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WRAP53β AND SCARNA2 ON THEIR JOURNEY TO REPAIR THE DNA

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WRAP53β and scaRNA2 on their journey to repair the DNA

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“Remember to look up at the stars and not down at your feet”

Professor Stephen Hawking

To my sister Christina
and to my parents Soula and Giannis

ABSTRACT

WRAP53β is a multifunctional protein involved in several cellular processes like Cajal body formation, telomere maintenance and DNA repair. WRAP53β is responsible for targeting factors to specific cellular locations, and its very high affinity for the scaRNAs could implicate these molecules in similar biological functions.

In **Paper I** we established a new function of WRAP53β protein in DNA damage response. We showed that WRAP53β localizes rapidly at sites of DNA double strand breaks (DSBs) in an ATM, ATR, H2AX and MDC1 dependent manner. WRAP53β acts as a scaffold for the interaction between the E3 ligase RNF8 and the upstream repair factor MDC1. Subsequently, RNF8 initiates an ubiquitin cascade that triggers the accumulation of downstream repair proteins like 53BP1, BRCA1, RAD51 at the break site. Loss of WRAP53β inhibits both repair pathways (homologous recombination and non-homologous end joining) and this leads to accumulation of spontaneous DNA damage and ultimately to cell death.

In **Paper II** we introduced the proximity ligation assay (PLA), as a method for visualizing and analyzing factors that are recruited and form complexes at sites of DNA damage. By employing PLA we detected accumulation of WRAP53β in close proximity to γH2AX in an ATM, ATR dependent manner, a result we could also confirm by co-immunoprecipitation. With PLA we were able to observe the interaction of MDC1 and RNF8 that was abolished after depletion of WRAP53β or ATM inhibition. Finally, we monitored the phosphorylation of MDC1 that remained unaffected upon WRAP53β down-regulation.

In **Paper III** we showed that WRAP53β protein is phosphorylated in serine64 (pWRAP53β^{s64}) by ATM kinase in response to various types of DNA damage. Interestingly, pWRAP53β^{s64} is recruited to sites of DNA lesions and promotes its interaction with γH2AX. Furthermore, it stimulates the recruitment of the downstream factor 53BP1 and enhances repair with both HR and NHEJ pathways.

In **Paper IV** we identified the C/D box scaRNA2 as an important player of the DNA damage response. ScaRNA2 is recruited directly to sites of DNA damage and promotes the HR repair pathway by facilitating the recruitment of the HR repair factors at the DSBs. ScaRNA2 seems to act in combination with the C/D box RNP complex, as members of this complex accumulate also at sites of DNA lesions. The catalytic domain of this complex belongs to the methyltransferase fibrillarin. Interestingly, depletion of fibrillarin impairs the recruitment of specific repair factors and reduces the efficiency of HR repair.

To sum up, our data identify the Cajal body components WRAP53β and scaRNA2 as key regulators of the DNA repair process.

LIST OF SCIENTIFIC PAPERS

I. Henriksson S, Rassoolzadeh H, Hedström E, **Coucoravas C**, Julner A, Goldstein M, Imreh G, Zhivotovsky B, Kastan MB, Helleday T, Farnebo M. The scaffold protein WRAP53β orchestrates the ubiquitin response critical for DNA double-strand break repair. Genes Dev. 2014 Dec 15;28(24):2726-38. doi: 10.1101/gad.246546.114

II. Rassoolzadeh H, **Coucoravas C**, Farnebo M. The proximity ligation assay reveals that at DNA double-strand breaks WRAP53β associates with γH2AX and controls interactions between RNF8 and MDC1. Nucleus. 2015 Sep 3;6(5):417-24. doi: 10.1080/19491034.2015.1106675

III. **Coucoravas C**, Dhanjal S, Henriksson S, Böhm S, Farnebo M. Phosphorylation of the Cajal body protein WRAP53β by ATM promotes its involvement in the DNA damage response. RNA Biol. 2016 Oct 7:0 DOI:10.1080/15476286.2016.1243647

IV. **Coucoravas C**, Hrossova D, Bergstrand S, Dhanjal S, Pederiva C and Farnebo M. Cajal body-specific small nuclear RNA2 (scaRNA2) controls repair of DNA double-strand breaks. Manuscript

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LIST OF ABBREVIATIONS

4-OHT	4-hydroxytamoxifen
53BP1	Tumor suppressor p53-binding protein 1
Alt-EJ	Alternative end-joining
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
BER	Base excision repair
BRCA1	Breast cancer associated gene 1
BRCA2	Breast cancer associated gene 2
BrdU	Bromo-2-deoxyuridine
CAB box	Cajal body box
CAR	Chromatin associated RNA
CHD3	Chromodomain helicase DNA-binding protein 3
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CtIP	C-terminal binding protein 1(CtBP1) -interacting protein
D-loop	Displacement loop
DC	Dyskeratosis congenita
DD	Distabilization domain
DDR	DNA damage response
DKC1	Dyskerin 1
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase, catalytic subunit
DSB	Double-strand break
ER	Estradiol Receptor
FBL	Fibrillarin
FHA	Forkhead-associated
FTO	Fat mass and obesity-associated protein
GAR1	Glycine arginine rich protein 1
GFP	Green fluorescent protein
Gy	Gray
HP1	Heterochromatin protein 1
HR	Homologous recombination
Hstaf	Human selenocystein tRNA gene transcription activating factor

IR	Ionizing radiation
IRIF	Ionizing radiation-induced foci
Kap-1	KRAB-associated protein 1
KO	Knock-out
LacI	Lac repressor
LacO	Lac operator
LSm	Like Sm
MCP	MS2-coat protein
MDC1	Mediator of DNA damage checkpoint protein 1
METTL3	Methyltransferase-like 3
MMC	Mitomycin C
MMR	Mismatch repair
MMSET	Multiple myeloma SET domain-containing protein
Mre11	Meiotic recombination 11
mRNA	Messenger RNA
Nbs1	Nijmegen breakage syndrome 1
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NHP	Non-histone chromosome protein
NHP2	Nucleolar Protein Family A member 2
NHP2L1	NHP2-Like protein 1
NOP10	Nucleolar Protein Family A member 3
NOP56	Nucleolar protein 56
NOP58	Nucleolar protein 58
Nt	Nucleotide
NuRD	Nucleosome remodeling and histone deacetylation
PAR	Poly (ADP-ribose)
PARP	Poly (ADP-ribose) polymerase
PLA	Proximity ligation assay
RBP	RNA binding protein
RCA	Rolling circle amplification
RCP	Rolling circle product
RDS	Radioresistant DNA synthesis

RNA	Ribonucleic acid
RNaseA	Ribonuclease A
RNF168	Ring finger protein 168
RNF8	Ring finger protein 8
RNP	Ribonucleoprotein
RPA2	Replication protein A2
rRNA	Ribosomal RNA
scaRNA	Small Cajal body-specific RNA
scaRNP	Small Cajal body-specific Ribonucleoprotein
Sm	Smith
SMA	Spinal muscular atrophy
smFISH	Single molecule RNA FISH
SMN	Survival of motor neuron
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
snRNP	Small nuclear Ribonucleoprotein
SSA	Single-strand annealing
SSB	Single-strand break
ssDNA	Single-stranded DNA
SUV39H	Suppressor of variegation 3-9 homologue
TCAB1	Telomerase Cajal body protein 1
TERC	Telomerase RNA component
TIP60	60 kDa Tat-interactive protein
tRNA	Transfer RNA
Unrip	Unr-interacting protein
USP	Ubiquitin-specific protease
UTR	Untranslated region
UV	Ultraviolet
WRAP53	WD40-encoding RNA antisense to p53
WT	Wild type
XLF	XRCC4-like factor
XRCC	X-ray repair cross-complementing protein
ZNF143	Zinc Finger Protein 143

1 INTRODUCTION

1.1 DNA damage and repair

DNA integrity is constantly threatened by numerous factors that can be either exogenous/environmental (Ultra-violet irradiation (UV), ionizing irradiation (IR) etc.), or derived from internal cellular processes (replication stress, metabolic function). Thousands of those damaging events occur daily, and once a cell loses its ability to repair these lesions efficiently, genome stability is lost [1].

The fate of a cell that has accumulated unrepaired DNA damage is either to become senescent or apoptotic [2]. This might lead to several neurodegenerative diseases and premature aging [3]. Alternatively, the cell can undergo malignant transformation [4].

There are several types of DNA damage where the most lethal are DNA double strand breaks (DSBs). These lesions can arise upon collapse of the replication fork, during processing of inter-strand crosslinks or through exposure to IR, which is widely used to treat cancer [5, 6]. Cells have developed multiple repair mechanisms to repair their DNA damage. Non-homologous end joining (NHEJ) and homologous recombination (HR) are the main pathways used to repair DSBs [7]. In addition, other types of DSB repair named alt-EJ (alternative end-joining) and single strand annealing (SSA) also exist and such repair often causes genome rearrangements and oncogenic alterations [8]. Base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR) are used to repair single stranded DNA (ssDNA) damage [7, 9-11].

1.2 DNA damage response (DDR)

DNA damage activates a cellular response termed the DNA damage response (DDR). This response pathway is comprised of DNA damage sensors, signal transducers, mediators and effectors [12]. Proteins such as the Mre11, RAD50 and Nbs1 (forming the MRN complex), as well as the Ataxia telangiectasia mutated (ATM) and Ataxia telangiectasia and Rad3 related (ATR) kinases initially sense damaged DNA [12]. Accumulation of ATM and ATR at such sites promotes phosphorylation of the neighboring histone variant H2AX in serine 139 (γH2AX) [13]. This event initiates the repair cascade where proteins involved accumulate at the break site in a stepwise manner. One of the first factors to be recruited is MDC1, which directly interacts with γH2AX [14]. MDC1 is an adaptor protein that via multiple interactions recruits downstream proteins to the DSBs [15]. These include the crucial E3 ubiquitin ligases RNF8 and RNF168 that catalyze ubiquitylation of damaged chromatin [15].

Ubiquitylation refers to the process where a small protein named ubiquitin is attached to lysine residues on other proteins, including histones in chromatin. This modification of damaged chromatin associates with alterations in the chromatin structure, which subsequently promotes the accumulation of the downstream DDR factors like p53 binding protein 1 (53BP1), breast cancer associated gene 1(BRCA1) and RAD51 at the break site

[16].

1.3 Non-Homologous End Joining (NHEJ)

NHEJ is the primary DSB repair mechanism in mammalian cells, as it is functional throughout the cell cycle. The activity of NHEJ is focused on the re-ligation of damaged DNA ends. The type of DSB influences the quality of the subsequent repair by NHEJ. For example, breaks with blunt ends or complementary overhangs do not require processing before end-joining and the initial DNA sequence can be restored. Other types of DSBs cannot re-ligate directly and require processing and DNA synthesis to generate proper substrates for DNA ligation. IR-induced DSBs usually require processing/trimming of the broken DNA ends before end-joining can occur. The actual procedure of DNA end-processing can lead to mutagenic events, by adding or losing genetic information, making NHEJ an error-prone DSB repair pathway [17, 18].

The main NHEJ machinery is comprised of the proteins Ku70, Ku80, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XRCC4 (X-ray cross complementing group 4), XLF (XRCC4-Like Factor), and the DNA ligase IV [19]. Ku70 and Ku80 bind strongly to DNA in a sequence-independent manner and their affinity for DNA is much higher in the presence of DSBs. Ku70 and Ku80 have the ability to form a ring-shaped heterodimer, which immediately binds very strongly and wraps around both damaged DNA ends [20, 21]. The Ku70/80 heterodimer can act as a scaffold for the recruitment of downstream NHEJ factors to the DNA damage site, including the nuclease Artemis, DNA polymerase and ligase necessary for end-joining [17]. Upon binding to DSBs, Ku70/80 heterodimers recruit and activate DNA-PKcs to form the DNA-PK holo-enzyme [22]. DNA-PK is now able to bring the broken DNA ends in close proximity in order to start the synapsis procedure, which is crucial for end-joining [23]. The final step of NHEJ is the actual ligation of the processed DNA ends, which is performed by the DNA ligase IV in collaboration with XRCC4 and the XLF factor [24, 25]. Another very important component of NHEJ repair pathway is the exo/endonuclease Artemis. The endonuclease activity of Artemis is activated after being phosphorylated by the DNA-PKcs. Artemis cleaves DNA structures in order to resolve potential problems arising from incompatible DNA ends, such as hairpins and 5'/3'-overhangs [26, 27].

