Live Cell Dynamics of Homology-Directed DNA Double-Strand Break Repair

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Abstract

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Amanda Jane Vines
2021

It is crucial that DNA damage is repaired efficiently in order to maintain genome integrity. Double-strand breaks, or DSBs, are the most harmful type of DNA damage, and thus their repair is tightly regulated. Fission yeast is a prime model system for studies of such repair processes as they favor homology-directed repair (HDR) over other more error-prone mechanisms. My work seeks to better interrogate homology-directed repair of DSBs in a temporally and spatially controlled manner, expanding on previous studies using primarily genetic outcome-based assays of repair. This is necessary in order to probe the complex dynamics of interhomologue repair in living cells. I have developed a microscopy-based assay in live diploid fission yeast to determine the dynamics and kinetics of an engineered, site-specific interhomologue repair event. My data indicate a highly efficient homology search in this system. Surprisingly, I observe not one but multiple site-specific and Rad51-dependent co-localization events between the DSB and donor. This and other observations suggest that efficient interhomologue repair in fission yeast often involves multiple strand invasion events that are regulated by Rqh1. In the absence of Rqh1, successful repair requires a single strand invasion event, suggesting that multiple strand invasion cycles reflect ongoing synthesis-dependent strand annealing (SDSA). However, failure to repair is also more likely in Rqh1 null cells, which could reflect increased strand invasion at non-homologous sites. This has implications for the molecular etiology of Bloom syndrome, caused by mutations in BLM (the human
ortholog of Rqh1) and characterized by aberrant sister chromatid crossovers. Additionally, I monitored DSB repair dynamics under a variety of perturbations such as loss of repair factors or manipulations of the donor sequence. I found that fission yeast HDR is largely robust to these changes; chromatin mobility is not necessarily tied to repair efficiency; and donor sequence alterations can greatly affect associations with the DSB during homology search. Lastly, I discuss the implications of multiple strand invasions in HDR processing and also call for further work to expand on my research. In particular, it would be powerful to include complementary assays to assess sequence changes and strand invasion intermediates upon DSB induction in my system.
Live Cell Dynamics of Homology-Directed DNA Double-Strand Break Repair

A Dissertation
Presented to the Faculty of the Graduate School
of
Yale University
in Candidacy for the Degree of
Doctor of Philosophy

By
Amanda Jane Vines

Dissertation Director: Megan C. King

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Chapter 1: Introduction

*Genome integrity is crucial to preserve biological life*

Deoxyribonucleic acid (DNA) is an essential biological molecule. Its functional interconnectedness with ribonucleic acid (RNA) and protein forms the central dogma of molecular biology, a simplified yet assistive view of information transfer and utility in cells. In order for a cell to achieve homeostasis, it is imperative that it possesses mechanisms to repair its DNA after damage from intracellular or extracellular sources. If not efficiently repaired, a variety of outcomes are possible. In less essential portions of the genome, this damage in the form of point mutations or other slight alterations can go unnoticed by the cell and perpetuate to future generations. More rarely, some mutations, or even duplication or deletion of coding sequences, can promote advantageous adaptation. However, un repaired or mis-repaired DNA can drive loss of genome integrity, uncontrolled growth, and/or death at the cellular level, leading to disease, cancer and/or death on an organismal level. The furtherance of life is tethered to the faithful conveyance of genomes through time (cell and organismal lifetimes) and space (cell division and organization) despite these ever-present threats.

**DNA damage: causes, types and solutions**

DNA is a complex, information-rich molecule, and as such there are myriad ways in which this information can be altered, damaged, recombined or removed entirely that have long been a focus of research and clinical treatments. The below list is by no means exhaustive but meant to impress upon the reader
the enormity and complexity of ever-present dangers to DNA through which cells must (and do) doggedly persevere.

Exogenous threats to DNA

Some of the earliest characterized (Puck and Marcus, 1956; Witkin, 1956) sources of DNA damage are external to the cell, such as UV and ionizing radiation. UV radiation from sunlight can photochemically activate C4 and C6 carbons within adjacent pyrimidine bases, forming thymine dimers. Photoactivation can also result in altered bases such as 8-oxoguanine (produced by C8 hydroxylation), which binds to adenine rather than cytosine. On an organismal level, these and other photoactivation reaction products can accumulate and result in a number of different skin cancers if unrepaired (Ichihashi et al., 2003; Cadet and Douki, 2018). Ionizing radiation (IR), consisting of alpha, beta, and gamma rays in the environment (or X-rays in a medical treatment context), results in a broad array of direct and indirect deleterious effects on a DNA molecule, including free radicals, lost or damaged bases, and single- and double-stranded breaks (Obe et al., 1992; Desouky et al., 2015; Chatterjee and Walker, 2017). Reactive chemical compounds introduced naturally through diet/environment or in the lab also modify and damage DNA structure. Alkylating agents, notably ethyl methanesulfonate (EMS) and methyl methanesulfonate (MMS) used regularly in studies of DNA repair, as well as crosslinking agents also result in altered nitrogenous bases via SN1 or SN2 reactions with their nucleophilic sites (Wyatt and Pittman, 2006; Chatterjee and Walker, 2017).
**Endogenous threats to DNA**

Other types of DNA damage result from threats already within the nucleus due to normal metabolic and homeostatic processes. Reactive oxygen species (ROS) are a set of highly reactive molecules formed by photoactivation by UV light or as byproducts of metabolism. Though more stable than its cousin molecule RNA, DNA also has its own inherent instability and is susceptible to ROS. Several bonds in all four main bases of DNA are vulnerable to oxidative damage and hydrolytic attack, in particular the bond between a purine and its deoxyribose sugar base (Lindahl, 1993; Cadet and Richard Wagner, 2013). As a result, oxidation and depurination are quite common, each estimated to occur as much as 10,000 times per day in a mammalian cell (Lindahl and Nyberg, 1972; Ames *et al.*, 1993; Nakamura *et al.*, 1998). A number of lesions can also result from errors in the replication of DNA. These range from simple base misincorporation by polymerase error to more severe types of DNA damage from replication fork stalling or collapse, namely single strand breaks and double strand breaks (DSBs) (Friedberg, 2003; Helleday, 2003; Chatterjee and Walker, 2017).

**Intersecting pathways to repair DNA damage**

Given the many distinct ways in which DNA can be damaged, there might conceivably be a separate pathway to repair each one. However, cells are much more efficient in use of their resources. Some DNA damage response (DDR) pathways can process multiple types of DNA damage, so it is no surprise that these pathways often work in place of or in concert with one another and can
have shared substrates, steps and repair factors, of which a few of the copious examples are enumerated below.

The myriad effects of oxidative DNA damage from ROS, chemical agents or IR require that the nucleotide excision repair, base excision repair, mismatch repair, and DSB repair pathways operate in close temporal and spatial proximity (Friedberg, 2003; Chalissery et al., 2017). Each of these pathways can also be invoked for stalled replication forks, depending on the damage the replication fork encounters (Friedberg, 2003; Bryant et al., 2009; Syeda et al., 2014). Break-induced replication (BIR), most commonly encountered in bacteria and budding yeast or upon replication stress in mammals, is a versatile means of resolving DNA damage from one-ended double strand breaks at replication forks (Malkova, 2018). It has slower kinetics than some other repair pathways to repair DSBs since it is often error-prone, but nonetheless it can promote replication restart and lengthening of telomeres with Rad51-dependent and -independent variations (Malkova et al., 2005; Costantino et al., 2014; Roumelioti et al., 2016; Malkova, 2018). Rad54, a rather unique protein in the Snf2 helicase family that translocates on double-stranded DNA (dsDNA) but does not boast classical unwinding properties of helicases, acts in multiple steps of homology-directed repair, including promoting chromatin remodeling, stabilizing or destabilizing the nucleoprotein filament, mediating Rad51-dependent strand exchange, and facilitating Holliday junction branch migration (Alexeev et al., 2003; Mazin et al., 2003; Heyer et al., 2006; Li et al., 2007; Wright and Heyer, 2014). DNA polymerase δ is a workhorse of DNA synthesis contributing to homology-directed
repair, with demonstrated involvement in HR, BIR, microhomology-mediated end-joining (MMEJ) and alternative end-joining (altEJ) (Lee and Sang, 2007; Maloisel et al., 2008; Meyer et al., 2015; McVey et al., 2016; Donnianni et al., 2019). The human RecA ortholog RAD51, required for homology-dependent mechanisms of DSB repair (elaborated on below), also acts in other ways to preserve genome integrity, such as to resolve stalled replication forks in a manner not dependent on its strand exchange function (Mason et al., 2019).

As suggested already, this interconnectedness of repair pathways extends to those repairing the most severe form of DNA damage, double strand breaks (DSBs), which are the focus of my research.

**Homologous recombination: the poster child of homology-directed repair**

Homology-directed repair (HDR) denotes a set of conserved, high-fidelity mechanisms for repairing DSBs. Uniting them is the requirement for a sequence homologous (perfectly matching) or homeologous (very similar but not perfectly matching) to the break site to use as a donor template for repair. Distinguishing them are the repair factors employed (to some degree) and the means in which this donor is identified and utilized for repair. The complex nature of these pathways defies a full description, but relevant snapshots focused on key events and proteins in budding and fission yeast are outlined below and illustrated in Figure 1.

Homologous recombination (HR) is one of two main pathways (along with non-homologous end joining, or NHEJ) that the cell can use to repair DSBs. HR is an important method for repairing DSBs that utilizes a homologous sequence
(a donor sequence) in the genome as a template for new synthesis that ultimately repairs the DSB. HR is preferentially carried out in the S or G2 phases of the cell cycle (when the sister chromatid is available as a template for repair), whereas NHEJ, a more error-prone pathway, typically occurs during G1 prior to DNA replication (Takata et al., 1998; Ferreira and Cooper, 2004; Symington and Gautier, 2011; Renkawitz et al., 2014; Ceccaldi et al., 2016). Both HR and NHEJ have a classically understood mechanism of repair and several alternate pathways, but my work will focus primarily on HR and SDSA. In brief, following recognition and end resection of the DSB, faithful repair by HR requires a Rad51-dependent homology search by the resultant nucleoprotein filament to locate a homologous donor sequence as a template for new synthesis. A more detailed explanation of each step can be found below.
Figure 1. Overview of homology-directed DSB repair.
Certain fission yeast proteins highlighted in the text are represented; other repair factors and details of alternate pathways are excluded for simplicity (see text for additional information). Diagram is not to scale.

