Identification and characterization of novel factors in the DNA damage response

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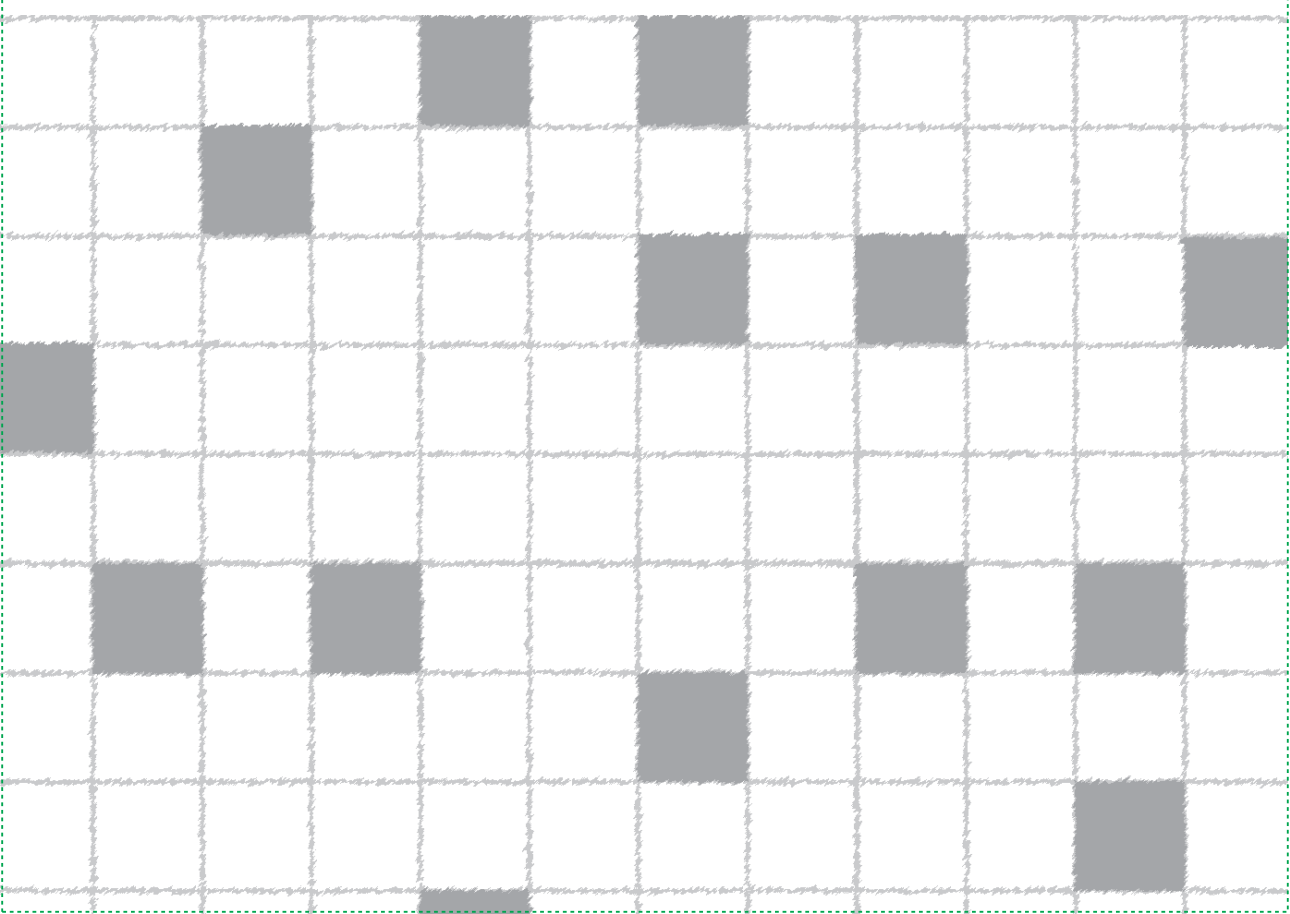

CHAPTER 1

General introduction

The cellular responses to DNA damage

The genetic information in our cells is constantly challenged by several sources that can cause DNA damage. These include exogenous sources such as ionizing radiation (IR), ultraviolet radiation (UV), alkylating compounds, viral infections and chemotherapeutic drugs, as well as endogenous sources such as aberrant DNA replication or reactive oxygen species (ROS) that are generated as a byproduct of cell metabolism [1]. Amongst the various lesions that can occur, DNA double-strand breaks (DSBs) are considered as most dangerous [2]. Furthermore, during DNA replication the genome is particularly vulnerable to lesions that block replication fork progression and prolonged stalling of replication forks can lead to fork collapse, DSBs and genome instability [3]. Cells combat these lesions and provide surveillance to the genome by activation of the DNA damage response (DDR). The DDR is a complex evolutionary conserved cellular network that has the ability to sense, signal and repair DNA lesions in time and space while coordinating gene expression, modulating chromatin structure, regulating cell-cycle progression or in the case of irreparable DNA damage, promote apoptosis [4]. An inefficient or inaccurate DDR may lead to mutations and genome instability, contributing to the development of diseases such as cancer, neurodegenerative disorders and ageing.

DSB repair

DNA double-strand break repair pathways

In somatic cells, two main pathways dictate the repair of DSBs either by homologous recombination (HR) or non-homologous end joining (NHEJ). Canonical non-homologous end joining (cNHEJ) is the dominant repair pathway in human cells, which is active throughout the entire cell cycle. This repair process is initiated by the binding of Ku70/Ku80 (Ku) heterodimers to the broken ends, followed by activation of DNA-PKcs and recruitment of APLF via its conserved Ku-binding motif (KBM). Ku and DNA-PKcs recruit and activate several enzymes involved in the processing of DNA ends, including the Artemis nuclease and DNA polymerases μ and λ. Finally, the broken ends are sealed in a mainly error-free manner by the assembly of the XLF-XRCC4-LIG4 complex, which is stimulated by PAXX [5, 6]. HR is the error-free repair pathway, which is active in the S and G2 phase of the cell cycle. It is initiated by extensive end resection of the broken ends by endo- and exo-nucleases such as MRE11, CtIP, DNA2 and EXO, resulting in the formation of 3'overhangs at the DSB ends, which become coated by the ssDNA-binding complex RPA. This is followed by the BRCA1-dependent loading of PALB2, which facilitates the assembly of BRCA2 and the recombinase RAD51, the latter of which catalyzes HR by using the sister chromatid as a template for repair. When cNHEJ or HR are disabled, DSB repair can also occur via alternative non-homologous end joining (aNHEJ) or single-strand annealing (SSA). During aNHEJ, microhomology is used that ranges between 2 base pairs (bp) and 20 bp, which seals the broken ends in an error-prone and in a manner dependent on the XRCC1-Ligase III complex and POLQ [6]. Particularly, the repair of blunt DNA ends is favored by POLQ-mediated end joining [7]. Alternatively, in the case of more extensive end-resection (> 20 bp) microhomology usage may lead to deleterious, RAD52-dependent repair of DSBs via single-strand annealing (SSA) (Figure 1 and [8].

