Control of repair activities at DNA double strand breaks and telomeres

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GENERAL DISCUSSION

The DNA damage response and DNA repair

The integrity of the DNA in our cells is continuously threatened by various sources of damage. Especially DNA double strand breaks (DSBs), in which the two broken DNA ends are no longer attached, are dangerous if left unrepaired. While cellular integrity relies on efficient repair of damaged DNA, unwanted repair activities at natural chromosome ends need to be prevented. This is accounted for by telomeres. Telomeres consist of long stretches of repetitive DNA sequences, bound by a highly specialized protein complex, shelterin. This together protects chromosome ends from being recognized as damaged DNA. When chromosomes lose the protective function of telomeres, or when a cell encounters a DNA double strand break, a DNA damage response (DDR) is activated. Interestingly, this response is highly similar at chromosome internal DSBs and at deprotected telomeres. The initial damage recognition relies on sensor proteins, and this is followed by activation of DNA damage kinases. The signal is then further amplified and a large set of effector proteins is activated. Ultimately, DNA repair proteins are activated and recruited in order to resolve the damage. With the research described in this thesis we aimed to increase our mechanistic understanding of the DNA damage response and DNA repair, both at DNA DSBs and at deprotected telomeres. In order to do so, we set out to identify novel factors that play a role in DNA repair, and studied their function in the repair of broken DNA. First, we focused on the role of chromatin in the joining of broken DNA (**Chapter 2 to Chapter 4**). Second, we aimed to increase our mechanistic understanding of the control of DNA end-resection and DNA repair pathway choice (**Chapter 5 to Chapter 8**). Importantly, these two parts are closely connected; one way in which chromatin status can impact on DNA repair pathway choice is illustrated by the work described in **Chapter 4**.

I. Chromatin mediated control of DNA end-joining

Chromatin changes impact on the efficiency of NHEJ at deprotected telomeres

The DNA in cells is packed around histone proteins, and further compacted as chromatin. Chromatin by itself has a dynamic nature, it can be highly compacted or more relaxed and accessible. This plasticity of the chromatin is affected by many different factors, such as whether or not a region is actively transcribed. The chromatin is subjected to various modifications that can impact on biological processes involving DNA. This includes DNA methylation, post-translation modifications (PTMs) of histones, and the incorporation of histone variants. Also in the response to DNA DSBs, the chromatin surrounding the damage plays an important role. Indeed, many ongoing efforts are aimed at understanding how DNA repair is regulated at different locations throughout the genome, and by different chromatin modifications. We found that the efficiency of NHEJ at deprotected telomeres is dependent on H3K36me2 mediated by the methyltransferase MMSET (**Chapter 2,** Figure 1a) as well as on the chromatin remodeler CHD2 and incorporation of the H3.3 histone variant (**Chapter 3,** Figure 1b). Importantly, the cellular depletion of a histone modifying enzyme could have widespread effects. This is illustrated by MMSET (**Chapter 2**). The primary catalytic activity of MMSET is

directed towards H3K36, which it can mono- and di-methylate (H3K36me1/me2)[1]. Although trimethylation of H3K36 (H3K36me3) is exclusively performed by SETD2, the depletion of MMSET thereby likely also reduces the substrate for H3K36me3 (**Chapter 2**)[2,3]. H3K36me2 marks active chromatin, and dysregulation of H3K36me2 distribution, for instance by overexpressing MMSET, leads to changes in gene expression[1]. Indeed, in various studies, MMSET has been linked to both transcriptional activation as well as transcriptional repression[1,3,4]. PTMs at histones can also function as a platform to recruit proteins involved in DNA repair, illustrated by 53BP1 that can directly bind H4K20me2, further described below[5]. For H3K36me2 it seems that NBS1 can directly bind[6]. Finally, MMSET also interacts with various proteins, such as HDAC1 and HDAC2, and can methylate non-histone substrates such as PTEN[3,4,7,8]. MMSET can thereby directly (through H3K36me1/2), or indirectly (though its protein-interactions, or through affecting H3K36me3) affect gene expression, or change chromatin states independently of gene expression regulation. Furthermore, MMSET can impact on various processes through affecting non-histone substrates. The mechanism by which MMSET affects NHEJ-mediated repair of deprotected telomeres can therefore be a combination of multiple independent effects. We found that MMSET affects DNA repair at deprotected telomeres through its ability to promote H3K36me2. Furthermore, we have found that MMSET-dependent H3K36me2 does not impact on the recognition of deprotected telomere ends as damaged DNA nor affects NHEJ at the level of DNA end-resection (**Chapter 2**). Any effect, either directly, or indirectly, mediated by MMSET-dependent H3K36me2 is therefore likely further downstream in the DNA repair cascade.