1.4 Homologous Recombination (HR)

The most important characteristic of HR is that it offers an error-free repair mechanism by using an undamaged template to restore any information lost at the break site. The template used is found either on a sister chromatid, a homologous chromosome, or a non-homologous chromosome, as a repeated sequence. The template most commonly used, is the sister chromatid, which is why HR is active during late S and G2 phases of the cell cycle when DNA replication is completed and an intact sister chromatid is available. HR starts with a 5' to 3' end-resection to generate ssDNA. The first step of the resection is initiated by the MRN complex and the C-terminal binding protein 1(CtBP1) - interacting protein (CtIP) nuclease,

which is followed by a process that involves invasion of the undamaged sequence and extension of the resected DNA [28]. The second step involves binding of the ssDNA by the replication protein A (RPA) [29], which protects the ssDNA from further processing. The strong binding of RPA to ssDNA does not allow formation of secondary hairpin structures [30, 31]. Subsequently, RPA is evicted and replaced by RAD51, with the assistance of recombination mediator proteins, like the breast cancer associated gene 2 (BRCA2) and the five RAD51 paralogs [32, 33]. RAD51 loading on ssDNA promotes the creation of a filament with the ability to invade intact DNA in the search for sequence homology. This will generate a displacement loop (D-loop) formation, which is necessary to produce a Holliday junction and a heteroduplex molecule [34]. The repair will continue by using the undamaged sister chromatid strand as a template, followed by ligation of the DNA ends. Very often more than one Holliday junction is formed. In the final step of HR the Holliday junctions are dissolved in order to complete the process, which will give rise to either a crossover or a non–crossover product, depending on the direction of resolution [35].

1.5 DNA Repair Pathway choice

It is still unclear how exactly the cells decide which DSB repair mechanism to use. Beside the cell cycle phase, the repair factors themselves appear to affect this choice. For example, the NHEJ factor 53BP1 is upon recruitment to DSBs negatively regulating resection in G1 phase, thus promoting NHEJ repair. In S phase, however, BRCA1 stimulates the removal of 53BP1 from DSBs, allowing resection and HR. Consequently, cells with mutated BRCA1 loose their ability to induce HR in S phase and as a result improper NHEJ occurs at replication-associated DSBs, leading to genome rearrangements [36, 37].

Other factors that affect repair pathway choice include chromatin state (euchromatin vs heterochromatin) and damage complexity [38, 39]. Euchromatin tends to be more transcriptionally active and a DNA lesion within these areas should preferentially be repaired with an error-free method like HR. It has been shown that some repair proteins are associated with specific chromatin markers. Actively transcribed regions rich in H4K16 acetylation (H4K16ac) have been shown to block 53BP1 from binding to H4K20me2 and instead promote recruitment of BRCA1 and repair by HR [40, 41]. Additionally, the euchromatin marker H3K36me3 plays a role as a scaffold for HR associated factors in the S/G2 phase, while DNA damaged in heterochromatin areas lacking this marker recruit NHEJ factors [42]. Moreover, it is very important that the damaged regions are not transcribed, as this may give rise to an erroneous transcript. Recent studies show that damaged euchromatin during the G1 phase undergoes a heterochromatinisation procedure that blocks DSB repair in this phase. This allows these lesions to be repaired by HR during the G2 phase instead [43]. The formation of heterochromatin is induced by a repressive complex comprised of the histone methyltransferase SUV39H1 responsible for the methylation of H3 at lysine 9, the heterochromatin protein 1 (HP1) and the transcription factor Kap-1. Kap-1 recruits CHD3 (chromodomain helicase DNA binding 3) that is part of the Nucleosome Remodelling and Deacetylase (NuRD) complex and its presence is associated with compacted chromatin [44].

This complex as a whole is known to promote H3K9me3 formation of euchromatin areas [43], and this modification is essential for the recruitment of the acetyltransferase Tip60, which promotes the activation of ATM [45]. However, since such compacted structures will inhibit efficient DSB repair, it is important that the chromatin rapidly reinstates to a more loose architecture. Persistent chromatin condensation will negatively impact repair and recovery from DNA damage [46]. The de-compaction procedure is mainly guided by activated ATM through a mechanism that involves the phosphorylation of Kap-1, resulting in the release of the CHD3 complex [47] and relaxation of the chromatin structure [48]. Altogether, this indicates that the chromatin state is very important for DNA repair efficiency and choice of repair pathway.

1.6 WRAP53 & Cajal Bodies

The gene *WRAP53 (WD40-encoding RNA antisense to p53)* was identified in our laboratory as an antisense regulator of the p53 tumor suppressor and a critical player in the DNA DSB repair cascade [49-52]. The *WRAP53* gene encodes at least two functional products. The first one is called WRAP53α and is an antisense transcript that stabilizes the p53 tumor suppressor gene by interacting with the p53 RNA through a sequence of perfect complementarity (Fig. 1). This stabilization of p53 by WRAP53α is required for the induction of the p53 protein upon DNA damage [50]. The second transcript encoded by *WRAP53* is called WRAP53β and gives rise to a multifaceted and evolutionary conserved protein (Fig.1). The most important structural characteristic of this protein is its WD40 domain, which is predicted to contain seven repeats and has the capacity to interact simultaneously with multiple binding partners in a non-exclusive manner [53]. WRAP53β is located both in the cytoplasm and the nucleus; and is highly enriched in sub-nuclear organelles named Cajal bodies [52].

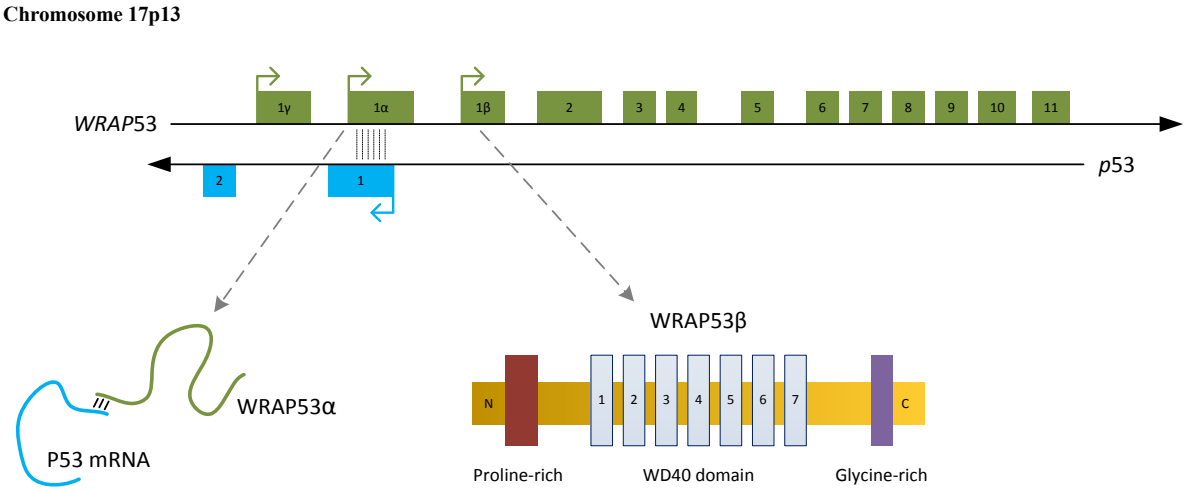

Figure 1: Schematic representation of the *WRAP53* gene, with the two functional products WRAP53α and WRAP53β depicted.

Cajal bodies were first described more than 100 years ago by the Spanish scientist Santiago Ramon y Cajal [54]. They can vary from 1 to 10 per nucleus and this number is positively correlated with the transcription and splicing activity of the cell [55]. Cajal bodies are

enriched in ribonucleoprotein (RNPs) complexes, including small nuclear RNPs (snRNPs), small Cajal body specific RNPs (scaRNPs) and the telomerase RNP complex, involved in splicing and telomere maintenance [55]. Cajal bodies are a meeting point and maturation area for these complexes. Moreover, the survival of motor neuron (SMN) complex and specific factors implicated in 3' end processing of histone mRNA also locate to Cajal bodies [56, 57]. A marker of Cajal bodies is the coilin protein, which interacts with several Cajal body factors and function as a platform to promote the formation of these organelles.

WRAP53β is an essential Cajal body factor that similar to coilin plays a central role in the formation and maintenance of these organelles. In the absence of WRAP53β, Cajal bodies are disrupted and cannot be reformed. High over-expression of WRAP53β also disrupts Cajal bodies for unclear reasons [52]. Importantly, WRAP53β has the ability to guide several factors to Cajal bodies and loss of this protein will lead to their mis-localization. For example, the SMN protein, whose inherited mutations cause the neurodegenerative disorder spinal muscular atrophy (SMA), is imported into the nucleus and targeted to Cajal bodies by WRAP53β [52]. Under normal conditions SMN binds to WRAP53β in the cytoplasm and together they enter the nucleus by interacting with the nuclear pore receptor importin β. Lack of WRAP53β leads to an accumulation of SMN in the cytoplasm and mis-localization of nuclear SMN to the nucleolus [52]. Mis-localization of SMN and in particular loss of this protein in Cajal bodies can lead to defective splicing [58], which could explain why SMA patients with problematic binding of SMN to WRAP53β show much more severe symptoms [52].

Another group of molecules that depend on WRAP53β for their localization in Cajal bodies are the small Cajal body specific RNAs (scaRNAs) [59]. These RNAs will be described in more detail later on in the text.

WRAP53β is also important for the localization of coilin and the telomerase holo-enzyme to Cajal body. Loss of WRAP53β results in their mis-localization from Cajal bodies to the nucleolus similar to SMN. In the case of telomerase, WRAP53β associates with the telomerase RNA component (TERC RNA) and thereby promotes the localization of the entire telomerase enzyme to Cajal bodies and further on bring this enzyme to telomeres [60]. TERC is a 451 nucleotide (nt) H/ACA scaRNA and WRAP53β binds the CAB box in this RNA.

Germline mutations in WRAP53β, which inactivate the ability of this protein to recruit telomerase to telomeres, result in a rare progressive disease called dyskeratosis congenita (DC) [61]. This disease is defined by bone marrow failure, premature ageing, cancer susceptibility and some of the symptoms include mucosal leukoplakia, skin hyperpigmentation and nail dystrophy. Although, some of these symptoms can be connected to short telomeres caused by deficient WRAP53β, other properties of DC, like age of onset and the severity of this syndrome seem to be related to impairment of other cellular functions [61]. Indeed, accumulation of DNA damage, indicating problematic repair has been linked to

the progression of this disease [62]. This comes in accordance with the recent findings of our group demonstrating the involvement of WRAP53β in DNA DSB repair. In this setting, WRAP53β targets the ubiquitin ligase RNF8 to the DNA breaks by mediating its interaction with the upstream factor MDC1 [49].

1.7 Chromatin Associated RNAs and DNA repair

The increasing information about the intersection of RNA with chromatin gives evidence that the functional aspects of RNAs on DNA and chromatin is significant and could reflect the tip of the iceberg. RNAs are being involved in several epigenetic processes like dosage compensation, RNA interference-mediated heterochromatin assembly and gene silencing, and programmed DNA elimination. The exact mechanism of action of all these chromatin associated RNAs (CARs) is not always clear but very often they function through a RNA-binding protein (RBP) [63].

This information encouraged us to explore the role of RNAs in DNA repair, as it is a cellular process where RNA and chromatin could collide. Indeed coding and non-coding RNAs are being more and more linked in the DNA repair process. Non-coding RNAs can be associated with DNA repair in an indirect or a direct manner. They can function indirectly through interaction with RBPs that can act either in cis, by being recruited to the damage site, or in trans, by manipulating the levels of DDR-related genes [64]. Recent studies showed that DNA damage-induced foci are sensitive to RNaseA treatment and require locally produced small non-coding RNAs to be formed [65]. Furthermore, it was shown that endogenous RNA transcripts could play a role as a template during homologous recombination in yeast Saccharomyces cerevisiae [66, 67]. Lastly, recent findings point out the critical effect of RNA post-transcriptional modifications at the sites of damage, supporting the idea of the importance of RNA in DNA repair [68].

The high affinity of WRAP53β for the family of non-coding scaRNAs made us raise the question whether WRAP53β could collaborate with scaRNAs during DNA repair.