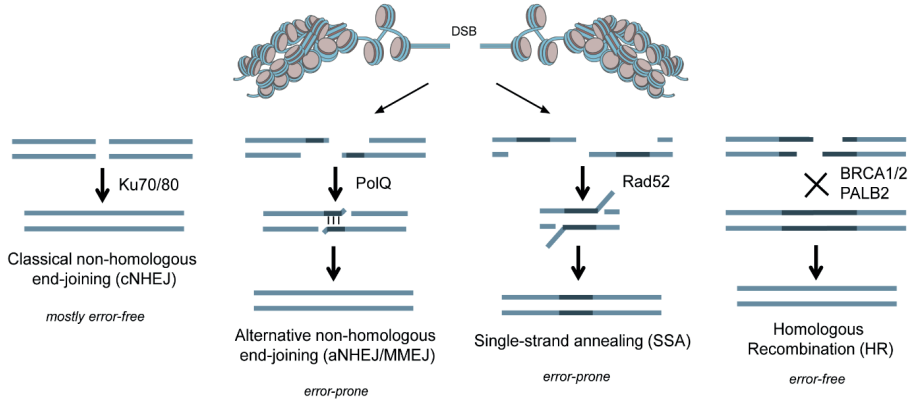

Figure 1. Overview of the different DSB repair pathways. DSBs that arise in chromatin can be repaired via two main pathways homologous recombination (HR) or non-homologous end joining (NHEJ). Classical NHEJ (cNHEJ) relies on the Ku78/Ku80 heterodimer and is mostly error-free. HR on the other hand, is a high fidelity repair pathway dependent on BRCA1/2-PALB2-RAD51, which loads RAD51. Subsequently, RAD51 invades the sister chromatid that serves as a template for repair. When HR and cNHEJ are disabled, DSB repair relies on the more error-prone pathway alternative NHEJ (aNHEJ), in which microhomology is used by PolQ to guide repair. In the case of more extensive resection, DSBs can be repaired via the RAD52-dependent highly error-prone single-strand annealing (SSA) pathway.

DSB repair: pathway choice

DSB pathway choice is tightly regulated by several mechanisms which ensure that HR is strictly regulated in S/G2 phase. During this phase, CDK-specific phosphorylation of CtIP enables its resection activity [9]. Furthermore, during G1 phase the interaction of BRCA1 with PALB2 is inhibited by the suppressive ubiquitylation of the BRCA1 interacting domain in PALB2, impairing HR by limiting BRCA1-PALB2-BRCA2-RAD51 complex formation [10]. In addition, the KU-binding protein CYREN inhibits NHEJ in S/G2 phase by protecting breaks with overhangs [11]. Also, in G1 phase TIRR-mediated recruitment of 53BP1 to DSBs inhibits DNA end-resection through 53BP1's effectors RIF1, MAD2L2, REV7 and the Shieldin complex, thereby impairing HR and to favor NHEJ. [12]. The 53BP1-RIF1-Shieldin complex mediates the recruitment of the CTC1-STN1-TEN1 (CST) complex to DSBs. The CST complex and Polα promote the fill-in of resected DSB ends, which may be protected against DNA end resection by Shieldin [13]. Remarkably, it has also been reported that small tandem duplications (TD) are generated by CST-Polα at DSBs containing 3' overhangs in a 53BP1 and shieldin dependent manner, leading to Ku-dependent cNHEJ [14]. Finally, it was shown that ZMYM3 facilitates the recruitment of ABRA1 and BRCA1 to DSBs, thereby antagonizing the HR-suppressive effects of the

BRCA1-A complex and facilitating the loading of RAD51 and HR [15]. In addition to the above-mentioned mechanisms, DSB repair pathway choice also depends on the complexity of the DSB ends. During cNHEJ, compatible DNA ends are not processed, and incompatible DNA ends are minimally processed in a ligation-competent complex or otherwise protected by Ku70/Ku80 and DNA-PKcs [5]. This type of repair also plays an important role during V(D)J recombination and class-switch recombination (CSR), which are processes occurring during lymphocyte differentiation that ensure the generation of a diverse class of antibodies in order to eliminate pathogens [16]. During V(D)J recombination, RAG1 and RAG2 induce DSBs between the V, D and J segments, and the conserved recombination signal sequences (RSS) of the Ig and T cell receptor (TCR). This is followed by the repair of these DSBs by NHEJ, resulting in the diversification of antigen receptor genes [17]. A key step during B cell maturation is isotype switching of immunoglobulins (Igs) through CSR. This process relies on the formation and repair of DSBs induced by activation-induced (cytidine) deaminase (AID) at conserved motifs within the switch (S) regions, which are upstream of the constant regions of antibody heavy chains [18]. Two S regions are joined by NHEJ upon break formation, resulting in loss of intervening DNA between the S regions, and consequently a change in the class of Igs expressed by a B cell.

An important step in response to DSBs is the activation of the ATM kinase. ATM is recruited to DSBs by the MRE11-RAD50-NBS1 (MRN) complex, where it initiates a signaling cascade by phosphorylating histone variant H2A.X. Phosphorylated H2AX, also referred to as γH2AX, then serves as a platform to load MDC1 [19]. The ATM-dependent phosphorylation of MDC1 leads to the recruitment of the E3 ubiquitin ligase RNF8, which ubiquitylates histone H1 and allows the accrual of another E3 ubiquitin ligase RNF168. RNF168 further decorates the DSB-flanking chromatin with H2AK15ub. The simultaneous association of 53BP1 to the RNF8/RNF168 dependent H2AK15ub, and H4K20me2 via its tudor domain, results in 53BP1 dependent cNHEJ [20]. While high levels of H4K20me2 in G1 cells promote 53BP1 recruitment, this mark is gradually diluted during S-phase and lowers 53BP1 binding at replicated areas of the genome, leading to a switch in HR [21]. On the other hand, the BRCA1-associated RING domain protein 1 (BARD1) recruits BRCA1 to DSBs by engaging H2AK15ub. The binding of BARD1 to H2AK15Ub and unmethylated H4K20 via its BRCT-domain-associated ubiquitin-dependent recruitment motif (BUDR) and Ankyrin repeat domains respectively, promotes BRCA1-BARD1-dependent DSB repair via HR during replication (Figure 2 and [22]).

