatin-1S	40 μ M	Abcam	221984

2.1.8 Antibodies

Table 12. List of primary antibodies used for immunostaining

Epitope	Product no.	Host	Dilution	Provider
MSI1	MBL-D270-3	rat	1:500	MBL
MSI2	NBP2-67547	rabbit	1:500	Novus Biologicas
FXR1	sc-374148	mouse	1:50	Santa Cruz
gammaH2AX (S139+140)	ab22551	mouse	1:200	Abcam
Phalloidin647	647P1-33	-	1:400	Dyomics
Flag (M2)	F3165	mouse	1:1000	Sigma Aldrich
Lotus Tetragonolobus Lectin (LTL) - FITC	FL-1321-2	-	1:250	Vector laboratories
E-Cadherin	610182	mouse	1:500	BD biosciences
THP	AB733	sheep	1:100	Sigma Aldrich

Table 13. List of primary antibodies used for western blot

Epitope	Product no.	Host	Dilution	Provider
MSI1	14-9896-82	rat	1:5000	Invitrogen
MSI2	NBP2-67547	rabbit	1:5000	Novus Biologicas
Flag (M2)	F1804-1MG	mouse	1:10000	Sigma Aldrich
β -tubulin (H-235)	Sc-9104	rabbit	1:500	Santa Cruz
β -tubulin (E7)	E7	Mouse	1:1000	DSHB
EGFP (B-2)	Sc-9996	mouse	1:2000	Santa Cruz

Table 14. List of secondary antibodies used within this work

Epitope	Product no.	Host	Dilution	Application	Provider
Biotin-SP-conjugated AffiniPure donkey anti-rat	712-065-150	Donkey	1:500	IHC	Jackson ImmunoResearch
Biotin-SP-conjugated AffiniPure donkey anti-rabbit	711-065-152	Donkey	1:500	IHC	Jackson ImmunoResearch

continued on next page

Epitope	Product no.	Host	Dilution	Application	Provider
donkey anti mouse A488	715-545-150	Donkey	1:500	IF	Jackson ImmunoResearch
donkey anti mouse Cy3	715-165-150	Donkey	1:500	IF	Jackson ImmunoResearch
donkey anti rabbit Cy3	711-165-152	Donkey	1:500	IF	Jackson ImmunoResearch
donkey anti rabbit A488	711-545-152	Donkey	1:500	IF	Jackson ImmunoResearch
donkey anti rat Alexa647	712-605-153	Goat	1:500	IF	Jackson ImmunoResearch
Cy TM 3 AffiniPure Donkey anti-rat IgG (H+L)	712-165-153	Donkey	1:500	IF	Jackson ImmunoResearch
anti-rat IgG (H+L) HRP	112-035-167	Goat	1:30000	WB	Jackson ImmunoResearch
anti-mouse IgG (H+L) HRP	115-035-003	Goat	1:30000	WB	Jackson ImmunoResearch
anti-rabbit IgG (H+L) HRP	111-035-003	Goat	1:30000	WB	Jackson ImmunoResearch
IRDye® 680RD anti-mouse IgG	926-68070	Goat	1:10000	WB	LI-COR Biosciences
IRDye® 800CW anti-rabbit IgG	926-32211	Goat	1:10000	WB	LI-COR Biosciences

2.1.9 Enzymes

Table 15. List of enzymes used within this work

Enzyme	Product no.	Provider
Q5 High Fidelity DNA Polymerase	M0491L	New England Biolabs
MluI	R0198S	New England Biolabs
NotI	R0189S	New England Biolabs
T4 Polynucleotide kinase	EK0032	Thermo Fisher Scientific
TURBO TM Dnase	AM2239	Thermo Fisher Scientific
Ambion TM RNase I	AM2295	Thermo Fisher Scientific
FastAP Thermosensitive Alkaline Phosphatase	EF0652	Thermo Fisher Scientific
Murine RNase Inhibitor	M0314L	New England Biolabs
T4 RNA Ligase 1	M0437M	New England Biolabs
Proteinase K	P8107S	New England Biolabs
SuperScript TM III Reverse Transcriptase	18080044	Thermo Fisher Scientific
ExoSAP-IT TM PCR Product Cleanup Reagent	78201.1.ML	Thermo Fisher Scientific
5' Deadenylase	M0331S	New England Biolabs
GoTaq® G2 Hot Start Polymerase	M7401	Promega

2.1.10 Consumables

Table 16. List of consumables used within this work

Consumable	Product no.	Provider
10 cm dish for Agar Plates	82.1473	Sarstedt
384 well plates	785290	Greiner BioOne
96-well plate, sterile, f-bottom, with lid	655180	Greiner BioOne
Acclaim™ 5µm PepMap™ 300 µ-Precolumns™ Cartridge Columns	11362223	Thermo Fisher Scientific
Adhesive qPCR tape	951.999	Sarstedt
Advanced PAP Pen	Z672548	Merck
AMPure XP Reagent	A63881	Beckman Coulter
Biosphere Filter Tip 10 µl, sterile	70.1116.210	Sarstedt
Biosphere Filter Tip 1000 µl, sterile	70.762.211	Sarstedt
Biosphere Filter Tip 20 µl, sterile	70.1116.215	Sarstedt
Biosphere Filter Tip 200 µl, sterile	70.760.211	Sarstedt
Black silk, 6-0, sterile, 100m	14719	Agtho's AB
Blotting paper (Type BF4, 580 x 580)	FT-2-521-580580G	VWR
Cell culture dishes (10-cm)	430167	Corning
Cell culture dishes (12-well)	3513	Corning
Cell culture dishes (15-cm)	430599	Corning
Cell culture dishes (6-well)	3516	Corning
Cell lifter	3008	Corning
Combs (10 well, 1 mm) for acrylamide gels	NC3010	Invitrogen
Combs (12 well, 1 mm) for acrylamide gels	NC3012	Thermo Fisher Scientific
Coverslips (round 18 mm Nr. 1.5)	631-0153	VWR
D1000 Reagents	5067- 5583	Agilent Technologies
D1000 ScreenTape	5067- 5582	Agilent Technologies
DNA LoBind® Tubes, 1.5 mL	30108051	Eppendorf
Dynabeads™ MyOne™ Silan	37002D	Thermo Fisher Scientific
Dynabeads™ Protein G	10004D	Thermo Fisher Scientific
Eppendorf tubes (1.5 mL)	72.690.001	Sarstedt
Gel cassette (1 mm)	NC2010	Invitrogen
Histosette® I	M499-11	Simport
Luer slip syringe (2 mL)	300185	BD
Luer slip syringe (3 mL)	309658	BD
Microfuge Tube Polypropylene (1.5 mL)	357448	Beckman Coulter
Millipore Immobilon-P Transfer Membranes	T831.1	Carl Roth
Mini Trans-Blot® Filter Paper	1703932EDU	Bio-Rad
Needle 20G x 1½"	301300	BD
Needle 23 G x 1	305762	BD
Nunc Cryotube™	368632	Thermo Fisher Scientific
NuPAGE™ 4 to 12%, Bis-Tris gels	NP0321PK2	Thermo Fisher Scientific
PCR Soft-tubes 0.2 mL 8 Tubes/Flat Caps, clear	710970	Biozym
PCR Soft-tubes 0.2 mL assorted colours	711088	Biozym

continued on next page

Consumable	Product no.	Provider
PCR Soft-tubes 0.2 mL clear	710920	Biozym
Pipette tips (1000 µl blue)	70.760.002	Sarstedt
Pipette tips (200 µl yellow)	70.762	Sarstedt
Polypropylene conical tube (15 mL)	227261	Greiner BioOne
Polypropylene conical tube (50 mL)	227261	Greiner BioOne
Protran Nitrocellulose Membranes	16533	Schleicher & Schuell
Safe Lock 1,5 mL Eppendorf tubes	211-2130	VWR
Screw cap tubes	72.692.005	Sarstedt
Stripettes (10 mL)	4101	LMS
Stripettes (25 mL)	4251	LMS
Stripettes (5 mL)	4051	LMS
Stripettes (50 mL)	4501	LMS
SuperFrost®/Plus microscope slides	7695002	Th.Geyer Group
Syringe (Plastipak™ 1 mL)	7392/2007	BD
TipOne (0.1-10 µl XL), sterile	S1110-3810	Starlab
TipOne (101-1000 µl graduated), sterile	S1111-6811	Starlab
TipOne (1-200 µl beveled), sterile	S1111-1816	Starlab
TipOne Pipette Tip 10 µl, refill	S1111-3700	Starlab
TipOne Pipette Tip 1000 µl, refill	S1111-6700	Starlab
TipOne Pipette Tip 200 µl, refill	S1111-1700	Starlab
Weighing tray 140 x 140 mm	2159.1	Carl Roth
Weighing tray 89 x 89 mm	2150.1	Carl Roth

2.1.11 Equipment

Table 17. List of equipment used within this work

Equipment	Model/Product no.	Provider
96S Super Magnet	A001322	Alpaqua
Aesculap Isis rodent shaver	GT421	Agntho's AB
Amersham Typhoon Biomolecular Imager	IP	Cytiva
Autoclave	V-150	Systec
AutoClip® Applicator	12020-09	Fine Science Tools
AxioCam	MRm	Zeiss
Axio observer microscope	200M	Zeiss
BD FACSAria™ III Cell Sorter	-	BD Biosciences
Centrifuge (refrigerated)	5810 R	Eppendorf
Crosslinker	CL-3000	LTF Labortechnik
Dumont #55 forceps	14098	WPI
DynaMag™-2 Magnet	12321D	Thermo Fisher Scientific
EASY-nLC™ 1200 System	LC140	Thermo Fisher Scientific
Eppendorf Research® plus, 8-channel, variable, 10 – 100 µL	3122000035	Eppendorf
Fusion Solo chemiluminometer	60-FU-SOLO	PeqLab
Hamilton syringe (50 µl Type 705)	549-1155	VWR

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Equipment	Model/Product no.	Provider
Heatingblock	TH 21	Ditabis
Heraeus B12 Function Line incubator	50042307	Kendro Laboratory Products
Homogenisator	Precellys® 24	Bertin instruments
Incubator	BD 115	Binder
IncuCyte® S3	-	Sartorius
Innova Incubator Shaker	4400	New Brunswick Scientific
Laminar Flow Cabinet	HS12	Heraeus
Leica Embedding Station	EG1150	Leica
MacMix Tube Rotator	MX100	Miltenyi Biotech
MacMix Tube Rotator	MX100	Miltenyi Biotech
Micro Serrefine Clamps, Straight/13mm	18055-04	Fine Science Tools
Microcentrifuge	5424	Eppendorf
Microcentrifuge (refrigerated)	5415 R	Eppendorf
Micro-Serrefine Clamp Applicator with Lock	18056-14	Fine Science Tools
Microtome	RM2235	Leica
Minicentrifuge	521-2844	VWR
Nanodrop Spectrophotometer	1000	PeqLab
Odyssey® CLx Imager	-	LI-COR
Operating scissor	501754	WPI
Orbitrap Exploris™ 480 Mass Spectrometer	BRE725533	Thermo Fisher Scientific
PerfectBlue™ Gelsystem Maxi M "Revolution"	700-0435	PeqLab
PerfectBlue™ Gelsystem Maxi S Plus	700-0538	PeqLab
Pipetboy acu	155 015	Integra Biosciences AG
Pipetman Pipette set (P2, P10, P100)	F167500	Gilson
Pipetman Pipette set (P20, P200, P1000)	F167300	Gilson
Powerpac 200 Power supply	1655052	Bio-Rad
Powerpac 3000 Power supply	1655057	Bio-Rad
Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer	IQLAAEGAAPFALGMBDK	Thermo Fisher Scientific
Real-time PCR System	QuantStudio 12K Flex	Thermo Fisher Scientific
Resolve microscope	-	BICO
Retractor	WP67418	World Precision instruments
Semidry Electroblotting System	Owl™ HEP-1	Thermo Fisher Scientific
Shaker	KS 260	IKA
Slidescanner	S360	Hamamatsu
Sonicator	Bioruptor® Pico	Diagenode Diagnostics
Staining jar	MARI4200004	Marienfeld
Storage phosphor screen	28-9564-74	VWR
Suction pump	181-0067DE	VWR
Suction pump (cell culture)	HLC	DITABIS

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Equipment	Model/Product no.	Provider
TapeStation	Agilent 4200	Agilent technologies
TCS SP8	-	Leica
Thermal Cycler	C1000 Touch	Bio-Rad
Thermomixer Comfort 1.5 mL Thermoblock	5360 000.011	Eppendorf
UltiMate 3000	-	Thermo Fisher Scientific
UV Transilluminator system	MW312	Intas
Vertical Electrophoresis Cell	Mini-PROTEAN® Tetra electrophoresis system	Bio-Rad
Vortex Mixer	444-1372	VWR
Water bath	WNB 22	Memmert
Water bath (for paraffin sections)	HI1210	Leica
Water cooler	B02010003	Diagenode Diagnostics
XCell SureLock™ Mini-Cell electrophoresis system	100601-1408	Invitrogen

2.1.12 Software and online tools

Table 18. List of software used within this work

Software	Version	Provider
Adobe illustrator 2023	27.2	Adobe
GraphPad Prism9	9.1.0	GraphPad Software Inc.
ImageJ/Fiji	v1.54b	Wayne Rasband
INTAS GelDoc	2019	Intas
LAS	4.13	Leica
Mausoleum	7.3.8 b4	Dr. H.-E. Stöfler
MaxQuant	1.5.2.8.	Computational Systems Biochemistry ²²⁵
Mendeley	1.19.8	Elsevier
Microsoft Office Suite	Professional Plus 2016	Microsoft
Nanodrop 1000	3.8.1	Thermo Scientific
Perseus	1.6.1.1/1.6.15.0	Computational Systems Biochemistry ²²⁶
QuantStudio	1.4.1	Applied Biosystems
ZEN Blue	3	Zeiss

Table 19. List of online tools used within this work

Online Tool	Website
Benchling	https://www.benchling.com/
Ensembl	https://www.ensembl.org
The Human Protein Atlas	https://www.prvoteinatlas.org/
NCBI	https://www.ncbi.nlm.nih.gov/
NCBI Blast	https://blast.ncbi.nlm.nih.gov/Blast.cgi
NCBI PubMed	https://pubmed.ncbi.nlm.nih.gov/

2.2 Methods

2.2.1 Cloning

2.2.1.1 Cloning PCR

2.2.1.1.1 Expression vectors

The pcDNA6/V5-HisB backbone (Invitrogen) has been previously modified through QuikChange site-directed mutagenesis and PCR amplification (Q5® High-Fidelity DNA Polymerase) to remove the MluI restriction site from the CMV promoter region and insert a 3xFlag sequence into the multiple cloning site. MSI1 and MSI2 cDNA with MluI and NotI restriction sites was obtained from mouse brain (MSI1) and mIMCD3 (MSI2) cDNA through PCR (Q5® High-Fidelity DNA Polymerase). MluI-MSI1-NotI was amplified using the primers Msi1_MluI_fwd and Msi1_NotI_rev, MluI-MSI2-NotI was amplified using the primers Msi2_MluI_fwd and Msi2_NotI_rev. Amplification was performed according to the manufacturers' instructions. The following annealing temperatures were used: MluI-MSI1-NotI (65°C), MluI-MSI2-NotI (72°C). Detailed vector maps are listed in the appendix (Figure A.1/ Figure A.2).

2.2.1.1.2 TALEN genome editing vectors

The pDonor MCS Rosa26 (Addgene Plasmid #37200) backbone has been previously modified through QuikChange site-directed mutagenesis to remove HindIII, KpnI and two NotI restriction enzyme sites. PCR amplification was used to insert a CMV promoter driven expression cassette and an EF1a (Elongation factor 1-alpha) promoter driven puromycin selection cassette between the two Rosa26 homology arms. The expression cassette was modified to enable expression of a gene of interest with N-terminal as well as C-terminal 3xFlag or EGFP tag. To generate N-terminal tagged MSI1 or MSI2 pDonor Rosa26 plasmids, MSI1 and MSI2 cDNA was amplified as described in 2.2.1.1.1. MSI1 cDNA missing the stop codon sequence (MluI-MSI1-noStop-Not) was used for cloning of pDonor 3xFlag (Cterm) Rosa26 plasmids and was amplified by PCR from 3xFlag.MSI1 pCND6 plasmids using the primers Msi1_MluI_fwd and mMsi1_nostop_NotI_rev and an annealing temperature of 58°C. All PCRs were performed using Q5® High-Fidelity DNA Polymerase according to the manufacturer's instructions. Detailed vector maps are listed in the appendix (Figure A.3 - Figure A.6).

2.2.1.2 Restriction enzyme digestion

MSI1 and MSI2 insert DNA and target vectors were digested with MluI and NotI (NEB). In a 30 µl reaction volume 1 µg DNA was mixed with 1.5 µl MluI, 1.5µl NotI, 3 µl NEBuffer™ 3.1 and

23 µl H₂O. After incubation at 37°C overnight 5 µl TriTrack DNA loading dye was added and the digested DNA was analyzed via 1% gel electrophoresis (2.2.1.3).

2.2.1.3 Agarose gel electrophoresis

Agarose was dissolved as a 1% or 2% solution in 1x TAE buffer and poured into a gel cast system. Horizontal gel electrophoresis was performed by applying an electric field of 5 to 8 V/cm depending on the size of the system. A 1kb DNA ladder was used on 1% agarose gels and 2% agarose gels were analyzed with a 50bp DNA ladder. Documentation of the agarose gels was accomplished through the Intas UV Transilluminator system. DNA was extracted from gels by excision using a scalpel under UV light and application of the GeneJET Gel Extraction Kit according to the manufacturer's instructions.

2.2.1.4 Ligation

Sticky end ligation of the MSI1/MSI2 amplicons into pCDNA6 or pDonor Rosa26 plasmids was performed using the T4 DNA ligase. Linearized vector and amplicons were added to the reaction in a 1:1 ratio and mixed with 1 µl T4 DNA ligase buffer (10x), 1 µl T4 DNA ligase and filled up to 10 µl with H₂O. Two control reactions were set-up lacking amplicon and amplicon+T4 DNA ligase. All reactions were incubated for 4 hours at RT.

2.2.1.5 Bacterial transformation and plasmid DNA isolation

The ligated plasmids (2.2.1.4) were transformed into chemocompetent DH10B T1 phage-resistant *E.coli* cells. 25 µl DH10B cells were added to the ligation/control reactions and incubated 10 minutes on ice. Heat shock for 45 seconds at 42°C was followed by incubation on ice for 2 minutes. After adding 175 µl SOC medium bacteria were grown for 30 minutes at 1000 rpm and 37°C. 100 µl of the bacteria/SOC medium solution were spread on LB agar with 100 µg/ml ampicillin, dried and incubated overnight at 37°C. Colony growth was compared between plates with bacteria transformed with the ligation reaction, and plates with bacteria transformed with control reactions. Based on the number of grown colonies on control plates an appropriate number of colonies on ligation plates was used to inoculate 3 ml LB medium + 100 µg/ml ampicillin and bacteria were grown at 37°C overnight. Plasmid DNA was isolated using the GeneJet Plasmid Miniprep Kit according to manufacturer's instructions using 1.5 ml of the bacterial cultures. DNA concentration was assessed using the Nanodrop 1000 spectrophotometer. 300 ng plasmid were used for a restriction enzyme digestion with MluI/NotI as described in 2.2.1.2, which was analyzed via 1% agarose gel electrophoresis (2.2.1.3). Confirmation of the sequence was done with Sanger sequencing at the Microsynth Seqlab GmbH and the sequencing primers (pcDNA6 (-mlu) fsp1/ pcDNA6 (-mlu) rsp1) listed in Table

5. 500 ng plasmid were mixed with 3 μ l sequencing primer (10 μ M) and filled up to 15 μ l with H₂O. Sequencing reads were aligned using Benchling. Maps of all plasmids generated within this study were attached in the appendix (Figure A.1 - Figure A.6).

2.2.2 Cell culture

2.2.2.1 Culturing of immortalized cell lines

Cells were grown at 37°C and 5% CO₂ in standard CO₂ incubators and handled under sterile conditions within a laminar-flow hood. Propagation of cells was accomplished by washing with PBS, adding 1 ml trypsin and incubation for 5-10 minutes at 37°C. Trypsin was inactivated by resuspending the cells in cultivating medium containing fetal bovine serum (FBS). Cells were seeded in pre-warmed medium into fresh culture dishes. Human embryonic kidney (HEK) 293T cells were cultivated in DMEM medium with GlutaMAX and 10% FBS. Inner medulla collecting duct (mIMCD3) cells were kept in DMEM/F12 medium supplemented with 10% FBS, 2 mM GlutaMAX and 1% Penicillin/Streptomycin. mIMCD3 cells were previously subcloned and characterized. Subclone #8 (mIMCD3#8) showed increased ciliation and expression of distal tubule specific markers compared to the heterogeneous population of mIMCD3 cells. Cultivation of cell lines generated via TALEN genome editing was done in mIMCD3 medium which was supplemented with 1 μ g/ml puromycin (mIMCD3 selection medium).

2.2.2.2 Transfection of HEK293T cells

HEK293T cells were seeded to a confluency of 30% in 10 cm dishes the day before transfection with calcium phosphate. 5 μ g plasmid were mixed with 500 μ l 0.25 M CaCl₂ solution before 500 μ l 2x HEBS were added while mixing. The solution was added dropwise to the HEK293T cells and transfection was stopped after 6-8 h through changing the medium. Cells were harvested 48 h after transfection.

2.2.2.3 TALEN genome editing

2.2.2.3.1 Transfection

Gene editing utilizing customized transcription activator-like effector nucleases (TALENs) was reported previously²²⁷. Integration of MSI1 and MSI2 transgenes into the Rosa26 locus of mIMCD3#8 cells was done applying the same system with the plasmids generated in 2.2.1 and listed in Table 9. A 3xFlag.EGFP mIMCD3#8 cell line was previously generated. All cell lines generated and used within this work are listed in Table 10. mIMCD3#8 cells were seeded to a confluency of 40-50% in 6-well plates the day before transfection. Transfection was performed using Lipofectamine™ 2000. To generate transgenic cell lines 0.4 μ g TALEN-mROSA26 ELD,

0.4 µg TALEN-mROSA26 KKR and 0.8 µg pDonor Rosa26 plasmids (Figure A.3 - Figure A.6) were diluted in 150 µl Opti-MEM® (Table 20). Two controls contained either 1.6 µg pDonor Rosa26 plasmid or 0.8 µg TALEN-mROSA26 KKR and 0.8 µg pDonor Rosa26 plasmid. The diluted plasmid DNA was mixed with 150 µl Opti-MEM® containing 6 µl Lipofectamine™ 2000 and incubated for 20 minutes at room temperature. The transfection mixtures were added dropwise to the mIMCD3#8 cells. After 48 h, the medium was changed and the cells were transferred into 10 cm dishes. Selection was started 48 h after splitting the cells into 10 cm dishes by changing the medium to mIMCD3 medium supplemented with 1 µg/ml puromycin. Cells were grown for 6-7 days while changing the medium every second day. When reaching 80-90% confluence cells were single cell sorted as described in 2.2.2.3.2.

Table 20. Composition of transfection reactions used for cell line generation

Components	Amount for transgenic cell line	Amount for control #1	Amount for control #2
TALEN-mROSA26 ELD	0.4 µg	-	-
TALEN-mROSA26 KKR	0.4 µg	-	0.8 µg
pDonor Rosa26	0.8 µg	1.6 µg	0.8 µg
Opti-MEM®	150 µl	150 µl	150 µl

2.2.2.3.2 Single cell sorting

Single cell sorting was performed using the BD FACSAria™ III Cell Sorter at the Max Planck Institute for Biology of Ageing FACS facility. The cells were washed with PBS, incubated in 1 ml trypsin for 5-10 minutes at 37°C and mixed with 5 ml mIMCD3 selection medium. The cells were centrifuged at 1500 rpm for 5 minutes at RT, the supernatant was removed and the pellet was carefully resuspended in 1 ml FACS buffer. The cells were single cell sorted into 96 well plates with fresh and pre-warmed mIMCD3 selection medium using a 100 µm nozzle. After sorting, the cells grew for 13-14 days at 37°C before they were split into 2x 96 well plates.

2.2.2.3.3 Genotyping

Genomic DNA from confluent 96 wells was extracted by washing with PBS and adding 30 µl quantilyse buffer. The cells were shaken 15 minutes at 1000 rpm at RT and 30 µl were transferred to PCR tubes. After incubation at 50°C for 30 minutes the lysates were incubated at 90°C for 10 minutes. Genotyping was performed using the GoTaq® Hot Start Polymerase according to the manufacturers' instructions. The wild type allele was detected using the primers typ_ROSA_wt_fp and typ_ROSA_wt_rp and integration into the Rosa26 locus was detected using the primers R26_beforeHA_Talen_fp and Typ_mTmG_tg_rp. Both PCRs were

run with an annealing temperature of 55°C. PCR products were analyzed via 1% agarose gel electrophoresis as described in 2.2.1.3.

2.2.2.4 Freezing and thawing of cells

Cells, which showed a successful integration into the Rosa26 locus by genotyping (2.2.2.3.3) were expanded and frozen when reaching 80-90% confluence in a 10 cm dish. After washing with PBS and incubation with 1 ml trypsin for 5-10 minutes at 37°C cells were mixed with 5 ml mMCD3 selection medium, transferred to a 15 ml falcon and centrifuged at 1500 rpm for 5 minutes at RT. The supernatant was removed and the pellet resuspended in 1 ml freezing medium. The cells were initially frozen in a cryogenic vial in Mr. Frosty™ Freezing Containers at -80°C and then transferred into nitrogen tanks for long-term storage. For thawing, cells were removed from liquid nitrogen, quickly warmed-up and resuspended in 5 ml warm cultivation medium. After centrifugation at 1500 rpm for 5 minutes at RT, the supernatant was removed and the pellet was dissolved in growth medium and transferred into 10 cm dishes. All cells were tested for mycoplasma contamination using the PCR Mycoplasma Test Kit.

2.2.2.5 Induction of cellular stress pathways

2.2.2.5.1 Induction of heat shock

Cells were seeded to a confluency of 50-60% in 24-well plates with coverslips and differentiation was initiated by serum starvation (DMEM/F12 medium supplemented with 2 mM GlutaMAX and 1% Penicillin/Streptomycin) for 24 h. After 24 h, heat shock was induced through incubation at 45°C and 5% CO₂ for four hours. Control cells were incubated for four hours at 37°C and 5% CO₂.

2.2.2.5.2 Induction of hypoxia

Cells were seeded to a confluency of 50-60% in 24-well plates with coverslips and differentiation was initiated by serum starvation (DMEM/F12 medium supplemented with 2 mM GlutaMAX and 1% Penicillin/Streptomycin) for 24 h. After 24 h, hypoxia was induced through incubation at 37°C and 1% O₂ for 6 hours. Control cells were incubated for 6 hours at 37°C and 5% CO₂.

2.2.2.5.3 Cisplatin treatment

Cells were seeded to a confluency of 50-60% in 24-well plates with coverslips and differentiation was initiated by serum starvation (DMEM/F12 medium supplemented with 2 mM GlutaMAX and 1% Penicillin/Streptomycin) for 24 h. After 24h, cells were treated with Cisplatin for 10 hours through incubation in starvation medium supplemented with 20 µM Cisplatin.

Unstimulated controls were cultivated in starvation medium without Cisplatin supplementation. Cisplatin NeoCorp (2 mg/ml) was ordered from the pharmacy of the University hospital cologne.

2.2.2.5.4 Hydrogen peroxide (H₂O₂) stimulation

Cells were seeded to a confluency of 50-60% in 24-well plates with coverslips and differentiation was initiated by serum starvation (DMEM/F12 medium supplemented with 2 mM GlutaMAX and 1% Penicillin/Streptomycin) for 24 h. After 24h, cells were treated with H₂O₂ for 6 hours through incubation in starvation medium supplemented with 0.6 mM H₂O₂. Unstimulated controls were cultivated in starvation medium without H₂O₂ supplementation.

2.2.2.5.5 Paraquat stimulation

Cells were seeded to a confluency of 50-60% in 10-15 cm dishes and differentiation was initiated by serum starvation (DMEM/F12 medium supplemented with 2 mM GlutaMAX and 1% Penicillin/Streptomycin) for 24 h. After 24 h, cells were stimulated with paraquat for 6 h through incubation in starvation medium supplemented with 10 mM paraquat. Unstimulated controls were cultivated in starvation medium without paraquat supplementation.

2.2.2.6 siRNA-mediated knockdown

Cells were seeded to a confluency of 50-60% in 6-well plates and transfected with 40 nM *Msi2* siRNA (ON-TARGETplus SMARTpool, L-040079-00-0005, Dharmacon) or control siRNA (ON-TARGETplus Non-targeting Control pool, D-001810-10-05, Dharmacon) per well, using Lipofectamine™ RNAiMAX transfection reagent.

Table 21. Composition of siRNA transfection reactions

Components	Solution A	Solution B
LipoRNAiMAX	6 µl	-
siRNA (20 µM)	-	4.8 µl
Opti-MEM®	200 µl	200 µl

Solutions A and B were prepared as described in Table 21 and incubated at room temperature for 5 minutes. Solution A was mixed into solution B and incubated for 20 minutes at room temperature before the mixture was added dropwise to the cells. For live-cell imaging, after 24h, cells were washed with 2 ml PBS per well and incubated with 500 µl trypsin for 5 minutes. Cells were resuspended with 1.5 ml mIMCD3 medium and seeded à 10000 cells per well into 96-well plates. For RNA extraction and cell lysis, cells were grown for 48h before harvesting and processing as described in 2.2.6 and 2.2.2.7.

2.2.2.7 Cell lysis

Cell lysates were harvested from confluent 6-wells by removing the growth medium and washing with ice cold PBS on ice. After removing the washing PBS, 1 ml of ice cold PBS was added and cells were detached using a scraper. The cell suspension was transferred into a tube on ice and centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was removed and the pellet was resolved in 80 µl modified RIPA buffer. Lysis was performed for 15 minutes on ice before centrifugation at 14000 rpm for 20 minutes at 4°C. The cell lysates were transferred into new tubes and mixed with 16 µl 5x Laemmli sample buffer. Cooking at 95°C for 5 minutes was followed by centrifugation at 14000 rpm for 2 minutes. Cell lysates were stored at -20°C.

2.2.2.8 Live-cell imaging

2.2.2.8.1 Determination of paraquat-induced cell death

For live cell imaging, cells were seeded into 96 well plates with a density of 10000 cells per well and differentiated through serum starvation for 24 h as described in 1.2.2.4. Cells were simultaneously stimulated with 10 mM paraquat and different combinations of cell death inhibitors listed in Table 11. Control cells were only stimulated with cell death inhibitors. DIYO-1 was added to a concentration of 150 nM to all cells in order to monitor cell death. Live cell imaging was performed with the IncuCyte® S3 (37°C, 5% CO₂). A 20x objective was used to acquire four images per well every 2 hours over a period of 24 hours. Dead cells were imaged using the green channel (exposure time 300 ms) and cell density was monitored by phase-contrast images. Analysis was performed using the IncuCyte® Cell-by-Cell Analysis Software Module and Prism software.

2.2.2.8.2 Paraquat stimulation and siRNA-mediated knockdown

Cells were transfected with siRNA as described in 2.2.2.6 and seeded into 96 well plates with a density of 10000 cells per well. Cells were stimulated with paraquat for 24 hours and cell death was monitored by adding DIYO-1 with a concentration of 150 nM per well. Cells were stimulated with paraquat through incubation in mMCD3 medium supplemented with 10 mM paraquat. Control cells were cultivated in mMCD3 medium without paraquat supplementation. Live cell imaging was performed with the IncuCyte® S3 (37°C, 5% CO₂). A 10x objective was used to acquire four images per well every 2 hours over a period of 24 hours. Dead cells were imaged using the green channel (exposure time 300 ms) and cell density was monitored by phase-contrast images. Analysis was performed using the IncuCyte® Cell-by-Cell Analysis Software Module and Prism software.

2.2.2.9 Immunofluorescence staining of cells

Cells were grown in 24 well plates on round glass coverslips until reaching a confluence of 60-80%. The growth medium was removed, the cells were carefully washed with PBS and fixed in 4% formaldehyde for 15 minutes. After washing 3x 5 minutes with PBS the cells were blocked in 5% normal donkey serum (NDS) in PBT-X for 1 h at RT. The cells were washed 3x 5 minutes with PBS and incubated with the first antibodies diluted in 5%NDS/PBT-X as listed in Table 12 overnight at 4°C. Three times washing with PBS was followed by incubation with fluorescently labeled secondary antibodies (Table 14) and Hoechst (1:1000) for 45 minutes at RT. The coverslips were washed 3x 5 minutes with PBS and mounted using Prolong Diamond antifade reagent. Imaging was performed using the TCS SP8 Confocal microscope and image analysis was performed with ImageJ/Fiji.

2.2.3 SDS polyacrylamide gel electrophoresis

Proteins in cell lysates were analyzed and separated via SDS polyacrylamide gel electrophoresis (SDS-PAGE) and western blot. To prepare a polyacrylamide gel with 12% separating gel, 200 µl 10% APS and 30 µl TEMED were added to 20 ml separating gel buffer (Table 4), poured into a gel cassette and covered with isopropanol. After 30 minutes the isopropanol was removed and the stacking gel was added by mixing 5 ml stacking gel buffer (Table 4) with 50 µl 10% APS and 7.5 µl TEMED. Depending on the sample size a 10-well or 12-well comb was inserted and the stacking gel polymerized for 15 minutes. SDS-PAGE was performed using the XCell SureLock Mini-Cell Electrophoresis System filled with 1x running buffer. 10-20 µl of the cell lysates (2.2.2.7) were loaded together with 5 µl PageRulerPlus onto the polyacrylamide gel and separated by applying 180 V for 1.5 hours.

2.2.4 Chemiluminescent western blot

After performing SDS-PAGE as described in 1.2.3, proteins were transferred onto a Millipore Immobilon-P polyvinylidene difluoride (PVDF) membrane using the Owl™ HEP-1 semi-dry electroblotting system. The PVDF membrane was activated in methanol for 30 seconds before washing in 1x transfer buffer. The gel was removed from the cassette and incubated together with filter paper in 1x transfer buffer. The blot was assembled in the following order: filter paper, membrane, polyacrylamide gel, filter paper. The transfer was performed at 12 V for 1 hour. After blotting, the membrane was incubated with 5% BSA/1x protein wash buffer for 1 h at RT. Rinsing the membrane 3x 10 minutes with 1x protein wash buffer was followed by incubation with the first antibody diluted in 1x protein wash buffer as listed in Table 4. The membrane was washed 3x 10 minutes with 1x protein wash buffer and incubated with the Horseradish peroxidase (HP) -conjugated secondary antibody (Table 14) diluted 1:30000 in 1x protein wash

buffer. The membrane was rinsed 3x 10 minutes with 1x protein wash buffer. Chemiluminescent protein detection was accomplished by incubating the membrane with ECL detection solution or SuperSignal West Femto Chemiluminescent Substrate. The signal was visualized using the PeqLab Fusion Solo chemiluminometer.

2.2.5 Fluorescent western blot

After performing SDS-PAGE as described in 2.2.3, proteins were transferred onto PVDF membrane as described in 2.2.4. After blotting, the membrane was incubated in 1x ROTI®Block solution for 1h at RT. The membrane was rinsed 3x 10 minutes with 1x LICOR wash buffer and incubated with the first antibody diluted in 1x LICOR wash buffer as listed in Table 12. After washing the membrane 3x 10 minutes with 1x LICOR wash buffer, the membrane was incubated with the fluorescently-labeled secondary antibody diluted in 1x LICOR wash buffer for 45 minutes in the dark (Table 14). Finally, the membrane was rinsed 3x 10 minutes in 1x LICOR wash buffer and 1x 5 minutes with PBS. Fluorescent protein detection was performed using the odyssey system and densitometric analysis was performed using the Image Studio™ software (version 5.2).

2.2.6 RNA extraction

Cell pellets were resuspended in 700 µl Trizol and incubated for 15 minutes at RT. RNA extraction for quantitative PCR (qPCR) analysis and mRNA sequencing was performed using the Direct-zol™ RNA Miniprep Plus kit including DNaseI digestion. RNA was eluted in 25-50 µl H₂O depending on the initial size of the cell pellet. RNA concentrations were measured using the Invitrogen™ Qubit™ RNA Broad Range assay kit and quality was assessed using Nanodrop 1000 spectrophotometer according to manufacturer's instructions.

2.2.7 Reverse transcription and qPCR

Up to 2 µg RNA were transcribed into cDNA using the High-Capacity cDNA Reverse Transcription kit according to manufacturer's instructions. As control, one sample was lacking the reverse transcriptase (-RT). PrimeTime™ qPCR Probes targeting MSI1, MSI2 and POLR2A are listed in Table 6. qPCR reactions were set-up mixing 0.5 µl PrimeTime™ qPCR Probes with 5 µl TaqMan™ Universal PCR Master Mix, 25-200 ng cDNA and filling up to 11 µl with H₂O. qPCR was run in technical triplicates and control samples either contained genomic DNA, -RT reaction or H₂O. qPCR was performed using the QuantStudio 12K Flex Real-time PCR System and data was analyzed with Quantstudio and Prism software. Statistical significance was assessed by applying the student's t-test and data are presented with +/- standard error of the mean (SEM).

2.2.8 Interactome

2.2.8.1 Cell lysis

Paraquat stimulation was performed as described in 2.2.2.5.5. Per replicate one paraquat-stimulated and one unstimulated 10 cm dish per cell line were harvested. Cells were washed with ice cold PBS on ice. The PBS was removed, 5 ml of ice cold PBS was added and cells were harvested by scraping. 500 µl of the cell suspension was transferred into a tube for whole mRNA sequencing. The remaining cell suspension and the mRNA sequencing samples were centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was removed, the pellets were frozen in liquid nitrogen and stored at -20°C. Pellets were retrieved from -20°C and resuspended in 750 µl IP buffer. Cell lysates were homogenized by passing three times through a 23G syringe. Incubation for 15 minutes on ice was followed by centrifugation at 14000 rpm for 20 minutes at 4°C. Cell lysates were transferred into new tubes on ice.

2.2.8.2 Immunoprecipitation

The cell lysates (2.2.8.1) were incubated with 10 µl dynabeads protein G coupled to M2 Flag antibodies overnight at 4°C. Per sample, 10 µl dynabeads protein G were coupled to 1 µg M2 Flag antibody. To prepare the coupled dynabeads protein G, 10 µl beads were washed twice with IP buffer using a DynaMag-2 Magnet. Beads were briefly centrifuged, magnetically separated and the IP buffer was removed completely. The beads were resuspended in 10 µl IP buffer per sample and incubated with 1 µg M2 Flag antibody on a tube rotator for 45 minutes. The coupled beads were washed twice with 500 µl IP buffer, the IP buffer was removed completely after the last washing step and the beads were resuspended in 10 µl IP buffer per sample. Cell lysates and beads were incubated on a tube rotator overnight at 4°C. The beads were washed three times with 900 µl IP buffer. After the last washing step the buffer was removed completely and the beads were resuspended in 80 µl SP3 buffer to prepare the lysates for single-pot, solid-phase-enhanced sample preparation (SP3)²²⁸.

2.2.8.3 SP3 sample preparation

Samples in SP3 buffer were incubated 3 minutes at 95°C before cooling down on ice for 1 minute. Using the DynaMag-2 Magnet the lysate was separated from the beads and transferred into a new tube. Reduction with 5 mM DTT for 30 minutes at 55°C was followed by alkylation with 40 mM CAA (chloroacetamide) for 30 minutes at RT in the dark. The lysates were centrifuged at 14000 rpm for 20 minutes at RT and submitted to SP3²²⁸ and mass spectrometry in the CECAD proteomics core facility.

2.2.8.4 Mass spectrometry and bioinformatics analysis

All samples were analyzed by the CECAD proteomics facility on a Q Exactive Plus Orbitrap mass spectrometer that was coupled to an EASY nLC (both Thermo Scientific). Peptides were loaded with solvent A (0.1% formic acid in water) onto an in-house packed analytical column (50 cm length, 75 μ m inner diameter, filled with 2.7 μ m Poroshell EC120 C18, Agilent). Peptides were chromatographically separated at a constant flow rate of 250 nL/min using the following gradient: initial 3 % solvent B (0.1% formic acid in 80 % acetonitrile), 3-5% B within 1.0 min, 5-30% solvent B within 119.0 min, 30-50% solvent B within 19.0 min, 50-95% solvent B within 1.0 min, followed by washing and column equilibration. The mass spectrometer was operated in data-dependent acquisition mode. The MS1 survey scan was acquired from 300-1750 m/z at a resolution of 70,000 and 20 ms maximum injection time. The top 10 most abundant peptides were isolated within a 1.8 Th window and subjected to HCD fragmentation at a normalized collision energy of 27%. The AGC target was set to 5e5 charges, allowing a maximum injection time of 110 ms. Product ions were detected in the Orbitrap at a resolution of 35,000. Precursors were dynamically excluded for 10.0 s. All mass spectrometric raw data were processed with Maxquant ²²⁵ (version 2.0.3.0) using default parameters against the Uniprot canonical mouse database (UP589, downloaded 05.08.2020) with the match-between-runs option enabled between replicates. Follow-up analysis was done in Perseus 1.6.15 ²²⁶. Protein groups were filtered for potential contaminants and insecure identifications. Remaining IDs were filtered for data completeness in at least one group and missing values imputed by sigma downshift (0.3 σ width, 1.8 σ downshift). Afterwards, FDR-controlled two-sided t-tests were performed. Differential analysis was performed using the R package limma ²²⁹. Here, the abundance of proteins; measured in IP samples generated from MSI1.3xFlag or 3xFlag.MSI2 mIMCD3#8 cell lines, was compared to their abundance in IP samples generated from wildtype mIMCD3#8 cells. Deming regression was performed using the R package deming ²³⁰. Significantly enriched proteins (adjusted p value $\leq$ 0.05; log₂ fold change >0) were defined as protein interactors of MSI1/MSI2. Known Musashi interactors were downloaded from Biogrid ²³¹. Venn diagrams were generated using DeepVenn ²³². GO enrichment analysis was done using the R package clusterProfiler (function enrichGO) and visualization was performed using the R package GOplot and custom R and bash scripts ^{233,234}. Enriched GO terms were clustered using Revigo ²⁴².

2.2.9 Proteome

2.2.9.1 Sample processing

mIMCD3#8 cells (WT) were stimulated with paraquat as described in 2.2.2.5.5. For each replicate one 10 cm dish of mIMCD3#8 cells was stimulated with paraquat and one 10 cm dish

served as unstimulated control. Cell pellets were lysed in 300 µl Urea buffer and chromatin was degraded using sonication with the Bioruptor (10 min, 30 sec on/30 sec off) at 4°C. Lysates were centrifuged for 15 minutes at 20000 x g at 4°C and supernatants were transferred into new tubes. Protein concentration was measured using the Qubit™ Protein assay kit. 50 µg protein lysate per sample were transferred to a new tube and reduced by incubation with 5 mM DTT for 1 h at 25°C. The lysates were alkylated by adding 40 mM CAA and incubating for 30 minutes in the dark. Samples were diluted with 50 mM TEAB to reduce urea to a concentration below 2 M. Trypsin was added in an enzyme:substrate ratio of 1:50 and incubated overnight at 25°C. Enzymatic digestion was stopped by adding formic acid to a final concentration of 1% and the samples were centrifuged for 5 min at 14000 rpm. Peptides were purified using SDS-RP (styrenedivinylbenzene reverse phase sulfonate) StageTips. StageTips were equilibrated by adding 20 µl methanol and centrifuging for 1 min at 2600 rpm followed by adding solvent B (0.1% formic acid in 80 % acetonitrile) and repeating the previous centrifugation step. 20 µl solvent A (0.1% formic acid in water) were added and the StageTips were centrifuged 1.5 minutes at 2600 rpm. This step was repeated once with a centrifugation time of 2 minutes. Samples were loaded onto the stage tips and centrifuged at 2600 rpm for 5 minutes. StageTips were washed by adding 30 µl solvent A and centrifuging at 2600 rpm for 3 minutes. Washing with 30 µl solvent B through centrifugation at 2600 rpm for 3 minutes was repeated twice before the StageTips were dried completely and stored at 4°C.

2.2.9.2 Mass spectrometry and bioinformatics analysis

Samples were analyzed by the CECAD Proteomics Facility on an Orbitrap Exploris 480 (Thermo Scientific, granted by the German Research Foundation under INST 1856/71-1 FUGG) mass spectrometer equipped with a FAIMSpro differential ion mobility device that was coupled to an UltiMate 3000. Samples were loaded onto a precolumn (Acclaim 5 µm PepMap 300 µ Cartridge) for 2 min at 15 µl flow before reverse-flushed onto an in-house packed analytical column (30 cm length, 75 µm inner diameter, filled with 2.7 µm Poroshell EC120 C18, Agilent). Peptides were chromatographically separated at a constant flow rate of 300 nL/min and the following gradient of solvent B (0.1% formic acid in 80 % acetonitrile): initial 6% B (0.1% formic acid in 80 % acetonitrile), up to 32% B in 72 min, up to 55% B within 7.0 min and up to 95% solvent B within 2.0 min, followed by column wash with 95% solvent B and re-equilibration to initial condition. The FAIMS pro was operated at -50V compensation voltage and electrode temperatures of 99.5 °C for the inner and 85 °C for the outer electrode. MS1 scans were acquired from 399 m/z to 1001 m/z at 15k resolution. Maximum injection time was set to 22 ms and the AGC target to 100%. MS2 scans ranged from 400 m/z to 1000 m/z and were acquired at 15 k resolution with a maximum injection time of 22 ms and an AGC target of 100%. DIA scans covering the precursor range from 400 - 1000 m/z and were acquired in 60

x 10 m/z windows with an overlap of 1 m/z. All scans were stored as centroid. Samples were analyzed in DIA-NN 1.8.1 (Demichev 2020). For library generation a Swissprot mouse canonical database was used and settings, matching the acquisition parameters, as well as the match-between-runs function were applied. The match-between-runs function refines the generated library as samples are directly used for a second search of the sample data. Data was processed using DIA-NN ⁴⁴ and the functions “-report-lib-info” and “-relaxed-prot-inf”. Moreover, the following output settings were used: filtered at 0.01 FDR, N-terminal methionine excision enabled, maximum number of missed cleavages set to 1, min peptide length set to 7, max peptide length set to 30, min precursor m/z set to 400, max precursor m/z set to 1000, cysteine carbamidomethylation enabled as a fixed modification. The output files were further filtered on library q-value and global q-value ≤ 0.01 and at least two unique peptides per protein using R (4.1.3). Finally, LFQ values were calculated using the DIA-NN R-package. Differential analysis was performed using the R package limma ²²⁹. Here, expression of proteins in PQ-treated mIMCD3#8 cells was compared to their abundance in unstimulated mIMCD3#8 cells. Proteins with an adjusted p value ≤ 0.05 were considered as differentially regulated.

2.2.10 mRNA sequencing

Samples for mRNA sequencing were harvested as described in 2.2.2.7. RNA was extracted as described in 2.2.6. mRNA sequencing was performed by the Cologne Center for Genomics. Libraries were generated by applying the Illumina® Stranded TruSeq® RNA sample preparation Kit using 200 ng total RNA as input material. After poly-A selection (using poly-T oligo-attached magnetic beads), mRNA was purified and fragmented using divalent cations under elevated temperature. Random primers were used to reverse transcribe the RNA, followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. Indexing adapters were ligated after end repair and A-tailing. After purification, cDNA libraries were created by amplification with 15 PCR cycles. cDNA libraries were validated and quantified (Agilent Tape Station) and equimolar amounts of library were pooled. The pool was quantified by using the Peqlab KAPA Library Quantification Kit and the Applied Biosystems 7900HT Sequence Detection System. Sequencing was performed on an Illumina NovaSeq6000 sequencing instrument with a PE100 protocol. Sequencing reads were trimmed with Trimmomatic (version 0.36) ²³⁵ using default parameters. Additionally, the first ten bases were cropped because of bad quality. The trimmed reads were mapped to the GRCm38 mouse reference genome with STAR (version 2.6.0) ²³⁶ using default parameters. The analysis of differential gene expression was performed using the R package DESeq2 (version 1.26.0) ^{237,238}. DESeq2 is an R-package that offers the statistical framework to estimate variance-mean dependence in read count data from high-throughput sequencing data. It furthermore uses the negative binomial distribution to test for differential expression and provides adjustment of the

p-values. Differential gene expression analysis was used to compare gene expression after PQ exposure to gene expression in unstimulated conditions in wildtype mIMCD3#8 cells. Genes with adjusted p-value < 0.05 were determined as differentially expressed.

