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Identification and characterization of novel factors in the DNA damage response

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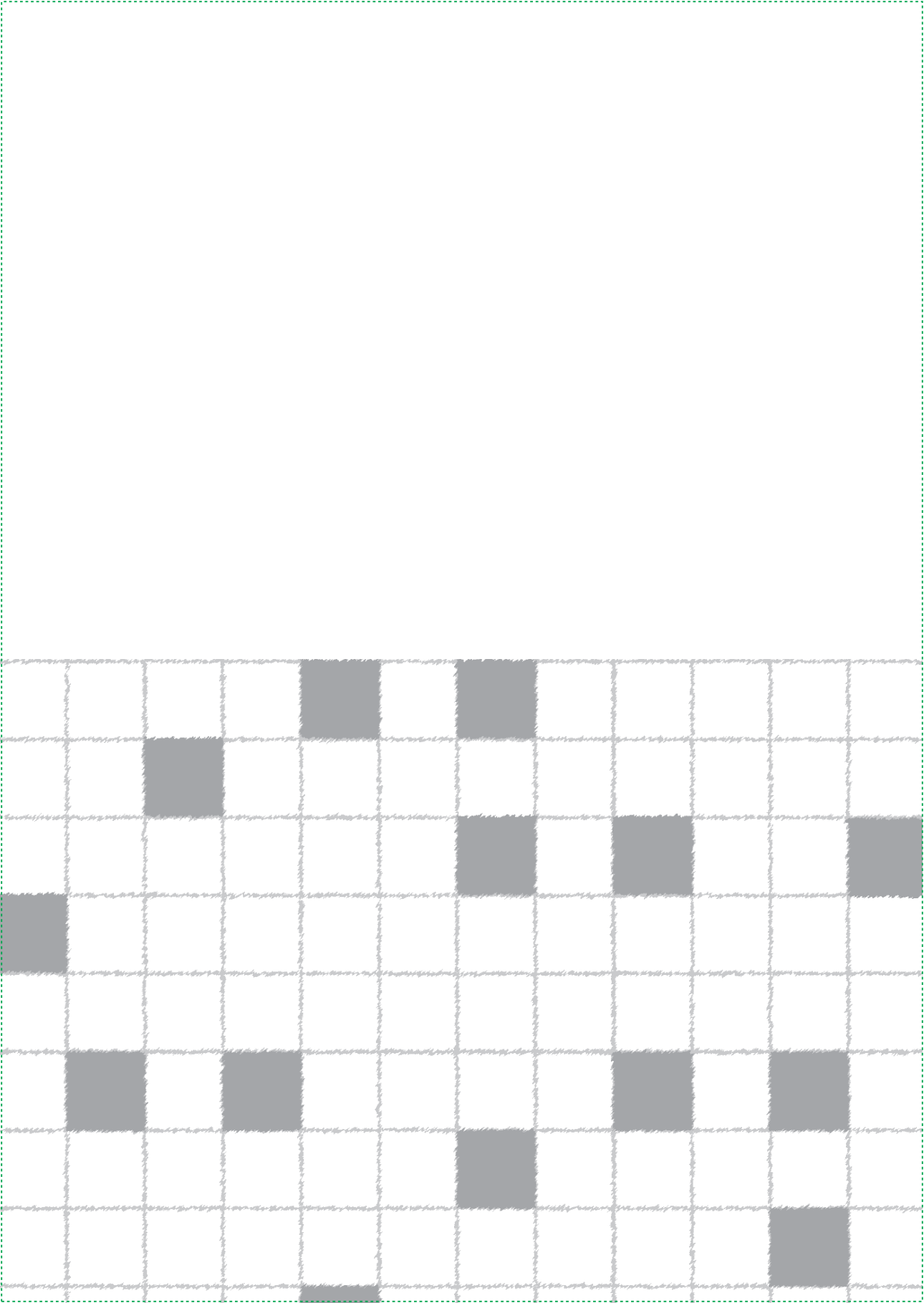
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CHAPTER 6