1.8 Small Cajal body-specific RNAs (scaRNAs)

ScaRNAs belong to a family of around 20 members that guide post-transcriptional modifications on small nuclear RNAs (snRNAs). ScaRNAs are related both in structure and function with small nucleolar RNAs (snoRNAs) that are involved in modification of ribosomal RNA (rRNAs). The main differences of scaRNA vs snoRNAs are their localization and target RNAs; while snoRNAs are mainly localized in the nucleolus and guide modifications of rRNAs, scaRNAs are mostly found in Cajal bodies and guide modifications of snRNAs [69, 70]. However, scaRNAs and snoRNAs associate with the same core proteins that catalyze the modifications on r/snRNAs.

1.8.1 scaRNA sub-groups

There are two main classes of scaRNAs - C/D or H/ACA box scaRNAs (Fig. 2). The C/D box scaRNAs contain the C (RUGAUGA) and D (CUGA) motifs in their 5' and 3' ends, and guide 2'-O-methylation of target snRNAs. Very often there are two C/D boxes named C/D and C'/D' boxes, respectively. This class of scaRNAs associate with four core proteins; the methyltransferase fibrillarin (FBL), NOP56, NOP58 and NHP2L1/15.5K. Upstream of the D or D' box there is a stretch of 10-21 nt complementary to the target RNA. The methyltransferase fibrillarin catalyzes the 2'-O-ribose methylation of precisely the 5th nt upstream the D or D' box (Fig. 2A).

The H/ACA box scaRNAs contain the H (ANANNA) and ACA boxes and catalyze the isomerization of uridine into pseudouridine. They associate with the pseudouridine synthase dyskerin (DKC1), GAR1, NHP2 and NOP10 [71]. H/ACA box scaRNAs contain two hairpins and two single-stranded sequences that represent the two boxes (Fig. 2B). Each hairpin forms a bulge called recognition loop, and that is complementary to the RNA target. The first unpaired base of the loop represents the uridine targeted for pseudouridinylation. This uridine is always located 14 to 16 nt upstream the H or ACA box. ScaRNAs often contain two H/ACA boxes, two C/D boxes, or a combination of them, while snoRNAs usually contain only one of these boxes.

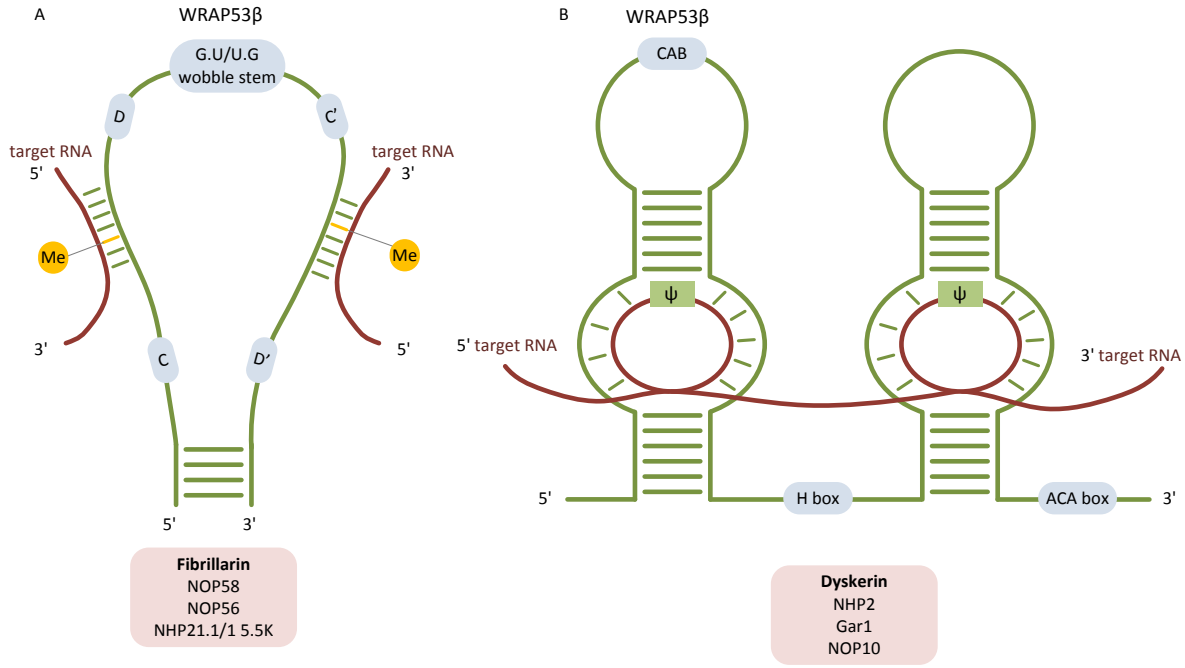

Figure 2: Illustration of the two sub classes of scaRNAs, A) C/D box scaRNA B) H/ACA box scaRNA

1.8.2 scaRNA localization

The localization of scaRNAs in Cajal bodies is important for their function, since their target snRNA also localize to this organelle, increasing the chance of interaction. A specific sequence in scaRNAs, called the CAB box mediates their recruitment to Cajal bodies (Fig. 2). This sequence is bound by the WRAP53β protein, which thereby targets them to Cajal

bodies. The CAB box in H/ACA scaRNAs is four nucleotides long and is present either on the 5' or 3' hairpin terminal loops. This tetranucleotide sequence is ugAG, where the AG dinucleotide is highly conserved and possibly found in all H/ACA and mixed domain C/D-H/ACA scaRNAs (Fig. 2B) [72]. Although C/D scaRNAs lack this consensus CAB box, they instead contain a GU- or UG-dinucleotide-rich repeat sequences, called the G.U/U.G wobble stem, predicted to form a terminal stem-loop of the RNA apical hairpin (Fig. 2A). This novel Cajal body localization element is critical for the interaction of C/D box scaRNPs with WRAP53β, and their subsequent localization to Cajal bodies [73].

1.8.3 C/D box scaRNA2

Most scaRNA genes in vertebrates are located in introns of host genes. These intronic scaRNAs are spliced out from the host pre-mRNA and then matured by trimming of their 3' and 5' ends [74]. However, some scaRNAs, including scaRNA2 (C/D scaRNA) and TERC/scaRNA19, the telomerase RNA (H/ACA scaRNA) are not encoded by host genes but contain their own promoters. For scaRNA2, the promoter is located 161 nt upstream of the transcription start site (TSS) and contains specific information used to initiate the transcription by RNA polymerase II. There are four evolutionary conserved cis-acting elements that promote transcription of human scaRNA2: a TATA box, which is important but not necessary as mutation of this element results in a new transcription start site without affecting the transcription per se; a hStaf/ZNF143 binding site that is known to be associated with the expression of snRNAs [75]; and finally two other uncharacterized motifs X and Z present only in mammalian scaRNA2 promoters that act in a species-specific manner as they are absent from promoters of other species (i.e. Xenopus and pufferfish) [76].

1.9 RNA Modifications

The decoding of the epitranscriptome is a major challenge and achievement for the RNA field. There are more than 170 different known RNA modifications with around 50 of them present in mammalian cells [77]. The most commonly modified RNAs are the transfer RNAs (tRNAs), while the most common modification is the N^6-methyladenosine (m^6A) that can occur at least at 3 locations in every mRNA on average. m^6A has been proven to stimulate mRNA processing and transport in mammalian cells [78]. There have been identified three different group of enzymes linked with the m^6A modification. First are the enzymes that catalyze the modification and they are called "writers", second there are the proteins that identify these modifications and they are called "readers", and lastly there are the enzymes that remove this modification and they are named "erasers" [79]. These proteins might be responsible for the orchestration of other kind of post-transcriptional modifications.

Furthermore, it is very important to investigate the biological function of all these modifications. It has been proven that RNA modifications can affect protein diversity, as variations in the epitranscriptome can lead to alternative splicing and translation of

different protein isoforms [80]. RNA modifications can also affect the stability of RNA. Finally, post-transcriptional modifications can play a crucial role in the folding of RNAs by changing the secondary structure and thus influence the potential RNA or protein-binding partners. On that matter, very recent publication has reported enriched m^6A modification induced at DSBs. This modification might change the conformation of the RNAs located to the breaks and alter their binding capacities [68].

1.9.1 snRNAs: scaRNA targets

SnRNAs, are the known RNA targets of scaRNAs. This is a class of highly transcribed non-coding RNAs located in the nucleus. They are divided in two main categories the Sm and the Lsm snRNA, according to their structure and binding protein partners. The Sm group is composed by U1, U2, U4, $U4_{atac}$, U5, U7, U11 and U12 while Lsm group contains U6 and $U6_{atac}$. The majority of those RNAs are part of the spliceosome core machinery, that is responsible for the removal of introns from precursor mRNAs [81]. Post-transcriptional modifications of the snRNAs are essential for proper spliceosome function and pre-mRNA splicing [82].

1.9.2 snRNA modifications

The modified sites on snRNAs are well conserved and distributed in functionally important loci involved in RNA–RNA and RNA–protein interactions. Thus, modification of snRNAs alters their structure and enhances interactions with RNA and proteins important for the function of snRNA in pre-mRNA splicing [69] [83, 84]. For example, pseudouridylation of the U2 snRNA stabilizes its interaction with intronic pre-mRNAs and induces a structural change that promotes the splicing reaction. U2 snRNAs lacking post-transcriptional modifications create a dysfunctional U2 snRNP complex that leads to a defective assembly of the spliceosome resulting in impaired splicing [85].

The strong affinity of WRAP53β for scaRNAs and the ability of WRAP53β to target scaRNAs to their site of action, indicate that these factors may collaborate in other cellular processes like DNA repair. Potentially, WRAP53β could recruit scaRNAs to DNA DSB sites where these RNAs could be involved in guiding modifications on target DNA, RNA or proteins (e.g. histones).

2 MATERIAL AND METHODS

2.1 DNA damaging agents and ionizing radiation induced foci (IRIF)

There are several DNA damaging agents used in both scientific research and in the clinic. DNA damaging agents can be divided into different groups according to the type of damage they induce. Alternatively, they are often characterized as chemical or physical agents [86]. There is a great variety of different types of chemical agents that are widely used, all with different properties and mode of action. These agents are commonly applied to study the different DNA repair pathways [86, 87]. On the other hand, there are primarily two different sources of physical agents; UV radiation and IR [88]. By using X-rays or γ-rays as source of irradiation, DNA damage occurs by two pathways, through direct and indirect action. Direct action occurs when a charged particle such as an ion or an electron directly passes through the DNA, which results in a DNA break. Indirect action occurs when highly reactive OH radicals are produced from ionize water molecules that are in close proximity to DNA which eventually damages the DNA [89, 90]. These actions results in the formation of single strand breaks (SSB) or DSB when a base is damaged or when the sugar phosphate backbone is interrupted. For every Gray of X-ray irradiation there is approximately an induction of 3000 base damages, 1000 SSBs and 20-40 DSBs [91, 92].

IR is a commonly used method to induce DSBs, primarily because of the high amount of DSBs produced; but also because it is responsible for the formation of ionizing radiation induced foci (IRIF) [93]. IRIFs are patches of heavily damaged DNA that comprises thousands of accumulated DSB factors that includes checkpoint signaling and repair factors (Fig. 4A) [92, 94]. The accumulated factors that appear as IRIF allow visualization by immunocytological detection methods such as indirect immunofluorescence and live imaging. This enables the possibility to visually study the function of the repair factors, by i.e. exploring their kinetics, their localization to damage sites and with these data to pinpoint the function of those repair proteins in the DDR [49].

2.2 FokI system

Another method to examine recruitment of DDR factors is through the induction of site-specific DSBs by endonuclease, including FokI, I-SceI, I-PpoI and AsiSI. For the FokI system, we employed U2OS cells stably carrying a cassette of 256 repeats of the Lac Operator (LacO), kindly provided by Prof. Roger Greenberg (University of Pennsylvania). These cells stably express a mCherry-tagged Lac repressor (LacI) fused to the non-specific FokI endonuclease. This fusion protein also contains a destabilization domain (DD) and a modified estradiol receptor (ER) (ER-mCherry-LacI-FokI-DD) (Fig. 3) to allow inducible nuclear expression of ER-mCherry-LacR-FokI-DD by administration of the small molecule Shield-1 ligand that de-activates the DD-domain and 4-hydroxytamoxifen (4-OHT) to induce nuclear translocation of the fused protein. The high affinity between LacI and LacO enables binding of the fusion protein to the LacO and generation of hundreds of site specific DSBs in the LacO by FokI (Fig. 4B). Many breaks at the same site, allows

visualization of proteins at DSBs that otherwise are too few to form a detectable IRIF at only one break. It also enables us to follow the kinetics of repair factors to DNA breaks.