2.2.11 Polynucleotide kinase assay

RNA binding capacity of Musashi proteins was confirmed using T4 polynucleotide kinase assay²³⁹. HEK293T cells were transfected with pcDNA6 plasmids listed in Table 9 and as described in 2.2.2.2. Per replicate 10 x 10 cm dishes were transfected. Cells were harvested 48 h post-transfection by removing the growth medium and washing with ice cold PBS. 5 x 10 cm dishes were UV-C (254 nm) crosslinked at 150 mJ/cm² using a UV-C Crosslinker. The remaining 5 x 10 cm dishes served as non-crosslinked controls. Next, 2 ml PBS were added and the cells were harvested by scraping. After transfer into 15 ml falcons, cells were centrifuged at 1500 rpm / 4°C for 5 minutes, supernatant was removed, pellets were frozen in liquid nitrogen and stored at -80°C. Pellets were resuspended in 2 ml PNK lysis buffer, homogenized by passing through a 23G syringe three times and incubated on ice for 15 minutes. The lysates were centrifuged at 14000 rpm / 4°C for 20 minutes to pellet cell debris. DNA was digested by adding 2 µl/ml DNase I and RNA was partially digested by adding 40 U/ml RNase I and incubating 15 minutes at 37°C and 800 rpm on a thermomixer. Lysates were cooled down on ice for 5 minutes. Prior to cell lysis and DNA/RNA digestion, dynabeads protein G were coupled to M2 Flag antibodies as described in 2.2.8.2. Immunoprecipitation was performed by adding 50 µl M2 Flag-coupled dynabeads protein G to the cell lysates and incubating overnight at 4°C on a tube rotator. Beads were washed five times with 900 µl PNK wash buffer using a DynaMag-2 Magnet and twice with 900 µl PNK buffer. After the last washing, beads were centrifuged and PNK buffer was removed completely. Beads were resuspended in 60 µl PNK buffer supplemented with 5 mM DTT and 5 µl sample for SDS-PAGE and western blot analysis (2.2.4) were collected. [γ -³²P] ATP (SRP-501, Hartmann Analytics) was diluted to a radioactive concentration of 200 µCi/ml in PNK buffer + 5 mM DTT. To radioactively label RNA, cell lysates were incubated with 1.5 U/µl T4 polynucleotide kinase and 0.2 µCi [γ -³²P] ATP at 800 rpm and 37°C for 20 minutes on a thermomixer. Cell lysates were washed with 900 µl PNK buffer five times and the PNK buffer was removed completely after the last wash. Beads were resuspended in 20 µl PNK buffer and mixed with 7.5 µl NuPAGE™ LDS sample buffer + 3 µl 1 M DTT. Proteins were eluted from the magnetic beads for 5 minutes at 800 rpm and 70°C on a thermomixer. After cooling down on ice for 1 minute, 30 µl sample were loaded together with 5 µl PageRulerPlus onto a NuPAGE™ 4-12% Bis-Tris gel. SDS-PAGE was performed in a XCell SureLock Mini-Cell Electrophoresis System filled with 1x NuPAGE™ MOPS SDS Running Buffer at 180 V for 1.5 h. The protein gel was removed from the cassette and incubated in 1x NuPAGE™ Transfer Buffer + 10% methanol together with the nitrocellulose

membrane and filter paper. The blot was assembled in the following order: filter paper, membrane, gel, filter paper. Proteins were transferred onto nitrocellulose membrane using the Owl™ HEP-1 semi-dry electroblotting system and cold 1x NuPAGE™ Transfer Buffer + 10% methanol. Protein transfer was performed at 12 V for 1 h. The membrane was dried, packed into saran wrap and the radioactive signal was developed in a phosphorimager screen overnight. Images were acquired using the Amersham Typhoon Biomolecular Imager and analyzed via ImageJ/Fiji. After scanning, proteins on the membrane were detected and visualized by western blotting as described in 2.2.4.

2.2.12 seCLIP

2.2.12.1 Cell lysis and immunoprecipitation

Single-end enhanced crosslinking and immunoprecipitation (seCLIP) was performed as previously described under RNase-free conditions ²⁰⁰. Per replicate 12 x 15 cm dishes of MSI1.3xFlag mIMCD3#8 or 3xFlag.MSI2 mIMCD3#8 cells were seeded to a confluency of 70-80%. Cells were differentiated and 6 x 15 cm were stimulated with paraquat as described in 2.2.2.5.5 while the other 6 x 15 cm dishes served as unstimulated control cells. All cells were washed once with cold PBS and UV-C crosslinked at 400mJ/cm² on ice. 5 ml cold PBS were added and cells were harvested by scraping. After centrifugation at 1500 rpm / 4°C for 5 minutes the supernatant was removed, cells were frozen in liquid nitrogen and stored at -80°C. Cells were lysed in 1 ml seCLIP lysis buffer for 5 minutes on ice and sonicated 5 cycles (30 seconds on, 30 seconds off) at 4°C using the Bioruptor. Cell lysates were incubated with 10 U TurboDNase and 40 U RNase I shaking at 1200 rpm and 37°C for 5 minutes in a thermomixer. Samples were placed on ice for one minute before 440 U murine RNase inhibitor were added and cell debris were pelleted by centrifugation at 14000 rpm / 4°C for 20 minutes. Supernatant was transferred into new tubes. Prior to cell lysis dynabeads Protein G were coupled to M2 Flag antibodies. Per sample, 125 µl dynabeads protein G were washed twice with 900 µl seCLIP lysis buffer using the DynaMag-2 Magnet. seCLIP lysis buffer was removed completely, beads were resuspended in 100 µl seCLIP lysis buffer per sample and 10 µg M2 Flag antibody per 100 µl beads were added. Antibody was coupled for 45 minutes at RT on a tube rotator before beads were washed twice with 900 µl seCLIP lysis buffer. seCLIP buffer was removed completely and the beads were resuspended in 100 µl seCLIP lysis buffer per sample. Cell lysates were incubated with 100 µl M2-Flag coupled dynabeads protein G overnight at 4°C on a tube rotator.

2.2.12.2 Dephosphorylation of IP samples and 3' RNA linker ligation

IP Samples were retrieved from the tube rotator at 4°C and size-matched input (SMI) samples were collected. Per IP two SMI samples à 20 µl were transferred into new tubes and stored on ice. All washing steps were performed on ice. Remaining beads were washed twice with 500 µl high salt wash buffer. After a transition wash with 250 µl high salt wash buffer and 250 µl wash buffer the beads were washed three times with 500 µl wash buffer. The residual wash buffer was removed and the beads were resuspended in 50 µl dephosphorylation mix (1x FastAP buffer, 80 U murine RNase inhibitor, 4 U TurboDNase, 3 U FastAP™ Thermosensitive Alkaline Phosphatase, ad 50 µl H₂O). Beads were incubated at 1200 rpm and 37°C for 10 minutes in a thermomixer. 150 µl PNK master mix (1x PNK7 buffer, 40 U T4 polynucleotide kinase, ad 150 µl H₂O) were added and incubated with the beads at 1200 rpm and 37°C for 20 minutes in a thermomixer. 200 µl high salt wash buffer were added and supernatant was removed by placing the tubes on the magnet. After a transition wash with 500 µl high salt wash buffer and 500 µl wash buffer, samples were washed three times with wash buffer. Wash buffer was removed completely and the beads were resuspended in 25 µl 3' RNA linker ligation master mix (1.2 x RNA ligase buffer without DTT, 1.2 µM ATP, 3.6% DMSO, 0.024% Tween-20, 18% PEG 8000, 0.8 U murine RNase inhibitor, 72 U T4 RNA Ligase 1, ad 25 µl H₂O). Per sample 2.5 µl InvRIL 19 RNA adapter (40 µM) (Table 7) were added and incubated for 75 minutes at RT. During ligation, samples were mixed every 10 minutes.

2.2.12.3 SDS-PAGE and protein transfer

Samples were washed with 500 µl wash buffer followed by a transition wash in 500 µl wash buffer and 500 µl high salt wash buffer. Beads were mixed with 500 µl high salt buffer wash, magnetically separated and supernatant was removed. After a transition wash in 250 µl high salt washing buffer and 250 µl wash buffer, two final washing steps with 500 µl wash buffer were performed. Residual buffer was removed completely and beads were resuspended in 100 µl wash buffer. Per sample 20 µl beads were collected and transferred to a new tube for western blot analysis. The remaining beads were magnetically separated, the wash buffer was removed and the beads were mixed with 20 µl wash buffer. All IP and SMI samples were mixed with 10.5 µl NuPAGE™ LDS sample buffer + DTT (0.98x NuPAGE™ LDS sample buffer, 98 mM DTT) and proteins were eluted by incubation at 1200 rpm and 70°C for 10 minutes in a thermomixer. Samples were cooled on ice for 1 minute and centrifuged at 14000 rpm for 1 minute. All samples were loaded onto NuPAGE™ 4-12% Bis-Tris gel using magnetic separation of the beads. For the western blot gel, 15 µl of IP and SMI western blot samples were loaded together with 5 µl PageRulerPlus. For the RNA preparatory gels, IP and SMI RNA samples were loaded with their entire volume and samples were separated by lanes loaded

with 5 µl PageRulerPlus. SDS-PAGE was performed using the XCell SureLock Mini-Cell Electrophoresis System filled with 1x NuPAGE™ MOPS SDS Running Buffer and applying 150 V for 1.5 h. Western blot gels were processed as described in 2.2.3 and proteins were detected and visualized as described in 2.2.4. Protein transfer of RNA preparatory gels on nitrocellulose membranes was performed using the Mini-PROTEAN® Tetra electrophoresis system. Gels, filter papers, nitrocellulose membranes and sponges were incubated in cold 1x NuPAGE™ Transfer Buffer + 10% methanol. Blots were assembled onto the black side of the gel holder cassette in the following order: sponge, filter paper, gel, membrane, filter paper, sponge. Transfer was enabled by applying 30 V overnight at 4°C.

2.2.12.4 RNA isolation

The nitrocellulose membrane was washed in PBS and shortly dried on a filter paper before placing it on a glass plate. Based on the observed size in the western blot, the membrane was cut to excise MSI1.3xFlag or 3xFlag.MSI2 and all proteins ~75 kD larger than MSI1.3xFlag/3xFlag.MSI2 using a scalpel. The membrane was cut into ~2mm x 2mm squares and transferred into a tube on ice. 150 µl Proteinase K mix (24 U Proteinase K, ad 150 µl PKS buffer) were added to the membrane pieces. RNA was released by incubation at 37°C for 20 minutes and 50°C for 20 minutes at 1200 rpm in a thermomixer. Tubes were briefly centrifuged and the liquid was transferred into new tubes on ice. 55 µl H₂O were added to membrane, mixed and transferred into the corresponding tubes on ice. RNA was extracted using the RNA Zymo Clean & Concentrator-5 kit with a modified protocol. After adding 2 volumes of RNA binding buffer to the samples, 3.5 x volumes ethanol (100%) were mixed into the samples. Samples were loaded onto the Zymo-Spin™ IC Columns and centrifuged 30 seconds at 5000 x g. Per column 400 µl RNA prep buffer were added followed by centrifugation for 30 seconds at 5000 x g. Columns were washed with 500 µl RNA wash buffer repeating the previous centrifugation step. 500 µl RNA wash buffer were loaded and the column was centrifuged 30 seconds at 9000 x g. After a final washing step with 200 µl RNA wash buffer and centrifugation for 1 minute at 9000 x g, the column was centrifuged for 2 minutes at 9000 x g to remove residual buffer. RNA was eluted by adding 12 µl H₂O and centrifugation at 9000 x g for 1 minute. DNA LoBind tubes were used from here onwards. Eluates were reloaded onto the column and columns were centrifuged for 1 minute at 9000 x g. IP samples were stored at -80°C and SMI samples were further processed on ice.

2.2.12.5 Dephosphorylation of SMI samples and 3' RNA linker ligation

11 µl FastAP mastermix (1x FastAP buffer, 40 U murine RNase inhibitor, 2 U FastAP™ Thermosensitive Alkaline Phosphatase, ad 11 µl H₂O) were added to SMI samples and incubated at 37°C for 20 minutes shaking at 1200 rpm. Samples were mixed with 75 µl PNK

master mix (1.05 x PNK7 buffer, 5.26 mM DTT, 2 U TurboDNase, 40 U T4 PNK, ad 150 μ l H₂O) and incubated for 20 minutes at 37°C and 1200 rpm in a thermomixer. SMI RNA was purified with the RNA Clean & Concentrator-5 kit as described in 2.2.12.4. Per SMI sample, 5 μ l RNA were transferred into a new tube and stored at -80°C. The remaining 5 μ l RNA were mixed with 1.5 μ l 100% DMSO and 0.5 μ l 40 μ M invRIL19 RNA adapter (Table 7). The adapter was annealed by incubation at 65°C for 2 minutes and immediate cool down on ice for 1 minute. Per SMI RNA, 13.5 μ l 3' RNA ligation master mix (0.976 x RNA ligase buffer without DTT, 4.88 mM DTT, 0.976 mM ATP, 2.93% DMSO, 0.02% Tween-20, 14.63% PEG 8000, 12 U murine RNase inhibitor, 36 U T4 RNA ligase 1, ad 13.5 μ l H₂O) were slowly mixed into the tubes. Samples were incubated at RT for 60 minutes and mixed every 15 minutes. RNA was purified using Dynabeads™ MyOne™ Silan. Per sample 15 μ l Dynabeads™ MyOne™ Silan were magnetically separated and the supernatant was removed. Beads were washed with 75 μ l RNeasy Lysis Buffer (RLT) buffer and the RLT buffer was removed completely before the beads were resuspended in 62.5 μ l RLT+Tween-20 (RLTW) buffer. SMI RNA samples were carefully mixed with 61 μ l of Dynabeads™ MyOne™ Silan in RLTW buffer and 73 μ l 100% ethanol were added. Samples incubated for 15 minutes and were pipette mixed every 3 minutes. The beads were magnetically separated and the supernatant was removed. 1 ml 80% ethanol was added, the beads were carefully resuspended and transferred into a new tube. Dynabeads™ MyOne™ Silan were magnetically separated and the supernatant was removed. Beads were washed by resuspending in 900 μ l 80% ethanol, magnetic separation and removing the supernatant. The washing step was repeated once before the beads were dried for 3-5 minutes and resuspended in 9.5 μ l TT elution buffer. RNA was eluted by incubation at RT for 5 minutes and mixing every minute. Beads were magnetically separated and eluted RNA was transferred into PCR tubes on ice.

2.2.12.6 Reverse transcription and cDNA purification

IP sample RNA isolated in 2.2.12.4 was retrieved from -80°C and transferred into PCR strip tubes on ice. Per IP and SMI sample 1 μ l of 5 μ M InvAR17 RT primer and 1 μ l of 10 mM dNTPs were added and primer were annealed through incubation at 65°C for 2 minutes followed by immediate cool down on ice for 1 minute. 10 μ l RT master mix (1x first strand buffer, 5 mM DTT, 8 U murine RNase inhibitor, 120 U SuperScript III, ad 10 μ l H₂O) were added per sample. The reaction was mixed and cDNA synthesis was performed at 55°C for 40 minutes in a C1000 Touch Thermal Cycler. cDNAs were cleaned by adding 2.5 μ l ExoSAP-IT™ and incubation at 37°C for 15 minutes. After adding 1 μ l of 0.5 M EDTA, RNA was removed by adding 3 μ l of 1 M sodium hydroxide and incubation at 70°C for 10 minutes. The pH of the samples was readjusted by adding 3 μ l of 1 M hydrochloric acid. cDNAs were purified through Dynabeads™ MyOne™ Silan as described above. For the cDNA clean-up 5 μ l Dynabeads™

MyOne™ Silan were washed with 25 µl RLT buffer as described above and resuspended in 93 µl RLTW buffer. The cDNA samples were added to the beads in RLTW buffer and carefully mixed with 108 µl of 100% ethanol. After incubation for 10 minutes at RT and mixing every third minute beads were magnetically separated and supernatant was removed completely. Beads were carefully resuspended in 200 µl of 80% ethanol and transferred to a new tube. Samples were washed twice with 200 µl of 80% ethanol as described in 2.2.12.4 and dried for 3-5 minutes.

2.2.12.7 cDNA 5' adapter ligation

2.5 µl 5' adapter mix (4.66 µM InvRand3Tr3 adapter (Table 7), 7.77% DMSO, ad 2.5 µl TT elution buffer) were added to the dried beads. After incubation at 70°C for 2 minutes, the tubes were immediately placed on ice. Per sample 7.8 µl of 5' ligation master mix (0.097x RNA ligase buffer (NEB), 1.94 mM DTT, 0.97 mM ATP, 0.019% Tween-20, 17.48% PEG 8000, 30 U T4 RNA ligase 1, 15 U 5' deadenylase, ad 7.8 µl H₂O) were slowly added while mixing. Samples were incubated at RT overnight in a tube rotator. 5 µl TT elution buffer were added and the cDNAs were purified with Dynabeads™ MyOne™ Silan as described in 2.2.12.5. 2.5 µl of Dynabeads™ MyOne™ Silan were washed with 12.5 µl RLT buffer and resuspended in 47 µl RLTW buffer. 45 µl of Dynabeads™ MyOne™ Silan in RLTW buffer were added together with 45 µl of 100% ethanol to the samples. Washing was performed as described in 2.2.12.4. After the last washing step the beads were dried for 3-5 minutes and resuspended in 25 µl TT elution buffer. cDNA was eluted by incubation for 5 minutes at RT while mixing every minute and transferred to new tubes on ice.

2.2.12.8 seCLIP library amplification

A pilot PCR was performed to determine the optimal amount of library amplification cycles using the Q5® High-Fidelity DNA Polymerase. Per sample, 2x 30 µl Q5 master mix (1x Q5 Reaction Buffer, 200 µM dNTPs, 2 U Q5® High-Fidelity DNA Polymerase, ad 30 µl H₂O) was set-up. Per sample, the Q5 master mix was mixed with a unique pair of TruSeq combinatorial dual (CD) indexed DNA adapters (Table 7). 2.5 µl of 20 µM i5 (D501-D506) and 2.5 µl of 20 µM i7 (D701-D706) adapters were added to the reaction. 10 µl of SMI cDNA were mixed with 5 µl H₂O and added to the Q5 master mix. For amplification of IP cDNA, 12.5 µl were mixed with 2.5 µl H₂O and added to the Q5 master mix. As controls, one Q5 master mix per sample was mixed with 15 µl H₂O. Q5 PCR cycling conditions are listed in Table 22.

Table 22. PCR cycling conditions for seCLIP library amplification

Application	Step	Temperature [°C]	Duration [s]	Number of cycles
Library amplification	Denaturation	98	30	
	Annealing	68	30	
	Extension	72	40	go to step 1, 6 times
	Annealing	98	15	
	Extension	72	60	go to step 4, 21 times
	Final extension	72	60	
	Final hold	15	∞	

Beginning with cycle number 6, a 6 µl sample was collected at the end of the extension step of every third cycle to assess the amount of library produced by Q5 PCR. PCR samples were mixed with 1 µl of 6 x OrangeG loading buffer and analyzed via 3% agarose gel electrophoresis as described in 2.2.1.3. The agarose gel electrophoresis was performed at 200 V for 1 h. Amplification of the seCLIP libraries was performed with the in the pilot PCR determined amount of PCR cycles and the set-up described above.

2.2.12.9 seCLIP library purification and quantification

seCLIP libraries were purified using AMPure XP beads. AMPure XP beads were equilibrated to RT and 72 µl were added per sample. Samples were incubated with AMPure XP beads for 10 minutes at RT while mixing every 3 minutes. Beads were magnetically separated for 2 minutes using a 96S Super Magnet. Supernatant was removed and the beads were carefully washed twice with 200 µl of 80% ethanol. After the last washing step the ethanol was removed entirely and the beads dried for 3-5 minutes. seCLIP library was eluted by resuspending the beads in 20 µl PCR elution buffer and incubation at RT for 5 minutes. Beads were magnetically separated and the eluates were transferred into new tubes. Libraries were mixed with 5 µl of 6 x OrangeG loading buffer and loaded on a 3% low melting agarose gel together with a 50bp ladder. Agarose gel electrophoresis was performed at 80 V for 3 h. Libraries were excised by cutting the gel between 175-350 bp using a scalpel under UV light. DNA purification was performed using the QIAquick Gel Extraction Kit. Gel pieces were weighed and mixed with 6 x gel volumes QG buffer at RT. After the gels were completely dissolved 1x gel volume isopropanol was added and mixed. Samples were loaded onto the QIAquick columns and centrifuged at 10000 x g for 1 minute. Columns were washed by adding 500 µl QG buffer and centrifugation at 10000 x g for 1 minute. 750 µl PE buffer were added and the previous centrifugation step was repeated. The column was centrifuged at 10000 x g for 2 minutes to remove residual buffer and dried for 2 minutes. seCLIP libraries were eluted by adding 12.5 µl EB buffer to the columns and incubating for 15 minutes at RT. After centrifuging at fullspeed for 1 minute, the eluate was loaded onto the same column and centrifugation was repeated.

seCLIP libraries were quantified using the Qubit™ dsDNA HS-Assay-Kit according to manufacturer's instructions.

2.2.12.10 High-throughput sequencing

Single-end high-throughput sequencing of seCLIP libraries was performed by the Cologne Center for Genomics. Libraries were multiplexed at a concentration of 25 nmol per library in 150 µl EB buffer + 0.1% Tween-20. The seCLIPs library pool was quantified by using the Peqlab KAPA Library Quantification Kit and the Applied Biosystems 7900HT Sequence Detection System. The pool was then sequenced on the Illumina NovaSeq6000 platform with 101bp read length (read 1).

2.2.12.11 Bioinformatics analysis

Bioinformatics analysis was performed using the seCLIP analysis pipeline as previously published ²⁰⁰. This specialised seCLIP pipeline is a fully automated pipeline that identifies genomic locations of RBPs directly from fastq files. The pipeline trims adapters and adapter-dimers with cutadapt, maps to repeat elements with STAR and filters the reads. It proceeds with mapping the filtered reads, not mapping to repeat elements, to the given genome with STAR, removes PCR-duplicates with umi_tools, calls enriched peak regions (i.e. peak clusters) with CLIPPER and finally uses the size-matched input sample to normalize and calculate fold-change enrichment within enriched peak regions. Peaks in the output bed files were filtered for all downstream analyses for $-\log_{10}(\text{p-value}) \geq 3$ and $\log_2(\text{fold change}) \geq 3$, as recommended by the authors of the pipeline. Peaks and binding motifs were annotated using HOMER (function annotatePeaks.pl) ²⁴⁰. HOMER (Hypergeometric Optimization of Motif EnRichment) is a collection of command line tools for Motif Discovery and next-generation sequencing analysis. HOMER was originally written as a *de novo* motif discovery algorithm and is capable of identifying 8-20 bp motifs in high-throughput sequencing data. HOMER tools can work with ChIP-Seq, GRO-Seq, RNA-Seq, CLIP-Seq, Hi-C and various other types of sequencing data sets. We used specifically tools annotatePeaks.pl and findMotifsGenome.pl (with option -rna). Both work on the bed files of significant peaks (output from seCLIP pipeline, filtered as described earlier) and the given genome: AnnotatePeaks.pl processes each peak with the following two steps: 1) It determines the distance to the nearest transcription start site and annotates the peak with that gene. 2) It determines the genomic region of the centre of the peak. FindMotifsGenome.pl extracts the sequences of the regions and identifies motifs by comparing peaks to randomly selected background sequences. Differential analysis comparing binding sites enriched in PQ to binding sites enriched in no PQ conditions was performed using the R package DiffBind ²⁴¹. DiffBind is an R-package that works with aligned sequence reads (i.e. BAM files of IP and SMI) and the accompanying list of enriched loci identified by a peak

caller (i.e. bed files filtered for $-\log_{10}(\text{p-value}) \geq 3$ and $\log_2(\text{fold change}) \geq 3$ as described earlier). DiffBind performs a differential analysis and identifies differentially bound sites between these experimental conditions. DiffBind's statistics is based on established RNA-seq analysis R packages, like edgeR and DESeq2. Parameters for the normalization in DiffBind were set to `method=DBA_DESEQ2`, `normalize=DBA_NORM_NATIVE` and `background=TRUE`. Otherwise default settings were used. Results tables of DiffBind were filtered for $\text{FDR} < 0.05$. We used differential gene expression analysis of whole-cell mRNA-sequencing data generated from PQ-stimulated and unstimulated mIMCD cells (see 2.2.10 for more details) to filter significantly enriched binding sites for transcripts with unaltered abundance upon PQ stimulation. We continued our analysis with binding sites, which were not significant (adjusted p-value > 0.2) in the differential gene expression of the transcriptome and significant ($\text{FDR} < 0.05$) in the differential analysis (DiffBind) of the seCLIP. Venn diagrams were generated using DeepVenn²³². GO enrichment analysis was done using the R package clusterProfiler (function `enrichGO`)²³⁴. ClusterProfiler is an R-package that fully automatically performs gene functional annotations for various species using most recent versions of databases. It also offers a wide range of functions to perform enrichment analysis. We specifically used the function `enrichGO` which conducts a GO enrichment analysis with FDR control of a user given gene set and species. Enriched GO terms were filtered for biological relevant terms (excluded cell-type specific terms e.g. chondrocyte differentiation, post-synaptic development) and clustered using Revigo²⁴². Moreover, GO terms were filtered for a gene count $\geq 1\%$ of the total number of transcripts, which were used for the GO enrichment analysis.

2.2.13 Animal procedures

2.2.13.1 Mouse lines

Mice with floxed *Msi1* (MGI:5763184) and *Msi2* (MGI: 5588139) alleles were provided by Christopher Lengner and generation was described previously¹⁹¹. In floxed Musashi alleles the transcriptional start site as well as exons 1-2 (*Msi1^{flox}*) or exons 1-4 (*Msi2^{flox}*) are flanked with loxP sites. Ksp:Cre mice (MGI: 2665300) specifically express the Cre recombinase under the Cadherin-16 promotor in the distal tubules, loop of Henle and collecting duct of the kidney²⁴³. In Ksp:Cre^{ERT2} mice (MGI:3641108), a tamoxifen-inducible variant of the Cre recombinase is fused to a Cadherin-16 promotor region²⁴⁴. All mice were kept in a mixed C57BL6/N and C57BL6/J background.

2.2.13.2 Mouse holding and body weight measurement

Mice were housed under specific-pathogen-free conditions in group cages (five animals per cage) in the CECAD *in vivo* Research Facility. All mice received *ad libitum* water and food and

were kept within a 12-hour light/dark rhythm. All experimental procedures performed during this study were approved by State Agency for Nature, Environment and Consumer Protection North Rhine-Westphalia (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen VSG 81-02.04.2020.A153). Mice were weighed by animal caretakers in the CECAD *in vivo* Research Facility on a weekly basis.

2.2.13.3 HotSHOT DNA extraction

Genomic DNA was extracted from ear biopsies using HotSHOT (hot sodium hydroxide and Tris) extraction ²⁴⁵. The ear biopsies were incubated in 75 µl 1x base solution at 95°C for 30 minutes before 75 µl 1x neutralization solution were added. The genomic DNA was stored at -20°C.

2.2.13.4 Genotyping

Genotyping was performed using the GoTaq® G2 Flexi DNA polymerase system according to manufacturers' instructions. The *Msi1* flox allele was detected using the primers Msi1 3F-2 and Msi1 3R-2, the *Msi2* flox allele was detected using the primers Msi2 F2 and Msi2 R2 and the *Cre* allele was detected using the primers Cre fp and Cre rp. The following annealing temperatures were used for PCR reactions: *Msi1* flox (56°C), *Msi2* flox (58°C) and *Cre* (59°C). PCR products were analyzed via 1% (*Msi2* flox) and 2% (*Msi1* flox, *Cre*) agarose gel electrophoresis.

2.2.13.5 Sequencing of Msi1flox allele

Genomic DNA was extracted from ear biopsies of mice containing the *Msi1* flox allele using the Quick-DNA Miniprep Plus Kit (Zymo Research) according to manufacturer's instructions. A map of the *Msi1* flox allele and its generation was previously published ²¹⁶. Briefly, to develop the floxed *Msi1* allele a 3lox allele, harboring three loxP sites and a PGK-neomycin resistance cassette, was generated in embryonic stem cells using homologous recombination. The 3lox clones were electroporated with Cre recombinase and resulting 2lox clones were injected into blastocysts to set up a colony of mice with floxed *Msi1* alleles. The *Msi1*-3lox locus was detected by performing PCR using the Q5® High-Fidelity DNA Polymerase. The PGK cassette was detected using the primers Msi1_3loxP_fwd and Msi1_PGK_rev and the neomycin cassette was detected using the primers Msi1_Neo_fwd and Msi1_ex3_reverse1 (Table 8). Amplification was performed using an annealing temperature of 58°C. PCR products were analyzed via 2% agarose gel electrophoresis and excised from the gel using a scalpel under UV light. DNA was extracted using the GeneJET Gel Extraction Kit according to manufacturer's instructions and eluted in 24 µl H₂O. 12 µl of the purified PCR products were mixed with the 3

µl of primers (10 µM) used for amplicon generation and subjected to Sanger sequencing at the Microsynth Seqlab GmbH.

2.2.13.6 Tissue collection

Basic phenotyping of all mouse lines was assessed by sacrificing mice at indicated time points. Mice were euthanized by final blood withdrawal through cardiac puncture. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kgBW) and xylazin (20 mg/kgBW). The abdominal cavity was opened and a bilateral thoracotomy was performed. After blood was taken from the right ventricle, a syringe was inserted into the left ventricle and cardiac perfusion using PBS was performed. For histological analysis organs were harvested, cleaned with PBS and fixed in 4% formaldehyde for 24 h. After dehydration the organs were paraffin-embedded. For RNA isolation the tissue was harvested, cleaned with PBS and immediately frozen in liquid nitrogen.

2.2.13.7 Periodic acid–Schiff staining

Periodic acid–Schiff (PAS) staining was performed to allow histopathological analysis of the kidneys collected in 2.2.13.6. The paraffin-embedded kidneys were cut into 1.5-2 µm thick slices and the sections were dried overnight. After deparaffinization in xylene (2x 2 min), the tissue was hydrated using a descending ethanol row (2x2 min 100% ethanol, 2x2 min 95% ethanol, 2x2min 70% ethanol, and 2 min water). Oxidation of the tissue was performed using 0.9% periodic acid and after washing with water the sections were incubated with Schiff's reagent for 10 minutes. The tissue was rinsed in running tap water for 2 minutes and a nuclear counterstain was performed through incubation with Meyer's hematoxylin for 10 seconds. Washing with water for 3 minutes was followed by dehydration of the sections using an ascending ethanol row (2x2 min 70% ethanol, 2x2 min 95% ethanol, 2x2min 100% ethanol, and 2x2min xylene). The rehydrated tissue was embedded with Histomount, dried overnight and imaged using the Hamamatsu Slidescanner S360.

2.2.13.8 Immunohistochemistry staining

Immunohistochemistry was performed on 1.5-2 µm thick tissue slices. Similar to PAS staining, deparaffinization in xylene (2x 2min) was followed by rehydration of the tissue using a descending ethanol row (2x2min 100% ethanol, 2x2 min 95% ethanol, 2x2 min 70% ethanol, and 2 min TBS). Antigen retrieval was enabled by cooking in 10 mM citric acid buffer pH 6 (anti MSI1, MBL) or 10 mM Tris-EDTA buffer pH 9 (anti MSI2, Novus Biologicas/Biotechne) for 10 minutes. Once the tissue was cooled down to room temperature it was washed 1x 5min in TBS and incubated in 3% H₂O₂ for 15 minutes to block endogenous peroxidases. After rinsing with

water for 5 minutes a PAP pen was used to generate a hydrophobic barrier around the tissue. Avidin/Biotin blocking was performed by incubation in the avidin solution for 15 minutes, washing with TBS for 5 minutes and incubation with the biotin solution for 15 minutes. The sections were washed in TBS for 5 minutes and incubated with the primary antibodies (1:500 dilutions in 1% BSA/TBS) overnight at 4°C. The samples were rinsed 3x 5 minutes in TBS and incubated with 1:500 dilutions of biotinylated secondary antibodies in 1% BSA/TBS (MSI1: Biotin-SP-conjugated AffiniPure donkey anti-rat, MSI2: Biotin-SP-conjugated AffiniPure donkey anti-rabbit) for 45 minutes at room temperature. After washing 3x 5 minutes with TBS the samples were incubated with the ABC kit for 30 minutes at room temperature. The chromogenic signal was developed by applying the DAB chromogen mixture in substrate buffer (Dako) for 1-2 minutes before washing with water for 5 minutes. Staining of nuclei was accomplished by incubation with Meyer's hematoxylin for 10 seconds and rinsing in running tap water for 6 minutes. The tissue was rehydrated using an ascending ethanol row (2x2 min 70% ethanol, 2x2 min 95% ethanol, 2x2min 100% ethanol, and 2x2min xylene) and embedded using Histomount. After drying overnight the sections were imaged using the Hamamatsu Slidescanner S360.

2.2.13.9 Immunofluorescence staining

Sectioning, deparaffinization, rehydration and antigen retrieval was performed as described above (2.2.13.8). The tissue was washed in PBS and blocked in 5% NDS/PBS for 1 hour at room temperature. After rinsing with PBS 3x 5 minutes, the slices were incubated with the primary antibodies (dilutions in 5% BSA/PBS as listed in Table 12) overnight at 4°C. The samples were washed with PBS 3x 5 minutes at RT before incubation with 1:500 dilutions of fluorescently labeled secondary antibodies (Table 14) together with Hoechst (1:1000) in 5% BSA/PBS for 1 h in the dark. Washing with PBS 3x 5 minutes was followed by mounting with ProLong Diamond antifade reagent. Imaging was performed using the TCS SP8 confocal microscope (Leica).

2.2.13.10 Tamoxifen administration

At the age of 8 weeks Cre recombinase was induced in mice carrying the *KspCre^{ERT2}* allele. Tamoxifen was dissolved at a concentration of 20 mg/ml in 15% ethanol/sesame oil. Tamoxifen was administered through intraperitoneal injection five times at a dose of 1 mg /10 g BW over a time course of ten days. Mice were sacrificed at the age of 12 weeks as described in 2.2.13.6. Left kidneys, brain, eyes and intestine were processed for histological analysis as described in 2.2.13.8. Right kidneys were frozen in liquid nitrogen for further analysis.

2.2.13.11 Antisense oligonucleotide administration

Targeted inhibition of *MSI1* *in vivo* using antisense oligonucleotides (ASOs) was described previously²¹⁷. The ASO specifically targeting *Msi1* was ordered as listed in Table 10 (IDT) and dissolved in sterile PBS to a concentration of 5 µg/µl. At the age of 10 weeks, wild type C57BL6/N mice were either administered with the *Msi1* ASO at a concentration of 12.5 mg/kgBW or PBS as vehicle control. *Msi1* ASO was delivered through intravenous injection into the tail vein every third to fourth day over a time course of 11 days. 24 hours after the final administration the mice were sacrificed through terminal blood withdrawal as described in 2.2.13.6. The right kidney was harvested and processed for histological analysis as described in 2.2.13.8 while the left kidney, liver, testis and brain were harvested and frozen in liquid nitrogen for RNA extraction as described in 2.2.13.12.

2.2.13.12 RNA extraction

Frozen tissue was transferred into silica beads-containing screw cap tubes and 1 ml of Trizol was added. The tissue was grinded 3x 20 seconds (5 seconds pause between each cycle) at 4000 rpm using the Precellys® 24. Lysates were centrifuged at 12000 rpm for 5 minutes and the supernatant was transferred to a new tube. RNA was isolated using the Direct-zol™ RNA Miniprep Plus kit. RNA concentrations were measured using the Invitrogen™ Qubit™ RNA Broad Range assay kit and quality was assessed using Nanodrop 1000 spectrophotometer according to manufacturer's instructions.

2.2.13.13 Renal ischemia reperfusion injury

Renal ischemia reperfusion injury as a mouse model of acute kidney injury was described previously²⁴⁶. At the age of 12 weeks male mice were anaesthetized via intraperitoneal injection of ketamine (100 mg/kgBW) and xylazin (20 mg/kgBW). Mice were placed on a heated surgical pad before making a midline abdominal incision and inserting a retractor. The right ureter as well as the right renal artery and vein were ligated three times using black silk (6-0, sterile, 100m) and right nephrectomy was performed. Half of the right kidney was frozen in liquid nitrogen and the other half was fixed in 4% formaldehyde, dehydrated and paraffin-embedded. The left renal vein and artery were carefully dissected and ischemia was induced by administration of two micro serrefine clamps. After 40 minutes reperfusion was initiated by removing the clamps. Peritoneum and skin were closed and analgesic buprenorphine hydrochloride (0,1 mg/kgBW) was administered subcutaneously. Further buprenorphine hydrochloride was provided to the mice via drinking water (0,009 mg/ml). In sham mice only right nephrectomy without clamping of the left renal vein and artery was performed before peritoneum and skin were closed. All mice were euthanized 24 hours postoperative via *i.p.*

injection with ketamine/xylazin followed by final blood withdrawal through cardiac puncture. Half of the left kidney was frozen in liquid nitrogen and the other half was processed for histological analysis as described above for the right kidney.

2.2.13.14 Blood analysis

Blood was withdrawn via cardiac puncture as a terminal procedure when euthanizing the mice to collect tissue as described in 2.2.13.6. Blood was taken from the right ventricle using a heparin coated syringe and centrifuged at 10000 g for 10 minutes at room temperature. Plasma urea and creatinine were measured by the central laboratory of the University Hospital of Cologne using a Cobas C 702 (Roche Diagnostics).

2.2.14 Human kidney tissue

Human kidney tissue was obtained from tumor nephrectomies through the BioMaSOTA biobank of the University Hospital Cologne after approval of the project at hand by the ethics committee of the Medical Faculty, University of Cologne (#20-1206). Participants provided written informed consent and all procedures were executed following the Declaration of Helsinki and the good clinical practice guidelines by the International Conference on Harmonization.

3. Results

3.1 Expression of Musashi proteins in the kidney at baseline and in experimental mouse models of AKI

Little information is available on the function of MSI1 and MSI2 within the healthy and diseased kidney. The kidney is a highly complex organ, containing over 40 specialized cell types²⁴⁷. Pinpointing the intrarenal and subcellular localization of both Musashi proteins can help deciphering their function within the kidney. Available data on the expression of both Musashi proteins in mouse and rat kidneys showed that, although *Msi1* and *Msi2* mRNA was expressed in distinct tubular segments of the nephron, only MSI2, but not MSI1 protein was measured in kidneys of wild type rats (Figure 5). Immunofluorescence staining of wild type mouse kidneys revealed that, while MSI1 expression was restricted to distal tubular cells (Figure 7 a), MSI2 was expressed in proximal and distal tubules (Figure 7 b). On a subcellular level, MSI1 was expressed at the apical membrane and in the cytoplasm of tubular epithelial cells in kidneys from wild type mice (Figure 7 c). MSI2 was detected in the cytoplasm of all tubular epithelial cells and at the nuclear membrane in some tubular epithelial cells (Figure 7 d). Besides the tubular system, MSI2 signal was detected in glomerular podocytes.

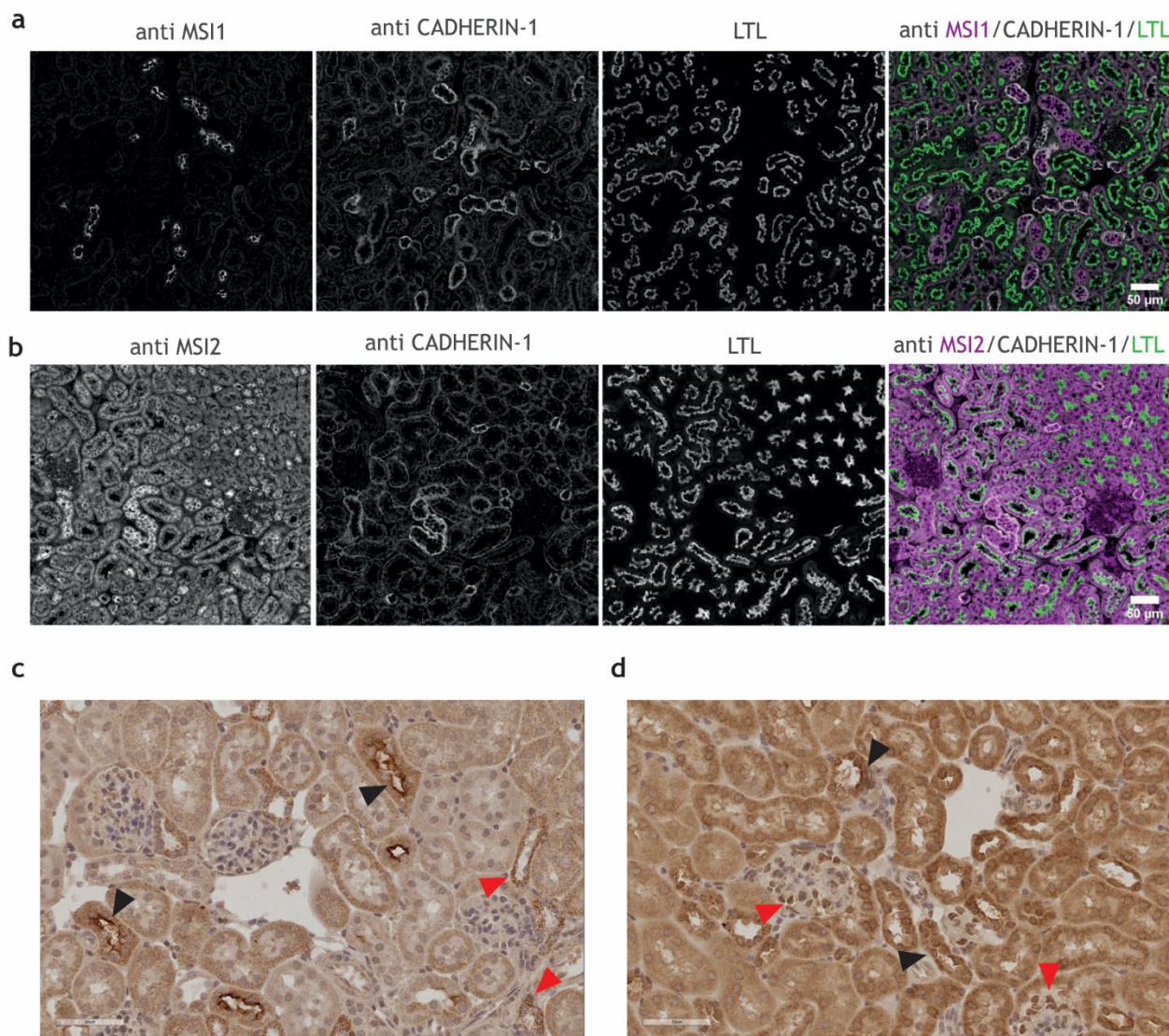


Figure 7. While MSI2 is detected across the entire nephron, MSI1 is expressed in distal tubular segments of mouse kidneys

a| MSI1 is expressed in distal, but not in proximal tubules. Immunofluorescence staining showed that MSI1 signal was absent from proximal tubule cells, but co-localized with the distal tubule marker CADHERIN-1. MSI1 was stained in combination with CADHERIN-1 as distal tubules marker and Lotus tetragonolobus Lectin (LTL) as proximal tubules marker. **b| MSI2 is expressed in distal and proximal tubules.** Immunofluorescence staining showed MSI2 signal co-localized with both, the proximal tubule marker LTL and the distal tubule marker CADHERIN-1. MSI2 was stained in combination with CADHERIN-1 and LTL. Images displayed in (a) and (b) were acquired with a TCS SP8 confocal microscope using a 20x objective. Scale bars indicate 50 μm. **c| MSI1 is expressed at the apical membrane and in the cytoplasm of tubular epithelial cells.** Immunohistochemistry staining showed MSI1 signal at the apical membrane (black arrows) and in the cytoplasm of tubular epithelial cells (red arrows) of wild type mouse kidneys. **d| MSI2 is localized in the cytoplasm and at the nuclear membrane of tubular epithelial cells.** Immunohistochemistry staining of MSI2 on wild type mouse kidney showed MSI2 signal in the cytoplasm and at the nuclear membrane of tubular epithelial cells (black arrows) and in glomerular podocytes (red arrows). Cell types were identified by an experienced nephropathologist. Brightfield images (c/d) were acquired with the Leica SCN400 slidescanner using the 40x objective. Scale bars indicate 50 μm.

Similar to mouse kidneys, MSI2 was expressed in distal tubular epithelial cells and glomerular podocytes in one human kidney biopsy specimen (Figure 8 a). MSI1 protein was not expressed in the human kidney biopsy specimen, as the same signal was obtained when stained with the secondary antibody only (Figure 8 a). Images of immunohistochemistry staining of MSI1 on human kidney biopsy specimens from three different patients (human protein atlas) showed low MSI1 expression at the membrane and within the cytoplasm of tubular epithelial cells in one human kidney biopsy specimen, while MSI1 expression in the other two biopsy specimens was absent (Figure 8 b).

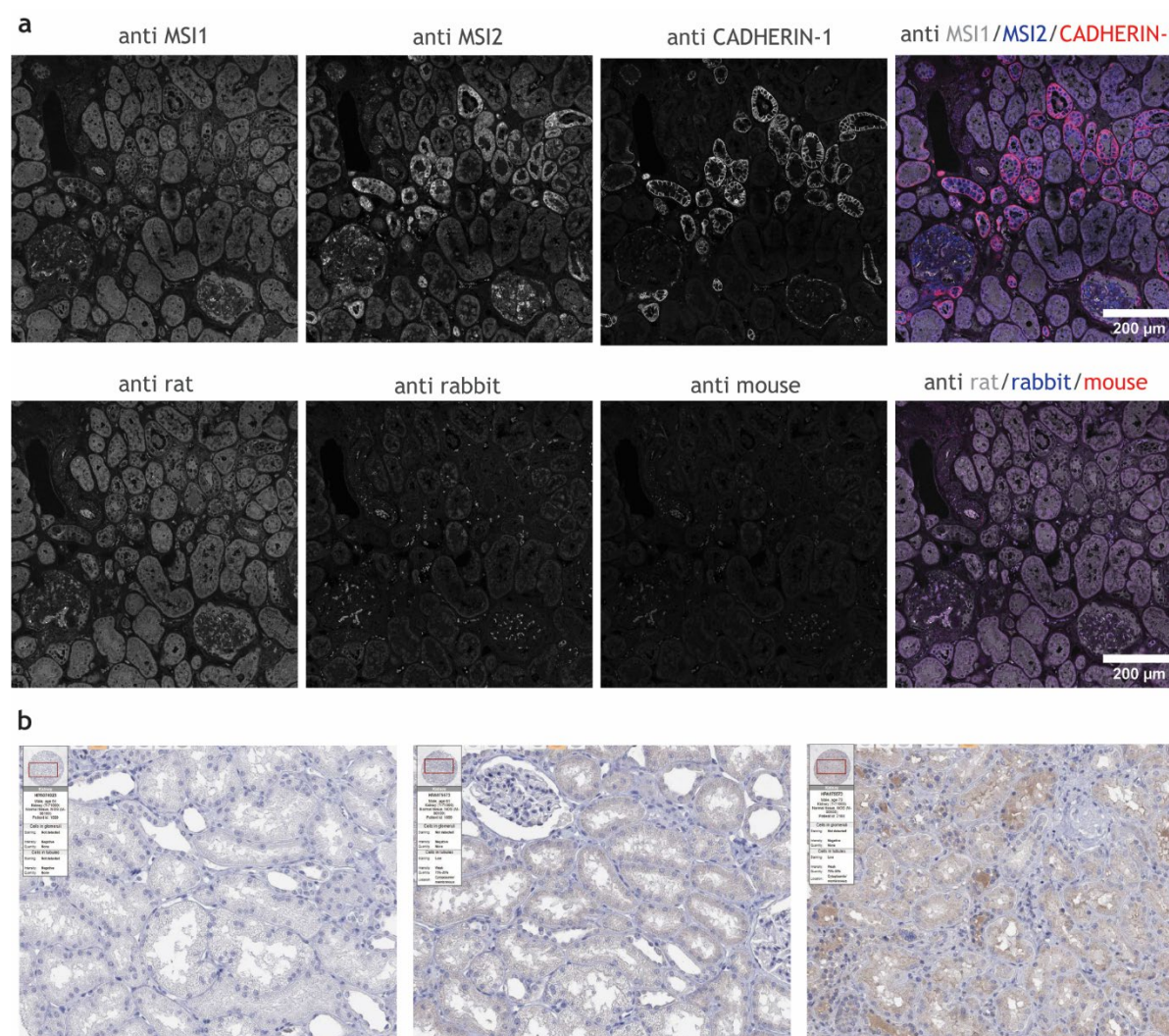


Figure 8. Only MSI2 is detected in distal tubules of human kidneys

a| While MSI1 is not detected, MSI2 is detected in distal tubules of human kidney biopsies. Immunofluorescence staining showed that MSI2 was expressed in distal tubular cells and glomerular podocytes of the human kidney. MSI1 expression was not detected in the human kidney biopsies, as the MSI1 signal intensity was similar to the control without primary antibody (below). MSI1 and MSI2 were co-stained with the distal tubule marker CADHERIN-1 on paraffin-embedded human kidney biopsies. Images were acquired with a TCS SP8 confocal microscope, using a 20x objective. Scale bar indicates 200 μm. **b| Low expression of MSI1 was detected in one human kidney biopsy specimen obtained from the human protein atlas.** MSI1 expression levels were low in human kidney biopsy specimens but varied between three patients. A weak expression of MSI1 was detected in the cytoplasm and membrane of tubular epithelial cells in the third patient (<https://www.proteinatlas.org/ENSG00000135097-MSI1/tissue/kidney#img>).

Previously published data of our own group showed that in transcriptomes of cisplatin-damaged mouse kidneys, *Msi1* was one of the most strongly upregulated RBPs compared to control mice ¹⁰⁴ (Figure 6). These findings were confirmed on protein level using immunohistochemistry staining, as MSI1 was highly expressed in cisplatin-damaged kidneys compared to controls (Figure 9 a). Moreover, immunofluorescence staining of MSI1 in kidneys after IRI confirmed an increased expression of MSI1 in a second experimental model of AKI and high expression of MSI1 was detected in necrotic tubules along the cortico-medullary border (Figure 9 b).

In contrast to *Msi1*, *Msi2* expression remained unchanged upon cisplatin damage in wild type mouse kidneys compared to controls (Figure 6). Immunohistochemistry staining of MSI2 in cisplatin damaged kidneys showed that MSI2 expression was not altered (Figure 9 a). Similarly, general MSI2 protein levels remained unchanged in kidneys after IRI, but necrotic tubules with high MSI1 expression were lacking MSI2 signal (Figure 9 b).

Although the data presented here indicate distinct roles of the two Musashi proteins in AKI, we decided to investigate both Musashi proteins considering the overlapping and redundant functions implicated by previous studies. MSI1 and MSI2 are homologous proteins, which share 75% overall amino acid sequence similarity ¹⁷⁹, and sequence similarity increases up to 91% when focusing on the RNA recognition motifs ¹⁷⁹. Unsurprisingly, MSI1 and MSI2 have been shown to bind to similar RNA targets in various cells and tissues ^{184,191–198}. The high similarity and the large overlap of RNA targets makes it likely that both Musashi proteins can compensate for each other in case one homologue is lost. Therefore, it was of interest to generate mouse lines with conditional loss of either MSI1 or MSI2, or simultaneous loss of both Musashi proteins, in specific tubular segments of the kidney. By subjecting these Musashi knockout mice to IRI, we intended to investigate the role of MSI1 in AKI, both in a wild type and a MSI2 loss-of-function background.