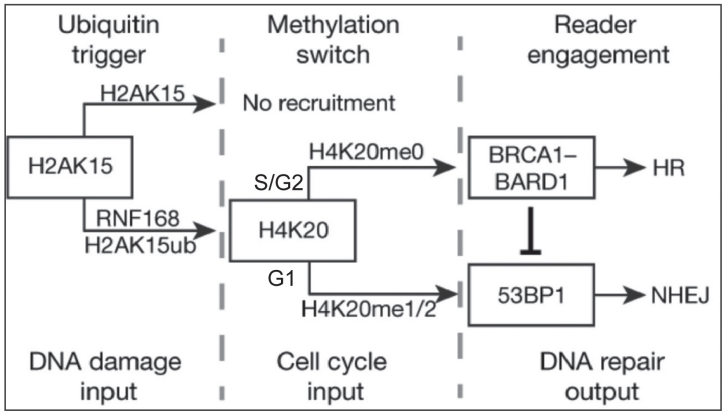

Figure. 2 DSB repair pathway choice by post-translational modifications (PTMs). Combinatorial H2AK15 and H4K20 PTMs govern the choice of the DSB repair pathway. On one hand, the DNA damage mediated recruitment of RNF168 results in the ubiquitylation of H2AK15 and recruitment of BRCA1-BARD1 to promote HR. On the other hand, high levels of H4K20me2 in G1 promote the recruitment of reader protein 53BP1 to mediate NHEJ. The simultaneous dilution of H4K20me2 during S/G2-phase leads to a switch in HR [22].

DSB repair in the context of chromatin

Efficient detection and repair of DSBs 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 at DSBs to facilitate repair [23, 24]. Over the past years it has become clear that besides the PTMs that are involved in regulating 53BP1 and BRCA1-BARD1 recruitment, several other PTMs and structural chromatin changes driven by ATP-dependent chromatin remodelers affect DSB repair. One key protein involved in circumventing the chromatin barrier during cNHEJ is PARP1. PARP1 is rapidly recruited to DSB sites where it promotes the local expansion of chromatin by the formation of PAR-chains on itself and its target proteins, including histones [25]. This results in the accumulation of DNA repair proteins which bind to PAR-chains directly [26] or to the exposed DNA, as was observed for several chromatin remodelers and cNHEJ factors that directly associate with DNA [27, 28]. Importantly, following DSB repair, the chromatin structure must be restored to its original state.

Chromatin remodeling is important for both HR and NHEJ, as it allows for the recruitment to DNA damage sites of factors involved in both these repair processes [29-31]. During short-range resection, chromatin is extensively remodeled to facilitate the loading of end-resection factors MRE11 and CtIP [32]. This is followed Fun30/

SMARCAD1 chromatin remodeling-driven long-range resection by the EXO1 and DNA2 nuclease. During this process, it is the Fun30/SMARCAD1-depent chromatin remodeling that counteracts the suppressive effect of 53BP1 on end-resection [33-35]. The Chromodomain Helicase DNA-binding (CHD) class of chromatin remodelers have also been implicated in DSB repair. Amongst these are CHD2 and CHD7, which have been shown to promote NHEJ [29, 30, 36]. CHD2 is recruited to sites of DNA damage by binding to PAR moieties associated with PARP1. Subsequently, it promotes DSB-induced chromatin expansion and deposits histone variant 3.3 to facilitate the efficient recruitment and proper functioning of the NHEJ machinery [30]. CHD7, on the other hand, does not bind PAR moieties but is recruited to DSBs via the PARP1-induced relaxation of damaged chromatin. CHD7 stimulates further chromatin relaxation and recruits HDAC1/2 for local chromatin de-acetylation and compaction. This allows for the efficient loading and correct positioning of KU and XRCC4/LIG4, thereby facilitating cNHEJ. Loss of CHD7 reinforces 53BP1 and shifts repair to mutagenic aNHEJ [29]. While the interplay between chromatin modulators and HR and cNHEJ has gained more insight over the last years, it remains unknown how they regulate aNHEJ and SSA. Future endeavors are required to understand the combinatorial effect of chromatin remodelers and histone modifications on DSB repair, particularly during aNHEJ and SSA.

DSB repair and transcription

DNA double strand breaks (DSBs) not only affect chromatin structure, but also the impact on other DNA-dependent processes such as transcription. Given the fact that DSBs occur in both inactive and actively transcribed regions, it is of utmost importance that transcription and DSB repair are coordinated properly in order to prevent collisions between the two processes [37]. Several studies have observed a direct link with transcription repression and DSB repair. In fact, HR and NHEJ factors are implicated in the repression of transcription upon DSBs. One such player is PARP1, which targets the RNAPII complex for PARylation in response to DSBs. This triggers an interaction between RNAPII and the NELF-E subunit of the negative elongation factor (NELF) complex, which leads to NELF-E dependent repression of transcription elongation in the vicinity of DSBs to promote efficient DSB repair [38]. Moreover, PARylated PARP1 is also responsible for the recruitment of KDM5A, which induces demethylation of H3K4me3. In turn, this modification allows for the recruitment of ZMYND8, which binds PAR and H4K16Ac to promote transcriptional repression and HR by facilitating the accrual

of the repressive NuRD complex [39]. PARP1 also functions with TIMELESS to recruit the E3 ubiquitin ligase complex FRRUC to DSBs. On one hand, FRUCC represses transcription by inducing the monoubiquitylation of H2AK119, while on the other hand it promotes the exchange of H2A with H2A.Z [40]. Two members of the PI3K kinase family, ATM and DNA-PK, have been shown to play crucial roles in the suppression of transcription upon DSBs. ATM dependent phosphorylation of transcription elongation factor ENL affects the accumulation and/or retention of Ku70 at DSBs [41]. Moreover, ATM can repress transcription in an RNF8/RNF168 dependent manner. RNF8 triggers the assembly of K11 linked ubiquitin moieties on histones H2A/H2AX in coordination with the Ube2S/C E2-conjugating enzymes to induce transcription silencing at DSBs by enforcing RNAPII pausing and repression of transcription of genes in close vicinity of a break [42-43]. On the other hand, ATM can also repress transcription in response to DSBs in a manner independent of RNF8/RNF168. This involves the ATM dependent phosphorylation of the PBAF subunit BAF180, which is crucial for the mono-ubiquitylation of H2A on K119, thereby promoting transcription silencing and NHEJ repair of DSBs [44]. More recently, the HECT E3 ubiquitin ligase WWP was identified as a critical mediator of DNA-PKcs-dependent transcription silencing at DSBs [24]. DNA-PK complex (consisting of Ku70/Ku80 and DNA-PKcs) and WWP2 are recruited to DSBs in actively transcribed genes. WWP2, in turn, modifies the RNAPII subunit RPB1 by K48 polyubiquitylation via DNA-PK. This triggers the recruitment of the proteasome at DSB sites and targets RNAPII for proteasomal degradation. Finally, this prevents collisions between the NHEJ factors and the transcription machinery, promoting efficient retention of the DNA-PK complex to repair the DSBs by NHEJ [24].

