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General discussion and future prospects

This dissertation discusses several aspects of DNA double strand break (DSB) repair and provides mechanistic insights in the occurrence and repair of DSBs during *C. elegans* development. DSB repair is crucial to ensure genome stability in developing animals, as unrepaired DSBs can result in extensive loss of genetic material and aneuploidy upon cell division. However, DSB repair is not error-free and can cause mutations and chromosome aberrations. In fact, mutagenic DSB repair can leave many genetic scars throughout development and ultimately promote malignant transformation (Aparicio *et al.* 2014). Eukaryotic cells possess many different DSB repair activities, some of which are intrinsically mutagenic (see Dutch summary, Figure 2). Cells need to tightly control the different DSB repair activities to limit the genetic consequences but also support efficient repair. Although the studies presented in this thesis provide further insight in the consequences of DSB repair pathway choice during animal development, still many questions remain to be addressed. Especially the temporal and spatial regulation of the different DSB repair pathways is poorly understood and will require future study. Furthermore, the molecular mechanisms that dictate repair template choice remain elusive. A selection of outstanding questions will be discussed in more detail below.

How to employ DSB repair in the right place and at the right time?

The consequences of mutagenic repair are mainly determined by the developmental context. For example, mutations in terminally differentiated somatic cells only affect the function of that particular cell, whereas mutations in germ stem cells may affect the fitness of the whole brood of the animal. It is thought that due to these different levels of evolutionary pressure, tissue-specific DSB repair modes could arise. In line with those concepts, *C. elegans* germ stem cells typically use error-free homologous recombination (HR) for DSB repair, while somatic cells rely on various error-prone DSB repair mechanisms (Clejan *et al.* 2006; Pontier and Tijsterman 2009). Interestingly, differential DSB repair activities are even present within the different germ line tissues. As described in Chapter 2, we found that certain germ cells depend heavily on COM-1 and EXO-1 for DNA end resection of DSBs, a crucial step in regulating DSB repair pathway choice. Interestingly, the requirement for these DNA end resection nucleases is regulated in a temporal and/or spatial fashion, given that certain germ cells at specific developmental stages were proficient in DNA end resection even in the absence of COM-1 and EXO-1. These specific germ cells are predicted to use the sister chromatid as a template for HR, whereas germ cells that require COM-1 and EXO-1 for DNA end resection use the homologous chromosome for repair (Hayashi *et al.* 2007). How early DSB processing factors are regulated during gametogenesis and how their activities are linked to chromosome organization and repair template availability is unknown to date. Similarly, we found LIN-61 to be required for HR in mitotic germ cells but not meiotic germ cells, again revealing tissue-specific DSB repair activities (Chapter 3). How these differential repair activities are controlled

and how malignant brain tumour domain proteins akin to LIN-61 regulate DSB repair on a mechanistic level requires further study.

How to choose between the good, the bad and the ugly?

The notion that DSB repair can have both benign and harmful consequences for animal development is well illustrated by the non-homologous end joining (NHEJ) pathway. As discussed in this thesis, NHEJ is vital to promote genome stability in somatic tissues (Chapter 4), but can also cause highly toxic repair products in germ cells (Chapter 2). Next to being toxic directly, NHEJ is also error-prone and thus creates mutations. A single DSB repair pathway can thus be "good", "bad" and "ugly" in light of genome maintenance.

The mutagenic attribute or the "ugly side" of DSB repair is adressed in more detail in Chapter 5 and 6, where we demonstrate that DSBs induced by unresolved replication barriers cause genomic deletions whose nature depends on sequence context and the mode of DSB repair. We have recently identified the major DSB repair pathway responsible for these deletions and named the pathway Theta-mediated end joining (TMEJ), as it required polymerase Theta/POLQ-1 (Koole *et al.* 2014; Roerink *et al.* 2014). In Chapter 5 we investigated the potential endogenous sources underlying TMEJ-mediated mutagenesis and found that a single unresolved DNA secondary structure, such as a G4-quadruplex, could serve as a continuous source of TMEJ substrates, ultimately leading to multiple deletions during *C. elegans* development. Subsequent in-depth analysis of repair footprints, revealed an alternative DSB repair mechanism that can compete with TMEJ for G4-induced deletion formation (Chapter 6). In contrast to TMEJ, this alternative homology driven repair (HDR) mechanism requires substantial sequence homology at both break ends. Because of these specific homology requirements, HDR is only feasible at highly repetitive loci. Nevertheless, the notion that TMEJ and HDR share the same substrates suggests an intimate connection between TMEJ and homology search mechanisms. Interestingly, genetic backgrounds that suffer from increased TMEJ-mediated deletions also show increased levels of HR intermediates (detected as RAD-51 foci), substantiating the idea that TMEJ and HR may act on similar substrates (Koole *et al.* 2014; Roerink *et al.* 2014). How cells decide between error-free repair via HR ("good") and mutagenic repair via TMEJ ("bad") is still an open question. As noted in Chapter 2, we identified a genetic factor, COM-1, that dictates the balance between HR ("good") and NHEJ ("bad") in germ cells. If COM-1 is also needed to prevent TMEJ to act on meiotic DSBs remains to be studied, but some level of genome instability remained in *com-1* animals that were deficient for NHEJ, suggesting that alternative end joining pathways may act on meiotic DSBs under these conditions (Chapter 2). Future genome-wide sequencing studies may reveal elevated level of TMEJ- and/or HDR-mediated mutagenesis in *com-1* deficient backgrounds.