Perspectives

PERSPECTIVES

All cellular organisms contain genomic DNA which provides the instructions for their correct development and functioning. Damage to this DNA may interfere with critical cellular processes such as transcription and replication and has the potential to drive mutagenesis. In turn, this may underlie inherited disorders and accelerate progression of diseases such as cancer and neurodegenerative disorders [1]. The protection of cells and organisms against these devastating effects of DNA damage relies on the DNA damage response (DDR), which comprises a complex network of signaling and repair pathways that coordinate the sensing, signaling and repair of DNA lesions while accommodating suitable adjustments in for instance chromatin structure and cell cycle progression [1, 2]. Not only does the DDR dictate the appropriate repair pathway for several types of DNA damage, including DNA double-strand breaks (DSB), it also modulates replication fork surveillance mechanisms in response to DNA replication stress (RS). While many core proteins have been studied in detail, the full repertoire of factors involved in these pathways remains unknown. Clearly, extending our knowledge on regulators of the DDR will contribute to our understanding of the development, and possibly the treatment, of the numerous disorders that are associated with defects in the DDR [3]. The research described in this thesis has successfully identified and characterized novel factors in DSB repair and the RS response, yet raises additional questions that provide a base for further research and discussion.

The contribution of ZnF proteins to DNA double-strand break repair

DNA-templated reactions such as transcription, DNA replication and DNA damage repair, rely on proteins that bind to DNA to locally regulate these processes. However, the accessibility of these proteins at DNA damage sites is complicated by the packaging of DNA into chromatin. ATP-dependent chromatin remodelers and post-translational modifications (PTMs) are therefore required to change chromatin structure and allow for DNA damage repair to occur [4, 5]. The spatiotemporal recruitment of DNA repair factors is not only regulated by PTM recognition and protein-protein interactions, but also DNA binding at DNA damage sites. One class of proteins that can associate with PTMs, proteins and/or DNA are zinc finger domain-containing proteins (ZnF proteins). Over the last couple of years, ZnF proteins have gained much attention because of their roles in DSB repair by non-homologous end joining (NHEJ) and homologous recombination (HR) (described in chapter 2).

ZnF proteins are categorized in different subfamilies based on their numerous structural folds, diverse binding modes and sequence recognition sites present in their ZnF domains. To date, more than 50 unique domains have been identified, several of which have been shown to be important for DSB repair due to their binding to for instance ubiquitin, poly(ADP-ribose) or DNA [6]. In addition, several ZnF domain families have been implicated in processes other than DSB repair. For instance, FYVE domain-containing proteins are mostly localized at endosomes and golgi compartments where they bind to phospho-inositides via their FYVE domain to regulate specific functions of proteins in these compartments [7]. C2H2-KRAB domain-containing proteins, on the other hand, are involved in the repression of foreign DNA transcription, primarily that of endogenous retroviruses, and bind to the regulatory regions of retrotransposons [8]. However, KRAB domain-containing ZnF proteins are also involved in transcription repression by binding in a sequence-specific manner to cis-regulatory elements and recruiting the KAP-1 corepressor to these loci [9]. This illustrates that ZnF proteins, even within the same domain family, exhibit diverse binding specificities and functions.

With regard to functions in DNA damage repair, it is interesting to note that several ZnF proteins ranked high in CRISPR/Cas9 screens aimed at identifying protein networks that protect cells against DNA damaging agents, including several chemotherapeutics [10]. In addition, several ZnF proteins were found to localize at sites of laser micro-irradiation induced DNA damage often in a manner dependent on PAR/PARP [11]. Although these screens highlight the potential importance of ZnF proteins in maintaining genome stability, the role of many of these ZnF proteins in DNA damage repair has remained uncharacterized thus far. Thus, further work is required to dissect their functional impact on the DSB response, but also in preventing human diseases such as cancer and neurodegenerative disorders [12]. The latter is of importance since mutations in ZnF proteins have been linked to broadly varying clinical consequences [12]. For instance, truncation and missense mutations in ZNF750 cause squamous cell carcinoma (SCC). Importantly, these mutations are located in the C2H2 domain, suggesting a critical role of this domain in mediating the tumor suppressor activity of ZNF750 [13]. Similarly, point mutations in the ZnF domain of tumor suppressor MDM2 have been linked to different tumours, including leukemia, hepatocellular carcinoma and osteosarcoma [14], whereas, mutations in E4F1, a ZnF protein that cooperates with PARP1 to promote DSB repair, have been associated with breast cancer [15]. This illustrates that although we have just begun to unravel the exact biological functions of ZnF proteins, their broad

implications in preventing human disease have already become apparent. It will be of interest to unravel to which extent their role therein relates to a function in the DDR, most notably DSB repair.