(A) Upon formation of a DSB, the MRN (Mre11-Rad50-Nbs1) complex binds to the DNA ends and Rad3 (not shown) activates the checkpoint. (B) Ctp1 is recruited and initial end resection is conducted by Mre11 and Ctp1. RPA binds to ssDNA as resection continues via Exo1 (or Rqh1 in Exo1’s absence). (C) Nucleoprotein filament formation ensues upon Rad52- and Rad54-induced recruitment of Rad51 (not represented in later steps for simplicity). (D) The nucleoprotein filament undergoes homology search; this is the
least well understood step of HDR and more mechanistic details are addressed throughout the thesis. (E) Upon an encounter with the homologous donor, the nucleoprotein filament invades to displace the strands and form a D loop, inducing synthesis of new nucleotides on the invading strand. (F) If the newly synthesized strand reinvents the original duplex, ligation of the DNA ends forms a double Holliday junction (dHJ), (G) the resolution of which can result in non-crossover (NCO) or crossover (CO) products. (H) Alternatively, and indeed more prominently in many eukaryotes, release of the donor strand and ligation to the original strand before completion of synthesis results in a non-crossover product through synthesis-dependent strand annealing (SDSA).
**Cell cycle and the DNA damage checkpoint**

First, the DSB is recognized by the Mre11-Rad50-Nbs1 (MRN) complex, a heterotrimer of regulatory proteins that bind the DSB ends and enlist ATM or ATR (Figure 1A) (Uziel et al., 2003; Kim et al., 2005). In mammals, ATM and ATR (Tel1 and Rad3 in fission yeast, respectively) regulate the DNA damage checkpoint and initiate a cascade that recruit repair factors to the DSB (Humphrey, 2000; Craven et al., 2002; Gobbini et al., 2013). Regulation at early stages of DSB recognition is one means of shunting DDR toward HDR or NHEJ. Activation of ATM/Tel1 typically occurs during G1 and leads to NHEJ, while ATR/Rad3 activity during S/G2 is an early step of HR (Maréchal and Zou, 2013; Weber and Ryan, 2015; Blackford and Jackson, 2017). Tellingly, Tel1 is dispensable for checkpoint activation in fission yeast while Rad3 is essential (Willis and Rhind, 2010), demonstrating their reliance on HDR during G2 (discussed further below).

**Resection: the fork in the DSB repair road**

DSB recognition is followed by bidirectional 5’ to 3’ exonuclease-dependent resection of one DNA strand at each end (Figure 1B). Nucleases Mre11 and Sae2/Ctp1 initiate the first phase of resection for a short stretch of ~300 nucleotides (Zhu et al., 2008a; Nicolette et al., 2010; Cannavo et al., 2019). A second phase of extended resection (usually several thousand nucleotides) results upon recruitment of nucleases Exo1 or Sgs1-Dna2 (Mimitou and Symington, 2008; Cejka, 2015). Each has characteristic efficiencies based on the model system studied and which repair factors are present. For instance, in
budding yeast (*Saccharomyces cerevisiae*), Exo1 and Sgs1-Dna2 operate in redundant pathways, and both must be deleted in order to significantly hinder extended resection (Mimitou and Symington, 2008; Zhu *et al.*, 2008a). This also seems true of EXO1 and DNA2-BLM in humans (Nimonkar *et al.*, 2011), though EXO1 may play a more dominant role. BLM can stimulate EXO1 processivity *in vitro* and *in vivo*, but EXO1 is still capable of resection independently of BLM (Nimonkar *et al.*, 2008). Also, deleting EXO1 alone can hinder resection in human cells (Zhou *et al.*, 2014). However, in fission yeast (*Schizosaccharomyces pombe*) Exo1 is distinctively the dominant nuclease for the second longer phase of resection; in its absence RecQ helicase 1 (Rqh1, the BLM ortholog in *S. pombe*) takes over and promotes an accelerated resection rate (Leland *et al.*, 2018a). Regulation of resection also factors heavily into repair pathway choice. More extensive resection inclines repair toward HR, while no or limited resection due to 53BP1/Crb2 activity or the presence of Ku70/Ku80 proteins at the DSB ends promotes classical NHEJ or MMEJ (Tomimatsu *et al.*, 2007; Shibata *et al.*, 2018; Wang *et al.*, 2018; Callen *et al.*, 2020). However, excessive resection can also be detrimental for faithful repair by HDR (Tinline-Purvis *et al.*, 2009; Lee *et al.*, 2015a), likely by promoting more error-prone pathways such as BIR (Tinline-Purvis *et al.*, 2009; Chung *et al.*, 2010).

Resected DNA is first bound by replication protein A (RPA) (Figure 1B), a heterotrimeric DNA-binding complex with very high affinity for single stranded DNA (ssDNA) (Kim *et al.*, 1992, 1994; Bochkarev *et al.*, 1997). RPA serves to protect the resected DNA from degradation until downstream repair factors are
recruited and loaded to ssDNA (Chen et al., 2013), in which recruitment and loading it also assists (Seeber et al., 2016). Like many other repair factors, its binding to DNA and association with other DDR proteins can be regulated by posttranslational modifications such as phosphorylation, ubiquitination and SUMOylation (Dou et al., 2011; Elia et al., 2015; Maréchal and Zou, 2015).

*Friend like me: a search for homology*

Rad51, a RecA family recombinase, is required for downstream steps of HR. Upon sufficient resection, Rad52 mediates loading of Rad51 to ssDNA (Sung, 1997; Shinohara and Ogawa, 1998; Sugiyama et al., 1998), along with the multifunctional assistor protein Rad54 (Sigurdsson et al., 2002; Li et al., 2007; Mazin et al., 2010). The resulting complex (Figure 1C) is called the nucleoprotein filament (sometimes referred to as the presynaptic filament). RecA and Rad51 induce changes in orientation of bases within the ssDNA upon binding, which is conducive to surveying dsDNA target sequences (Shibata et al., 2001; Takahashi et al., 2007).

The nucleoprotein filament then undergoes a Rad51-dependent search for a homologous donor sequence by movement along dsDNA surrounding the DSB (Figure 1D). Rad51 within the nucleoprotein complex drives strand invasion and formation of a displacement loop (D-loop) heteroduplex (Figure 1E) at presumptive dsDNA donor sequences in cooperation with Rad52 and Rad54 (Sung and Robberson, 1995; Petukhova et al., 1998; Shinohara and Ogawa, 1998). The resultant Holliday junction (Holliday, 1964; Szostak et al., 1983) can
progress along the DNA molecule via Rad51-dependent branch migration (Murayama et al., 2008).

This step of interrogating potential sequences for repair, called homology search, is relatively short given the availability of the sister chromatid, the favored template for HR across model organisms from budding yeast to mammalian cells (Kadyk and Hartwell, 1992; Takata et al., 1998; Hartsuiker et al., 2001; Ray and Langer, 2002; Fernandez et al., 2019). However, homology search can be long-range if the sister is not available and a homologous chromosome or other template is utilized (Barzel and Kupiec, 2008; Wright et al., 2018). In this case, a more extensive journey for the nucleoprotein filament to survey potential donor sequences is theorized and necessitated, but the precise means of this journey is poorly understood. One hypothesis is that the nucleoprotein filament undergoes 1D “sliding” along the exposed DNA, but this has so far been demonstrated mainly by RecA filaments in vitro in small spurts for short segments (<300 bp) (Ragunathan et al., 2012; Renkawitz et al., 2014). It is also less clear whether this is relevant in vivo (Adzuma, 1998). There is a large disparity between the theoretical time required to survey a vast genome in single increments (several weeks for even a relatively small bacterial genome) (Weiner et al., 2009) and the actual time taken for successful repair following long-range homology search, estimated to take as little as several hours in HR-favorable conditions (Aylon et al., 2003; Lisby et al., 2004; Barzel and Kupiec, 2008; Ohle et al., 2016). Even surveying a subset of the genome, as is theorized based on work examining restraints on chromatin movement (Weiner et al., 2009), would be time-intensive,
especially considering the potentially long contact times (several seconds) even at non-homologous sequences (Mani et al., 2009; Crickard et al., 2020). A non-mutually exclusive potential solution to the limitations of 1D sliding is 3D “intersegmental contact sampling” of multiple sequences by the nucleoprotein filament across distinct stretches of chromatin (Forget and Kowalczykowski, 2012; Renkawitz et al., 2014; Crickard et al., 2020). This alleviates the timing issue to a degree, but still leaves open questions of what regulates this sampling and the sensitivity of the RecA/Rad51 filament for particular sequences (discussed further below).

Early processing steps such as recruitment of MRX/MRN and nucleolytic activity of Mre11 only take several minutes in vitro on nucleosome-free DNA (Trujillo et al., 1998, 2003). These fast kinetics are also borne out in vivo at DSBs formed via irradiation in budding yeast (Lisby et al., 2004) and by irradiation or radiomimetic treatment in human cells (Uziel et al., 2003; Kim et al., 2005). Recruitment of Rad51, indicating sufficient resection for formation of a nucleoprotein filament, follows soon after, between 30 minutes and 2 hours in human cells following irradiation (Kim et al., 2005) or after formation of single-ended DSBs from replication fork collapse as monitored by super resolution microscopy (Whelan et al., 2018). Following homology search, synthesis is also likely to proceed quickly, since for instance elongation by DNA polymerase (Pol) δ has been measured at a rate of several hundred nucleotides per second in the presence of processivity factors (Bauer and Burgers, 1988; Mondol et al., 2019).
Homology search, the least well-studied and seldom directly monitored step of HR (Renkawitz et al., 2014; Haber, 2018), is therefore thought to be rate-limiting by process of elimination. However, the time required for homology search in vivo is still an outstanding question in the field. Due to the importance of homology search for my research, later in the introduction I will discuss further work to better understand the regulation of this as yet mysterious step of HDR.

Upon homology recognition, DNA synthesis is initiated (Figure 1E), usually by DNA Pol δ and/or ε (Holmes and Haber, 1999; Li et al., 2009; McVey et al., 2016). This results in reassembly of dsDNA at both the DSB and donor sequences by double Holiday junction (HJ) formation (Figure 1F), which can be processed by resolvases to complete repair (Figure 1G). The STR complex (Sgs1-Top3-Rmi1) promotes non-crossover resolution of HJs during mitotic recombination (Mullen et al., 2005; Bonner et al., 2016), while Yen1 or Mus81-Mms4 complex resolvases process HJs into non-crossover or crossover products (Hickson and Mankouri, 2011; Mazón and Symington, 2013).

**Synthesis-dependent strand annealing**

Homologous recombination is not the only repair pathway that cells can utilize following resection of a DSB. Others include BIR (repairing one-ended DSBs using replication and a homologous template) and single-strand annealing (SSA; annealing and synthesis of resected DNA ends in regions with repeated sequences resulting in deletion of intervening bases with no need for a homologous donor) (Costantino et al., 2014; Bhargava et al., 2016; Ceccaldi et al., 2016; Malkova, 2018). Another such pathway is synthesis-dependent strand
annealing (SDSA), which is arguably the most similar to HR and is the most relevant for my work. Rather than a single strand invasion event at the donor resulting in Holliday junction formation, resolution, synthesis and ligation of the entire resected break as in HR, SDSA involves limited synthesis at the donor, then strand invasion dissolution before synthesis is complete (Figure 1H). The released strand anneals back to the other resected end to continue synthesis and ligation, resulting in a non-crossover product (Nassif et al., 1994; Shinohara and Ogawa, 1995; Pâques and Haber, 1999). Indeed, though more recently defined than HR, SDSA is widespread in many eukaryotes and is likely the dominant mode of HDR in a variety of genetic contexts (Pâques et al., 1998; McVey et al., 2004; Wright et al., 2018), preserving genomic integrity more so than the chance for a crossover product with Holliday junction resolution following HR. It also carries implications that the number and persistence time of encounters between the DSB and donor involved in successful repair may vary more so than in HR, which is often assumed to have one long strand invasion/synthesis event.
Requirements for repair

Of particular interest for my work is the process of homology search introduced above (Figure 1D), so cryptically diagrammed due to the mysteries surrounding this step relative to the rest of HDR. A successful long-range homology search (i.e. with a non-sister chromatid donor) requires that (1) the distant DSB and donor loci are able to encounter one another within the nucleus; (2) the Rad51-bound nucleoprotein filament can drive strand invasion of potential donors (leading to formation of a displacement (D-) loop); and (3) the homologous sequence is used as the template for new synthesis. Despite the difficulty in observing these transient processes, a steadily growing body of work has arisen toward better defining the implementation of these prerequisites for homology search, sometimes with conflicting findings.