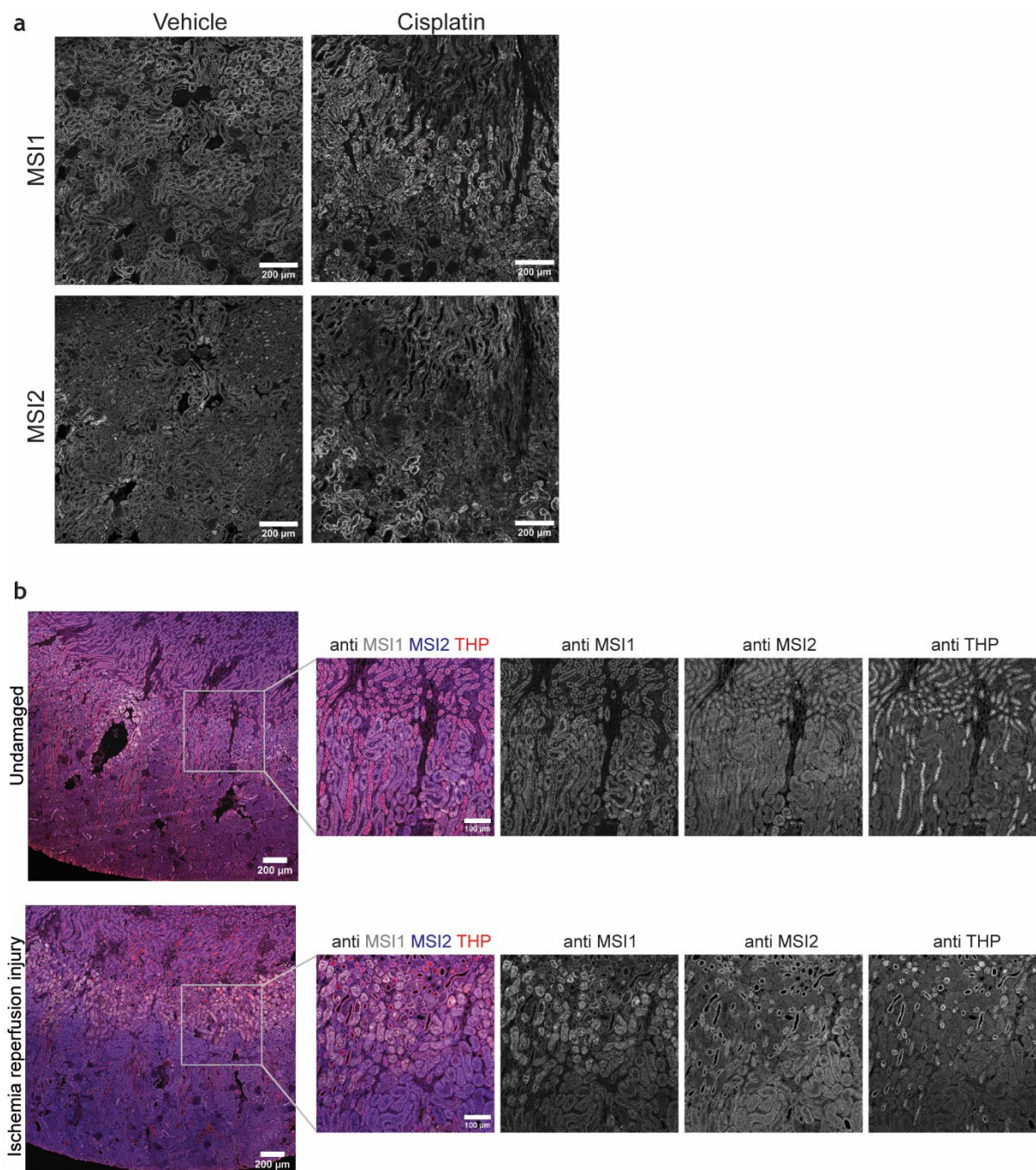


Figure 9. Expression of MSI1 is upregulated in mouse models of AKI

a| Increased detection of MSI1 in kidneys of cisplatin treated mice. Increased expression of MSI1 was detected in cisplatin-damaged mouse kidneys. Compared to MSI1 expression in kidneys from vehicle-injected animals, MSI1 signal was increased in cisplatin-damaged kidneys. Expression of MSI2 did not majorly change in cisplatin-damaged kidneys compared to vehicle controls. Shown are immunofluorescence staining of MSI1 and MSI2 in mouse kidney from cisplatin-treated and vehicle-treated animals. **b| MSI1 signal is increased in kidneys after ischemia reperfusion injury.** MSI1 was highly expressed in necrotic tubular cells at the cortico-medullary border of kidneys after IRI. Tubular cells with high MSI1 expression were largely lacking expression of MSI2. Shown are immunofluorescence staining of MSI1, MSI2 and Tamm Horsefall Glycoprotein (THP) on undamaged kidneys (top) and kidneys after IRI (bottom). Undamaged kidneys are right kidneys, which were removed during surgery before inducing IRI in left kidneys. THP is a marker for the thin ascending limb of the loop of Henle. Images were acquired with a TCS SP8 confocal microscope, using a 20x objective in (a) and a 40x objective in (b). Scale bars indicates 200 µm.

3.2 Generation of mouse models with loss of MSI1 in the kidney

3.2.1. Conditional *Msi1* knockout mouse models

To analyze the role of MSI1 in AKI, a conditional genetic knockout mouse model of *Msi1* in the kidney was generated. We decided on the generation of a conditional mouse model because, although studies showed that mice with conventional whole body knockout of *Msi1* are potentially viable, some mice developed severe hydrocephalies and died within 1-2 months of age¹⁷⁵. Based on the expression of MSI1 in the distal tubules and its high expression within tubular epithelial cells at the cortico-medullary border upon injury, KspCre²⁴³ mice were crossed with mice carrying floxed *Msi1* alleles¹⁹¹. Ksp-cadherin (cadherin 16) is specifically expressed in tubular epithelial cells and its expression in adult mouse kidneys is highest in the distal tubules, loop of Henle and collecting duct²⁴³. When crossing homozygous *Msi1* floxed animals with heterozygous *Msi1* floxed / KspCre transgenic mice, the probability of receiving conditional *Msi1* knockout animals (*Msi1*^{fl/fl} KspCre^{tg/wt}) was 25% (Figure 10 a). Within 119 born animals, only one mouse was a *Msi1* knockout animal (Figure 10 b). Heterozygous control animals (*Msi1*^{fl/wt} KspCre^{tg/wt}) made up over 44% of all animals and were therefore overrepresented across all litters. Of note, 26% of born mice died pre-weaning and before reaching the age of three weeks.

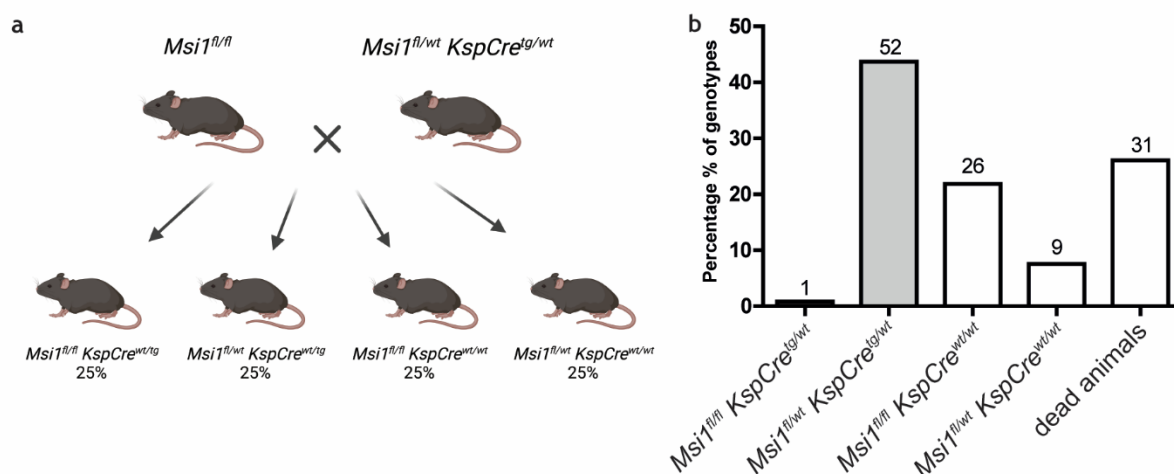


Figure 10. Conditional *Msi1* knockout mice are not viable

a| Mating scheme for the generation of mice with conditional *Msi1* knockout in tubular epithelial cells of the distal tubules, loop of Henle and collecting duct. Homozygous *Msi1* floxed mice²¹⁶ (*Msi1*^{fl/fl} KspCre^{wt/wt}) were crossed to KspCre²⁴³ transgenic, heterozygous *Msi1* floxed animals (*Msi1*^{fl/wt} KspCre^{tg/wt}). The probability for conditional knockout (*Msi1*^{fl/fl} KspCre^{tg/wt}) and heterozygous control (*Msi1*^{fl/wt} KspCre^{tg/wt}) animals was 25% for each genotype. Created with BioRender.com (OA253JEFOU)

b| Mice with conditional *Msi1* knockout in the kidney are not viable. While only one *Msi1* knockout animal was born across all litters, heterozygous control animals were overrepresented (44%). 26% of all mice died pre-weaning and before being reaching the age of three weeks.

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Depicted are Mendelian ratios for all animals generated by crossing mice according to the mating scheme displayed in (a). Shown is the percentage of each of the four possible genotypes and ungenotyped dead animals (less than three weeks of age) across all obtained litters. Exact animal numbers are indicated above the bars and genotypes of experimental groups (knockout vs. heterozygous control) are colored in black and grey.

Unpublished data from the laboratory which generated the *KspCre* transgenic mouse model²⁴³ suggested that the *KspCre* transgene was inserted within chromosome 5. As the *Msi1* gene is also located on chromosome 5, co-inheritance may explain the strong under-representation of conditional *Msi1* knockout animals. To avoid this co-inheritance, *KspCre* mice were replaced with tamoxifen-inducible *KspCreERT2* transgenic animals (Figure 11 a)²⁴⁴. Homozygous *Msi1* floxed animals were underrepresented with regards to the expected 25% (16% *Msi1^{fl/fl}* *KspCreERT2^{tg/wt}*, 12% *Msi1^{fl/fl}* *KspCreERT2^{wt/wt}*) (Figure 10 b). Unexpectedly, MSI1 was found to be expressed in distal tubules, loop of Henle and collecting duct of female (Figure 11 c) and male (Figure 11 d) *Msi1* knockout animals (*Msi1^{fl/fl}* *KspCreERT2^{tg/wt}*), similar to its expression in *KspCre* (*KspCreERT2^{tg/wt}*) and heterozygous control animals (*Msi1^{fl/wt}* *KspCreERT2^{tg/wt}*). Generating conditional *Msi1* knockout animals using the *KspCreERT2* mouse line was therefore not successful.

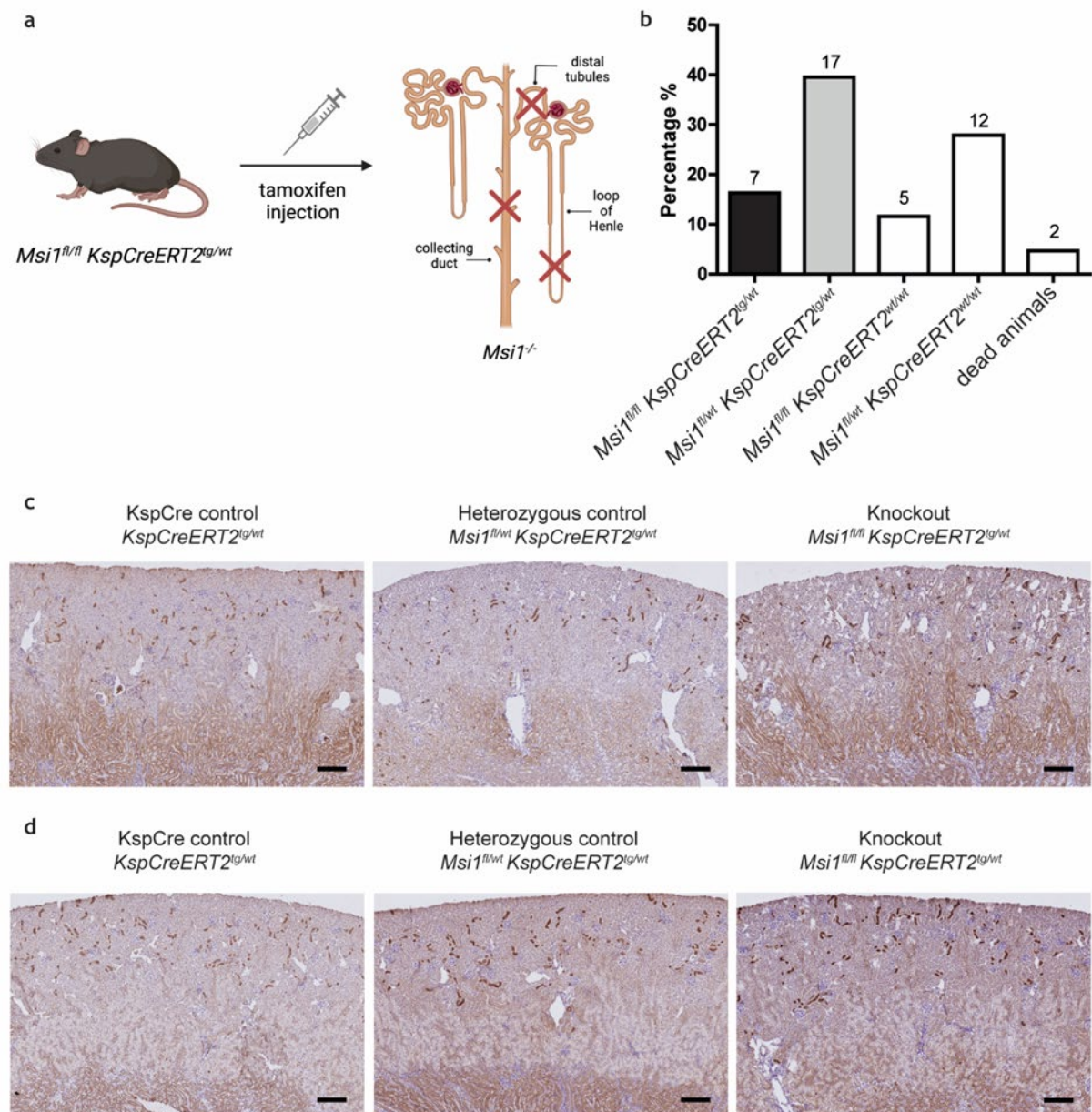


Figure 11. Inducible conditional *Msi1* knockout mice show MSI1 signal in distal tubules, loop of Henle and collecting duct

a| Tamoxifen administration induces KspCre recombinase and should lead to knockout of *Msi1* in distal tubules, loop of Henle and collecting duct. Schematic representation illustrates the tamoxifen-induced activation of KspCre recombinase which should result in knockout of *Msi1* in distal tubules, loop of Henle and collecting duct of the kidney. To induce a conditional knockout in the *Msi1*^{fl}/_{fl};KspCreERT2 mouse model, eight week old mice were intraperitoneally injected five times with tamoxifen over a period of ten days. Tamoxifen-induced activation of KspCre recombinase should result in knockout of *Msi1* in distal tubules, loop of Henle and collecting duct of the kidney. Created with BioRender.com (IV253JE8CZ) **b| Mice with conditional *Msi1* knockout in the kidney are underrepresented when compared to expected Mendelian ratios.** Homozygous *Msi1* floxed mice (*Msi1^{fl/fl} KspCreERT2^{tg/wt}* and *Msi1^{fl/fl} KspCreERT2^{wt/wt}*) were underrepresented when compared to expected Mendelian ratios. Based on the mating scheme provided in Figure 10 a, the theoretical probability for each of the genotypes was 25%. Depicted is the percentage of each of the four possible genotypes and ungenotyped dead animals (less than three weeks of age) across all obtained litters, when mating animals according to the scheme represented in Figure 10 a .

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Exact animal numbers are indicated above the bars and genotypes of experimental groups (knockout vs. heterozygous control) are colored in black and grey. **c| Male *Msi1* knockout animals express MSI1 in distal tubules, loop of Henle and collecting duct.** Immunohistochemistry staining demonstrated MSI1 expression in the distal tubules, loop of Henle and collecting duct of all experimental groups, including knockout animals. Depicted are kidneys from KspCre control animals (*KspCreERT2^{tg/wt}*), heterozygous control animals (*Msi1^{fl/wt} KspCreERT2^{tg/wt}*) and knockout mice. **d| Female *Msi1* knockout animals express MSI1 in distal tubules, loop of Henle and collecting duct.** Similar to male animals, MSI1 expression was detected in the distal tubules, loop of Henle and the collecting duct of female KspCre control, heterozygous control and knockout animals via immunohistochemistry staining. Depicted are the same experimental groups as described in (d). Brightfield images were acquired with a Hamamatsu S360 slidescanner using a 20x objective. Scale bar: 200 μ m.

Observation of litters obtained from crossing homozygous *Msi1* floxed mice (without *Cre* transgenes) revealed that 42% of all born animals died pre-weaning and before reaching the age of three weeks (Figure 12 a). In comparison, 0% of all mice generated from matings between homozygous *Msi2* floxed mice, died before the age of three weeks (Figure 12 b).

Floxed *Msi1* mice were purchased from the University of Pennsylvania¹⁹¹. For generation of the floxed *Msi1* allele, a *Msi1-3lox* allele was created in embryonic stem cells using homologous recombination. The *Msi1-3lox* allele consists of a loxP site upstream of the transcriptional start site and a PGK-Neo (phosphoglycerate kinase I (PGK) promoter-driven neomycin phosphotransferase gene) resistance cassette flanked by two loxP sites between exon 3 and 4 (Figure 12 c). *Msi1-3lox* positive clones were electroporated with *Cre* recombinase to generate the *Msi1* *fl* allele and injected into blastocysts to start a colony of mice with floxed *Msi1* allele. Two sets of primer flanking the PGK promoter (PCR#1) and the neomycin resistance gene (PCR#2) were designed and PCR was performed on genomic DNA retrieved from ear biopsies from *Msi1* knockout (KO, *Msi1^{fl/fl} KspCreERT2^{tg/wt}*), heterozygous control (Het, *Msi1^{fl/wt} KspCreERT2^{tg/wt}*) and KspCre (*Cre, KspCreERT2^{tg/wt}*) animals (Figure 12 d). To our surprise it was possible to amplify both parts of the PGK-Neo resistance cassette from genomic DNA of animals with floxed *Msi1* alleles (KO, Het), but not from mice solely expressing the *KspCreERT2* transgene (*Cre*). Sanger sequencing of the PCR products confirmed the PGK-Neo resistance cassette sequence (data not shown). These results demonstrate the existence of the PGK-Neo resistance cassette within the *Msi1* *fl* allele and indicate that the genomic composition of the *Msi1* *fl* allele rather resembles the structure of the *Msi1-3lox* allele. Crossing mice carrying this defective *Msi1* *fl* allele with *Cre*-transgenic animals will only lead to excision of the PGK-Neo resistance cassette without disrupting *Msi1* gene expression. These findings explain the missing *Msi1* knockout in *Msi1^{fl/fl} KspCreERT2^{tg/wt}* animals (Figure 11 c+d).

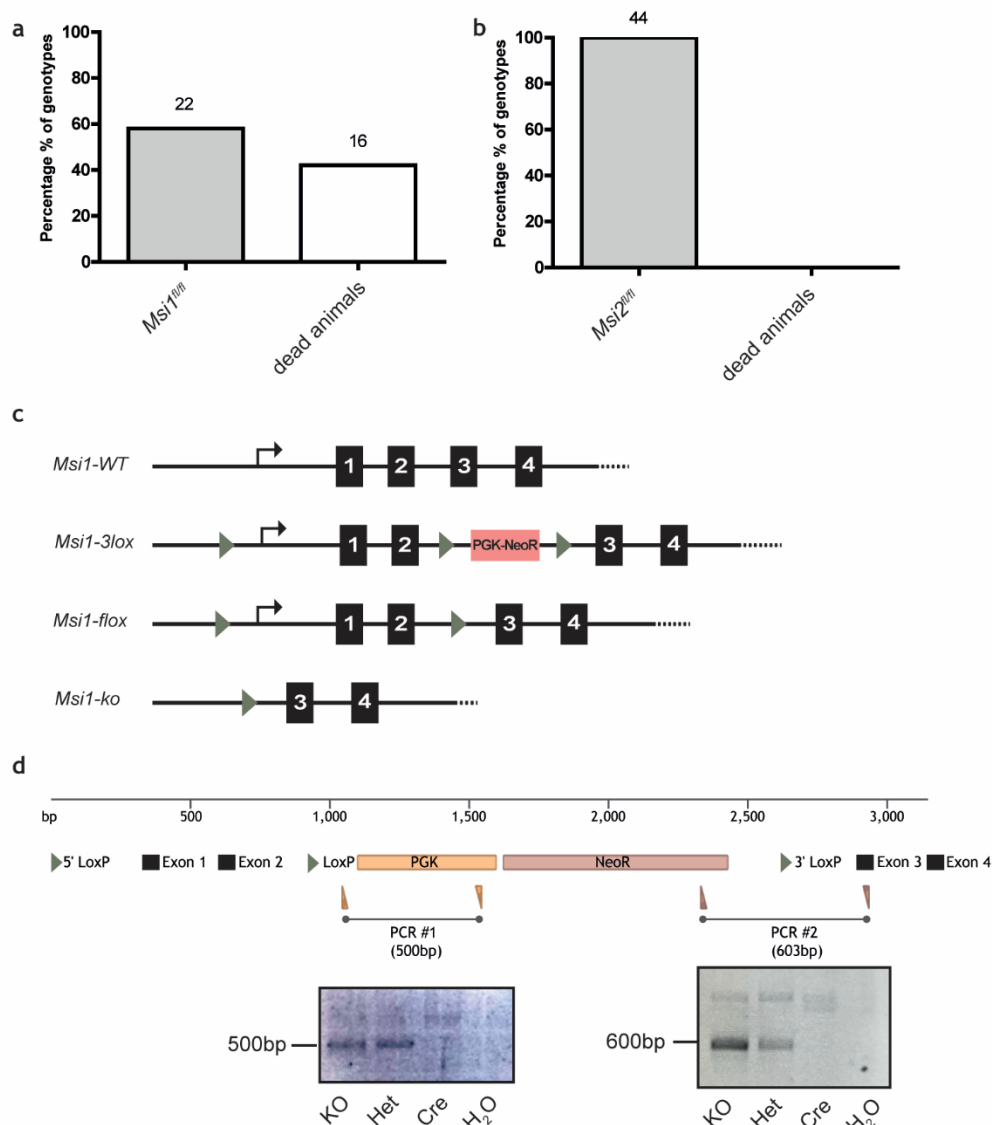


Figure 12. *Msi1* flox allele is defective

a| Over 40% of homozygous *Msi1* floxed mice die within three weeks of age. Observation of litters obtained from matings between homozygous *Msi1* floxed mice revealed that 42% of homozygous *Msi1* floxed (*Msi1^{fl/fl}*) animals died before reaching the age of three weeks. Shown is the distribution of homozygous *Msi1* floxed and dead animals (ungenotyped animals with an age less than three weeks, which died pre-weaning) across all litters generated from matings between homozygous *Msi1* floxed animals (without Cre transgenes). Exact animal numbers are indicated above the bars and homozygous *Msi1* floxed mice are colored in grey. **b| 100% of homozygous *Msi2* floxed animals survive.** In contrast to pre-weaning lethality observed in homozygous *Msi1* floxed mice, 100% of homozygous *Msi2* floxed animals survived. Depicted is the percentage of homozygous *Msi2* floxed animals across all litters generated from matings between homozygous *Msi2* floxed animals (without Cre transgenes). Exact animal numbers are indicated above the bars and homozygous *Msi2* floxed mice are colored in grey. **c| *Msi1* floxed alleles were generated via positive selection using incorporation of a PGK-neomycin resistance cassette in embryonic stem cells.** Mice with floxed Musashi alleles were generated by Christopher Lengner and purchased from the University of Pennsylvania²¹⁶. To develop the floxed *Msi1* allele, a *Msi1*-3lox allele, harboring three loxP sites (grey) and a PGK-neomycin resistance cassette (red), was generated in embryonic stem cells using homologous recombination. The 3lox clones were electroporated with Cre recombinase and the resulting *Msi1*-flox clones were injected into blastocysts to set up a colony of mice with floxed *Msi1* allele. Crossing homozygous *Msi1* floxed mice with Cre-transgenic animals should result in generation of mice harboring the conditional *Msi1*-ko allele.

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d| Successful PCR amplification of PGK-neomycin cassette from genomic DNA of *Msi1* floxed mice. PCR amplification of parts of the PGK and neomycin resistance cassette was only successful with genomic DNA from mice transgenic for the *Msi1*^{fl} allele, and not from animals exclusively expressing Cre-recombinase, indicating that the *Msi1* flox allele contains PGK-Neomycin resistance cassette, used for generation of the floxed *Msi1* allele. Indicated is the genomic localization of three loxP sites (grey), exons 1-4 (black) and the PGK (orange) Neomycin resistance (red) cassette. Two distinct sets of primers (indicated as triangles) were used for the amplification of PCR products flanking the PGK (PCR#1) and Neomycin resistance (PCR#2) cassette. Images at the bottom depict the corresponding PCR products using genomic DNA from KO (*Msi1*^{fl/fl}; *KspCreERT2*^{tg/wt}), het (*Msi1*^{fl/wt}; *KspCreERT2*^{tg/wt}) and Cre (*Msi1*^{wt/wt}; *KspCreERT2*^{tg/wt}) animals as input, as well as a H₂O negative control.

3.2.2 Post-transcriptional downregulation of *Msi1* expression *in vivo*

In order to study the role of MSI1 in AKI without utilizing a genetic knockout model, we aimed to reduce *Msi1* expression *in vivo* through administration of antisense oligonucleotides (ASOs). An ASO, targeting exon 15 of *Msi1* mRNA, was previously reported to specifically and efficiently downregulate *Msi1* expression *in vivo*²¹⁷. Wild type mice were injected four times with this *Msi1* ASO or PBS over a period of 11 days and sacrificed 24 hours after the last injection. *Msi1* expression was markedly reduced in testis, brain and significantly decreased in liver of ASO-treated animals compared to PBS-injected mice (Figure 13). *Msi1* expression in kidneys remained unchanged after ASO administration compared to injection with PBS. Of note, *Msi2* expression was slightly increased in testis and significantly reduced in liver, brain and kidneys after *Msi1* ASO administration, either indicating unspecific ASO binding or co-regulation of *Msi1* and *Msi2* expression. As application of ASOs failed to reduce *Msi1* expression in mouse kidneys, the role of *Msi1* in AKI could not be further studied *in vivo* within this work.

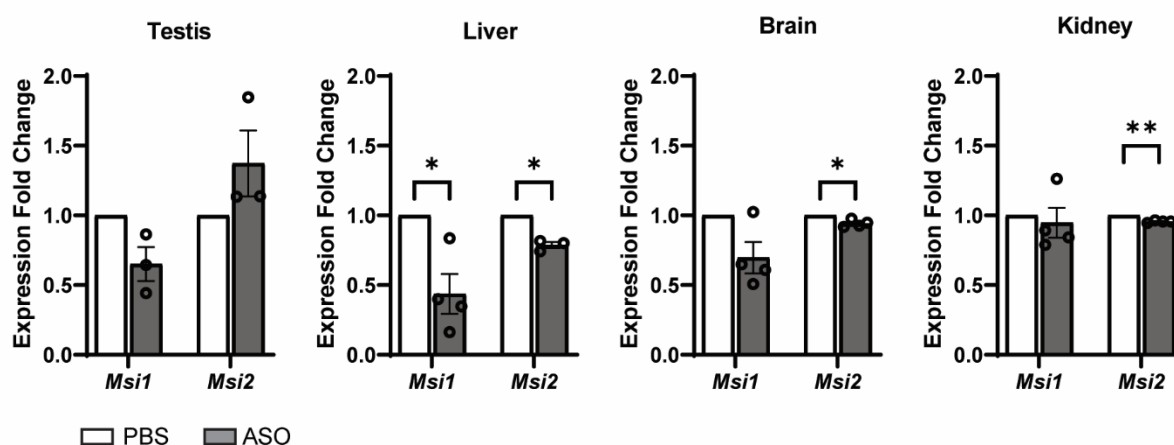


Figure 13. Downregulation of *Msi1* mRNA expression *in vivo* using antisense oligonucleotides

While *Msi1* expression was markedly decreased in testis, brain and liver of ASO-treated mice, *Msi1* mRNA levels within kidneys from ASO-administered animals remained almost unchanged.

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Except for an increased expression in the testis, *Msi2* mRNA levels were slightly reduced in liver, brain and kidney upon ASO treatment, indicating either unspecific ASO binding or co-regulation of *Msi1* and *Msi2* expression. qPCR data show *Msi1/Msi2* expression in organs from wild type mice injected with PBS or *Msi1* ASO. Mice were intravenously injected four times with either PBS or 12.5 mg/kgBW *Msi1* ASO over a period of 11 days. *Msi1/Msi2* expression after ASO administration within the indicated organs is depicted as expression fold change in comparison to PBS injected animals. Data is presented as mean $\pm$ SEM (n=3 - n=4, students t-test, * $p \leq 0.05$, ** $p \leq 0.01$).

3.3 Conditional *Msi2* knockout mouse model

3.3.1 Knockout validation and basic phenotyping

As previously mentioned MSI1 and MSI2 are homologous proteins and could therefore functionally compensate for each other upon the loss of the respective homologue. Although the conventional whole body knockout of *Msi2* in mice was shown to be viable²⁰², a conditional knockout mouse model was generated in order to be able to compare the single knockouts of *Msi1* and *Msi2* with each other and generate a conditional double knockout of both proteins in specific tubular segments of the kidney.

Homozygous *Msi2* *flox* animals were crossed with heterozygous *Msi2* *flox* and *KspCre* transgenic animals as described in Figure 10 a. Animals were born according to Mendelian ratios, although *Msi2* knockout mice (*Msi2*^{fl/fl}*KspCre*^{tg/wt}) were slightly underrepresented (21%) in relation to 25% genotype probability, which were expected (Figure 14 a). In male and female *Msi2* knockout mice, MSI2 protein was not expressed in the distal tubules, loop of Henle and collecting duct, as compared to heterozygous control animals (*Msi2*^{fl/fl}*KspCre*^{tg/wt}), where MSI2 was detected across the entire tubular system (Figure 14 b). Kidney morphology was evaluated through an experienced nephropathologist using PAS staining. Kidneys from male and female *Msi2* knockout animals did not show any morphological abnormalities compared to heterozygous control mice (Figure 14 c). While female *Msi2* knockout mice were slightly lighter than heterozygous control animals, no weight difference was observed for male mice within the first 12 weeks of age (Figure 14 d). Kidney function, as determined through serum creatinine and urea concentrations, did not differ in female and male *Msi2* knockout mice relative to heterozygous controls at the age of 12 weeks (Figure 14 e). In summary, conditional knockout of *Msi2* in the distal tubules, loop of Henle and collecting duct of male and female mice was successful and did not cause significant changes in body weight, kidney morphology and function at the age of 12 weeks.

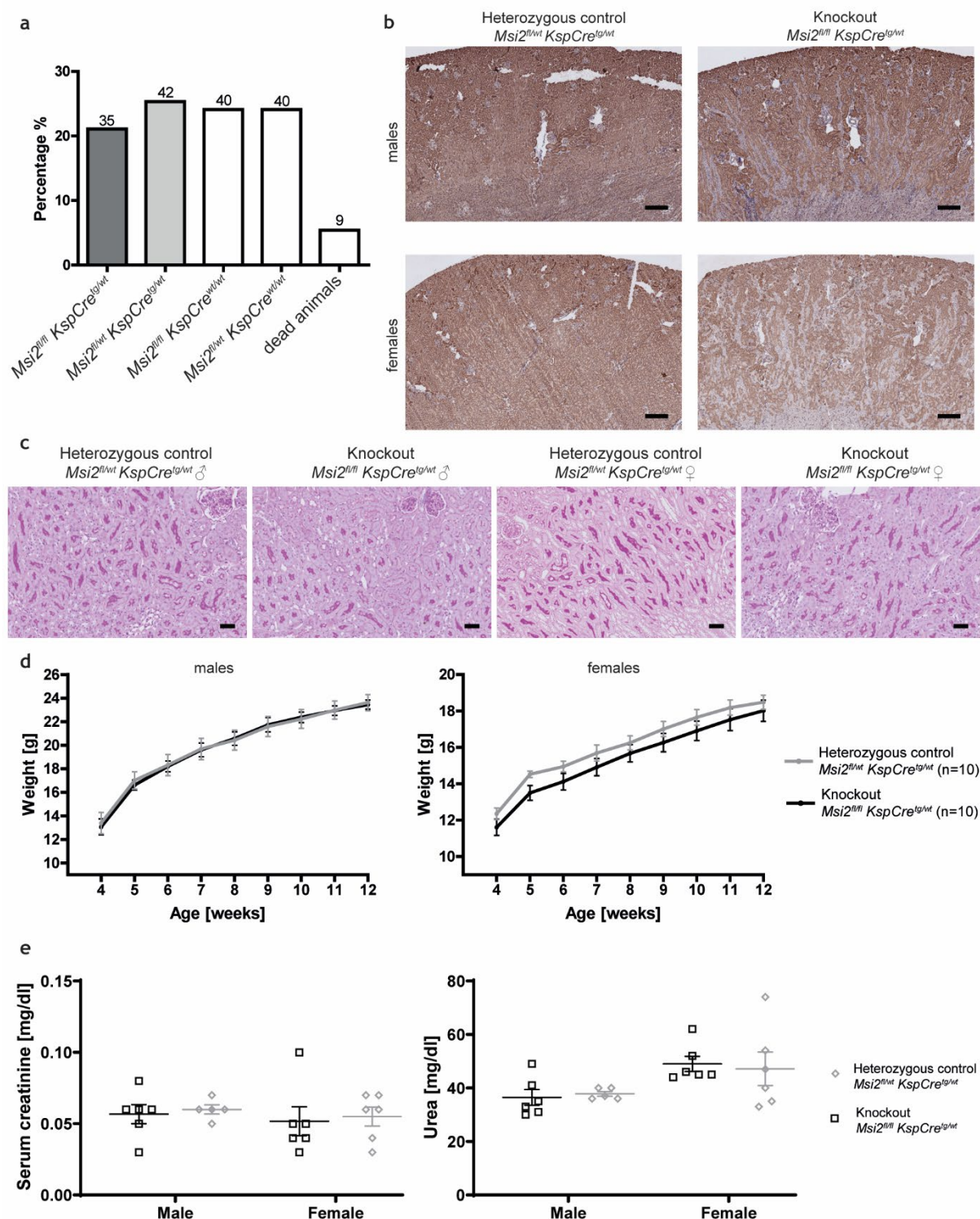


Figure 14. Conditional *Msi2* knockout mice show normal kidney function and morphology at the age of 12 weeks

a| Conditional *Msi2* knockout mice are slightly underrepresented compared to expected mendelian ratios. All animals were born according to Mendelian ratios, although with 21%, $Msi2^{fl/fl} KspCre^{tg/wt}$ animals were slightly underrepresented. Mice were bred according to the mating scheme provided in Figure 10 a. Depicted is the percentage of each of the four possible genotypes and ungenotyped dead animals (less than three weeks of age) across all obtained litters.

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Exact animal numbers are indicated above the bars and genotypes of experimental groups (knockout vs. heterozygous control) are colored in black and grey. The theoretical probability for each of the genotypes was 25%. **b| MSI2 is not expressed in distal tubules, loop of Henle and collecting duct of conditional *Msi2* knockout mice.** Immunohistochemistry staining showed that MSI2 expression was missing in the distal tubules, loop of Henle and collecting duct of knockout animals. Depicted are kidney slices from male and female heterozygous control animals (*Msi2^{fl/wt} KspCre^{tg/wt}*) and conditional knockout mice (*Msi2^{fl/fl} KspCre^{tg/wt}*) at the age of 12 weeks. **c| Conditional *Msi2* knockout mice show unaltered kidney morphology at the age of 12 weeks.** Kidney morphology was evaluated by an experienced nephropathologist who did not determine any structural alterations between the groups. Shown are PAS stainings on kidneys from male and female heterozygous control animals (*Msi2^{fl/wt} KspCre^{tg/wt}*) and conditional knockout mice (*Msi2^{fl/fl} KspCre^{tg/wt}*) at the age of 12 weeks. **d| Female conditional *Msi2* knockout animals are slightly lighter than heterozygous littermates.** While female knockout animals (black) were slightly lighter than heterozygous control mice (gray), no weight difference was detected for male animals. Animal caretakers of CECAD in vivo research facility monitored the weight on a weekly basis. **e| Conditional *Msi2* knockout mice show unaltered kidney function at the age of 12 weeks.** Male and female knockout animals (black squares) did not show differences kidney function compared to heterozygous control mice (grey diamonds). Kidney function of 12 week old animals was assessed using serum creatinine and urea measurements. Data are presented as mean $\pm$ SEM. Brightfield images were acquired with a Hamamatsu S360 slidescanner using a 20x objective. Scale bar represents 200 μ m in (b) and 50 μ m in (c).

In order to investigate whether the phenotype of *Msi2* knockout mice changes upon aging, animals were observed for more than one year and the final evaluation is ongoing. Similar to the age of 12 weeks, no morphological changes were determined in kidneys of male and female *Msi2* knockout mice compared to heterozygous control animals at the age of 52 weeks (Figure 15 a). In addition, no weight difference between male and female *Msi2* knockout and heterozygous control animals was observed within the first year of life (Figure 15 b). Finally, male and female *Msi2* knockout and heterozygous control mice depicted baseline serum creatinine and urea concentrations at the age of 52 weeks, indicating normal kidney function (Figure 15 c). In conclusion, *Msi2* knockout in the distal tubules, loop of Henle and collecting duct did not alter body weight, kidney morphology and function of male and female mice at the age of 52 weeks.

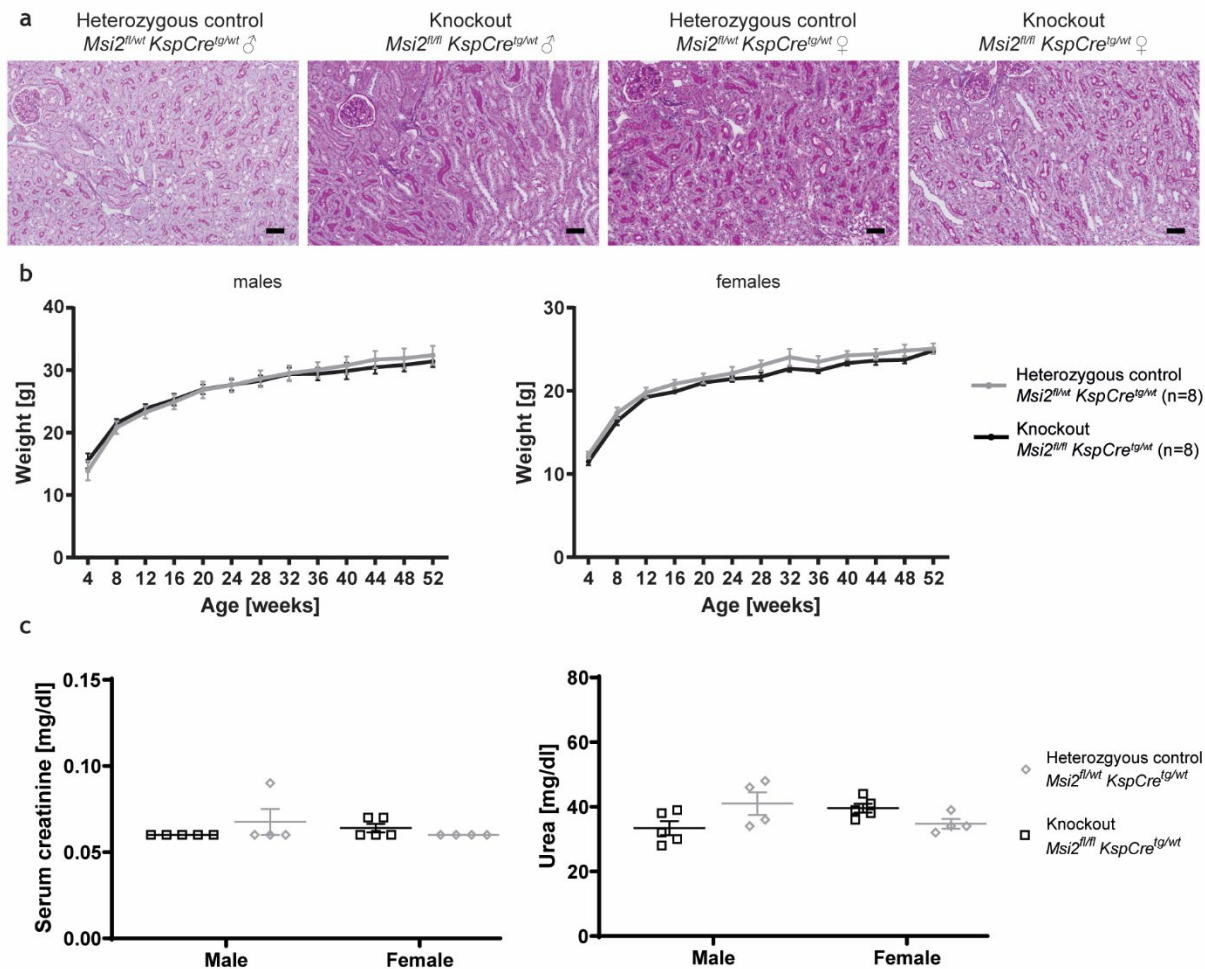


Figure 15. Conditional *Msi2* knockout mice show normal kidney function and morphology at the age of 52 weeks

a| Conditional *Msi2* knockout animals show unaltered kidney morphology upon aging. Kidney morphology was evaluated by an experienced nephropathologist who did not determine any structural alterations between the groups. Shown are PAS stainings on kidneys from male and female heterozygous control animals (*Msi2^{fl/wt} KspCre^{tg/wt}*) and conditional knockout mice (*Msi2^{fl/fl} KspCre^{tg/wt}*) at the age of 52 weeks. Brightfield images were acquired with a Hamamatsu S360 slidescanner using a 20x objective. Scale bar: 50 μ m **b| Conditional knockout of *Msi2* does not affect bodyweight of mice upon aging.** No weight differences in male and female knockout animals (black) were detected compared to heterozygous control mice (gray). Animal caretakers of CECAD in vivo research facility monitored weight on a weekly basis until the age of 52 weeks. Data are presented as mean $\pm$ SEM. **c| Kidney function remains unaffected by conditional *Msi2* knockout in mice upon aging.** Male and female knockout animals (black squares) did not show a difference in kidney function compared to heterozygous control mice (gray diamonds). Kidney function at 52 weeks was assessed by determining the serum creatinine and urea values. Data is presented as mean $\pm$ SEM.

3.3.2 Evaluation of conditional *Msi2* knockout animals after IRI

To analyze the role of MSI2 in AKI, conditional *Msi2* knockout mice were subjected to IRI. Renal IRI as an experimental model of AKI was previously published²⁴⁶. Briefly, during surgery the right kidney was removed and functions as an undamaged control kidney. After right nephrectomy, the left renal vein and artery were clamped for 40 minutes, inducing ischemia.

Removal of the clamp led to reperfusion injury of the left kidney. Mice were sacrificed 24 hours post-surgery.

Kidney function of *Msi2* knockout mice after IRI was compared to heterozygous control mice (*Msi2^{fl/wt}KspCre^{tg/wt}*), as well as to Cre control (*KspCre^{tg/wt}*) and wild type animals. In addition, in one group of *Msi2* knockout animals the right kidney was removed without inducing IRI in the left kidney (sham). Serum creatinine and urea concentrations were significantly increased in all experimental groups after IRI compared to sham animals (Figure 16 a). Moreover, serum creatinine concentrations in all *KspCre* transgenic animals (knockout, heterozygous control and Cre control) were significantly elevated compared to wild type mice after IRI. These findings indicate a mild but significant contribution of *KspCre* expression to decreased kidney function after IRI in mice. Kidney damage was evaluated based on PAS stainings on left kidneys (Figure 16 b). The degree of tissue damage was assessed through the following parameters: loss of brush border, vacuolization, epithelial flattening, loss of nuclei and necrosis, which altogether define the total damage score of the kidneys (Figure 16 c). While no difference between the groups was found for loss of brush border, loss of nuclei and vacuolization, epithelial flattening was significantly increased after IRI in all experimental groups compared to sham animals. Similarly, the total damage score was significantly elevated in all animals after IRI compared to sham controls. Interestingly, necrosis was significantly increased in all experimental groups, except for Cre control animals after IRI in comparison to sham mice. *Msi2* knockout and heterozygous control mice showed elevated levels of necrosis compared to Cre control and wild type animals, pointing towards a connection of *MSI2* expression in distal tubules, loop of Henle and collecting duct in IRI-induced necrosis.

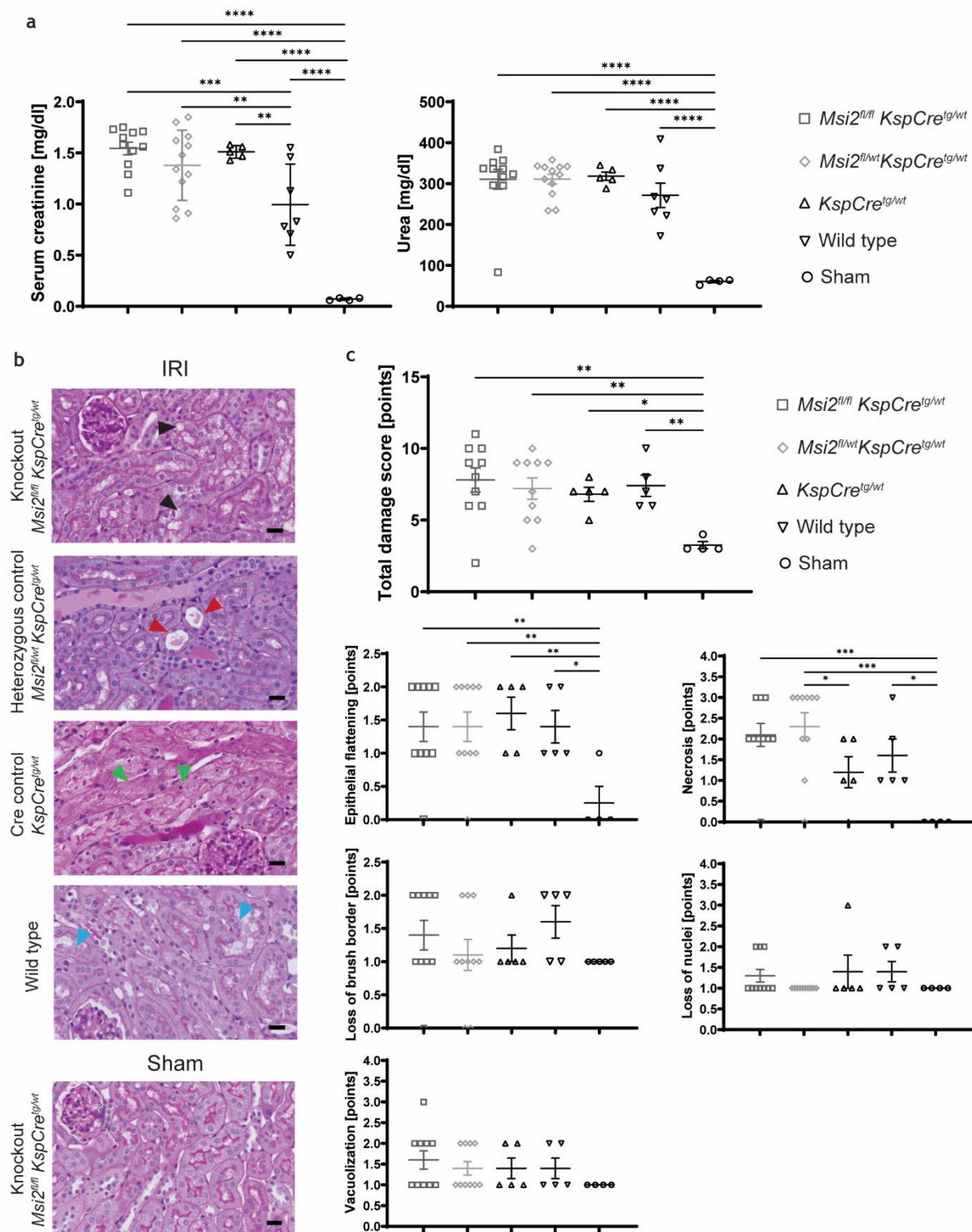


Figure 16. Conditional knockout of *Msi2* has no general effects on kidney dysfunction and morphology after IRI

a| Conditional loss of *Msi2* does not contribute to kidney dysfunction after IRI. Serum creatinine and urea concentrations were significantly increased in all experimental groups after IRI injury compared to sham animals (one-way ANOVA with Fisher's least significant difference (LSD), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). In addition, *KspCre* transgenic mice depicted significantly elevated serum creatinine concentrations compared to wild type animals after IRI, indicating that *KspCre* expression, but not loss of *Msi2*, contributes to kidney dysfunction after IRI.

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Depicted are serum creatinine and urea concentrations of conditional knockout (*Msi2^{fl/fl} KspCre^{tg/wt}*) and heterozygous control (*Msi2^{fl/wt} KspCre^{tg/wt}*) animals, as well as Cre-transgenic (*KspCre^{tg/wt}*) and wild type mice after IRI. A second group of knockout animals served as sham controls, in which the right kidney was removed without IRI. **b| Conditional *Msi2* knockout mice show similar kidney damage compared to experimental control groups after IRI.** Conditional loss of *Msi2* does not alter kidney damage after IRI, as tubular damage patterns were present to similar extent in all experimental groups after IRI. Displayed are conditional knockout (*Msi2^{fl/fl} KspCre^{tg/wt}*), heterozygous control (*Msi2^{fl/wt} KspCre^{tg/wt}*) animals, as well as Cre-transgenic (*KspCre^{tg/wt}*) and wild type mice after IRI. In sham control animals (*Msi2^{fl/fl} KspCre^{tg/wt}*) the right kidney was removed without IRI. Indicated with triangles are several parameters to assess kidney damage: vacuolization (black), loss of nuclei and epithelial flattening (red), necrosis (green) and loss of brush border (blue). Importantly, alterations in these parameters were observed throughout all experimental groups, but due to visualization purposes, only a distinct set of parameters is shown per kidney. Brightfield images were acquired with a Hamamatsu S360 slidescanner using a 20x objective. Scale bar: 20 μ m. **c| Conditional loss of *Msi2* does not contribute to general kidney damage after IRI.** Independent of the loss of *MSI2*, IRI significantly increased the total damage score in all experimental groups compared to right nephrectomy in sham animals (one-way ANOVA with Fisher's LSD, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Within the single parameters, IRI significantly increased epithelial flattening in all experimental groups compared to sham animals with right nephrectomy. Necrosis was significantly elevated in knockout mice, heterozygous control and wild type animals after IRI compared to sham control mice. In addition, necrosis was elevated in *Msi2* conditional knockout and heterozygous control mice compared to animals with endogenous *MSI2* expression after IRI. No significant alterations were detected for loss of brush border and nuclei and vacuolization. Kidney damage was evaluated using PAS stainings displayed in (b). Evaluation was done by a nephropathologist in a blinded manner according to degree of tubular damage score (mild=1, moderate=2, severe=3). Total damage score represents the sum of all injury parameters (loss of brush border, loss of nuclei, necrosis, vacuolization and epithelial flattening). Data are presented as mean $\pm$ SEM.