What is the mechanism and biological significance of TMEJ and HDR?

In recent years several alternative DSB repair mechanisms have been described that in contrast to NHEJ use complementary DNA sequences to seal DSBs. These alternative DSB repair mechanisms have received different names including alternative end joining (alt-EJ), micro-homology mediated end joining (MMEJ) or single strand annealing (SSA). To date the molecular characteristics and genetic requirements of these pathways are still ill defined and may encompass common mechanisms that include single-strand DNA exposure, annealing of complementary sequences and removal of non-complementary flaps. Depending on the availability, length and position of the complementary sequences, these alternative DSB repair mechanisms may require genetic factors that support DNA resection, annealing or nicking, respectively. In Chapter 6, we describe two alternative DSB repair mechanisms that are distinct from NHEJ and can act on G4-induced DSBs: HDR, which requires >4bp sequence homology and TMEJ, which does not require extensive homology but needs polymerase Theta/POLQ-1. Further research is needed to elucidate to which extent the earlier described alternative DSB repair activities involve TMEJ and/or HDR mechanisms.

Because genetic studies often use a limited amount of model substrates to measure DSB repair outcomes, pathway definitions and extrapolation of genetic requirements to a genome-wide level has proven to be problematic. To this end, genome-wide sequencing approaches will become attractive tools to evaluate the impact of alternative DSB repair pathways on genome maintenance and study their role at numerous genomic locations.

Under which conditions are DSBs repaired via TMEJ?

In this thesis we focused on TMEJ in the context of G4-induced DSBs, which because of their fixed genomic location proved to be a powerful approach to study the genetic consequences of low-frequency replication barriers (Chapter 5 and 6). Recently our lab has identified additional roles for TMEJ on DSBs derived from other sources, including transposition (unpublished data). By comparing the different genetic interactions and repair outcomes of TMEJ events triggered by various sources of DSBs, one should be able to identify the genetic features that are intrinsic to TMEJ reactions (e.g. frequent flank insertions) and distinguish these features from those that are provoked by the substrate (e.g. deletion size).

How to find new components and potential regulators of TMEJ?

Similar to NHEJ, TMEJ could be harmful, as it is not error-free and in case of multiple DSBs could lead to translocations. This latter attribute may also have clinical implications given that up-regulation of polymerase Theta is associated with poor prognosis in human cancers (Higgins *et al.* 2010). On the other hand, the ability of TMEJ to seal DSBs without the need of extensive sequence homology makes it a valuable pathway to repair DSBs genome-wide. Although in-depth genetic analysis of repair outcomes, as described in chapters 5 and 6,

provided important clues on the mechanism of TMEJ, complementary approaches such as unbiased screens to identify additional genetic factors required for TMEJ could provide vital insights into the mechanism and regulation of this pathway. In addition, targeted proteomics approaches could reveal new players of TMEJ as well as novel post-translational modifications on polymerase Theta itself.

As described in Chapter 4, we performed unbiased forward genetics screens to identify new regulators of NHEJ and found several mRNA binding factors to be required for efficient NHEJ in somatic cells. Subsequent transcriptome analysis resulted in the identification of specific mRNA splicing defects in several newly identified mutants, revealing a potential novel link between mRNA splicing and DSB repair. The identification of canonical NHEJ factors (e.g. CKU-70 and CKU-80) and novel factors such as THOC-5 and PNN-1 validated the screen and demonstrated the power of such unbiased approaches. Still, one drawback of forward genetics screens is the typically need for the obtained alleles to be homozygous viable, which hinders the identification of essential genes. We obtained a point mutation in *thoc-2* that causes a NHEJ defect in *C. elegans* but did not completely block the essential function of THO, given that *thoc-2* null mutants are sterile (Castellano-Pozo *et al.* 2012). Thus forward genetics screens as performed here can reveal essential genes in DSB repair, but the identification of such alleles typically requires substantial screening depth.

We have established various transgenic reporter systems that can measure TMEJ activity at G4 sites, which can be used to screen for new factors required for TMEJ. Given that many TMEJ factors may be intrinsically connected to DNA replication and HR (which are both essential processes in *C. elegans*), careful design of future screens is needed to acquire many alleles and thus potential hypomorphic mutations. Conversely, identification of viable alleles of HR or DNA replication factors could open new research avenues as they provide new tools to study these important biological processes in other developmental contexts.

Concluding remarks

As discussed in this thesis, the efficacy and choice of DSB repair pathways can have tremendous influence on the toxicity and mutagenicity of DSBs. Future research to delineate the different DSB repair modes is vital to understand the genetic consequences of DSBs for animal development. Ultimately, new insights concerning the endogenous sources and consequences of genomic instability in developing tissues could provide important clues on the origin and possible treatment strategies of cancer.

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