ZNF384, a novel NHEJ regulator

In this thesis, we uncovered a crucial role for the C2H2 type ZnF protein ZNF384 in ensuring the proper regulation of Ku70/Ku80, which are essential for DSB repair via NHEJ (described in chapter 3). ZNF384 regulates the functioning of Ku70/Ku80 by directly interacting with this complex. Our mass spectrometry analysis, however, revealed that ZNF384 not only interacts with Ku70/Ku80 but also with several other proteins including heat shock proteins (HSPA5, HSPA1B, HSPA8, HSPA9 and HSPH1) and replication factor C proteins (RFC1, RFC2, RFC4 and RFC4). Heat shock proteins are molecular chaperones that participate in important cellular processes such as protein folding. Their involvement in anti-cancer drug resistance have made them important targets in DNA damage repair [16]. In fact, several heat shock proteins (HSP) are implicated in DSB repair via HR and NHEJ [17]. For instance, HSPH1 also interacts with Ku70/Ku80, and promotes NHEJ in colorectal cancer cells [18]. This may suggest that ZNF384 protects genome stability in an even broader manner, for instance by the regulation of HSPs. The validation of these interactors by GFP pulldowns and laser micro-irradiation recruitment studies of GFP-tagged HSPs in the presence and absence of ZNF384 would be the first step in identifying the functional interplay between ZNF384 and HSPs in DNA damage repair. RFC complexes, on the other hand, are implicated in the unloading of PCNA during DNA replication [19]. The unloading of PCNA is tightly regulated to prevent premature termination of DNA replication. Since ZNF384 interacts with multiple components of the RFC complex, this may suggest that ZNF384 has a potential role in DNA replication.

In line with previous work, our DNA binding analysis revealed that ZNF384 has preference to bind to homopolymeric dA-dT DNA consensus elements in ssDNA (T-stretch), and does not associate with blunt dsDNA [20, 21]. Importantly, however, we also observed binding of ZNF384 to dsDNA with a 3' or 5' overhang. Consistent with its role in cNHEJ, dsDNA with 3' or 5' overhangs are better substrates for this type of NHEJ repair when compared to blunt dsDNA, which are preferentially repaired by polymerase theta-mediated end-joining [22]. It is important to note that all the DNA substrates used in this study contain the homopolymeric dA-dT DNA consensus

sequence. This raises the question whether ZNF384 binds to the ssDNA (T-stretch) and/or dsDNA moiety of these substrates with protruding ends. The emergence of a more detailed picture using structural biology analysis including Cryo-electron microscopy (cryo-EM) of recombinant ZNF384 bound to these substrates would provide insight into ZNF384's DNA binding mode [23]. Complementary to this, it would be interesting to confirm our *in vitro* results in cells by inducing DSBs with protruding or and blunt ends using the CRISPR/Cas9 nickase wildtype, and measure ZNF384 binding by chromatin-immunoprecipitation (ChIP) [24, 25]. To further extend on this, cell survival assays of ZNF384 depleted cells upon DSB induction with protruding *versus* blunt ends would provide insight into whether ZNF384 indeed promotes the repair of protruding ends. Taken together, future studies will definitively expand our knowledge on ZNF384's DSB end binding mode and NHEJ repair *in vivo*. Our current data reveal that the N-terminus of ZNF384 is responsible for a direct interaction with Ku70/Ku80 and promotes the loading of this complex at DSBs. However, the domains in Ku70/Ku80 required for their interaction with ZNF384 remain unknown. The identification of these domains will allow better insight into the mechanism by which ZNF384 mediates the contacts of Ku70/Ku80 with DSBs. Since the crystal structure of the Ku heterodimer has already been determined [26], structural analysis with ZNF384 bound to Ku70/Ku80 in the presence of protruding DNA substrates used in this study would definitely provide answers on how ZNF384 assists in Ku making DSB ends meet. However, it is important to note that the C2H2 motifs in ZNF384 have preference to bind T-rich DNA, whereas Ku70/Ku80 bind DSB ends in a sequence independent manner. Whether ZNF384 can also bind other sequences remains yet to be identified. Importantly, its substrate recognition properties are determined by the cysteines and histidine amino acids on each motif which coordinate zinc ions to mediate contact with DNA [20]. Therefore, amino acids substitutions in these residues may modulate ZNF384's DNA binding specificity to regions other than T-rich DNA. The generation of such mutants may significantly aid in determining whether ZNF384's DNA binding sequence specificity is required for the loading of Ku70/Ku80 at DSBs to promote cNHEJ.