In undamaged control kidneys, *MSI1* expression was unaltered between *Msi2* knockout and heterozygous control mice and overlapped in many tubular epithelial cells with expression of Tamm Horsfall Glycoprotein (THP), a marker for the thick ascending limb of the loop of Henle (Figure 17 a). After IRI, *MSI1* was highly expressed at the cortico-medullary border in *Msi2* knockout and heterozygous control mice (Figure 17 b). As expected, in undamaged control kidneys, *MSI2* expression was missing in distal tubules, loop of Henle and collecting duct of *Msi2* knockout animals, while it was uniformly expressed across the nephron in heterozygous control animals (Figure 17 a). Upon IRI, *MSI2* remained absent from distal tubules, loop of Henle and collecting duct in conditional *Msi2* knockout mice (Figure 17 b). Interestingly, in heterozygous control animals, *MSI2* was also absent from tubular epithelial cells at the cortico-medullary border with high *MSI1* expression upon IRI (Figure 17 b). Importantly, *MSI2* was expressed in tubular epithelial cells within this kidney area in undamaged control kidneys (Figure 17 a). Together these expression analyses (a) confirm the loss of *MSI2* in distal tubules, loop of Henle and collecting duct in conditional *Msi2* knockout animals and (b) indicate that, independently from *MSI2*, *MSI1* is upregulated in tubular segments around the cortico-medullary border upon IRI.

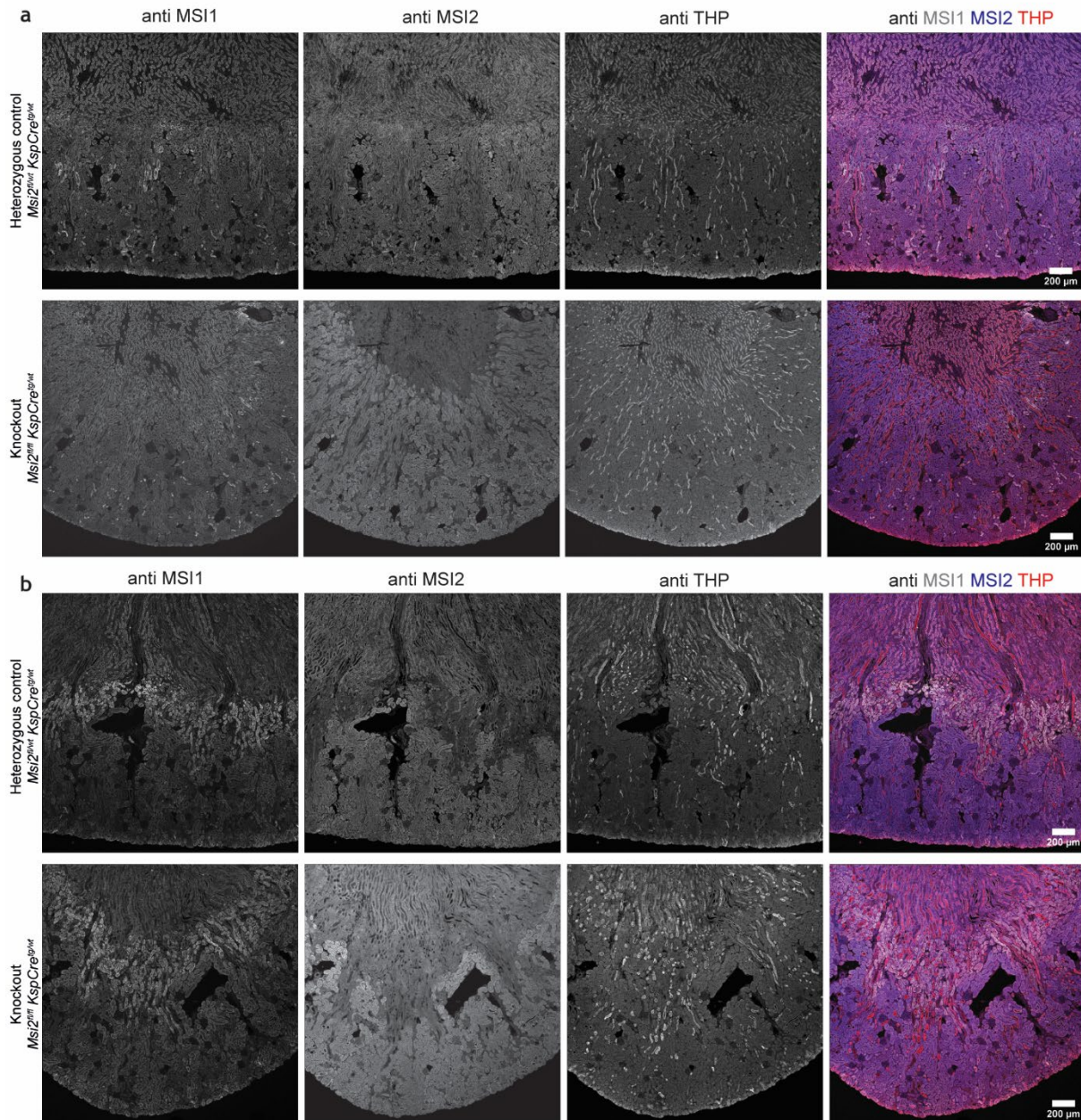


Figure 17. Independent of MSI2 expression, MSI1 is highly expressed at the cortico-medullary border upon IRI

a| Loss of MSI2 in distal tubules, loop of Henle and collecting duct does not affect expression of MSI1 in conditional *Msi2* knockout animals. While MSI2 remained expressed in the loop of Henle of heterozygous control mice, it was absent in conditional knockout animals. MSI1 expression across the nephron segments did not change in undamaged kidneys from conditional *Msi2* knockout animals. Immunofluorescence stainings were performed using anti MSI1, anti MSI2 and anti Tamm-Horsfall Glycoprotein (THP) on paraffin-embedded undamaged kidneys from conditional knockout mice (*Msi2^{fl/fl} KspCre^{tg/wt}*) and heterozygous control animals (*Msi2^{fl/wt} KspCre^{tg/wt}*). Undamaged kidneys are right kidneys, which were removed during surgery before inducing IRI in left kidneys. **b| Independent of MSI2 expression, high levels of MSI1 are detected at the cortico-medullary border after IRI.** While MSI2 was absent in the loop of Henle of knockout mice, MSI2 expression seemed to negatively correlate with MSI1 expression in both experimental groups. Areas with high MSI1 expression upon IRI showed decreased MSI2 signal, indicating a role of MSI1 upregulation in these tubules independent of MSI2. MSI1 was highly expressed at the cortico-medullary border in both, heterozygous control and *Msi2* conditional knockout animals, after IRI. Images were acquired with a TCS SP8 confocal microscope, using a 10x objective in (a) and a 20x objective (b). Scale bar presents 200 μm.

As described before (Figure 9), MSI1 expression was high at the cortico-medullary border upon IRI in kidney samples from wild type animals, obtained within studies of previous group members. These findings were validated in wild type animals subjected to IRI within this thesis (Figure 18 a). Upregulation of MSI1 in tubular epithelial cells at the cortico-medullary border was confirmed to be independent of MSI2 expression as it was observed in *Msi2* knockout mice, heterozygous control and wild type animals upon IRI (Figure 18 a). Higher magnification of the cortico-medullary border after IRI revealed that expression of MSI1 was especially high in necrotic tubular epithelial cells within this area (Figure 18 a'). Increased MSI1 protein levels in these necrotic cells negatively correlated with MSI2 signal independent of genetic *Msi2* expression status. Immunohistochemistry staining of undamaged kidneys and kidneys after IRI emphasized the IRI-dependent upregulation of MSI1 expression along the entire cortico-medullary border of the kidney, which was present in all experimental groups and is therefore independent of MSI2 expression (Figure 18 b). These results suggest a role of MSI1 in necrosis of tubular epithelial cells at the cortico-medullary border after IRI. Although the high expression of MSI1 in necrotic cells was induced independently of genetic *Msi2* expression, this finding was accompanied by markedly reduced MSI2 protein levels in injured tubular epithelial cells, even when MSI2 was endogenously expressed (wild type and heterozygous control animals). Only the conditional knockout of *Msi1* in the kidney can therefore reveal whether MSI1 upregulation at the cortico-medullary border is cause or consequence of tubular epithelial necrosis upon IRI. Obtaining such data will – considering the results described in Figure 12– depend on crossing an entirely new floxed *Msi1* allele with *KspCre* transgenic animals in both wild type and conditional *Msi2* knockout mice. While this experiment will be crucial in the future, it is beyond the scope of the thesis at hand, which was continued focusing on the molecular biology of Musashi proteins in cultured kidney epithelial cells.

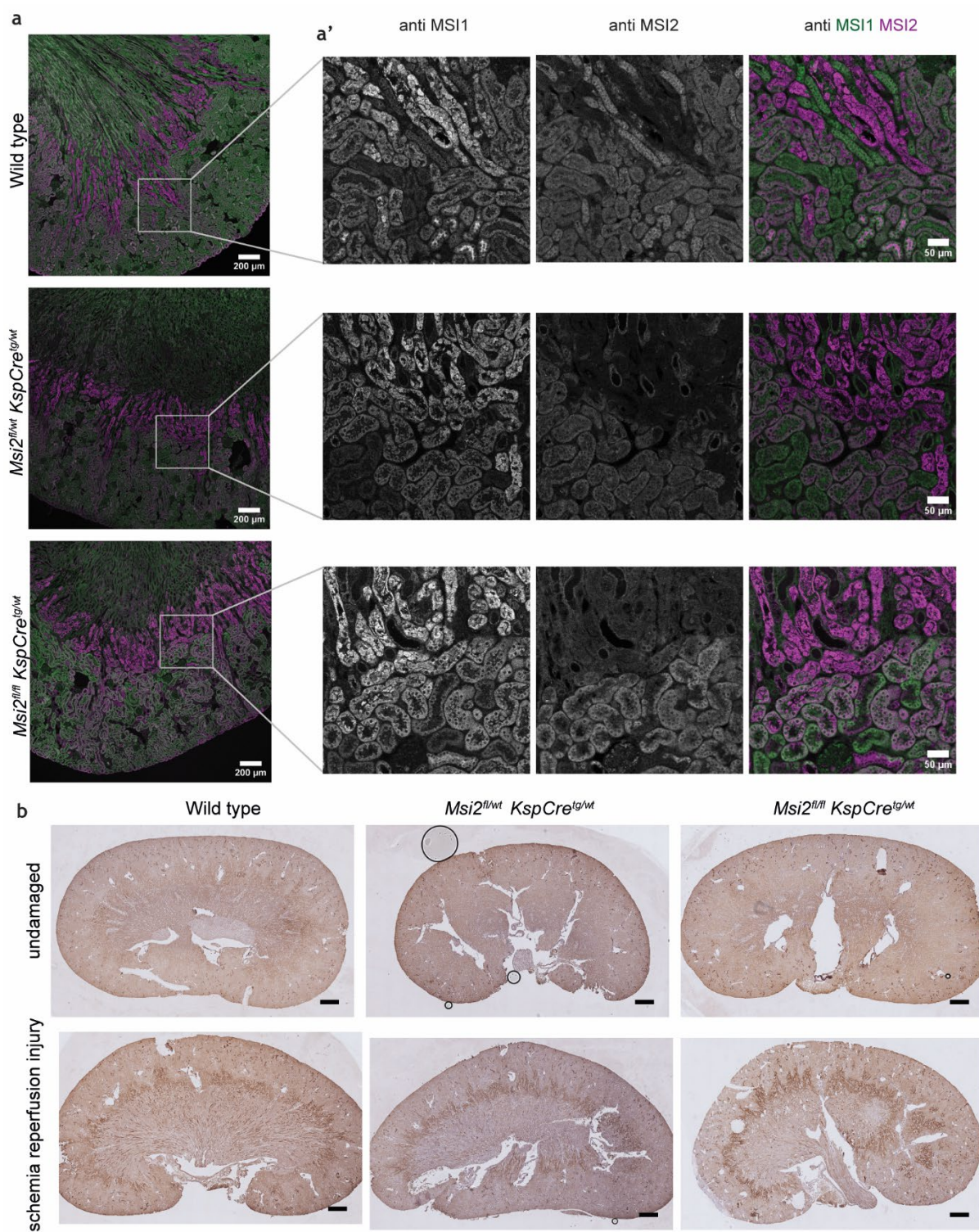


Figure 18. MSI1 is highly expressed in necrotic tubules upon IRI independent of MSI2 expression

a| IRI causes high expression of MSI1 in necrotic tubular epithelial cells. MSI1 was found to be highly expressed in necrotic tubular cells of wild type mice, as well as in *Msi2* heterozygous control and knockout animals. Independent of the genetic background, MSI2 was absent from necrotic tubules with high MSI1 expression. Paraffin-embedded kidneys from wild type, *Msi2* heterozygous control (*Msi2^{fl/wt} KspCre^{tg/wt}*) and conditional knockout animals (*Msi2^{fl/fl} KspCre^{tg/wt}*) after IRI were stained using anti MSI1/anti MSI2 antibodies.

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Images were acquired with a TCS SP8 confocal microscope, using a 10x objective. Shown in (a') is a zoom in perspective generated via ImageJ. Scale bar presents 200 μm in (a) and 50 μm in (a'). **b| MSI1 is highly expressed at the cortico-medullary border across the entire kidney after IRI.** MSI1 was highly expressed in necrotic tubules at the cortico-medullary border after IRI. Increased expression of MSI1 in necrotic tubules was detected in all experimental groups upon IRI and therefore independent of MSI2 expression status in the distal tubules, loop of Henle and collecting duct in mice. Depicted are immunohistochemistry stainings of MSI1 on undamaged control kidneys obtained from right nephrectomies and kidneys after IRI for the experimental groups wild type, *Msi2* heterozygous control and knockout. Images were acquired with a Hamamatsu S360 slidescanner using a 20x objective. Scale bar presents 500 μm .

3.4 Generation and validation of transgenic Musashi mIMCD cell lines

In order to elucidate the molecular biology of both Musashi proteins and their impact on cell biology in tubular epithelial cells, a murine inner medulla collecting duct cell line (mIMCD3) was used to generate gain- and loss-of-function models. All experiments were performed using the monoclonal mIMCD3#8 cell line, which was previously generated by our laboratory through single cell sorting of mIMCD3 cells and showed increased expression of distal tubule specific markers compared to the polyclonal cell line. Continuing, this clone#8 will be referred to as mIMCD cells. To facilitate immunoprecipitation using a Flag antibody, two mIMCD cell lines expressing MSI1 (MSI1.3xFlag mIMCD cells) and MSI2 (3xFlag.MSI2 mIMCD cells) with 3xFlag tag were generated (Figure 19 a). In order to study dynamic changes in subcellular localization, two additional mIMCD cell lines expressing MSI1 (EGFP.MSI1 mIMCD) and MSI2 (EGFP.MSI2 mIMCD) with an EGFP tag were created (Figure 19 a). Successful transgene integration into the *Rosa26* locus was confirmed via PCR (Figure 19 b). The genotype of all four cell lines was heterozygous for the integrated *Rosa26* allele, as besides the amplicon specific for integration, all cell lines showed the wild type specific amplicon (exemplified for MSI1.3xFlag and 3xFlag.MSI2 mIMCD cell lines in Figure 19 b).

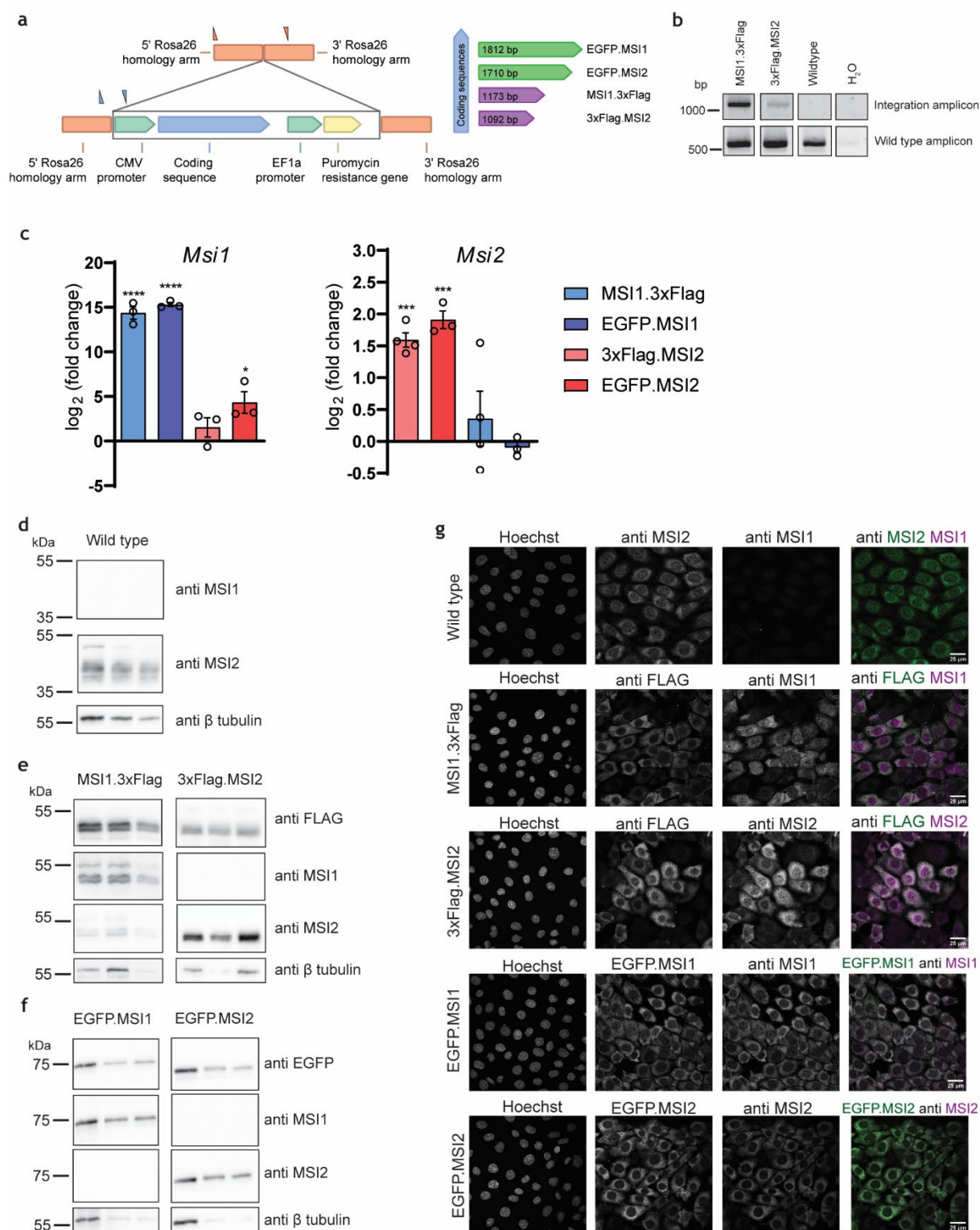


Figure 19. Successful generation and validation of mIMCD cell lines with integration of distinct Musashi transgenes

a| TALEN genome editing strategy used for the successful generation of transgenic mIMCD cell lines. Depending on the length of the coding sequence, between 3665 bp (MSI2.3xFlag) and 4385 bp (EGFP.MSI1) were inserted between the 5' Rosa26 homology arm and the 3' Rosa26 homology arm using TALEN genome editing. Illustrated is the wild type Rosa26 sequence (top) and an overview of the structure of the integrated region (bottom). The integrated region consists of a CMV promoter driven coding sequence and an EF1a promoter driven puromycin resistance gene, used for selection of integrated clones.

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Indicated with triangles are primer binding sites used for PCR amplification of the wild type amplicon (orange, top) and the integration amplicon (blue, bottom). Depicted on the right is an overview of the inserted coding sequences with their respective length, encoding the following fusion proteins: EGFP.MSI1 (1812 bp), EGFP.MSI2 (1710 bp), MSI1.3xFlag (1173 bp) and 3xFlag.MSI2 (1092 bp). A MSI1.3xFlag mIMCD cell line was created, as generation of a cell line expressing MSI1 with N-terminal 3xFlag-tag was not successful. **b) Successful validation of transgene insertion into *Rosa26* locus.** As expected, only MSI1.3xFlag and 3xFlag.MSI2 mIMCD cell lines showed an amplicon specific for the integrated *Rosa26* allele. All three cell lines showed an amplicon specific for the wild type *Rosa26* allele, indicating that MSI1.3xFlag and 3xFlag.MSI2 mIMCD cell lines are heterozygous for the integrated allele. Depicted are the amplicons specific for integrated (1015 bp) and wild type (588 bp) alleles derived from respective PCRs on genomic DNA retrieved from MSI1.3xFlag, 3xFlag.MSI2, wild type mIMCD cells and water after 1% agarose gel electrophoresis. EGFP.MSI1 and EGFP.MSI2 depicted amplicons specific for integration and wild type *Rosa26* alleles, demonstrating these cell lines were also heterozygous for the integrated *Rosa26* allele (data not shown). **c) Verification of increased mRNA expression levels of Musashi transgenes in mIMCD cell lines.** Expression of *Msi1* and *Msi2* mRNA was significantly upregulated in cell lines with single copy integration of the respective coding sequence. *Msi1* was also significantly upregulated in EGFP.MSI2 cells. Depicted are qPCR data showing the fold change (in log₂ scale) of *Msi1* mRNA expression (left) and *Msi2* expression (right) in the respective cell lines (MSI1.3xFlag and EGFP.MSI1 in blue and 3xFlag.MSI2 and EGFP.MSI2 in red) compared to expression in wild type mIMCD cells. Expression of *Msi1* and *Msi2* was normalized to expression of the house keeping gene *Polr2a* in the respective cell lines. Data are presented as mean $\pm$ SEM (n=3 - n=4, one-way ANOVA with Dunnett multiple comparison test, * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$). **d) MSI2, but not MSI1, is detected in lysates of wild type mIMCD cells.** While MSI1 was not detected in mIMCD cells, MSI2 was expressed in all three replicates. Depicted are western blots showing three lysates per cell line, stained with anti MSI1, anti MSI2 and anti β -tubulin antibodies. Expected protein sizes: MSI1 (39 kDa), MSI2 (35 kDa), β -tubulin (50 kDa). Staining of β -tubulin as housekeeping gene served as loading controls. **e) MSI1.3xFlag and 3xFlag.MSI2 fusion proteins are expressed in respective mIMCD cell lines.** In MSI1.3xFlag and 3xFlag.MSI2 mIMCD cell lines Flag signals and MSI1/MSI2 signals largely overlapped in the respective cell lines. In MSI1.3xFlag mIMCD3 #8 cell lines, the MSI2 antibody, weakly detected the MSI1.3xFlag fusion protein, indicating a cross-reactivity of the MSI2 antibody. Depicted are western blots showing three lysates per cell line, stained with anti Flag, anti MSI1 or anti MSI2 and anti β -tubulin antibodies. Expected protein sizes: MSI1.3xFlag (42 kDa), 3xFlag.MSI2 (39 kDa). **f) EGFP.MSI1 and EGFP.MSI2 fusion proteins are expressed in respective mIMCD cell lines.** In EGFP.MSI1 and EGFP.MSI2 mIMCD cell lines EGFP signals and MSI1/MSI2 signals largely overlapped in the respective cell lines. Depicted are western blots showing three lysates per cell line, stained with anti EGFP, anti MSI1 or anti MSI2 and anti β -tubulin antibodies. Expected protein sizes: EGFP.MSI1 (66 kDa), EGFP.MSI2 (62 kDa). **g) MSI1 and MSI2 fusion proteins are predominantly localized within the cytoplasm of respective mIMCD cell lines.** As expected, MSI1 was not expressed in mIMCD wild type cells. In contrast, MSI2 was expressed in the cytoplasm of mIMCD wild type cells. In MSI1.3xFlag mIMCD cells, Flag and MSI1 signals largely overlapped in the cytoplasm and in addition, MSI1 signal was detected within the nucleus of some cells. Similarly, Flag and MSI2 signals overlapped in the cytoplasm of 3xFlag.MSI2 mIMCD cells and MSI2 was also detected in the nuclei of some cells. Anti MSI1/MSI2 signals co-localized with EGFP.MSI1/EGFP.MSI2 expression in the cytoplasm of the respective cell lines. A nuclear signal for MSI1 or MSI2 was not detected in these cells. Images were acquired with a TCS SP8 confocal microscope, using a 63x objective. Scale bar: 25 μ m.

Msi1 and *Msi2* mRNA expression in the four transgenic mIMCD cell lines (MSI1.3xFlag, EGFP.MSI1, 3xFlag.MSI2 and EGFP.MSI2) was determined using qPCR analysis (Figure 19 c). Expression of *Msi1* was significantly increased in MSI1.3xFlag and EGFP.MSI1 mIMCD cell lines compared to wild type mIMCD cells (Figure 19 c). *Msi2* mRNA levels were significantly upregulated in 3xFlag.MSI2 and EGFP.MSI2 mIMCD cell lines in comparison to *Msi2* expression in wild type mIMCD cells (Figure 19 c).

Western blot analyses confirmed the absence of endogenous MSI1 (39 kDa) expression in wild type mIMCD cells (Figure 19 d). MSI2 (35 kDa) protein was detected as multiple bands

above 35 kDa in all three lysates of wild type mIMCD cells. The multiple MSI2 bands represent two MSI2 isoforms (ENSMUST00000092794.12 and ENSMUST00000107909.8) with additional site-specific phosphorylation ²⁴⁸. In MSI1.3xFlag mIMCD cells, anti MSI1 and FLAG signals overlapped, showing two bands at approximately 45 kDa in all replicates (Expected size of MSI1.3xFlag: 42 kDa) (Figure 19 e). Similar to MSI2, the additional band represents site-specific phosphorylation ²⁴⁹. FLAG and MSI2 antibodies detected the same band at approximately 40 kDa in 3xFlag.MSI2 mIMCD cells (Expected size of 3xFlag.MSI2: 39 kDa) (Figure 19 e). Similarly, to the FLAG-tagged cell lines, EGFP and MSI1/MSI2 signals overlapped in EGFP.MSI1 and EGFP.MSI2 mIMCD cell lines in the form of one band around 70 kDa (Expected size EGFP.MSI1: 66 kDa, EGFP.MSI2: 62 kDa) (Figure 19 f).

Besides confirming expression of Musashi proteins via western blot, the subcellular localization of both Musashi proteins was determined via immunofluorescence staining (Figure 19 g). While MSI1 was not expressed, MSI2 was detected in the cytoplasm of mIMCD wild type cells. MSI1/MSI2 signals largely overlapped with Flag signals in the cytoplasm of MSI1.3xFlag and 3xFlag.MSI2 mIMCD cell lines. In addition, MSI1 and MSI2 signals were detected in the nuclei of some cells within these cell lines and the signal did not overlap with the Flag staining. Similar to MSI1.3xFlag and 3xFlag.MSI2 cells, cytoplasmic expression of EGFP.MSI1/EGFP.MSI2 highly overlapped with anti MSI1/MSI2 stainings in EGFP.MSI1/EGFP.MSI2 mIMCD cell lines. In conclusion, generation of transgenic mIMCD cell lines expressing MSI1.3xFlag, 3xFlag.MSI2, EGFP.MSI1 and EGFP.MSI2 fusion proteins using TALEN genome editing was successfully validated on gene, transcript and protein level.

3.5 Subcellular localization of both Musashi proteins in response to diverse cellular stressors

Cells are constantly exposed to extrinsic and intrinsic stressors ²⁵⁰ and continuous activation of stress response pathways e.g. unfolded protein response in the ER ^{31,33}, mitochondrial oxidative stress ^{36,37} or DNA damage response ^{44,45} is implicated in the pathology of AKI. We aimed to investigate the molecular functions of both Musashi proteins in mouse kidney cells at baseline and after exposure to several types of stressors: heat, hypoxia, cisplatin and ROS-induced oxidative stress. Heat shock (45°C for four hours) induced the nuclear localization of MSI1 and MSI2 compared to their cytoplasmic localization under control conditions in EGFP.MSI1 and EGFP.MSI2 mIMCD cell lines (Figure 20 a). Hypoxia (1% O₂ for six hours) did not induce changes in cellular EGFP.MSI1/EGFP.MSI2 localization in mIMCD cell lines compared to normoxic conditions (Figure 20 b).

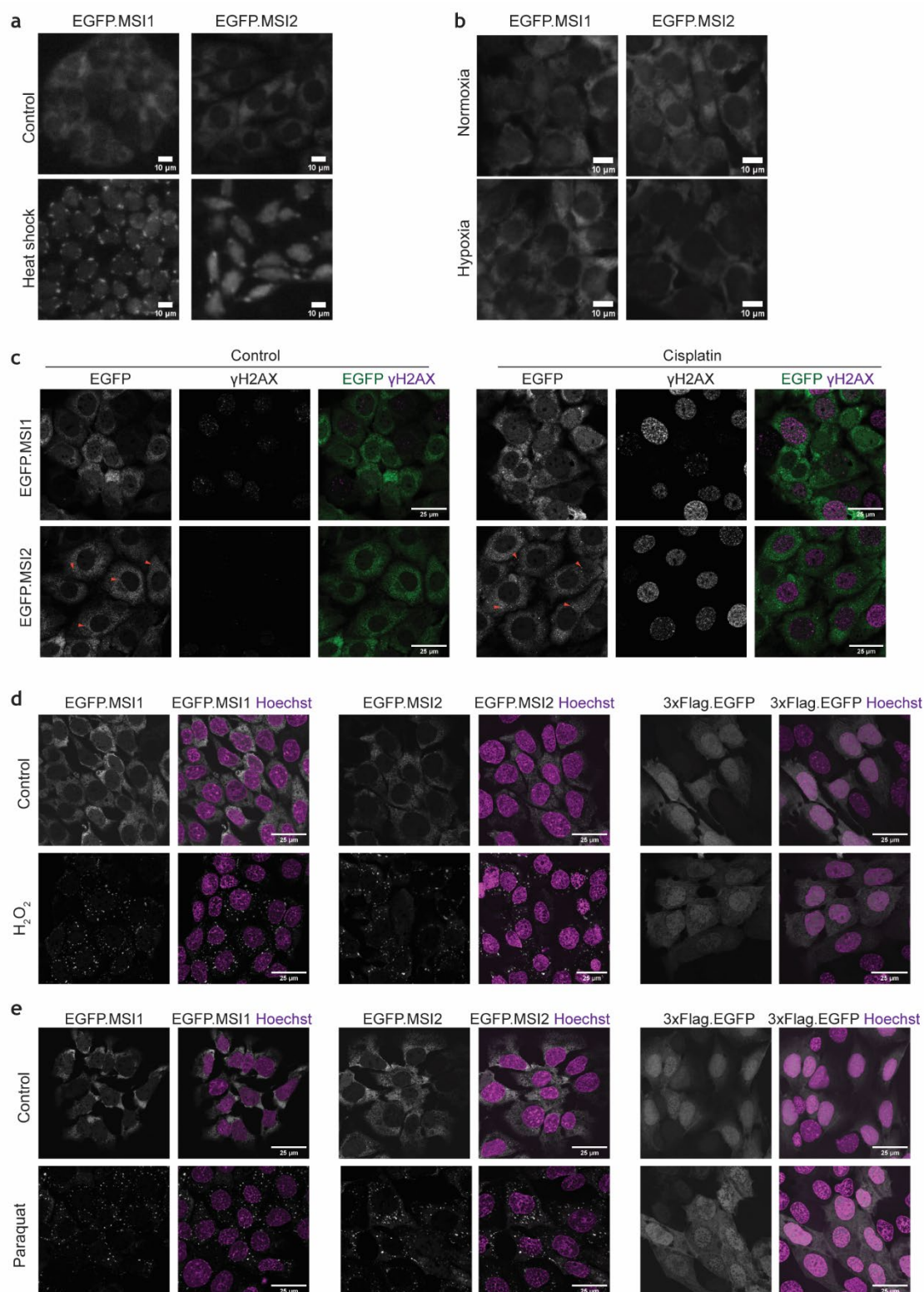


Figure 20. Stimulation of cellular stress pathways induces changes in subcellular localization of both Musashi proteins in mIMCD cells

a| Exposure of mIMCD cells to heat shock induces nuclear localization of EGFP.MSI1 and EGFP.MSI2. While EGFP.MSI1 and EGFP.MSI2 were localized in the cytoplasm under control conditions, heat shock induced the nuclear localization of EGFP.MSI1 and EGFP.MSI2. Images were acquired using the Resolve microscope (BICO) with a 10x objective. Scale bar: 10 μ m. **b| Exposure of mIMCD cells to hypoxia does not alter the subcellular localization of EGFP.MSI1 and EGFP.MSI2.** Similar to normoxic conditions, EGFP.MSI1 and EGFP.MSI2 remained expressed within the cytoplasm of mIMCD cells under hypoxia.

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Images were acquired using the Axio Observer microscope (Zeiss) with a 20x objective. Scale bar: 10 μm . **c| Cisplatin treatment leads to localization of MSI1 and MSI2 in some cytoplasmic granular structures in mIMCD cell lines.** Upon cisplatin treatment, phosphorylation of histone H2A variant H2AX (γH2AX) was increased in EGFP.MSI1 and EGFP.MSI2 cells. γH2AX phosphorylation is a DNA damage marker and shows effective cisplatin treatment of the cell lines. The cytoplasmic localization of EGFP.MSI1 and EGFP.MSI2 in mIMCD cell lines remained unchanged upon cisplatin treatment. To some extent, localization of EGFP.MSI1/EGFP.MSI2 in granular structures in the cytoplasm of cisplatin-treated cells was visible (red arrows). **d| H_2O_2 treatment induces localization of MSI1 and MSI2 in many cytoplasmic granular foci in mIMCD cell lines.** H_2O_2 stimulation induced strong localization of EGFP.MSI1 and EGFP.MSI2 in cytoplasmic granular foci compared to their uniform expression pattern across the cytoplasm under control conditions. Localization of 3xFlag.EGFP did not change upon H_2O_2 treatment. **e| Paraquat stimulation leads to localization of MSI1 and MSI2 in cytoplasmic granular structures in mIMCD cell lines.** Similar to H_2O_2 treatment, exposure of EGFP.MSI1/EGFP.MSI2 cells strongly induced localization of EGFP.MSI1 and EGFP.MSI2 in cytoplasmic granular structures. In contrast, the cellular localization of 3xFlag.EGFP was unaltered by paraquat treatment. Images presented in (c)-(e) were acquired using the TCS SP8 confocal microscope with a 63x objective. Scale bars: 25 μm .

MSI1 was strongly upregulated upon treatment with the nephrotoxic drug cisplatin in mouse kidneys (Figure 9), but cisplatin administration in tubular epithelial cells did not cause major changes in EGFP.MSI1/EGFP.MSI2 localization patterns (Figure 20 c). In a few cells, a localization of EGFP.MSI1/EGFP.MSI2 in granular foci within the cytoplasm was visible after cisplatin administration. To confirm that cisplatin treatment of mIMCD cells was effective, DNA damage was determined through immunofluorescence staining of γH2AX . Although cisplatin did not extensively influence MSI1/MSI2 distribution in cells, it effectively induced DNA damage as shown by increased levels of γH2AX compared to untreated cells (Figure 20 c).

Furthermore, the influence of ROS-induced oxidative stress on the subcellular localization of both Musashi proteins in mIMCD cells was examined using H_2O_2 and paraquat (PQ) stimulation. A major redistribution of EGFP.MSI1 and EGFP.MSI2 was noticeable after treatment of mIMCD cells with H_2O_2 (Figure 20 d) and PQ (Figure 20 e). Both treatments induced a strong localization of MSI1 and MSI2 in granular compartments within the cytoplasm of both cell lines. In untreated control cells, MSI1 and MSI2 were evenly expressed across the cellular cytoplasm. Localization of 3xFlag.EGFP alone did not change upon H_2O_2 and PQ stimulation.

3.6 In-depth characterization of the impact of oxidative stress on Musashi proteins

3.6.1 PQ induces recruitment of Musashi proteins to cytoplasmic stress granules

Considering the marked impact of PQ treatment on the subcellular localization of EGFP-tagged Musashi proteins, this stressor was used for a more detailed characterization. Similar to EGFP.MSI1/EGFP.MSI2 mIMCD cell lines, PQ stimulation induced strong localization of endogenous MSI2 into granular structures within the cytoplasm of mIMCD wild type cells

(Figure 21 a). Interestingly, MSI2-positive granules overlapped with expression of the stress granule marker FMR1 autosomal homolog 1 (FXR1), suggesting MSI2 was recruited into stress granules (SGs) after oxidative stress induction via PQ. In line with these findings, PQ treatment of 3xFlag.MSI2 (Figure 21 b) and MSI1.3xFlag (Figure 21 c) mIMCD cells led to localization of 3xFlag.MSI2/MSI1.3xFlag into FXR1-positive SGs. In conclusion, PQ-induced oxidative stress triggers localization of endogenous MSI2, as well as both Musashi EGFP/3xFlag fusion proteins to cytoplasmic SGs.

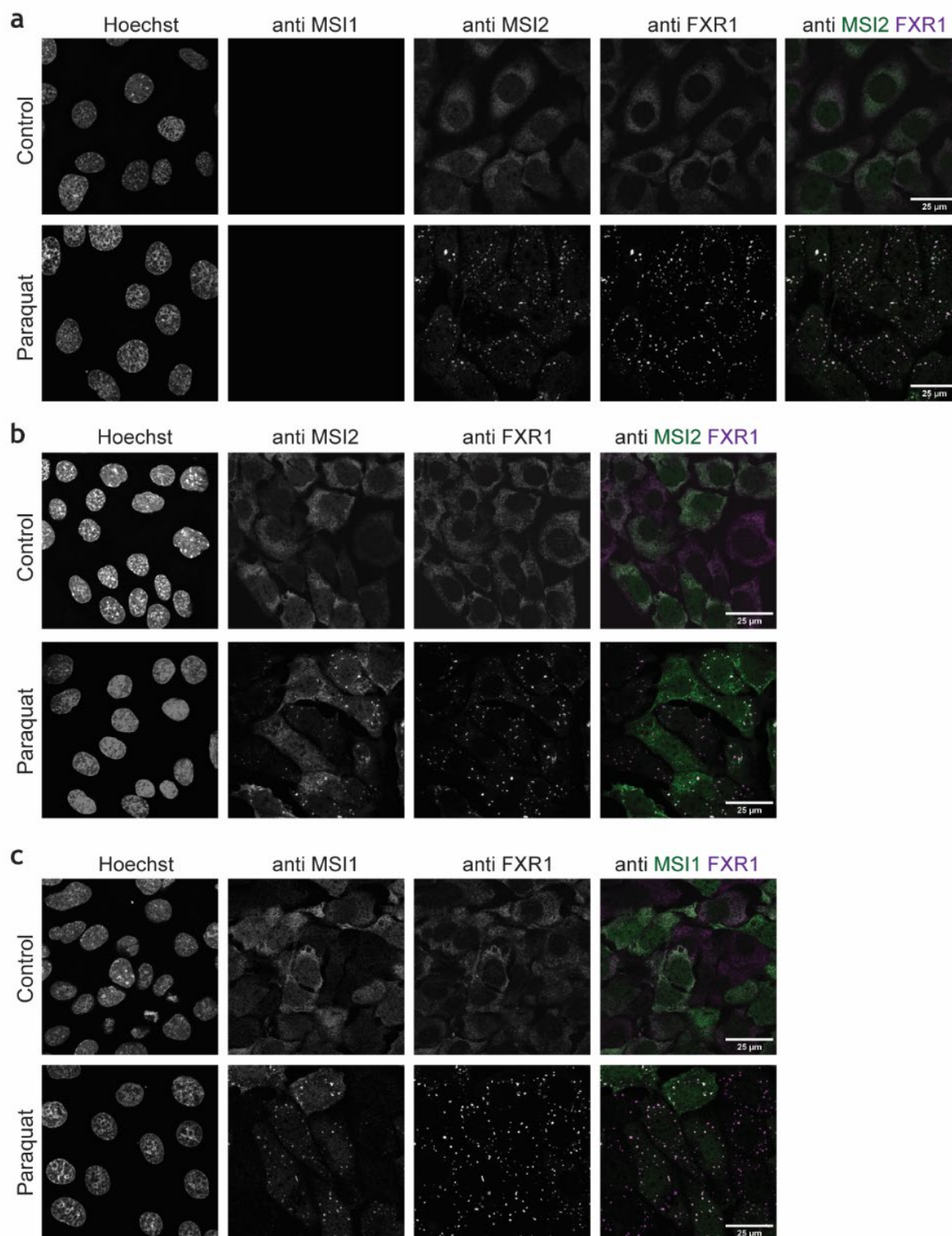


Figure 21. PQ stimulation triggers the localization of both Musashi proteins in cytoplasmic SGs in mIMCD cells

a) Exposure of wild type mIMCD cells to PQ induces localization of MSI2 in SGs. Treatment of wild type mIMCD cells with PQ triggered the localization of endogenously expressed MSI2 into cytoplasmic granules. MSI2 signal overlapped with FXR1 expression, a SG marker, indicating localization of MSI2 in SGs upon PQ stimulation. MSI1 was not expressed in wild type mIMCD cells.

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b| PQ treatment triggers localization of MSI2 to SGs in MSI2.3xFlag mIMCD cells. PQ stimulation induced localization of MSI2 in granular structures in the cytoplasm of 3xFlag.MSI2 mIMCD cells. MSI2-positive granular structures expressed the SG marker FXR1, confirming localization of MSI2 to SGs upon PQ treatment. **c| MSI1 is localized in SGs in MSI1.3xFlag mIMCD cells upon PQ stimulation.** Similar to the localization of MSI2 in mIMCD cells, MSI1 localized in FXR1-positive cytoplasmic SGs upon PQ stimulation of MSI1.3xFlag mIMCD cells. All images were acquired with a TCS SP8 confocal microscope, using a 63x objective. Scale bars present 25 μ m.

3.6.2 PQ induces ferroptosis-mediated cell death in mIMCD wild type cells

To investigate the consequences of continuous PQ stimulation on cellular survival, wild type mIMCD cells were simultaneously treated with PQ and distinct combinations of cell death inhibitors. While Emricasan inhibited apoptosis, Ferrostatin-1 was used to block ferroptosis (Figure 22 a). Furthermore, pyroptosis was prevented by treatment with CRID3 and VX-765 and necroptosis was blocked through stimulation with Nec-1s and GSK-872 (Figure 22 a). Besides single administration of all cell death inhibitors, a combinatorial treatment with Emricasan was utilized to avoid that cells shift towards apoptosis.

Over a time-course of 24 hours, PQ treatment strongly induced cell death in wild type mIMCD cells (w/o inhibitors / + PQ) compared to unstimulated conditions (w/o inhibitors / - PQ) (Figure 22 b-e). Inhibiting ferroptosis through stimulation with Ferrostatin-1 fully ameliorated cell death of mIMCD3 cells (Figure 22 b). Treatment with Ferrostatin-1 alone, and in combination with Emricasan, reduced the number of dead cells throughout the entire 24 h of PQ stimulation even below the level of unstimulated cells (Figure 22 b). In comparison, exclusive inhibition of apoptosis through treatment with Emricasan only delayed cell death, as the numbers of dead cells progressively increased beginning approximately 16 h after stimulation with PQ was started.

Similarly, combined blockage of apoptosis and pyroptosis only delayed death of mIMCD3 cells, as the number of dead cells treated with Emricasan and VX-765/CRID3 was reduced compared to unstimulated cells (until 22 h after beginning PQ stimulation) but progressively increased over time (Figure 22 c). Moreover, while single treatment with CRID3 showed no effect on PQ-induced death of mIMCD3 cells, stimulation with VX-765 increased the number of dead cells compared to the level in cells without any cell death inhibitor (w/o inhibitors / + PQ).

Inhibiting RIPK-1 mediated necroptosis together with apoptosis delayed PQ-triggered death of mIMCD cells, similar to combinatorial blockage of apoptosis and pyroptosis observed before (Figure 22 d). Treatment with Emricasan and GSK-872 reduced the number of dead cells below the amount observed in unstimulated cells, but cell death progressively increased over-time. Stimulation with GSK-871 and Nec-1s alone, and the combination of Nec-1s with Emricasan had no effect on PQ-induced cell death.

Quantification of cell death, 24 h after the start of PQ exposure, showed that (a) PQ significantly increased death in mIMCD cells (w/o inhibitors / + PQ) compared to untreated cells (w/o inhibitors / - PQ) and (b) blocking ferroptosis alone (Ferrostatin-1) and in combination with apoptosis (Ferrostatin-1+Emricasan) significantly reduced cell death compared to untreated cells (Figure 22 e).

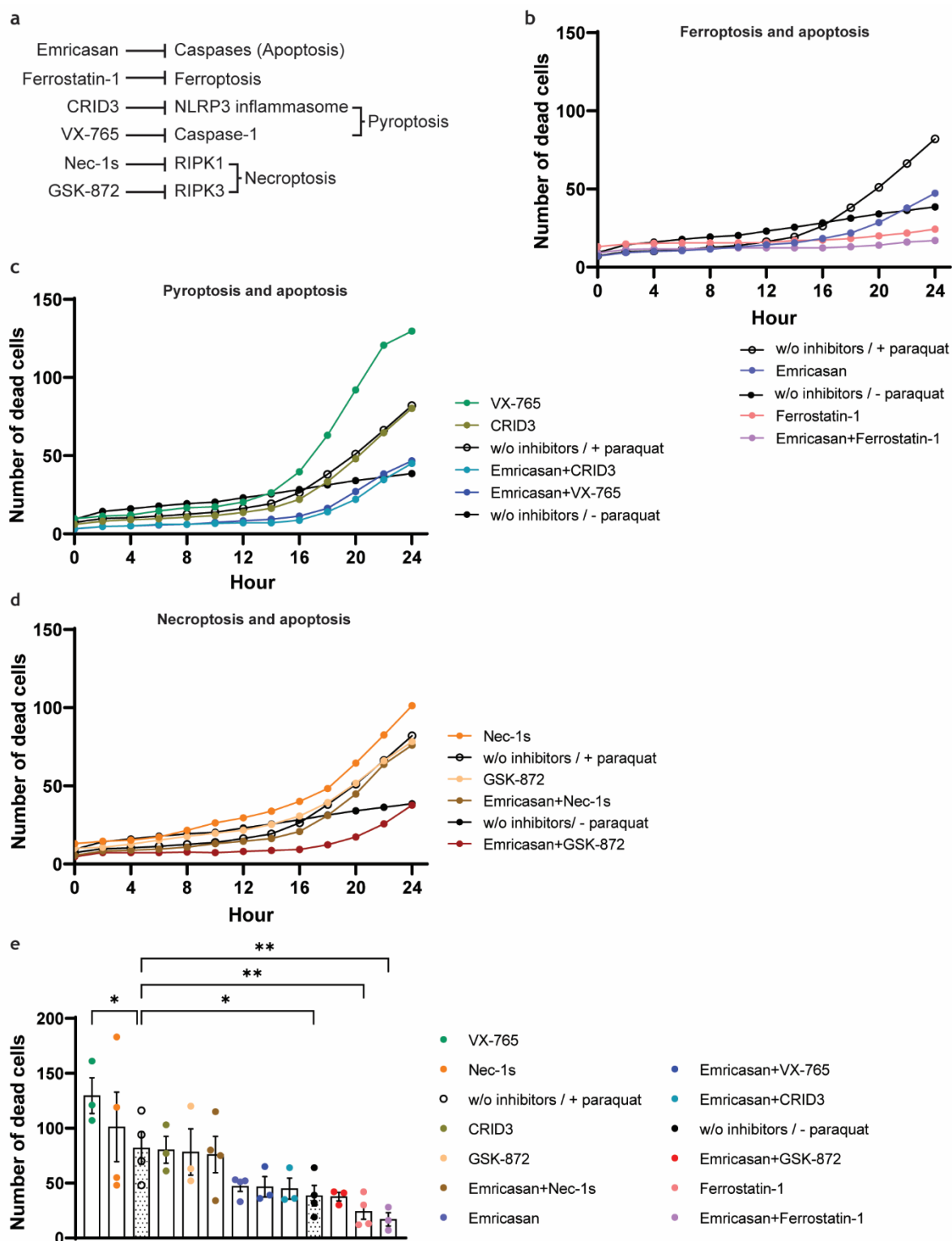


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Figure 22. Continuous exposure to PQ induces ferroptotic cell death in mIMCD cells

a| PQ-induced cell death was determined using different cell death inhibitors. Emricasan is a pan-caspase inhibitor of apoptosis. Ferrostatin-1 is a ROS scavenger and inhibits ferroptosis. Pyroptosis is prevented by the NLRP3 inflammasome inhibitor CRID3 and through caspase-1 inhibition mediated by VX-765. Necrostatin-2 (Nec-1s) and GSK-872 inhibit necroptosis through RIPK1/RIPK3 inactivation. **b| Preventing ferroptosis alone and in combination with apoptosis fully protects mIMCD wild type cells from PQ-induced cell death.** Over 24 h exposure to PQ, treatment with Ferrostatin-1 alone and in combination with Emricasan fully ameliorated death of wild type mIMD3#8 cells, as the number of dead cells was continuously reduced below the level of unstimulated cells (w/o inhibitors / - PQ). In contrast, after 16 h of stimulation with PQ, the number of dead cells progressively increased in cells treated with Emricasan and without any cell death inhibitor (w/o inhibitors / + PQ). Depicted is the number of dead mIMCD wild type cells (DIYO-1 positive green fluorescent signal), treated with distinct cell death inhibitor combinations, during continuous exposure to PQ over a time course of 24 hours. Green fluorescence was monitored with the IncuCyte® S3 using the 20x objective. Data are presented as mean (n=3). **c| Simultaneous inhibition of pyroptosis and apoptosis delayed death of mIMCD wild type cells during continuous PQ stimulation.** Combinatorial treatment of VX-765 and CRID3 with Emricasan reduced the number of dead cells below the level of unstimulated cells until 22 h stimulation with PQ. Cell death was only delayed and not fully ameliorated in comparison to unstimulated cells, as the number of dead cells progressively increased over the time-course of 24 h stimulation with PQ. Treatment with CRID3 alone did not prevent PQ-induced cell death, as the number of dead cells was similar to the number of dead cells which were not treated with cell death inhibitors (w/o inhibitors / + PQ). Single administration of VX-765 elevated the number of dead cells compared to untreated cells (w/o inhibitors / + PQ). Data was acquired and visualized as described in (b). **d| Inhibition of apoptosis together with RIPK3-dependent necroptosis delayed PQ-induced mIMCD cell death.** Simultaneous administration of GSK-872 and Emricasan decreased the number of dead cells below the number of dead cells in unstimulated conditions. Cell death was delayed, but not fully prevented, as in contrast to unstimulated cells, the number of dead cells progressively increased during 24 h of exposure to PQ. Treatment with GSK-872 and Nec-1s alone, and the combination of Nec-1s with Emricasan, did not prevent PQ-induced cell death, as the number of dead cells was similar to the number of dead cells which were not treated with cell death inhibitors (w/o inhibitors / + PQ). Data was acquired and visualized as described in (b). **e| Inhibition of ferroptosis significantly reduces PQ-induced mIMCD cell death after 24 hours.** Treatment with Ferrostatin-1 and its combinatorial treatment with Emricasan fully ameliorated cell death after 24 hours of PQ stimulation, as observed through significant reduction of the number of dead cells below the number determined in unstimulated cells (w/o inhibitors / - PQ). In general, 24 hours exposure to PQ significantly increased cell death of mIMCD wild type cells (w/o inhibitors / + PQ) compared to unstimulated cells (w/o inhibitors / - PQ). Stimulation with VX-765 significantly increased cell death in mIMCD cells compared to cells without any cell death inhibitor (w/o inhibitors / + PQ). Data was acquired as described in (b) and is represented as mean $\pm$ SEM (n=3, one-way ANOVA with Fisher's LSD, * $p \leq 0.05$, ** $p \leq 0.01$).

