CHAPTER 1

Introduction
DNA damage and repair

Our cells are constantly exposed to exogenous and endogenous stresses, which can alter genome integrity. UV, ionizing radiation, reactive oxygen species or chemicals produce a wide range of DNA damage, ranging from base alkylation, pyrimidine dimer, inter strand-crosslink, single-strand break (SSBs) or double-strand break (DSB) (reviewed in Lord and Ashworth, 2012). All of these types of DNA damage can alter gene transcription, DNA replication and if unrepaired, mutations associated with cancer and/or cell death. In order to maintain genome integrity, cells have evolved mechanism to repair DNA damage. Among all the types of DNA damage, DNA breaks (SSBs and DSBs) are most problematic. Generally SSBs are efficiently repaired via base excision repair or nucleotide excision repair (reviewed in Wilson and Bohr, 2007), but an SSB can easily be converted into a DSB during DNA replication (Kuzminov, 2001). It is commonly thought that a DSB is the most hazardous DNA lesion since a single unrepaired DSB is enough to kill a cell (Resnick and Martin, 1976; Weiffenbach and Haber, 1981). Therefore cells have evolved multiple DSB repair pathways. DSBs are repaired via two major pathways, known as nonhomologous end joining (NHEJ) and homology-directed repair (HDR) (reviewed in Li and Xu, 2016)

DNA double-strand break repair pathways

DSB repair pathways

NHEJ directly ligates the two DNA extremities together with no need for sequence homology and is considered a mutagenic-prone pathway (Guirouilh-Barbat et al., 2004). However, HDR is a more accurate form of repair since it involves the use of undamaged homologous sequences as a donor template, and it is inhibited in G1 phase, due to the absence of the donor sister chromatids (Rothkamm et al., 2003; Orthwein et al., 2015).

HDR can be separated into three pathways: homologous recombination (HR), which is dependent on strand-invasion, single-strand annealing (SSA), which involves the annealing of complementary RPA-coated single-stranded DNA (ssDNA) with more than 30 bp of sequence homology, and microhomology-mediated end joining (MMEJ), which is similar to SSA but requires only 5 to 25 bp of homology for annealing before ligation. SSA and MMEJ are considered as error-prone mechanisms because they result in deletion of sequences at the break site (reviewed in Jasin and Rothstein, 2013). For the remainder of this thesis, only HR will be further discussed. Unless otherwise indicated, genes and proteins mentioned are from the budding yeast
Saccharomyces cerevisiae, but HR is a highly evolutionarily conserved mechanisms.

**Homologous recombination**

The HR pathway can be divided into three subpathways: double-strand break repair (DSBR), synthesis-dependent strand annealing (SDSA) and break-induced replication (BIR) (Figure 1). The first step of HR is detection of the DSB by HR proteins, and this step is similar to all the three HR pathways. Recognition of the DSB is mediated by the MRX complex, composed of Mre11, Rad50 and Xrs2, which is recruited along with Sae2 to the DSB site (Paull TT, 1998; Trujillo et al., 1998; Trujillo and Sung, 2001; Lengsfeld et al., 2007). MRX/Sae2 initiates short tracks of 5′–3′ end resection (Clerici et al., 2005; Mimitou and Symington, 2008, 2009; Zhu et al., 2008). MRX-mediated end resection is inhibited by the competing factor Ku (Ku70 and Ku80), which favors NHEJ over HR (Clerici et al., 2008; Mimitou and Symington, 2010; Shim et al., 2010). Further end resection, leading to an extensive 3′ overhang up to 50 kb in length, is catalyzed by the Exo1 and/or Dna2 nucleases (Mimitou and Symington, 2008; Shim et al., 2010; Zhu et al., 2008). Dna2 functions together with the Sgs1 helicase complex (Sgs1, Top3, Rmi1 or STR) to unwind DNA prior to Dna2-mediated cleavage (Zhu et al., 2008).