Also CHD2 and H3.3 are important for efficient telomere-NHEJ (**Chapter 3**). Importantly, similar to the loss of MMSET, the depletion of CHD2 or H3.3 does not impact on the initial damage signaling by γH2AX. Indeed, CHD2 and H3.3 eventually impact on the assembly of downstream NHEJ-factors Ku and XRCC4 at damaged chromatin. Whether the loss of MMSET also reduces Ku and XRCC4 recruitment to either DSBs or deprotected telomeres will be interesting to further study. Whether there is crosstalk among the mechanisms by which MMSET, CHD2 and H3.3 incorporation facilitate efficient NHEJ would also be interesting to further explore. H3.3 is associated with sites of active chromatin as well as with sites that are transcriptionally silent, such as telomeres[9]. H3.3 contributes to the telomeric H3.3K9 mark, and thereby contributes to a heterochromatic state at telomeres[10]. It will be interesting to know if H3.3 could also be a substrate for global or (sub)telomeric H3.3K36me2, potentially mediated by MMSET[10]. This seems feasible, as it was already shown that H3.3 can contribute to H3.3K36me2 and H3.3K36me3[11]. Furthermore, it was shown that a H3.3K36M mutation, in which H3.3K36 can no longer be methylated, inhibits MMSET and reduces H3K36me2/3 levels genome wide[11]. The expression of H3.3K36M thereby affected gene expression, and was found to reduce expression of ATR and BRCA1 DNA repair genes[11]. Although we did not observe an effect on BRCA1 expression or recruitment to deprotected telomeres in the absence of MMSET (**Chapter 2**) it is possible that H3.3 incorporation differentially impacts DNA repair depending on the genomic location, whether this is in telomeres or in active genes.