While it is evident that transcription becomes silencing occurs at DSBs, transcription termination is another crucial process for transcription regulation at DSBs. This process involves the combined action of several different enzymes that target nascent RNAs, leading to transcription termination and RNAPII removal from chromatin. The 5'-3' exoribonuclease XRN2 and the RNA/DNA helicase Senataxin (SETX) are examples of these enzymes [45]. SETX interacts with RNAPII and thereby promotes the cleavage of nascent RNA at poly(A) sites in a manner dependent on XRN2. This in turn, allows for the release of the pre-mRNA and the degradation of the remaining chromatin-associated RNA [46].

While transcription silencing and transcription termination at DSBs is better understood, mechanisms that promote transcription restart following the completion of DSB repair are still very poorly understood. Given that chromatin structure changes dramatically

surrounding DSB sites [47, 48], restoring the pre-existing chromatin state is foremost important to restart transcription following DSB repair. For instance, the removal of H2AK119Ub by the deubiquitinase USP16 is crucial for transcription restart after DSB repair [43]. Counterintuitively, WWP2-DNA-PKcs trigger the proteasomal degradation of RNAPII [24]. Further studies are needed to resolve how restart of transcription is achieved following RNAPII pausing and/or degradation and the completion following DSB repair in different chromatin contexts.

Replication stress

DNA replication

The ability of cells to survive and proliferate depends on the faithful duplication of their DNA via DNA replication during each cell cycle. Inaccurate or incomplete DNA replication can result in genomic instability, cell death and cancer. In eukaryotes, replication origins are licensed through the assembly of the pre-replication complex (pre-RC). This process is dependent on the six-subunit origin recognition complex (ORC), which provides a platform for the loading of the minichromosome maintenance complex (MCM) in a Cdc6- and Cdt1-dependent manner in G1. Upon S-phase entry, cyclin-dependent kinases (CDKs), and Dbf4-Cdc7 (DDK) activate a subset of replication origins. This is followed by the formation and activation of the CMG (CDC45-MCMs-GINS) helicase complex, which unwinds the DNA duplex, forming a replication bubble in which DNA synthesis is initiated by the replication machinery [49]. DNA replication can be initiated stochastically at thousands of individual replication origins, which form bidirectional forks. It is of utmost importance that cells balance accuracy and speed, as well as the consumption of resources such as nucleotides and replication factors, to complete DNA replication. Therefore, the firing of replication origins is strictly regulated and divided into early replication origins and late replicating origins. Also, not all licensed origins fire during an unperturbed S-phase, but can be activated following replication stress to ensure the completion of DNA replication [3, 50].

ATR signaling

Replication stress (RS) is defined as the stalling or slowing of replication fork progression or DNA synthesis. Several different sources can induce RS, including hydroxyurea (HU)-induced uncoupling of the MCM helicases from the replicative polymerases, leaving behind nicks or gaps with stretches of single-stranded DNA (ssDNA). DNA lesions caused

by UV light or chemical mutagens, DNA secondary structures, replication-transcription conflicts (TRCs), common fragile sites (CFS) and oncogene-induced replication stress are common DNA polymerase-blocking lesions [3]. ATR is the major kinase responding to RS, which coordinates a wide range of cellular processes including preserving the RPA pool, suppression of new origin firing, preventing fork collapse and promoting fork restart at stalled replication forks before cells enter mitosis (Figure 3 and [51]). RPA-coated ssDNA generated by DNA polymerase-helicase uncoupling during fork stalling triggers the activation of ATR by its binding partner ATRIP which binds RPA directly. This results in the recruitment of the ATR activating protein TOPBP1, which is recruited to ssDNA-dsDNA 5' junctions through its interaction with the MRE11-RAD50-NBS1 (MRN) complex and the 9-1-1 (RAD9-RAD1-HUS1) complex [52]. TOPBP1 also interacts with RHINO, and this interaction is required for the full activation of ATR upon replication stress [53]. On the other hand, ATR can be activated via ETAA1, which is, unlike TOPBP1, recruited to RPA-coated ssDNA via a direct interaction with RPA. This interaction is required for the activation of ATR via its ATR activation domain (AAD) and the subsequent activation of the S-phase checkpoint kinase CHK1 [54].

Fork reversal

Protection of ssDNA by RPA and checkpoint signaling is not sufficient to prevent fork collapse under prolonged replication stress conditions. In fact, RPA is highly dynamic on ssDNA and is displaced in a timely manner by RAD51, thereby promoting replication fork reversal [55]. During fork reversal the three-way fork junction is migrated backward to displace and anneal the nascent DNA strands to form a 'chicken foot' structure (Figure 3). This process is catalyzed by several enzymes including the SNF2 family of chromatin remodelers SMARCAL1, ZRANB3 and HLTF, as well as the RecQ helicases BLM and RECQ1 [54]. SMARCAL1 travels with the replication fork and remodels forks with persistent ssDNA gaps into reversed forks in an ATR dependent manner. ZRANB3 interacts with polyubiquitinated PCNA to promote fork remodeling and HLTF affects fork reversal directly via its HIRAN domain [56]. Fork reversal has the following benefits on replication fork fidelity: 1) backtracking and annealing of nascent DNA strands prevents replication fork collapse, 2) preventing the formation of excessive amounts of ssDNA and allowing more time for the repair of replication impediments which can be repositioned back on the dsDNA template, and 3) generating a Holliday junction with a single-ended DSB that can be repaired via HR [57]. On the other hand, reversed forks

are common substrates for nucleolytic degradation by several nucleases such as MRE11, CtIP, DNA2 or EXO1, which act predominantly in the absence of fork protection factors RAD51, BRCA1 and BRCA2. This ultimately leads to nascent DNA strand shortening, fork collapse and chromosomal aberrations [56].