Interplay between ZNF384 and DNA-PKcs during cNHEJ

To our surprise we found that ZNF384 promotes the loading of APLF and XRCC4/LIG4 in a manner dependent of PARP1 and Ku, but independently of DNA-PKcs. This is in contrast with previous work suggesting that XRCC4/LIG4 assembly depends on the

recruitment and activation of DNA-PKcs by Ku-bound DNA-ends [27]. We found that DNA-PKcs activity remained intact in Ku70/Ku80 knockdown human cells and *Ku80* KO mouse embryonic stem cells. This may suggest that the presence of DNA, regardless of Ku, is sufficient for DNA-PKcs activation [28]. This is in line with a recent study showing that Ku and XRCC4/LIG4, XLF and PAXX are sufficient, while DNA-PKcs is dispensable for bringing broken DNA ends together *in vitro* in a process called DNA-end synopsis [29]. Extending our epistasis analysis to decipher the interplay between ZNF384 and PAXX or XLF, as well as the interplay between Ku and DNA-PKcs, would be an important next step to gain insight into the functioning of the NHEJ apparatus. Interestingly, DNA-bound Ku is essential for the initial recruitment of PAXX at DSBs [30]. This may suggest that ZNF384 and PAXX cooperate during NHEJ, in which ZNF384 ensures the initial loading of Ku70/Ku80 and PAXX promotes Ku70/Ku80 retention at DSBs. Furthermore, another study reported that destabilization of the APLF or XLF interaction with Ku80 impairs NHEJ due to defects in loading of XRCC4 and Ku [31]. Whether these observations are linked to defects in DNA-PKcs recruitment and/or activation remains unclear. To this end, it would be interesting to measure NHEJ of protruding DSB ends using an *in vitro* NHEJ reconstitution system with ZNF384 and combinations of other NHEJ components, including Ku70/Ku80, DNA-PKcs, APLF, PAXX, XLF or XRCC4/LIG4, all of which have been purified previously [32, 33]. In addition, atomic force microscopy (AFM) has been proven to serve as a powerful tool to study protein-DNA interactions and end-tethering by NHEJ proteins, and thus may be a valuable add-on to the *in vitro* studies [34]. Finally, deciphering the spatiotemporal recruitment of the NHEJ apparatus in cells would be an indispensable extension to this research. Since our data reveal that there is redundancy between ZNF384 and DNA-PKcs, it may be fact that they act mutually exclusive at DSBs. Genome-wide recruitment studies of these and other NHEJ components by ChIP-sequencing at AsiSI- and CRISPR/Cas9 nickase-induced DSBs may determine which NHEJ factors act in concert at these distinct DNA breaks [35, 36].

ZNF384: an oncogene with therapeutic potential

ZNF384 has been implicated in various cancers by acting as an oncogene. For instance, overexpression of ZNF384 results in hepatocellular carcinoma tumor growth via the upregulation of cyclin D1 expression [37]. Furthermore, it has been reported that overexpression of ZNF384 can promote metastasis in melanoma cells [37]. More