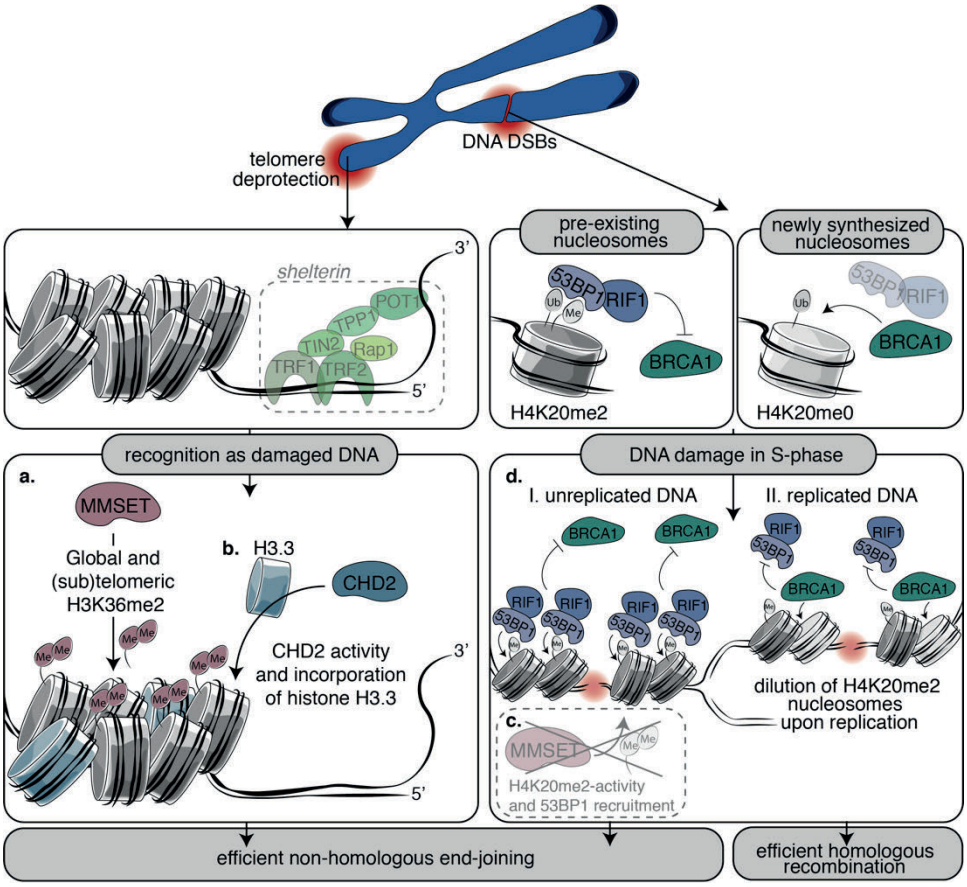

Figure 1. Chromatin changes impact on DNA repair efficiency and DNA repair pathway choice. a. MMSET is important for efficient NHEJ at uncapped telomeres through promoting H3K36me2, both globally and at the (sub)telomeres. MMSET is not involved in the initial recognition of deprotected telomeres as damaged DNA. MMSET-dependent H3K36me2 therefore likely affects DNA repair steps that act further downstream. **b.** CHD2 and H3.3 contribute to efficient NHEJ of deprotected telomeres. CHD2 is a chromatin remodeler and was found to incorporate H3.3 at sites of damage. This thereby promotes the recruitment of downstream DNA repair factors such as XRCC4 towards the damage. A similar mechanism may be at play at deprotected telomeres. **c.** In line with previous research we do not observe an effect of MMSET on 53BP1 recruitment towards sites of damage. **d.** During S-phase, unreplicated (I) and replicated DNA (II) are present at the same time. The incorporation of newly synthesized and therefore unmodified nucleosomes into newly replicated DNA dilutes the presence of H4K20me2. This thereby reduces the affinity of 53BP1 towards the chromatin. By doing so, BRCA1 can more efficiently be recruited and DNA repair is steered towards HR. At unreplicated DNA, high levels of H4K20me2 will promote repair through NHEJ.

H4K20me2: SET a mark for 53BP1

53BP1 plays a central role in promoting DNA repair through NHEJ by inhibiting DNA end-resection. 53BP1 is recruited to the chromatin surrounding a DSB where it directly binds to two defined chromatin marks. First, 53BP1 interacts with H4K20me2 through its tandem tudor domains[5]. Second, 53BP1 binds to ubiquitylated H2A (H2AK15ub), a DNA damage-dependent mark put on by the RNF8/RNF168 signaling cascade[12]. H4K20me2 is widely spread, and pre-exists on >80% of the nucleosomes[13]. Whether H4K20me2 levels would change upon damage,

or how changes in H4K20me2 contribute to 53BP1 binding and DNA repair pathway choice throughout the cell cycle was largely unclear. Interestingly, MMSET was initially identified to facilitate the recruitment of 53BP1 to DNA damage sites by promoting a local accumulation of H4K20me2 (Figure 1c)[14,15]. However, it was subsequently shown that in various cell lines 53BP1 recruitment appeared unperturbed in the absence of MMSET[16,17]. Moreover, the catalytic activity of MMSET was found directed towards H3K36me1 and H3K36me2, rather than H4K20me2[1,18]. Also we did not find that MMSET impacts on the recruitment of 53BP1 to deprotected telomeres and did not observe an effect on H4K20me2-levels in the absence of MMSET (**Chapter 2**, Figure 1c). Indeed, H4K20-methylation is mediated by SETD8, SUV4-20H1 and SUV4-20H2 methyltransferases[19].

Interestingly, the fluctuation of H4K20me2 levels throughout the cell cycle appeared important in directing either 53BP1 or BRCA1 binding towards the chromatin (**Chapter 4**, Figure 1d). Especially in the S-phase of the cell cycle, the presence of both un-replicated and replicated DNA requires both NHEJ and HR to be employed at the same time while ensuring that their activities are restricted to the correct genomic location. The binding of 53BP1 needs to be controlled so that DSBs at non-replicated DNA are efficiently recognized and repaired through NHEJ. Conversely, at replicated DNA, repair through HR is preferred. The H4K20me2 mark seems instrumental in directing this difference; newly synthesized nucleosomes that are incorporated into the replicated DNA are unmodified (H4K20me0) and thereby dilute the abundance of H4K20me2 (**Chapter 4**)[20]. This limits the accumulation of 53BP1 on chromatin, and thereby allows BRCA1 and the HR machinery to act specifically at replicated DNA. In addition to the dilution of H4K20me2 upon replication, additional mechanisms are in place to regulate 53BP1 recruitment towards the chromatin. 53BP1 binding is negatively regulated by acetylation at H4K16 by TIP60, which reduces the binding of 53BP1 with H4K20me2 and thereby contributes to the antagonistic recruitment of 53BP1 versus BRCA1[17,21]. In addition, L3MBTL1 and JMJD2A/KDM4A mask H4K20me2 in the absence of DNA damage and get displaced upon damage induction[22-24]. This thereby prevents 53BP1 binding to undamaged chromatin that inherently contains high H4K20me2 levels prior to S-phase. Interestingly, 53BP1 forms a dimer which is promoted by DYNLL1 and that is required for its accumulation at DNA damage sites[25,26]. Whether the dilution of H4K20me2 also impacts on the ability of 53BP1 to form a stable dimer onto the chromatin would be interesting to address. Furthermore, it was recently shown that 53BP1 can form a large protein compartment through phase separation. These 53BP1 compartments thereby can function as a scaffold to recruit additional activities or proteins[27]. Dilution of H4K20me2-presence could therefore potentially also indirectly affect the ability of 53BP1 to efficiently form these droplet-like clusters.