Homology search: donors, distances and drives

The sister chromatid available after replication is the most common template for repair (Kadyk and Hartwell, 1992; San Filippo et al., 2008; Mimitou and Symington, 2009) and is in close proximity to the DSB, as sister chromatids are tethered during S phase by the cohesion complex (Seeber et al., 2016; Haber, 2018). In this case, the template is identical to the original sequence and homology search is likely to be temporally and spatially efficient. However, HR can also take place using the homologous chromosome sequence as the template (in the case of a diploid cell) or an ectopic donor sequence. In each case, homology search is comparatively more extensive (long-range) than that to a sister chromatid and is expected to be less efficient (Pâques and Haber, 1999;
Mehta and Haber, 2014). Use of an alternate donor, likely not perfectly homologous to the original sequence, can lead to loss of heterozygosity or genome instability (San Filippo et al., 2008; Renkawitz et al., 2014; Kowalczykowski, 2015). Therefore, high fidelity repair hinges on the accurate choice of a homologous donor.

Understanding the length and degree of homology within the donor DNA necessary for this choice is a growing area of research. *In vitro* studies by Eric Greene’s group with DNA curtains bound by Rad51 as pseudo-nucleoprotein complexes suggested that a minimum of 8 nucleotides of microhomology at the donor are required for prolonged interactions between the nucleoprotein filament and dsDNA (Qi et al., 2015), and that this could reflect a property of highly sensitive base triplet recognition by Rad51 (Lee et al., 2015b). However, it is not yet clear how this may contribute to faithful repair *in vivo*, and this 8-nt length requirement has been contested. For instance, homology recognition by sliding RecA filaments *in vitro* is feasible with only 6 nucleotides of homology (Ragunathan et al., 2012). Similarly, in a Rad51-dependent BIR assay by the Haber group, as much as every sixth base pair could be mismatched in the donor from the original sequence and still generate a recombination product, the reconstitution of a split *S. cerevisiae ura3* open reading frame (ORF) (Anand et al., 2017). Non-homologous tails between split *ura3* ORFs with overlapping homology did not fully deter recombination, though the likelihood of BIR decreased as tail length increased (Anand et al., 2017). Further, when examining RecA activity upon ssDNA and dsDNA segments by FRET, the lifetime of
synapses formed by the nucleoprotein filament with DNA targets is greatly influenced by the degree of homology of the ssDNA to the target sequence (Mani et al., 2009; Danilowicz et al., 2017), but ATP-hydrolysis-dependent strand exchanges with as long as 75bp recognition sequences remain reversible (Danilowicz et al., 2017).

These studies are informative as to the capabilities of RecA- or Rad51-driven sequence interrogation during homology search on non-nucleosomal DNA. Nevertheless, ultimately the contribution of 3D nuclear architecture along with chromatin organizing proteins and modifications, not to mention the remainder of HDR repair factors, within living cells must also be taken into account. Even in vitro in a recent DNA curtain assay, DNA partially loaded with nucleosomes was sufficient to require remodeling, bypass the sequence, or arrest homology search by a Rad54-loaded nucleoprotein filament (Crickard et al., 2020), and in vivo such interactions are likely compounded and otherwise regulated.

Some in vivo studies of HDR strive to define the sequence-level requirements for a successful homology search by providing cells with several contemporaneous potential donors for repair, or providing one of several sequences with varying amounts of homology. These could to some degree emulate situations of template choice leading to disease-relevant loss-of-heterozygosity (LOH) (see section on cancer below). For example, the Jinks-Robertson group constructed a series of cassettes in budding yeast with inverted repeats that could crossover to reconstitute a his3 intron (Datta et al., 1996). The
rates of His\(^+\) prototroph formation were then compared in strains with either 100%, 91%, or 77% homologous intronic sequences. Even in WT, the rates of recombination were as much as \(~40\)-fold (91%) or \(2000\)-fold (77%) decreased compared to a 100% homologous sequence (Datta \textit{et al.}, 1996; Spell and Jinks-Robertson, 2003). Additionally, the Haber group utilized overlap of homologous or homeologous sequences from \textit{ura3} flanking an HO-induced DSB in budding yeast as an SSA assay. They found that 97% homologous regions (~220bp long) were six times less likely to recombine than 100% homologous flanking regions (Sugawara \textit{et al.}, 2004). In a competition variation of the SSA assay within the same study, recombination products were more likely from a distant, perfectly homologous (100%) donor than a closer homeologous (97%) donor (Sugawara \textit{et al.}, 2004). Similar extreme reduction in recombination efficiency upon small percent increases in homeology have also been observed in mammalian cells (Bhattacharjee \textit{et al.}, 2014).

Many \textit{in vivo} assays to assess the stringency of homology search utilize exogenous integrated cassettes, which aid in evaluating the specificity of repair and/or allow for easy screening methods, such as viability on selective media. Importantly, in these cases the DSB and ectopic donor regions often do not share homology beyond the cassette itself. However, work from several groups indicates that surrounding homology (or lack thereof) can play a large role in acceptance or rejection of a sequence for HDR, even if an ectopic donor sequence is fully homologous or greatly divergent from the original. In a budding yeast gene conversion assay, when provided with two potential donor
sequences, one with homology to the region immediately adjacent to the DSB and one with homology to more distant flanking sequences several kilobases away, ~40% of recombination events were with the latter donor, and this proportion only increased upon reducing the DSB-adjacent homology length (Inbar and Kupiec, 1999). Also in budding yeast, about half of recombinants utilized a homeologous donor at the allelic locus rather than a homologous donor placed ectopically on the same chromosome (Wang et al., 2017). In mouse fibroblasts, regions of high homology surrounding a locally mismatched segment allowed such segments to be utilized for gene conversion when they might normally be rejected (Chapman et al., 2017; Li et al., 2018).

There may also be a relationship between long-range homology search efficiency and proximity between the DSB and donor. These correlated when an ectopic inducible DSB in S. cerevisiae was monitored for repair from 24 different donor sequences integrated throughout the genome – more proximal donors were more prone to be utilized (Lee et al., 2015a). Additionally, DSB repair foci nearer to one another in mammalian cells were more likely to merge in a live cell assay monitoring rare translocation events (Roukos et al., 2013). Inducible DSBs in another live cell mammalian translocation assay appeared static, limiting their possible repair partners to proximal chromatin regions (Soutoglou et al., 2007). However, the relationship between DSB and donor proximity and repair efficiency, especially as it pertains to the mobility of these regions, is not quite so straightforward.
Chromatin mobility and DNA damage

Chromatin mobility would be expected to facilitate the encounter rate between a DSB and its distant donor. Correspondingly, chromatin mobility often increases upon DSB induction both locally at the DSB and globally in budding yeast and human cells (Miné-Hattab and Rothstein, 2012; Seeber et al., 2013), and in Arabidopsis, it was shown that homologous loci are closer together and colocalize more frequently upon IR or zeocin treatment to produce DSBs (Hirakawa et al., 2015). However, the degree of induced mobility may be influenced by a number of factors.

The activity of repair proteins can greatly influence mobility, most centrally chromatin remodelers (discussed in Chapter 3) but also DSB recognition proteins or checkpoint activators such as NBS1 and ATM (Tobias et al., 2013; Becker et al., 2014; Aymard et al., 2017). Some genomic regions are inherently less mobile in response to DSB induction, for instance due to tethering at the nuclear envelope or local compaction of chromatin (Chubb et al., 2002; Bystricky et al., 2004; Zimmer and Fabre, 2019). Additionally, whether a DSB has persisted long enough to activate checkpoint arrest influences the extent of chromatin mobility (Miné-Hattab et al., 2017; Zimmer and Fabre, 2019). Indeed, chromatin mobility can be induced by activation of the checkpoint response in the absence of damage in budding yeast (Bonilla et al., 2008), and even when chromatin mobility is induced by a break it appears to be random rather than directed (Seeber and Gasser, 2017; Mekhail, 2018), despite evidence for involvement of
nuclear microtubules in this movement (Lottersberger et al., 2015; Lawrimore et al., 2017; Zhurinsky et al., 2019). An exception to the randomness of DSB movement is the migration of DSBs after a period of DSB recognition and arrested repair processes due to lack of repair factor recruitment or failure of alternate repair pathways. This has been demonstrated for heterochromatic DSBs in flies (Chiolo et al., 2011; Ryu et al., 2015), excessively resected DSBs in fission yeast (Swartz et al., 2014), and rDNA in budding yeast and human cells (Torres-Rosell et al., 2007; van Sluis and McStay, 2017; Horigome et al., 2019). On a whole, this work draws into question whether chromatin mobility is a driver or simply a byproduct of the prolonged repair response to DSBs requiring cell cycle arrest.

Chromatin mobility is often, but not always, associated with DSB formation. Interestingly, an initial decrease in mobility within the first hour following DSB induction has been observed in budding yeast with a single DSB (Saad et al., 2014). This early reduction in mobility may contribute to early repair events using “local” donor sequences such as the sister chromatid. If needed, increased local and global mobility following cell cycle arrest may then facilitate interactions with alternative sequences that are less desirable templates but still allow for later successful DSB repair following initially unproductive local attempts.

DSB induction at euchromatin is also not the unique instigating cause of chromatin mobility. In mammalian cells, DSB induction through the telomerase-independent alternative lengthening of telomeres (ALT) pathway promotes
mobility of chromosome ends (Cho et al., 2014, 2017), though the processing and outcome of telomeric DSBs are markedly different from those in euchromatin despite shared protein factors and similar DNA structures (Marcomini and Gasser, 2015). DNA damage induced by irradiation in budding yeast during G1 phase (in which NHEJ is promoted over HR) can also result in increased chromatin mobility (Smith et al., 2019). Chromatin mobility as a response to other types of DNA damage apart from the contribution of DSBs has yet to be clearly demonstrated (likely due to the relatively transient nature of such damage over DSBs), but could possibly be invoked as a byproduct of changes to the stiffness of chromatin or activity of non-HDR repair factors (Seeber et al., 2018). Thus, while increased mobility may aid in long-range homology search during HDR, it is also likely a fundamental response to DDR and/or checkpoint arrest, not necessarily unique to HDR pathways or even DSB formation.

Taken together, these reports suggest that the potential influence of chromatin mobility on DNA damage repair is as yet unclear. More work to understand the role of chromatin mobility in assays with location- and time-sensitive measurements is obligatory.

_Cohesion of sister chromatids during HDR_

Cohesion of sister chromatids can contribute to HR efficiency and the choice of the template for HR. The acetyltransferase Eco1 promotes cohesion establishment, not only between sister chromatids but also at sites throughout the genome, in response to DSBs in budding yeast (Ström et al., 2004; Ünal et al., 2007). Spontaneous DSBs result in increased mobility of surrounding
chromatin upon loss of cohesion via Eco1 degradation in budding yeast (Dion et al., 2013). These findings have implications for both short- and long-range homology search.