interestingly, ZNF384 is found to be rearranged in 48% of mixed phenotype acute leukemia (MPAL) patients [38]. These chromosomal rearrangements involve the fusion of ZNF384 with transcription regulators and epigenetic modifiers such as TCF3, EP300, TAF15 and CREBBP. However, the underlying disease-causing mechanism related to the expression of these fusion proteins remains unclear. Given the role of ZNF384 in NHEJ, one possibility would be to express these ZNF384 fusion proteins in our U2OS Flp-In/Trex ZNF384 KO cells and examine their effect on NHEJ. Another hallmark of acute leukemia are defects in class switch recombination (CSR) and V(D)J recombination [39], both of which are processes that rely on cNHEJ [40]. A step towards understanding the disease mechanism of MPAL would be to investigate the effect of ZNF384 and these ZNF384-fusion proteins on CSR and V(D)J recombination. Finally, C2H2 ZnF proteins have become attractive therapeutic targets. A recent study screened the human C2H2 proteome for degradation by thalidomide analogs, which induces the ubiquitylation and degradation of C2H2 containing transcription factors (e.g. IKZF1) by targeting this domain to the substrate receptor of the CRL4_{CRBN} E3 ligase [41]. These analogs are clinically approved therapies for the treatment of multiple myeloma and other hematologic malignancies. Whilst ZNF384 function in health and disease mechanism becomes better understood, developing small molecule inhibitors to target ZNF384 may be worthwhile in the future.

ZNF384 and other ZnF proteins in DSB repair

Besides ZNF384, several other ZnF proteins have been implicated in NHEJ. For instance, APLF and ZBTB24 possess distinct ZnF domains (PBZ in APLF and C2H2 in ZBTB24) that are required for the build-up of a functional NHEJ complex by binding to auto-mono(ADP-ribosyl)ated (MAR) PARP3, PARylated PARP1, respectively, at DSBs [42, 43]. Another C2H2 ZnF protein, ZNF281, is recruited to DSBs via PARP activity and through its C2H2-domain. Whether the latter involves its binding to PAR or DNA is unclear. Its C2H2-domain also interacts with XRCC4, thereby facilitating XRCC4 recruitment and cNHEJ [44]. In addition, ZNF281 binds to the *XRCC4* promotor via its C2H2-domain, thereby controlling XRCC4 expression and cNHEJ [45]. It is therefore tempting to speculate that ZNF384 may cooperate with other ZnF proteins to promote efficient NHEJ repair. In fact, our data suggest that ZNF384 cooperates with ZnF protein PARP1, to promote the efficient loading of XRCC4. Possibly, this involves the PARP1-dependent PARylation of ZNF384, which is important for XRCC4 recruitment [42].

Moreover, previous studies reported that other ZnF proteins are also recruited to sites of DNA damage in a manner often dependent on PARP/PAR, indicating their importance in NHEJ or other DNA repair pathways [11, 46]. This raises the question as to why different ZnF proteins are required for efficient NHEJ repair. We speculate that their versatile substrate recognition features may help to load and accommodate the different cNHEJ repairosome components at DSBs, thereby promoting efficient DSB repair.

Several ZnF proteins have been implicated in DNA repair pathways other than NHEJ and HR [6]. For instance, the C2H2 domain-containing protein Aprataxin, encoded by the *APTX* gene that is frequently mutated in the neurological disorder ataxia oculomotor apraxia, catalyzes the removal of adenylate residues from 5' termini at nicks and gaps in duplex DNA to promote efficient ligation via the DNA repair proteins XRCC1 and XRCC4 [47]. Moreover, ZNF451 aids in the resolution of trapped TOP2 cleavage complexes (TOP2cc) by stimulating SUMOylation of TOP2cc via the activity of TDP2, a protein involved in the resolution of TOP2-dependent protein-DNA crosslinks [48]. Interestingly, several ZnF proteins, including ZSCAN4, ZNF827 and TZAP, have also been implicated in telomere maintenance [49]. While ZSCAN4 is required for telomere extension during the early stages of embryogenesis [50], ZNF827 binds telomeres and recruits the NuRD chromatin remodeling complex to facilitate HR and telomere elongation mediated by the alternative lengthening of telomeres (ALT) pathway [51]. Furthermore, TZAP contains 11 C2H2 motifs, three of which bind specifically to canonical telomeric repeats TTAGGG. By binding to these repeats, TZAP promotes the rapid deletion of telomeres to prevent them from becoming extremely elongated, thereby preventing genome instability [52]. Taken together, these studies highlight the significant contribution of ZnF proteins in different genome integrity pathways. However, with regards to DSB repair, their roles in pathways other than HR or NHEJ (for instance aNHEJ and SSA), as well as their roles in DSB repair pathway choice remains to be elucidated.