DNA damage tolerance (DTT) pathways

A pathway acting in parallel to fork reversal is PrimPol dependent repriming. PrimPol is an RNA/DNA primase that is recruited to replication forks via its interaction with RPA coated ssDNA under conditions of fork stalling induced by UV irradiation or HU treatment. Primpol leaves behind ssDNA gaps during repriming on the leading strand, which are enlarged by MRE11 and EXO1. This in turn, results in the loading of RAD51 to promote HR-mediated post-replicative repair (PRR) (Figure 3 and [58, 59]. Importantly, depletion of RAD51 or fork reversal factors such SMARCAL1 or HLTF leads to PrimPol mediated repriming on the leading strand (Figure 3 and [58, 59]. Thus, the balance between fork reversal factors such as RAD51-SMARCAL1 and PrimPol, determines the choice between fork reversal and repriming and thereby controls the fate of the replication fork. When the replication fork obstacle (usually a DNA lesion) is present on the lagging strand, the replication machinery can bypass these DNA lesions by using so-called DNA damage tolerance (DDT) pathways, which comprise translesion synthesis (TLS) and template switching (TS) (Figure 3). During TLS, replication across a DNA lesion is resumed by low fidelity DNA polymerases including Y-family polymerases (POLH, POLI, POLK and REV1), B-family polymerase Pol ζ and A-family polymerases POLQ and POLN. Each polymerase has the ability to recognize a specific type of DNA lesion, which can either be bypassed in a relatively error-free mode, for example by POLN, or via an error-prone mechanism by Pol ζ and REV1 [60]. TS is the more faithful DDT pathway in which the stalled replication fork uses the newly synthesized strand as a template to avoid the lesion via pathways that are less well understood [61]. The choice between the DDT pathways is regulated by PCNA, the replicative sliding clamp that is essential for DNA replication. In response to RS, PCNA is either mono- or polyubiquitylated on Lysine 164 (K164). TLS is mediated by the RAD18 dependent mono-ubiquitylaton of PCNA for the loading of TLS polymerases, whereas TS is regulated by HLTF and SHPRH dependent polyubiquitylation of PCNA [62]. However, the exact mechanism by which the PCNA ubiquitylation status triggers TLS or TS remains unknown.

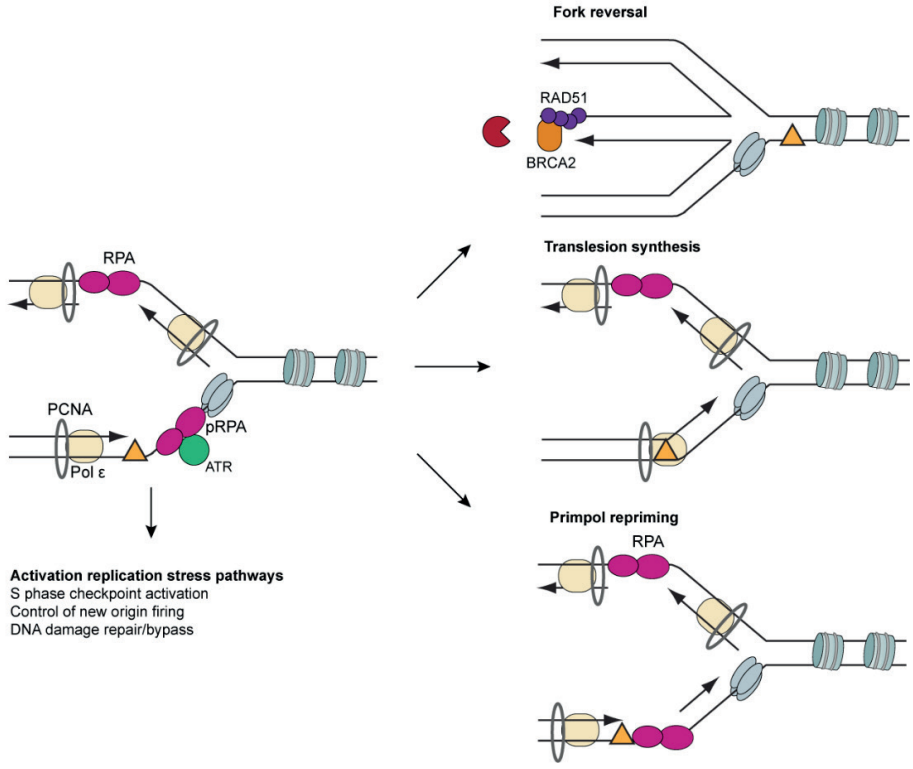

Figure. 3 Fork repair mechanisms. Polymerase-helicase uncoupling leads to the activation of the S-phase checkpoint which prevents new origin firing. Alternatively, fork reversal, translesion synthesis (TS), fork repriming may act to overcome DNA lesions caused by polymerase-helicase uncoupling.

Break induced replication (BIR)

The resumption of DNA replication at stalled forks is mediated by several different mechanisms. One of the most favorable mechanisms of fork restart is the resumption of semiconservative DNA synthesis supported by the CMG helicase. However, when the CMG helicase is disassembled or lost, a collapsed fork is prone to cleavage by the MUS81 nuclease resulting in a one-ended DSB [63]. This is followed by resection and strand invasion of the DNA end using the undamaged opposite strand, which can resume DNA synthesis using a modified replisome on a migrating D-loop during a process called break induced replication (BIR). The molecular mechanism of BIR is, however, poorly understood in mammalian cells. While canonical BIR relies on RAD51, several

studies have reported RAD51-independent BIR in mammalian cells, which relies on RAD52 and may be occurring at common fragile sites with large amounts of RS [54]. Furthermore, BIR occurs during mitotic DNA synthesis (MiDAS). MiDAS completes DNA synthesis at replication origin poor chromosomal regions referred to as 'common fragile sites' during mitosis in a MUS81-dependent manner. BIR is considered an error-prone pathway, which can lead to genomic rearrangements such as the formation of tandem duplications and copy number variations. Moreover, defects in BIR can also lead to impaired restart of reversed replication forks in the absence of BRCA2 [64]. Future work is required to better understand the genomic context in which BIR occurs, and which factors drive this important process.

Transcription-replication conflicts

Co-directional and head-on transcription-replication conflicts

During the S-phase of each cell cycle, eukaryotic cells are the most vulnerable as the replication and transcription machineries co-exist on the same DNA template. It is inevitable that the two machineries could interfere with each other, giving rise to transcription-replication conflicts (TRCs). Depending on the orientation of transcription relative to the direction of the replication forks, TRCs may occur in a head-on (HO) or co-directional (CD) orientation (Figure 4). During HO collisions, the transcription machinery and replication machinery progress in opposite directions. CD collisions, on the other hand, are caused by replisomes that encounter RNA polymerases that progress in the same direction [65]. Studies in bacteria and eukaryotes have shown that HO collisions induce pausing and blockage of the replication fork to a greater extent than CD collisions do. Consequently, HO collisions are considered more toxic.

