Mechanisms underlying mutational outcomes of DNA double-strand break repair

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CHAPTER 5

Mechanisms underlying mutational outcomes of DNA double-strand break repair

This thesis addresses the repair of DNA double-strand breaks (DSBs) that arise in different contexts, both artificially inflicted DNA damage and spontaneously arising breaks. When worms are grown for multiple generations without inflicting DSBs artificially, we observe accumulation of DNA repair footprints after whole genome sequencing (Kamp, van Schendel et al. 2020). We call these type of experiments mutation accumulation (MA) experiments. The major type of mutations we identify in MA experiments are base substitutions: change of 1 nucleotide without the loss or addition of other nucleotides (for example, an A is changed to a G in the DNA sequence). The loss of DNA sequences (deletions) is low in wild-type worms: about 1 deletion arises in 30 generations of worms.

BRCAness in worms

In contrast to the low deletion rate in wild-type worms, worms that lack the orthologue of BRCA1 (encoded by *brc-1*) or its binding partner BARD1 (encoded by *brd-1*), accumulate a deletion every 3-4 generations. Moreover, they accumulate base substitutions at an increased rate and sometimes obtain a rare form of structural variation: tandem duplications. A tandem duplication is a stretch of DNA that is doubled without loss of nucleotides, and this copied stretch is placed immediately adjacent to the original DNA. The mutations in genomes of *brc-1* and *brd-1* worms were very similar to mutations observed in BRCA1 deficient human tumors: small deletions with an overrepresentation of homology usage, increased base substitutions and tandem duplications with a median size of 11 kb (Nik-Zainal, Davies et al. 2016, Davies, Glodzik et al. 2017, Menghi, Barthel et al. 2018, Koh, Degasperi et al. 2021). The mutational spectrum in BRCA1-deficient context is called BRCAness. Because of the similarities between the mutations in BRC-1/BRCA1 deficient worms and tumors, we hypothesized a pathway that is conserved from worm to man was responsible for the generation of these mutations.

Because BRC-1 and BRD-1 play a crucial role in DSB repair pathway homologous recombination (HR, Figure 1), mutating the genes encoding these proteins leads to impaired DSB repair. Repair of DSBs via HR usually results in the restoration of the original DNA sequence: it usually does not result in deletions or insertions. The increase in deletions suggested that DSB repair was performed in a more error prone fashion than HR. The best known error prone DSB repair pathway is non-homologous end-joining (NHEJ, Figure 1). However, NHEJ acts on breaks in different contexts than HR: HR repairs breaks in cells that are replicating their DNA or have just finished replication before cell division. The break ends are resected: single-strand overhangs are needed for invasion of the sister chromatid (or homologous chromosome) to copy the DNA needed to repair the break. NHEJ is mainly active in cells that are not replicating their DNA and does not act on these resected break ends. It has however been shown that a protein called 53BP1 is a regulator of break end processing (Bunting, Callén et al. 2010). 53BP1 can

keep the break ends blunt instead of single-stranded, and thereby stimulates repair of DSBs using NHEJ instead of HR. BRCA1 counteracts 53BP1 at break sites (Densham, Garvin et al. 2016), therefore, it could be hypothesized that in the absence of BRCA1/BRC-1, 53BP1 steers break repair to NHEJ instead of HR. A potential orthologue of 53BP1 has been identified in *C. elegans* (Ryu, Kang et al. 2013), but its role in break end maintenance has not been confirmed yet. Interestingly, loss of 53BP1 rescues embryonic lethality but not the HR defect of BRCA1 total knockout mice (Chen, Li et al. 2020). The lack of a proper 53BP1 orthologue could be an explanation for the fact that BRCA1 deficiency does not lead to lethality in *C. elegans*.