3.6.3 Expression of Musashi proteins increases susceptibility to PQ-mediated cell death

PQ triggered a specific relocalisation of both Musashi proteins into cytoplasmic SGs. At the same time, long-term PQ stimulation over a time-course of 24 hours, induced ferroptotic cell death of mIMCD cells. Based on these findings, the potential role of Musashi proteins in PQ-induced ferroptosis in mIMCD cells was further investigated.

To examine PQ-induced cell death in relation to MSI1 and MSI2 expression levels, siRNA-mediated knockdown of *Msi2* mRNA in wild type and MSI1.3xFlag mIMCD cells was utilized. Western blot analysis showed that MSI2 levels were strongly decreased in wild type and MSI1.3xFlag mIMCD cells upon siRNA-mediated knockdown compared to cells, transfected with control siRNA (Figure 23 a/b).

Next, PQ-induced cell death of mIMCD cells under varied expression levels of MSI1 and MSI2 was examined. PQ treatment triggered elevated cell death in MSI1.3xFlag mIMCD cells, which were either transfected with control siRNA or not transfected at all, compared to the remaining experimental groups (Figure 23 c). Importantly, these MSI1.3xFlag cells (siRNA control / without siRNA) were the only cells expressing both Musashi proteins. Among the other experimental groups, MSI2 expression was decreased in MSI1.3xFlag cells with *Msi2* knockdown, wild type cells only expressed MSI2 and wild type cells with *Msi2* knockdown showed significantly decreased MSI2 levels. These results indicate that expression of both Musashi proteins rendered mIMCD cells more susceptible to PQ-induced cell death. Quantification of cell death after 24 hours supported these observations, as the level of dead cells was significantly increased in MSI1.3xFlag mIMCD cells with control siRNA and without siRNA, compared to MSI1.3xFlag and wild type mIMCD cells with *Msi2* knockdown (Figure 23 d). In conclusion, mIMCD cells expressing both Musashi proteins (MSI1.3xFlag + w/o siRNA / control siRNA) were more susceptible to PQ-induced cell death than mIMCD cells expressing only MSI1 (MSI1.3xFlag + *Msi2* siRNA), only MSI2 (wild type w/o siRNA / control siRNA) and mIMCD cells lacking both Musashi proteins (wild type + *Msi2* siRNA).

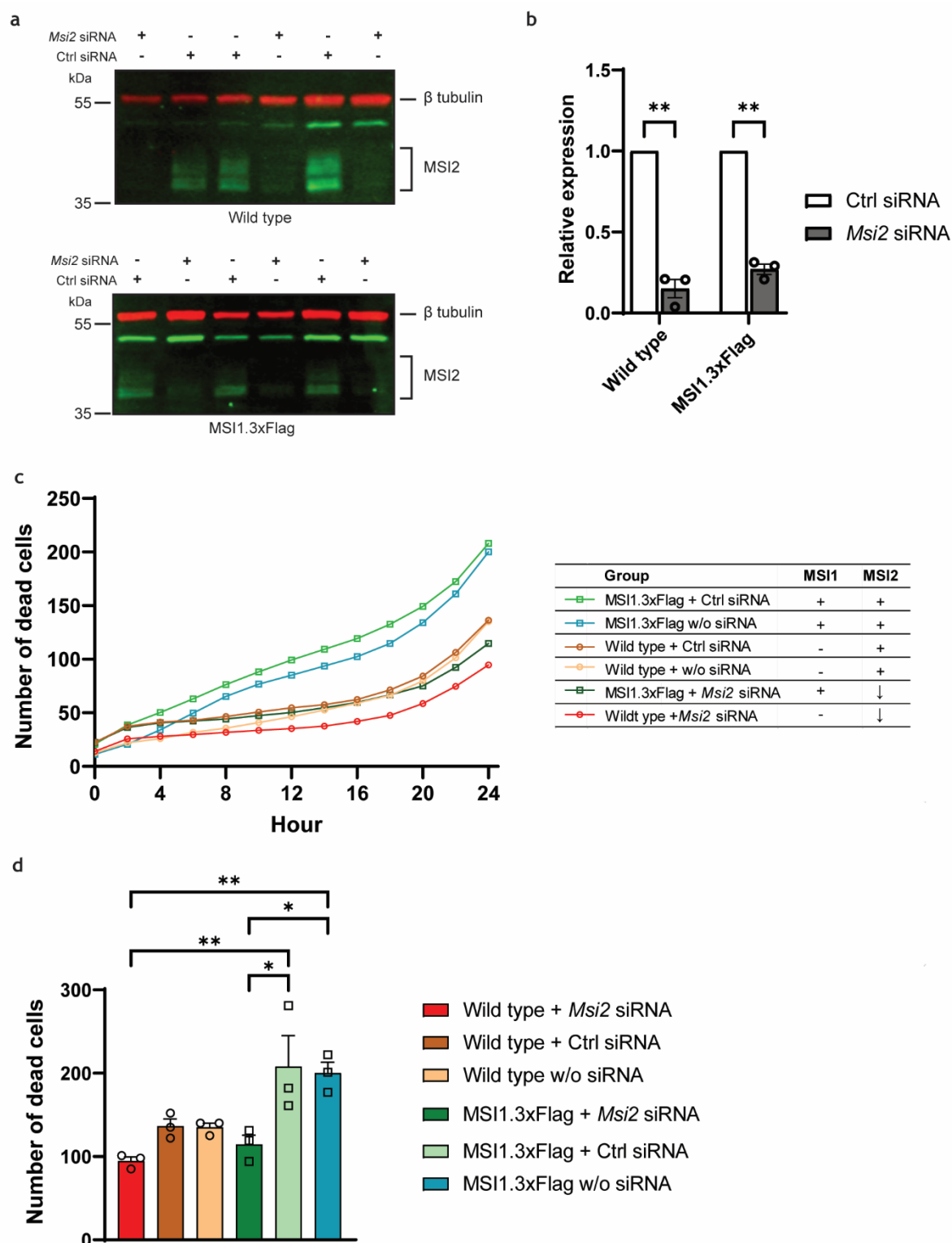


Figure 23. Expression of both Musashi proteins renders mIMCD cells more susceptible to PQ-induced cell death

a| Expression of MSI2 is reduced upon siRNA-mediated *Msi2* knockdown in wild type and MSI1.3xFlag mIMCD cells. Expression of MSI2 in both cell lines was markedly reduced upon transfection with *Msi2* siRNA compared to transfection with control siRNA. Wild type (top) and MSI1.3xFlag mIMCD cells were either transfected with *Msi2* siRNA or control siRNA. MSI2 and β-tubulin expression were determined by multiplex western blotting using the odyssey system.

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b| MSI2 expression is significantly downregulated upon siRNA-mediated knockdown in wild type and MSI1.3xFlag mIMCD cells. MSI2 expression was significantly reduced upon treatment with *Msi2* siRNA in wild type and MSI1.3xFlag mIMCD cells in comparison to treatment with control siRNA. Shown is the MSI2 protein expression fold change in wild type and MSI1.3xFlag mIMCD cells treated with *Msi2* siRNA and control siRNA, determined by densitometric analysis of the western blots depicted in (a). MSI2 expression was normalized to β -tubulin expression. MSI2 expression upon *Msi2* knockdown is shown relative to its expression in the siRNA control. Data are presented as mean $\pm$ SEM (n=3, students t-test, ** $p \leq 0.01$). **c| Long-term PQ exposure leads to increased cell death of mIMCD cells expressing both Musashi proteins.** Over a period of 24 hours of long-term PQ exposure, MSI1-3xFlag cells with siRNA control and without siRNA showed markedly increased numbers of dead cells compared to the other experimental groups, indicating that expression of both Musashi proteins contributes to PQ-induced death of mIMCD cells. In contrast to the remaining experimental groups, MSI1.3xFlag cells with siRNA control / without siRNA represent the only cells expressing MSI1 and MSI2 at the same time. Expression of MSI2 was significantly decreased in MSI1.3xFlag cells with *Msi2* knockdown, wild type mIMCD cells generally lack MSI1 expression and upon *Msi2* knockdown in wild type cells MSI2 expression was significantly decreased. Cell death during 24 hours PQ exposure was monitored for MSI1.3xFlag and wild type mIMCD cells treated with either *Msi2* siRNA, control siRNA or without siRNA. Depicted are the killing curves for the respective experimental groups. A table indicates the expression status of MSI1 and MSI2 within the experimental groups. Dead cells were detected using DIYO-1 and green fluorescence was monitored with IncuCyte® S3 using the 10x objective. Data are presented as mean (n=3). **d| Number of dead cells is significantly increased in cells expressing both Musashi proteins.** After 24h of PQ stimulation, cell death was significantly increased in MSI1.3xFlag cells without siRNA/control siRNA compared to MSI1.3xFlag and wild type mIMCD cells with siRNA-mediated *Msi2* knockdown. As MSI1.3xFlag mIMCD cells expressed MSI1 and MSI2, (compared to wild type mIMCD cells, which only expressed MSI2) these data indicate that expression of both Musashi proteins renders cells more susceptible to PQ-induced cell death. Data are presented as mean $\pm$ SEM (n=3, one-way ANOVA with Tukey multiple comparison test, * $p \leq 0.05$, ** $p \leq 0.01$).

3.7 The effects of oxidative stress on the Musashi protein interactome

PQ-induced oxidative stress caused a strong localization of both Musashi proteins into cytoplasmic SGs. Moreover, continuous exposure to PQ triggered ferroptosis in mIMCD cells. In this context, expression of both Musashi proteins, rendered mIMCD cells more susceptible to PQ-induced cell death. As RBPs, MSI1 and MSI2 participate in the formation of ribonucleoprotein complexes and the composition of these complexes may change upon PQ-induced oxidative stress. Consequently, defining the protein and RNA network of both Musashi proteins in unstimulated mIMCD cells and examining possible alterations after PQ-induced oxidative stress can help in understanding their function in PQ-induced oxidative stress response.

3.7.1 Defining the protein interactome of both Musashi proteins in tubular epithelial cells at baseline

To determine the protein interactomes of MSI1 and MSI2, immunoprecipitation (IP) using the M2 Flag antibody in MSI1.3xFlag and 3xFlag.MSI2 mIMCD cell lysates, followed by mass spectrometry was performed. M2 Flag IP successfully enriched MSI1.3xFlag (Figure 24 a, left) compared to the level of MSI1.3xFlag in lysates before IP (input). Similarly, M2 Flag IP successfully enriched 3xFlag.MSI2, compared to 3xFlag.MSI2 levels in the input (Figure 24 a, right). Mass spectrometry identified MSI1 and 417 proteins significantly enriched in the M2

Flag IP in MSI1.3xFlag compared to wild type mIMCD cells (Figure 24 b). Within this group of MSI1 interactors, 28 proteins had previously been described to bind to MSI1²³¹. MSI2 and 296 interacting proteins were identified in the M2 Flag IP in 3xFlag.MSI2 compared to wild type mIMCD cells via mass spectrometry (Figure 24 c). Within this MSI2 interactome, 18 proteins were identified which had previously been reported to bind to MSI2²³¹.

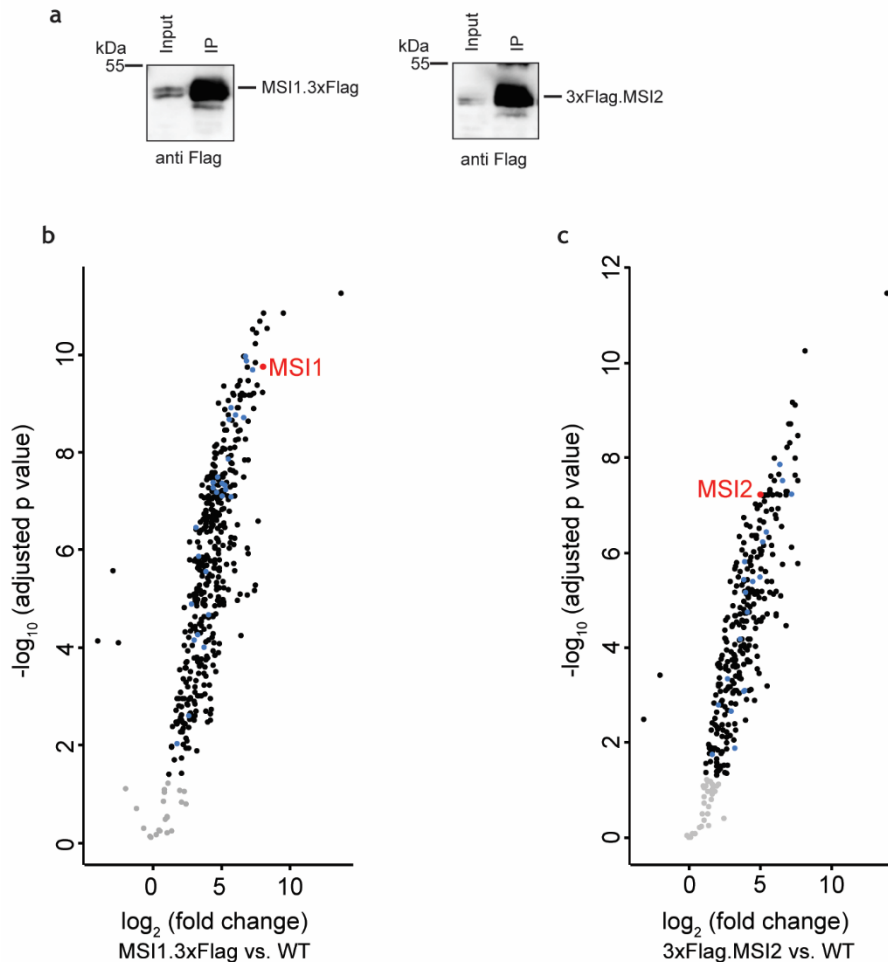


Figure 24. Defining the protein interactome of MSI1 and MSI2 in mIMCD cells at baseline

a) Successful enrichment of MSI1.3xFlag and 3xFlag.MSI2 via M2 Flag IP in unstimulated mIMCD cells. Western blots demonstrating successful enrichment of both Musashi proteins via IP with M2 Flag antibody in respective mIMCD cell lines. Depicted is IP of MSI1.3xFlag (42 kDa) (left) and 3xFlag.MSI2 (39 kDa) right, compared to expression within the lysate prior to IP (input). Western blot was performed using the M2 Flag antibody. **b) MSI1 and 417 proteins are significantly enriched after M2 Flag IP in unstimulated MSI1.3xFlag mIMCD cells.** MSI1 specifically interacted with 417 proteins in MSI1.3xFlag mIMCD cells. Within the MSI1 interactome, 28 proteins were identified which were previously reported to interact with MSI1. Depicted is a scatter plot showing all proteins identified via mass spectrometry after IP with M2 Flag antibody in MSI1.3xFlag mIMCD cells compared to wild type (WT) mIMCD cells. The log₂ fold change (MSI1.3xFlag vs. WT) is plotted on the x-axis and -log₁₀ adjusted p value is plotted on the y-axis. Visualized are non significant proteins (gray), significantly enriched proteins (adjusted p value ≤ 0.05) (black), known MSI1 interactors (blue) and MSI1 (red). **c) MSI2 and 296 proteins are significantly enriched after M2 Flag IP in unstimulated 3xFlag.MSI2 mIMCD cells.** MSI2 specifically interacted with 296 proteins in 3xFlag.MSI2 mIMCD cells. Within the MSI2 interactome, 18 proteins were identified, which were previously reported to interact with MSI2.

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Depicted is a scatter plot showing all proteins identified via mass spectrometry after IP with M2 Flag antibody in 3xFlag.MSI2 mIMCD cells compared to wild type (WT) mIMCD cells. The $\log_2$ fold change (3xFlag.MSI2 vs WT) is plotted on the x-axis and $-\log_{10}$ adjusted p value is plotted on the y-axis. Visualized are non-significant proteins (gray), significantly enriched proteins (adjusted p value ≤ 0.05) (black), known MSI2 interactors (blue) and MSI2 (red).

3.7.2 Defining the protein interactome of both Musashi proteins in tubular epithelial cells upon oxidative stress

To investigate whether the protein interactome of MSI1 and MSI2 is altered upon oxidative stress induction in mIMCD cells, the interactomes of MSI1 and MSI2 were analyzed after exposure to PQ. Upon exposure to PQ, MSI1 interacted with 421 proteins in mIMCD cells (Figure 25 a). The MSI1 interactomes of unstimulated and PQ-stimulated mIMCD cells largely overlapped (412 proteins). Five proteins (ACTN1, ANXA2, BLMH, LMNB1, PRPF19) exclusively interacted with MSI1 in unstimulated mIMCD cells (full protein names are indicated in Figure 25 a). This highly diverse group of proteins, exclusively interacting with MSI1 in unstimulated mIMCD cells, consisted of the cytoskeletal protein ACTN1, the phospholipid binding protein ANXA2, the inner nuclear membrane protein LMNB1, the ubiquitin ligase PRPF19 and the cytoplasmic cysteine peptidase BLMH. After stimulation with PQ, MSI1 exclusively interacted with nine proteins (see Figure 25 for full list of proteins). Within this group, ACTA1 / ACTA2 / ACTC1 / ACTG2, CTNNA1, LAD1 and TJP2 were associated with cellular adherence and cell junctions. Three of the proteins, exclusively interacting with MSI1 under PQ, were involved in translation: EIF4B, RPS16 and RPS19. Moreover, MSI1 interacted with the cytoplasmic chaperone HSP90AB1 and a histone variant HIST2H2AA1 / HIST2H2AC.

Interestingly, one third of the proteins interacting with MSI1 in unstimulated mIMCD cells (138) was identified as SG-associated proteins²⁵¹ (Figure 25 b). Upon exposure to PQ, the MSI1 SG protein network was not strongly altered (140) (Figure 25 c). EIF4B and RPS19 represented the only two SG-associated proteins, which were exclusively enriched in the MSI1 interactome of PQ-treated compared to unstimulated mIMCD cells. Importantly, PQ stimulation did not induce major alterations to the mIMCD proteome, when compared to unstimulated conditions (only two proteins (Chromatin licensing and DNA replication factor 1 and Cytochrome C oxidase subunit 8A) were differentially regulated, data not shown).

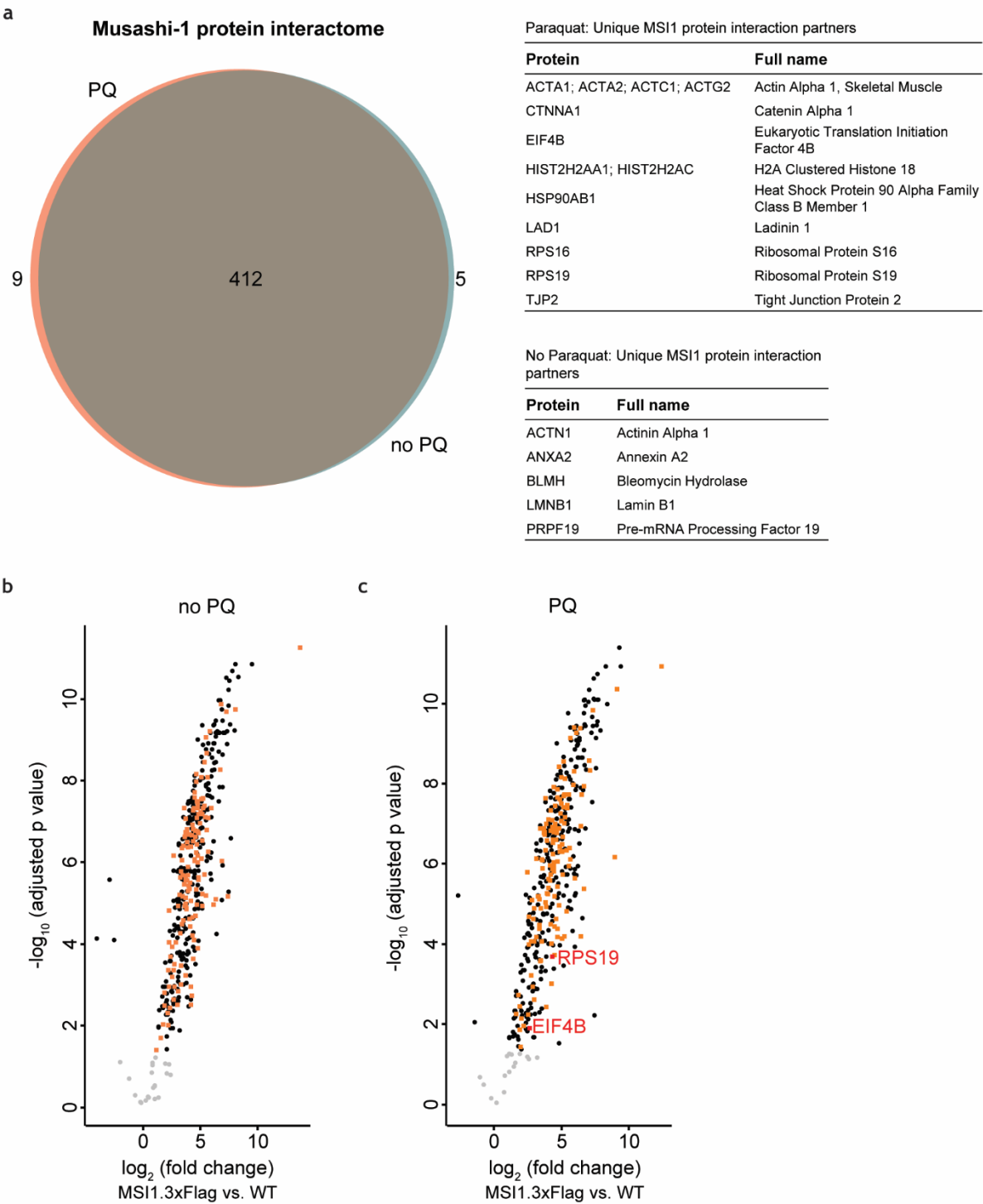


Figure 25. PQ exposure induces only mild changes to the MSI1 protein interactome

a| Comparison of MSI1 interactome after PQ stimulation vs. unstimulated conditions reveals no major changes in MSI1 interactome. The majority of MSI1-interacting proteins were identified under PQ exposure as well as in control conditions without PQ (no PQ) (412 proteins). Nine proteins (ACTA1/ACTA2/ACTC1/ACTG2, CTNNA1, EIF4B, HIST2HAA1/HIST2HAC, HSP90AB1, LAD1, RPS16, RPS19, TJP2) were identified to specifically interact with MSI1 under PQ exposure and five proteins only interacted with MSI1 without PQ stimulation (ACTN1, ANXA2, BLMH, LMNB1, PRPF19). Visualized is the overlap between protein interactors of MSI1 under PQ exposure vs. no PQ exposure as Venn diagram (left). The tables on the right depict proteins uniquely identified as MSI1 interactors in PQ (top) and no PQ (bottom). *continued on next page*

b| MSI1 interacts with SG proteins in control conditions (no PQ). One third (138 proteins) of the identified MSI1 protein interactors (417 proteins) were previously identified in SGs, indicating that MSI1 is already part of a SG protein network in unstressed conditions in mIMCD cells. Shown is a scatter plot visualizing all proteins identified via mass spectrometry after IP with M2 Flag antibody in MSI1.3xFlag vs wild type mIMCD cells in no PQ conditions. The $\log_2$ fold change (MSI1.3xFlag vs. WT) is plotted on the x-axis and $-\log_{10}$ adjusted p value is plotted on the y-axis. Visualized are non-significant proteins (gray), significantly enriched proteins (adjusted p value ≤ 0.05) (black) and SG proteins (orange). **c| PQ exposure does not induce major changes within the MSI1 SG protein network.** Upon PQ exposure, MSI1 was bound to a similar group of SG proteins (140) as observed in no PQ conditions (138). Only the proteins EIF4B and RPS19 were significantly enriched in the MSI1 interactome upon PQ compared to no PQ conditions. Shown is a scatter plot visualizing all proteins identified via mass spectrometry after IP with M2 Flag antibody in MSI1.3xFlag vs. wild type mIMCD cells after exposure to PQ as described in (b). Labeled proteins (red) are significantly (adjusted p value ≤ 0.05) enriched SG proteins exclusively interacting with MSI1 upon PQ exposure and not in no PQ conditions.

Similar to MSI1, the protein interactome of MSI2 did not extensively change upon PQ stimulation (Figure 26 a). MSI2 interacted with 306 proteins upon exposure to PQ and 13 of these proteins exclusively interacted with MSI2 in PQ conditions (see Figure 25 for full list). Six of these interactors were nuclear proteins, involved in nucleosome structure (HIST1H1A, H1F0 and HIST1H2BA / HISTH2BB / HIST1H2BF / HIST1H2BK / HIST1H2BP), splicing (PRPF8, SNRPD1) and nuclear export (ALYREF) (full protein names indicated in Figure 25). Besides these nuclear proteins, MSI2 exclusively interacted with three ribosomal proteins (RPS17, RPS19 and RPL38), exocytosis-related protein SCIN, mitochondrial phosphate transporter SCL25A3, tight junction protein TJP1 and the cytoplasmic dynein DYNC1H1. Only three proteins (BLMH, PRPF19, RAN/RASL2-9) were significantly and exclusively bound to MSI2 in unstimulated mIMCD cells. Interestingly, BLMH and PRPF19 were also identified to exclusively interact with MSI1 in unstimulated mIMCD cells. Besides BLMH and PRPF19, in PQ conditions, MSI2 interacted exclusively with the small GTPase Ran/RASL2-9, which is involved in nucleocytoplasmic shuttling.

Similar to MSI1, MSI2 interacted with many SG-associated proteins (107) already in unstimulated mIMCD cells (Figure 26 b). The MSI2-associated SG network did not change substantially upon PQ stimulation (109) (Figure 26 c). Analysis of the SG proteins bound to MSI2 within the respective cellular condition (no PQ or PQ) revealed that RAN was a SG protein, exclusively bound by MSI2 in unstimulated mIMCD cells (Figure 26 b). In contrast, H1F0 and RPS19 were SG-associated proteins bound by MSI2 exclusively after PQ stimulation of mIMCD cells (Figure 26 c).

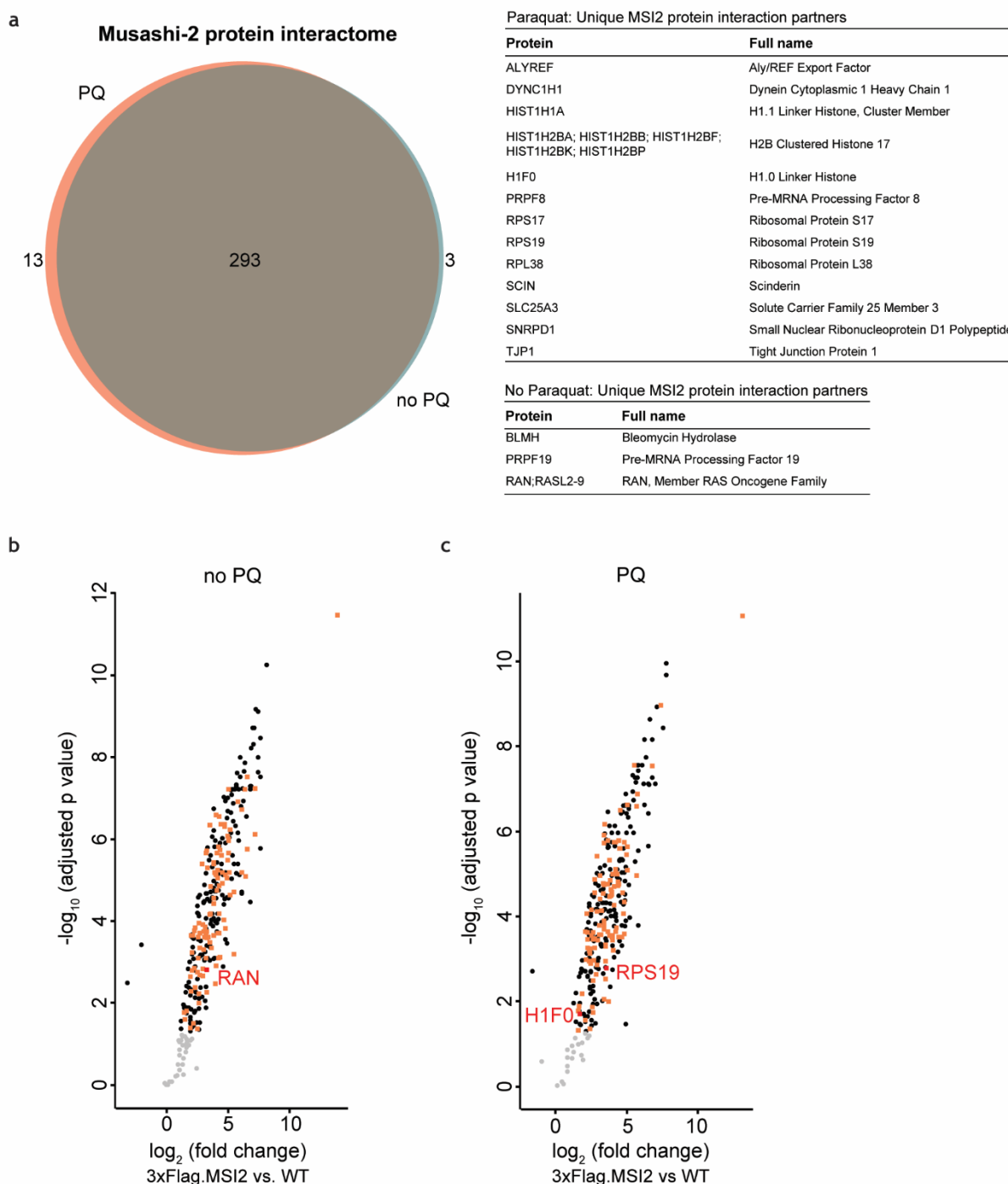


Figure 26. PQ exposure induces only mild changes to the MSI2 protein interactome

a| Comparison of MSI2 interactomes upon PQ stimulation vs. unstimulated conditions reveals no major changes in MSI2 protein interactome. The majority of MSI2 interacting proteins were simultaneously identified under PQ exposure and in no PQ conditions (293 proteins). 13 proteins (ALYREF, DYNC1H1, HIST1H1A, HIST1H2BA/HIST1H2BB/HIST1H2BF/HIST1H2BK/HIST1H2BP, H1F0, PRPF8, RPS17, RPS19, RPL38, SCIN, SLC25A3, SNRPD1, TJP1) were identified to specifically interact with MSI2 under PQ exposure and three proteins interacted with MSI2 exclusively in no PQ conditions (BLMH, PRPF19, RAN/RASL2-9). Visualized is the overlap between protein interactors of MSI2 under PQ exposure vs. no PQ exposure as Venn diagram (left). The tables on the right depict proteins uniquely identified as MSI2 interactors in PQ (top) and no PQ (bottom).

continued on next page

b| MSI2 interacts with SG proteins in no PQ conditons. One third (107 proteins) of the identified MSI2 protein interactors (296 proteins) were previously identified in SGs, indicating that similar to MSI1, MSI2 is already part of a SG protein network under unstressed conditions in mIMCD cells. RAN significantly interacted with MSI2 in control conditions and was not significantly enriched in the MSI2 interactome after PQ exposure. Shown is a scatter plot visualizing all proteins identified via mass spectrometry after IP with M2 Flag antibody in 3xFlag.MSI2 vs wild type mIMCD cells in no PQ conditions. The $\log_2$ fold change (3xFlag.MSI2 vs. WT) is plotted on the x-axis and $-\log_{10}$ adjusted p value is plotted on the y-axis. Visualized are non-significant proteins (gray), significantly (adjusted p value ≤ 0.05) enriched proteins (black) and SG proteins (orange). Labeled proteins (red) are significantly enriched SG proteins exclusively interacting with MSI2 in no PQ conditions and not after PQ exposure.

c| PQ exposure does not induce major changes within the MSI2 SG protein network. Upon PQ exposure, MSI2 bound to a similar group of SG proteins (109) as observed in no PQ conditions (107). Only the proteins H1F0 and RPS19 were significantly enriched in the MSI2 interactome upon PQ compared to control conditions (no PQ). Shown is a scatter plot visualizing all proteins identified via mass spectrometry after IP with M2 Flag antibody in 3xFlag.MSI2 vs. wild type mIMCD cells after exposure to PQ, as described in (b).

3.7.3 Comparison of MSI1 and MSI2 protein interactomes in tubular epithelial cells at baseline and upon oxidative stress

Comparison of the interactomes of MSI1 and MSI2 in unstimulated mIMCD cells revealed that both Musashi proteins shared a majority of the identified protein interactors (284) (Figure 27 a). While MSI1 bound to 133 proteins uniquely, MSI2 only interacted with 11 proteins, independently of MSI1, in unstimulated mIMCD cells (see Figure 26 for full list). Within these 11 proteins, six proteins were involved in cellular adherence and motility (CTNNA1, CTNNB1, MYH14, NES, SPTAN1 and TMOD3) (full names are indicated in Figure 26). Moreover, MSI2 bound exclusively to two translation initiation factors (EIF3H and EIF3I), the chaperon CCT3 and the ribosomal protein RPS16. MSI2 also exclusively interacted with PCBP2, one of the two central chaperons responsible for intracellular iron storage.

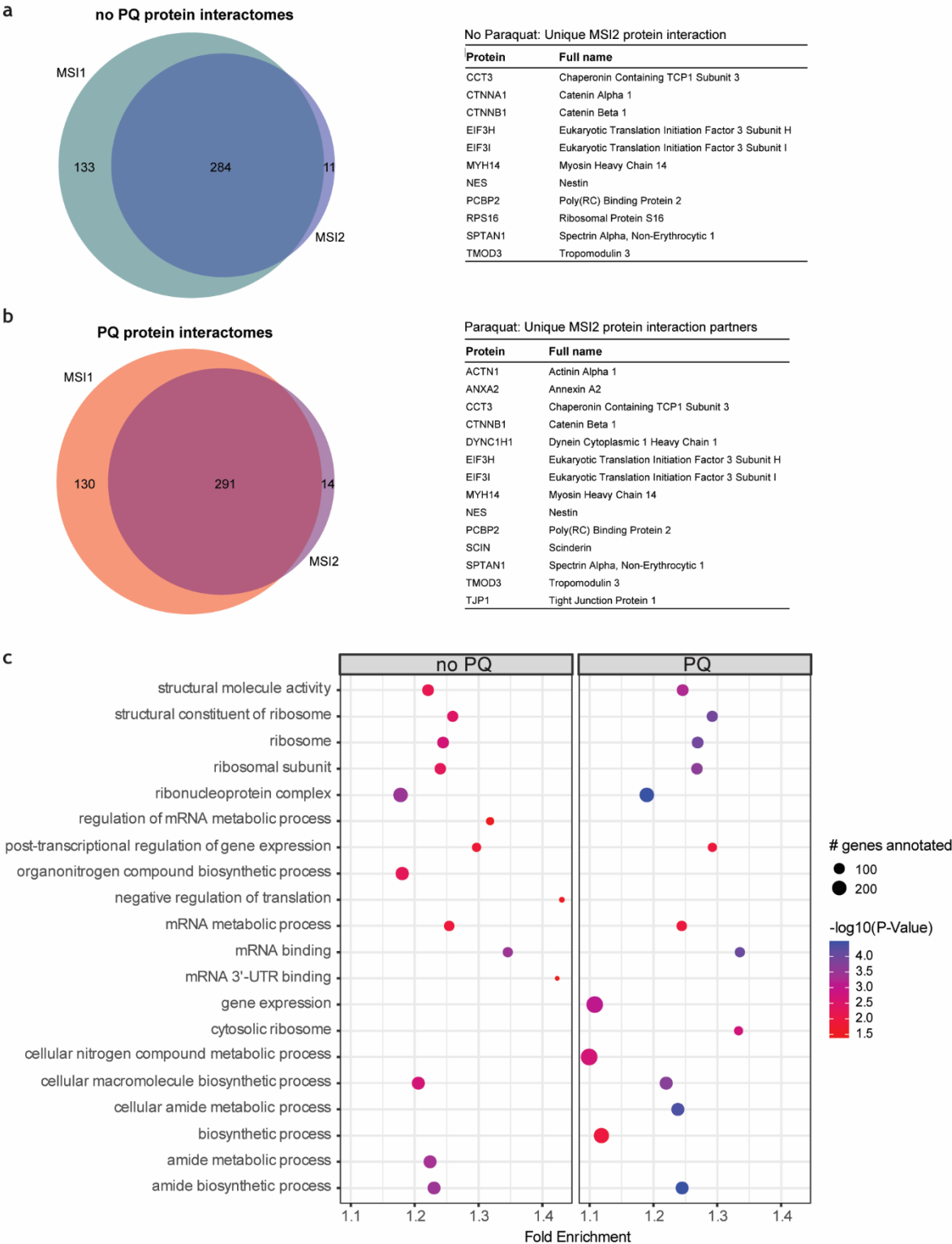


Figure 27. MSI1 interacts with more proteins than MSI2 and both Musashi proteins are part of a protein network regulating mRNA metabolism

a) MSI1 and MSI2 share the majority of their protein interacting partners in unstimulated mIMCD cells. While 284 proteins were bound to both Musashi proteins, MSI1 uniquely interacted with 133 additional proteins that were not identified within the MSI2 interactome under control conditions. 11 proteins were individually bound by MSI2 (CCT3, CTNNA1, CTNNB1, EIF3H, EIF3I, MYH14, NES, PCBP2, RPS16, SPTAN1 and TMOD3).

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Visualized is the overlap between protein interactors of MSI1 and MSI2 under no PQ conditions as Venn diagram (left). The table on the right depicts proteins uniquely identified as MSI2 interactors in unstimulated conditions. **b) Exposure to PQ has no major influence on the overlap of MSI1 and MSI2 interactomes.** After exposure to PQ, MSI1 bound to 130 proteins uniquely and shared 291 protein interaction partners with MSI2. 14 proteins were bound by MSI2 alone (ACTN1, ANXA2, CCT3, CTNNB1, DYNC1H1, EIF3H, EIF3I, MYH14, NES, PCBP2, SCIN, SPTAN1, TMOD3 and TJP1). Visualized is the overlap between protein interactors of MSI1 and MSI2 after PQ as Venn diagram (left). The table on the right depicts proteins uniquely identified as MSI2 interactors in PQ stimulated mIMCD cells. **c) Gene ontology (GO) enrichment analysis of common MSI1 and MSI2 protein interactors demonstrates participation of Musashi proteins in protein network regulating mRNA metabolism and translation.** GO enrichment analysis revealed that, independent of an external stressor e.g. PQ, the common interaction partners of MSI1 and MSI2 were enriched for GO terms involved in mRNA metabolism (e.g. mRNA 3'UTR binding, mRNA metabolism), translation (e.g. ribosome, amide biosynthetic process) and complex formation (e.g. structural molecule activity, ribonucleoprotein complex). Proteins, which were identified as common interactors of MSI1 and MSI2 under no PQ conditions and after PQ exposure were subjected to GO enrichment analysis. Significantly (q value ≤ 0.05) enriched GO terms (biological process, cellular component and molecular function) were clustered using Revigo²⁴². Depicted are the Top 15 enriched, clustered GO terms for the common Musashi interactors under no PQ and PQ conditions. GO enrichment analysis was performed using the R package clusterProfiler (function enrichGO) and visualization was done using the R package GOplot and custom R and bash scripts^{233,234}.

Similar to unstimulated conditions, the protein interactomes of MSI1 and MSI2 after PQ exposure largely overlapped (291 common proteins) (Figure 27 b). MSI2 bound to 14 proteins exclusively in PQ conditions. Out of these 14 proteins, nine proteins (CCT3, CTNNB1, EIF3H, EIF3I, MYH14, NES, PCBP2, SPTAN1 and TMOD3) were already identified as unique interactors of MSI2 in unstimulated mIMCD cells (when compared to PQ), indicating that these proteins exclusively bound to MSI2 independent of PQ stimulation. Among the remaining five proteins, DYNC1H1, SCIN and TJP1 were specifically and exclusively bound by MSI2 upon PQ stimulation when compared to all other datasets. The two remaining proteins, ACTN1 and ANXA2, were also bound by MSI1 in unstimulated mIMCD cells (FIGURE 20 a). In contrast to the small group of proteins, uniquely bound by MSI2 in PQ conditions, 133 proteins solely interacted with MSI1 after PQ exposure in mIMCD cells. Six proteins (ACTA1/ACTA2/ACTC1/ACTG2, HIST2H2AA1/HIST2H2AC, EIF4B, TJP2, LAD1 and HSP90AB1) were specifically and exclusively bound by MSI1 after PQ exposure compared to all other datasets. Similarly, LMNB1 was the only protein specifically and exclusively interacting with MSI1 in unstimulated mIMCD cells.

Seeing the large overlap of proteins bound by MSI1 and MSI2 in unstimulated and PQ-stimulated mIMCD cells, it was of interest to elucidate the cellular functions of these protein networks. To characterize the functions of common protein interactors of MSI1 and MSI2, GO enrichment analysis was performed using the proteins which bound to both Musashi proteins in unstimulated (285 common proteins) and PQ-stimulated (292 common proteins) mIMCD cells (Figure 27 c). GO enrichment analysis revealed that MSI1 and MSI2 interacted with proteins involved in mRNA metabolism (e.g. mRNA binding, mRNA metabolism), translation (e.g. ribosome, amide biosynthetic process) and complex formation (e.g. structural molecule

activity, ribonucleoprotein complex) in unstimulated and PQ-stimulated mIMCD cells. The most enriched GO terms were largely overlapping between the two conditions indicating that both Musashi proteins remain within a similar protein network, regulating post-transcriptional gene expression, independent of an external stimulus *e.g.* PQ.

3.8 The effects of oxidative stress on the Musashi RNA interactome

3.8.1 Successful generation of Musashi seCLIP libraries

Although both Musashi proteins were localized in cytoplasmic SGs after stimulation of mIMCD cells with PQ, the Musashi protein network was not strongly altered through PQ-induced oxidative stress. To elucidate the role of MSI1 and MSI2 in PQ-triggered oxidative stress in mIMCD cells, it was crucial to investigate potential alterations within the RNA interactome of both proteins, induced through PQ. Firstly, a T4 polynukleotide kinase assay performed in HEK293T cells overexpressing 3xFlag.MSI1 and 3xFlag.MSI2, confirmed that both Musashi proteins bind RNA (Figure 28 a). Secondly, single-end enhanced crosslinking and immunoprecipitation²⁰⁰ (seCLIP) was applied in unstimulated and PQ-stimulated MSI1.3xFlag and 3xFlag.MSI2 mIMCD cells to determine the RNA targets and binding sites of MSI1 and MSI2. seCLIP is based on high-throughput RNA sequencing of crosslinked RNA in IP samples (M2 Flag antibody IP in MSI1.3xFlag or 3xFlag.MSI2 mIMCD cell lines) and size-matched input (SMInput) samples (2% of IP). To prepare high quality seCLIP libraries, isolated RNA was reverse transcribed into cDNA and a pilot PCR was performed to determine the optimal amount of amplification cycles. Pilot PCR was successfully performed on SMInput and IP samples of MSI1.3xFlag (Figure 28 b) and 3xFlag.MSI2 (Figure 28 c) mIMCD cell lines, in unstimulated (no PQ) and PQ-stimulated (PQ) condition. In summary, four seCLIP libraries for MSI1 and four seCLIP libraries for MSI2 were generated. These libraries consisted of two replicates of SMInput and IP samples in PQ and no PQ conditions (seCLIP libraries for one replicate are shown in Figure 28 d). seCLIP libraries were subjected to high-throughput sequencing and IP samples were compared to SMInput samples to identify significantly enriched binding sites. Principal component analysis demonstrated clustering of the different samples and replicates, indicating high reproducibility of the identified binding targets within the experimental groups (Figure 28 d). Moreover, the well-clustered samples indicate differences in binding sites between MSI1 and MSI2 and no PQ vs. PQ conditions.

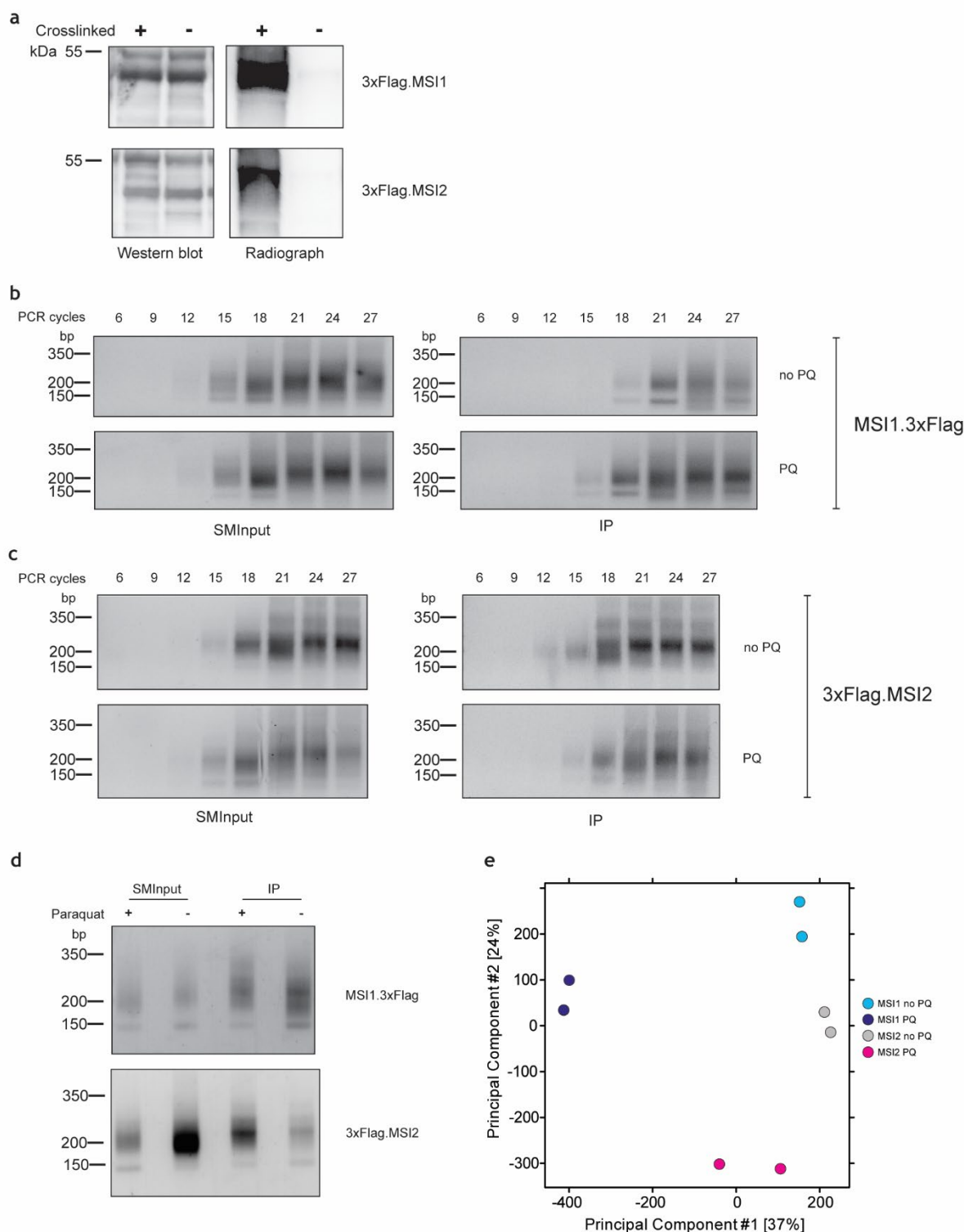


Figure 28. Successful verification of RNA binding properties of Musashi proteins and generation of seCLIP libraries

a| Confirmation of RNA binding by both Musashi proteins using T4 polynucleotide kinase (PNK) assay. Crosslinking allowed the detection of RNA covalently bound to overexpressed 3xFlag.MSI1 and 3xFlag.MSI2 proteins in HEK293T cells. Shown are western blots (left) and radiographs (right). The protein gel was loaded with lysates from HEK293T cells that were either transfected with 3xFlag.MSI1 pCDNA6 (top) or 3xFlag.MSI2 pCDNA6 (bottom). *continued on next page*

While 3xFlag.MSI1 (42 kDa) and 3xFlag.MSI2 (39 kDa) were expressed in crosslinked and non-crosslinked samples, radioactively labeled RNA was only detected in crosslinked samples at the same height as the corresponding proteins, confirming that 3xFlag.MSI1 and 3xFlag.MSI2 bound to RNA in HEK293T cells. **b| Determination of optimal amplification cycles for seCLIP library generation from RNA bound by MSI1 in mIMCD cells.** Based on the visualized pilot PCR the optimal amount of cycles used for the amplification of size-matched input (SMInput) controls was 14 (no PQ) and 13 (PQ). cDNA from IPs was amplified with 17 (no PQ) and 14 (PQ) cycles. In a pilot PCR, a range of 6-27 PCR cycles was used to amplify cDNA generated for SMInput controls (left) and IP samples (right). These samples were generated by IP with M2 Flag antibody from MSI1.3xFlag mIMCD cells, either in no PQ conditions (top) or after PQ exposure (bottom), using the seCLIP protocol²⁰⁰. PCR products of the pilot PCR were analyzed by agarose gel electrophoresis. Ideal cDNA libraries appeared as a smeared band between ~175 and 350 bp. **c| Determination of optimal amplification cycles for seCLIP library generation from RNA bound by MSI2 in mIMCD cells.** Based on the visualized pilot PCR the optimal amount of cycles used for the amplification of SMInput control cDNA was 14 (no PQ and PQ). cDNA from IPs was amplified with 15 (no PQ) and 16 (PQ) cycles. In a pilot PCR, a range of 6-27 PCR cycles was used to amplify cDNA generated for SMInput controls (left) and IP samples (right). These samples were generated by IP with M2 Flag antibody from 3xFlag.MSI2 mIMCD cells, either in control conditions (no PQ, top) or after PQ exposure (PQ, bottom), using the seCLIP protocol²⁰⁰. PCR products of the pilot PCR were analyzed by agarose gel electrophoresis. Ideal cDNA libraries appeared as a smeared band between ~175 and 350 bp. **d| Successful generation of cDNA libraries from RNA bound by MSI1 and MSI2 in mIMCD cells under control conditions and PQ exposure.** cDNA libraries were generated for SMInput and IP samples from RNA bound by MSI1 (top) and MSI2 (bottom) in mIMCD cells under control conditions (-) and after PQ stimulation (+). The optimal amount of amplification cycles was determined by pilot PCR as described above. Successful generation of the final cDNA libraries was analyzed via agarose gel electrophoresis. Ideal cDNA libraries appeared as a smeared band between ~175 and 350 bp. cDNA libraries were excised from the gel, DNA was purified and submitted to single-end high-throughput sequencing. **e| Evaluation of MSI1 and MSI2 seCLIP libraries displays good sample quality.** Principal component analysis (PCA) showed clustering of MSI1 and MSI2 seCLIP libraries, demonstrating high reproducibility of the resulting binding targets within the experimental groups. Depicted are two replicates per cell line (MSI1.3xFlag and 3xFlag.MSI2) and condition (PQ and no PQ). Shown are read counts in significant peaks (IP vs. SMInput) normalized to full library size.