Altogether, a complex picture emerges of many different factors and mechanisms that contribute to the affinity of 53BP1 for chromatin. Whether these mechanisms also impact 53BP1 recruitment towards deprotected telomeres in S-phase is unclear and would be interesting to study. The recognition of deprotected telomeres as damaged DNA by γH2AX is equal among G1 and S/G2-phase of the cell cycle[28]. The subsequent repair of deprotected telomeres upon TRF2 loss gives rise to chromosome-type fusions through NHEJ which rely on 53BP1[29]. These fusions occur predominantly in G1 and are repressed in S/G2-phase of the cell

cycle by various mechanisms[28]. In S/G2, NHEJ induced by TRF2-loss is suppressed by CYREN which thereby protects from chromatid-type fusions that would arise by NHEJ taking place after the DNA has been replicated[30]. Also CDK-activity inhibits NHEJ at deprotected telomeres in S/G2, as this would otherwise result in sister-fusions[28]. Whether reduced 53BP1 binding through regulation of H4K20me2-occupancy or availability at telomere-ends also contributes to protection from NHEJ in S/G2 will be interesting to further address.

II. Shieldin mediated control of DNA end-joining

MAD2L2 controls end-resection as a member of the shieldin complex

The control of DNA end-resection is a central factor in the decision point among different DNA repair pathways. Long-range end-resection to expose sequence homology is required for HR, but will severely impair NHEJ. Importantly, a large portion of DNA DSBs rely on NHEJ for their repair[31]. Engaging into end-resection at the wrong time, for instance in G1 when the sister-chromatid is not directly available, will result in the activation of alternative repair pathways such as microhomology mediated repair (MMEJ) or single-strand annealing (SSA)[32]. DNA repair through these alternative DNA repair pathways can lead to deletions or translocations and thereby impair genome integrity[32]. DNA end-resection is therefore tightly controlled through various cellular mechanisms, by ensuring that end-resection promoting activities are not yet activated in G1, as well as by recruiting protein complexes to sites of damage that inhibit end-resection. The latter is accounted for by the recruitment of 53BP1 to the chromatin surrounding the sites of DSBs together with its downstream effector RIF1. MAD2L2, also known as REV7, was identified to function downstream of 53BP1 and RIF1 in inhibiting DNA end-resection at DSBs and at uncapped telomeres[33,34]. Although MAD2L2 was described to interact with a variety of different proteins and function in various biological processes (**Chapter 5**), how this was connected to its role in inhibiting DNA end-resection was not immediately clear (**Chapter 6**). We therefore set out to identify novel MAD2L2 interactors using mass spectrometry approaches, and found two interactions that stood out: FAM35A (**Chapter 6, 7**), and TRIP13 (**Chapter 8**). In parallel, the loss of FAM35A, as well as a second novel factor called C20orf196, was found to promote resistance to PARP-inhibition in BRCA1-deficient cells (**Chapter 7**). FAM35A and C20orf196 loss therefore mimics MAD2L2-deficiency, and both appeared to function in the same pathway as MAD2L2, inhibiting end-resection. Indeed, we found that FAM35A and C20orf196 contribute to NHEJ at deprotected telomeres and inhibit end-resection (**Chapter 7**). Multiple parallel research efforts also identified a third novel protein, CTC-534A2.2, which functions upstream of MAD2L2 by bridging RIF1 and MAD2L2[35-37]. The complex is recruited to sites of damage, downstream of 53BP1 and RIF1, and functions to inhibit end-resection. Due to its ability to 'shield' the DNA ends from getting resected, this novel protein complex was called 'shieldin'. Except for MAD2L2, its individual components were renamed as SHLD1 (C20orf196), SHLD2 (FAM35A) and SHLD3 (CTC-534A2.2), as their participation in shieldin was the first characterized role for these 3 proteins[38].

Counteracting resection through shieldin

Two models have been proposed as to how shieldin counteracts DNA end-resection at DSBs (Figure 2a, b). First, the presence of shieldin could prevent nucleases from performing end-resection. The binding of shieldin could directly hide the free DNA-end from nucleases or through steric hinderance prevent access to nucleases (Figure 2a). Second, shieldin could recruit additional proteins containing enzymatic activity to actively counteract resection (Figure 2b). The finding that the CST complex is involved in PARPi-resistance downstream of shieldin (**Addendum I**)[39] suggests such a mechanism might be at play. CST is known to recruit the activity of Polα/Primase to telomeres in order to counteract over-resection of telomere ends. Importantly, the CST complex was found to bind to ssDNA with various different sequence compositions with relatively equal efficiency[40]. Furthermore, CST has been implicated in resolving replication stress genome-wide and in resolving G4-structures[41,42]. This is in line with a more general role in the DDR, rather than only binding telomeric sequences. At the damage sites, the recruitment and activity of shieldin/CST/Polα/Primase could promote fill-in synthesis, thereby shortening ssDNA overhangs, and promote DNA end-ligation through NHEJ. Indeed, it was found that toxic end-joining in BRCA1-deficient cells relies on the activity of