The resulting 3′ ssDNA overhang is first coated by the replication protein A (RPA), which is subsequently replaced by Rad51 with the help of Rad51 mediator proteins, ultimately forming a Rad51 nucleoprotein presynaptic filament (Ogawa et al., 1993). Rad52, Rad54, Rad55-Rad57 and the Shu complex (Csm2, Psy3, Shu1 and Shu2) facilitate presynaptic filament formation and its stabilization (Sung, 1997a; Sung et al., 2003; Sung, 1997b; Mankouri et al., 2007). Rad52 is the major recombination protein in yeast, involved in all kinds of recombination, and Rad52 possesses Rad51-dependent and -independent functions (Shinohara and Ogawa, 1998; Mott and Symington, 2011). During presynaptic filament formation, the Rad52 heptameric ring interacts with both Rad51 and RPA to stimulate RPA displacement by Rad51 (Stasiak et al., 2000; Davis and Symington, 2003; Shenohara et al., 1998). Rad52 can also catalyze the annealing of RPA-coated ssDNA independently of Rad51 (Mortensen et al., 1996). The Rad51 nucleoprotein filament initiates homology search, by invading and pairing with a donor DNA molecule, to form a displacement loop (D-loop). Gaps are filled by DNA polymerases, recruited within the D-loop to synthesize DNA using the donor DNA molecule as a template (Holbeck and Strathern, 1997).

The fate of the D-loop mainly depends on the second end of the DNA break, which dictates the choice between the three HR pathways. When the second end of the break is lost, repair is mediated by BIR. BIR requires extensive DNA synthesis by polymerase δ and is dependent on Pol32, a non-essential subunit of Polδ (Lydeard...
et al., 2007). Replication will proceed until the end of the donor chromatid or until the polymerase reaches another replication fork. If the second end remains in a close proximity, which is more often the case, migration of the D-loop can lead to the capture of the second end of the break by the displaced strand, which presumably requires the strand annealing activity of Rad52, and augmented by the presence of Rad59, leading to the formation of a double Holliday junction (dHJ) (Davis and Symington, 2001). dHJs can be removed via dissolution or resolution (Ii and Brill, 2005; Fabre et al., 2002). Resolution involves cleavage of the Holliday junctions by structure-specific endonucleases in a process that can result in a crossover product (where there is an exchange between the two DNA molecules) or a non-crossover product, while dissolution only leads to the formation of non-crossovers (Chaganti et al., 1974). Dissolution is mediated by the STR (Sgs1, Top3 and Rmi1) complex. It has been proposed that Sgs1 facilitates migration of the two HJs toward each other in order to form a single hemicatenane structure, which then can be removed by the action of Top3, a type I topoisomerase (Ira et al., 2003; Wu and Hickson, 2003). Recently, it has been shown that Top3 is required during the convergent migration of the dHJ (Chen et al., 2014). The role of Rmi1 is less clear. Rmi1 acts in a later stage after branch migration. Its role has been postulated to stimulate decatenation by stabilizing Top3 during cleavage of the ssDNA (Cejka et al., 2010).

Resolution is mediated by the interaction between 2 complexes: Mus81-Mms4 or Yen1 with Slx1-Slx4 (Schwartz et al., 2012; Ho et al., 2010; Sarbajna et al., 2014; Castor et al., 2013). Cleavage by Mus81-Mms4 or Yen1 can either lead to crossovers or non-crossovers products, depending on the site of cleavage.

Although most characterized for their role in DSB repair, HR proteins have also been found to facilitate the re-start of stalled replication forks, which will be discussed in the next section.
11

Introduction

Figure 1. Models for DNA double-strand break repair by homologous recombination. Adapted from (Symington et al, Genetics, 2014). DSB repair is initiated by 5’ to 3’ end resection by the MRX-Sae2 complex. Exo1 or Dna2-Sgs1 mediates more extensive end resection. The resulting 3’ overhang can then invade a donor DNA template (in red). This invasion step requires Rad51 and its mediators (Rad52, Rad55-Rad57, and the Shu complex), leading to the formation of a D-loop. In the synthesis-dependent strand annealing (SDSA) model, the invading strand can be displaced after limited DNA synthesis, and strand annealing activity is required to complete synthesis and repair. Strand annealing is dependent on Rad52. During D-loop migration, the second end of the break can be captured to form a double Holliday junction (dHJ) in the double-strand break repair (DSBR) model. In this model, dHJs can be either dissolved by the action of Sgs1-Top3-Rmi1, which will favor branch migration of the two HJs toward each other, resulting in the formation of a hemicatenane structure, which can be cleave by the action of Top3. Dissolution only results in non-crossover products. On the other hand, dHJs can be resolved by the action of endonucleases (Mus81-Mms4, Yen1, Slx1-Slx4). Resolution by cleavage in positions 1,2, 3 and 4 will lead to a non-crossover product, while cleavage in positions 1,2, 5 and 6 results in a crossover. If one end of the DSB is lost, repair proceed by break-induced replication (BIR). BIR-mediated DNA synthesis occurs by conservative replication and is dependent on Pol32.
Replication associated-DNA damage tolerance

DNA replication can be impaired by the presence of DNA damage on the template strand or by secondary structures, leading to stalled or collapsed forks.