R-loops and replication fork stability: the NSL-complex comes into focus

Transcription is a DNA-templated process that requires proper regulation. If not regulated properly or incorrectly, the RNAPII transcription machinery may stall and form obstacles to replication fork progression, leading to transcription-replication conflicts (TRCs) that mostly occur in a head-on orientation [53]. TRCs frequently involve the generation and persistence of harmful R-loops, which can be formed by RNAPII stalling and are a

source of genome instability [54]. In the recent years numerous studies have reported on the interplay between transcription and R-loops and its impact on genome instability. However, although through these studies the network of R-loop regulators is expanding, a full understanding of the context in which R-loops arise and cause TRC-associated genome instability is lacking.

Chromatin factors have emerged as important players in R-loop formation [55]. These factors include member of the MSL (male specific lethal) histone acetyltransferase (HAT) complex MOF, which has been reported to suppress replication fork stalling and R-loop formation [56]. In contrast, however, we found that MOF regulates H4K16Ac levels, but has no significant effect on R-loops or S-phase progression. MOF is shared within the non-specific lethal (NSL) complex which consists of four core members KANSL1, KANSL2, KANSL3 and PHF20 [57]. Interestingly, we found that KANSL3, in contrast to MOF, is dispensable for H4K16Ac, agreeing with a previous report [58, 59]. Furthermore, KANSL3 protects cells from HU-induced RS and is implicated in the restart of stalled replication forks. While previous studies have shed light on KANSL3's role in transcription [58, 60], our data suggest that KANSL3 interacts with proteins involved transcription and RNA splicing. Since defects in these processes are causally linked to R-loops, it is therefore more likely that KANSL3 promotes fork stability via its role in the suppression of aberrant R-loops [53]. To this end, it will be interesting to decipher the genomic localization at which these R-loops are enriched in KANSL3-depleted cells, and whether they occur at genes regulated by the NSL complex or also at other genes. In depth analysis of the R-loop distribution by DRIP-sequencing, as well as RNA sequencing to study altered mRNA expression, in KANSL3 depleted cells will therefore be required [58]. Components of the MSL and NSL complex, however, are essential in mammalian cells [61], and siRNA knockdown experiments are not ideal for these type of large scale experiments. Therefore, the endogenous tagging of the NSL complex using CRISPR/Cas9 with the auxin inducible degron in OsTIR1 expressing cells would be a more feasible approach to rapidly and transiently deplete this complex [62]. Given that KANSL3 depleted cells suffer from RS-induced fork collapse, we speculate that this is due to transcription-replication conflicts (TRCs) occurring in the head-on direction (HO), in which the transcription machinery and replication machinery progress in opposite directions [63]. HO collisions induce pausing and blockage of the replication fork and are therefore considered more toxic. Our data suggest that KANSL3 affects replication fork stability/recovery following stress and we speculate that this is due to R-loop induced head-on TRCs.