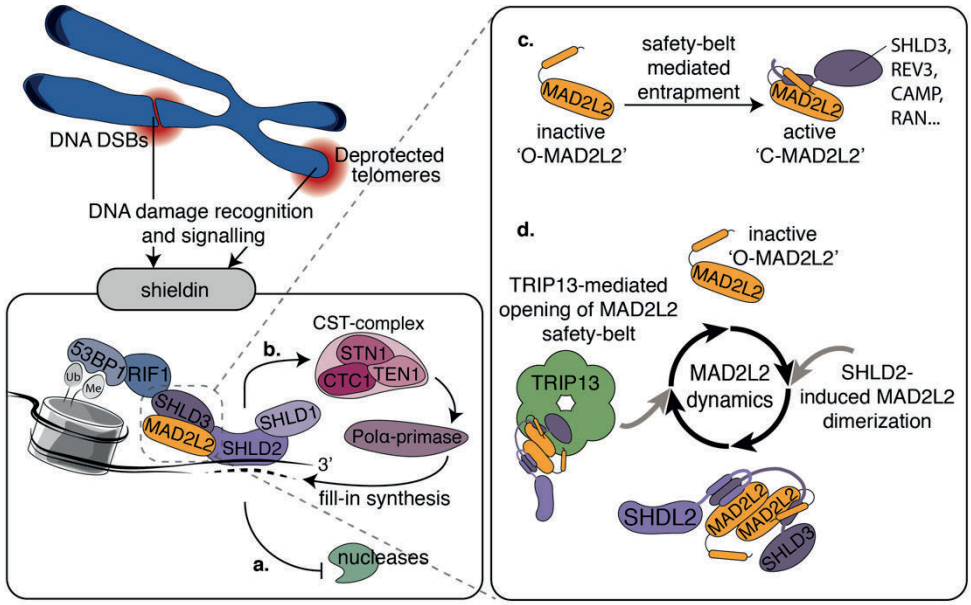

Figure 2. MAD2L2 inhibits 5' end resection as part of the shieldin complex. a. MAD2L2 is recruited towards DSBs downstream of 53BP1/RIF1 as a member of the shieldin complex. By binding to DSBs, shieldin might prevent nucleases from access and thereby protect from further resection. **b.** The shieldin complex interacts with the CST complex and recruits CST to sites of damage. Together with Polα/Primase, the CST complex may counteract resection by performing fill-in synthesis. **c.** MAD2L2 interacts with different proteins through a safety-belt mechanism, in which it entraps interaction partners through its C-terminus. This C-terminal portion of MAD2L2 can be in an 'open' state ('O-MAD2L2') and is closed ('C-MAD2L2') when it entraps a partner protein. **d.** MAD2L2 structural features are important in shieldin complex (dis)assembly. Within the shieldin complex, MAD2L2 interacts with SHLD3 through its safety-belt. Next, SHLD2 induces the dimerization of MAD2L2, and this thereby forms a complex consisting of 1 SHLD3 protein, 1 SHLD2 protein, and a dimer of MAD2L2. This complex interacts with TRIP13, an AAA+ ATPase that facilitates the opening of the MAD2L2 safety-belt. TRIP13 is therefore important for the disassembly of the shieldin complex. This might also be important for regulating the pool of 'O-MAD2L2' that can engage in different protein complexes.

Polα/Primase[39]. So rather than 53BP1, RIF1 and shieldin passively inhibiting any end-resection from happening, the involvement of the CST complex suggest that end-resection is actively counteracted through fill-in synthesis.