DNA polymerases have difficulty using damaged DNA as template during DNA synthesis. One important, but still poorly characterized, pathway to overcome replication obstacles is known as DNA damage tolerance (DDT) or post-replication repair (PRR) (Branzei, 2011). DTT allows DNA to be synthesized past a lesion so that lesion removal can occur through classical DNA damage repair pathways after replication. Two main PRR sub-pathways have been described (Figure 2). First, translesion synthesis (TLS) relies on the temporary replacement of the replicative DNA polymerase by a special TLS polymerase. TLS polymerases lack the 3' to 5' proofreading exonuclease activity of classical polymerases, and can recognize modified bases to bypass the lesion. Due to their low fidelity, the TLS polymerases are highly mutagenic (reviewed in Sale et al., 2012). On the other hand, a higher fidelity PRR sub-pathway, known as template switch (TS) or error-free PRR, allows the newly-synthesized DNA strands from both chromatid to pair with each other, allowing one to be used as a template for the other to replicate past the lesion, a step which can involve HR proteins (Ball et al., 2009; Gangavarapu et al., 2007).

The choice between TLS and error-free PRR relies depends on the degree of PCNA ubiquitylation (Hoege et al., 2002; Ulrich and Jentsch, 2000; Stelter and Ulrich, 2003). When replication forks encounter DNA damage, the PCNA sliding clamp is monoubiquitylated by the action of Rad6 and Rad18, E2 and E3 ubiquitin ligases, respectively. Monoubiquitylation of PCNA at lysine 164 activates TLS by interaction of a TLS polymerase (Pol ζ, Rev1 and Pol η in yeast) with PCNA. Further polyubiquitylation of PCNA at lysine 63 by Rad5/Mms2 activates error-free PRR. Rad5 recruitment at replication forks also mediates the uncoupling of leading and lagging strand synthesis due to the helicase activity of Rad5 (Blastyak et al., 2007). The unwinding of DNA promotes the annealing of the nascent DNA strands. It has been proposed that when the lesions occur on the leading strand, lagging strand synthesis will continue, allowing the nascent DNA strand to anneal with the blocked strand. Replication over the damage will proceed by the transient regression of the replication forks, forming a four-way junction intermediate called a “chicken foot” structure (reviewed in Li and Xu, 2016). On the other hand, if the lesion is located on the lagging strand, template switch is mediated by the annealing and invasion of the ssDNA into the newly formed sister chromatid, by the action of Rad52, Rad51, Rad54, similar to the SDSA mechanism (Blastyak et al., 2007; Gangavarapu et al., 2007).
Figure 2. Replication-associated DNA damage bypass mechanisms. Adapted from (Ghosal and Chen, Transl Cancer Res, 2013). When a replication fork encounter DNA damage, the sliding clamp PCNA is monoubiquitylated at lysine 164 (K164) by Rad6-Rad18. Monoubiquitylation of PCNA favors the recruitment of a translesion synthesis polymerase to transiently replace the normal replicative polymerase and synthesize DNA over the lesion. On the other hand, monoubiquitylation of PCNA K164 can also lead to the polyubiquitylation of K63 by the Rad5-Ubc13-Mms2 complex. Polyubiquitylation of K63 drives repair toward a template switch mechanism, which involves the use of the sister chromatid to bypass the lesion. If the lesion occurs on the leading strand template, Rad5 will be recruited to mediate repair. However, if the damage is located on the lagging strand template, repair will be mediated by Rad51 and Rad52.