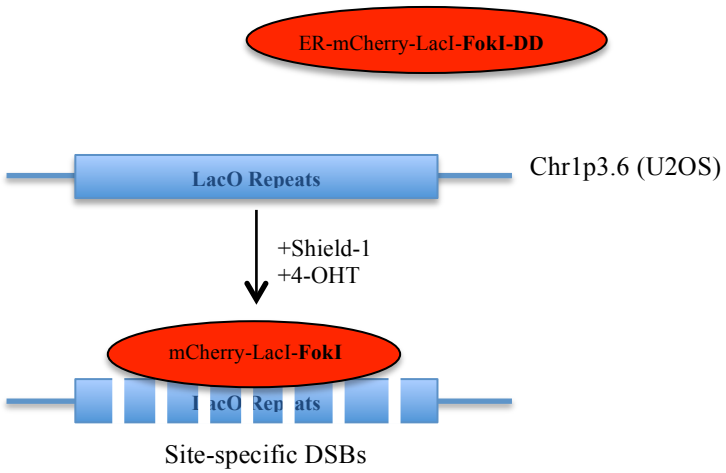

Figure 3: Schematic representation of the FokI system

2.3 Laser micro-irradiation

An alternative approach to visualize accumulation of DNA repair factors at sites of DNA damage is by laser micro-irradiation. This method induces local damage with precise irradiation of 1-2.5 µm within the nuclear DNA (Fig. 4C). Different laser types, wavelengths, energy output and photosensitization can be used [92, 95], as exemplified below. Laser micro-irradiation is a powerful tool to study spatiotemporal protein dynamics in the DNA repair process and additionally provides the opportunity to visualize factors that are unable to form IRIF. Furthermore, by incorporating Green fluorescent protein (GFP) fused with a protein of interest, the dynamics of this protein can be studied within seconds after micro-irradiation by live imaging [95]. There are different types of laser used for induction of localized damage, such as UV-A, UV diode laser and YAG laser. However, the laser type that induces damage that resembles the IRIFs is UV-A, which wavelength ranges between 337 – 390 nm [94]. 24 hours prior such experiment cells need to be pre-sensitized with 5-Bromo-2-deoxyuridine (BrdU) which upon irradiation will stimulate a photochemical reaction that will cause DNA damage [96].

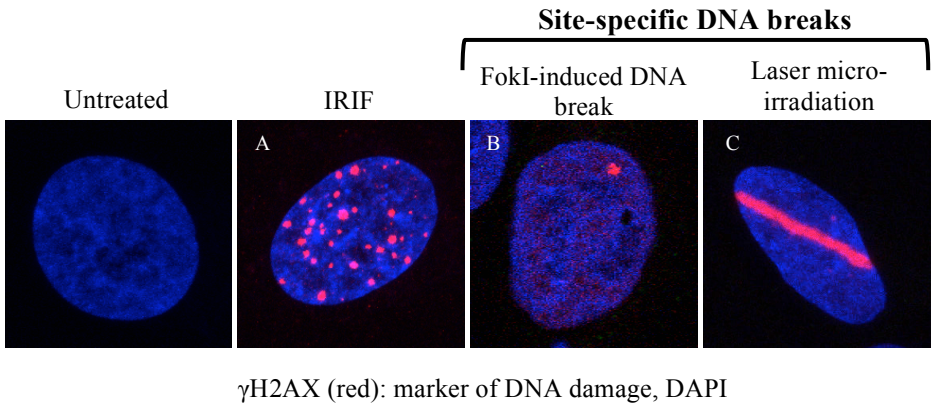

Figure 4: Different methods to induce DSBs in cells.

2.4 Single molecule RNA FISH (smFISH)

Fluorescent *in situ* hybridization is a well-established method for visualization of DNA and RNA sequences in fixed cells. In this context we used a signal amplification scheme in order to enhance the sensitivity and specificity of this method. In our case, the detection of scaRNA2 molecule at sites of damage was a challenge. On one hand, it was the small size of this RNA and on the other hand, only a small pool of this RNA was actually localized at DSBs. In order to amplify the signal, we designed 10 DNA probes (~30 nt) with a 10 nt gap in between them, covering the whole scaRNA2 molecule (Fig. 5). Each of these probes is equipped with a 5' and 3' linker sequence (Fig. 5). Double-labeled DNA oligos complementary to the linker sequence are used to visualize the probe (Fig. 5). This enables substantial signal amplification that allows detection of single RNA molecules.

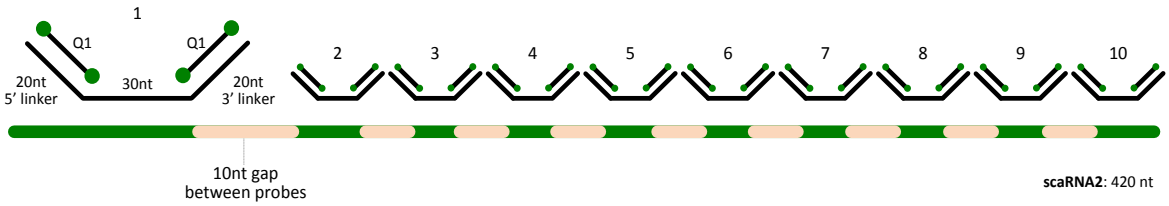

Figure 5: Schematic representation of smFISH experimental design that depicts the 10 DNA probes and the double-labeled fluorescent oligos for scaRNA2.

2.5 Visualization of MS2-tagged RNA with GFP-tagged MS2 coat protein (GFP-MCP)

Although the smFISH method has high specificity and sensitivity, it can be mainly used on fixed cells. In order to study the kinetics of RNA molecules in live cells we employed a GFP-MCP system in combination with an MS2-tagged RNA[97]. This method is based on the high affinity between the coat protein of the bacteriophage MS2 and a specific 19 nt stem-loop structure (MS2 sequence: CGTACACCATCAGGGTACG) [98, 99]. Co-transfection of GFP-MCP and RNA-MS2 will result in their binding and the GFP will allow detection of the RNA in live cells by microscopy (Fig. 6).

The challenge with this approach is the correct design and insertion of the MS2 loop. These extra nucleotides must be added carefully; otherwise they can interfere with the secondary structure of the RNA and subsequently with its function. It is advised to add this sequence in a predicted stem loop structure of the RNA, away from functional domains and regulatory regions. In our case we used a software that predicts RNA secondary structures, called mFold [100]. Correct insertion of the MS2 loop will result in a functional RNA. This method can be combined for example with laser-microirradiation or as in our case with FokI cells in order to follow the trafficking of a specific RNA molecular at sites of DNA damage.

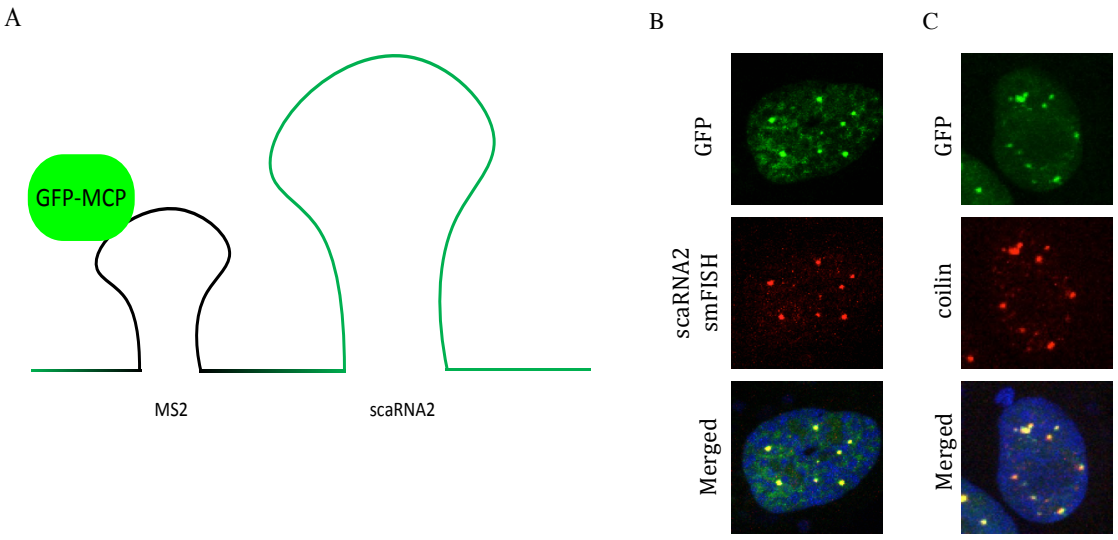

Figure 6: A) Illustration of GFP-MCP and RNA-MS2 loop. B) GFP signal contained scaRNA2 as confirmed with smFISH against scaRNA2. C) GFP signal accumulates in Cajal bodies overlapping with coilin showing correct localization of the scaRNA-MS2 construct.

2.6 Proximity Ligation Assay (PLA)

Proximity ligation assay (PLA) is a dual target recognition method that was developed to increase the *in situ* specificity and sensitivity of protein detection and quantification using conventional microscope in immunocytochemistry and immunohistochemistry settings [101-103]. Additionally, PLA enables the visualization of protein-protein interaction or association within one complex, when these factors are in close proximity (up to 40 nm apart) and moreover can detect posttranslational modification of proteins in cells and tissues [104].

There are two different approaches of PLA, the direct and the indirect method. For direct PLA, primary antibodies are conjugated with short DNA oligonucleotides, whereas the indirect starts with the use of conventional primary antibodies to target the proteins of choice and subsequently the use of secondary antibodies that are conjugated with DNA probes. These short DNA oligonucleotides are referred to as proximity probes and if the probes are in close proximity they will act as template for hybridization and joining of the probes into a circular DNA molecule by adding a DNA ligase. The circular DNA serves then as a primer for the rolling circle amplification (RCA). Adding a DNA polymerase results in a long single stranded DNA product, covalently attached to one of the probes, which is called rolling circle product (RCP). Since the RCP is attached to one of the probes, it reveals the localization of the targeted protein(s). The RCP is then visualized by hybridizing of multiple fluorescent detection oligonucleotides to the repeated RCP sequence, allowing for visualization under a microscope (Fig. 7) [102, 103].

Over the years, the method has been widely used in research and also been modified and implemented in different visualization techniques. For example, the method has been

combined with flow cytometry to have a quantitative analysis of protein-protein interaction and post-translational modifications [103]. By combining padlock probes and PLA, individual mRNA molecules and protein complexes could be detected *in situ* simultaneously [105]. Additionally, modifying PLA by implementation of an extra circle-forming oligonucleotide upon ligation, can enable the detection and scoring of different chromatin fibers in proximity [106].