In the case where the sister chromatid is available for repair (short-range), cohesion between the sisters would be expected to facilitate efficient HR. However, if repair using the sister chromatid as a template is not successful and thus the homologous chromosome or an ectopic sequence is required (long-range), cohesion would likely reduce the ability of the nucleoprotein filament to probe both homologous and non-homologous sequences. These hypotheses are supported in sundry DDR assays. For instance, cohesin loss or mis-regulation is associated with repair preferentially using the homologous chromosome over the sister chromatid. This occurs in the context of mating-type locus repair in rad50Δ fission yeast (Hartsuiker et al., 2001) and rad50Δ budding yeast (Seeber et al., 2016), as well as upon irradiation in budding yeast deficient in cohesin subunit mcd1 (Covo et al., 2010). Additional cohesion between an intact and a broken sister chromatid may promote faithful short-range homology search and repair (Litwin et al., 2018). However, cohesin surrounding a DSB may also block repair factors from accessing the DNA ends (McAleenan et al., 2013), which is perhaps why loading of cohesin is observed less prominently at regions immediately adjacent to the DSB (Ström et al., 2004; Ünal et al., 2007).

To date, study of DSB dynamics during the process of long-range homology search has primarily focused on the DSB itself; still unasked is the question of whether specific remodeling or altered dynamics of the donor
sequence may facilitate its capture during homology search. While the factors that are involved in remodeling at the DSB site are well established (Daley et al., 2014), little is known about the events occurring at the donor sequence prior to strand invasion.

Cancer therapeutics and DSB repair mechanism choice and efficiency

Drugs that introduce DNA lesions have proven to be effective chemotherapeutics. In addition, therapeutics that modulate repair pathway choice and efficiency are a growing area of intervention in cancer treatment. Notably, poly(ADP–ribose) polymerase (PARP), which promotes repair of single-stranded nicks thereby lessening the burden on the HR pathway (Bryant et al., 2009), is the target of multiple inhibitors (PARPi) approved or in clinical trials to treat HR-deficient BRCA1/2-deficient ovarian and breast cancers (Ledermann et al., 2012; Mirza et al., 2016), and may also be useful for treatment of tumors with mutational signatures indicative of an HR deficiency (Patel et al., 2011).

As mentioned above, the selection of the donor sequence template for new DNA synthesis also plays a critical role in the fidelity of DSB repair. Short-range homology search leading to recombination using the sister chromatid as a template is essentially error-free. However, in cases where processing of a DSB is slow and/or in which multiple potential homologous or homeologous sequences exist in the genome, long-range homology search to use these alternative templates can drive LOH or genomic rearrangements (San Filippo et al., 2008; Daley et al., 2014; Kowalczykowski, 2015; Cannan and Pederson, 2016). Thus, identifying and targeting factors that specifically influence long-range homology search could reduce genomic rearrangements that are
hallmarks, and potentially drivers, of cancer. Understanding the mechanisms that influence DSB repair pathway choice, efficiency and outcome is crucial to developing cancer treatments, particularly for patients with germline or somatic mutations in DSB repair factors (Pennington et al., 2014; Xu et al., 2015; Cunniff et al., 2017).
Considerations for interrogating HDR

As is clear so far, HDR has been studied using varied model systems, assays and biological contexts. Each has distinct advantages and caveats for understanding the maintenance of genome integrity, the most relevant of which for my work are elaborated on here.

Influence of model system on repair pathway and relevance for disease

Much of the work on HDR described above is generalizable across model systems, but there are also distinct differences in repair pathway choice and efficiency. Budding yeast spend about equal periods of time in G1 phase as in S and G2 phases (Hartwell, 1974) so it is fitting that they are implemented for studies on both pathways (Haber, 2016; Talhaoui et al., 2016; Abugable et al., 2017). Human cell lines, another oft-used model system in the field, can vary in the relative proportions of their cell cycle phases based on cell type and speed of cycling, but G1 is generally much longer than G2 (Cooper GM, 2000). Correspondingly NHEJ is favored, to the point where a homology-directed pathway is thought by many to be used only when NHEJ proves unsuccessful (Her and Bunting, 2018; Shibata et al., 2018).

Conversely, fission yeast experience relatively short G1 and S phases, together typically lasting no longer than 25-30% of the cell cycle in normal conditions (Mitchison and Walker, 1959; Hoffman et al., 2015). Their entry into G2 is very early and coincident with cytokinesis, an example of the unique morphological correlations that prompted their use in early studies characterizing the cell cycle (Mitchison, 1957; Nurse, 1975, 1990). Spending the majority of
their cell cycle in G2 suggests that *S. pombe* favor HR and its associated homology-driven pathways over NHEJ, and this is supported experimentally (Goedecke *et al.*, 1994; Ferreira and Cooper, 2004; Raji and Hartsuiker, 2006). Furthermore, the repair pathways of *S. pombe* are conserved with more complex eukaryotes (Lehmann, 1996; Lambert *et al.*, 2003; Deshpande *et al.*, 2009).

Fission yeast have several added practical advantages. Thanks to the work of Urs Leupold, Murdoch Mitchison, Paul Nurse, Susan Forsburg, Paul Russell and many others, facile genetic tools and standards of cultivation have been developed to take advantage of the laboratorial strengths of fission yeast. These include their potential for haploid or diploid states, propensity to use HDR (resulting in efficient transformations and stable expression of tagged proteins) and ease and economy of growth in the lab (Hoffman *et al.*, 2015; Hayles and Nurse, 2016).

Studies in fission yeast HDR also have inherent relevance for clinical work in preventing human disease and cancer. Admittedly, to fully recapitulate HDR with a perfectly homologous donor at the sister chromatid would be ideal, but such a system is not conducive to visualizing or otherwise monitoring the kinetics of repair since no determinable sequence or structural DNA changes during the events in question could be detected. However, this is not the process of interest in a disease context. Given that human cells are prone to resort to HDR later in the repair process following unsuccessful NHEJ repair (as discussed above), the use of a homologous donor rather than the sister chromatid is actually more akin to repair events leading to LOH and promoting further genome instability. Fission
yeast also have the advantage over budding yeast of closer sequence homology and functional similarity of their repair proteins with those in humans (Koken et al., 1991; Ogawa et al., 1993; Monahan et al., 2008; Hoffman et al., 2015). Together, these characteristics indicate that fission yeast are an ideal model system in which to study HDR.

*A need for live cell DSB assays*

Much of this introduction has been spent describing our current understanding of HDR from several main types of experiments. The strength of biochemical assays in studying HDR is the ability to define specific activation and binding properties of repair factors, in relationship with each other and/or with ss- and dsDNA. Less evident from this research alone are the implications of these interactions within the complex nuclear environment of living cells. Similarly, *in vivo* studies in which the main readout is cell viability or a sequencing product of repair intermediates or outcomes have the advantage of assessing the ultimate efficacy of various repair conditions, but (in the case of cell viability) lose temporal information of processes during repair and (in the case of sequencing) are analyzed outside the nuclear context. Combined, these techniques are much more powerful, but still do not fully describe the dynamics between DSBs and their homology search partners during repair. Additionally, these methods are restricted to monitoring trends in populations of cells and often fail to capture differences between individual cells.

*In vivo* studies of HDR monitoring recruitment of repair factors to a DSB upon global (e.g. radiation, chemical) or site-specific (e.g. nuclease) damage
induction have also long been a staple of the field, and have also been informative as to roles for nuclear architecture in the DNA damage response, such as repression of HDR in heterochromatin (Chiolo et al., 2011; Ryu et al., 2015; Caridi et al., 2017). However, to date very few of these in vivo studies have monitored dynamics at the homologous donor sequence in mitotic DSB repair, the most notable of which was conducted in budding yeast at the mating type locus (Houston and Broach, 2006). As a facile research organism more reliant on HR than budding yeast, fission yeast is an advantageous system in which to develop an HDR assay in live cells to concurrently observe the donor sequence and DSB during repair, in order to better define the kinetics of the poorly understood homology search step described above.

To address this unmet need in the field, in this dissertation I describe the development of a microscopy-based assay in diploid fission yeast to determine the dynamics and kinetics of an engineered, interhomologue repair event. Although the initial distance between DSB and donor sequence predicts the time to their first physical encounter, it fails to predict the time to repair. Instead, repair efficiency is dictated by the number of strand invasion events, with most repair requiring multiple strand invasion cycles. In the absence of Rqh1, successful repair requires a single strand invasion event, suggesting that multiple strand invasion cycles reflect ongoing SDSA. Additionally, examining DSB repair in the absence of other factors that influence small RNA processing, chromatin mobility, and mismatch repair demonstrates the complexity inherent in regulation of these processes to promote efficient repair. Altering the donor sequence utilized or
observing a different sequence during repair also shift the dynamics of HDR in fission yeast. My work therefore reveals the spatial and temporal events that influence HDR outcomes in living cells.
Chapter 2: Developing a site-specific DSB assay in diploid fission yeast

Much of the work presented in this chapter is adapted from my manuscript in revision: Vines AJ, Cox KL, Leland BA and King MC, MBoC 2020. “Homology-directed repair involves multiple strand invasion cycles in fission yeast.”

A microscopy assay to study interhomologue repair in living fission yeast

In response to the need for more live cell assays to better understand HDR, in particular the poorly defined homology search step, I designed a microscopy-based system to induce site-specific DSBs in diploid fission yeast. I also conducted several tests to establish a baseline for DSB repair in WT fission yeast and demonstrate that my observations result from the DSB induction itself rather than spontaneous events.

Temporal and spatial control over DNA damage adjacent to mmf1

I employed the region surrounding mmf1 (S. pombe Chr II 3442679-3442191) (Figure 2) as a gainful location at which to induce DSB and monitor homology search and repair. Several factors contributed to this decision.

First, I strove to monitor a eukaryotic region of the genome that is within the nuclear interior to best allow for long-range homology search, as opposed to heterochromatic regions at the periphery of the nuclear envelope, which have been shown to downregulate HDR pathways (Chiolo et al., 2011; Jakob et al., 2011; Dion and Gasser, 2013; Lemaître and Soutoglou, 2015). According to chromosome conformation capture data and modeling, mmf1 is a centrally located gene in S. pombe nuclei (Tanizawa et al., 2010; Leland, 2017).
Chromosomally, it is located in a densely protein-coding region of Chr II. It is also far from major \textit{S. pombe} heterochromatin sites, just under 1.8MB downstream from the centromere, ~1.3MB downstream from the nearest mating-type locus and a little over 1MB upstream from the telomere (Wood \textit{et al.}, 2002).