Interestingly, the involvement of fill-in activity implies that ssDNA is already present at the site of damage. This is in line with the notion that the SHLD2 member of the shieldin complex binds ssDNA, and that the ability of SHLD2 to bind ssDNA is essential in shieldin recruitment to sites of damage[37,43,44]. *In vitro*, SHLD2 binds ssDNA substrates that are relatively long (60nt), but not substrates that are shorter (30nt)[44]. This does not only suggest that ssDNA is needed, but also implies that shieldin is required at breaks that already have quite a substantial amount of ssDNA-overhang. The CST complex has also been shown to bind ssDNA, but not dsDNA, and requires the amount of ssDNA to be 32nt or longer in order to efficiently bind[40]. Moreover, the CST complex was found to bind ssDNA-dsDNA junctions[41]. The CST complex is tethered to shieldin, and the presence of shieldin is thereby essential for CST recruitment to DSBs[39]. However, whether the ability of CST to directly bind ssDNA is important for the recruitment or function of CST/Polα/Primase at DSBs is currently unclear and will be important to uncover. Irrespective of whether CST itself also needs to bind ssDNA, the preference of SHLD2 for long ssDNA substrates suggests that the amount of fill-in synthesis performed by CST is relatively long. Since the processivity of Polα/Primase seems to be rather short, it has been suggested that other polymerases could further aid in fill-in synthesis[45,46]. Interestingly, it was recently shown that REV3, that together with MAD2L2 forms Polζ (**Chapter 5)**, can interact with SHLD3 and localize to sites of damage[46]. REV3 has a well described role in translesion synthesis as Polζ but also has been implicated in HR as well as in NHEJ. Indeed, similar to MAD2L2, REV3-loss impairs class switch recombination (CSR), a biological process that relies on NHEJ[33,34,47]. During CSR, the DNA breaks that are generated contain a ssDNA overhang. This overhang requires processing prior to end-ligation, and Polζ was suggested to contribute to the fill-in reaction[47]. However, unlike MAD2L2, the depletion of REV3 does not enhance ssDNA formation around CSR break-sites and does not render BRCA1-deficient cells resistant to PARP1-inhibition[34]. Moreover, MAD2L2-loss could still further enhance end-resection in a REV3-deficient background, indicating that MAD2L2 controls end-resection in a REV3-independent manner[33]. Together this indicates that MAD2L2 and REV3 might act at different levels in controlling NHEJ-efficiency. Whether Polζ can function in concert with other polymerases to facilitate fill-in synthesis therefore is an interesting possibility that needs to be further explored. This is also interesting considering the role of MAD2L2, as it would suggest that MAD2L2 functions in two distinct protein complexes during NHEJ-repair, shieldin and Polζ. How this would be regulated at the level of MAD2L2 (perhaps involving TRIP13, **chapter 8** and further discussed below), would also be interesting to study.

Also, it is unclear whether it is actually necessary to completely fill in resected ends, or if a certain amount of overhang can still be present at the damage site. NHEJ can only take place at DSBs that have an overhang of 4nt or less[48]. However, deprotected telomeres, eventually also repaired by NHEJ, already contain a long 3' overhang. This single-stranded 3'-overhang is retained in ligase4-deficient cells in which NHEJ is severely impaired[49]. At unprotected telomeres, the ssDNA-overhang therefore seems to be removed during, rather

than prior to, the actual end-ligation. It is therefore unclear why NHEJ would actually be inhibited by extending the 3' overhang at deprotected telomere ends[50]. Perhaps inhibiting resection at telomere ends protects from the loss of telomeric repeats. Altogether, it could also be possible that in addition to protecting from further overhang extension, and performing a certain amount of fill-in synthesis, shieldin would facilitate the recruitment of additional end-processing enzymes and NHEJ proteins.

Single stranded binding modules in repair through NHEJ

The underlying DNA substrate at a DSB seems important in determining what repair complexes are required. This is suggested to be the case in the regulation of two biological processes that rely on DNA end-joining through NHEJ, CSR and long-range V(D)J recombination. While both are controlled through 53BP1-mediated NHEJ, only CSR, and not V(D)J recombination, requires the activity of MAD2L2[36]. This likely reflects the underlying DNA substrate; in CSR the DNA ends contain ssDNA, while the DNA ends that require ligation in V(D)J recombination do not. The presence of ssDNA thereby seems to dictate whether shieldin binding ssDNA and CST/Polα/Primase promoting fill-in synthesis is required[36]. Also in the ligation of deprotected telomere-ends the activity of shieldin and the CST complex (and thereby potentially Polα/Primase) is required (**Chapter 7**, **Addendum I**)[33,35,39,43,51], which is in line with the single-stranded 3' G-rich overhang present at telomere-ends.

Interestingly, three different ssDNA-binding protein complexes now seem involved in binding ssDNA at DSBs; RPA, shieldin and CST. The presence of ssDNA, for instance at stalled replication forks, is rapidly detected and coated by RPA which thereby functions as a sensor, and activates ATR. Furthermore, RPA can get phosphorylated by ATM, ATR and DNA-PKcs, which eventually can also restrict further end-resection[52,53]. It will be interesting to further study how these three ssDNA-binding complexes determine who binds when and whether there is active competition ongoing. Indeed, loss of either CST or shieldin leads to increased ssDNA bound by RPA, potentially previously occupied by either or both complexes, and ATR activation[33,34,38,54]. Interestingly, it seems that the SHLD2 and SHLD1 components of shieldin are very lowly abundant[35]. RPA is rate limiting in the cell, up to a point that additional formation of ssDNA will no longer be bound by RPA[55]. Also the cellular abundance of the CST complex seems to affect the response to replication stress, such that cells lacking CST are more sensitive, while cells overexpressing CST are more resistant to replication stress-inducing drugs[42]. It is therefore also interesting to consider the relative abundance of these different ssDNA binding protein complexes and how their abundance would impact on DNA repair. Interestingly, both RPA and CST have been described to associate with and dissociate from ssDNA in a dynamic manner[41]. This dynamic exchange could allow for the subsequent recruitment of repair proteins, or could facilitate their relocation towards potential other sites of damage within the cell.