3.8.2 Characterization of RNA targets of MSI1 in tubular epithelial cells at baseline and upon oxidative stress

MSI1 interacted with a similar number of binding sites in two biological replicates of unstimulated mIMCD cells (10570 common binding sites), as determined through comparison of IP and SMInput samples (Figure 29 a). In PQ-stimulated mIMCD cells, MSI1 interacted with 6530 binding sites when comparing the overlap of the two biological replicates. In total, 17922 binding sites were enriched in unstimulated conditions, and 17399 binding sites were enriched in PQ-stimulated conditions.

Analysis of the distribution of binding sites identified for MSI1 in unstimulated mIMCD cells revealed predominant binding to 3'UTRs (70.1%) in target RNAs (Figure 29 b). Together with introns (22.3%), 3'UTRs made up over 90% of the target binding regions of MSI1 in no PQ conditions. After exposure to PQ, MSI1 bound more frequently to 3'UTRs (83.1%) in target RNAs and binding to introns was markedly decreased (3.0%). Moreover, PQ increased the binding of MSI1 to exons from 1.4% in no PQ conditions to 5.4%.

Binding motif analyses revealed that MSI1 preferably bound to a core motif containing the bases UAG in unstimulated MSI1 mIMCD cells (Figure 29 c). When binding to 3'UTRs, exons

and introns, the UAG trinucleotide was the predominant sequence bound by MSI1, as it was identified in >50% of the binding sites. Overall, MSI1 bound to 35% of all targets via the UAG trinucleotide in no PQ conditions. Upon PQ stimulation, MSI1 continued to bind to 3'UTRs, exons and introns via UAG, but binding sites containing the UAG trinucleotide were less frequently bound compared to no PQ conditions. Moreover, across all binding sites, UAG was lost as the predominant sequence motif upon PQ treatment. The identified top binding motif was a more random sequence, which was detected in 10% of all binding sites. Altogether, these data suggest that PQ causes MSI1 to bind more frequently to 3'UTRs and exons and that the preferred trinucleotide UAG is less frequently bound by MSI1 within these binding sites.

Next, the identified binding sites were assigned to the corresponding RNAs. MSI1 bound to 5264 different RNAs in unstimulated mIMCD cells and MSI1 interacted with 4178 transcripts after exposure to PQ (Figure 29 d). A majority of these transcripts (3327) was bound by MSI1 in both conditions.

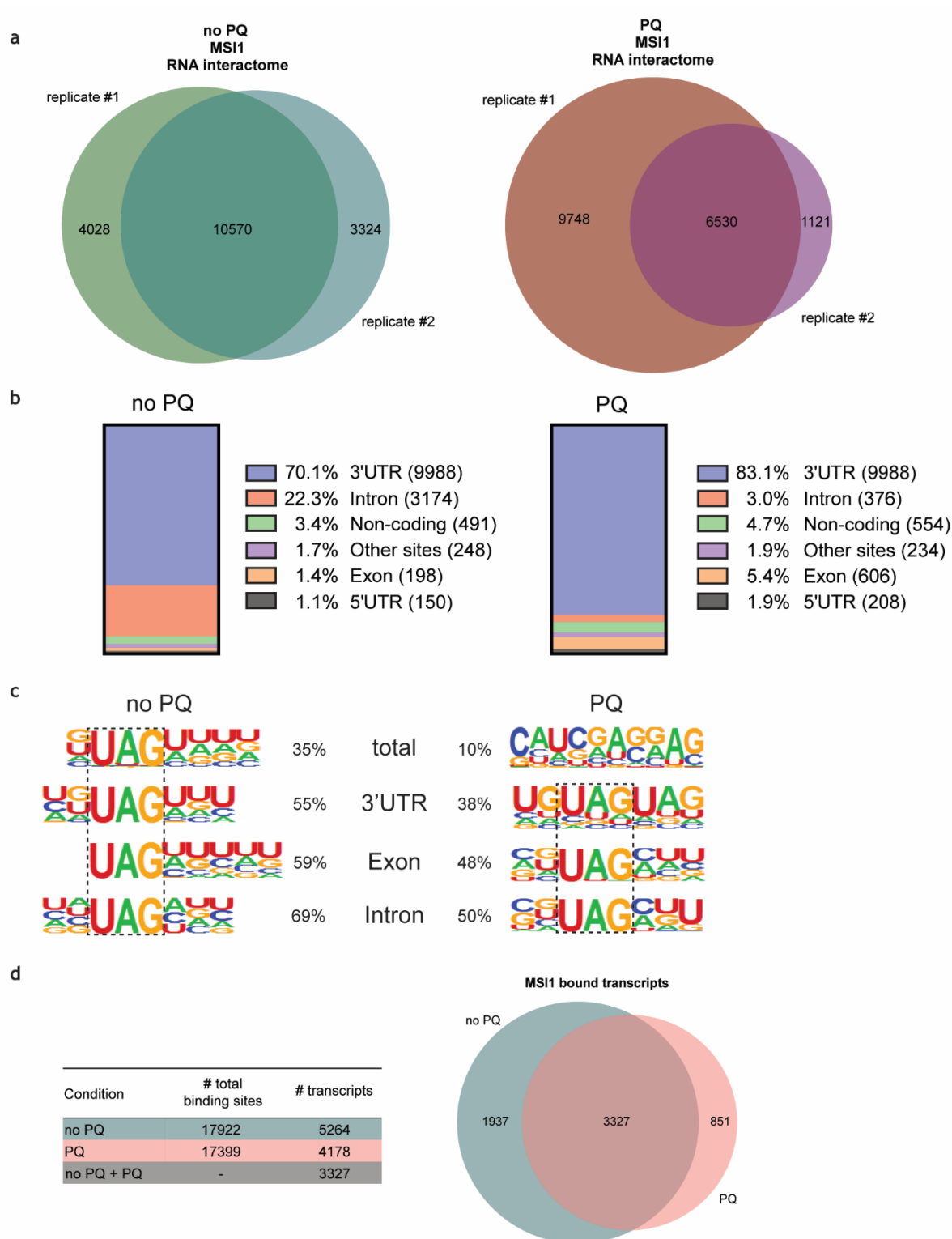


Figure 29. Characterization of MSI1 RNA interactome

a) Overlap of significant binding sites in no PQ and PQ MSI1 seCLIP libraries. In no PQ MSI1 seCLIP libraries the two replicates showed a major overlap in significantly enriched binding sites (10570 common binding sites) (IP vs. SMIInput). In PQ MSI1 seCLIP libraries replicate #2 (7651) showed a markedly smaller amount of significant binding sites compared to replicate #1 (16278). Analysis of the seCLIP libraries was supported by an experienced bioinformatician. Peak calling was performed as previously described²⁰⁰ and peaks were annotated with corresponding binding sites using HOMER (annotatePeaks.pl)²⁴⁰. Binding sites identified in IP samples were compared to SMIInput samples.

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The overlap of significantly enriched binding sites between the two replicates within the two conditions (no PQ and PQ) is visualized as Venn diagram. **b| PQ increases the binding of MSI1 to 3'UTR and exons within target RNAs.** Binding site analyses of MSI1 seCLIP libraries revealed that MSI1 increasingly bound to 3'UTR and exons within transcripts after exposure to PQ. Upon PQ treatment, binding to intronic RNA regions was markedly decreased when compared to untreated control conditions (no PQ). Depicted as stacked bar chart is the average proportion of each binding site bound by MSI1 within the two replicates in no PQ (left) and PQ (right). The average percentage of significantly enriched binding sites over SMIInput is listed on the right with average binding site numbers indicated in brackets. **c| Analyses of the MSI1 binding motifs reveals UAG as predominant bound trinucleotide in target RNAs.** In no PQ conditions, UAG was the core trinucleotide bound by MSI1 in the 3'UTR, exons, introns and across all binding sites in target RNAs (total). Similarly, UAG was identified within the top binding motifs of MSI1 in 3'UTR, introns and exons of bound transcripts upon PQ stimulation of mIMCD cells. Interestingly, UAG as the core trinucleotide bound by MSI1, was not identified in the top motif across all binding sites (total) after PQ stimulation. Significantly enriched binding motifs across the indicated binding sites were identified and visualized using HOMER²⁴⁰ (findMotifsGenome.pl with the option rna). Represented is the most significantly enriched, top binding motif (rank 1) of MSI1 within the indicated binding sites in one replicate per condition (PQ and no PQ). When present, the core UAG trinucleotide is highlighted with a dashed line within the binding motifs. Listed percentages show the fraction of binding sites containing the indicated binding motif. **d| MSI1 binds to distinct sets of transcripts under all conditions.** MSI1 bound to more transcripts under no PQ conditions (5264) compared to PQ exposure (4178) and 3327 transcripts were bound by MSI1 in both conditions. Depicted is the comparison of the all MSI1 bound transcripts (both replicates) under no PQ and PQ as Venn diagram. Importantly, multiple significantly enriched binding sites can be contained in one single RNA, explaining the strong reduction from binding sites to transcript numbers. Significantly enriched binding sites and corresponding transcripts are summarized in a table on the left.

3.8.3 Characterization of RNA targets of MSI2 in tubular epithelial cells at baseline and upon oxidative stress

MSI2 interacted with a similar number of binding sites when comparing two biological replicates of unstimulated mIMCD cells (10198 common binding sites) (Figure 30 a). The amount of significantly enriched binding sites within the first replicate (16386) was larger compared to the second replicate (12732). MSI2 interacted with a total of 18920 binding sites in unstimulated mIMCD cells. In comparison, MSI2 bound to a slightly smaller number of binding sites in PQ-stimulated mIMCD cells (16067 binding sites identified in total). MSI2 interacted with 7721 binding sites within both biological replicates of PQ-stimulated mIMCD cells. However, in the first PQ replicate, more binding sites (14277) were significantly enriched compared to the second replicate (9511).

Binding site analysis revealed that MSI2 predominantly bound to 3'UTRs (67%) and introns (25.1%) within target RNAs in unstimulated mIMCD cells (Figure 30 b). PQ induced increased binding of MSI2 to 3'UTRs (75.7%) and exons (3.1% in PQ compared to 1.1% in no PQ) and led to reduced binding of MSI2 to introns (13.7%).

MSI2 primarily bound the UAG trinucleotide in target RNAs (Figure 30 c). UAG was the preferred binding site of MSI2 in approximately every second 3'UTR, exon and intron bound in unstimulated conditions. Overall, 24% of all binding sites possessed the core UAG trinucleotide in unstimulated MSI2 mIMCD cells. MSI2 bound to the UAG sequence with similar frequencies

upon exposure to PQ. Only introns were bound less frequently at the UAG site (40% in PQ compared to 55% in no PQ).

In summary, alike MSI1, MSI2 primarily interacted with UAG and PQ caused MSI2 to bind less to introns (25.1% in no PQ and 13.7% in PQ) and more frequently to exons (1.1% in no PQ and 3.1% in PQ). Identification of the transcripts belonging to the significantly enriched binding sites (overlap of both replicates) revealed that MSI2 bound to 5318 distinct RNAs in no PQ conditions (Figure 30 d). After PQ stimulation, MSI2 bound to 4514 RNAs, highlighting that similar to MSI1, MSI2 bound to more transcripts in unstimulated conditions compared to PQ conditions.

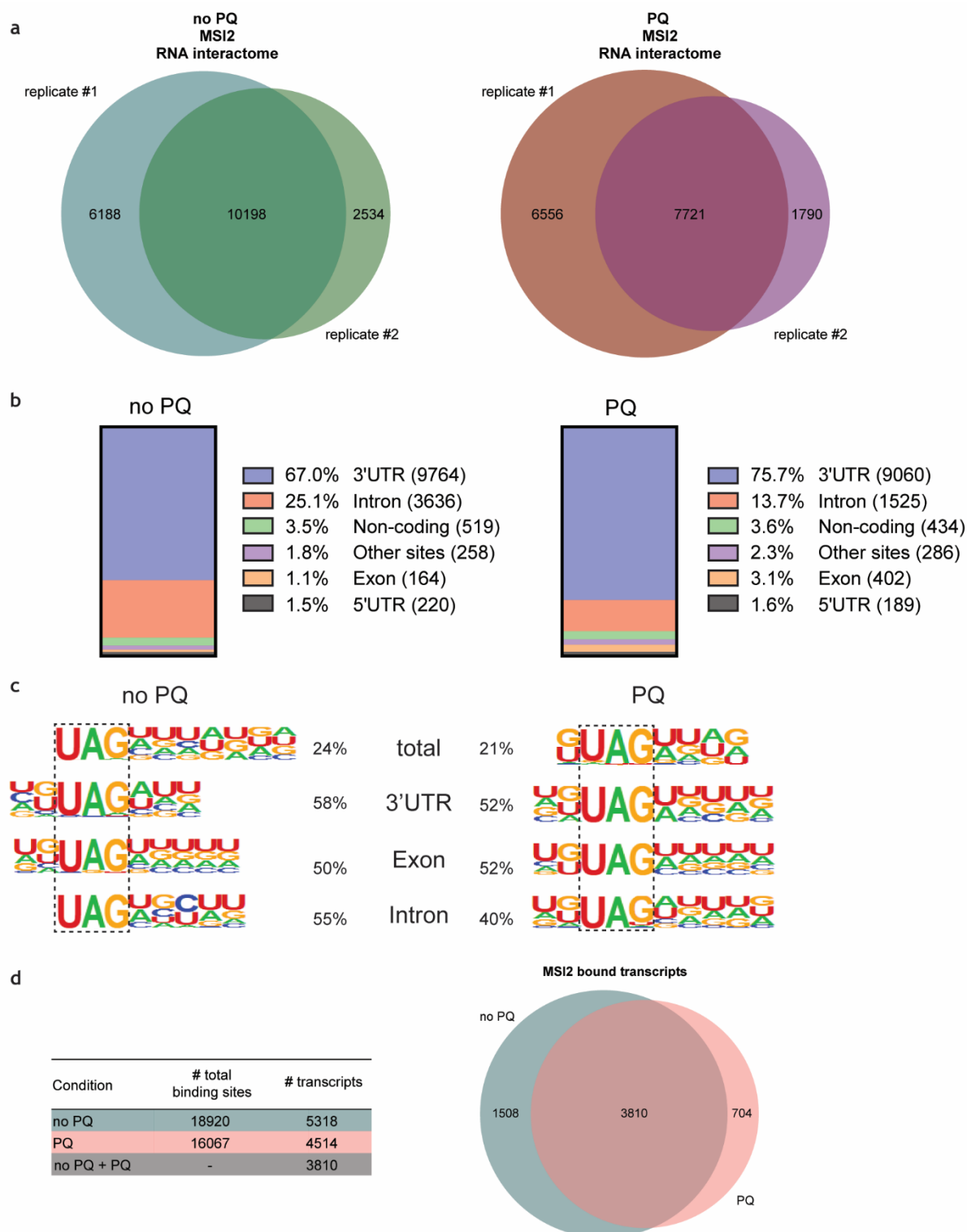


Figure 30. Characterization of MSI2 RNA interactome

a| Overlap of significant binding sites in no PQ and PQ MSI2 seCLIP libraries. In MSI2 seCLIP libraries, generated in unstimulated conditions, the two replicates showed a major overlap in significantly enriched binding sites (10198 common binding sites) (IP vs. SMIInput). The overlap of significant binding sites between the two MSI2 seCLIP libraries after PQ exposure was smaller (7721 common binding sites) compared to no PQ conditions. Analysis of the seCLIP libraries was supported by an experienced bioinformatician.

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Peak calling was performed as previously described²⁰⁰ and peaks were annotated with corresponding binding sites using HOMER (annotatePeaks.pl)²⁴⁰. Binding sites identified in IP samples were compared to SMInput samples. The overlap of significantly enriched binding sites between the two replicates within the two conditions (no PQ and PQ) is visualized as Venn diagram. **b| PQ increases the binding of MSI2 to 3'UTR and exons of target RNAs.** Similar to MSI1, binding site analysis of MSI2 seCLIP libraries revealed that MSI2 increasingly bound to 3'UTR and exons of target RNAs after exposure to PQ. Also, upon PQ treatment, binding to intronic RNA regions was markedly decreased when compared to untreated control conditions (no PQ). Depicted as stacked bar chart is the average proportion of each binding site bound by MSI2 within the two replicates in no PQ (left) and PQ (right). The average percentage of significantly enriched binding sites over SMInput is listed on the right with average binding site numbers indicated in brackets. **c| Analysis of the MSI2 binding motifs reveals UAG as predominant bound trinucleotide in target RNAs.** Similar as MSI1, UAG was identified as the core trinucleotide bound by MSI2 in the 3'UTR, exons, introns and over all binding sites in target transcripts (total) in both conditions. In contrast to MSI1, UAG remained part of the top binding motif across all bound RNAs (total) after PQ stimulation. Significantly enriched binding motifs across the indicated binding sites were identified and visualized using HOMER²⁴⁰ (findMotifsGenome.pl with the option rna). Represented is the top binding motif (rank 1) of MSI2 within the indicated binding sites in one replicate per condition (PQ and no PQ). The core UAG trinucleotide is highlighted with a dashed line within the binding motifs. Listed percentages show the fraction of binding sites containing the indicated binding motif. **d| MSI2 binds to distinct sets of transcripts under all conditions.** Similar to MSI1, MSI2 bound to more RNAs under no PQ conditions (5318) compared to PQ exposure (4514) and 3810 transcripts were bound by MSI2 in both conditions. Depicted are all MSI2-bound transcripts under no PQ and PQ (both replicates) as Venn diagram. Importantly, multiple significantly enriched binding sites can be contained in one single RNA, explaining the strong reduction from binding sites to transcript numbers. Significantly enriched binding sites and corresponding transcripts are summarized in a table on the left.

3.8.4 Comparison of MSI1 and MSI2 RNA interactomes in tubular epithelial cells at baseline and upon oxidative stress

As mentioned before, both Musashi proteins are highly similar and have been found to bind to a largely, but not completely, overlapping group of target RNAs in different cell types^{179,184,191–198}. In line with these observations, the RNA interactomes of MSI1 and MSI2 largely overlapped (4207 common transcripts) in unstimulated mIMCD cell lines (Figure 31 a). Both Musashi proteins uniquely bound to a smaller set of RNAs in no PQ conditions (MSI1: 1057 RNAs; MSI2: 1111 RNAs). After exposure to PQ, both Musashi proteins interacted with 3103 common transcripts (Figure 31 b). MSI2 bound to a larger set of RNAs uniquely (1411 RNAs) compared to MSI1 (1075 RNAs) in PQ conditions. These results implicate that the RNA interactomes of both Musashi proteins could be altered in response to external stimuli e.g. oxidative stress induced through PQ. We therefore aimed to investigate how PQ influences the composition of the RNA interactome of MSI1 and MSI2 compared to unstimulated conditions.

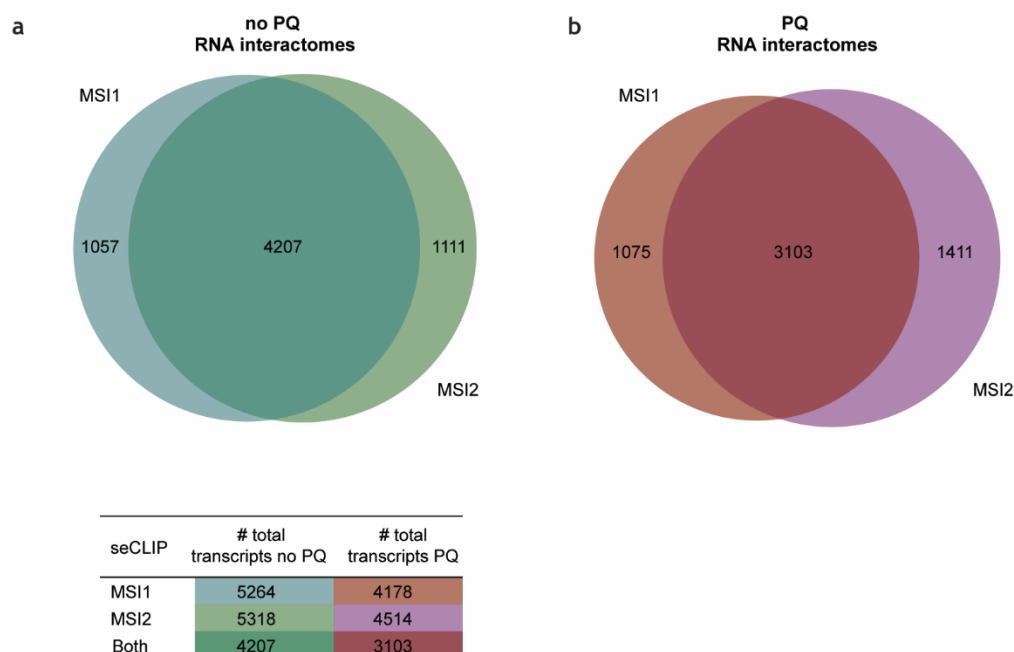


Figure 31. Both Musashi proteins bind to largely overlapping sets of RNAs at baseline and upon oxidative stress

a| In unstimulated mIMCD cells, MSI1 and MSI2 bind to a largely, but not completely, overlapping set of target RNAs. Both Musashi proteins bound to a common set of 4207 transcripts under no PQ conditions. MSI1 (1057) and MSI2 (1111) bound to a similar amount of RNAs uniquely. Depicted is the comparison of the total number of MSI1- and MSI2-bound transcripts in unstimulated mIMCD cells (both replicates, no PQ) as Venn diagram. The number of transcripts bound by MSI1 in each condition is summarized in the table below. **b| After PQ stimulation in mIMCD cells MSI2 binds more RNAs uniquely compared to MSI1.** While MSI2 bound to 1411 RNAs uniquely under PQ conditions, MSI1 only interacted with 1075 transcripts exclusively. Both Musashi proteins bound to an overlapping set of 3103 transcripts after PQ exposure. The number of transcripts bound by MSI1 and MSI2 in each condition is summarized in the table below.

3.8.5 Defining alterations within the RNA interactome of MSI1 induced by oxidative stress

3.8.5.1 Identifying alterations in RNA binding sites bound by MSI1 upon oxidative stress

To examine alterations within the MSI1 RNA interactome induced upon PQ-mediated oxidative stress, the abundance of binding sites detected after PQ exposure was compared to their abundance in unstimulated mIMCD cells (Figure 32 a). Binding sites with a positive fold change are enriched, indicating that MSI1 interacted more with these binding sites in PQ-stimulated compared to unstimulated cells. In contrast, binding sites with a negative fold change are under-represented in PQ-stimulated conditions, meaning that MSI1 interacted less with these binding sites in PQ-stimulated cells.

Differences in the interaction of MSI1 to certain binding sites upon PQ treatment can have two explanations: (1) altered transcript availability due to expression changes of the target RNA or

(2) altered binding of MSI1 to the target RNAs as a consequence of PQ stimulation. Consequently, we added differential gene expression analysis using whole-cell transcriptomes of unstimulated and PQ-stimulated wild type mIMCD cells to determine PQ-induced changes of transcript abundance (Figure 32 b). Importantly, we excluded all differentially expressed MSI1-bound transcripts, independent of the direction of regulation.

Using this approach 881 binding sites were identified, which were enriched upon PQ treatment and of which the expression of binding-site carrying transcripts was unaltered upon PQ exposure in wild type cells (visualized in red in Figure 32 a). 377 binding sites, which were under-represented after PQ stimulation, belonged to RNAs the abundance of which remained unchanged through PQ treatment in wild type cells (visualized in green in Figure 32 a). Further analyses were restricted to these binding sites and they are referred to as enriched upon PQ treatment (PQ^{up}) and under-represented upon PQ treatment (PQ^{down}) hereafter. In summary, twice as many MSI1 binding sites were enriched in PQ conditions compared to binding sites, which were under-represented upon PQ treatment

Binding site analysis showed that MSI1 primarily interacted with 3'UTRs (53.4%), exons (22.8%) and introns (10.3%) in those RNAs, which were under-represented in PQ-stimulated conditions (Figure 32 c). Similarly, PQ enriched target sites, were mainly found in 3'UTRs (61.9%), exons (19.5%) but in contrast, the interaction with introns was slightly decreased (6.0%) (Figure 32 c). In comparison, in general (IP vs. SMInput), MSI1 predominantly bound to 3'UTRs in no PQ (70.1%) and PQ conditions (83.1%) , and only a small amount of binding sites laid in exons (no PQ: 1.4%, PQ: 5.4%) (Figure 29 b). In summary, these findings demonstrate that altered binding of MSI1 in response to PQ stimulation was accompanied by increased binding of exons, compared to the low binding of MSI1 to exons, which was observed in general.

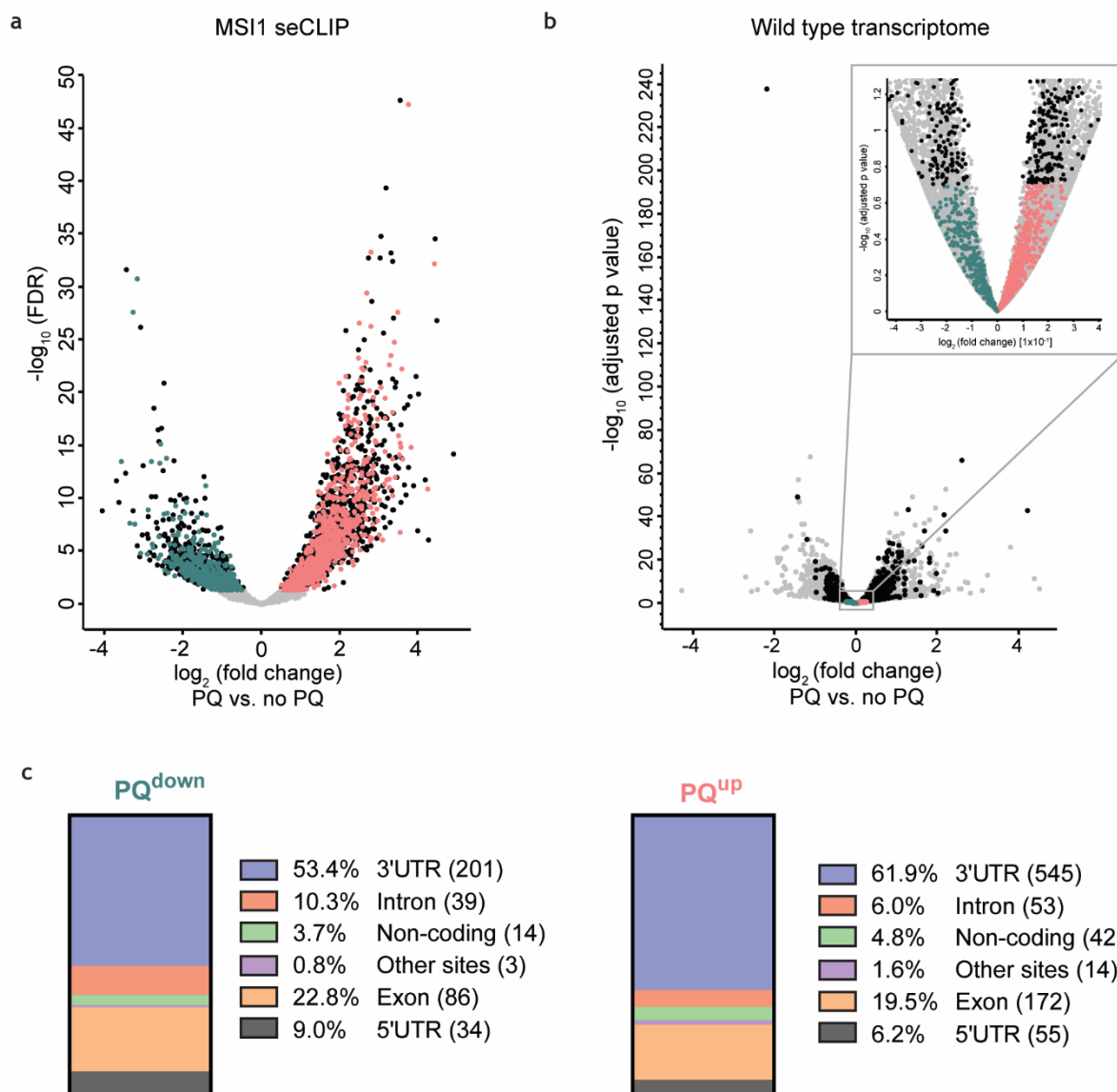


Figure 32. MSI1 binding site interactions are altered upon PQ stimulation

a) seCLIP based identification of changes in MSI1 binding site preferences upon PQ treatment.

While 881 binding sites were identified to be enriched in PQ conditions (PQ^{up}), 377 binding sites were under-represented in PQ conditions (PQ^{down}). PQ^{up} and PQ^{down} binding sites are binding sites in which expression of binding-site containing transcripts was not differentially regulated by PQ treatment in wild type mIMCD cells (see (b) for more information). Shown is a scatter plot visualizing the differential binding site analysis of MSI1 seCLIP libraries, comparing significantly enriched binding sites under PQ exposure to no PQ conditions. The log₂ fold change (PQ vs. no PQ) is plotted on the x-axis and -log₁₀ false discovery rate (FDR) is plotted on the y-axis. Highlighted are non-significant binding sites (gray) and significantly enriched binding sites, in which expression of binding site-containing transcripts was differentially regulated by PQ stimulation in wild type mIMCD cells (black). Colorized are significantly enriched binding sites, in which the expression of binding site-containing transcripts was not altered upon PQ stimulation of wild type mIMCD cells. Interaction of MSI1 to these binding sites was increased upon PQ stimulation (red) or decreased upon PQ stimulation (green) in mIMCD cells. Differential binding was assessed using the R package DiffBind²⁴¹. **b) Differential gene expression analysis reveals PQ-induced alterations of RNA expression in wild type mIMCD cells.** Differential gene expression analysis was performed on RNA-seq data of PQ-stimulated and unstimulated mIMCD cells. MSI1 target sites, in which expression of binding site-containing transcripts was unaltered due to PQ stimulation were identified through applying a stringent significance cut-off (adjusted p value ≥ 0.2).

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Depicted is a scatter plot showing the differential transcript expression between PQ-stimulated and unstimulated wild type mIMCD cells. The $\log_2$ fold change (PQ vs. no PQ) is plotted on the x-axis and $-\log_{10}$ adjusted p value is plotted on the y-axis. Highlighted are non-significant transcripts (gray) and significantly dysregulated MSI1-bound transcripts (black). Colorized are non-significant MSI1-bound transcripts, enriched in PQ conditions (PQ^{down} (turquoise)) and under-represented in PQ conditions (PQ^{up} (rose)). A zoom in perspective highlights the non-significant MSI1-bound transcripts. The analysis of differential gene expression was performed using the R package DeSeq2 (version 1.26.0)^{237,238}. **c) MSI1 predominantly interacts with 3'UTRs and exons in significantly enriched PQ^{up} and PQ^{down} binding sites.** Binding sites, which were significantly under-represented in PQ-stimulated conditions (PQ^{down}) were found in over 90% in 3'UTRs (53.4%), exons (22.8%) and introns (10.3%). Similarly, 3'UTRs (61.9%), exons (19.5%) and introns (6.0%) made up over 80% of all binding site locations significantly enriched in PQ conditions (PQ^{up}). Binding sites were annotated and proportions are depicted as stacked bar chart for significantly under-represented binding sites in PQ (left) and significantly enriched binding sites in PQ (right). The percentage of respective binding sites and exact numbers (brackets) is indicated on the right. Binding sites were annotated using HOMER (annotatePeaks.pl)²⁴⁰.

3.8.5.2 Characterizing alterations within the RNA interactome of MSI1 induced upon oxidative stress

Next, the transcripts belonging to PQ^{up} and PQ^{down} MSI1 binding sites were identified and functionally characterized. Binding sites, which were under-represented upon PQ stimulation (377) belonged to 342 RNAs. Binding sites, which were enriched upon PQ stimulation (881) were assigned to 643 transcripts (Figure 33 a). The number of transcripts enriched after PQ stimulation was twice as large as compared to the number of RNAs, which were under-represented upon PQ stimulation. As described before, one MSI1-bound transcript can contain multiple binding sites (e.g. in 3'UTR, exons, introns), which were either enriched or under-represented after PQ treatment. 123 MSI1-bound transcripts were identified, which contained multiple binding sites that were either enriched or over-represented after PQ stimulation in mIMCD cells.

GO enrichment analysis of PQ^{down} transcripts revealed their involvement in chromatin remodeling (histone H2A monoubiquitination, PcG protein complex, nuclear ubiquitin ligase complex), nuclear membrane structure (nuclear membrane organization, nuclear pore) and cell division (cell division site, spindle microtubule) (Figure 33 b). Moreover, MSI1 interacted less with RNAs upon PQ stimulation that were associated with vesicular transport (transcytosis, negative regulation of calcium ion transmembrane transporter activity) and autophagy (regulation of autophagosome assembly, autophagosome membrane). Interestingly, PQ^{down} transcripts were also highly enriched for the GO terms cell redox homeostasis and cytoplasmic stress granule.

PQ^{up} MSI1-bound RNAs were largely related to DNA replication (cell cycle DNA replication, regulation of telomere maintenance via telomerase, 2'-deoxyribonucleotide metabolic process) and cell division (attachment of spindle microtubules to kinetochore, kinetochore) (Figure 33 c). Furthermore, transcripts enriched upon PQ stimulation, were connected to chromatin

remodeling (histone monoubiquitination, PcG protein complex), vesicular transport (retromer complex) and cytoskeletal rearrangement (positive regulation of filopodium assembly, myosin binding, dynein complex). Similar to PQ^{down} RNAs, “cytoplasmic stress granules” was one of the most upregulated GO terms associated with PQ^{up} transcripts.

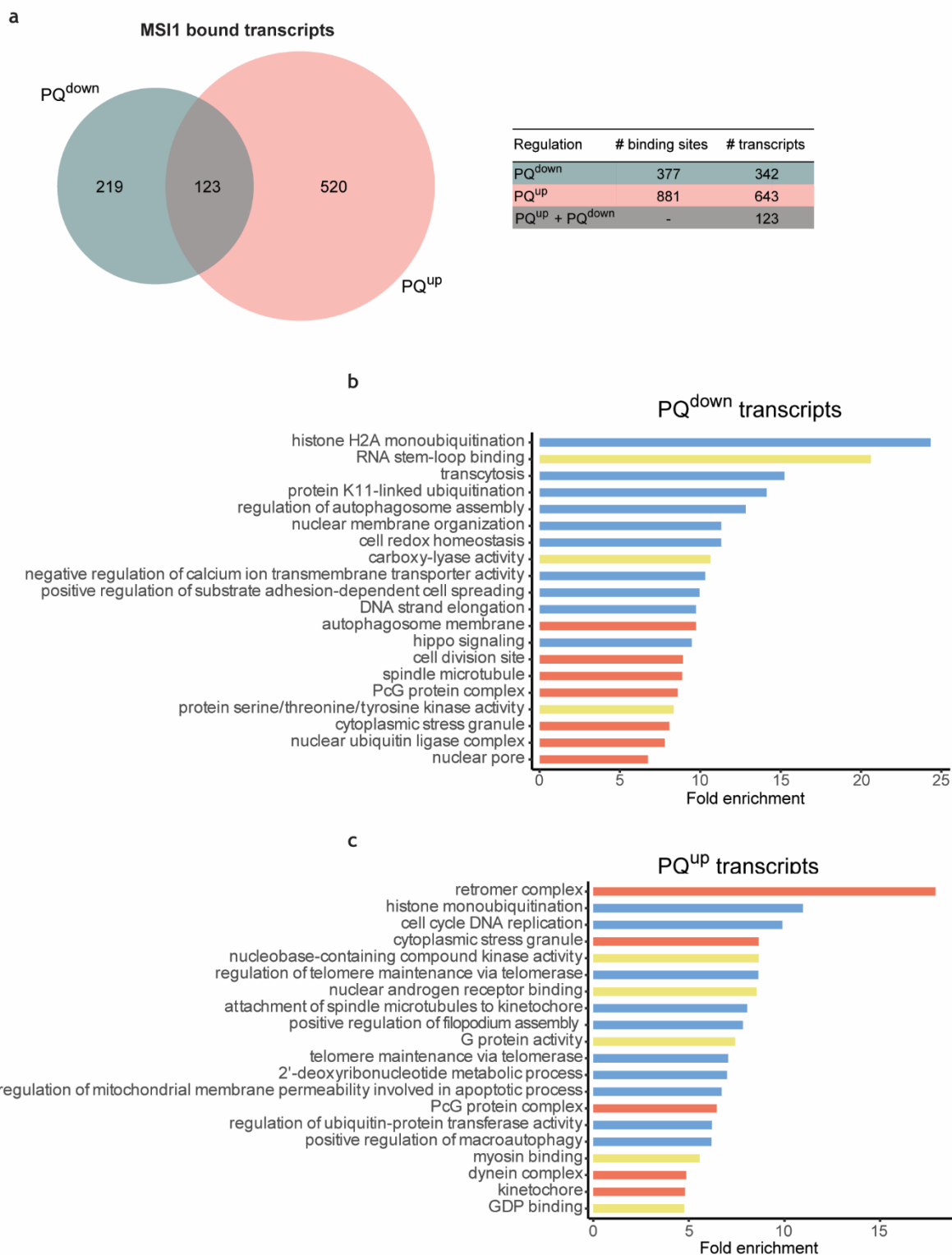


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Figure 33. Many MSI1-bound transcripts are enriched upon PQ stimulation and related to broad cellular functions

a| Twice as many MSI1-bound transcripts are enriched upon PQ stimulation compared to transcripts which are under-represented after exposure to PQ. While 643 transcripts were enriched upon PQ stimulation, 342 RNAs were under-represented after exposure to PQ in mIMCD cells. 123 transcripts contained multiple binding sites that were either enriched or under-represented upon PQ stimulation. The overlap of PQ^{up} and PQ^{down} transcripts is visualized as Venn diagram and exact numbers of PQ^{up} and PQ^{down} binding sites and corresponding transcripts are listed in the table on the right. **b| MSI1-bound RNAs, under-represented upon PQ stimulation, are involved in chromatin remodeling, cell division and nuclear membrane structure.** PQ^{down} transcripts were enriched for GO terms involved in chromatin remodeling (histone H2A monoubiquitination, PcG protein complex, nuclear ubiquitin ligase complex), nuclear membrane structure (nuclear membrane organization, nuclear pore) and cell division (cell division site, spindle microtubule). Besides these predominantly nuclear processes, RNAs under-represented after exposure to PQ, were associated with RNA binding (RNA stem-loop binding), vesicular transport (transcytosis, negative regulation of calcium ion transmembrane transporter activity) and autophagy (regulation of autophagosome assembly, autophagosome membrane). Lastly, PQ^{down} transcripts were highly enriched for the GO terms cell redox homeostasis and cytoplasmic stress granule. GO enrichment analysis on under-represented transcripts after PQ stimulation was performed using the R package clusterProfiler (function enrichGO). Enriched GO terms were filtered for biological relevant terms (excluded cell-type specific terms e.g. chondrocyte differentiation, post-synaptic development) and clustered using Revigo²⁴². Moreover, GO terms were filtered for a gene count $\geq 1\%$ of the total number of transcripts, which were used for the GO enrichment analysis. Depicted as bar graphs are the Top20 significantly enriched biological processes (blue), molecular functions (yellow) and cellular components (red). **c| MSI1-bound transcripts, enriched after PQ stimulation, are associated with DNA replication, cell division and chromatin remodeling.** PQ^{up} transcripts were associated with DNA replication (cell cycle DNA replication, regulation of telomere maintenance via telomerase, 2'-deoxyribonucleotide metabolic process) and cell division (attachment of spindle microtubules to kinetochore, kinetochore). Moreover, RNAs enriched after PQ stimulation were involved in chromatin remodeling (histone monoubiquitination, PcG protein complex), vesicular transport (retromer complex) and cytoskeletal rearrangement (positive regulation of filopodium assembly, myosin binding, dynein complex). Cytoplasmic stress granules and positive regulation of macroautophagy were also among the most enriched GO terms for PQ^{up} transcripts. GO enrichment analysis on under-represented transcripts after exposure to PQ was performed using the R package clusterProfiler (function enrichGO). Enriched GO terms were filtered and clustered as described above. Depicted as bar graphs are the Top20 significantly enriched biological processes (blue), molecular functions (yellow) and cellular components (red).

3.8.6 Defining alterations within the RNA interactome of MSI2 induced by oxidative stress

3.8.6.1 Identifying alterations in RNA binding sites bound by MSI2 upon oxidative stress

To investigate whether RNA binding of MSI2 was similarly influenced through PQ stimulation, as observed for MSI1, differential analysis on MSI2 binding sites, comparing their abundance after PQ exposure to the abundance in unstimulated conditions (see 3.8.5 for details of the analysis) was performed (Figure 34 a). Similar to MSI1, we used differential gene expression analysis on whole-cell RNA-seq data retrieved from PQ-stimulated and unstimulated wild type mIMCD cells, to identify enriched and under-represented binding sites belonging to RNAs with unchanged expression upon PQ treatment (Figure 34 b). The terminology for such binding sites is PQ^{up} for binding sites enriched upon PQ stimulation and PQ^{down} for binding sites under-represented upon PQ treatment. MSI2 is considered to interact more with PQ^{up} and less with

PQ^{down} binding sites. Using these criteria, 59 binding sites were identified as enriched upon PQ stimulation (visualized in red in Figure 34 a) and 21 binding sites were found to be under-represented after exposure to PQ (visualized in green Figure 34 a).

Analysis of PQ^{down} binding sites revealed that MSI2 primarily interacted with introns (50.8%) and exons (30.5%) and less with 3'UTRs (11.9%) (Figure 34 c). Similarly, over 80% of all PQ^{up} binding sites were found in introns (47.7%) and exons (33.3%), and 3'UTRs made up only 9.5% of all binding site locations. In comparison, binding region analysis of general MSI2 binding sites (IP vs. SMIinput) showed that MSI2 mainly interacted with 3'UTRs (no PQ: 67%, PQ: 75.7%) and introns (no PQ: 25.1%, PQ: 13.7%), and that exons (no PQ: 1.1%, PQ: 3.1%) were bound less frequently (Figure 30 b). These findings demonstrate that, similar to MSI1 (Figure 32 c) altered binding of MSI2 in response to PQ stimulation is accompanied by increased binding of exons, compared to the low binding of MSI2 to exons, which was observed in general. Moreover, PQ^{up} and PQ^{down} MSI2 binding sites were largely found in introns, while for MSI1 these binding sites mainly laid within 3'UTRs (Figure 32 c).

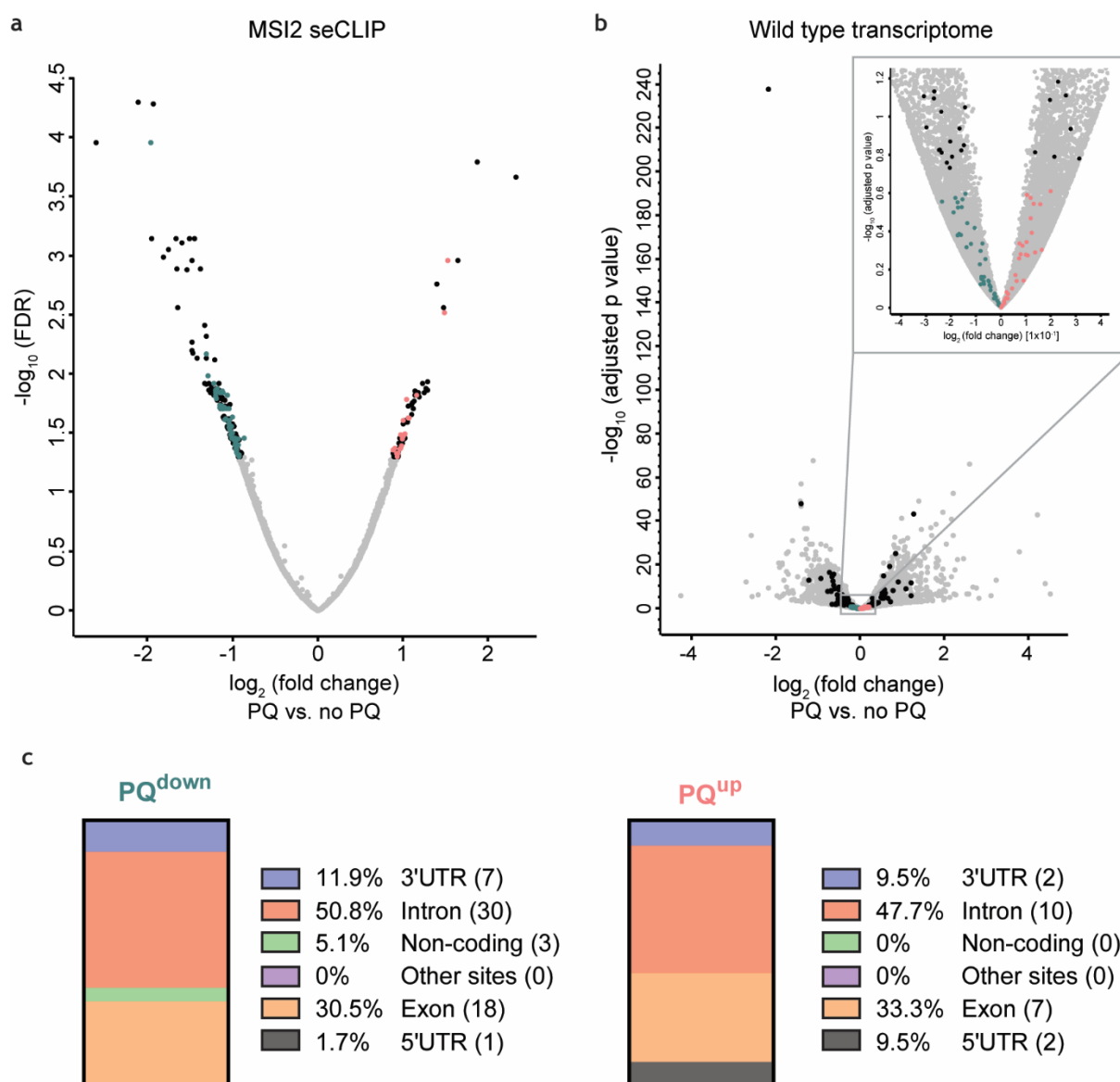


Figure 34. MSI2 binding site interactions are only slightly altered upon PQ stimulation

a) seCLIP based identification of changes in MSI2 binding site preferences upon PQ treatment.