We wondered whether NHEJ would be responsible for the structural variations we found in *brc-1* and *brd-1* worms. To investigate this, we repeated the MA experiments with *brc-1* and *brd-1* mutants, but this time with animals that were also deficient for NHEJ, by knocking out the core NHEJ genes *lig-4* and *cku-80*. After whole genome sequencing of the genomes of the double mutants, we observed that mutations accumulated at a similar rate as in *brc-1* and *brd-1* worms, showing that NHEJ did not affect mutation formation in *brc-1* and *brd-1* deficient worms. While we did not see any effect of NHEJ on the mutation rate, this does not mean that BRC-1/BRD-1 substrates are never repaired *via* NHEJ. It is possible that some DSBs are repaired *via* NHEJ in an error-free fashion. It was recently shown that polymerase alpha can fill in 3' overhangs to enable blunt ligation of breaks by NHEJ (Schimmel, Muñoz-Subirana et al. 2021). This could lead to error free repair when the original break was a blunt break that was resected. However, the Shieldin complex, which is necessary for polymerase alpha recruitment to break ends (Schimmel, Muñoz-Subirana et al. 2021), is not conserved in *C. elegans* (Setiaputra and Durocher 2019). It is therefore unclear whether polymerase alpha is also recruited to resected break ends in worms.

The deletions observed in *brc-1* worms and BRCA1 deficient tumors are comparable to the mutations generated by polymerase theta-mediated end-joining (TMEJ, Figure 1). Similar to TMEJ footprints in cultured mammalian cells (Schimmel, Kool et al. 2017), the deletions in cancer genomes showed significant homology usage (Koh, Degasperi et al. 2021). In order to investigate the role of TMEJ in the formation of these type of deletions, we knocked out polymerase theta, the core TMEJ player, in *brc-1* and *brd-1* worms. We observed that knocking out polymerase theta (by mutation of the *polq-1* gene) in *brc-1* and *brd-1* worms prevented the accumulation of structural variations (deletions and tandem duplications), showing that polymerase theta plays a crucial role in the formation of these mutations and is an important driver of BRCAness (Kamp, van Schendel et al. 2020). This is also reflected in the viability and IR sensitivity of *brc-1 polq-1* mutants compared to *brc-1* single mutants (Kamp, van Schendel et al. 2020). The dependence on polymerase theta is also shown in tumors: HR deficient epithelial ovarian cancers depend on TMEJ for their survival (Ceccaldi, Liu et al. 2015). Of note, not only

BRCA1 deficient cells rely on polymerase theta, and the need for polymerase theta is associated with high levels of replication associated breaks (Feng, Simpson et al. 2019). Interestingly, NHEJ-deficient murine tumors show a similar mutational footprint as HR-deficient murine tumors, suggesting that these cancer cells might also depend on TMEJ (Ratnaparkhe, Wong et al. 2018). The structural variations associated with BRCA1 loss are thus likely to be a general signature of increased polymerase theta action, instead of a specific hallmark of HR deficiency.