Second, a well-understood locus within \textit{S. pombe} would be beneficial to improve upon previous studies of HDR rather than “reinvent the wheel” in terms of defining traits of repair pathways in a newly analyzed region. For that reason alone, the mating-type region (composed of several expressed and silent loci just over 2.1MB down \textit{S. pombe} Chr II) would be a top contender. Much of our early understanding of recombinational repair comes from the mating-type locus in \textit{S. cerevisiae} (Haber, 1995; Tsukuda \textit{et al.}, 2009b; Thon \textit{et al.}, 2019) since mating type switching results from repair of a DSB, and this is also true in \textit{S. pombe} (Klar \textit{et al.}, 1991; Arcangioli, 2000). However, recombinational repair events at mating-type loci may not be indicative of events at “typical” eukaryotic regions, given their (at least partially) heterochromatic nature and unique cell-cycle dependent regulation. In the King lab, our practical familiarity with the \textit{mmf1} region makes it a good candidate in a similar vein to the mating-type locus without the associated complications. Previously, we have analyzed HDR in live \textit{S. pombe} through visualizing resection at the \textit{mmf1} locus (Leland and King, 2014; Leland \textit{et al.}, 2018b). Through my work, we have furthered our understanding of HDR at this region from regulation of resection to homology search events near \textit{mmf1}.
With the choice of a locus made, I took advantage of two staples of HDR studies to induce and visualize a DSB and its donor sequence. First, site-specific endonucleases allow for spatial and, along with an inducible promoter, temporal control of DSB formation. Two of the most commonly-used nucleases to study HDR are HO and I-SceI, both derived from \textit{S. cerevisiae}. I-SceI undergoes site-specific cleavage to produce a DSB and remains active long after induction (Haber, 2016). HO endonuclease causes replication-associated DSBs during late G1/S phase (Nasmyth, 1993; Lisby \textit{et al.}, 2001) and can be induced quickly (within an hour) with systems such as the uracil-sensitive \textit{Purg}1 promoter (Watt \textit{et al.}, 2008; Watson \textit{et al.}, 2011), yet it is also speedily degraded following expression (Haber, 2002). This provides precise temporal control of DSB induction in concert with the cell cycle to promote HDR. I-Ppol, derived from the slime mold \textit{Physarum polycephalum} and seeing increasing use in DDR studies (Monnat \textit{et al.}, 1999; Sunder \textit{et al.}, 2012), is another option. However, there is an endogenous I-Ppol cut site in each of the \textasciitilde150 rDNA copies in \textit{S. pombe} (Sunder \textit{et al.}, 2012), making it impractical for viewing a single site-specific break in euchromatin without arduously removing these sites. Given this, HO best serves my purposes.

Second, the integration of bacterial operator arrays (e.g. \textit{lacO}, \textit{tetO}) and concurrent expression of their inhibitor binding partners (e.g. LacI, TetR) tagged with fluorescent proteins have greatly aided many fields of research, including HDR, in a variety of model systems (Tatebe \textit{et al.}, 2001; Bertram and Hillen, 2008; Rohner \textit{et al.}, 2008; Smith \textit{et al.}, 2015; Klein \textit{et al.}, 2019). The King lab
has developed methods for efficient integration of *lacO* repeats in fission yeast (Leland and King, 2014), and along with LacI-GFP expression I have utilized these methods to monitor specific gene loci in my system.

*A match made in New Haven: using diploid fission yeast to recapitulate interhomologue repair*

In order to monitor the timing and dynamics of long-range homology search, I took advantage of the *mat2*-102 mating type mutant of *S. pombe* (Egel, 1973; Bodi *et al.*, 1991) to generate stable diploids. This allowed me to monitor DSB repair that utilizes an endogenous donor on the homologous chromosome, which is preferable over ectopic cassettes, a frequent donor in HDR experiments as described in the introduction.

In all cases (unless described otherwise), one of the haploid strains (mated to make a diploid) contains a site-specific HO endonuclease cut site adjacent to the *mmf1* gene, expresses Rad52-mCherry and has a floxed marker at the *urg1* gene that facilitates efficient Cre-mediated integration of the HO endonuclease such that it is regulated by the uracil-regulated *urg1* promoter (Watson *et al.*, 2011) (Figure 2A). The other haploid strain has a 10.3 kb array of *lacO* repeats integrated adjacent to *mmf1* and expresses LacI-GFP (Figure 2B and Figure 3A). Cells therefore have a single GFP focus and a diffuse distribution of Rad52-mCherry in the absence of HO endonuclease expression when visualized by fluorescence microscopy (Leland *et al.*, 2018a) (Figure 3A).

Admittedly, there are caveats to using diploid strains for my system. During natural growth, *S. pombe* prefer to remain in a haploid state, so when mating to produce diploids they must be selected for. Upon mating *h*—with *mat2*-
I selected for stable diploids by plating to EMM-Ura+Nat. As a secondary confirmation measure, I screened imaged cells to confirm the presence of both diffuse Rad52-mCherry nuclear signal (and/or a Rad52-mCherry focus signaling a DSB) and a LacI-GFP focus before considering them for analysis.

Bryan Leland in our group showed previously in haploid cells that such a system induces a site-specific and irreparable DSB during S-phase on both replicated copies upon addition of uracil to the growth media (Leland et al., 2018a). In my diploid system, the induced DSB can undergo interhomologue repair (Figure 3B), with the DSB searching the nuclear volume and utilizing the homology near mmf1 on the lacO array-containing homologous chromosome as the donor sequence (Figure 3A). Corroborating what Bryan observed previously in haploid cells (Leland et al., 2018), in my diploid assay DSB induction and end resection lead to the recruitment of Rad52-mCherry, a proxy for the formation of the nucleoprotein filament that facilitates homology search and strand invasion, in ~15% of cells (Figure 3C).

**Spontaneous vs. HO-induced DSBs have unique characteristics**

While all cells display transient and dim Rad52-mCherry foci during S-phase (prior to cytokinesis), I hypothesized that the formation of a Rad52-mCherry focus at the site-specific DSB could be inferred by progressive and long-lived (>15 minutes) Rad52 loading induced at S phase. Indeed, cells without the induction of HO nuclease demonstrate only sporadic Rad52-mCherry loading (Figure 4A). The percent of frames (taken every 5 minutes) in which a Rad52-mCherry focus is observed is significantly higher for cells (n=47) with HO
nuclease induction than without (Figure 4B). This interpretation was further validated experimentally (see below).

WT DSBs largely demonstrate efficient homology search and faithful repair

An example of the time course of repair timing and chromatin dynamics within the 3D nuclear context is presented in Figure 5A. Images were acquired at 5 minute intervals for 3 hours after addition of uracil to induce expression of the HO nuclease. The lacI-GFP marking the donor sequence can be monitored throughout the movie. In this example, persistent Rad52-mCherry loading occurs at 40 minutes following nuclear division and persists up to 100 minutes following nuclear division (65 minutes total). Colocalization between the Rad52-mCherry loaded DSB and the donor sequence first occurs at 90 minutes post nuclear division, with Rad52 eviction 10 minutes later (100 minutes post nuclear division). The relationship between loss of a persistent Rad52-mCherry focus and repair was affirmed by monitoring subsequent cell division (see example, Figure 5B).

Additional examples of WT DSBs (Figure 6A-D) demonstrate cell-to-cell variety in timing of repair and colocalizations between the DSB and donor. Such variations in individual cells are less evident in population-based studies of DSB repair, especially those primarily monitoring genetic outcome.
Experimental design for the repair of a site-specific DSB in diploid fission yeast. A haploid strain with an (A) genotype and a haploid strain with a (B) genotype (both either WT or with deletions/modifications described in text and Table 3) were mated to make the diploid strain used for imaging as described in Methods. Other modifications to visualize DNA damage are described in the text and Figure 3A. Modified from a figure by Bryan Leland, approximately to scale.

(A) A recognition site for the HO nuclease is integrated near the *mmf1* gene on Chr II in *mat2-102* strains. (B) On Chr II in *h* strains there is a *lac* operator array integrated ~ 5 kb from *mmf1*.
Figure 3. A fission yeast model system to monitor homology search during interhomologue repair in single, living cells.

(A) Schematic (not to scale) of a diploid fission yeast nucleus with the modifications to Chr II described in Figure 2 before and after induction of a DSB. The other assay components include expression of LacI-GFP, Rad52-mCherry, and HO nuclease from the uracil-regulated urg1 promoter. (B) Interhomologue repair (mitotic recombination) is the dominant mode of homology-directed repair in diploid fission yeast. The proportion of cells expressing the HO endonuclease that undergo interhomologue repair, as determined by HO cut site marker loss assay (see Methods). Data from 8 biological replicates each containing between 50 and 200 colonies. (C) Proportion of WT cells with a site-specific DSB is similar to that seen previously (Leland et al., 2018a) for the inducible HO/urg1 expression system. Proportions of WT cells with a site-specific DSB from 5 technical replicates (4 separate inductions) (n > 200 per replicate except for a replicate of n = 101).
Figure 4. Induction of a site-specific DSB in WT cells during S phase has properties distinct from short-lived, non-specific DNA damage.

(A) Spontaneous DSBs are short-lived and occur at random times in the cell cycle in WT cells without HO nuclease expression. WT cells were prepared and imaged as if induced but without transformation of the plasmid containing the HO endonuclease. Each row represents one individual representative cell, and each circle represents a time point taken every 5 minutes. Time points shown are between 50 minutes before and 50 minutes following cytokinesis, denoting the beginning of G2 (the observation window for the first two cells were shorter than 50 minutes following cytokinesis). Blue circles: nucleus did not contain a Rad52-mCherry focus in that frame. Pink circles: nucleus contained a Rad52-mCherry focus in that frame. 

(B) WT cells with HO-induced DSBs have a significantly greater proportion of G2 frames with a Rad52-mCherry focus than WT cells with spontaneous DSBs. Frames in which cells were in G2 phase were analyzed for the presence of a Rad52-mCherry focus (only cells with at least one frame
with a Rad52 focus throughout the observation window were included). Data represent percentages from individual cells in G2 for at least 5 frames. ****p < 0.0001, Kolmogorov-Smirnov test of cumulative distributions (of percentages from individual cells). WT w/ HO: n=47, WT w/o HO: n=47.
Figure 5. Efficient homology search and subsequent repair during interhomologue repair in fission yeast.

(A) Representative cell undergoing repair of the HO-induced DSB (see Supplementary Figure 2 for additional representative cells). Below the images the events are indicated as blue circles (no Rad52-mCherry focus), pink circles (Rad52-mCherry focus present but not colocalized with the donor), or yellow (Rad52-mCherry focus present and colocalized with the donor). Contrast of Rad52-mCherry signal adjusted according to the full histogram of intensities where indicated. Scale bar = 1µm. (B) Cells observed to induce the site-specific DSB followed by repair reenter the cell cycle as indicated by subsequent cell division, validating successful repair. Representative example of a WT cell in which cell division is observed after repair of the HO-induced DSB. Time before and after nuclear division is noted for each frame, and cytokinesis is estimated to have followed the observation window by 5 to 10 minutes. Images were acquired every 5 minutes (columns) (see Methods for details). Contrast of Rad52-mCherry signal adjusted according to the full histogram of intensities where indicated. Scale bar = 1µm.
Figure 6. WT DSB cells exhibit a range of repair times, as well as number and length of colocalization events. (A-D) Representative cells undergoing repair of the HO-induced DSB. Repair times ranged widely in WT DSB cells (see Figure 9A). Additionally, cells might (A) exhibit multiple colocalizations during repair, (B-C) have single colocalizations with long periods of no colocalization, or (D) have no colocalization. Time of nuclear division was estimated based on cytokinesis in brightfield images (A-B), (D) or denoted as 0 minutes (C). Images were acquired every 5 minutes (columns) and are indicated relative to nuclear division (see Methods for details). Contrast of Rad52-mCherry signal adjusted according to the full histogram of intensities where indicated. Scale bar = 1µm.
Controls to support experimental design

As this system relies on inferring on-target, site-specific DSBs, I also carried out several controls to rigorously test whether the dynamics that I observe indeed reflect homology-directed repair and can be meaningfully interpreted. The \textit{rad51}\textDelta strain discussed below (and the additional KO strains discussed in Chapter 3) have the WT DSB induction system and are induced as described above and in Methods.