Mutations or deregulation of MSL and NSL proteins are associated with severe human diseases including cancer. For instance, KANSL2 is reportedly upregulated in glioblastoma and drives the stem cell-like features of glioblastoma cells [64]. KANSL1 haploinsufficiency, on the other hand, impairs the activity of MOF to acetylate histone H4 on lysine 16 [65], a defect that has been associated with a wide range of malignancies including skin carcinogenesis [66]. It is also interesting to note that MOF is more stable when associated with the NSL complex, but that cancer cells that suffer from decreased expression of MOF (e.g. ovarian cancer) still display profound levels of NSL activity, while H4K16Ac levels are reduced [58]. However, a recent study reported that H4K16Ac is not required for cell proliferation and differentiation, which are common hallmarks of cancer, questioning how loss of MOF affects cancer development [58]. Our data suggest that MOF does not influence R-loop levels or fork stability, which could otherwise also affect carcinogenesis. Therefore, we infer that low MOF-expressing cancer cells suffer from defects in other MOF-dependent processes such as fatty acid oxidation and mitochondrial respiration, or apoptosis [67]. Moreover, as the catalytic subunit of NSL, MOF also catalyzes H4K5 and H4K8 acetylation, which are important marks for the initiation of transcription of housekeeping genes and for cell survival [58]. Whether loss of this function impacts cancer development is largely unclear. Our data suggest that KANSL3-depleted cells display increased levels of R-loops and fork instability as measured by pRPA (S4/S8), and suffer from reduced DNA synthesis. It is tempting to speculate that these phenotypes reflect a loss of MOF/NSL's acetylation activity towards H4K5 and H4K8, rather than MOF/MSL-dependent H4K16Ac. To this end, an epistasis analysis between members of the NSL and MSL complexes would be a starting point to better understand their interplay in these processes. Ultimately, our data reveal a dual role for the NSL-complex in which on one hand MOF promotes the formation of H4K16Ac which is dispensable for R-loop homeostasis and fork stability. On the other hand, KANSL3 suppresses R-loop formation, and may thereby prevent collisions between the replication fork and the transcription machinery that can lead to replication fork arrest/collapse. Although, the mechanism leading to this dual function within the NSL complex remains to be determined, it would be interesting to decipher whether R-loops and TRCs occur at genomic regions distinct from those enriched for H4K16Ac.

Deciphering the role of exonuclease ERI1 in R-loop suppression and fork stability

The 3'-to-5' exonucleolytic degradation and processing of a variety of RNAs is an essential feature of RNA metabolism in all cells [68]. This involves the activity of RNA processing enzymes during ribosomal RNA maturation, polyadenylation, small RNA production, and histone mRNA regulation. On the other hand, RNA can also exist in RNA-DNA hybrids or R-loops. Several RNA binding proteins are implicated in the processing of R-loops such as RNaseH1 and RNaseH2 [69]. We uncovered a novel function of the DEDDh exonuclease family member ERI1 as a suppressor of R-loop formation and replication fork instability. However, how ERI1 mechanistically impacts these processes remains to be resolved. Our mass spectrometry analysis revealed that ERI1 has various interaction partners including mRNA binding proteins (RNPS1 and PNN), RNA helicases (DHX15), chromatin remodelers (SMARCA5 and CHD4), histone proteins (HP1BP3, H1F0 and H2AFY), RNA splicing factors (THRAP3) and the DNA repair protein PARP1 (Table S1 in Chapter 5). Although, the interaction for the majority of these factors needs to be confirmed, the data may already indicate that ERI1 maintains fork stability in a broader manner than our current study indicates. Interestingly, we also successfully validated the interaction between ERI1 and PARP1 interaction by GFP pulldowns, although it needs to be confirmed reciprocally and endogenously. We found that ERI1 recruits rapidly but transiently at laser micro-irradiation induced DNA damage, in a manner dependent on the activity of PARP. This could be a consequence of the PARylation of ERI1 or the binding of ERI1 to PAR chains directly, which should be further investigated by for instance GFP-ERI1 pulldowns under denaturing conditions followed by western blot analysis and *in vitro* PAR-binding assays using recombinant ERI1 respectively [42]. ERI1 is recruited to (stalled) replication forks as measured by isolation of proteins on nascent DNA (iPOND), and the next logical step would be to investigate whether this recruitment is dependent on PAR/PARP1. PARP1 associates with (stalled) DNA replication forks and PARP1 auto-PARylation triggers fork reversal, a mechanism that ensures fork stabilization upon RS [70, 71]. In addition, it functions as a sensor of unligated Okazaki fragments during unchallenged DNA replication, and facilitates their repair [72]. Investigating the potential functions of ERI1 in these processes and the spatiotemporal modulation of the ERI1-PARP1 interaction could shed light on its contribution to replication fork stability.