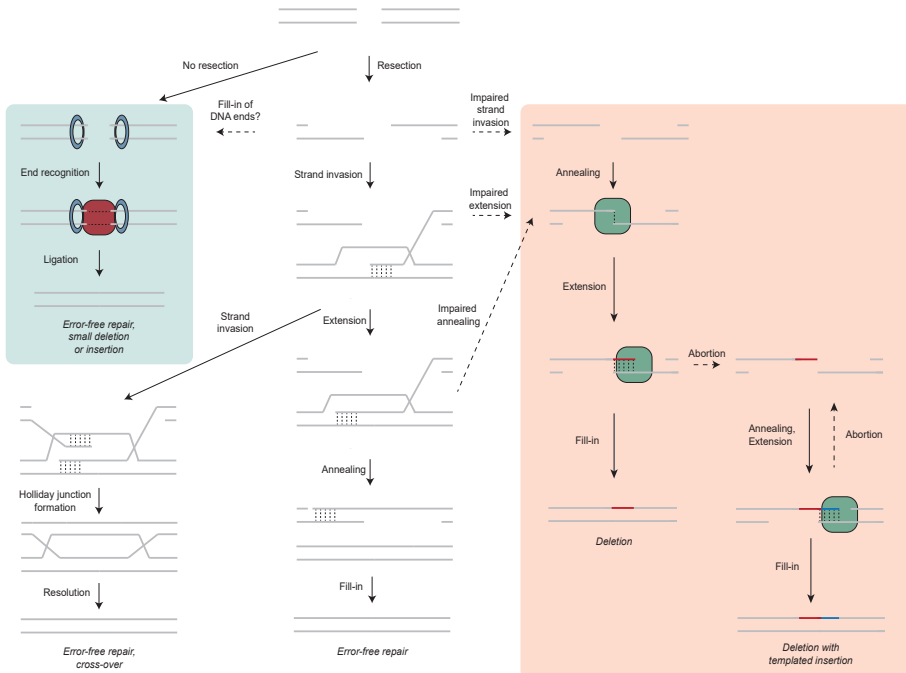

Figure 1 Overview of double-strand break repair Model depicting potential break repair outcomes. Error prone mechanisms are highlighted in blue (non-homologous end-joining, NHEJ) and red (polymerase theta-mediated end-joining, TMEJ). Resected break ends are primarily repaired via error free homologous recombination (HR). After strand invasion, breaks can be repaired without errors by annealing of the extended strand to the other break end in a mechanism called synthesis-dependent strand annealing. Alternatively, when the other break end also invades the repair template, a double holiday junction is formed, and breaks are repaired after resolution by nucleases, leading to error free repair of cross-over of genetic sequences from the template and repair substrate. Novel DNA interactions are depicted with dotted lines. When break ends are blunt, CKU-70/80 dimers (blue rings) recognize the break ends, and ligase 4 (red box) joins the break ends together to seal the break. It is unknown whether break ends can be repaired via NHEJ when HR has been initiated. When HR cannot be completed (e.g. because of absence of *brc-1*, impaired extension because of a damage or secondary structure in the template or impaired annealing because lack of helicase activity), the break can be repaired by polymerase theta (green box). Break ends are annealed using one or more homologous nucleotides of the two break ends, and polymerase theta extents one break end. Other polymerases complete the repair reaction by fill-in synthesis. Infrequently, the repair reaction is aborted after extension by polymerase theta, and the extended break end is used for re-annealing. This can lead to a templated insertion.

Of note, the increase of base substitutions was not affected by NHEJ or TMEJ. Therefore, the origin of the base substitution is likely another substrate than DSBs. Previous work showed that deletions and base substitutions are generated via different mechanisms in BRCA1 deficient cells and the source of base substitutions are proposed to be DNA helix distorting lesions that stall replication (Pathania, Nguyen et al. 2011, Zamborszky, Szikriszt et al. 2017).

The origin of microhomology-mediated deletions

While most deletions observed in *brc-1* and *brd-1* are dependent on polymerase theta for their generation, a few are not: in the genomes *brc-1 polq-1* and *brd-1 polq-1* mutant animals, a small number of deletions arise (Kamp, van Schendel et al. 2020). The observed deletions can be divided in two classes: large deletions (>10kb) that are remarkably larger than the deletions observed in *brc-1* and *brd-1* single mutants, of which the majority is not larger than 100 bp. These large deletions are not characterized by homology. It is possible that these deletions arise by NHEJ, but if NHEJ was indeed active at the DSBs in *brc-1* or *brd-1* germlines, it is unknown why NHEJ did not generate smaller deletions. These class of deletions also arises in genomes of *polq-1* single mutants, suggesting that polymerase theta protects against extensive DNA loss at DSBs (van Schendel, Roerink et al. 2015).

The other class of deletions observed in *brc-1 polq-1* and *brd-1 polq-1* genomes are deletions within the size range observed in genomes *brc-1* and *brd-1* single mutants, but these deletions are all characterized by extensive homology at their junctions (Kamp, van Schendel et al. 2020). The occurrence of these deletions in polymerase theta deficient situations suggested that a pathway other than TMEJ can also be responsible for microhomology-mediated end-joining (MMEJ), and this form of MMEJ can be distinguished by TMEJ by footprint analysis. This form of MMEJ does not leave insertions in the DNA and uses more than 5 nucleotides of homology.

It was not the first time that polymerase theta independent MMEJ footprints were observed. In yeast, polymerase theta in not conserved, but MMEJ footprints are present (Boulton and Jackson 1996). In mammalian cells depleted of polymerase theta, footprints with extensive homology at the junctions have been observed (Kelso, Lopezcolorado et al. 2019). In *C. elegans,* we observed that breaks induced by G4 structures, which are usually repaired by polymerase theta, can be repaired via another mechanism when substantial homology is present surrounding the break site (Kamp, Lemmens et al. 2021). This shows that the presence of homologous stretches can lead to bypass of the need for polymerase theta, probably because polymerase theta's major role is to extent one break end with the other break end as a template, leading to similar homologous stretches. When these stretches are already present, polymerase theta is thus unnecessary.