Establishing a baseline for \textit{mmf1} colocalization

First, I determined the likelihood that the two \textit{mmf1} loci would, at the diffraction limit of the light microscope, be found colocalized due to random fluctuations of the chromosomes in the absence of DSB induction. To this end, I, along with rotating graduate student Dahyana Arias, generated a diploid strain in which a \textit{lacO} array was integrated at both copies of \textit{mmf1} (Figure 7A-B) and assessed the frequency at which the two \textit{lacO} foci were found to be coincident. Under our imaging conditions, we found that the two \textit{lacO}-GFP-LacI foci cannot be resolved in \textasciitilde10\% of frames during G2 (the cell cycle stage when we monitor repair (Leland \textit{et al.}, 2018a), the majority of the \textit{S. pombe} cell cycle) (Figure 7C). This is in stark contrast to the analysis of an aggregated cohort of WT cells with DSBs (\textit{n}=21), in which Rad52-mCherry foci colocalized with the LacI-GFP-tagged donor sequence in \textasciitilde35\% of 5 minutes frames (Figure 7C). Thus, the majority of colocalization events between the Rad52-mCherry-loaded DSB and the donor sequence require the presence of the DSB.
Colocalizations are largely dependent on Rad51

To further test if the observed colocalization events are driven by strand invasion, I examined cells lacking Rad51 (see example, Figure 8A), which as discussed above is required for homology search and strand invasion during HDR. Colocalization events between the induced DSB and donor sequence were strongly attenuated in rad51Δ cells (Figure 7C), nearly to the level observed in the absence of damage in the control 2 lacO cells (Figure 7C) despite persistent Rad52-mCherry loading. This suggests that most encounters between the DSB and donor sequence occur due to homology search via Rad51. I also analyzed the relative time of DSB-donor sequence colocalization in individual cells in all three conditions: WT, 2 lacO and rad51Δ (Figure 8B). I observe that colocalization events in control 2 lacO cells without DNA damage and rad51Δ cells with DSB induction are short-lived compared to a broad distribution of lifetimes in WT cells, a conclusion reinforced by the difference in cumulative probability of colocalization frequency (Figure 8C).
Figure 7. Colocalization of the DSB and donor sequence is driven by DSB formation.

(A) Experimental design for monitoring of *mmf1* at both homologous chromosome II loci in the absence of DSB induction (2 lacO at *mmf1* background). On both copies of Chr II there is a lac operator array integrated ~ 5 kb from *mmf1* (see Figure 2B and Methods), and lacI-GFP is expressed to visualize both homologs. The DSB was not induced with the HO/urg1 system. (B) Chr II homologs near the *mmf1* gene undergo minimal colocalization in the absence of an induced DSB. Z stack images of a nucleus from a representative 2 lacO at *mmf1* cell (see Methods and Figure 7A). Imaged as described in Methods, with 5 minutes between each time frame (columns) and labeled relative to nuclear division. Contrast adjusted to the full histogram of intensities where indicated. Scale bar = 1 µm. (C) Colocalization of homologs near *mmf1* is largely dependent on DSB induction and Rad51. Frames in which cells were in G2 phase were analyzed for colocalization of the DSB and donor (for WT and *rad51*Δ (see Figure 8A), only cells judged to have persistent, site-specific DSBs (see Methods) were included) and averaged as a total percentage across all cells. Colocalization is that of the DSB (Rad52-mCherry) and donor sequence (LacI-GFP bound to lacO repeats at *mmf1*) (WT
and rad51Δ) or both Chr II homologs in the absence of damage (2 lacO at mmft). *p < 0.05, ****p < 0.0001, Kolmogorov-Smirnov test of cumulative distributions (of percentages from individual cells). WT: n=26, 2 lacO at mmft: n=129, rad51Δ: n=23.
Figure 8. Colocalization of the DSB and donor sequence is largely Rad51-dependent.

(A) The DSB induced by HO endonuclease is persistent in rad51Δ cells. Z stack images of a representative rad51Δ induced cell imaged with 5 minutes between each time frame (columns) and labeled relative to nuclear division. Contrast of Rad52-mCherry signal adjusted according to the full histogram of intensities where indicated. Scale bar = 1µm.

(B-C) Colocalization between the DSB and donor sequence is far more prevalent in WT cells than in rad51Δ cells or for cells with two lacO arrays at mmf1 in the absence of damage. (B) Relative frequency histograms of percentages of G2 frames with colocalization in individual 2 lacO at mmf1 control cells (n = 129), rad51Δ DSB cells (n = 23), and WT DSB cells (n = 21) (>5 G2 frames per cell). Colocalization is for the DSB (Rad52-mCherry) and donor sequence (LacI-GFP bound to lacO repeats at mmf1) (WT and rad51Δ) or both Chr II homologs in the absence of damage (2 lacO at mmf1). (C) Cumulative frequency histograms of data in Figure 8B.
Evidence in WT supporting a tendency for multiple encounters during HDR

Timing of DSB repair in WT fission yeast

The characteristic time required to successfully orchestrate HDR is fission yeast is still an outstanding question. Near complete recovery of a site-specific I-Ppol-induced DSB at a “generic” locus or at the rDNA repeats in fission yeast took place within 4 hours as measured by qPCR (Ohle et al., 2016), but no data was reported for times less than 4 hours. Other assay systems often employ donor sequences with only short-range homology to the DSB or utilize a mini-chromosome (as described in the introduction).

By contrast, here I monitor repair between true homologous chromosomes. In addition, here I specifically monitor the time from the onset of long-range DSB end resection (~125 resected bps) (Leland et al., 2018a) to the eviction of Rad52, which corresponds more closely to the period of homology search. Consistent with this, I find that repair as defined here is highly efficient. The mean time to repair is ~50 minutes, although there is substantial cell-to-cell variation with a standard deviation of ~20 minutes (Figure 9A).

A site-specific DSB promotes multiple encounters with the homologous donor

My initial expectation was that repair time corresponds to a single colocalization event reflecting strand invasion of the donor sequence by the DSB, new synthesis and ultimate repair. In this case, I would expect that (1) the time to the first encounter and the time to repair are correlated, if not equivalent, and (2) the initial distance between the loci and the time to repair are correlated (Lee et al., 2015a). However, in my assay I observed that the time to the first encounter
and the time to repair are not correlated (Figure 9B). Additionally, I found that the initial distance between the DSB and donor sequence does not correlate with repair time (Figure 9C), suggesting that an encounter per se is not the rate-limiting factor of homology search leading to repair.

Instead, I frequently observe multiple colocalization events in individual cells over the course of DSB repair (Figure 10A). I therefore examined if the initial distance between the DSB and donor sequence correlates with the time to the initial colocalization event. Indeed, my analysis confirmed such a relationship (Figure 10B).

Given that most colocalization events are Rad51-dependent (Figure 7C and Figure 8B-C), I infer that many cells undergo multiple strand invasion events between the DSB and donor sequence prior to repair. If true, I would expect repair time to be tied to the number of strand invasion events. Indeed, I observe a positive correlation, supporting this interpretation (Figure 10C).

*Frame rate impacts on number of encounters observed*

Ideally, I would observe these events with no lost time between images to fully understand these dynamics, though technical challenges prevent this. The somewhat arbitrary time scale for imaging of 5 minutes between each frame was chosen to allow for periodic monitoring of DSB and donor dynamics over the total time course of repair while balancing the effects of photobleaching on the fluorophores, mCherry in particular. This time interval also maximizes the number of fields that can be sequentially imaged on the microscope. However, in the course of 5 minutes I could be missing encounters in those cells for which no
DSB-donor interactions were observed. I wondered whether I would observe more and/or longer individual encounters between the DSB and donor if the cells were imaged at shorter time intervals.

When WT DSB cells are imaged at 2 minute intervals (2.5 times more frequently), I do observe a shift (of ~ 1 encounter per 30 minute window) toward more colocalization events with the donor sequence (Figure 11A-B). However, I also observe that more sampling leads to an increase in random encounters as revealed by similar analysis of the 2 lacO at mmt1 uninduced control strain (Figure 11A-B). This suggests that I may be missing some transient encounter events while using 5 minute time points, so in WT cells where I observe no colocalizations at 5 minute intervals (e.g. Figure 6D) there may still be encounters that are too brief or transient to be resolved at that frame rate. These 30 minute snapshots were obtained early in G2 to maintain consistency of cell cycle/DSB induction timing across cells with a spectrum of repair times, so there may also be more encounters later in G2 in some cells upon further DSB processing.

Together, this work demonstrates that I have developed a system to monitor Rad51-dependent repair events following resection of site-specific DSBs in live diploid fission yeast. During this repair, I observed encounters between the DSB and donor that are largely damage induction- and Rad51-dependent. In Chapter 3, I describe my findings from repair events in strains containing HDR mutants or modifications of the donor sequence with the aim of better understanding regulation of these homology search events.
Figure 9. HDR in fission yeast is efficient relative to previous estimates and does not support the correlation of a single colocalization event with repair.

WT cells were imaged as described in Methods. Data points represent individual cells.

(A) Repair of WT fission yeast cells is highly efficient in my induced DSB system. Time to repair was measured as the time in minutes from the first appearance of a site-specific DSB (persistent rad52-mCherry focus) to its disappearance for at least three consecutive frames (5 minute intervals, n = 25). Mean = 51.2, standard deviation = 19.4.

(B) Timing of the first encounter between the DSB and donor sequence and timing of repair are not correlated. Time to first encounter is the difference between the first frame when Rad52-mCherry is visualized and the first colocalization event. Time to repair was measured as in Figure 3A. Linear regression: p value = 0.3757, R^2 = 0.04641 (n = 16).

(C) Timing of repair and initial distance are not correlated. Initial distance between the DSB and donor sequence was measured as the 3-D distance between the centers of the Rad52-mCherry (DSB) and lacI-GFP (donor) foci in the first frame after appearance of a site-specific (persistent) Rad52-mCherry focus. Time to repair was measured as in Figure 3A. Linear regression: p value = 0.3498, R^2 = 0.03809 (n = 24).
Figure 10. HDR in fission yeast frequently involves multiple encounters between the DSB and donor sequence.

WT cells were imaged as described in Methods. Data points in (B-C) represent individual cells. 

(A) Many WT DSB cells experience multiple colocalization events during repair, with variability in repair timing as well as number and length of colocalizations. Graph of colocalization events of Rad52-mCherry (DSB) and lacI-GFP (donor) foci in representative WT DSB cells. Each row represents one individual cell, and each circle represents a time point taken every 5 minutes. Blue circles: time from nuclear division to Rad52-mCherry loading. Pink circles: time from Rad52-mCherry loading to unloading for at least 3 consecutive frames. Yellow circles: Colocalization of the DSB (Rad52-mCherry) and donor (LacI-GFP bound to lacO repeats at mmf1) foci. See Figure 6 for additional representative cells. (B) Timing of the first encounter between the DSB and donor sequence is correlated with their initial distance. Time to first encounter is the difference between the first frame when Rad52-mCherry is visualized and the first colocalization event. Initial distance was measured as in Figure 9C. Linear regression: p value = 0.0126, R² = 0.3141 (n = 18). (C) The number of individual encounters is
correlated with the timing of repair in individual cells. # of visualized encounters indicates
the number of separate encounters (one or more consecutive frames (at 5 min. intervals)
with colocalization) between the DSB and donor. Time to repair was measured as in
Figure 9A. Linear regression: p value = 0.0109, R^2 = 0.25 (n = 20).
Figure 11. Preliminary more frequent imaging of WT DSB and 2 lacO at mmf1 cells reveals an increase in observed encounters.