ERI1 contains a conserved SAF-box, Acinus and PIAS (SAP) domain, which binds to double-stranded DNA and RNA. However, it is unclear whether the SAP domain primarily anchors ERI1 to RNA or additionally recruits proteins to RNA-dependent complexes that modify substrates such as the histone mRNA 3' stem-loop [73]. Alternatively, the SAP domain may prevent the degradation of RNA [74]. In order to establish whether this domain is responsible for ERI1's recruitment at stalled replication forks, investigating the recruitment of SAP domain mutants in iPOND experiments will be required in the future [75]. Furthermore, ERI1 contains an exonuclease domain which is involved in the processing of RNA. Mutations in this domain result in fork instability upon HU-induced RS. Since ERI1 is involved in the regulation of R-loops, which are often associated with transcription-replication conflicts (TRCs), we speculate that R-loops might be responsible for the observed RS in ERI1-depleted cells. To investigate the role of ERI1 in RNA processing and fork stability, several future experiments are required for instance: 1) *in vitro* R-loop formation assays using purified ERI1 wildtype and exonuclease dead to determine whether ERI1 processes R-loops and if this is dependent on its exonuclease domain [76]. 2) RNaseH1 overexpression experiments to study whether fork stability can be restored in ERI1-depleted cells and 3) proximity ligation assay (PLA) between PCNA, as a marker for replication, and RNAPII to evaluate the occurrence of TRCs in absence of ERI1 [55].

The fidelity of DNA polymerases is challenged by the 10-200 fold higher abundance of ribonucleotide triphosphates (rNTPs) versus DNA deoxyribonucleotide triphosphates (dNTPs) in mammalian cells [77]. Therefore, rNTPs are frequently mis-incorporated into genomic DNA during normal DNA replication, leading to replication stress, mutagenesis and DNA breaks [78]. RNaseH2 is the main enzyme that removes these rNTPs via a process called ribonucleotide excision repair (RER) [79]. Since ERI1 has a wide range of potential RNA substrates (e.g. ribosomal RNA, histone mRNAs and small regulatory RNAs), it may be possible that ERI1 is responsible for the removal of miss incorporated ribonucleotides during DNA replication, and thereby maintains fork stability. Genome-wide mapping of ribonucleotides by for instance Ribose-seq in both control and ERI1-depleted cells may provide insight into whether ERI1 is involved in RER [78]. On the other hand, ERI1 is required for the degradation of histone mRNAs at the end of DNA replication [80, 81]. In fact, ERI1 binds to the 3' and 5' stem-loop arms of the histone mRNAs to ensure their degradation at the end of each S-phase [82]. The balance of DNA and histone synthesis is important since increased levels of histones

can cause chromosome loss and genome instability [83]. We speculate that the genome instability observed in ERI1-depleted cells is a consequence of TRCs caused by R-loops. To this end, deciphering the genomic locations that are enriched for R-loops in these cells (using DRIP-sequencing), and uncovering whether changes in R-loop levels are affected by changes in histone incorporation (using chromatin occupancy after replication-seq (ChOR-seq)) would be worthwhile future endeavors [84]. Moreover, ERI1 activity is restricted by SLBP, a key protein recruited to the histone 3' UTR, where it stabilizes and promotes the translation of histone mRNAs. At the end of S-phase, SLBP is degraded by the proteasome, permitting rapid degradation of histone mRNAs by ERI1 [85]. Artificial tethering of SLBP at a genomic site enriched for R-loops could serve as an alternative approach to investigate whether inhibition of histone mRNA degradation is linked to an increase in R-loops [86].

Replication dependent histone supply is required for S-phase entry and DNA replication [87, 88]. This raises the question as to how impaired histone mRNA degradation as previously observed in ERI1 deficient cells, results in the formation of R-loops and replication stress [80]. It is tempting to speculate that disruption of histone mRNA degradation could disturb nucleosome assembly which relies on local recycling of parental histones along with deposition of newly synthesized histones in S-phase. Defects in this process are linked to developmental disorders and ageing [89]. Moreover, impaired nucleosome assembly is associated with delays in PCNA unloading and R-loop formation [87, 90]. Further work will improve our understanding on whether ERI1 suppresses R-loops and replication stress by directly acting at R-loops, or via its role in histone mRNA processing. Ultimately, this will extend our mechanistic insights into how histone biosynthesis is linked to R-loops and fork stability, and how defects in these processes can cause disease.

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