Several different scenarios have been suggested for how TRCs can occur. For instance, the MCM2-7 helicase unwinds the parental DNA ahead of the replisome. This could lead to a frontal clash of the helicase with the RNA polymerase and may lead to the inactivation of the helicases [66]. Furthermore, transcription and replication require unwinding of the DNA. During transcription, this leads to positive and negative supercoiling upstream and downstream of the RNA polymerase causing torsional stress which is relieved by DNA topoisomerase enzymes. Indeed, depletion of Topoisomerase I in mammalian cells increases negative supercoiling in actively transcribed regions which results in replication fork stalling and DNA breaks [67]. In addition to torsional stress, non-B structures on DNA are also common sources of TRCs. Such non-B DNA structures are formed at a

particular DNA sequence (e.g. repetitive sequences or at common fragile sites) and exist in the form of hairpins, triplex DNA, G-quadruplexes or alternatively as RNA-DNA hybrids (R-loops) [65].

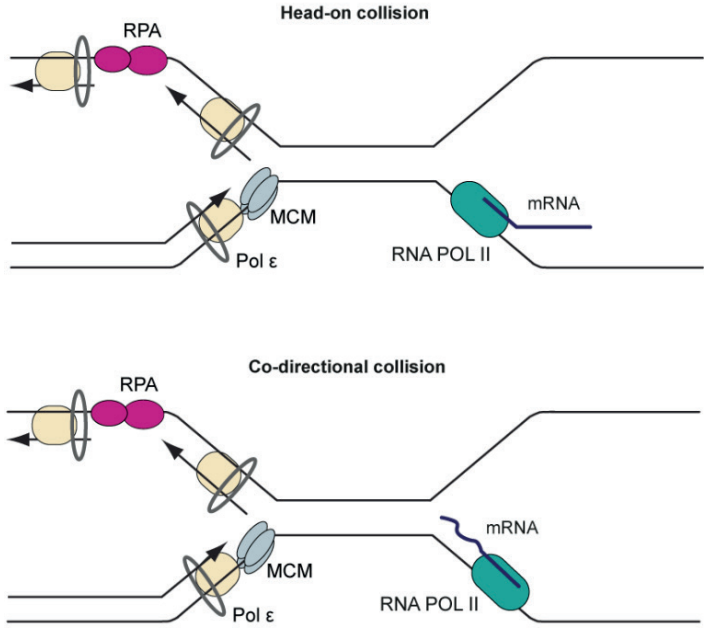

Fig. 4 Transcription-replication conflicts (TRCs). Progression of RNA polymerase (RNAPII) and a replication fork in the opposite direction leads to head-on collisions (HO), which induce pausing and blockage of the replication fork, and may lead to fork collapse and the formation of DNA breaks. Progression of RNAPII and a replication fork in the same direction leads to co-directional (CD) collisions if the fork moves faster than RNAPII. CD collisions can be resolved by the displacement of RNAPII from DNA.

RNA-DNA hybrids

R-loops are important players in a number of physiological processes. These structures are formed due to nascent RNA base pairing with its template DNA behind the RNA polymerase leaving behind the non-templated strand as ssDNA. Their presence in the genome is required during DNA replication at the start of leading strand synthesis, as well as during Okazaki fragment synthesis at the lagging strand. In addition, R-loops can also be a source of genome instability. Consequently, the factors that resolve or prevent their formation such as RNA binding proteins, helicases, nucleases and DNA replication and repair factors, are of utmost importance for genome maintenance [68].

Genome wide mapping studies revealed that R-loops are primarily located at promoters of RNA polymerase II (RNAPII) transcribed genes. Here, they activate transcription by the inhibition of transcriptional repressors such as DNA-methylation enzymes (DNMT), or act as binding sites for transcription factors and chromatin remodelers. Alternatively, R-loops can also repress transcription by the induction of repressive chromatin marks [69]. Furthermore, R-loops are also involved in efficient transcription termination by stalling RNAPII downstream of the poly-adenylation tail. This is followed by the recruitment of the R-loop helicases senataxin (SETX) and DHX9 which resolve R-loops and promote the release of nascent RNA for degradation by the exonuclease XRN2 leading to transcription termination [68].

Unscheduled R-loop formation can be a source of genomic instability as the ssDNA generated during this process can be susceptible to DNA damage. Furthermore, when R-loops are not removed properly they can cause TRCs. Recent studies on an engineered bacterial genome and a mammalian episomal system have shed light on the orientation of TRCs and how they influence the levels of R-loops [70, 71]. Interestingly, both studies revealed that HO collisions enhance R-loops formation due to increased topological stress, whereas CD collisions contribute to resolving R-loops, the latter which occurs via a mechanism that remains largely unknown. R-loops can also cause transcriptional stress by the excessive stalling, arrest or backtracking of RNAPII. It is unclear what distinguishes R-loops that promote transcription from those that cause transcription stress. One possibility is that regulatory R-loops are rapidly degraded in cells and therefore do not persist long enough to block RNAPII [72].

Cells have evolved several mechanisms to prevent or remove unscheduled R-loops in order to maintain genomic stability. One such mechanism involves the prevention of newly transcribed RNA to hybridize with DNA. This mechanism is driven by mRNA binding proteins (RBPs), which promote the processing and export of the RNA, thereby impeding hybrid formation [73]. Indeed, mutants of the THO/TREX protein complex involved in transcription and mRNA export, initially identified in yeast, showed increased R-loop-mediated genomic instability [74]. Another mechanism is the relieve of DNA supercoiling at transcription sites. During this process, specialized topoisomerase enzymes such as TOP1 prevent the formation of R-loops by relieving negative supercoiling behind RNA polymerases [67]. More recently, chromatin factors have emerged as important players in R-loop homeostasis. An interaction between the THO and SIN3A histone deacetylase complex (HDAC) was described to regulate acetylation of histones to prevent

the accumulation of harmful R-loops [75]. Furthermore, depletion of the SWI/SNF complex member BRG1 in mammalian cells increases R-loop dependent DNA breaks and TRCs [76]. Besides factors that suppress R-loop formation, cells are also equipped with enzymes that directly remove R-loops such as RNase H1 and RNase H2 that cleave the RNA strand in the hybrid. In addition, Rnase H2 is also involved in the removal of mis-incorporated ribonucleotides in a process called ribonucleotide excision repair (RER) [77]. While Rnase H1 is mainly involved in the removal of R-loops following replication stress, Rnase H2 is required to remove R-loops in a post-replicative manner [78]. In addition, several helicases are involved in resolving R-loops, either by translocating along the RNA strand, unwinding double-strand regions and displacing proteins, or by acting locally at R-loops [79]. Examples of such helicases are DDX5, which is stimulated by BRCA2, aquarius (AQR), and Senataxin (SETX) [80]. *In vitro* studies of Sen1, the yeast ortholog of SETX, revealed that this protein displays 5' to 3' translocation activity, as well as 5' to 3' unwinding properties along DNA and RNA. Loss of RNase H and SETX activity was shown to cause R-loop-induced replication stress and genomic instability [73]. Interestingly, ectopic expression of Rnase H enzymes supresses replication stress in cells accumulating R-loops, which indicates that R-loops interfere with replication fork progression [70]. Furthermore, R-loops can be processed by nucleotide excision repair (NER) factors XPF and XPG. While it remains unclear how XPF and XPG recognize and process R-loops, one possibility may be that they may recognize and cleave both strands of an R-loop, removing the R-loop and generating a DSB. Another possibility could be that the transcription-coupled NER (TC-NER) machinery is recruited to R-loops during DNA replication and generates a ssDNA gap that is converted into a DSB [81].