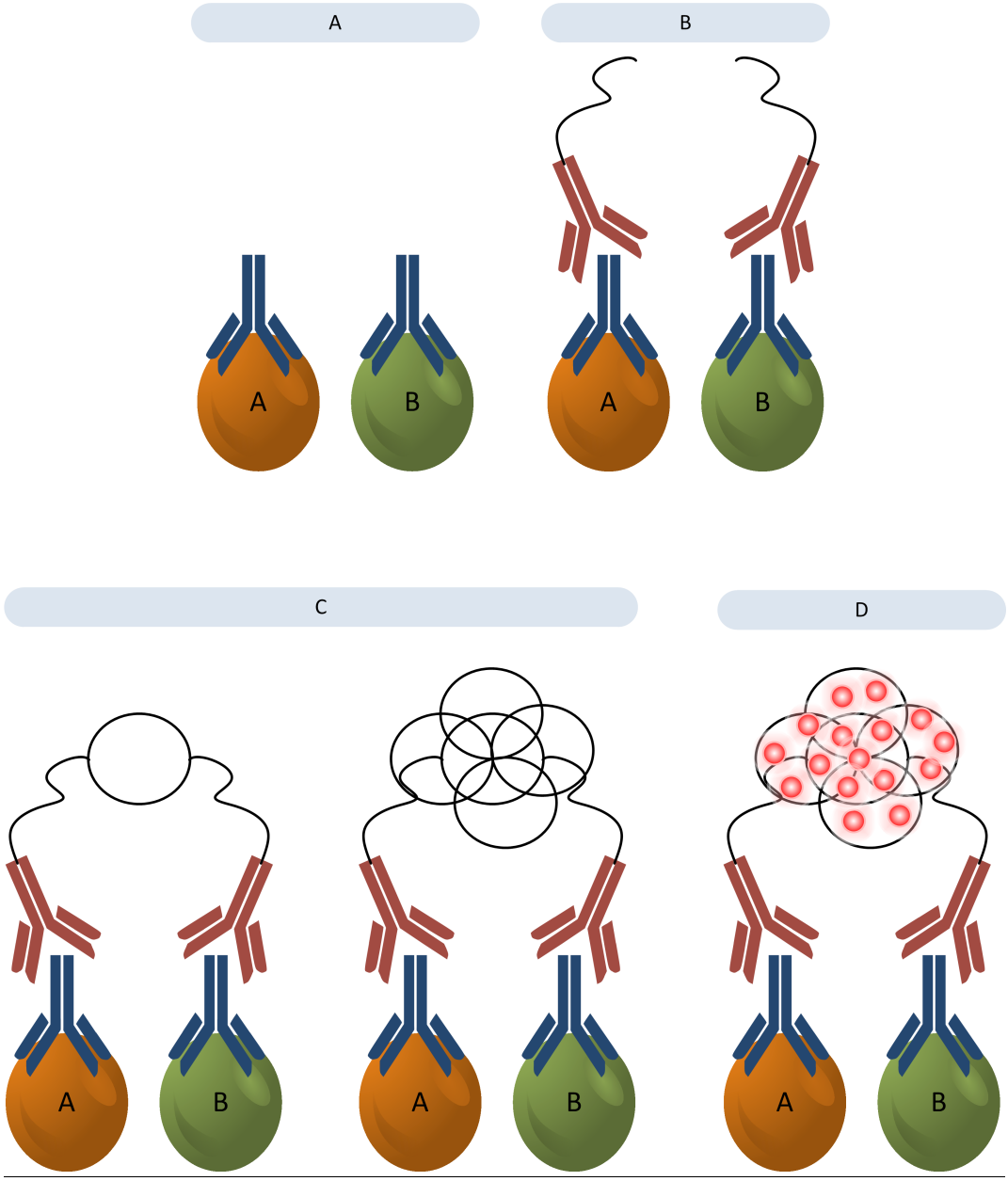

Figure 7: Schematic representation of Proximity Ligation Assay. A) Conventional antibodies raised in different species, recognize the proteins of interest. B) Secondary antibodies containing PLA probes bind to primary antibodies. C) The PLA probes that are in proximity initiate rolling circle DNA synthesis. D) The circular DNA is labeled with fluorescent-tagged complementary oligonucleotides.

3 AIMS OF THIS THESIS

The overall aim of this thesis is to identify and characterize the function of WRAP53β and scaRNAs in DNA repair

The specific aims of each paper were:

Paper I: To elucidate the molecular mechanism of WRAP53β in DNA DSB repair

Paper II: To further investigate the role of WRAP53β in DNA DSB repair by the PLA method

Paper III: To investigate the phosphorylation of the WRAP53β protein on serine residue 64 and its role in the response to DNA damage

Paper IV: To examine the function of scaRNA2 in DNA DSB repair

4 RESULTS AND DISCUSSION

4.1 Paper I - The scaffold protein WRAP53β orchestrates the ubiquitin response critical for DNA double-strand break repair.

Paper I was based on several observations implicating WRAP53β in the DDR. A large-scale proteomic analysis of proteins phosphorylated in response to DNA damage revealed WRAP53β as a target of ATM and ATR [107]. Furthermore, two independent genome-wide screens, one identifying proteins involved in genome stability by monitoring the phosphorylation of the histone variant H2AX, and the other designed to identify proteins involved in HR by using the DR-GFP reporter assay, revealed that WRAP53β is a protein associated with these processes [108, 109]. Finally, inherited mutations in the WRAP53 gene resulting in amino acid substitutions in the WRAP53β protein has been described to cause the congenital disorder Dyskeratosis Congenita (DC), and patients suffering from this disease show signs of premature aging and have a predisposition for malignancies, characteristics that are linked to impaired DNA repair[61].

In order to study the possible involvement of the WRAP53β protein in the DDR cascade, we initially employed a laser microirradiation to induce local DNA damage and check if this protein can accumulate at sites of such damage. WRAP53β was rapidly recruited at DNA breaks, indicating that it is involved early in the DDR pathway. Furthermore, we were able to visualize IRIF of WRAP53β, which co-localized and had similar kinetics with γH2AX foci. Our next step was to investigate if the accumulation of WRAP53β to DNA lesions was dependent on any of the three main kinases implicated in early steps of DNA repair[110]. According to our results, DNA-PK and ATR did not affect the recruitment of WRAP53β while inhibition of ATM significantly impaired this accumulation. In addition, WRAP53β failed to be recruited at the break site after the knock down of H2AX and MDC1. To explore the spatial and temporal dynamics of WRAP53β, we performed a ChIP experiment using an I-PpoI endonuclease to induce site specific DSBs. This approach revealed that WRAP53β is not only accumulated at the exact damage site but also in the surrounding area, up to 6kb from the DNA breakage point.

Our next approach was to elucidate the molecular mechanism of WRAP53β in the DNA repair process. Since WRAP53β is known to be involved in intracellular trafficking we raised the question whether this protein is responsible for the recruitment of DDR factors to DNA breaks. Interestingly, after knocking down WRAP53β, the repair proteins 53BP1, BRCA1 and RAD51 were unable to be recruited to DSBs while the upstream factors γH2AX and MDC1 were not affected. Since 53BP1, BRCA1 and RAD51 are recruited in an ubiquitinylation dependent manner, we further explored the recruitment of the E3 ligases RNF8 and RNF168 at the DNA damaged sites. Indeed, neither of the two E3 ligases were able to be efficiently recruited to DSBs after depletion of WRAP53β resulting in significant loss of DNA-associated ubiquitinylation.

As the accumulation of MDC1 at DSBs was unaffected by depletion of WRAP53β, while the RNF8 recruitment that is immediately downstream of MDC1 was abolished, we concluded that the potential function of WRAP53β was in-between these proteins in the DNA repair cascade. To obtain additional information on how WRAP53β recruits RNF8, we explored the potential binding of WRAP53β with both MDC1 and RNF8. WRAP53β is indeed a binding partner of both MDC1 and RNF8 and their interaction was enhanced upon induction of damage, although they bound even in cells lacking irradiation. To investigate further these interactions, we employed a series of MDC1 deletion constructs and tested the binding to WRAP53β. The MDC1 deletion mutant missing the phosphopeptide recognition domain FHA was unable to bind WRAP53β, while the rest of the deletions did not alter the binding. Similarly, we observed that WRAP53β binds to the N-terminus of RNF8 and specifically the amino acids 1-38, while MDC1 interacts with the amino acids 39-140 of the FHA domain of RNF8. Thus, WRAP53β binds the FHA domains of MDC1 and RNF8.

To investigate whether WRAP53β promotes the interaction between MDC1 and RNF8, we checked the binding pattern between WRAP53β, MDC1, RNF8 and γH2AX. By depleting each one of these factors and immunoprecipitating WRAP53β we observed that MDC1 binds WRAP53β independent of RNF8 and H2AX and that WRAP53β interacts with RNF8 independent of MDC1 and H2AX. However, MDC1 and RNF8 interaction after IR was completely abolished upon WRAP53β depletion. Of note, WRAP53β did not affect the phosphorylation of MDC1, a modification crucial for the interaction of MDC1 to RNF8.

To determine the domain of WRAP53β that binds MDC1 and RNF8 the N-terminus, WD40 domain and C-terminus of WRAP53β was separately tested for binding. By immunoprecipitation experiments we concluded that the WD40 domain of WRAP53β was sufficient and necessary for the binding of both MDC1 and RNF8. In connection to these findings, we decided to divide this domain into different combination of repeats. This approach revealed that both MDC1 and RNF8 have a preference to interact with repeats 2 and 3 of the WD40 domain. Altogether these observations show that WRAP53β interacts with both MDC1 and RNF8 through its WD40 domain, and thus promoting their binding and accumulation of RNF8 at the break sites.

To assess the effect of WRAP53β depletion on the repair of DSBs, we examined the kinetics of γH2AX foci after exposure to IR. Cells lacking WRAP53β showed a significant number of residual γH2AX foci 24h upon IR indicating a problematic repair of DNA DSBs. To examine the involvement of WRAP53β in the HR and NHEJ repair pathway, we employed two GFP-reporter cell lines (DR-GFP and EJ5-GFP) that can respectively measure their efficiency. Our results showed that by knocking down WRAP53β there was a reduction of 74% of HR and 41% of NHEJ. Moreover, irradiated cells lacking WRAP53β, demonstrated an extended G2/M arrest sign of problematic recovery due to impaired DNA repair.

Lastly, non-irradiated WRAP53β depleted cells showed signs of spontaneous damage that was measured with the formation of γH2AX foci. Same results were obtained with comet assay where cells lacking WRAP53β showed a larger amount of sporadic DNA lesions.

To conclude, in this paper number we identify WRAP53β as a new player in DNA DSB repair that mediates the interaction between MDC1 and RNF8, and thus promotes ubiquitylation of the damaged chromatin that stimulates the recruitment of the downstream DDR factors.

4.2 Paper II - The proximity ligation assay reveals that at DNA double-strand breaks, WRAP53β associates with γH2AX and controls interactions between RNF8 and MDC1.

Paper II was initiated to explore the possibility of visualizing DNA repair factors at sites of damage using the proximity ligation assay and to examine whether this method could detect accumulation of factors unable to form IRIF at breaks.

To investigate if PLA is a suitable method to detect factors at DSBs, we initially applied this method to visualize the interaction between γH2AX and MDC1, two factors that are known to interact upon IR [14]. Indeed a distinct amount of γH2AX- MDC1 PLA signals was detected in irradiated cells while no signals were found in non-irradiated cells. Depletion of either MDC1 or γH2AX or inhibition of H2AX phosphorylation with an ATM inhibitor resulted in a significant reduction of PLA signals, pointing out the sensitivity and specificity of this method. To further investigate the specificity, we checked for the interaction of γH2AX and 53BP1 that again showed significant amount of PLA signals in irradiated cells. Similarly we detected γH2AX-53BP1 PLA signals in micro-irradiated cells that were located specifically at the laser stripe.

Our next step was to explore whether PLA could detect the association between γH2AX and WRAP53β. As we described in paper I, WRAP53β is recruited at sites of damage facilitating the interaction between MDC1 and RNF8. Although we used several methods to confirm this recruitment (ChIP and immunofluorescence) there was only one WRAP53β antibody capable of detecting IRIF of this protein. Here we set out to use PLA between a WRAP53β antibody that didn't detect IRIF of WRAP53β and γH2AX. Interestingly, irradiated cells showed PLA signals while non-irradiated cell lacked such signals. Moreover, WRAP53β-γH2AX PLA signals were observed at laser stripes.

To test weather WRAP53β and γH2AX directly interacted, we immunoprecipitated WRAP53β or γH2AX in two cell lines and indeed we observed that those proteins can interact and this interaction is enhanced upon DNA damage. To pinpoint the region of interaction, we employed the same deletion constructs of WRAP53β used in paper I, however, all of these constructs failed to bind γH2AX. Therefore, we used another set of deletion constructs lacking smaller parts of the WRAP53β protein. Most of these constructs also failed to co-precipitating γH2AX, except for a variant lacking only 15 amino acids in the C-terminus of WRAP53β, which indeed retained the capacity to bind γH2AX. Furthermore, constructs with disease mutations of WRAP53β associated with Dyskeratosis Congenita were all unable to bind γH2AX.

To understand the order of interaction, we employed siRNA and inhibitors. This revealed that the association between WRAP53β and γH2AX is dependent on ATM, but not MDC1 known to be required for IRIF of WRAP53β, indicating that two pools of WRAP53β may be active at DSBs.