Regulation of shieldin assembly

How the recruitment or potential activity of the shieldin complex and its effectors is regulated towards different breaks and in space and time is an important question. We found that at least part of shieldin regulation is taken care of by TRIP13 (**Chapter 8**). Also specialized structural

features of MAD2L2 appear to play an essential role in shieldin composition and function (**Chapter 8**). Within the shieldin complex, MAD2L2 directly interacts with SHLD3, as MAD2L2 wraps its C-terminal safety belt structure around a REV7 (MAD2L2)-binding motif (RBM) on SHLD3 (Figure 2c)[36,46,56]. Together MAD2L2 and SHLD3 then interact with SHLD2, which in turn interacts with SHLD1. We found that in order to interact with SHLD2 (and thereby SHLD1), MAD2L2 needs to form a dimer. Importantly, this dimerization is not only important for shieldin complex assembly, but also for the interaction of shieldin with TRIP13 (**Chapter 8**). TRIP13 in turn seems important in regulating shieldin stability and assembly. TRIP13 functions by promoting the opening of the safety belt of HORMA-domain containing proteins, which include MAD2 and MAD2L2 (**Chapter 5**, Figure 2d)[57,58]. By doing so, it enables the release of the interacting protein. This indeed seems to be the case in shieldin complex formation. In shieldin, TRIP13 acts towards MAD2L2, mediating release of SHLD3 and thereby dissociation of the entire shieldin complex[57]. Through promoting the disassembly of shieldin, TRIP13 might ensure that the shieldin complex dissociates from the break-site when repair is completed or when fill-in synthesis is sufficiently promoted by CST/Polα/Primase activity. The precise signal that determines when TRIP13 is required to disassemble the shieldin complex is interesting to further explore. *In vitro*, the presence of MAD2L2 forming a dimer, together with the presence of full-length SHLD3 is sufficient for the interaction with TRIP13 (**Chapter 8**). It is therefore possible that there is ongoing activity of TRIP13 towards shieldin, that thereby facilitates continuous disassembly of the shieldin complex. Conversely, shieldin assembly does not require a DNA damage signal, and shieldin proteins thereby seem to form a stable complex[35-37,43,59]. A cycle of (spontaneous) assembly and (TRIP13-mediated) disassembly could facilitate shieldin binding and releasing its ssDNA substrate at the DSB. Eventually, this might allow that shieldin gets outcompeted by other ssDNA binding complexes, or loses its binding affinity as a result of a reduction in ssDNA to bind. However, this does not exclude that additional signals are necessary to direct TRIP13 activity towards facilitating the disassembly of shieldin. This could include PTMs on shieldin components or on TRIP13 itself, or the presence of additional proteins that signal for termination of shieldin binding and activity. It would also be interesting to study how TRIP13 levels or activity are regulated throughout the cell cycle. It is likely that a stable shieldin complex is most important in G1 phase when cells rely on efficient NHEJ, while in S/G2 phase repair through HR is preferred and 53BP1/RIF1/shieldin binding to DSBs is disfavoured (**Chapter 4**). Active disassembly of shieldin by TRIP13 might therefore be predominant in S/G2-phase of the cell cycle. It is also unclear if the disassembly of shieldin is regulated at the site of damage, or whether reduced ssDNA binding substrate and thereby affinity would passively dissociate the entire shieldin complex from the site of damage, followed by disassembly by TRIP13 outside of the DNA break. The finding that in the absence of TRIP13, SHLD2, SHLD3 and MAD2L2 are more stable at sites of damage could suggest that the disassembly takes place at the break-site, but could also simply be indicative of more stable shieldin complexes being present in the nucleus to respond to the damage (**Chapter 8**)[57]. Finally, the regulation of MAD2L2 through TRIP13-mediated opening of the MAD2L2 safety belt could be essential in regulating the cellular pool of 'open-MAD2L2' that is needed to engage MAD2L2 in different protein complexes. MAD2L2 interacts with multiple proteins through its

safety belt, including SHLD3 and REV3, but also CHAMP1, RAN and IpaB (**Chapter 5,** Figure 2c). The active cycling of MAD2L2 between an 'open' and 'closed' protein bound state facilitated by TRIP13 safety belt opening could be instrumental in MAD2L2 interacting with its different partner proteins and thereby mediating various cellular responses.