R-loops and DSB repair

Several recent studies have shown that R-loops can also form at DSBs, where they can both inhibit and promote repair. For instance, the loss of RNase H enzymes in budding yeast is associated with an increase in RAD52 foci, indicative of persistent breaks [82]. In contrast, a study in fission yeast showed that R-loops are enriched at *I-Ppol* induced DSBs, and that both overexpression and deletion of Rnase H impair HR, indicating the importance of a temporary R-loop near the break site for efficient repair [83]. To this end, an important observation is the fact that R-loops may impact DNA end resection. In budding yeast, persistent R-loops block resection at the break site adjacent to the R-loop [84]. On the other hand, factors that promote resection, including Sae2 in budding

yeast and its ortholog CtIP in mammalian cells, have been shown to promote R-loops resolution, illustrating the complex relationship between R-loops and resection [85].

R-loops also contribute to an alternative type of HR in which a homologous RNA molecule instead of a homologous DNA molecule is used as the template for DSB repair [86]. There is evidence that this type of HR occurs in G0/G1 cell cycle phase of post-mitotic neurons. During this process, R-loops may guide RNA-templated HR by promoting RAD52 recruitment to DSBs [87]. Although the usage of RNA as a template for HR has been observed in both yeast and human cells, the physiologic relevance of R-loops herein needs to be resolved [86]. Interestingly, DNA damage-induced long non-coding RNAs (dilncRNAs) which are transcribed from broken DNA ends have also been implicated in R-loop formation [88]. dilncRNAs pair to the resected DNA ends during S/G2 phase and form R-loops, which are recognized by BRCA1. This is followed by BRCA2-mediated recruitment of RNase H2, which degrades R-loops to ensure efficient HR-mediated repair [89]. Finally, R-loops can also be generated as a consequence of transcription inhibition induced upon DNA damage [37]. Indeed, pausing of RNAPII distal to the DNA damage site has been associated with increased R-loop accumulation [71, 90]. Altogether, these findings highlight the complexity of R-loops and their link to genome stability and pinpoint the importance of R-loop processing factors, genomic location and transcriptional status as determinants on how they affect DSB repair.

DSB repair, replication stress response and cancer therapy

Recent technological advances in the field (e.g. CRISPR/Cas9 based screens), have enabled us to explore synthetic lethal interactions relevant to DNA repair and replication in normal cells and cancer cells [91]. Synthetic lethality describes the cellular condition in which a defect in either one of two genes has little or no effect, but the combination of both gene defects results in death (synthetic lethality) or sickness (synthetic sickness) [92]. The ever-growing focus on exploring synthetic lethality has stemmed from the success of PARP inhibitors (PARPi) in the clinic for the treatment of tumors with mutations that disrupt BRCA1/BRCA2 genes or other HR factors. Mechanistically, PARPi does not only block PARP1 activity for the repair of DNA lesions such as single-stranded DNA breaks or DNA replication intermediates (e.g. unligated okazaki fragments), but also traps PARP1 on to damaged DNA [93]. PARPi-induced trapping of PARP1 on DNA possesses a block to the replisome and thereby causes cytotoxicity. In order to resolve these blocks and resume cell cycle progression, cells require functional HR. Defects in

HR lead to the persistence of DNA damage, thereby causing cell death. However, over the past years, studies in mouse models and patients have shown that PARPi responses are frequently associated with resistance. Intriguingly, PARPi resistance is often correlated with resistance to platinum-based agents such as cisplatin [94]. Several resistance mechanisms have been described which can be classified into four main categories: (1) influence of the cellular availability of the inhibitor, (2) direct impact on the activity and abundance of PAR chains, (3) reactivation of HR and (4) the influence of replication fork protection [12].

Long-term treatment of *Brca1*-deficient breast tumors in mouse murine models with PARPi displayed resistance due to overexpression of transporter genes *Abcb1a* and *Abcb1b* encoding P-glycoprotein efflux pumps. Indeed, coadministration of the P-glycoprotein inhibitor reversed their resistance to PARPi [95]. Upregulation of ABCB1 was also observed in chemotherapy-treated ovarian and breast cancer cells as a result of chromosomal translocations involving these genes [96]. However, whether these translocations are present in BRCA1/2 mutated tumors, and whether P-glycoprotein efflux pumps are upregulated in PARPi-resistant tumors needs to be addressed in the future.

PARylation induced by PARP1 is a reversible and transient modification of proteins by the covalent addition of PAR upon DNA damage [97]. While depletion of PARP1 is synthetic lethal with BRCA1/2 deficiency, the cytotoxicity caused by PARPi treatment is much larger than that following genetic depletion of PARP1 [98, 99]. Therefore, mutations in PARP1 that influence PARP1 trapping induce PARPi resistance, even in HR deficient cells [100]. A mechanism related to PARP inhibition involves the inhibition of PAR glycohydrolase (PARG), which is the enzyme that degrades PAR chains in cells. Genetic screens performed using *Brca2* mutated mouse mammary tumors revealed that loss of PARG represented a major PARPi resistance in these tumors. PARG loss partially restored PARylation in PARPi-treated cells, also diminished PARP1 trapping on DNA, and partially rescued DNA damage signaling caused by PARP1 [101].

The majority of HR-disrupting mutations such as in BRCA1/2 are frameshift mutations or single nucleotide mutations. The occurrence of secondary- or reversion mutations, would enable frame-restoration and potentially restore protein activity and HR. Interestingly, these reversion mutations are often showing a microhomology signature, suggesting that they result from the repair of DSBs via aNHEJ [102]. The identification of mutational signatures has allowed for the identification of several synthetic lethal interactions to

therapeutically exploit BRCA-deficient tumors. There are several examples of synthetic lethal interactions described in recent years. For instance, FANCD2 supports POLQ mediated TMEJ and protects stalled replication forks from degradation. Consequently, loss of FANCD2 leads to cell death in BRCA1/2 deficient cells [103]. More recently, the POLQ inhibitor ART558 was found to effectively target BRCA-defective cancers and even enhance the effect of the PARPi treatment [104]. Furthermore, FEN1 is involved in the 5'flap processing following aNHEJ. It also plays a role in long-patch SSB repair. FEN1 inhibition or downregulation results in increased replication stress due to compromised 5'flap processing and BER in BRCA-deficient cells, leading to cell death [105]. Another feature of PARPi resistance in BRCA1, but not BRCA2 deficient cells, is the loss of resection inhibitors by factors that regulate NHEJ including 53BP1 and its downstream factors in the Shieldin complex [106]. Interestingly, the POLQ inhibitor ART558 could overcome the PARPi resistance observed in 53BP1/Shieldin defective cells, due to DNA nuclease-mediated hyperresection [104]. In addition to 53BP1/Shieldin, CRISPR/Cas9 screens have enabled the identification for other suppressors of PARPi resistance in BRCA1 deficient cells, including DYNLL1 and its transcriptional activator ATMIN [107]. DYNLL1 associates with DNA end resection factors, including MRE11, and these factors to promote DNA end-resection. Indeed, loss of DYNLL1 leads to increased resection in BRCA1 deficient cells and thereby causing PARPi resistance [107].