In continuation of these findings, we wanted to confirm the interaction of WRAP53β with MDC1 and RNF8 with the use of PLA. WRAP53β showed PLA signals with both MDC1 and RNF8 confirming their interaction, and those signals were independent of IR and phosphorylation by ATM. Subsequently, we employed PLA to study the interaction of MDC1 and RNF8. Under normal conditions irradiated cells formed PLA signals between MDC1 and RNF8, while knock down of WRAP53β, MDC1, RNF8 or the use of ATM inhibitor significantly down-regulated this interaction. Finally, we explored the interaction of MDC1 and ATM and the effect of WRAP53β depletion in this context. PLA signals between MDC1 and ATM were unaffected by knock down of WRAP53β, confirming that the latter has no effect in MDC1 phosphorylation.

In summary, in paper II we introduce PLA as a method to monitor the localization and the interaction of proteins accumulated at DSBs. Strikingly, our observation that WRAP53β binds γH2AX independent of MDC1, while it cannot form IRIF in the absence of MDC1 might reveal the presence of two different pools of WRAP53β that can interact independently of each other with either γH2AX directly or MDC1. Our observations from paper I that WRAP53β is present both at the exact site of damage but also in the surrounding area. This difference in localization might propose interaction with difference DDR factors in each case. Moreover, the kinetics of WRAP53β at the break point was much slower in comparison with time spent in distal regions from the damage.

4.3 Paper III - Phosphorylation of the Cajal body protein WRAP53β by ATM promotes its involvement in the DNA damage response.

In paper III, we investigated the post-translational modifications of WRAP53β and especially phosphorylation by the protein kinase ATM as a response to DNA damage. As mentioned before, WRAP53β has been shown to be a potential target of ATM and ATR [107], and specifically its serine residue 64 (S64). An evolutionary preserved SQ motif at this position reveals a potential biological importance of this site. To study whether WRAP53β is actually phosphorylated at S64, we generated a phospho-specific antibody that targets precisely this site (pWRAP53β^{s64}). Indeed this antibody showed phosphorylation of Flag-WRAP53β in response to DNA damaging agents, including IR, UV, hydroxyurea, camptothecin and mitomycin C. In order to confirm the specificity of this antibody, we generated a phospho-mutant construct where we exchanged the serine at position 64 with an alanine (S64A). The pWRAP53β^{s64} antibody was unable to recognize the S64A construct proving its precision.

One additional interesting observation was that the kinetics of pWRAP53β^{s64} were very similar to the time-course of WRAP53β accumulation at DSBs. Next step was to pinpoint

which exact protein kinase was responsible for this phosphorylation. We employed inhibitors against ATM, ATR and DNA-PK and after treating cells with either IR or UV we observed that ATM inhibition completely suppressed phosphorylation of WRAP53β^{64}. Moreover, depletion of WRAP53β abrogated this modification confirming again the specificity of the pWRAP53β^{64} antibody. This phosphorylation was faster following exposure to IR in comparison to UV in agreement with the slower formation of γH2AX foci after UV. Depletion of known binding factors of WRAP53β (H2AX, MDC1, RNF8) did not affect its phosphorylation proving that these proteins are not involved in this process. Furthermore, we were able to detect phosphorylation of endogenous WRAP53β although the signal was very weak, proposing that only a small fraction of this protein is actually phosphorylated at S64.

To study if pWRAP53β^{64} is recruited at DSBs, we used three independent methods. Initially, we used the FokI cell line that induces DSBs after binding to the LacO. Indeed pWRAP53β^{s64} was accumulated at the sites of such damage in similar pattern as WRAP53β. Our next approach was to use laser micro-irradiation and again pWRAP53β^{64} was rapidly recruited to the laser stripes. Finally, we studied if pWRAP53β^{s64} was able to form IRIF, and while we were unable to visualize endogenous pWRAP53β^{s64} at break sites, we could observe IRIF in a cell line that stably over-expressed Flag-WRAP53β, that clearly overlapped with γH2AX. Phosphorylation of over-expressed WRAP53β was also observed to form foci after exposure to UV that co-localized with replication protein A2 (RPA2).

We also explored whether the localization of WRAP53β in Cajal bodies was influenced by its phosphorylation. When we over-expressed the WT or the phospho-mutant S64A of WRAP53β both of them accumulated in Cajal bodies, the latter indicating that phosphorylation is not required for this accumulation. Rather it appears like phosphorylation stimulates exit from this organelle, since pWRAP53β was rarely detected in Cajal bodies by the pWRAP53β^{s64} antibody.

We next tested the importance of WRAP53β phosphorylation for interactions with its known binding partners γH2AX, MDC1 and RNF8. Strikingly, the S64A mutant was unable to interact with γH2AX while the binding to MDC1 and RNF8 was unaffected, the latter in accordance with our previous finding that MDC1 and RNF8 bind to WRAP53β independent of DNA damage and ATM.

In paper I, we concluded that WRAP53β is important for the recruitment of the downstream factors 53BP1, BRCA1 and RAD51 to DSBs. Here, we examined the influence of WRAP53β phosphorylation at S64 in this context by testing whether the S64A mutant could restore to recruitment of 53BP1 to DSBs in cells lacking endogenous WRAP53β. Notably, the S64A variant failed to restore the recruitment of 53BP1 to sites of DNA damage. Furthermore, this mutant also failed to restore proper clearance of γH2AX foci 24h after DNA damage. Since WT WRAP53β could restore both 53BP1 foci and clearance of γH2AX, we conclude that phosphorylation of WRAP53β promotes its function during DNA repair. Lastly, we employed the DR-GFP and EJ5-GFP cell lines to measure the efficiency of HR and NHEJ

respectively and we observed that by over-expressing the WT but not the S64A variant of WRAP53β mutant we were able to enhance both repair pathways.

In conclusion, we demonstrated that WRAP53β is phosphorylated at serine 64 by the protein kinase ATM as a response to DNA damage, and this modification is important for the recruitment of WRAP53β to DSBs and its interaction with γH2AX. Moreover, phosphorylation of WRAP53β was crucial for the recruitment of the downstream factor 53BP1 and the appropriate function of the HR and NHEJ repair pathways.

4.4 Paper IV - Cajal body-specific small nuclear RNA2 (scaRNA2) controls repair of DNA double-strand breaks

In this study we investigated the involvement of scaRNAs and their C/D box related proteins in the response to and repair of DNA damage. Three observations made us interested in exploring the link between scaRNAs and DNA repair. First, scaRNAs are often encoded by host genes involved in DNA repair and chromatin remodeling indicating similar function of the scaRNAs. Second, scaRNAs bind WRAP53β, known to be involved in DNA repair. Third, WRAP53β guides scaRNAs to Cajal bodies and telomeres [59, 60, 111-113] and considering the fact that WRAP53β localizes to sites of damage it appears likely that this protein could guide scaRNAs to DSBs.

In this study we focused on one member of this family; scaRNA2. The reason behind this choice was that this scaRNA binds strongly to WRAP53β, it associates with chromatin for unknown reasons and moreover it is transcribed by an independent promoter, fact that reduced the risk of indirect effect by co-depletion of the host gene.

Our initial approach to identify if scaRNA2 is a factor involved in DDR was to follow the kinetics of residual γH2AX foci 24h after exposure to IR. For this matter we used U2OS cells containing or depleted of scaRNA2 with by GapmeRs. Interestingly, a significant amount of residual γH2AX foci was still present 24 h after IR in cells lacking scaRNA2. To confirm this result, we generated two CRISPR/Cas9 scaRNA2 KO cell lines (U2OS and MCF7), where we observed again a delayed recovery of γH2AX foci after IR. Another approach was to check the cell survival in irradiated cells depleted from scaRNA2 by clonogenic cell survival assay. Indeed cells depleted of scaRNA2 showed reduced formation of colonies following exposure to IR.

Our next step was to elucidate if the involvement of scaRNA2 in DNA repair was direct and for this reason we employed three different approaches to check if scaRNA2 is recruited to sites of damage. First, we laser micro-irradiated U2OS cells followed by smFISH for scaRNA2. Strikingly, endogenous scaRNA2 accumulated at the laser stripes and co-localized with γH2AX. To confirm the specificity of this signal, we performed single staining of scaRNA2 after laser micro-irradiation, again showing accumulation of scaRNA2 at laser stripes. Of note, the detection of scaRNA2 at laser stripes could fluctuate from 0-30% between runs making quantification difficult. In that context, it should also be mentioned that detection of WRAP53β at laser stripes also varied depending on antibody was used.

An alternative approach was to study the accumulation of scaRNA2 at FokI-induced DSBs. Interestingly, smFISH revealed that scaRNA2 located exactly at the break site or in the surrounding area in 25% of the cells. As a third approach we performed live-cell imaging to follow scaRNA2 kinetics to FokI-induced breaks. MS2-tagged scaRNA2 (scaRNA2-MS2) and GFP-tagged MCP (GFP-MCP) were co-expressed in cells. This approach revealed that scaRNA2-MS2 rapidly was recruited to the FokI-induced breaks and stayed there for approximately one hour, kinetics that are similar with other DNA repair proteins in a similar model system [114].

In order to explore if scaRNA2 collaborates with WRAP53β in the assembly of the repair factors at the DBSs, we used irradiated cells expressing or depleted of scaRNA2 (either with GapmeRs or CRISPR/Cas9 KO) and stained for various DDR factors. Interestingly, depletion of scaRNA2 did not affect factors like γH2AX, MDC1, conjugated ubiquitin (recognized by FK2 antibody) and the ubiquitin-dependent NHEJ factor 53BP1, but it significantly reduced the accumulation of repair proteins involved in HR like BRCA1, RAD51 and RPA2. A small but not significant decrease was observed in the recruitment of WRAP53β to breaks. The fact that WRAP53β still could be recruited could explain why the ubiquitin response was still functional in cells lacking scaRNA2. Similar impaired recruitment of HR factors was also observed in FokI cell depleted of scaRNA2.

To confirm that the impairment of HR factors accumulation caused by scaRNA2 depletion was not a result of an off-target effect, we re-introduced scaRNA2-MS2 into scaRNA2 KO cells by using a plasmid that also contained the GFP-MCP construct. Re-introduction rescued the accumulation of BRCA1 and RAD51 at DSBs. Moreover, as scaRNA2 is involved in post-transcriptional modification of the U2 spliceosomal RNA (snU2 RNA) that is implicated in splicing, we wanted to make sure that depletion of scaRNA2 would not affect the levels of the repair factors that showed impaired accumulation at breaks when scaRNA2 was depleted. No such alterations was detected in cells depleted of scaRNA2. Furthermore, the E3 ligase RNF8 that is commonly affected in cells with impaired splicing was also stable in the absence of scaRNA2 [115].

Next, we employed the DR-GFP reporter cell line to measure HR efficiency after knocking down scaRNA2. Strikingly, depletion of scaRNA2 reduced the HR efficiency by 76%, a measurement comparable with the 75% reduction upon depletion of RAD51, a protein known to be implicated in this pathway.

The C/D box containing scaRNA2 forms a ribonucleoprotein (RNP) complex by interacting with four core proteins; fibrillarin, NOP56, NOP58 and NHP2L1. To further examine the function of scaRNA2 in DDR, we wondered whether it is recruited at the sites of damage as a complex. To answer this question we used laser micro-irradiated cells and immunostained for the scaRNP subunits. Interestingly, three of these proteins showed accumulation at laser stripes, in accordance with a recent study showing recruitment of HA-tagged NOP56 and fibrillarin to laser stripes in a PARP-dependent manner [116]. Moreover, fibrillarin has been

shown to accumulate in perinucleoar caps with members of the HR repair machinery in response to rDNA damage [117].

As fibrillarin is the catalytic subunit of the scaRNP complex, we examined whether its depletion would affect the repair of DNA breaks in a similar manner as scaRNA2 depletion. Indeed, depletion of fibrillarin resulted in delayed recovery from IR-induced DNA damage and significantly impaired HR repair. Moreover, loss of fibrillarin resulted in problematic recruitment of RAD51 protein at DSBs, while accumulation of MDC1 and 53BP1 was relatively unaffected.

In paper IV, we introduce scaRNA2 and the C/D box core proteins as new players in the DDR. These molecules appear to accumulate at sites of damage, facilitate the recruitment of HR factors and the subsequent repair by HR. The involvement of RNA-modifying enzymes like fibrillarin in DNA repair indicates that RNA modification plays a broader role in signaling DNA damage. Indeed, recently it was reported that RNAs at the sites of damage are methylated at the 6 position of adenosine (m^6A). This modification is catalyzed by the METTL3 methyltransferase (methyltransferase-like 3) and the demethylase FTO (fat mass and obesity-associated protein) [68]. In this study m^6A-modified RNAs at the sites of damage promote the recruitment of DNA polymerase kappa [68]. This raises the possibility that RNAs 2'-O-methylated by scaRNA2 and associated C/D complex could be implicated in the repair of DNA DSBs by promote the recruitment of factors important for HR.