G4s surrounded by homologous stretches provided us with the opportunity to test candidates for polymerase theta-independent MMEJ. We found that helicase Q, encoded by the *helq-1* gene, is responsible for all polymerase-theta independent MMEJ at G4-induced breaks(Kamp, Lemmens et al. 2021). Besides MMEJ of G4-induced breaks, MMEJ of DSBs induced by the endonuclease I-SceI was also mediated by Helicase Q(Kamp, Lemmens et al. 2021).

The role of helicase Q in microhomology-mediated end-joining

Investigation of DSB repair footprints revealed the role of HELQ in MMEJ. We therefore now have two proteins of which the mutational signature has been elucidated in *C. elegans*: polymerase theta and helicase Q. While helicase Q's mutational signature consist of deletions with extensive homology at the junctions, polymerase theta's signature consists of deletions with (templated) insertions or simple deletions with less than 6 nucleotides of homology (Koole, van Schendel et al. 2014, Roerink, van Schendel et al. 2014, van Schendel, van Heteren et al. 2016, Kamp, van Schendel et al. 2020). Interestingly, deficiency of helicase Q also influenced the footprints generated by polymerase theta: while the proportion of insertions is usually low in TMEJ footprints, it is elevated in helicase Q deficient backgrounds. Closer inspection of these insertions showed that they often consisted of repetitive fragments, and these fragments seemed to be copied of the deletion flanks. While these templated insertions were described previously in TMEJ (Koole, van Schendel et al. 2014, Schimmel, van Schendel et al. 2019), these are usually not repetitive. Repetitive templated insertions were observed previously in plants (van Kregten, de Pater et al. 2016), in which helicase Q is not conserved.

The increase in insertions during TMEJ in absence of helicase Q, shows that helicase Q's role is not restricted to one MMEJ pathway, but can influence break repair in a broader way. Recent work shows that helicase Q removes RPA from single-stranded (ss) DNA (Jenkins, Northall et al. 2021), providing a potential explanation for the (repetitive) insertions at TMEJ deletions: the single stranded overhangs of DSBs contain RPA, and this needs to be removed to allow polymerase theta to polymerize along the break ends. When polymerase theta encounters the RPA molecules bound to the ssDNA, it lets loose of the DNA, and the repair reaction reinitiates with the partly extended strand (Figure 1). This hypothesis is also compatible with the requirement of helicase Q for end-joining using extensive homology: when the homologous stretches are covered by RPA, they cannot anneal to each other, preventing MMEJ. Thus, we propose helicase Q processes DNA ends to allow annealing of homologous nucleotides.

The role of helicase Q in HR

MMEJ is not the only repair mechanism in which single stranded break ends anneal to each other: also during the final steps of synthesis-dependent strand annealing (SDSA) route of HR, ssDNA ends are aligned to repair breaks. Defects in this step can be detected via two different reporters: the single strand annealing (SSA) and homology-directed repair (HDR) reporter (see introduction for details). The HDR reporter gives a general measure of SDSA efficiency, while the SSA reporter mimics the final step of the SDSA pathway: annealing of the single-stranded break ends. Helicase Q deficiency leads to an almost complete absence of reporter activity in both reporters, showing that helicase Q plays an important role in the annealing step of the SDSA pathway.

Similar to *brc-1* and *brd-1* animals, we performed a mutation accumulation experiment with *helq-1* mutants. Because *brc-1* and *helq-1* both play a major role in the high fidelity repair pathway SDSA, an increase in mutations was expected in the genomes of both mutants. Interestingly, knocking out these genes lead to a different mutational footprint. While the major type of structural variation in *brc-1* genomes were deletions, *helq-1* animals mainly accumulated tandem duplications in their genomes. This difference illustrates that BRC-1 and helicase Q play different roles in SDSA. While BRC-1 exerts its function in the initial steps of the pathway, before strand invasion (Chen, Nievera et al. 2008, Cruz-Garcia, Lopez-Saavedra et al. 2014, Zhao, Steinfeld et al. 2017), helicase Q functions at the final steps, post strand invasion (Ward, Muzzini et al. 2010, Adelman, Lolo et al. 2013).