(A) Number of encounters within 30 minute windows in early G2 from individual cells (during a DSB in WT cells). WT 2 min: n = 7, WT 5 min: n = 28, 2 lacO at mmf1 2 min: n = 24, 2 lacO at mmf1 5 min: n = 103. *p<0.05, n.s. = not significant, Kolmogorov-Smirnov test of cumulative distributions.

(B) Comparison of cumulative frequency of encounters within 30 minute windows in early G2 from individual cells (during a DSB in WT cells). Same n values as in (A).
Chapter 3: Impact of repair factors and donor on fission yeast DSB repair

 Portions of the work presented in this chapter are adapted from my manuscript in revision: Vines AJ, Cox KL, Leland BA and King MC, MBoC 2020. “Homology-directed repair involves multiple strand invasion cycles in fission yeast.” The strains discussed in this chapter have the WT DSB induction system and are mated and induced as described above and in the Methods, with no other modifications unless delineated in the text.

Roles of an anti-recombinase in HDR

The outcome of homology search is also impacted by the regulation of strand invasion by the nucleoprotein filament as it samples potential templates. Factors such as the BLM helicase (Sgs1 in S. cerevisiae and Rqh1 in S. pombe) are thought to dissolve D-loops, thereby driving non-crossover repair events (Hope et al., 2007; Oh et al., 2007; Lorenz et al., 2014). Rqh1 likely promotes non-crossover products by favoring synthesis-dependent strand annealing (SDSA), in which (as described in Chapter 1) strand invasion leads to new synthesis followed by dissolution of the D-loop, strand annealing that spans the initial DSB site, and repair (Symington et al., 2014; Symington, 2016). However, direct observation of this Rqh1 activity has not yet been possible.

Based on the prevalence of multiple encounters between the DSB and donor sequence and variability in repair timing, I next considered whether these kinetics reflect anti-recombination pathways that enforce HDR fidelity and/or non-crossover repair by SDSA. To address this, I tested the impact of deleting the S.
*pombe* RecQ helicase, Rqh1, orthologous to human BLM. On a whole across various model systems, RecQ helicasess such as Rqh1 act in several capacities during HDR. Rqh1 is established to dissolve D-loops (Van Brabant *et al.*, 2000; Bachrati *et al.*, 2006; Hope *et al.*, 2007) and also contributes to DSB end resection in some contexts (Nanbu *et al.*, 2015; Yan *et al.*, 2019). However, Bryan Leland in the King Lab previously demonstrated that Rqh1 is dispensable for end resection in fission yeast DSB cells (Leland *et al.*, 2018a). Thus, the primary role(s) for Rqh1 in fission yeast HDR are downstream of resection and likely involve regulation of strand invasion structures.

*Cells lacking Rqh1 display a bimodal repair phenotype*

In cells lacking Rqh1, I observe two distinct repair outcomes. In one subset of cells I observe very rapid repair (Figure 12A), while in another I observe highly persistent DSBs (Figure 12B). Indeed, the rate of productive repair within 90 minutes of initial Rad52-mCherry loading falls from over 65% in WT cells to ~40% in *rqh1Δ* cells (Figure 13A), suggesting that loss of Rqh1 negatively impacts repair as a whole. However, *rqh1Δ* cells that execute repair do so faster on average than for WT cells (Figure 13B).

*DSBs in *rqh1Δ* cells often repair after a single encounter with the donor*

Given Rqh1’s role in D-loop disassembly, I next examined if the more rapid repair reflected a higher likelihood that a strand invasion event leads to repair. Indeed, I observe far fewer encounters between the DSB and donor in *rqh1Δ* cells that successfully repair, both in the population as a whole (Figure 13C) and within individual cells, where I often fail to visualize colocalization prior
to repair within the 5 minute frame rate (Figure 13D) (though data from observing WT DSBs at 2 minute intervals (Figure 11A-B) suggests these encounters could be occurring faster than can be resolved at 5 minute intervals). Thus, repair in \(rqh1\Delta\) cells is bimodal, being either more efficient than in WT cells (often involving a single colocalization event) or failing entirely within my experimental observation window.

**Implications for HDR and Bloom syndrome**

Based on the above, I suggest that the observed dissolution of D-loops by Rqh1 in my system likely reflects its contribution to promoting repair by SDSA. It may also facilitate rejection of strand invasion intermediates with non-homologous or homeologous sequences; the latter could explain why I often observe concomitant repair failure and lack of colocalization events in cells lacking Rqh1 (Figure 14A-B). Indeed, expression of mutated forms of Sgs1 (the ortholog of BLM and Rqh1) abrogated colocalization events between a DSB and the repair template in budding yeast (Piazza *et al*., 2017). More broadly, new insights into the highly transient nature of D-loop processing in budding yeast (Piazza *et al*., 2019) support the possibility of short-lived encounters that are regulated by Rqh1.

In patients with mutations in BLM, mitotic and meiotic crossover events are greatly increased, leading to genome instability and cancer predisposition among other symptoms (Brosh, 2013; Arora *et al*., 2014). My observations suggest that defects in the ability to promote non-crossover repair by SDSA combined with an accumulation of dead-end repair intermediates could both
contribute to disease etiology, consistent with the observation that mutated alleles of Rqh1 lead to the “cut” phenotype in fission yeast treated with DNA damaging agents (Stewart et al., 1997).

Recent work from other groups to define antagonists of Rqh1 further supports my findings that the strand dissolution function of Rqh1 in HDR is crucial for faithful repair. For instance, Rad9(53BP1/Crb2)-mediated antagonism of the budding yeast BLM/Rqh1 ortholog Sgs1 results in increased crossover products of HDR (Ferrari et al., 2020), and the histone chaperone chromatin assembly factor 1 (CAF-1) opposes Rqh1 at stalled replication forks to promote template switching (Pietrobon et al., 2014). It will be intriguing to further examine these and other regulators of Rqh1 activity in live cell fission yeast homology search.
Figure 12. DSB cells lacking Rqh1 display a bimodal repair phenotype.  
(A) Successful repair events are often relatively short in *rqh1Δ* DSB cells. Z stack images of a representative *rqh1Δ* DSB cell nucleus showing productive repair (persistent loss of Rad52-mCherry signal for at least 3 frames). Imaging as described in Methods with 5 minutes between each time frame (columns). Contrast of Rad52-mCherry signal adjusted to the full histogram of intensities where indicated. Scale bar = 1µm. (B) Failure to repair DSBs efficiently is more prevalent in *rqh1Δ* cells with the induced DSB. Z stack images of a representative *rqh1Δ* DSB cell nucleus showing repair failure (persistence of Rad-52mCherry signal >90 min). Imaging as described in Methods with 5 minutes between each time frame (columns). Contrast of Rad52-mCherry signal adjusted according to full histogram of intensities where indicated. Scale bar = 1µm.
Figure 13. DSB cells lacking Rqh1 have fewer encounters between the DSB and donor during repair.

(A) Cells lacking Rqh1 are less likely to undergo efficient DSB repair than WT cells. Total percentage of WT (n = 37) and rqh1Δ (n = 37) cells with an induced DSB that repair within 90 minutes. (B) Cells lacking Rqh1 have much shorter successful repair times. Stacked (no data hidden) relative frequency histograms of time to repair (10 minute bins) in WT cells (pink, n = 21; see Figure 3A) and rqh1Δ cells (orange, n = 16) with the induced DSB. (C) Cells lacking Rqh1 have a significantly smaller proportion of G2 frames with a colocalization per cell compared to WT. Quantification of colocalization of DSB (Rad52-mCherry) and donor sequence (LacI-GFP bound to lacO repeats at mmf1). Frames in which cells were in G2 phase were analyzed for colocalization of DSB and donor (for WT, rad51Δ, and rqh1Δ, only DSB cells were included) and assembled as a total percentage across all cells. Colocalization is that of the DSB (Rad52-mCherry) and donor sequence (LacI-GFP bound to lacO repeats at mmf1) (WT and rad51Δ) or both Chr II homologs in the absence of damage (2 lacO at mmf1). *p < 0.05, ****p < 0.0001, Kolmogorov-Smirnov test of cumulative distributions (of percentages from individual cells). WT: n=26, 2 lacO at mmf1: n=129, rad51Δ: n=23, rqh1Δ: n=37. (D) Cells lacking Rqh1 have significantly fewer encounters between the DSB and donor per cell relative to WT. # of encounters per repair indicates the number of separate encounters (one or more consecutive frames at 5 min. intervals with colocalization) between the DSB and donor in individual WT (n = 25) or rqh1Δ (n = 10) cells. *p = 0.0143, Kolmogorov-Smirnov test of cumulative distributions.
**Figure 14. Model for Rqh1 influence on homology search dynamics in *S. pombe* HDR.**

Arrow weight indicates relative likelihood of sustained strand invasion or repair outcome. Arrow length indicates relative speed of heteroduplex extension or time to repair outcome.

(A) In WT cells, Rad51 drives strand invasions and Rqh1 antagonizes them for all potential donors. Rad51 promotion of heteroduplex extension is stabilized at a homologous sequence, while Rqh1 dissolution activity is stronger in the absence of homology. Therefore, many cells experience faithful repair, but at the cost of sometimes invading a template multiple times before completion of synthesis. (B) In *rqh1Δ* cells, Rad51 is not antagonized by Rqh1 and is more prone to persist at both homologous and non-homologous sequences. This results in faster repair upon invading homologous sequences, but also a propensity to strand invade non-homologous sequences for greater unproductive periods, leading to the bimodal repair phenotype I observe in these cells.
Small RNA processing in DSB repair

Small RNAs with a regulatory function directed toward DNA are most often discussed in the context of RNA interference (RNAi) pathways present in many eukaryotes (Carthew, 2001; Bailis and Forsburg, 2002; Grosshans and Slack, 2002; Kalantari et al., 2016). Briefly, RNA produced from a genomic site destined for down-regulation is cleaved by the exonuclease Dicer into ~22nt long dsRNA segments called microRNA (miRNA). These segments are then loaded into Argonaute, a component of the RNA-induced silencing complex (RISC). This complex mediates sequence-specific silencing by interference with or destruction of mRNA corresponding to the dsRNA loaded into Argonaute. In *S. pombe*, this pathway is primarily involved in heterochromatin formation in addition to gene silencing (Martienssen et al., 2005; Martienssen and Moazed, 2015; Chang et al., 2012; Goto and Nakayama, 2012; Bhattacharjee et al., 2019). Single-copy genes of the main RNAi pathway genes, including Dicer, have facilitated the study of this pathway in *S. pombe*, and have also facilitated my study of these factors in HDR.