4.4.1 Additional data about the function of scaRNA2

In the course of this study, we acquired a plethora of data related, or not to the DNA repair function of scaRNA2. Here we take the opportunity to present some of them outside the scope of this paper.

During the study we performed RNA immunoprecipitation (RIP) experiments to explore potential new binding partners of scaRNA2. One of our striking findings was that scaRNA2 had very high affinity for the heterochromatin histone markers H3K9me2/3, as well as for the histone methyltransferase SUV39H2 responsible for the methylation of H3K9me2/3, indicating that scaRNA2 could promote H3K9 methylation by for example guiding the enzyme to site of action. Intriguingly, we observed that depletion of scaRNA2 enhanced H3K9me2/3 methylation pointing to an opposite function of scaRNA2, i.e. the scaRNA2 may inhibit the SUV39H2 enzyme and that loss of scaRNA2 removed this inhibitory function leading to elevated H3K9 methylation.

Interestingly, both homologs of the SUV39H methyltransferase have been implicated in DNA repair. SUV39H1 is part of the kap-1/HP1/suv39h1 complex that is rapidly recruited to DSBs in euchromatin to heterochromatinizes them [43]. SUV39H2 was reported to methylate K134 of H2AX and promote its phosphorylation by ATM during DDR [118]. To further explore the involvement of scaRNA2 in this context, we examined whether loss of this RNA alters phosphorylation of H2AX shortly after induction of DNA damage. However, we did not observe reduced levels of H2AX phosphorylation upon scaRNA2 depletion. If anything,

loss of scaRNA2 led to elevated levels of gH2AX 24h post irradiation, again indicating that scaRNA2 could inhibit the function of SUV39H2 in DNA repair.

We also examined whether fibrillarin could be responsible for the elevated histone methylation upon loss of scaRNA2, since this enzyme previously was shown to methylate not only RNA but also histones [119]. However, knockdown of fibrillarin did not affect the levels of H3K9me2/3.

We next observed that depletion of scaRNA2 showed similar up-regulation of the heterochromatin markers H3K9me2/3 both in irradiated and non-irradiated cells, suggesting that the connection between scaRNA2 and these markers were not linked to the role of scaRNA2 in DNA repair. Intriguingly, when we performed a DNase I assay, we observed that chromatin from cells lacking scaRNA2 were less susceptible to cleavage, indicating compact chromatin. This compaction was only observed when cells lacking scaRNA2 had been irradiated. Thus, this could mean that a compacted chromatin environment in cells lacking scaRNA2 prevents recruitment of HR factors, leading to dysfunctional HR repair.

Indeed, some repair proteins are associated with specific chromatin markers. For example, H4K16 acetylation (H4K16ac) which is correlated with actively transcribed regions, blocks 53BP1 binding to H4K20me2 and instead recruits BRCA1, thus promoting HR [40, 41]. Another euchromatin marker H3K36me3 acts as a scaffold for the recruitment of HR factors in the S/G2 cell cycle phase, while breaks induced in inactive genes, that are not associated with this marker, recruit NHEJ proteins [42]. The fact that histone modifications can antagonize them [120], explains that perpetual high levels of H3K9me3 due to scaRNA2 depletion can block the presence of other markers important for HR.

Altogether, these data propose an alternative mechanism of function for scaRNA2 in regulating the chromatin dynamics and the epigenome independent of DNA damage. This could be interesting to explore in the context of DNA repair and further investigation is warranted.

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6 REFERENCES

1. Ciccia, A. and S.J. Elledge, *The DNA damage response: making it safe to play with knives.* Mol Cell, 2010. **40**(2): p. 179-204.
2. Vicencio, J.M., et al., *Senescence, apoptosis or autophagy? When a damaged cell must decide its path--a mini-review.* Gerontology, 2008. **54**(2): p. 92-9.
3. Jackson, S.P. and J. Bartek, *The DNA-damage response in human biology and disease.* Nature, 2009. **461**(7267): p. 1071-8.
4. Collado, M., et al., *Tumour biology: senescence in premalignant tumours.* Nature, 2005. **436**(7051): p. 642.
5. Jackson, S.P., *Sensing and repairing DNA double-strand breaks.* Carcinogenesis, 2002. **23**(5): p. 687-96.
6. Roos, W.P. and B. Kaina, *DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis.* Cancer Lett, 2013. **332**(2): p. 237-48.
7. Hoeijmakers, J.H., *Genome maintenance mechanisms for preventing cancer.* Nature, 2001. **411**(6835): p. 366-74.
8. Howard, S.M., D.A. Yanez, and J.M. Stark, *DNA damage response factors from diverse pathways, including DNA crosslink repair, mediate alternative end joining.* PLoS Genet, 2015. **11**(1): p. e1004943.
9. Aguilera, A. and B. Gomez-Gonzalez, *Genome instability: a mechanistic view of its causes and consequences.* Nat Rev Genet, 2008. **9**(3): p. 204-17.
10. Heyer, W.D., K.T. Ehmsen, and J. Liu, *Regulation of homologous recombination in eukaryotes.* Annu Rev Genet, 2010. **44**: p. 113-39.
11. Friedberg, E.C., *How nucleotide excision repair protects against cancer.* Nat Rev Cancer, 2001. **1**(1): p. 22-33.
12. Marechal, A. and L. Zou, *DNA damage sensing by the ATM and ATR kinases.* Cold Spring Harb Perspect Biol, 2013. **5**(9).
13. Durocher, D. and S.P. Jackson, *DNA-PK, ATM and ATR as sensors of DNA damage: variations on a theme?* Curr Opin Cell Biol, 2001. **13**(2): p. 225-31.
14. Stucki, M., et al., *MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks.* Cell, 2005. **123**(7): p. 1213-26.
15. Mailand, N., et al., *RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins.* Cell, 2007. **131**(5): p. 887-900.
16. Kolas, N.K., et al., *Orchestration of the DNA-damage response by the RNF8 ubiquitin ligase.* Science, 2007. **318**(5856): p. 1637-40.
17. Lieber, M.R., *The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway.* Annu Rev Biochem, 2010. **79**: p. 181-211.

18. Lieber, M.R., et al., *Mechanism and regulation of human non-homologous DNA end-joining.* Nat Rev Mol Cell Biol, 2003. **4**(9): p. 712-20.

19. Critchlow, S.E. and S.P. Jackson, *DNA end-joining: from yeast to man.* Trends Biochem Sci, 1998. **23**(10): p. 394-8.

20. Dynan, W.S. and S. Yoo, *Interaction of Ku protein and DNA-dependent protein kinase catalytic subunit with nucleic acids.* Nucleic Acids Res, 1998. **26**(7): p. 1551-9.

21. Walker, J.R., R.A. Corpina, and J. Goldberg, *Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair.* Nature, 2001. **412**(6847): p. 607-14.

22. West, R.B., M. Yaneva, and M.R. Lieber, *Productive and nonproductive complexes of Ku and DNA-dependent protein kinase at DNA termini.* Mol Cell Biol, 1998. **18**(10): p. 5908-20.

23. Weterings, E., et al., *The role of DNA dependent protein kinase in synapsis of DNA ends.* Nucleic Acids Res, 2003. **31**(24): p. 7238-46.

24. Ahnesorg, P., P. Smith, and S.P. Jackson, *XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining.* Cell, 2006. **124**(2): p. 301-13.

25. Grawunder, U., et al., *Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells.* Nature, 1997. **388**(6641): p. 492-5.

26. Ma, Y., et al., *Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination.* Cell, 2002. **108**(6): p. 781-94.

27. Ma, Y., K. Schwarz, and M.R. Lieber, *The Artemis:DNA-PKcs endonuclease cleaves DNA loops, flaps, and gaps.* DNA Repair (Amst), 2005. **4**(7): p. 845-51.

28. Symington, L.S. and J. Gautier, *Double-strand break end resection and repair pathway choice.* Annu Rev Genet, 2011. **45**: p. 247-71.

29. Sugiyama, T., E.M. Zaitseva, and S.C. Kowalczykowski, *A single-stranded DNA-binding protein is needed for efficient presynaptic complex formation by the Saccharomyces cerevisiae Rad51 protein.* J Biol Chem, 1997. **272**(12): p. 7940-5.

30. Fanning, E., V. Klimovich, and A.R. Nager, *A dynamic model for replication protein A (RPA) function in DNA processing pathways.* Nucleic Acids Res, 2006. **34**(15): p. 4126-37.

31. Chen, H., M. Lisby, and L.S. Symington, *RPA coordinates DNA end resection and prevents formation of DNA hairpins.* Mol Cell, 2013. **50**(4): p. 589-600.

32. Liu, J., et al., *Human BRCA2 protein promotes RAD51 filament formation on RPA-covered single-stranded DNA.* Nat Struct Mol Biol, 2010. **17**(10): p. 1260-2.

33. Yonetani, Y., et al., *Differential and collaborative actions of Rad51 paralog proteins in cellular response to DNA damage.* Nucleic Acids Res, 2005. **33**(14): p. 4544-52.

34. McIlwraith, M.J., et al., *Human DNA polymerase eta promotes DNA synthesis from strand invasion intermediates of homologous recombination.* Mol Cell, 2005. **20**(5): p. 783-92.

35. Ip, S.C., et al., *Identification of Holliday junction resolvases from humans and yeast.* Nature, 2008. **456**(7220): p. 357-61.

36. Bothmer, A., et al., *53BP1 regulates DNA resection and the choice between classical and alternative end joining during class switch recombination.* J Exp Med, 2010. **207**(4): p. 855-65.

37. Bunting, S.F., et al., *53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks.* Cell, 2010. **141**(2): p. 243-54.

38. Kakarougkas, A., et al., *Opposing roles for 53BP1 during homologous recombination.* Nucleic Acids Res, 2013. **41**(21): p. 9719-31.

39. Shibata, A., et al., *DNA double-strand break repair pathway choice is directed by distinct MRE11 nuclease activities.* Mol Cell, 2014. **53**(1): p. 7-18.

40. Hsiao, K.Y. and C.A. Mizzen, *Histone H4 deacetylation facilitates 53BP1 DNA damage signaling and double-strand break repair.* J Mol Cell Biol, 2013. **5**(3): p. 157-65.

41. Tang, J., et al., *Acetylation limits 53BP1 association with damaged chromatin to promote homologous recombination.* Nat Struct Mol Biol, 2013. **20**(3): p. 317-25.

42. Aymard, F., et al., *Transcriptionally active chromatin recruits homologous recombination at DNA double-strand breaks.* Nat Struct Mol Biol, 2014. **21**(4): p. 366-74.

43. Ayrapetov, M.K., et al., *DNA double-strand breaks promote methylation of histone H3 on lysine 9 and transient formation of repressive chromatin.* Proc Natl Acad Sci U S A, 2014. **111**(25): p. 9169-74.

44. Stanley, F.K., S. Moore, and A.A. Goodarzi, *CHD chromatin remodelling enzymes and the DNA damage response.* Mutat Res, 2013. **750**(1-2): p. 31-44.

45. Sun, Y., et al., *Histone H3 methylation links DNA damage detection to activation of the tumour suppressor Tip60.* Nat Cell Biol, 2009. **11**(11): p. 1376-82.

46. Burgess, R.C., et al., *Activation of DNA damage response signaling by condensed chromatin.* Cell Rep, 2014. **9**(5): p. 1703-17.

47. Goodarzi, A.A., T. Kurka, and P.A. Jeggo, *KAP-1 phosphorylation regulates CHD3 nucleosome remodeling during the DNA double-strand break response.* Nat Struct Mol Biol, 2011. **18**(7): p. 831-9.

48. Goodarzi, A.A., et al., *ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin.* Mol Cell, 2008. **31**(2): p. 167-77.

49. Henriksson, S., et al., *The scaffold protein WRAP53beta orchestrates the ubiquitin response critical for DNA double-strand break repair.* Genes Dev, 2014. **28**(24): p. 2726-38.

50. Mahmoudi, S., et al., *Wrap53, a natural p53 antisense transcript required for p53 induction upon DNA damage.* Mol Cell, 2009. **33**(4): p. 462-71.