Inhibiting polymerase theta or helicase Q as a potential cancer treatment

Similar to polymerase theta, deficiency in helicase Q leads to decreased survival of BRC-1 deficient animals. However, while polymerase theta is an ideal target for inhibition in BRCA1-deficient cancers, inhibition of helicase Q should be considered with more caution. The emergence of tandem duplications in the absence of helicase Q function would be a hazard in the non-transformed cells of cancer patients, and might lead to secondary cancers. In contrast, polymerase theta deficiency does not lead to increased mutations in genomes (van Schendel, Roerink et al. 2015), and inhibitors for polymerase theta are now being developed (Zatreanu, Robinson et al. 2021, Zhou, Gelot et al. 2021).

The origin of tandem duplications

The accumulation of tandem duplications in genomes of *helq-1* (and to a lesser extent in *brc-1*) animals prompted us to think about the mechanisms leading to tandem duplications. A

TD is an alteration of a genomic sequence, detectable as two adjacent copies of the original sequence. Tandem duplications come in different sizes: small tandem duplications are frequently identified in human genomes (Messer and Arndt 2007), but large tandem duplications, spanning kilobases or even megabases, also arise in human genomes (Marques-Bonet, Girirajan et al. 2009), especially in malignant contexts, like cancer (Menghi, Inaki et al. 2016, Nik-Zainal, Davies et al. 2016).

The mechanism responsible for small TDs (<1 kb) has recently been elucidated (Schimmel, Muñoz-Subirana et al. 2021): When two single strand breaks are in close proximity, a DSB with 3' overhangs can arise. Polymerase alpha can be recruited to polymerize along these overhangs, generating double-stranded DNA ends that are substrates for NHEJ. Because both overhangs are filled in with new nucleotides, the sequence between the two single stranded gaps is doubled, leading to a tandem duplication after NHEJ.

Larger tandem duplications arise in contexts where HR cannot function properly. The tandem duplication size differs between genetic drivers (Menghi, Barthel et al. 2018). BRCA1 deficiency led to tandem duplications of 11 kb, both in tumors and in *C. elegans* (Menghi, Barthel et al. 2018, Kamp, van Schendel et al. 2020). One requirement for a tandem duplication to arise in contexts where HR is compromised, is that strand invasion has to be performed to copy the DNA that is part of the duplication. Because BRCA2 is absolutely required for strand invasion, whereas BRCA1 is not, tandem duplications do not arise in BRCA2 deficient tumors, while they are an important signature of BRCA1 deficient tumors (Menghi, Inaki et al. 2016, Nik-Zainal, Davies et al. 2016, Willis, Frock et al. 2017, Menghi, Barthel et al. 2018). Other examples of proteins important in the suppression of tandem duplications in *C. elegans* are SMC-5 and SMC-6 (Meier, Volkova et al. 2021), which facilitate HR by keeping the sister chromatids tethered together (Bickel, Chen et al. 2010). Potentially, the receiving break end is not in close proximity at the end of the HR reaction to allow proper annealing, and alternative end-joining is necessary. Another suppressor of tandem duplications is the protein RTEL (León-Ortiz, Panier et al. 2018, Meier, Volkova et al. 2021), which is important for disassembly of D-loops, the structure that arises after strand invasion in HR (Barber, Youds et al. 2008). It is possible that failure in disassembly of D-loops leads to superfluous extension of the invaded strand, which is not compatible with the break end on the other side of the break, leading to alternative annealing. Defective annealing is also a likely explanation for the tandem duplications observed in *brc-1* and *helq-1* mutants, because of incomplete resection or defective protein unloading at the receiving break end, respectively (Kamp, van Schendel et al. 2020, Kamp, Lemmens et al. 2021).