Shieldin and shelterin protect DNA ends

With the discovery of the shieldin complex, cells appear to have two distinct protein complexes that are important in protecting DNA ends: the shelterin complex specifically shields natural chromosome ends at telomeres from getting recognized by the DDR-machinery and processed by DNA repair activities, and the shieldin complex shields broken DNA ends or shelterin-free telomeric DNA ends from getting resected (Figure 3). Interestingly, both shieldin and shelterin seem to bind combined structures of ssDNA and dsDNA to fully elicit their function. Shieldin requires both upstream binding by 53BP1/RIF1, connecting it to dsDNA through PTMs on histone proteins (**Chapter 4**), as well as ssDNA binding by the SHLD2-component (**Chapter 7**). For shelterin, TRF2 and TRF1 bind dsDNA with high affinity, while the POT1-component binds to ssDNA[60]. Indeed, SHLD2 contains OB-folds at its C-terminus and thereby resembles the ssDNA binding proteins RPA and POT1[37,44]. Also the CST complex members contain such OB-folds and CST was shown to bind ssDNA-dsDNA junctions[41]. We hypothesize that in addition to their protective function, both shelterin and shieldin function as central hubs, recruiting additional proteins and activities at the different DNA ends. For shelterin, this includes interactions with DNA repair proteins such as DNAPK/Ku and ERCC1/XPF that either interact with TRF1, TRF2 or generally associate with shelterin[61]. At least for some of the proteins associated with shelterin it is thought that they have a structural function and contribute to telomere maintenance[61]. Also the shieldin complex recruits additional proteins harbouring activities. The shieldin complex recruits CST/Polα/Primase to facilitate fill-in DNA synthesis and recruits TRIP13 to mediate its (dis)assembly.

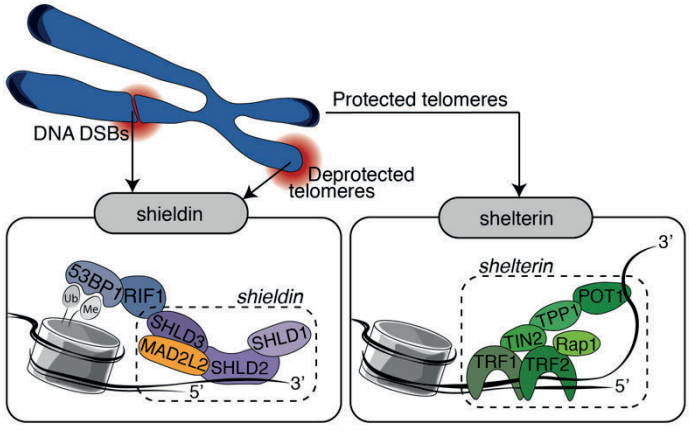

Figure 3. Shieldin and shelterin protect DNA ends. Two distinct protein complexes shield DNA ends. The shelterin complex (right) shields the ends of natural chromosomes and prevents the telomeres from getting recognized as damaged DNA. A second protein complex, shieldin (left), is recruited towards DNA DSBs and deprotected telomeres. At sites of damage, the shieldin complex protects the DNA ends from getting extensively resected.

Furthermore, SHLD3 can interact with REV3 although it is unclear if this happens in conjunction with the entire shieldin complex, and how this is regulated around MAD2L2 forming a dimer together with SHLD3[46]. As described above, it is also possible that additional repair proteins are recruited to DSBs via shieldin. Further research might uncover additional protein-protein interactions mediated by the shieldin complex.

III. Outlook: Control of DNA end-joining

Better understanding of chromatin- and shieldin-mediated DNA end-joining is important to increase our fundamental biological knowledge on cellular processes, such as repair of damaged DNA, CSR and V(D)J, but is also important from a clinical perspective. MMSET is found dysregulated in cancer, and is upregulated in multiple myeloma due to a t(4;14)+ translocation. While MMSET-deficiency impairs NHEJ, we found that its overexpression actually enhances NHEJ (**Chapter 2**). This implies that the cellular levels of MMSET need to be tightly regulated in order to properly control DNA repair efficiency. This is important in the response to various therapeutic agents used for cancer treatment. Often, treatment strategies rely on inflicting DNA damage that is better tolerated by non-cancerous cells compared to fast growing cancer cells. It was found that the upregulation of MMSET, possibly through facilitating efficient NHEJ, renders cells resistant to DNA-damage inducing therapeutic agents[62]. Also, we found that cells overexpressing MMSET are more resistant to irradiation (**Chapter 2**). Understanding the molecular basis of cancer development thereby uncovers potential tumor vulnerabilities, but can also identify what treatment strategies will be less beneficial, or if a combination of treatments is required. Conversely, the search for factors that render BRCA1-deficient tumors resistant to PARP-inhibitors has greatly advanced the molecular understanding on DNA repair pathway choice (**Chapter 7, Addendum I, Chapter 8**). Future research will shed further light on how the many different proteins involved in DNA repair coordinate their actions at the right time and place to properly direct DNA repair.

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