51. Mahmoudi, S., et al., *WRAP53 promotes cancer cell survival and is a potential target for cancer therapy.* Cell Death Dis, 2011. **2**: p. e114.

52. Mahmoudi, S., et al., *WRAP53 is essential for Cajal body formation and for targeting the survival of motor neuron complex to Cajal bodies.* PLoS Biol, 2010. **8**(11): p. e1000521.

53. Stirnimann, C.U., et al., *WD40 proteins propel cellular networks.* Trends Biochem Sci, 2010. **35**(10): p. 565-74.

54. Gall, J.G., *The centennial of the Cajal body.* Nat Rev Mol Cell Biol, 2003. **4**(12): p. 975-80.

55. Cioce, M. and A.I. Lamond, *Cajal bodies: a long history of discovery.* Annu Rev Cell Dev Biol, 2005. **21**: p. 105-31.

56. Carvalho, T., et al., *The spinal muscular atrophy disease gene product, SMN: A link between snRNP biogenesis and the Cajal (coiled) body.* J Cell Biol, 1999. **147**(4): p. 715-28.

57. Novotny, I., et al., *In vivo kinetics of U4/U6.U5 tri-snRNP formation in Cajal bodies.* Mol Biol Cell, 2011. **22**(4): p. 513-23.

58. Zhang, Z., et al., *SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing.* Cell, 2008. **133**(4): p. 585-600.

59. Tycowski, K.T., et al., *A conserved WD40 protein binds the Cajal body localization signal of scaRNP particles.* Mol Cell, 2009. **34**(1): p. 47-57.

60. Venteicher, A.S., et al., *A human telomerase holoenzyme protein required for Cajal body localization and telomere synthesis.* Science, 2009. **323**(5914): p. 644-8.

61. Zhong, F., et al., *Disruption of telomerase trafficking by TCAB1 mutation causes dyskeratosis congenita.* Genes Dev, 2011. **25**(1): p. 11-6.

62. Gu, B.W., M. Bessler, and P.J. Mason, *A pathogenic dyskerin mutation impairs proliferation and activates a DNA damage response independent of telomere length in mice.* Proc Natl Acad Sci U S A, 2008. **105**(29): p. 10173-8.

63. Bernstein, E. and C.D. Allis, *RNA meets chromatin.* Genes Dev, 2005. **19**(14): p. 1635-55.

64. Dutertre, M., et al., *DNA damage: RNA-binding proteins protect from near and far.* Trends Biochem Sci, 2014. **39**(3): p. 141-9.

65. Francia, S., et al., *Site-specific DICER and DROSHA RNA products control the DNA-damage response.* Nature, 2012. **488**(7410): p. 231-5.

66. Keskin, H., et al., *Transcript-RNA-templated DNA recombination and repair.* Nature, 2014. **515**(7527): p. 436-9.

67. Storici, F., et al., *RNA-templated DNA repair.* Nature, 2007. **447**(7142): p. 338-41.

68. Xiang, Y., et al., *RNA m(6)A methylation regulates the ultraviolet-induced DNA damage response.* Nature, 2017. **543**(7646): p. 573-576.

69. Kiss, T., *Small nucleolar RNA-guided post-transcriptional modification of cellular RNAs.* EMBO J, 2001. **20**(14): p. 3617-22.

70. Kiss, T., et al., *Biogenesis and intranuclear trafficking of human box C/D and H/ACA RNPs.* Cold Spring Harb Symp Quant Biol, 2006. **71**: p. 407-17.

71. Kiss, T., *Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions.* Cell, 2002. **109**(2): p. 145-8.

72. Richard, P., et al., *A common sequence motif determines the Cajal body-specific localization of box H/ACA scaRNAs.* EMBO J, 2003. **22**(16): p. 4283-93.

73. Marnef, A., et al., *Targeting vertebrate intron-encoded box C/D 2'-O-methylation guide RNAs into the Cajal body.* Nucleic Acids Res, 2014. **42**(10): p. 6616-29.

74. Kiss, A.M., et al., *Human box H/ACA pseudouridylation guide RNA machinery.* Mol Cell Biol, 2004. **24**(13): p. 5797-807.

75. Schaub, M., et al., *Staf, a promiscuous activator for enhanced transcription by RNA polymerases II and III.* EMBO J, 1997. **16**(1): p. 173-81.

76. Gerard, M.A., et al., *The scaRNA2 is produced by an independent transcription unit and its processing is directed by the encoding region.* Nucleic Acids Res, 2010. **38**(2): p. 370-81.

77. Boccaletto, P., et al., *MODOMICS: a database of RNA modification pathways. 2017 update.* Nucleic Acids Res, 2018. **46**(D1): p. D303-D307.

78. Camper, S.A., et al., *Effect of undermethylation on mRNA cytoplasmic appearance and half-life.* Mol Cell Biol, 1984. **4**(3): p. 538-43.

79. Liu, N. and T. Pan, *RNA epigenetics.* Transl Res, 2015. **165**(1): p. 28-35.

80. Roundtree, I.A., et al., *Dynamic RNA Modifications in Gene Expression Regulation.* Cell, 2017. **169**(7): p. 1187-1200.

81. Valadkhan, S., *snRNAs as the catalysts of pre-mRNA splicing.* Curr Opin Chem Biol, 2005. **9**(6): p. 603-8.

82. Yu, Y.T., M.D. Shu, and J.A. Steitz, *Modifications of U2 snRNA are required for snRNP assembly and pre-mRNA splicing.* EMBO J, 1998. **17**(19): p. 5783-95.

83. Karunatilaka, K.S. and D. Rueda, *Post-transcriptional modifications modulate conformational dynamics in human U2-U6 snRNA complex.* RNA, 2014. **20**(1): p. 16-23.

84. Newby, M.I. and N.L. Greenbaum, *Sculpting of the spliceosomal branch site recognition motif by a conserved pseudouridine.* Nat Struct Biol, 2002. **9**(12): p. 958-65.

85. Newby, M.I. and N.L. Greenbaum, *A conserved pseudouridine modification in eukaryotic U2 snRNA induces a change in branch-site architecture.* RNA, 2001. **7**(6): p. 833-45.

86. Woods, D. and J.J. Turchi, *Chemotherapy induced DNA damage response: convergence of drugs and pathways.* Cancer Biol Ther, 2013. **14**(5): p. 379-89.

87. Cheung-Ong, K., G. Giaever, and C. Nislow, *DNA-damaging agents in cancer chemotherapy: serendipity and chemical biology.* Chem Biol, 2013. **20**(5): p. 648-59.

88. Rastogi, R.P., et al., *Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair.* J Nucleic Acids, 2010. **2010**: p. 592980.

89. Herskind, C. and O. Westergaard, *Inactivation of a Single Eucaryotic Gene Irradiated in Vitro in Transcriptionally Active Chromatin Form.* Radiat Res, 1986. **106**(3): p. 331-344.

90. Herskind, C. and O. Westergaard, *Variable protection by OH scavengers against radiation-induced inactivation of isolated transcriptionally active chromatin: the influence of secondary radicals.* Radiat Res, 1988. **114**(1): p. 28-41.

91. Maier, P., et al., *Cellular Pathways in Response to Ionizing Radiation and Their Targetability for Tumor Radiosensitization.* Int J Mol Sci, 2016. **17**(1).

92. Polo, S.E. and S.P. Jackson, *Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications.* Genes Dev, 2011. **25**(5): p. 409-33.

93. Rothkamm, K., et al., *DNA damage foci: Meaning and significance.* Environ Mol Mutagen, 2015. **56**(6): p. 491-504.

94. Bekker-Jensen, S., et al., *Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks.* J Cell Biol, 2006. **173**(2): p. 195-206.

95. Drexler, G.A. and M.J. Ruiz-Gomez, *Microirradiation techniques in radiobiological research.* J Biosci, 2015. **40**(3): p. 629-43.

96. Lukas, C., J. Bartek, and J. Lukas, *Imaging of protein movement induced by chromosomal breakage: tiny 'local' lesions pose great 'global' challenges.* Chromosoma, 2005. **114**(3): p. 146-54.

97. Querido, E. and P. Chartrand, *Using fluorescent proteins to study mRNA trafficking in living cells.* Methods Cell Biol, 2008. **85**: p. 273-92.

98. Dundr, M., *Nucleation of nuclear bodies.* Methods Mol Biol, 2013. **1042**: p. 351-64.

99. Peabody, D.S., *The RNA binding site of bacteriophage MS2 coat protein.* EMBO J, 1993. **12**(2): p. 595-600.

100. Zuker, M., *Mfold web server for nucleic acid folding and hybridization prediction.* Nucleic Acids Res, 2003. **31**(13): p. 3406-15.

101. Fredriksson, S., et al., *Protein detection using proximity-dependent DNA ligation assays.* Nat Biotechnol, 2002. **20**(5): p. 473-7.

102. Soderberg, O., et al., *Characterizing proteins and their interactions in cells and tissues using the in situ proximity ligation assay.* Methods, 2008. **45**(3): p. 227-32.

103. Leuchowius, K.J., I. Weibrecht, and O. Soderberg, *In situ proximity ligation assay for microscopy and flow cytometry.* Curr Protoc Cytom, 2011. **Chapter 9**: p. Unit 9 36.

104. Maszczak-Seneczko, D., et al., *In Situ Proximity Ligation Assay (PLA) Analysis of Protein Complexes Formed Between Golgi-Resident, Glycosylation-Related Transporters and Transferases in Adherent Mammalian Cell Cultures.* Methods Mol Biol, 2016. **1496**: p. 133-43.

105. Weibrecht, I., et al., *In situ detection of individual mRNA molecules and protein complexes or post-translational modifications using padlock probes combined with the in situ proximity ligation assay.* Nat Protoc, 2013. **8**(2): p. 355-72.

106. Chen, X., et al., *Chromatin in situ proximity (ChrISP): single-cell analysis of chromatin proximities at a high resolution.* Biotechniques, 2014. **56**(3): p. 117-8, 120-4.

107. Matsuoka, S., et al., *ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage.* Science, 2007. **316**(5828): p. 1160-6.

108. Adamson, B., et al., *A genome-wide homologous recombination screen identifies the RNA-binding protein RBMX as a component of the DNA-damage response.* Nat Cell Biol, 2012. **14**(3): p. 318-28.

109. Paulsen, R.D., et al., *A genome-wide siRNA screen reveals diverse cellular processes and pathways that mediate genome stability.* Mol Cell, 2009. **35**(2): p. 228-39.

110. Stiff, T., et al., *ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation.* Cancer Res, 2004. **64**(7): p. 2390-6.

111. Henriksson, S. and M. Farnebo, *On the road with WRAP53beta: guardian of Cajal bodies and genome integrity.* Front Genet, 2015. **6**: p. 91.

112. Mahmoudi, S., et al., *Wrap53, a natural p53 antisense transcript required for p53 induction upon DNA damage.* Molecular cell, 2009. **33**(4): p. 462-71.

113. Mahmoudi, S., et al., *WRAP53 is essential for Cajal body formation and for targeting the survival of motor neuron complex to Cajal bodies.* PLoS biology, 2010. **8**(11): p. e1000521.

114. Goldstein, M., et al., *Nucleolin mediates nucleosome disruption critical for DNA double-strand break repair.* Proc Natl Acad Sci U S A, 2013. **110**(42): p. 16874-9.

115. Pederiva, C., et al., *Splicing controls the ubiquitin response during DNA double-strand break repair.* Cell Death Differ, 2016. **23**(10): p. 1648-57.

116. Izhar, L., et al., *A Systematic Analysis of Factors Localized to Damaged Chromatin Reveals PARP-Dependent Recruitment of Transcription Factors.* Cell Rep, 2015. **11**(9): p. 1486-500.

117. van Sluis, M. and B. McStay, *A localized nucleolar DNA damage response facilitates recruitment of the homology-directed repair machinery independent of cell cycle stage.* Genes Dev, 2015. **29**(11): p. 1151-63.

118. Sone, K., et al., *Critical role of lysine 134 methylation on histone H2AX for gamma-H2AX production and DNA repair.* Nat Commun, 2014. **5**: p. 5691.

119. Tessarz, P., et al., *Glutamine methylation in histone H2A is an RNA-polymerase-I-dedicated modification.* Nature, 2014. **505**(7484): p. 564-8.

120. Kouzarides, T., *Chromatin modifications and their function.* Cell, 2007. **128**(4): p. 693-705.