While small tandem duplications are a result of NHEJ (Schimmel, Muñoz-Subirana et al. 2021), larger ones (spanning kilobases) are a result of polymerase theta (Kamp, van Schendel et al. 2020, Kamp, Lemmens et al. 2021). The junctions of large tandem duplications are similar to deletion junctions generated by polymerase theta, and without polymerase theta, these large tandem duplications do not arise. The difference in end-joining pathways required for small versus large tandem duplications can be explained by the nature of the break ends. While polymerase alpha made the break ends (near-)blunt before ligation in case of small tandem duplications, the larger tandem duplications result from breaks with large single-stranded overhangs.

Hierarchy of DNA repair pathways

The increase in tandem duplications in *helq-1* animals illustrates that repair of DSBs via HR is preferred over TMEJ: while TMEJ is active in the cells where DSBs occur, it will not generate deletions immediately, but will only join the break ends after strand invasion and extension. Polymerase theta is thus not immediately competitive with HR, but will act when HR cannot be completed. This is the case when the final annealing step is not possible, or when the sister chromatid cannot be used as a template because it contains DNA damage or a secondary structure (Koole, van Schendel et al. 2014, Lemmens, van Schendel et al. 2015, Kamp, Lemmens et al. 2021).

In BRC-1 deficient animals, polymerase theta does act directly on DSB before strand invasion and extension: while deletions do not require extension for their formation, about ten times more deletions than tandem duplications arise in their genomes (Kamp, van Schendel et al. 2020). One potential explanation for this phenomenon is that BRC-1 inhibits TMEJ before strand invasion, but a more likely explanation is that strand invasion is partially impaired in *brc-1* animals, because of impaired resection. An unanswered question is which mechanism or entity prevents polymerase theta from repairing HR substrates when HR is available.

Besides the occurrence of TDs in genomes of *helq-1* mutants, the dominance of helicase Q over TMEJ is also reflected in SSA reporter experiments(Kamp, Lemmens et al. 2021): the number of TMEJ signature deletions was significantly higher in helicase Q deficient conditions compared to helicase Q proficient conditions. This indicates that the breaks induced in this reporter are preferentially repaired via helicase Q mediated annealing, and TMEJ can back-up repair. Altogether, the data in *brc-1* and *helq-1* deficient animals indicate that TMEJ serves as an alternative break repair mechanism when HR cannot be completed (Figure 1).

In line with this, it was shown that HR and TMEJ share the need for an initial end resection step to DSBs (Truong, Li et al. 2013). In contrast to TMEJ and HR, NHEJ is obstructed by

DNA resection (Lemmens, Johnson et al. 2013, Yin and Smolikove 2013). NHEJ is therefore less likely to directly compete with HR than TMEJ: NHEJ's substrate is a different type of DSB. While channeling breaks from being HR substrates to NHEJ substrates seems possible in mammalian cells by polymerase alpha-mediated fill in synthesis (Schimmel, Muñoz-Subirana et al. 2021), it is not known yet whether this is also occurring in *C. elegans*.

NHEJ deficiency does lead to an increase in SDSA and SSA in the HDR and SSA reporter respectively (Pontier and Tijsterman 2009, Johnson, Lemmens et al. 2013). This does not automatically mean that NHEJ and HR compete directly at the breaks induced in these reporters. It is likely that breaks that are usually repaired via NHEJ, remain unrepaired in NHEJ deficient contexts until resection can take place. When these breaks get resected, SDSA and SSA can take place. In this case, NHEJ deficiency thus leads to a larger number of substrates for homology-directed repair.

Identification of factors influencing non-homologous end-joining

In order to identify genetic factors involved in NHEJ and its regulation, we made use of the knowledge that SSA reporter activity increases in the case of NHEJ deficiency. The SSA reporter can thus function as an indirect NHEJ assay. We combined the SSA reporter with a novel reporter: the NHEJ reporter. This reporter was only expressed in the pharyngeal muscle cells, which are not replicating after the worms hatch from their egg. Therefore, the NHEJ reporter provides a unique opportunity: to study breaks specifically in terminally differentiated cells. The NHEJ reporter is completely specific for NHEJ: only when a break was induced in the reporter and repaired by erroneous NHEJ, GFP and LacZ was expressed in the pharynx. SSA reporter activity can only be observed in replicating cells, therefore we were able to combine the reporters and read out NHEJ and SSA at the same time within one animal.