While 21 binding sites were identified to be enriched in PQ conditions (PQ^{up}), 59 binding sites were under-represented in PQ conditions (PQ^{down}). PQ^{up} and PQ^{down} binding sites are binding sites in which expression of binding site-containing transcripts was not differentially regulated by PQ treatment in wild type mIMCD cells (see (b) for more information). Shown is a scatter plot visualizing the differential binding site analysis of MSI2 seCLIP libraries, comparing significantly enriched binding sites under PQ exposure to no PQ conditions. The log₂ fold change (PQ vs. no PQ) is plotted on the x-axis and -log₁₀ false discovery rate (FDR) is plotted on the y-axis. Highlighted are non-significant binding sites (gray) and significantly enriched binding sites, in which expression of binding site-containing transcripts was differentially regulated by PQ stimulation in wild type mIMCD cells (black). Colorized are significantly enriched binding sites, in which the expression of binding site-containing transcripts was not altered upon PQ stimulation of wild type mIMCD cells. Interaction of MSI2 to these binding sites was increased upon PQ stimulation (red) or decreased upon PQ stimulation (green) in mIMCD cells. Differential binding was assessed using the R package DiffBind²⁴¹. **b) Differential gene expression analysis reveals PQ-induced alterations of RNA expression in wild type mIMCD cells** Differential gene expression analysis was performed on RNA-seq data of PQ-stimulated and unstimulated mIMCD cells. MSI2 target sites, in which expression of corresponding transcripts was unaltered due to PQ stimulation were identified through applying a stringent significance cut-off (adjusted p value ≥ 0.2).

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Depicted is a scatter plot showing the differential transcript expression between PQ-stimulated and unstimulated wild type mIMCD cells. The $\log_2$ fold change (PQ vs. no PQ) is plotted on the x-axis and $-\log_{10}$ adjusted p value is plotted on the y-axis. Highlighted are non-significant transcripts (gray) and significantly dysregulated MSI2-bound transcripts (black). Colorized are non-significant MSI2-bound transcripts, enriched in PQ conditions (PQ^{down} (turquoise)) and under-represented in PQ conditions (PQ^{up} (rose)). A zoom in perspective highlights the non-significant MSI2-bound transcripts. The analysis of differential gene expression was performed using the R package DeSeq2 (version 1.26.0)^{237,238}. **c| MSI2 predominantly interacts with introns and exons in significantly enriched PQ^{up} and PQ^{down} binding sites.** Binding sites, which were significantly under-represented in PQ-stimulated conditions (PQ^{down}) were primarily found introns (50.8%) and exons (30.5%). Similarly, introns (47.7%) and exons (33.3%) made up over 80% of all binding site locations significantly enriched in PQ conditions (PQ^{up}). Binding sites were annotated and proportions are depicted as stacked bar chart for significantly under-represented binding sites in PQ (left) and significantly enriched binding sites in PQ (right). The percentage of respective binding sites and exact numbers (brackets) is indicated on the right. Binding sites were annotated using HOMER (annotatePeaks.pl)²⁴⁰.

3.8.6.2 Characterizing alterations within the RNA interactome of MSI2 induced upon oxidative stress

Identification of the transcripts belonging to MSI2-bound PQ^{up} and PQ^{down} binding sites showed that 50 RNAs were enriched after PQ stimulation and 20 transcripts were under-represented after PQ exposure in mIMCD cells (Figure 35 a). Two RNAs were identified which contained multiple binding sites (*e.g.* in 3'UTRs or exons) that were classified either as PQ^{up} or PQ^{down} binding sites. These transcripts were *Katna1* (Katanin catalytic subunit A1), encoding a protein involved in microtubule organization²⁵² and *Wac* (WW domain containing adaptor with coiled-coil), a linker protein associated with chromatin remodeling and transcription²⁵³. While one PQ^{up} binding site was located in an exon of *Katna1*, two PQ^{down} binding site were part of a second exon and an intron of *Katna1*. For *Wac*, one PQ^{up} and one PQ^{down} binding site was contained in two different exons.

GO enrichment analysis showed that, similar to MSI1, MSI2-bound PQ^{down} RNAs were mainly involved in chromatin remodeling (chromatin binding, ATPase complex, pericentric heterochromatin) and cytoskeletal rearrangement (positive regulation of plasma membrane bounded cell projection assembly, actin cytoskeleton organization) (Figure 35 b). Moreover, intracellular signaling pathways (negative regulation of GTPase activity, negative regulation of canonical Wnt signaling pathway, regulation of MAPK cascade) were among the most enriched GO terms in PQ^{down} transcripts bound by MSI2. Besides these functions, PQ^{down} RNAs were connected to transcriptional regulation (positive regulation of transcription by RNA polymerase III) and protein degradation (regulation of protein catabolic process, negative regulation of phosphorus metabolic process).

Transcripts increasingly bound by MSI2 after PQ stimulation were predominantly involved in actin cytoskeletal rearrangement (actin filament, Rho protein signal transduction, small GTPase binding), motility (polar microtubule, microtubule-severing ATPase activity,

lamellipodium organization) and cellular adhesion (regulation of protein localization to cell-cell junction, intrinsic component of the cytoplasmic side of the plasma membrane) (Figure 35 c). Moreover PQ^{up} RNAs were involved in chromatin remodeling (CHRAC, histone H2B conserved C-terminal lysine ubiquitination, PTW/PP1 phosphatase complex) and protein modification (protein O-linked fucosylation, protein monoubiquitination).

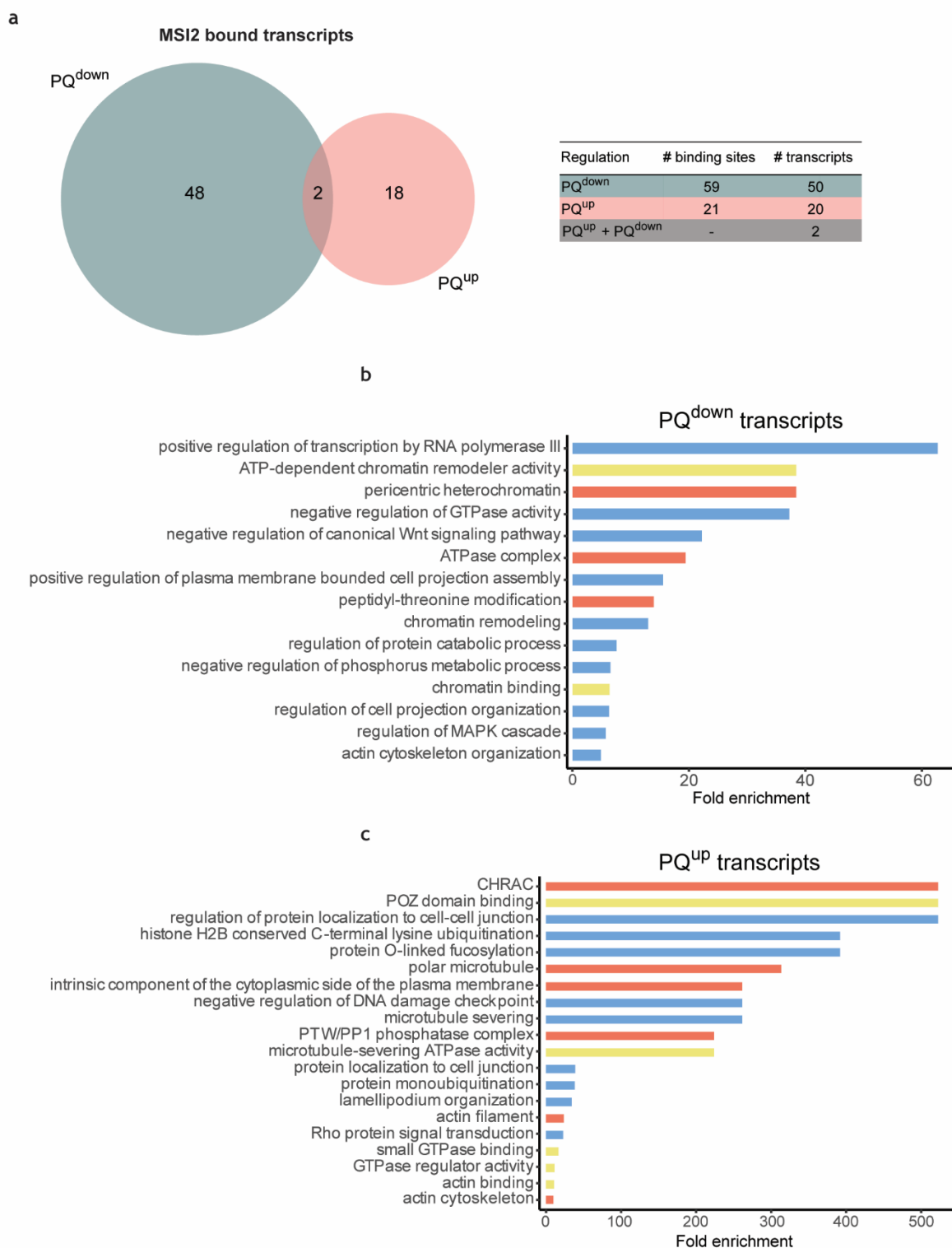


Figure 35. More MSI2-bound RNAs are under-represented after PQ exposure and associated with broad cellular functions

a| Twice as many MSI2-bound transcripts are under-represented after exposure to PQ compared to transcripts which are enriched after PQ stimulation. Few MSI2-bound RNAs were enriched (PQ^{up}) and under-represented (PQ^{down}) after PQ stimulation in mIMCD cells. While 50 transcripts were under-represented after exposure to PQ, 20 RNAs were enriched upon PQ stimulation. Two transcripts (*Katna1* and *Wac*) were identified in which multiple binding sites were either enriched or under-represented after PQ stimulation.

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The overlap of enriched and under-represented transcripts after PQ stimulation are visualized as Venn diagram and exact numbers of binding sites and corresponding transcripts are listed in the table on the right. **b| MSI2-bound transcripts, under-represented after PQ stimulation are involved in chromatin remodeling, cytoskeletal rearrangement and intracellular signaling cascades.** PQ^{down} transcripts were majorly enriched for GO terms related to chromatin remodeling (chromatin binding, ATPase complex, pericentric heterochromatin) and cytoskeletal rearrangement (positive regulation of plasma membrane bounded cell projection assembly, actin cytoskeleton organization). Besides these processes, RNAs which were under-represented after exposure to PQ were associated with distinct intracellular signaling pathways (negative regulation of GTPase activity, negative regulation of canonical Wnt signaling pathway, regulation of MAPK cascade) and protein degradation (regulation of protein catabolic process, negative regulation of phosphorus metabolic process). Of note, positive regulation of transcription by RNA polymerase III was the most enriched GO term. GO enrichment analysis on PQ^{down} transcripts was performed using the R package clusterProfiler (function enrichGO). Enriched GO terms were filtered for biological relevant terms (excluded cell-type specific terms e.g. chondrocyte differentiation, post-synaptic development) and clustered using Revigo²⁴². Depicted as bar graphs are the Top15 significantly enriched GO terms consisting of biological processes (blue) and molecular functions (yellow) and cellular compartments (red). **c| PQ^{up} RNAs are associated with chromatin remodeling, mitosis/DNA replication and transcriptional regulation.** MSI2-bound transcripts, which were enriched upon PQ stimulation were predominantly involved in actin cytoskeletal rearrangement (actin filament, Rho protein signal transduction, small GTPase binding), motility (polar microtubule, microtubule-severing ATPase activity, lamellipodium organization) and cellular adhesion (regulation of protein localization to cell-cell junction, intrinsic component of the cytoplasmic side of the plasma membrane). Moreover, PQ^{up} transcripts were associated with chromatin remodeling (CHRC, histone H2B conserved C-terminal lysine ubiquitination, PTW/PP1 phosphatase complex) and protein modification (protein O-linked fucosylation, protein monoubiquitination). GO enrichment analysis on enriched transcripts after PQ stimulation was performed using the R package clusterProfiler (function enrichGO). Enriched GO terms were filtered and clustered as described above. Depicted as bar graphs, are the Top20 significantly enriched biological processes (blue), molecular functions (yellow) and cellular compartments (red).

4. Discussion

4.1 Musashi proteins portray distinct expression patterns in mouse and human kidneys

Musashi-1 and Musashi-2 are expressed across many different tissues and cell types and are found to be localized within the cytoplasm and nucleus *e.g.* in photoreceptor cells ^{184,212}, intestinal epithelial cells ^{254,255}, neural stem cells ²⁵⁶, hepatocytes ²⁵⁷ and many more ^{213,258}. Importantly, some reports suggest that dynamic nucleocytoplasmic shuttling and redistribution of MSI1/MSI2 regulates the function of Musashi proteins ²⁵⁹ *e.g.* during spermatogenesis ¹⁸⁰ or retinal development ¹⁸⁵. In microdissected renal tubules from mouse kidneys, low expression of *Msi1* mRNA was detected in the loop of Henle and outer medullary collecting duct (OMCD) (Figure 5 a). Proteomics data from the same group, collected in microdissected rat kidneys, failed to detect MSI1 protein along the tubular system (Figure 5 b). One study examined the role of MSI1 in kidney fibrosis and showed expression of MSI1 in proximal and distal tubules of mouse kidneys ²²².

We found MSI1 expressed in mouse distal tubules but not in proximal tubules (Figure 7 a). Within tubular epithelial cells, MSI1 was specifically localized within the cytoplasm and at the apical membrane (Figure 7 c). The specific localization of MSI1 at the apical plasma membrane was not previously reported. MSI1 is a well-characterized RBP, characterized to primarily interact with 3'UTRs and inhibit or activate translation of target RNAs ^{177,203,210,211}. RBPs, associated with intracellular membranes of certain organelles *e.g.* mitochondria or endoplasmic reticulum, are thought to play a major role in organelle-coupled translation ²⁶⁰, but little is known about the function of RBPs at the plasma membrane. Some RBPs are associated with the formation of microvesicles, a type of extracellular vesicles consisting of lipids, proteins and nucleic acids, important for intercellular communication ²⁶¹. Microvesicles form through budding of the plasma membrane and contain a variety of RNA molecules ²⁶². Localization of MSI1 at the apical membrane could indicate an involvement of MSI1 in sequestering target RNAs into microvesicles in tubular epithelial cells in the kidney. While extracellular vesicles have been increasingly linked to kidney disease in recent years ²⁶³, a potential connection of MSI1 and microvesicles and its role within the kidney has to be studied further.

Msi2 mRNA was measured within all tubular segments of mouse kidneys and the expression was highest in medullary segments of the loop of Henle (MTAL, DTL1, DTL3, ATL) and the inner medullary collecting duct (IMCD) ²²³ (Figure 5 a). Interestingly, mRNA levels in mouse kidneys did not match protein expression along the tubular system of rats, as MSI2 was mostly detected in straight proximal tubules at the cortical-medullary rays (PTS2) and in the outer

stripe of the outer medulla (PTS3)²²⁴ (Figure 5 b). In line with the mRNA expression profile, MSI2 was expressed in proximal and distal tubules in mouse kidneys (Figure 7 b) and localized within the cytoplasm and at the nuclear membrane (Figure 7 d). The distinct expression patterns of MSI1 and MSI2 along the tubular system indicate independent roles of both Musashi proteins within the kidney.

While we did not detect MSI1 expression in one human kidney biopsy specimen, low levels of MSI1 were visible in one of three human kidney biopsy specimens retrieved from the human protein atlas (Figure 8 a/b), suggesting expression variability of MSI1 within the human kidney. Gene expression variability, caused through genetic polymorphism and genome plasticity, is known across all human tissues and causes phenotypic variability within the human population²⁶⁴. In human kidneys, variable gene expression of several housekeeping genes was detected between many individuals²⁶⁵, highlighting that also *Msi1* expression in the human kidney could be highly variable within the human population. Moreover, previous data of our lab showed a strong upregulation of *Msi1* after cisplatin treatment in mice¹⁰⁴, suggesting that *Msi1* might be a damage-associated gene in humans as well and expression could be increased in kidney disease. As MSI1 and MSI2 are homologous proteins, it is also possible that MSI2 compensates for the function of MSI1 within the human kidney. In contrast to mouse kidneys, where MSI2 was detected in proximal and distal tubular cells and expressed across the entire tubular system (Figure 7 b), MSI2 was expressed in distal tubules and glomerular podocytes in the human kidney biopsy (Figure 8 a). Both Musashi proteins are conserved across many species^{179,266}. The general overlap of gene expression between mouse and human kidneys is considered to be higher compared to conservation between other tissues *e.g.* adipose tissue or stomach^{267,268}. However, gene expression levels and the expression profiles within different nephron segments can vary strongly between mouse and human kidneys²⁶⁹. Exclusive expression of MSI2 in human distal tubules and glomerular podocytes compared to its uniform expression across the entire nephron in mice could be explained by these species-specific differences in gene expression.

4.2 MSI1 is upregulated in mouse models of AKI

In cisplatin-damaged mouse kidneys, *Msi1* mRNA was highly upregulated compared to controls, while *Msi2* mRNA levels remained unchanged (Figure 6). This upregulation was confirmed on protein level, as MSI1 was highly expressed in tubular epithelial cells at the cortico-medullary border upon cisplatin damage (Figure 9 a). As expected from the mRNA levels, MSI2 was ubiquitously expressed across the nephron and MSI2 levels did not change upon cisplatin damage. Interestingly, strong expression of MSI1 was not exclusively induced by cisplatin damage, but also upon ischemia reperfusion injury (IRI) in mouse kidneys (Figure

9 b), suggesting a general role of MSI1 in murine AKI independent of the initiating insult. MSI2 expression did not change upon IRI, emphasizing that MSI1 may have a function in AKI that is independent of MSI2. MSI1 was highly expressed in necrotic tubular epithelial cells at the cortico-medullary border upon IRI, as evident through disrupted plasma membranes and loss of nuclei. Of note, MSI1-positive necrotic cells were lacking MSI2 expression (Figure 9 b). Persistent tubular injury triggers activation of cell death either through apoptosis or regulated necrosis pathways e.g. necroptosis or ferroptosis ²⁷⁰. In recent years, necroptosis and ferroptosis were proven to play a major role in the pathophysiology of AKI ^{54,55,271,272}. In contrast to apoptosis, regulated necrosis is accompanied by a pronounced pro-inflammatory reaction, which contributes to kidney damage in AKI ²⁷³. Nevertheless, apoptosis was shown to contribute to tubular epithelial cell death upon injury and confer kidney dysfunction *in vivo* ^{52,53}. Both Musashi proteins have been extensively studied in the context of various cancers and knockdown of either *Msi1* ^{274,275}, *Msi2* ^{276,277} or both proteins ^{278,279} induced apoptosis in multiple cancer cells *in vitro* and *in vivo*. Similarly, in non-cancerous cells, knockdown or deletion of *Msi1* or *Msi2* triggered apoptosis e.g. in osteoclasts ²⁸⁰ and *Drosophila* ovaries ²⁸¹. Consequently, overexpression of Musashi proteins inhibited apoptosis in multiple cell types from different species ^{182,282–284}. A connection of Musashi proteins to regulated necrosis has not been reported yet. We aimed to investigate whether (a) high expression of MSI1 in necrotic tubular epithelial cells is connected to tubular epithelial cell death and kidney dysfunction in mouse models of AKI, (b) MSI1 upregulation is cause or consequence of tubular epithelial cell injury and (c) Musashi proteins fulfill an independent or compensatory role within the healthy kidney and AKI.

4.3 Targeting *Msi1* gene expression in the kidney

4.3.1 Establishing conditional *Msi1* knockout mice to study the function of MSI1 in mouse models of AKI

To analyze the role of MSI1 in mouse models of AKI we wanted to generate a conditional *Msi1* knockout mouse model with genetic deletion of *Msi1* in the distal tubules, loop of Henle and collecting duct of the kidney and subject it to IRI. Crossing of *Msi1* flox and *KspCre* transgenic animals did not generate conditional *Msi1* knockout animals, possibly through genetic linkage between *Msi1* and the *KspCre* transgene on chromosome 5, which is only resolved upon chromosomal recombination during meiosis and significantly decreases the probability of knockout animals ²⁸⁵ (Figure 10).

While crossing of *Msi1* flox mice with tamoxifen-inducible *KspCreERT2* transgenic animals resulted in the generation of potential *Msi1* knockout mice, immunohistochemistry staining of

MSI1 demonstrated that the protein was still present in distal tubules, loop of Henle and collecting duct of knockout animals (Figure 11).

The unexpected expression of MSI1 in conditional knockout mice was explained through the presence of a PGK-Neo cassette within the floxed *Msi1* allele that potentially caused pre-weaning lethality to mice carrying solely the *Msi1* flox allele (Figure 12). Importantly, many studies reported on serious off-target effects of PGK-neo cassette expression in mice^{286–289} and these off-target effects resulted in lethality in two independent mouse lines^{290,291}. Similarly, expression of the PGK-neo cassette could explain the high lethality of homozygous *Msi1* flox mice (Figure 12 a) and the underrepresentation of homozygous *Msi1* floxed animals within our two mouse lines (Figure 10 b and Figure 11 b). Furthermore, these results indicate that the floxed *Msi1* allele in our animals in reality resembles the genetic structure of the *Msi1-3lox* loci, containing one loxP site before the transcriptional start site and a floxed PGK-Neomycin resistance cassette between exons 2 and 3 (Figure 12 c). Breeding of these animals with Cre-transgenic mice would result in generation of the *Msi1* flox allele, and not the *Msi1* knockout allele. This hypothesis explains the missing knockout of *Msi1* in the *Msi1flox*;KspCreERT2 mouse line (Figure 11 c/d). In conclusion, the floxed *Msi1* allele was shown to be genetically and functionally defective and is therefore unsuitable for the generation of kidney-specific knockout mice to study the role of MSI1 in AKI.

4.3.2 Post-transcriptional targeting fails to reduce *Msi1* expression in the kidney

While it was impossible to study the role of MSI1 in AKI in a genetic *Msi1* knockout mouse model, we utilized antisense oligonucleotide (ASO) technology to reduce *Msi1* mRNA expression *in vivo*. ASOs are small (~18–30 nucleotides), synthetic, single-stranded nucleic acid oligomers which bind to target mRNAs and form an RNA-DNA heteroduplex. As a result, RNaseH is recruited leading to mRNA cleavage and degradation²⁹². ASOs targeting exon 15 of human *Msi1* mRNA were previously published and efficiently downregulated *Msi1* expression in patient-derived xenograft tumors in mice²¹⁷. Sequence conservation between human and mouse *Msi1* is high and the sequence of the selected *Msi1* ASO shows 100% complementarity with the targeted locus of exon 15 in mice. After systemic administration, highest ASO concentrations are generally detected in the liver and kidney^{293,294}. Moreover, ASOs have been shown to efficiently reduce the expression of different target mRNAs in mouse kidneys^{295–298}.

Administration of ASOs in wild type mice resulted in a marked reduction of *Msi1* mRNA expression in testis, brain and liver compared to PBS-injected animals (Figure 13). Expression of *Msi1* in the kidney remained unchanged upon treatment with ASOs compared to the control group. One explanation for the absence of reduction of *Msi1* mRNA levels in the kidney after

ASO administration is that expression of *Msi1* in the kidney is generally low and restricted to specific tubular epithelial cells in distal segments. ASOs are rapidly taken up by proximal tubular cells through endocytosis but uptake in the distal tubular sections is significantly lower, observed by a 10-fold difference between ASO concentrations within the cortex compared to the medulla²⁹³. In our mouse model, major uptake of ASOs by the proximal tubules could result in low *Msi1* ASO concentration in distal segments of the kidney, which consequently prohibited downregulation of *Msi1* in exactly these tubular sections. Of note, administration of *Msi1* ASOs induced a minor but significant reduction in *Msi2* mRNA levels in brain and kidney and a 0.2-fold decrease within the liver compared to the PBS control group (Figure 13). As the *Msi1* ASO specifically binds to exon 15 within the *Msi1* mRNA and not within the *Msi2* transcript, these observations could indicate a positive correlation between *Msi1* and *Msi2* expression, which was previously reported²⁹⁹. Importantly, the positive correlation of *Msi1* and *Msi2* mRNA levels detected in brain, kidney and liver was not true for testis, in which *Msi2* expression was increased, while *Msi1* mRNA levels were decreased upon ASO administration, indicating that co-regulation of Musashi proteins could be tissue specific. As administration of *Msi1* ASOs did not lead to a significant reduction of *Msi1* mRNA levels within the kidney, this approach could not be utilized to study the role of MSI1 in AKI *in vivo*.

To assess the functional role of MSI1 in AKI, it is inevitable to examine the consequences of loss of MSI1 on kidney function in mouse models of AKI. We generated two putative conditional *Msi1* knockout mouse lines, which were dysfunctional due to defects within the *Msi1* flox allele. Moreover, pharmacological inhibition of *Msi1* expression within the kidney was not successful, probably due to decreased bioavailability of the *Msi1* ASO within the distal parts of the nephron. In order to address this research question within future projects, a new *Msi1* flox mouse model was obtained²¹⁷, which is currently crossed with *KspCre* transgenic animals in order to generate conditional *Msi1* knockout mice.

4.4 Targeting MSI2 gene expression in the kidney

4.4.1 Conditional *Msi2* knockout mice display normal kidney physiology

Deletion of *Msi2* in the distal tubules, loop of Henle and collecting duct in mice did not induce significant changes in body weight or kidney morphology and function in male and female mice at 12 weeks (Figure 14) and upon aging at 52 weeks (Figure 15). These findings fit to a study reporting that the conventional whole body knockout of *Msi2* in mice is viable, but leads to decreased body size and birth frequency²⁰².

4.4.2 MS11 is upregulated upon IRI independent of MS12

To investigate the role of MS12 in AKI, mice with conditional knockout of *Msi2* in the distal tubules, loop of Henle and collecting duct were subjected to IRI. Kidney function was significantly decreased in *Msi2* knockout animals upon IRI compared to sham animals with undamaged kidneys (Figure 16 a). However, kidney dysfunction in *Msi2* knockout mice was similar to *Cre*-transgenic control animals (Figure 16 a). All *Cre*-transgenic animals showed significantly increased serum creatinine concentrations compared to wild type mice after IRI. These results indicate that, independent of *Msi2* expression, the *KspCre* transgene alone increases kidney dysfunction after IRI. Side effects of Cre recombinase expression have been reported for many Cre transgenic mouse lines^{300–304}. To our knowledge, no studies on off-target effects within the *KspCre* mouse line exist. Other studies in which *KspCre* transgenic animals were investigated in the context of IRI did not include control mice, which only expressed the *KspCre* transgene, in their experiments^{305–307}. Whether the *KspCre* transgene alone altered kidney function after IRI in these studies can therefore not be finally assessed. After IRI, kidney damage was increased to a similar extent in *Msi2* knockout and control mice compared to sham animals (Figure 16 c). Interestingly, necrosis was elevated in *Msi2* knockout and heterozygous control animals compared to Cre control mice after IRI, suggesting that loss of *Msi2* in the distal tubules, loop of Henle and collecting duct may be associated with elevated necrosis in tubular epithelial cells after IRI. MS11 was upregulated in tubular epithelial cells at the cortico-medullary border in *Msi2* knockout and heterozygous control mice in response to IRI (Figure 17). High expression of MS11 in necrotic tubules was associated with low MS12 expression and was detected across the entire cortico-medullary border, independent of the genotype (Figure 18). These findings confirm a robust upregulation of MS11 in necrotic tubular epithelial cells at the cortico-medullary border, which is independent of MS12. Only a genetic knockout of *Msi1* in the kidney can decipher whether this upregulation is cause or consequence of tubular damage and necrosis in mouse models of AKI. Moreover, generation of a double conditional knockout of *Msi1* and *Msi2* in the kidney will give further insights into the potential compensatory role of both proteins in AKI.

4.5 Dynamic alterations in subcellular expression of Musashi proteins under cellular stress

4.5.1 Successful generation of transgenic mIMCD cell lines

The molecular functions of both Musashi proteins were examined through several *in vitro* studies in murine inner medullary collecting duct (mIMCD) cells. Because these cells did not endogenously express *Msi1*, transgenic cell lines were generated using TALEN genome

editing³⁰⁸. Four different cell lines with single copy integration of coding sequences of Musashi-Flag and Musashi-EGFP fusion proteins into the *Rosa26* locus of mIMCD cells were successfully created (Figure 19). For these cell lines, genomic integration was verified and expression on the transcript and protein level was demonstrated. As expected from previous reports^{184,212,213,254–258}, both Musashi proteins were primarily localized within the cytoplasm and nucleus in MSI1.3xFlag, 3xFlag.MSI2, EGFP.MSI1 and EGFP.MSI2 mIMCD cell lines. Western blot analysis revealed that fusion tag (3xFlag, EGFP) and endogenous Musashi signals nicely overlapped in all four transgenic cell lines (Figure 19 c). Of note, integration of an N-terminal 3xFlag-tagged MSI1 fusion protein in mIMCD cells was not successful and we therefore decided on the generation of a mIMCD cell line with C-terminal 3xFlag MSI1 fusion protein (MSI1.3xFlag).

4.5.2 Subcellular localization of Musashi proteins is altered by heat shock, but not by hypoxia

Cells are constantly challenged with external (e.g. pathogens, toxins, osmotic stress / heat stress) and internal (e.g. metabolic, oxidative, genotoxic stress) stimuli interfering with cellular homeostasis²⁵⁰. Depending on the type and duration of cellular stress, distinct pro-survival stress response pathways are activated^{309,310}. Unresolved cellular stress and sustained activation of cellular stress response pathways are implicated to play a role in AKI, such as unfolded protein response in the ER^{31,33}, mitochondrial oxidative stress^{36,37} or DNA damage response^{44,45}. We hypothesized that Musashi proteins could play a role in stress response signaling induced in tubular epithelial cells upon AKI and subjected EGFP.MSI1 and EGFP.MSI2 mIMCD cells to diverse external stressors (Figure 20).

Heat shock induces the heat shock response pathway, a pro-survival signaling cascade, which is activated in response to general proteotoxic stress within the cytoplasm³¹¹. Upon heat shock, localization of both Musashi proteins shifted from the cytoplasm (control conditions) to the nucleus (Figure 20 a). Heat shock response (HSR) revolves around activation and nuclear translocation of heat shock transcription factor 1, which regulates gene expression of various target genes involved in proteotoxic stress response, e.g. molecular chaperones and ubiquitin³¹². Both Musashi proteins have been shown to participate in alternative splicing in nuclei of photoreceptor cells before¹⁸⁴. Nuclear localization of MSI1 and MSI2 upon heat shock suggests a similar transcriptional role of MSI1 and MSI2 in HSR in mIMCD cells. In contrast to heat shock, hypoxia did not induce an obvious relocalization of either Musashi protein in mIMCD cells (Figure 20 b). Similar to heat shock, hypoxia triggers transcriptional and post-transcriptional gene regulation through activation of the hypoxia-inducible factors (HIFs)⁸⁸.

However, based on their unaltered cytoplasmic localization, Musashi proteins seem not to partake in hypoxia-induced transcriptional adaptation in mIMCD cells.

4.5.3 Localization of Musashi proteins in cytoplasmic granules upon exposure to cisplatin and ROS-induced oxidative stress

4.5.3.1 Cisplatin induces DNA damage and localization of Musashi proteins in cytoplasmic granules

Exposure to cisplatin triggers a multitude of cellular stress response pathways in tubular epithelial cells including DNA damage response, oxidative stress and ER stress³¹³. In mIMCD cells, treatment with cisplatin induced DNA damage as demonstrated through increased phosphorylation levels of histone H2A variant H2AX (γ H2AX) (Figure 20 c). Although a pronounced relocalization of Musashi proteins, as observed upon heat shock, was missing upon cisplatin administration, MSI1 and MSI2 were detected in granules within the cytoplasm. Cytoplasmic granular compartments mainly represent SGs and processing bodies (PBs). These membraneless organelles form through LLPS and are involved in post-transcriptional and translational control^{133,314}. While SGs dynamically assemble upon translational arrest, induced upon various environmental stressors *e.g.* oxidative stress or osmotic stress^{133,315}, PBs are constitutively present within the cytoplasm³¹⁶. Moreover, SGs and PBs differ through their content^{133,314}. Both are cytoplasmic mRNP granules, which contain mRNAs, RBPs and ribosomal subunits. However, PBs primarily house mRNA decay proteins and SGs contain proteins involved in translational initiation^{133,314}. The formation of cytoplasmic granular foci upon cisplatin treatment was previously reported in osteosarcoma cells³¹⁷, and even more interestingly, in proximal tubular epithelial cells³¹⁸. In both studies, granule formation upon cisplatin treatment was significantly lower compared to other stressors *e.g.* glycolysis inhibition or treatment with arsenite. In osteosarcoma cells, granule formation upon cisplatin treatment was linked to translational inhibition³¹⁷. Moreover, granule formation was irreversible and therefore different from SGs, which are dynamically disassembled once stress is resolved. In addition, cisplatin prevented the formation of canonical SGs induced through arsenite treatment in osteosarcoma cells³¹⁷. In proximal tubule cells, inhibition of SG formation rendered cells more susceptible to cisplatin-induced cell death³¹⁸. Altogether these studies associate cisplatin with the formation of cytoplasmic granules and suggest that SG formation protects from cisplatin-induced cell death, possibly through mediating translational inhibition. Our findings indicate that both Musashi proteins are part of cytoplasmic granular structures, formed upon cisplatin treatment in mIMCD cells.

4.5.3.2 Exogenous stimulation with hydrogen peroxide markedly induces localization of Musashi proteins to cytoplasmic granular compartments

Hydrogen peroxide (H_2O_2) is a major ROS species, which is mainly generated through reduction of molecular oxygen within the mitochondria³¹⁹. In cellular physiology, H_2O_2 is essential for redox signaling, primarily mediated through oxidation of different peroxidases³²⁰. An imbalance in redox signaling through excessive production of ROS is defined as oxidative stress. Persistent oxidative stress (oxidative distress) results in damage of nucleic acids, proteins and lipids, ultimately triggering cell death when oxidative stress is not resolved³²¹. Unsurprisingly, oxidative distress contributes to pathophysiology of many human diseases³²² including AKI^{41–43,84–86}. Upon H_2O_2 treatment, a marked relocalization of both EGFP-tagged Musashi proteins in cytoplasmic granular foci compared to control cells was visible (Figure 20 d). Several studies showed formation of cytoplasmic granular structures upon H_2O_2 treatment and identified these cytoplasmic foci as SGs^{323–325}. Although the exact mechanism is unknown, at lower concentrations (<0.5 mM), H_2O_2 seemed to inhibit SG formation³²⁴, while higher H_2O_2 concentrations (>0.5 mM) triggered the assembly of uncommon, irreversible SGs^{323,325}. We treated mIMCD cells with 0.6 mM H_2O_2 and detected a visible localization of Musashi proteins in cytoplasmic granules, which were positive for the SG marker FXR1 (data not shown), indicating that Musashi proteins are part of irreversible SGs which form under high H_2O_2 concentrations. However, it was not investigated whether the MSI-positive cytoplasmic granules assemble irreversibly or resolve once H_2O_2 is removed.

4.5.3.3 Paraquat (PQ) triggers localization of Musashi proteins in SGs and is associated with AKI

Paraquat (PQ, 1,1'-dimethyl-4,4'-bipyridylium dichloride) is a widely used herbicide, known for its high toxicity within the lung³²⁶. Besides causing pulmonary fibrosis³²⁶, PQ intoxication is associated with the development of AKI^{327–329} and PQ-induced AKI is connected to increased mortality in patients^{330,331}. On a cellular level, PQ is reduced to PQ radicals by various NADPH oxidases^{332,333}. As electron donors, PQ radicals react with molecular oxygen and produce a plethora of ROS, thereby inducing oxidative stress and cellular damage, e.g. through DNA damage and lipid peroxidation^{334,335}. Similar to H_2O_2 , PQ led to localization of MSI1 and MSI2 in cytoplasmic granular foci (Figure 20 e). PQ was shown to induce cytoplasmic granule formation in neuroblastoma and HeLa cells^{336,337} and bovine oocytes³³⁸. Interestingly, in HeLa cells, PQ induced formation of irreversible SGs, similar as observed upon cisplatin³¹⁷ and H_2O_2 ^{323,325} treatment *in vitro*. In mIMCD cells, the cytoplasmic granules formed upon PQ treatment, expressed the SG protein FXR1^{133,339} (Figure 21 a-c). FXR1-positive SGs were also positive for both Musashi proteins, confirming that MSI1 and MSI2 were localized in SGs upon PQ

stimulation of tubular epithelial cells. In summary, induction of oxidative stress either through H_2O_2 or through PQ, triggered the localization of both Musashi proteins into cytoplasmic SGs in multiple mIMCD cell lines. Several reports showed that PQ potently induced oxidative stress, as evident through mitochondrial dysfunction, lipid peroxidation and apoptosis *in vitro* and *in vivo* ^{340–344}. Based on these reports and our results, we hypothesized that Musashi proteins might play a role in injury and death of tubular epithelial cells, triggered upon PQ-induced oxidative stress.

4.6 PQ induces cell death in tubular epithelial cells

4.6.1 PQ triggers ferroptosis in tubular epithelial cells

Monitoring of cell death revealed that over a time course of 24 hours, continuous exposure to PQ markedly increased mIMCD cell death (Figure 22 b-e). To unravel the exact cell death pathways which were triggered by PQ in mIMCD wild type cells, cells were stimulated with PQ and diverse cell death inhibitors, either exclusively or as combination with the apoptosis inhibitor Emricasan (Figure 22 a). Combinatorial inhibition was necessary because all cell death pathways are interconnected ^{345,346} and especially blocking apoptosis through general caspase inhibition (e.g. via Emricasan ³⁴⁷) was shown to shift cell death towards activation of other pathways e.g. necroptosis or pyroptosis ^{348,349}.

Blocking ferroptosis alone, through administration of the antioxidant scavenger Ferrostatin-1 ⁶⁰, markedly reduced death of mIMCD cells over the entire time course of 24 h of PQ stimulation (Figure 22 b/e). Only combinatorial inhibition of ferroptosis together with apoptosis was able to reduce the number of dead cells even more than observed with single blockage using Ferrostatin-1. Neither blocking pyroptosis alone (Figure 22 c/e), through treatment with the NLRP3 inhibitor CRID3 ³⁵⁰ or the caspase-1 inhibitor VX-765 ³⁵¹, nor inhibiting necroptosis alone (Figure 22 d/e) through administration of the RIPK1 inhibitor Nec-1s ³⁵² or the RIPK3 inhibitor GSK-872 ³⁵³, reduced cell death to a similar extent as observed when inhibiting ferroptosis. Combinatorial inhibition of apoptosis (Emricasan) together with pyroptosis (CRID3/VX-765) (Figure 22 c/e) or RIPK1-mediated necroptosis (GSK-872) (Figure 22 d/e) delayed death of mIMCD cells but was not able to fully prevent PQ-triggered cell death as observed through Ferrostatin-1 treatment (Figure 22 b/e). Cell death was only delayed, because although apoptosis, pyroptosis and necroptosis were inhibited through the respective combinatorial treatments, ferroptosis remained active and may have contributed to the progressive increase in the number of dead cells over time.

These findings identify ferroptosis as the major cell death pathway induced upon PQ exposure of mIMCD cells. PQ was previously connected to ferroptosis in neuroblastoma cells ^{354,355}.

Mitochondria were identified as the primary source of ROS production upon PQ intoxication *in vivo*³⁵⁶ and mitochondrial dysfunction is a major consequence of PQ-induced oxidative stress^{357–359}. Ferroptosis is associated with mitochondrial fragmentation⁶⁰ and targeting mitochondrial dysfunction using a small molecule compound blocked ferroptosis in osteosarcoma cells³⁶⁰. Interestingly, the same small molecule compound also prevented PQ-induced mitochondrial damage³⁶⁰. Moreover, blocking mitophagy *in vivo* protected from PQ-induced ferroptosis within the myocardium³⁶¹. These reports pinpoint towards a mechanism in which mitochondrial damage induced through PQ triggers ferroptosis. As ferroptosis plays a major role in tubular epithelial cell death observed in AKI^{54,59,61}, a deeper understanding of the interconnection between PQ and ferroptosis could evolve in novel treatment options of AKI³⁶². Of note, several reports connected PQ intoxication with AKI^{330,331}. Besides the possible interconnection of PQ, ferroptosis and AKI, these results underline how PQ stimulation can be utilized as a tool to study some aspects of the molecular pathophysiology of AKI in cell culture.

4.6.2 Expression of both Musashi proteins renders tubular epithelial cells more susceptible to PQ-induced cell death

Over a time course of 24 hours, PQ exposure induced cell death most potently in mIMCD cells, expressing both Musashi proteins, compared to cells which expressed only MSI1/ MSI2 or low levels of MSI2 (Figure 23). These results demonstrate that expression of both Musashi proteins rendered cells more susceptible to PQ-triggered cell death, indicating that MSI1 and MSI2 might play a role in PQ-triggered ferroptosis of mIMCD cells. The expression of both Musashi proteins is upregulated in many types of cancer and correlates with metastasis, therapy resistance and poor prognosis^{188,196,363–366}. In malignant cells, both Musashi proteins have been shown to regulate many pro-survival signaling pathways including epithelial-mesenchymal transition, cell cycle progression and self-renewal capacity^{215,363,364,367}. Consequently, reducing MSI1 or MSI2 levels promoted apoptotic cell death in many cancer cells^{274–276,278,368}. In contrast, overexpression of MSI1 inhibited apoptosis in glioblastoma and cervical cancer cells^{365,369}. However, in mIMCD cells, expression of both Musashi proteins was associated with elevated cell death compared to single expression of MSI1 or MSI2. These findings indicate that while overexpression of Musashi is connected to cancer cell growth and prevents apoptotic cell death in malignant cells, MSI1 and MSI2 could play a contrary role in PQ-triggered cell death of tubular epithelial cells. The different implications of Musashi proteins in cancer cells and tubular epithelial cells could not only originate from the aberrant nature of cancer cells but also from the cell death pathways involved. PQ exposure primarily induced ferroptosis in mIMCD cells and apoptosis, which is promoted by knockdown of Musashi proteins in cancer cells, was initiated only to a smaller extent (Figure 22). While apoptosis is triggered by death-receptor activation or expression of pro-apoptotic BH3-only proteins and

cell demolition is mediated through caspases ³⁷⁰, ferroptosis depicts a completely different concept of programmed cell death. During ferroptosis, accumulation of iron and/or ROS overwhelms the cellular antioxidant machinery consisting of the cystine/glutamate antiporter (System Xc⁻), glutathione peroxidase 4 and glutathione ³⁷¹. This imbalance in redox signaling leads to excessive lipid peroxidation, loss of membrane integrity and triggers ferroptotic cell death. Both Musashi proteins were found to bind and regulate the fate of multiple target RNAs in a cell and context-specific manner ^{184,191–198}, which highlights that the function of MSI1 and MSI2 in ferroptosis in mIMCD cells, could differ from its role in apoptosis in cancer cells.

4.7 PQ-induced oxidative stress results in small alterations of Musashi protein interactomes

As RBPs, MSI1 and MSI2 participate in the formation of ribonucleoprotein complexes to regulate the fate of target RNAs in a context-dependent manner ^{203,210}. Interestingly, it was reported that the protein interactome of MSI1 was dynamically altered upon progesterone stimulation of *Xenopus* oocytes ²¹¹ and a dysregulated MSI2 protein network was identified in leukemia cells ³⁷². We hypothesized that the relocalization of MSI1 and MSI2 into SGs upon PQ-induced oxidative stress was accompanied by dynamic alterations within their protein interactomes.

4.7.1 Identification of known interactors within the protein interactomes of both Musashi proteins in tubular epithelial cells

At baseline, MSI1 interacted with 417 proteins and MSI2 bound to 296 proteins in mIMCD cells (Figure 24) and many of the identified interactors were previously identified to bind to MSI1 or MSI2 ²³¹. Research on protein-protein interactions within the Musashi protein family has largely focused on MSI1 ^{203,209–211,373}. Within the MSI1 protein, the C-terminal end has been identified to be essential for binding to e.g. poly(A) binding protein ²⁰³ or germ line development defective-2 ²¹⁰ and MSI1 was shown to indirectly activate or repress translation of target RNAs through these protein-protein interactions. Interestingly, MSI2 had less binding affinity to both of these proteins, indicating that MSI1 and MSI2 also engage in distinct protein-protein networks independent of each other ^{210,211}. In line with these observations, we found MSI1 to interact with 30% more proteins in mIMCD cells at baseline, suggesting that MSI1 may be part of more, or larger, ribonucleoprotein complexes in tubular epithelial cells compared to MSI2.

4.7.2 Both Musashi proteins are integral part of a large SG protein network in unstimulated and PQ-stimulated tubular epithelial cells

Although both Musashi proteins localized in cytoplasmic SGs upon oxidative stress induction via PQ, PQ did not induce major alterations within the MSI1 and MSI2 protein networks, as for both proteins the no PQ and PQ interactomes largely overlapped (Figure 25 a / Figure 26 a). Interestingly, one third of the identified interactors of MSI1 and MSI2 were SG-associated proteins ²⁵¹ and MSI1 and MSI2 bound to these proteins in unstimulated and PQ-treated mIMCD cells (Figure 25 b / Figure 26 b). It was previously shown that large SG networks pre-exist in unstressed cells, which enables rapid formation of SGs upon cellular stressors ³⁷⁴. Our findings suggest that both Musashi proteins are an integral part of these SG networks in tubular epithelial cells and may participate in the formation of SGs upon PQ-induced oxidative stress. Importantly, MSI1 and MSI2 were detected in SGs in human induced pluripotent stem cells ³⁷⁴, human osteosarcoma cells ³⁷⁵ and mouse brain lysates ³⁷⁶, indicating that both Musashi proteins might be essential components of SGs in different species and cell types. Of note, persistent existence of SGs is connected to protein aggregation and associated with neurodegenerative disorders ^{377,378}. Interestingly, both Musashi proteins were found to form toxic aggregates with tau protein in brains from patients with different neurodegenerative diseases ^{220,221}, indicating that MSI1 and MSI2 might be linked to pathologic protein accumulation within unresolved SGs. Lastly, one study reported that SGs form in proximal tubule cells of mice upon IRI and that disabled SG formation rendered cells more sensitive to cisplatin-induced cell death ³¹⁸. More data need to decipher the role of SGs in AKI and whether both Musashi proteins contribute to SG formation in tubular epithelial cells.

4.7.3 Identification of common and unique protein-protein interactions of MSI1 and MSI2 in tubular epithelial cells

Comparison of the MSI1 and MSI2 protein interactomes demonstrated that the interactors of both Musashi proteins largely overlapped in no PQ and PQ conditions (Figure 27 a/b). However, MSI1 bound to approximately 30% more proteins in both conditions compared to MSI2. As described before, MSI1 was shown to interact with some proteins via the C-terminal end, while MSI2 did not interact with the same proteins ^{210,211}. It is therefore possible that MSI1 interacts with many proteins via its C-terminal protein binding domain in mIMCD cells. Of note, IPs were performed in 3xFlag.MSI2 and MSI1.3xFlag mIMCD cells and it is possible that the attachment of 3xFlag to the N- or C-terminal protein end of MSI2/MSI1 modulates its binding affinity to protein partners.

As anticipated from the general role of both Musashi proteins as RBPs, MSI1 and MSI2 interacted with a network of proteins involved in mRNA metabolism (e.g. mRNA 3'UTR binding, mRNA metabolism), translation (e.g. ribosome, amide biosynthetic process) and complex formation (e.g. structural molecule activity, ribonucleoprotein complex) in unstimulated and PQ-stimulated mIMCD cells (Figure 27 c). As no major alterations within the respective protein interactomes upon PQ stimulation were detected, it was coherent that the most enriched GO terms largely overlapped between the two conditions and primarily indicated the functional role of both Musashi proteins in regulating the fate of target RNAs as part of large ribonucleoprotein complexes.

Comparison of the no PQ and PQ interactomes of MSI1 (Figure 25) and MSI2 (Figure 26) revealed that both proteins either exclusively or cooperatively interacted with certain groups of proteins associated with e.g. cellular adhesion and nuclear functions such as pre-mRNA processing.

MSI1 and MSI2 interacted with several proteins related to cellular adhesion (e.g. CTNNA1/CTNNB1, TJP1/TJP2, ACTN1) either exclusively or cooperatively in no PQ and PQ conditions in tubular epithelial cells (Figure 25 and Figure 26). While MSI1 has been shown to regulate cellular adhesion through RNA binding in medulloblastoma¹⁹⁶ and glioblastoma cells¹⁹², MSI2 was demonstrated to regulate migration and motility via interaction with its target RNAs in keratinocytes¹⁹⁴. However, to our knowledge, protein-protein interaction of MSI1 and MSI2 with cellular adhesion proteins have not yet been reported and these findings suggest that Musashi proteins may contribute to the maintenance of structural integrity of tubular epithelial cells. Of note, we demonstrated that, within the mouse kidney, MSI1 was localized at the apical membrane of tubular epithelial cells, highlighting that further studies need to investigate the role of both Musashi proteins, but especially MSI1, in epithelial polarity and adhesion.

Besides cellular adhesion proteins, MSI1 and MSI2, alone or together, interacted with a row of nuclear proteins in unstimulated and PQ-stimulated mIMCD cells (Figure 25 and Figure 26). These protein interactors were e.g. involved in nucleosome structure (HIST2H2AA1 / HIST2H2AC, HIST1H1A, H1F0 and HIST1H2BA / HISTH2BB / HIST1H2BF / HIST1H2BK / HIST1H2BP) and splicing (PRPF8, PRFP19, SNRPD1), indicating a potential function of MSI1 and MSI2 e.g. in chromatin rearrangement and pre-mRNA processing. Although both Musashi proteins were mainly localized in the cytoplasm, MSI1 and MSI2 were also detected within the nucleus of mIMCD cells (Figure 19 g). Supporting these findings, both Musashi proteins have been demonstrated to be involved in alternative splicing in photoreceptor cells of mice^{183,184,212}. Moreover, both Musashi proteins were also reported to localize in the nucleus of a tau inducible

HEK (iHEK) model ²⁵⁸. Here, Musashi proteins formed toxic nuclear aggregates with tau proteins, which affected chromatin remodeling and nuclear integrity, fitting to the interaction of MSI1 and MSI2 with histones in mIMCD cells. Furthermore, these toxic tau oligomers prevented the interaction of MSI1 with LMNB1 and increased the binding of MSI2 with LMNB1, thereby significantly destabilizing nuclear morphology and transport in iHEK cells ²²⁰. These reports are interesting, because across all interactome data sets, LMNB1 exclusively interacted with MSI1 in unstimulated mIMCD cells, suggesting a similar function of MSI1 in maintaining nuclear integrity in tubular epithelial cells. Together these findings indicate a role of MSI1 and MSI2 in pre-mRNA processing and maintaining chromatin and nuclear stability in tubular epithelial cells.