Sister chromatid exchanges

DNA repair involving the use of an undamaged sister chromatid can lead to the formation of sister chromatid exchanges (SCEs). Defined as the exchange between two identical sister chromatids, elevated levels of SCE indicate genome instability, a hallmark of cancer. However, how spontaneous SCEs arise in cells remain unclear. Because SCEs do not result in any change in DNA sequence, they have been difficult to study. Their first visualization involved radiography of tritium-
labeled chromosomes in plants. However, resolution was poor and did not allow for quantification of SCEs (Taylor, 1958). The discovery of the thymidine analogue bromodeoxyuridine (BrdU) has led to two major improvements in the visualization and mapping of SCEs. The first amelioration relies on the differential staining of the heavy BrdU-labeled chromatid (lighter color) and the chromatid containing the parental strand (darker) after being replicated two times in the presence of BrdU. Due to the differential staining of the sister chromatid, SCEs are visualized by the exchange between dark to light or light to dark chromatids (Perry and Wolff, 1974). Although this technique has been highly informative for the quantification of SCEs, and for their use in measuring genome instability by measuring the rate of SCE formation in mutants or cancer cells, they did not allow precise mapping of the position of SCEs.

Recently, a new approach based on DNA next-generation sequencing technology allows the mapping of SCEs at a higher resolution (Figure 3). This method, called Strand-seq, is based on BrdU incorporation into newly synthesized DNA strands followed by selective degradation of the BrdU-containing nascent strands to isolate the parental strands for the construction of directional sequencing libraries of a single cell (Falconer E, 2012).

Because of their small genome, visualization of SCEs in yeast by microscopy is not possible. Therefore, visualization of SCEs in yeast relies on genetic markers that are integrated at specific loci or on plasmids. These techniques rely on the ability of cells to grow on selective media after an SCE has occurred. Although these studies have been highly informative, they possess some limitations. First, the marker is only present at a specific locus, which may not be representative of the rest of the genome. Moreover, integrating a marker in the genome is not always neutral. Adding some DNA sequences at a specific locus could modify the natural environment, such as the chromatin state, and therefore could modify the recombinogenic potential of that specific locus. Furthermore, genetic marker-based assays used to measure SCEs are designed to only measure unequal SCEs. Because unequal recombination events can lead to genetic alterations, equal SCEs, which are neutral, are thought to occur more frequently (González-Barrera S, 2003). Until now, only one report has measured equal SCEs in yeast, using an inverted repeat containing plasmid (González-Barrera S, 2003), and while this is a very elegant system, plasmids are known to behave differently in terms of recombination compared to chromosomes. Thus, although these assays have been beneficial for the genetic characterization of SCE formation, a more physiological approach to study spontaneous SCEs is missing in yeast.
Figure 3. Schematic representation of a sister chromatid exchange and its visualization using Strand-seq. Cells are replicated in presence of BrdU, which is incorporated into newly synthesized DNA. If a DSB is formed, repair can proceed by DSBR leading to the formation of a dHJ. If the dHJ is resolved in a manner leading to the formation of an SCE, after cell division, each daughter cell will inherit a chromatid containing both Watson (orange) and Crick (blue) original DNA template strands in a reciprocal pattern. In Strand-seq, only the original template strands are sequencing. The reads are then aligned to the reference genome and the BAIT program is used for visualization of the data.
CHAPTER 1

Thesis overview

Recombination proteins are involved in multiple repair pathways and therefore play a major role in protecting genome integrity. Rad52 is one of the main recombination proteins, with a well-characterized function in the repair of DSBs but has more poorly defined roles in the repair of other types of damage. During my PhD work, I have been interested to understand the role of recombination proteins outside of their role in DSB repair.

Deletion of *RAD52* has been found to accelerate replicative senescence in the absence of telomerase. However, its role in preventing accelerated senescence still remains unknown. In the Chapter 3, I describe our work in this area, and propose a model of how Rad52 can prevent accelerated senescence. Chapter 4 describes a recently-developed technique called Strand-seq, which we have applied to yeast to measure and map spontaneous SCE events genome-wide and at a single-cell level. In addition, we characterized genes involved in the formation of spontaneous SCEs. Moreover, we discuss how our data strongly suggests that most spontaneous SCEs do not originate from DSBs, and how we should re-think the role of HR proteins outside of DSB repair.

The work in both Chapters 3 and 4 benefitted from the use of *rad52* separation-of-function mutants that lack the strand-annealing activity of Rad52. In Chapter 5, I present some preliminary data that further exploits these mutants to more carefully determine the role of Rad52 in different repair pathways.

Finally, in Chapter 6, I discuss my work as a whole in a more general context, and outline my thoughts for potential follow-up studies to my works.
References


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Hey Chromatid!!
Do you know what telomeres are?

They are the extremities of chromosomes. Like feet and hands!

"Many facets of homologous recombination at telomeres"?

I don't know! Let's read about it?