We performed an unbiased forward genetic screen in nematodes carrying the transgenic NHEJ/SSA reporter (Kamp, Lemmens et al. 2022). We isolated seven bona fide NHEJ mutants, three of these contained mutations in the NHEJ factors *cku-70* and *cku-80*. The other four mutants carried mutations in genes of the conserved THO ribonucleoprotein complex (*thoc-2*, *thoc-5* and *thoc-7*) and in *pnn-1*. Both the THO complex and PNN play a role in RNA processing (Wang, Lou et al. 2002, Li, Lin et al. 2003, Chi, Wang et al. 2013). Intriguingly, defective THO complex function is known to result in genome instability in various species, including yeast, worms and humans, which can be partly explained by THO's role in preventing the formation of RNA:DNA hybrids throughout the genome (Huertas and Aguilera 2003, Dominguez-Sanchez, Barroso et al. 2011, Castellano-Pozo, Garcia-Muse et al. 2012). We found that deficiency of PNN and the THO complex also leads to sensitivity to ionizing

radiation in somatic tissues, but not in the germline. This is similar to the response of animals defective in *cku-70* and *cku-80*, and pointed towards a role for the THO complex and PNN in NHEJ. Besides CKU-70 and CKU-80, *C. elegans* also codes for ligase 4, which is essential for joining the break during NHEJ. We did not identify ligase 4 deficient nematodes using the screen, which indicates that the screen was not saturated. It is therefore possible that replication of this screen might lead to the identification of novel NHEJ factors.

Crosstalk between RNA processing and DNA repair

To identify the mechanism by which the THO complex influences NHEJ efficiency, a suppressor screen was performed in *thoc-5* mutants. We discovered that mutated *smg-1* rescues the NHEJ defect in THO mutants (Kamp, Lemmens et al. 2022). SMG-1 is essential for the control of RNA quality: it plays an essential role in nonsense-mediated decay (NMD). Our findings indicate that defective RNA processing can affect DSB repair. This might have an evolutionary explanation: modern day organisms store their genetic information in DNA-based genomes, and the information is transferred to RNA to encode protein sequences (Crick 1970). However, it is generally believed that RNA-based genomes preceded DNA, and DNA evolved from RNA *via* reverse transcription (Gilbert 1986). Nowadays multiple proteins that safeguard genome stability have evolved, which work in specialized pathways to repair different types of DNA damage. Other proteins, like SMG-1, have evolved to ensure RNA stability. The major NMD components (SMG-1, SMG-2, SMG-8 and SMG-9) can be traced back to at least the last eukaryotic common ancestor (Causier, Li et al. 2017).

SMG proteins also have roles in other cellular processes besides NMD, including pathways controlling DNA synthesis, cell cycle progression, DNA damage signaling and telomere maintenance (Isken and Maquat 2008). SMG-1 belongs to the phosphoinositide 3-kinase-related kinase (PIKK) family, which comprises of structurally similar kinases that respond to diverse stresses, like metabolic stress and DNA damage (Lempiainen and Halazonetis 2009) and SMG-1 has been proposed to function between RNA and DNA surveillance mechanisms to ensure the integrity of the gene expression program (Abraham 2004). This functional crosstalk could have benefits: several lines of evidence indicate that DNA damage signaling can change RNA expression; either by controlling RNA transcription, processing and/or stability(Wickramasinghe and Venkitaraman 2016, Burger, Ketley et al. 2019). This could facilitate proper repair. However, our study shows it can also have detrimental defects: altered RNA signaling leads to inhibition of NHEJ, which in non-replicating cells is the only pathway known to repair DSBs.

Interestingly, SMG mutants are hypersensitive to ionizing radiation-induced DSBs in the germline (González-Huici, Wang et al. 2017), similar to HR and TMEJ mutants. This hypersensitivity could suggest that SMG proteins stimulate repair of DSBs via HR or TMEJ. In THO deficient animals, in which SMG-1 seems to be more active, we could investigate the role of SMG-1 (Kamp, Lemmens et al. 2022). When performing footprint analysis at breaks induced in NHEJ defective THO mutants and ligase 4 mutants, we did observe an increase in homology usage in ligase 4 mutants, but not in THO mutants (Chapter 4). This suggests that SMG-1 does not stimulate TMEJ, but it is possible it stimulates error free HR. Alternatively, it is possible THO deficiency leads to impaired TMEJ in addition to impaired NHEJ. Therefore, we cannot draw conclusions about SMG-1's role in stimulation of TMEJ or HR based on this data.