While the above mentioned groups contained proteins interacting with MSI1 or MSI2 in no PQ and PQ conditions, one protein, Bleomycin hydrolase (BLMH), exclusively interacted with MSI1 and MSI2 in unstimulated mIMCD cells (Figure 25 and Figure 26). BLMH is a cytoplasmic cysteine peptidase, which besides its aminopeptidase activity, possesses hydrolase activity and is majorly involved in the conversion of homocysteine-thiolactone into homocysteine (Hcy) ³⁷⁹. Hcy-thiolactone is generated through metabolization of Hcy by methionyl-tRNA synthetase in an error-editing process ³⁸⁰. Proteotoxic effects, mediated through Hcy-thiolactone, are thought to contribute largely to the pathological state of hyperhomocysteinemia (elevated plasma homocysteine) e.g. observed in vascular disease ^{381–383}. Consequently, BLMH e.g. protected from Hcy-thiolactone induced neurotoxicity ³⁸⁴. Of note, hyperhomocysteinemia was shown to accelerate AKI in various animal models ^{385,386} and within the kidney, BLMH was associated with diverse cellular processes including energy metabolism, detoxification and antioxidant defenses, which were dysregulated by hyperhomocysteinemia ³⁸⁷. Interaction of MSI1 and MSI2 with BLMH could indicate that Musashi proteins play a role in BLMH-mediated protection from Hcy-thiolactone-mediated cellular injury. Moreover, this specific interaction was not detected upon PQ-induced oxidative stress in mIMCD cells. The PQ-triggered localization of MSI1/MSI2 in cytoplasmic SGs may perturb the interaction of MSI1 and MSI2 with BLMH under oxidative stress. Whether this interaction is e.g. needed to protect cells from additional proteotoxic stress, mediated through excessive amounts of Hcy-thiolactone, needs to be examined more closely to draw further conclusions on the functional interaction of these proteins.

Although MSI1 bound to more proteins exclusively compared to MSI2, some proteins were also detected to specifically interact with MSI2 and not with MSI1 (Figure 27 a/b). One of these proteins was the chaperon PCBP2 (Poly(rC)-binding protein 2), which was exclusively bound by MSI2 in unstimulated and PQ-stimulated mIMCD cells. PCBP2 is a chaperon which is central to iron storage and iron accumulation and iron-mediated lipid peroxidation are

hallmarks of ferroptosis⁶⁰. mIMCD cells were more susceptible to PQ-induced cell death when both Musashi proteins were expressed (Figure 23 c/d). Importantly, PCBP2 is a multifunctional RBP, which, besides iron storage, is involved in mRNA stabilization and transcriptional/translational control³⁸⁸. Together, these findings indicate that MSI2 binds to PCBP2, maybe as part of a larger ribonucleoprotein complex, which regulates metabolism of target RNAs. Doing so, MSI2 might play a more important role in PQ-triggered ferroptosis compared to MSI1 e.g. through prohibiting PCBP2 from storing intracellular iron, causing iron accumulation and ferroptotic cell death. Clearly, more experiments are needed to prove whether a functional interplay of MSI2 and PCBP2 plays a role in the higher susceptibility of mIMCD cells to PQ-induced ferroptotic cell death.

4.8. Both Musashi proteins bind to functionally related target RNAs and PQ markedly alters the context-specific RNA interactome of MSI1

Although both Musashi proteins were localized in cytoplasmic SGs upon PQ-induced oxidative stress in mIMCD cells, the protein interactomes of MSI1 and MSI2 were not largely altered. Both Musashi proteins were integral parts of large networks of SG-related proteins in unstimulated tubular epithelial cells and remained in complexes with the same SG proteins after exposure to PQ. Moreover, MSI1 and MSI2 bound to a largely overlapping group of proteins, which was connected to complex formation, mRNA metabolism and translation in unstimulated and PQ-stimulated mIMCD cells. Consequently, defining the RNA interactomes of MSI1 and MSI2 in unstimulated and PQ-stimulated mIMCD cells can help elucidate the role of both Musashi proteins in oxidative stress response of tubular epithelial cells. After confirming general RNA-binding capacity of Musashi proteins, specific RNA-binding sites and RNA targets were identified using seCLIP (Figure 28 a-d). The quality of seCLIP libraries was high based on the general assumption that 18 PCR cycles represent the upper limit of amplification cycles before libraries consist majorly of PCR duplicates and data complexity and quality decrease²⁰⁰. Reproducibility of the results was confirmed by good group separation in a PCA (Figure 28 e).

4.8.1 MSI1 and MSI2 bind to well-established binding sites and motifs in tubular epithelial cells

A similar amount of binding sites was identified for MSI1 and MSI2 in unstimulated and PQ-stimulated conditions in mIMCD cells (Figure 29 / Figure 30). In general, the overlap between the replicates was good, although the number of identified binding sites in one MSI1 PQ sample was smaller due to a decreased amount of sequencing reads (12 million reads instead of 20 million reads) (Figure 29 a). For MSI2, more binding sites were identified in unstimulated

compared to PQ-stimulated conditions, indicating slightly decreased RNA binding of MSI2 in response to oxidative stress in mIMCD cells (Figure 30 a). However, the binding sites identified for RBPs can vary depending on the organism, cell type, amount of replicates and the utilized CLIP technique. For MSI1 the amount of identified binding sites varies strongly between human and mouse datasets. MSI1 iCLIP (Individual-nucleotide resolution UV crosslinking and immunoprecipitation) in human glioblastoma cells ¹⁹² identified 90225 and eCLIP (enhanced crosslinking and immunoprecipitation) in human medulloblastoma cells identified 98920 binding sites ¹⁹⁶. In comparison, one study determined the RNA targets of MSI1 within the mouse retina and identified 10161 binding sites via eCLIP ¹⁸⁴. We found a similar amount of binding sites (17922 binding sites) within the MSI1 seCLIP libraries generated from unstimulated mouse tubular epithelial cells, indicating that MSI1 interacts with a similar amount of binding sites in retinal and tubular epithelial cells of mice. An analogous number of binding sites was identified for MSI2 (18920 binding sites) in unstimulated mIMCD cells. Information on exact amounts of identified bindings sites of MSI2 was only available for a HITS-CLIP (High-throughput sequencing of RNA isolated by CLIP) dataset of MSI2 in human hematopoietic stem cells, where over 25000 peaks were significantly enriched in one replicate ¹⁹⁷, demonstrating that MSI2 bound to a comparable number of binding sites in tubular epithelial and hematopoietic stem cells.

Analysis of the binding site distribution showed that MSI1 and MSI2 predominantly interacted with 3'UTRs in unstimulated and PQ-stimulated mIMCD cells (Figure 29 b / Figure 30 b). Many studies reported that both Musashi proteins bind to 3'UTRs of mRNAs, destabilize target transcripts and thereby repress translation ^{177,194,195,198,203}. However, it was also demonstrated that MSI1 interacts with 3'UTRs in order to activate translation of target RNAs ^{210,211}. It will be interesting to analyze how binding of 3'UTRs by MSI1 and MSI2 influences translation of target RNAs in tubular epithelial cells and whether the direction of translational regulation changes upon oxidative stress induction via PQ. Introns represented the second largest group of binding sites identified for MSI1 and MSI2 in both conditions (Figure 29 b / Figure 30 b). Interestingly, both Musashi proteins bound less to introns upon PQ stimulation in mIMCD cells. In general, binding to introns suggests a participation of MSI1 and MSI2 in pre-mRNA processing within the nucleus of tubular epithelial cells. Involvement of MSI1 and MSI2 in pre-mRNA processing is supported by the nuclear localization observed for both Musashi proteins in mIMCD cells (Figure 19 g) and the interaction of MSI1 and MSI2 with splicing proteins (Figure 25 / Figure 26). Moreover, MSI1 and MSI2 were both shown to regulate alternative splicing of target pre-mRNAs in photoreceptor cells ^{184,212}. Altogether, these findings strongly suggest a connection of MSI1 and MSI2 to splicing of target RNAs in tubular epithelial cells. Decreased binding to introns upon PQ stimulation indicates that MSI1 and MSI2 are less involved in splicing under oxidative stress, which is likely attributed to their localization in cytoplasmic SGs in PQ

conditions. While 5'UTRs, exons, non-coding regions and other binding sites made up only a minor part of MSI1/MSI2 binding site locations in unstimulated mIMCD cells (<10%), it is noteworthy that the binding to exons slightly increased for both Musashi proteins under PQ stimulation. This shift from intron to exon binding observed upon PQ stimulation underlines that oxidative stress may interfere with the nuclear functions of Musashi proteins at baseline and induce their interaction with exons within RNAs in the cytoplasm *e.g.* in order to store them in SGs. Further experiments, *e.g.* RNA *in situ* hybridization ³⁸⁹, need to underline whether the transcripts bound within exons are truly localized in SGs and if Musashi proteins interact specifically with these RNAs or RNA storage into SGs occurs more randomly.

Binding motif analyses demonstrated that MSI1 and MSI2 preferably bound a UAG trinucleotide in a majority of binding sites located in 3'UTRs, exons and introns in unstimulated and PQ-stimulated mIMCD cells (Figure 29 c / Figure 30 c). Across all binding sites, UAG was part of the top ranked binding motif of MSI2 within both conditions. While the same was true for MSI1 in no PQ conditions, the most significant binding motif upon PQ stimulation did not contain UAG. These findings hint towards a larger distribution of binding motifs of MSI1 upon PQ stimulation and oxidative stress in mIMCD cells. Of note, less variability of binding motifs was detected for binding motifs located in exons, introns and 3'UTRs bound by MSI1 in PQ conditions, as these datasets were markedly smaller compared to the group of total binding sites. Binding sites were identified via HOMER ²⁴⁰ through comparison to randomly picked sequences within the dataset, which decreases the probability of identifying rare motifs in smaller datasets. Importantly, UAG was identified as the core motif, bound by MSI1 and MSI2 across different cell types and organisms ^{184,186,191–196,198,390} and one study indicated that UAG appears to be bound by Musashi proteins independent of its function as stop codon ¹⁹⁸.

4.8.2 Both Musashi proteins bind to a largely overlapping group of RNAs in tubular epithelial cells

Across both conditions (no PQ and PQ) MSI1 and MSI2 interacted with a similar amount of RNAs (Figure 29 d / Figure 30 d). Of note, both Musashi proteins interacted with more transcripts in unstimulated compared to PQ-stimulated mIMCD cells, indicating that oxidative stress altered the size of the RNA interactomes of MSI1 and MSI2. Overall, MSI1 bound to 6115 distinct RNAs within both conditions. In comparison, MSI1 interacted with over 10000 transcripts within the mouse retina (eCLIP) ¹⁸⁴ and with over 9000 different transcripts in human glioblastoma cells (iCLIP) ¹⁹², emphasizing that the amount of bound transcripts can vary depending on cell type, organism and utilized CLIP technique. MSI2 interacted with 6022 transcripts and a similar number of RNAs was identified as MSI2 targets within the mouse intestinal epithelium, as determined by HITS-CLIP analysis ¹⁹³.

Several studies reported on the largely, but not completely, overlapping group of transcripts, which are bound by both Musashi proteins, especially in mouse tissues *e.g.* the intestinal epithelium or the retina^{184,191,193}. In line with these reports, MSI1 and MSI2 indeed interacted with a largely overlapping group of transcripts in unstimulated and PQ-stimulated tubular epithelial cells (Figure 31). However, within both conditions MSI1 and MSI2 also interacted with a substantial number of RNAs individually. Upon exposure to PQ, MSI2 bound exclusively to more transcripts compared to MSI1. Based on these results, we concluded that MSI1 and MSI2 indeed participate in similar ribonucleoprotein networks in tubular epithelial cells, but that both Musashi proteins also partake in distinct complexes, independent of each other. These complexes might differ largely through the amount and type of RNA bound. The transcripts contained within these complexes might be specific to MSI1 or MSI2 and may be dynamically altered upon oxidative stress induction.

4.8.3 PQ stimulation influences binding site interaction and binding region preferences of both Musashi proteins

To determine distinct alterations induced within the RNA interactomes of MSI1 and MSI2 through stimulation with PQ and subsequent exposure to oxidative stress, the abundance of binding sites identified in no PQ conditions was compared to their abundance upon PQ stimulation (Figure 32 a / Figure 34 a). We focused our analysis on those binding sites, which belonged to RNAs in which expression levels were generally unaltered by PQ stimulation in mIMCD cells. These transcripts were identified through differential gene expression analysis of whole-cell RNA-seq data of mIMCD cells comparing no PQ and PQ conditions (Figure 32 b / Figure 34 b). Importantly, all differentially expressed RNAs were excluded, independent of the direction of regulation induced upon PQ exposure. However, especially downregulated transcripts, which contain binding sites that were enriched in MSI1/MSI2 binding and vice versa are of particular interest for future analysis as these RNAs are subjected to marked changes in binding affinities of MSI1/MSI2 upon PQ stimulation.

Using this approach, binding sites enriched upon PQ stimulation (PQ^{up}) and binding sites, which were under-represented upon PQ stimulation (PQ^{down}) in mIMCD cells were identified. While Musashi proteins are considered to bind more to PQ^{up} sites, interaction of MSI1/MSI2 is significantly decreased with PQ^{down} sites. When comparing PQ^{up} and PQ^{down} sites of MSI1 and MSI2, strong differences in numbers of enriched and under-represented sites were revealed. Approximately 15-times more PQ^{up} and PQ^{down} sites were detected for MSI1 as compared to MSI2, indicating that PQ induces stronger alterations within the MSI1 RNA interactome, compared to the mild changes observed within the MSI2 RNA interactome. Importantly, multiple comparative analyses have revealed adaptations of RBPomes between distinct cell

types ¹¹⁹, during developmental processes ³⁹¹ or as response to external stimuli ¹²², but little is known about dynamic alterations within RNA interactomes of specific RBPs. Recently, a comparative multiomic analysis revealed MSI1 to bind to cancer-selective RNA targets in pediatric medulloblastoma cells compared to normal neural stem cells ¹⁹⁶, but more evidence on alterations within Musashi protein RNA interactomes *e.g.* in response to external stressors, is missing. To our knowledge, this work is the first to investigate (a) the general RNA interactome of Musashi proteins in tubular epithelial cells and (b) specific alterations within the interaction of MSI1 and MSI2 to target RNAs in response to PQ-induced oxidative stress.

Not only did the number of PQ^{up} and PQ^{down} sites differ quite strongly between MSI1 and MSI2, but the distribution of binding regions of PQ^{up} and PQ^{down} sites varied between both Musashi proteins (Figure 32 b / Figure 34 b). While MSI1 PQ^{up} and PQ^{down} sites were mainly found in 3'UTRs and exons (over 75%), MSI2 PQ^{up} and PQ^{down} sites were more frequently part of introns and exons (over 80%). For both Musashi proteins, PQ^{up} and PQ^{down} sites were more frequently located within exons, compared to low binding of exons observed in general (Figure 29 b / Figure 30 c). PQ^{down} sites are under-represented upon PQ exposure, which also means these binding sites are enriched in unstimulated mIMCDs. Increased binding of exons indicates that Musashi proteins might be involved *e.g.* in transport or subcellular localization of target RNAs. Besides exons, MSI1 PQ^{up} and PQ^{down} sites were located in 3'UTRs, indicating that MSI1 regulates stability of these target RNAs, as previously reported ^{177,203,210,211,392}. In contrast to MSI1, MSI2 PQ^{up} and PQ^{down} sites were also more frequently located in introns, suggesting that MSI2 participates in pre-mRNA processing of enriched RNAs in no PQ and PQ conditions. These findings are in line with the hypothesis that both Musashi proteins are generally involved in splicing in tubular epithelial cells as described before.

4.8.4 Oxidative stress induces stronger alterations within the MSI1 RNA interactome

The number of PQ^{up} transcripts bound by MSI1 was 30-times larger compared to MSI2, and the number of PQ^{down} transcripts bound by MSI1 was seven times larger compared to MSI2 (Figure 33 a / Figure 35 a). These results demonstrate that PQ stimulation and oxidative stress induced robust alterations within the MSI1 RNA interactome, while only mild changes were observed for the MSI2 RNA interactome. Together with the observations that, independent of the cellular context, MSI1 interacted with more proteins compared to MSI2, these findings suggest that (a) MSI1 participates in the formation of more or larger RNP complexes in tubular epithelial cells and (b) oxidative stress does not majorly alter the protein but the RNA composition of these RNP complexes. As both Musashi proteins localized in SGs upon PQ stimulation, the stronger alterations within the MSI1 RNA interactome suggests that MSI1 might sequester specific RNAs into SGs upon oxidative stress in tubular epithelial cells.

However, these data were generated from whole-cell lysates and not purified SGs and therefore more experiments *e.g.* through SG isolation or RNA *in situ* hybridization have to demonstrate that these RNAs are truly more abundant in SGs upon PQ stimulation. Of note, we also identified MSI1/MSI2-bound RNAs, in which multiple binding sites were either enriched or under-represented upon PQ stimulation in mIMCD cells. These RNAs are highly interesting to study further, as MSI1 and MSI2 switch their binding region preference within these transcripts as response to PQ stimulation in mIMCD cells. More experiments need to elucidate on the functional consequence of this binding region switch of MSI1/MSI2, induced by oxidative stress in tubular epithelial cells.

For both Musashi proteins, PQ^{up} and PQ^{down} RNAs were associated with chromatin remodeling and rearrangements of the actin cytoskeleton (Figure 33 b+c / Figure 35 b+c). Interestingly, both Musashi proteins also interacted with proteins involved in nucleosome structure and cellular adhesion (Figure 25 and Figure 26), indicating that MSI1 and MSI2 participate in these processes through protein-protein interactions and via controlling the fate of process-related RNAs. It will be important to uncover the exact transcripts behind the single GO terms and how the interaction of Musashi proteins with particular RNAs, enriched or under-represented upon PQ stimulation, exactly influences chromatin remodeling and cellular adhesion. Noteworthy, only MSI1-bound PQ^{up} and PQ^{down} transcripts were associated with cellular redox homeostasis and cytoplasmic SGs, emphasizing a potentially stronger involvement of MSI1 in the cellular response to oxidative stress in tubular epithelial cells. In contrast, only MSI2-bound PQ^{down} transcripts were connected to the regulation of Wnt and MAPK signaling. Multiple studies showed that MSI2 regulates Wnt and MAPK signaling and thereby promotes epithelial-mesenchymal transition and migration in diverse cancer cells ^{193,257,277,393,394}. Importantly, as described before, MSI1 and MSI2 showed different binding region preferences in PQ^{up} and PQ^{down} transcripts. Further analyses have to unravel the functional consequences of *e.g.* increased binding of MSI1 to exons in RNAs involved in cytoplasmic SGs or enhanced interaction of MSI2 with introns in transcripts involved in Wnt signaling on tubular epithelial cell biology.

5. Conclusion

Musashi proteins are evolutionarily conserved RBPs, which have been extensively studied for their role in stem cells and for their oncogenic properties in diverse types of cancers^{188,189}. Besides one study showing that MSI1 was downregulated in kidney fibrosis and downregulation was associated with cell cycle arrest in tubular epithelial cells, little information on the role of MSI1 and MSI2 within the kidney is available²²². Previous data of our own group found *Msi1* mRNA, but not *Msi2* mRNA, highly upregulated in kidneys of mice after cisplatin administration, a mouse model of AKI¹⁰⁴. In the work presented here, upregulation of MSI1 at the cortico-medullary border was demonstrated in two mouse models of AKI and it was shown that the high expression of MSI1 in necrotic tubules within this kidney area is independent of MSI2. Further research needs to show whether conditional loss of MSI1 within the kidney generally influences kidney function and alters kidney dysfunction upon AKI in mice. These *in vivo* experiments will be complemented by generating mice with loss of both Musashi proteins within the kidney and investigating how this simultaneous loss influences kidney damage in mouse models of AKI. Moreover, this thesis demonstrated that paraquat (PQ) stimulation represents a useful tool to study important pathophysiological mechanisms of AKI *e.g.* oxidative stress or ferroptotic cell death *in vitro*. To the best of knowledge, this work is the first to examine the role of MSI1 and MSI2 in response to PQ-induced oxidative stress in tubular epithelial cells. Given their role as RBPs, the protein and RNA interactomes of both Musashi proteins were determined at baseline and after PQ stimulation. MSI1 and MSI2 were shown to be an integral part of a large SG protein network already at baseline. Consequently, oxidative stress led to robust localization of Musashi proteins in cytoplasmic SGs in tubular epithelial cells. Based on these findings it remains to be investigated whether Musashi proteins are essential for SG assembly in tubular epithelial cells and whether PQ induces formation of conventional or persistent SGs. Moreover, it will be interesting to elucidate the role of SGs in the kidney *in vivo* and whether *e.g.* persistence of SGs contributes to injury and death of tubular epithelial cells and how Musashi proteins might be involved in this process. While the protein interactomes of MSI1 and MSI2 remained largely unchanged upon oxidative stress, stronger alterations within the composition of MSI1 RNA interactome were determined upon PQ stimulation. An in-depth, integrative analysis of our protein and RNA interactome data can help unravel the participation of Musashi proteins in distinct ribonucleotide complexes, which might play a role in oxidative stress responses of tubular epithelial cells. Lastly, examining how MSI1 and MSI2 may contribute to splicing of particular pre-mRNAs in tubular epithelial cells can help in deciphering their role within the healthy and diseased kidney. These insights will be of crucial importance to target specific RNA-interactions of Musashi proteins as a potential strategy to ameliorate AKI in the future.

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VI. Publications

Kaiser, Rainer W. J., Michael Ignarski, Eric L. Van Nostrand, Christian K. Frese, Manaswita Jain, Sadrija Cukoski, Heide Heinen, Melanie Schaechter, **Lisa Seufert**, Konstantin Bunte, Peter Frommolt, Patrick Keller, Mark Helm, Katrin Bohl, Martin Höhne, Bernhard Schermer, Thomas Benzing, Katja Höpker, Christoph Dieterich, Gene W. Yeo, Roman Ulrich Müller, and Francesca Fabretti. *A Protein-RNA Interaction Atlas of the Ribosome Biogenesis Factor AATF*. *Scientific Reports* 9(1) (2019).

Esmailie, Reza, Michael Ignarski, Katrin Bohl, Tim Krüger, Daniyal Ahmad, **Lisa Seufert**, Bernhard Schermer, Thomas Benzing, Roman Ulrich Müller, and Francesca Fabretti. *Activation of Hypoxia-Inducible Factor Signaling Modulates the RNA Protein Interactome in Caenorhabditis Elegans*. *IScience* 22:466–76 (2019).

Lisa Seufert, Thomas Benzing, Michael Ignarski, and Roman Ulrich Müller. *RNA-Binding Proteins and Their Role in Kidney Disease*. *Nature Reviews Nephrology* 2021 18:3 18(3) (2021).

Hoyer-Allo, Karla Johanna Ruth, Martin Richard Späth, Susanne Brodesser, Yiyi Zhu, Julia Binz-Lotter, Martin Höhne, Hella Brönneke, Katrin Bohl, Marc Johnsen, Torsten Kubacki, Katharina Kiefer, **Lisa Seufert**, Felix Carlo Koehler, Franziska Grundmann, Matthias J. Hackl, Bernhard Schermer, Jens Brüning, Thomas Benzing, Volker Burst, and Roman Ulrich Müller. *Caloric Restriction Reduces the Pro-Inflammatory Eicosanoid 20-Hydroxyeicosatetraenoic Acid to Protect from Acute Kidney Injury*. *Kidney International* 102(3):560–76 (2022).

Späth, Martin R., K. Johanna R. Hoyer-Allo, **Lisa Seufert**, Martin Höhne, Christina Lucas, Theresa Bock, Lea Isermann, Susanne Brodesser, Jan-Wilm Lackmann, Katharina Kiefer, Felix C. Koehler, Katrin Bohl, Michael Ignarski, Petra Schiller, Marc Johnsen, Torsten Kubacki, Franziska Grundmann, Thomas Benzing, Aleksandra Trifunovic, Marcus Krüger, Bernhard Schermer, Volker Burst, and Roman-Ulrich Müller. *Organ Protection by Caloric Restriction Depends on Activation of the De Novo NAD⁺ Synthesis Pathway*. *Journal of the American Society of Nephrology: JASN* (2023).

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VIII. Erklärung

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.

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Work & Professional Experience

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03/2016 - 12/2016	Master thesis, Department of orthopedics, Prof. Dr. Franz Jakob, University of Würzburg <i>Role of anabolic therapy in the hematopoietic cancer multiple myeloma</i>
04/2014 - 08/2014	Bachelor thesis, Institute of human genetics, Prof. Dr. Michael Schmid, University of Würzburg <i>Molecular cytogenetic analysis of the XY-chromosomes of the North American frog species Hyla femoralis</i>

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10/2011 - 09/2014	Julius-Maximilians University of Würzburg Bachelor's program: Biochemistry

X. Appendix

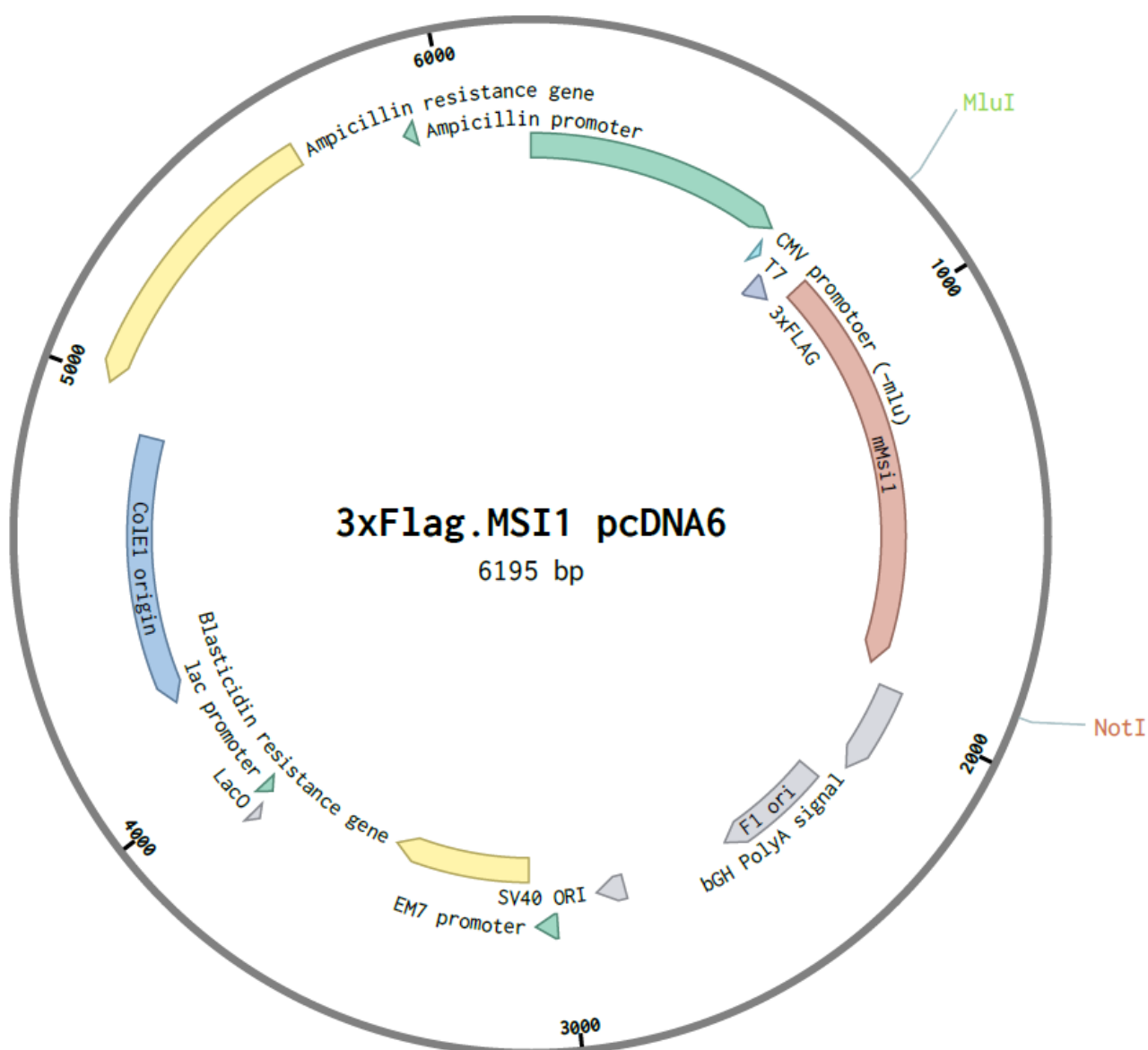


Figure A.1. Map of 3xFlag.MSI1 pcDNA6 plasmid

MSI1 amplicon was inserted into the modified pcDNA6 /V5-His B backbone (Invitrogen) by MluI and NotI restriction enzyme digest and T4 ligation as described in 2.2.1. Expression of 3xFlag.MSI1 in mammalian cells is driven by a CMV promoter. Bacterial selection is enabled by expression of an ampicillin resistance gene. Mammalian selection is enabled by expression of a blasticidin resistance gene.

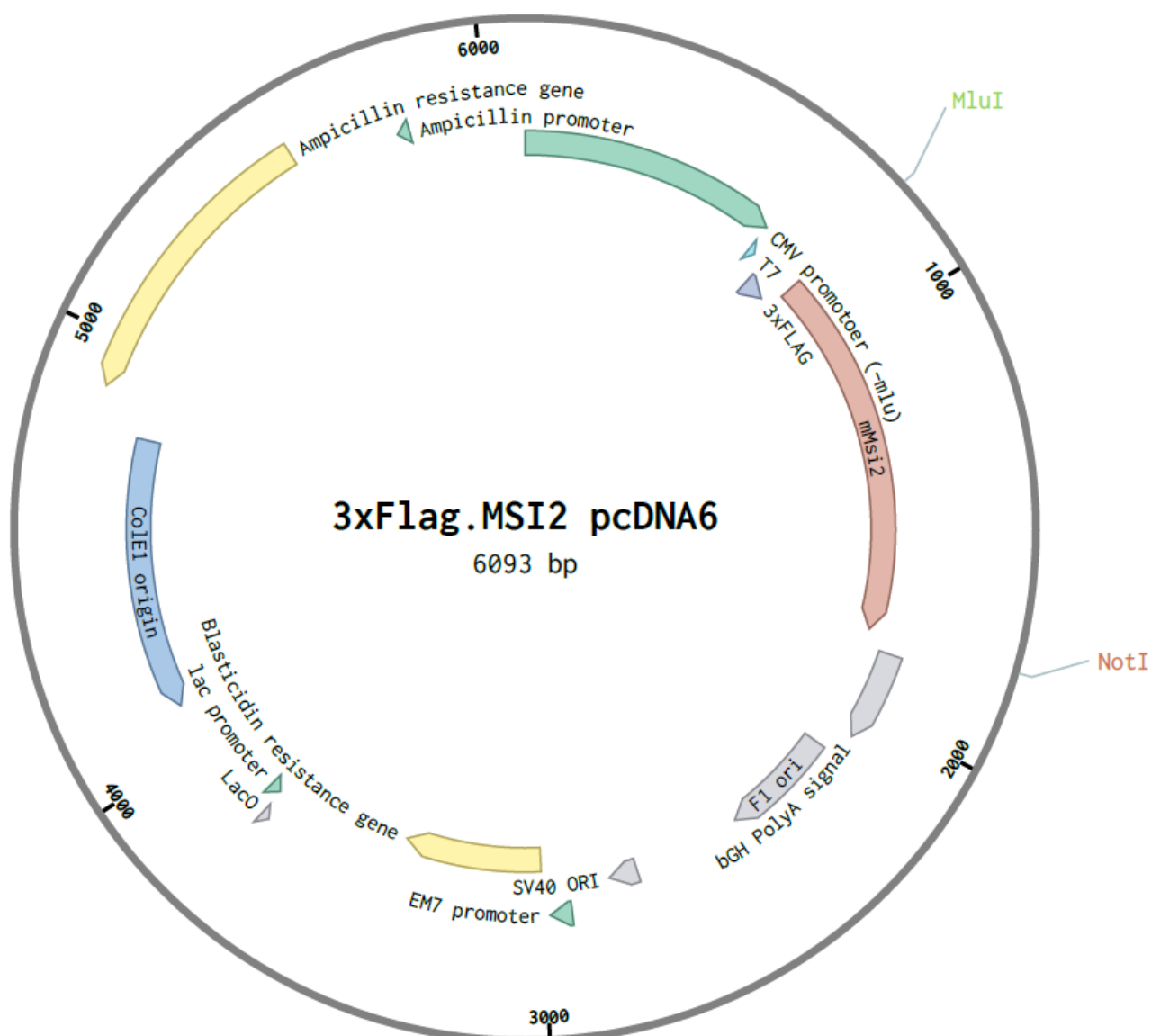


Figure A.2. Map of 3xFlag.MSI2 pcDNA6 plasmid

MSI2 amplicon was inserted into the modified pcDNA6 /V5-His B backbone (Invitrogen) by MluI and NotI restriction enzyme digest and T4 ligation as described in 2.2.1. Expression of 3xFlag.MSI2 in mammalian cells is driven by a CMV promoter. Bacterial selection is enabled by expression of an ampicillin resistance gene. Mammalian selection is enabled by expression of a blasticidin resistance gene.

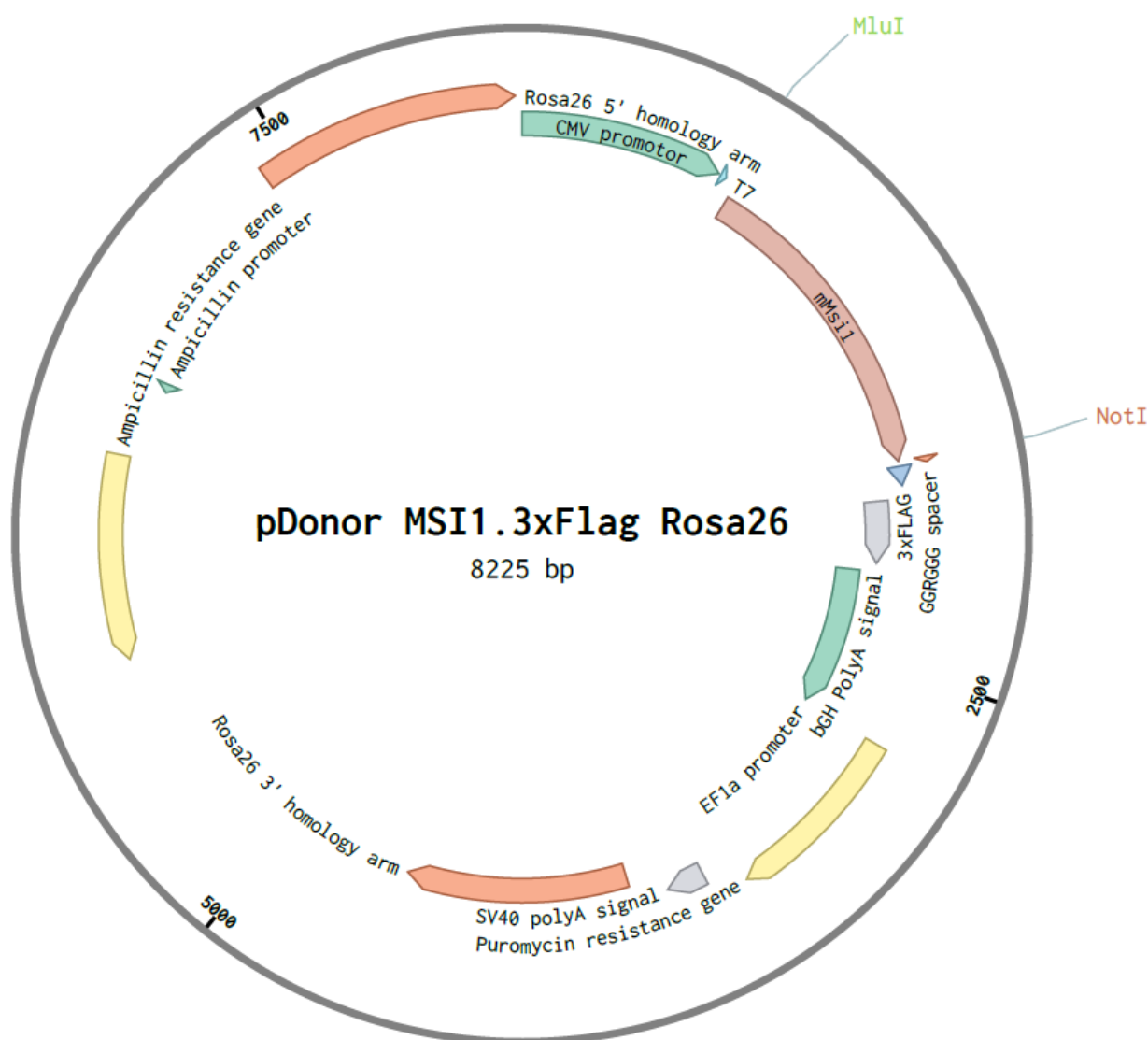


Figure A.3. Map of pDonor MSI1.3xFlag Rosa26 plasmid

MSI1 amplicon was inserted into the modified pDonor MCS Rosa26 (Addgene Plasmid #37200) by MluI and NotI restriction enzyme digest and T4 ligation as described in 2.2.1. Expression of MSI1.3xFlag in mammalian cells is driven by a CMV promoter. Integration into the Rosa26 locus of mouse cells is enabled by the two homology arms flanking the expression cassette. Bacterial selection is enabled by expression of an ampicillin resistance gene. Mammalian selection is enabled by an EF1a promoter-driven expression of a puromycin resistance gene.

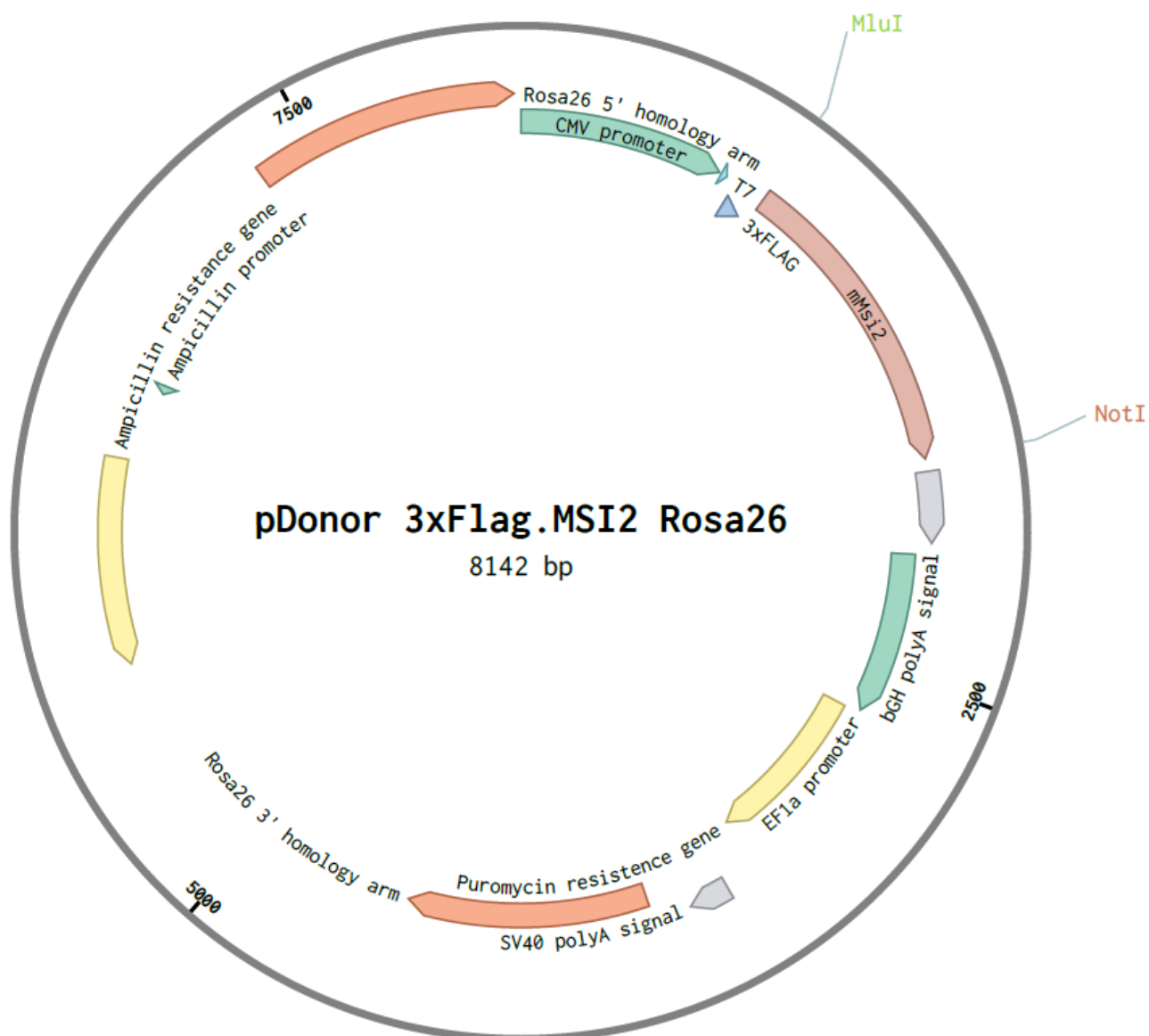


Figure A.4. Map of pDonor 3xFlag.MSI2 Rosa26 plasmid

MSI2 amplicon was inserted into the modified pDonor MCS Rosa26 (Addgene Plasmid #37200) by MluI and NotI restriction enzyme digest and T4 ligation as described in 2.2.1. Expression of 3xFlag.MSI2 in mammalian cells is driven by a CMV promoter. Integration into the Rosa26 locus of mouse cells is enabled by the two homology arms flanking the expression cassette. Bacterial selection is enabled by expression of an ampicillin resistance gene. Mammalian selection is enabled by an EF1a promoter-driven expression of a puromycin resistance gene.

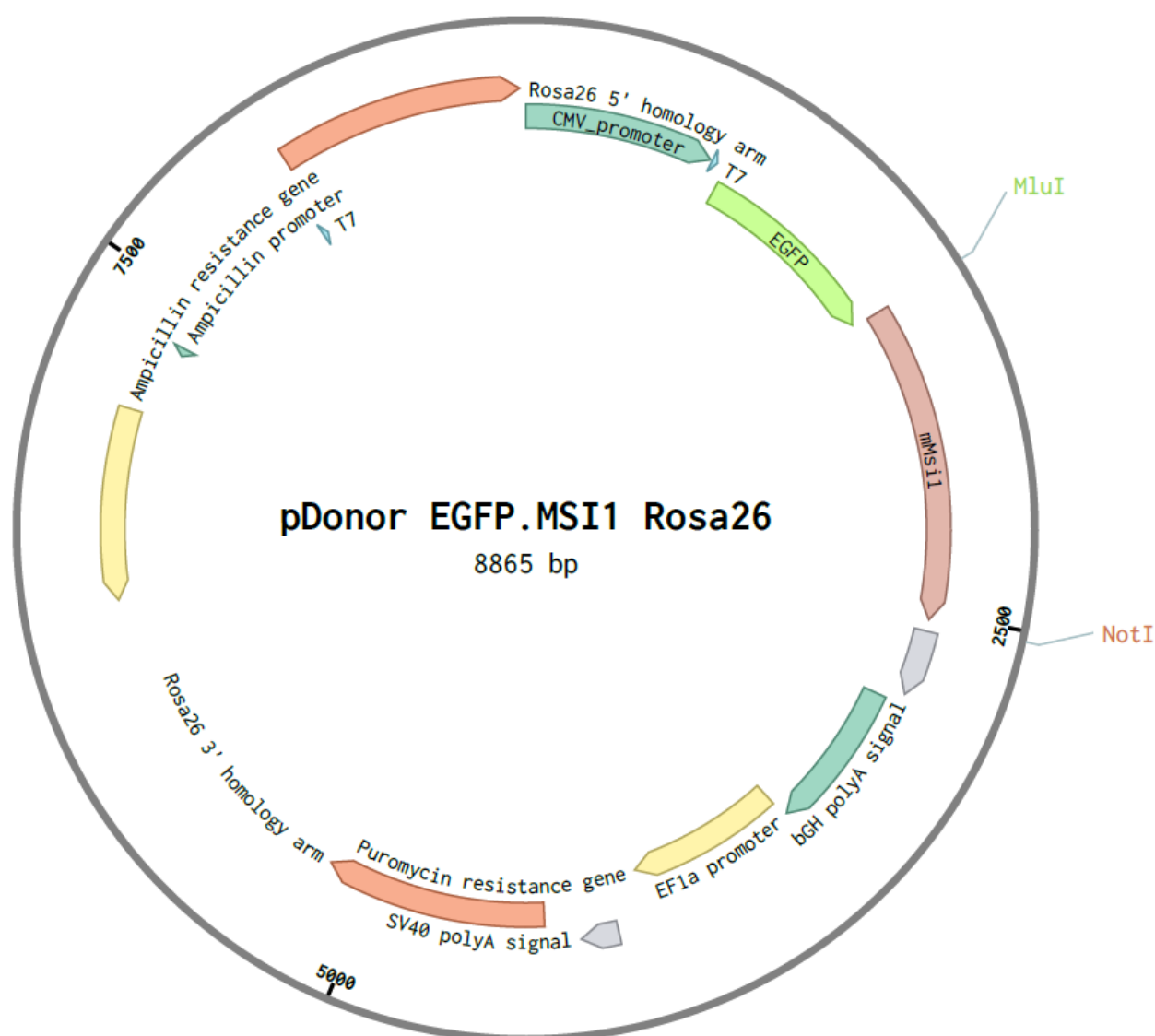


Figure A.5. Map of pDonor EGFP.MSI1 Rosa26 plasmid

MSI1 amplicon was inserted into the modified pDonor MCS Rosa26 (Addgene Plasmid #37200) by MluI and NotI restriction enzyme digest and T4 ligation as described in 2.2.1. Expression of EGFP.MSI1 in mammalian cells is driven by a CMV promoter. Integration into the Rosa26 locus of mouse cells is enabled by the two homology arms flanking the expression cassette. Bacterial selection is enabled by expression of an ampicillin resistance gene. Mammalian selection is enabled by an EF1a promoter-driven expression of a puromycin resistance gene.

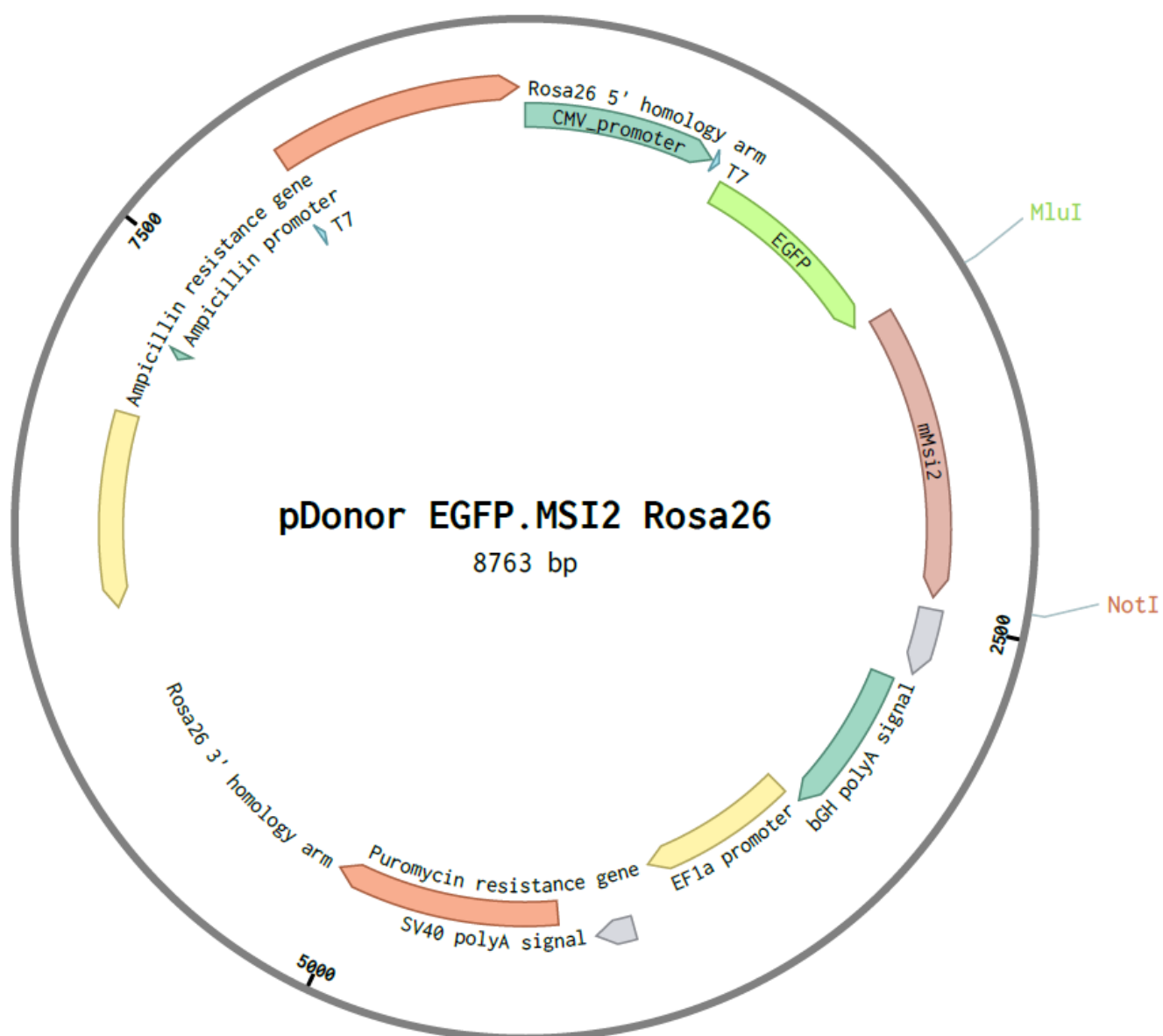


Figure A.6. Map of pDonor EGFP.MSI2 Rosa26 plasmid

MSI2 amplicon was inserted into the modified pDonor MCS Rosa26 (Addgene Plasmid #37200) by MluI and NotI restriction enzyme digest and T4 ligation as described in 2.2.1. Expression of EGFP.MSI2 in mammalian cells is driven by a CMV promoter. Integration into the Rosa26 locus of mouse cells is enabled by the two homology arms flanking the expression cassette. Bacterial selection is enabled by expression of an ampicillin resistance gene. Mammalian selection is enabled by an EF1a promoter-driven expression of a puromycin resistance gene.