Finally, the restoration of replication fork protection also serves as a mechanism for PARPi resistance. In addition to their role in HR, BRCA1/2 also protect stalled replication forks [108]. BRCA1/2 loss leads to nascent strand shortening, fork collapse and chromosomal aberrations due to nucleases such as MRE11 and MUS81, which act upon the deprotected fork. Sepcifically, EZH2 and PTIP were found to mediate PARPi sensitivity by recruiting MUS81 and MRE11 to stalled forks [109, 110]. In addition, RADX is involved in the stabilization and resolution of stalled forks which prevents MUS81-dependent fork cleavage and inhibits RAD51 mediated fork remodeling [111]. Fork remodeling is another process required for MRE11-dependent degradation of nascent DNA in BRCA1/2 deficient human cells, and depletion of fork reversal factors SMARCAL1, ZRANB3 and HLTF cause PARPi resistance [112].

More recently, another mechanism of BRCA1/2 deficiency and synthetic lethality has been described. This mechanism underlies the accumulation of ssDNA gaps in BRCA1 deficient cells, a phenomenon that was found to correlate with PARPi sensitivity. Interestingly, LIG3 loss promotes the formation of MRE11 mediated post-replicative

ssDNA gaps in BRCA1 deficient and BRCA1/53BP1 double deficient cells. Moreover, LIG3 depletion enhances the efficacy of PARPi treatment in Brca1-deficient mammary tumors in mice, suggesting LIG3 as a potential therapeutic target [113, 114]. CRISPR/Cas9 screens also allowed the identification of the PAR-binding chromatin remodeler ALC1/CHD1L as a key determinant of PARPi toxicity in HR-deficient cells. ALC1 loss results in decreased chromatin accessibility, thereby impairing the loading of base excision repair (BER) factors. This results in the accumulation of replication associated unrepaired ssDNA gaps, increased PARP trapping and a critical dependence on HR repair. Therefore, targeting ALC1 alone or as a PARPi sensitizer could be specifically employed in HR deficient tumors.

AIMS AND OUTLINE

The genetic information in our cells has to be properly protected in order to allow its faithfull duplication prior to it being transferred to daughter cells during cell division. This process is challenged by the continuous induction of DNA damage by exogenous and endogenous sources. To circumvent adverse effects of DNA damage, cells activate a DNA damage response (DDR) that consist of pathways that detect and repair lesions, as well as pathways leading to the activation of checkpoints that delay cell cycle progression to allow for repair of DNA damage. Insight into the regulation of repair and signaling cascades, particularly those that counteract DSBs and RS, may improve our understanding of the DDRs and how their dysfunction causes devastating diseases such as neurodegenerative disorders and cancer [4].

In **Chapter 1** this thesis I provide an overview of DDR mechanisms with a focus on DSB repair and RS. This chapter describes the core factors involved in HR, cNHEJ, aNHEJ and SSA and how these mechanisms are key in repairing DSBs and preventing genome instability. Furthermore, it describes how DSB repair pathway choice is regulated in the context of chromatin. This is followed by a description of how DSB repair is linked with transcription regulation at sites of DSBs. Moreover, this chapter also describes mechanisms that help to preserve DNA replication fork integrity in response to RS, with a particular emphasis on the role transcription-replication conflicts therein. Finally, this chapter describes the link between these pathways and cancer, focus on their exploitation in cancer therapy.

In **Chapter 2**, I highlight the importance of zinc-finger domain-containing proteins in a diverse range of cellular processes, including DSB repair. ZnF-domains exist in 5% of all

human proteins and bind to a large variety of substrates such as DNA, RNA and several PTMs including PAR, ubiquitin and SUMO [28]. Importantly, ZnF-domain containing proteins have been shown to be important players in DSB repair, demonstrating their importance as caretakers of the genome and beyond.

In **Chapter 3**, I describe a new role of ZnF-protein ZNF384 in DSB repair. ZNF384 is a member of the C2H2 family of ZnF proteins which binds DNA ends *in vitro* and is recruited to DSBs *in vivo*. The accumulation of ZNF384 at DSBs requires PARP1-dependent chromatin expansion, which allows ZNF384 to bind to the exposed DNA via its C2H2 motifs. ZNF384 interacts with core NHEJ proteins Ku70/Ku80 via its N-terminus domain, thereby promoting Ku70/Ku80 assembly and the accrual of downstream factors including APLF and XRCC4/LIG4. In conclusion, this chapter highlights the role of ZNF384 as a 'Ku-adaptor' that binds broken DNA and Ku70/Ku80 to facilitate cNHEJ.

In **Chapter 4**, I focus on the molecular basis of the non-specific lethal (NSL) chromatin remodeling complex in R-loop homeostasis, and I specifically describe the role of the NSL complex member KANSL3 in this process [76]. KANSL3 protects cells from replication stress by promoting the restart of stalled replication forks, which may arise due to the formation of R-loop -induced TRCs. Indeed, KANSL3 has a suppressive role on R-loop formation, thereby preventing collisions between the replication fork and transcription machinery. Future work is required to gain insight into how KANSL3 affects R-loop formation and consequently replication fork stability/recovery following stress. Future work is required to gain mechanistic insight into how the dual role of KANSL3 protects genomic integrity.

In **Chapter 5**, I focus on the RNA processing enzyme ERI1. ERI1 is an evolutionary conserved 3'-5' exonuclease that participates in several RNA processing pathways, such as those in ribosomal RNA processing and the degradation of histone mRNAs. Here, I describe a novel function of ERI1 in suppressing R-loop formation and preserving replication fork stability. Together, my findings unveil a novel role of the RNA-binding and RNA-processing protein ERI1 in protecting cells form R-loop induced replication fork instability.

Finally, In **Chapter 6** I discuss how these findings contribute to our understanding of genome surveillance mechanisms, highlighting unresolved issues and making recommendations for follow-up studies.

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