Homologous recombination and RNA

Recent work shows that RNA:DNA hybrids, which accumulate in THO mutants, can either stimulate (Ngo, Grimstead et al. 2021, Ouyang, Yadav et al. 2021) or hinder (Ortega, Mérida-Cerro et al. 2021) repair via HR. RNA-templated HR that is independent of BRCA1 and BRCA2, but dependent on RAD52 and Cockayne Syndrome protein B has also been shown (Wei, Nakajima et al. 2015, Teng, Yadav et al. 2018). When RNA is used as a repair template, a polymerase with reverse transcriptase activity is necessary. Yeast replicative polymerases have been shown to possess reverse transcriptase *in vitro* (Storici, Bebenek et al. 2007), polymerase zeta has been proposed to reverse transcribe transcript RNA at sites of DNA damage (Meers, Keskin et al. 2020), and more recently, polymerase theta has been shown to possess reverse transcriptase activity and function in RNA-templated DSB repair (Chandramouly, Zhao et al. 2021). While the frequency of RNA-templated repair in organisms has to be assessed and the functional relevance has to be determined, there are scenarios in which RNA-templated DSB repair can be beneficial. For example, when a break occurs in a gene in a terminally differentiated cell. When cells are terminally differentiated, breaks cannot be repaired via classical HR, because the lack of replication. While DSBs can be repaired via NHEJ, RNA-templated HR is potentially less error prone. However, usage of spliced mRNA as a template might lead to exclusion of intron sequences in the genome. This type of mutation is observed in neurons of Alzheimer's disease patients (Lee, Siddoway et al. 2018).

Investigating break repair in context

To investigate DSB repair, researchers are dependent on the available tools (or have to create novel ones). While in the last few years, a lot of progress has been made in techniques to induce

breaks at specific sites, we are not able to visualize these breaks directly in live cells. We can approach visualization by tagging a subset of proteins we expect to be present at break sites, but we cannot see the all proteins and the DNA ends at once.

In our studies in *C. elegans*, we mainly made use of mutational footprint analysis. In reporters and after using CRISPR/Cas9, we know the exact break site, but because we only see the end product, we can only infer the potential processing steps. Therefore, combining different assays is essential to strengthen conclusions.

Combining different assays to investigate the same question is especially essential in studies of DSB repair pathway choice. In these studies, often genetic reporters are used to study the interplay of the different DSB repair pathways. However, local parameters at these assays may influence the outcomes of the experiments. When a reporter is established in a way that multiple sequence repeats are created, end-joining using homologous stretches is enabled. It is then essential that these outcomes are also quantified, or that the experiment is repeated in different sequence contexts.

A challenge in the DNA repair field remains the quantification of error free repair. Reporters rely on erroneous repair of DSBs, and during footprint analysis, it is impossible to distinguish DNA that has not been broken from DNA that has been repaired without errors. MA line frequency is also not a reliable proxy for frequency of error free repair, because too many factors, like selection, influence frequency to give an accurate estimate of error free repair. A high confidence measure of error free repair would lead to give more insight in the fates of DSBs.

Conclusion

We have found that the (mutational) repair outcome of a DSB depends on the context in which it occurs. When cells are not replicating, DSBs are repaired via NHEJ. NHEJ efficiency can be affected by defective RNA processing. In replicating cells, the preferable mechanism for DSB repair is HR. When canonical HR cannot be executed, because the repair template is not available (at G4-induced breaks, for example) or when not all HR factors are present (in BRCA1 deficient situations), alternative annealing is needed. This is carried out via TMEJ, or when homologous nucleotides are available, via HELQ-1 mediated annealing of these homologous stretches. Finally, we have found that large TDs can arise when break ends cannot anneal properly after the extension step in HR.

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