StRNAger Things: the multifaceted roles of RNA in HDR

Among the many outstanding mysteries in the field of HDR, and in particular the mechanisms of homology search between homologous chromosomes or other distant loci, two of the most compelling are the means by which a homologous sequence is so quickly identified and utilized by repair factors, as well as the molecular requirements for accessing the donor sequence. These ambiguities could both be addressed at least in part by a diffusible...
mediator between the DSB and donor sequence with the capability of competing with the partner DNA strand for binding. The most compelling candidate for such a mediator is non-coding RNA (ncRNA) produced at the DSB by spurious transcription (Wei et al., 2012) – this possibility is attractive due to sequence specificity for targeting to the donor sequence, and indeed has been a growing topic of investigation for the last decade. Such RNA goes by several names based on the context of their discovery and activity (ddRNA, diRNA, etc.) so for simplicity I will use small RNAs or ncRNA as catch-all terms.

Direct interaction of RNA with DNA at DSB sites can drive recombination and/or repair in S. cerevisiae (Keskin et al., 2014; Onozawa et al., 2014; Sollier and Cimprich, 2015), and a role for ncRNA produced at the DSB in promoting HR has been demonstrated in both Arabidopsis (Wei et al., 2012) and mammalian cells (Francia et al., 2012). In this context, Dicer, the endonuclease responsible for processing dsRNA into miRNA, also supports DSB repair in zebrafish, Drosophila, and human cells (D'Adda di Fagagna, 2014). Additionally in human and murine cells, Dicer is phosphorylated upon DNA damage and accumulates at DSBs (Burger et al., 2017; Burger and Gullerova, 2018). RNA transcripts maintained on chromatin may also contribute to homologous pairing during meiosis (Ding et al., 2012), and a study in S. cerevisiae even invoked RNA as a potential template for HR instead of DNA (Keskin et al., 2014).

Evidence for early loss of cohesion at donor sequence during repair

As described above in Chapter 1, loss of cohesion between sister chromatids may be favorable for DSB repair involving long-range homology
search to provide greater access to the Rad51-Rad52-ssDNA nucleoprotein filament. In preliminary studies of a DSB repair event at ade3, the King lab found evidence for loss of cohesion (i.e. resolution of two LacI-GFP foci rather than one) at the donor sequence chromosome copy prior to colocalization of the donor with the DSB (data not shown). Control sites in other loci non-homologous with the DSB did not undergo this same cohesion loss (data not shown). Additionally, in my system, loss of cohesion is largely dependent on DSB induction and Rad51 (Figure 15A). Together, this work suggests that there is a diffusible factor that enables communication between the DSB site and donor sequence distinct from the deposition of γH2A and recruitment of other repair factors (Renkawitz et al., 2013). We hypothesized that this mediator could be small RNAs produced adjacent to the DSB and that this site-specific loss of cohesion could facilitate long-range homology search by making the homologous dsDNA more accessible to the Rad51 nucleoprotein filament.

**Loss of cohesion and delayed early homology search in the absence of Dicer**

Because of the requirement in some organisms for small RNAs produced at a DSB to be processed by Dicer for their activity in HDR regulation, I sought to better understand the role of small RNAs in my system by knocking out Dicer (dcr1 in *S. pombe*) in my WT DSB assay. In doing so, I hypothesized that loss of cohesion at the donor upon DSB formation, if it is indeed reflective of the activity of small RNAs processed by Dicer, would decrease relative to WT. I also surmised that if this small RNA activity at the donor was the primary means of Dicer contributing to an efficient long-range homology search and repair, the time
to the first encounter would increase while the total number and/or longevity of encounters would decrease, leading to an increase in the time to repair.

When I knocked out Dicer (dcr1) in my system, I did see a significant decrease in the number of individual loss of cohesion events during a DSB, denoted by consecutive frames in which two LacI-GFP foci are resolvable (Figure 15B). However, the overall number (Figure 15A) and cumulative probability (Figure 15C) of G2 frames showing loss of cohesion during repair is comparable to WT. Together with the above data, this suggests less frequent but potentially longer loss of cohesion events.

*Time to repair and mobility are largely unaltered in dcr1Δ DSB cells*

Similar to WT (Figure 10A), dcr1Δ DSBs often exhibit multiple encounters between the DSB and donor (Figure 16A), thus homology search as a whole still seems efficient. I do observe DSBs earlier (relative to nuclear division) in dcr1Δ cells than in WT (Figure 16B), suggesting faster resection and/or a reduction in barriers to Rad52-mCherry loading. Similar early loading of Rad52 upon replication stress-induced DNA damage was observed previously in dcr1Δ fission yeast (Zaratiegui *et al.*, 2011). Earlier loading of repair factors to form a nucleoprotein filament might be expected to facilitate a more efficient homology search in the absence of Dicer.

However, the time to the first encounter is overall not significantly different (though it approaches significance with longer times in dcr1Δ cells) between dcr1Δ and WT cells (Figure 16C). At the same time, far fewer dcr1Δ DSB cells than WT DSB cells exhibit very early colocalization in the first observable frame.
Together, these observations suggest a less efficient early homology search. As a whole however, this does not seem to bear greatly on overall repair efficiency, as there is no significant difference in dcr1Δ and WT DSB repair times (Figure 16E). This is not entirely unexpected given the lack of correlation between early encounter and repair times in WT (Figure 9B), but still raises the question of what role then, if any, Dicer has in regulating these aspects of HDR in fission yeast.

Through its RNAi activity, Dicer is known to alter nucleosome modifications, which influence chromatin state and mobility (Matzke et al., 2004; Zentner and Henikoff, 2013; Gursoy-Yuzugullu et al., 2016; Gutbrod and Martienssen, 2020). Its loss can result in lagging chromosomes during mitosis and meiosis correlated with defects in cohesin recruitment (Hall et al., 2003; Gutbrod and Martienssen, 2020), and loss of cohesin promotes chromatin mobility (as described in Chapter 1). Additionally, if Dicer-processed RNA:DNA hybrids forming at the DSB and/or donor sequence were contributing to a more efficient homology search, there may be an associated increase in mobility between these loci. To assess these potential changes in mobility upon loss of Dicer in my system, I measured the 3D distance between the DSB (Rad52-mCherry focus) and the donor sequence (LacI-GFP focus) during early time points in both WT and \textit{dcr1Δ} DSB cells. As may be expected, there is no shift in the distance between the DSB and donor in the first frame when Rad52-mCherry is observed when examining \textit{dcr1Δ} versus WT cells (Figure 17A). Despite a slight shift toward less overall mobility, there is also no significant difference
between absolute changes in DSB and donor mobility between the first and second Rad52-mCherry frames in these two groups (Figure 17B). The mobility of these loci could still have directionality (e.g. the WT loci move closer together while the dcr1Δ loci move farther apart, or vice versa). However, that is not the case when I examine the distance changes as positive (farther apart in the second frame than the first frame) or negative (closer together in the second frame than the first frame), either in WT or dcr1Δ DSB cells (Figure 17C). In sum, these data suggest that *S. pombe* Dicer has no great effect on chromatin mobility in the context of early DSB repair events, or such effects may come into play later during repair. Alternatively, time intervals shorter than 5 minutes may be required to observe distinct mobility shifts, or observing a heterochromatic DSB where RNAi-responsive cohesin recruitment and chromatin modifications are more prevalent may result in greater mobility changes.

*A nuanced role for Dicer in fission yeast HDR*

Given the demonstrated roles for small RNAs in faithful HDR in other organisms, often requiring Dicer activity, I was surprised to see a more subtle effect upon loss of Dicer in my diploid fission yeast assay. Prior to the start of my graduate work there were no comparable studies of the effects of small RNA regulation during HDR in fission yeast. However, in 2016, Ohle and colleagues demonstrated that transient RNA:DNA hybrids can regulate HR in fission yeast. In the absence of RNase H which specifically degrades RNA:DNA hybrids, RPA recruitment to ssDNA following end resection is largely blocked due to persistent hybrids, while RNase H overexpression allows promiscuous resection, resulting
in sequence loss adjacent to DSBs (Ohle et al., 2016). Upon DSB formation via I-Ppol (which produces ~150 endogenous DSBs in S. pombe rDNA repeats, in addition to an engineered site within euchromatin on Chr II), there was a mild reduction in survival in dcr1Δ (~50% survival) compared to WT (~75% survival) (Ohle et al., 2016). Though this result was not interrogated further in the paper, it does suggest an effect of Dicer on long-term resolution of DNA repair resulting in cell death. For instance, in the presence of copious resection upon RNA:DNA hybrid loss, HDR could be directed toward alternate, less faithful pathways for resolution such as BIR. In my system and the above, it may also be that such RNA:DNA hybrids are only partly regulated by Dicer. The presence, or at least HDR activity, of RNA:DNA hybrids at DSBs in S. pombe has been contested in the context of reparable damage from replication fork collapse (Zhao et al., 2018), which is similar to my assay in that HO endonuclease also causes replication-associated DSBs during late G1/S phase (Nasmyth, 1993; Lisby et al., 2001).

One possible explanation for the subtle response to Dicer loss in my assay is the multiplicity of roles for small RNAs in DDR. Recently, a wide array of regulatory roles for small ncRNA in HDR have been proposed and discussed (D’Adda di Fagagna, 2014; Michelini et al., 2018; Thapar, 2018; Puget et al., 2019). For instance, the mere presence of RNA:DNA hybrids formed by ncRNA at resected ssDNA could be protective and promote HR over NHEJ (Aguilera and Gómez-González, 2017). It has been demonstrated in fission yeast that RNA:DNA hybrids compete with binding of RPA to resected DNA (Ohle et al.,
2016). Rad52-mCherry recruitment to the DSB appears to be faster in dcr1Δ cells in my system (Figure 16B), so if such RNA:DNA hybrids are dependent on Dicer their removal by loss of Dicer could facilitate faster RPA and subsequent Rad52 binding. This would not fully explain the reduction in viability upon Dicer loss in Ohle et al. however, unless accelerated repair kinetics are refractory to successful HDR in this case. Contrary to the notion that RNA:DNA hybrids prevent repair factor binding, Dicer-processed DNA damage induced RNAs in mammalian cells have also been implicated in recruitment of the DDR signaling modification γH2A.X and its dependent repair factors (Francia et al., 2016) as well as Rad51 (Gao et al., 2014) to DSBs rather than solely competing for binding. RNAs transcribed from and/or adjacent to DSBs and other types of DNA damage like ROS may also contribute to repair factor recruitment through their posttranscriptional modifications such as m⁵C (Chen et al., 2020) or m6A (Zhang et al., 2020). However, a similar phenomenon dependent on Dicer-processed RNAs is unlikely in my system due to the robustness of DSB induction and repair in dcr1Δ cells relative to WT.

Another possibility (not mutually exclusive with the above) is that longer ncRNAs that do not require Dicer processing are contributing to repair. As study of these regulators of gene expression increases, a number of specific long non-coding RNAs (lncRNAs) have been implicated in DSB repair through HR and NHEJ, and their loss or overexpression is correlated with genome instability and cancer (Sharma et al., 2015; Dianatpour and Ghafouri-Fard, 2017; Michelini et al., 2017; Dangelmaier et al., 2019). Beyond these particular examples, long non-
coding RNAs transcribed from the DSB itself (dubbed dilncRNAs) have also been shown to promote DSB repair through recruitment of repair factors or small RNAs, for which they can also act as precursors (D’Alessandro et al., 2018; Caron et al., 2019; Domingo-Prim et al., 2020). However, as with small RNAs, prolonged accumulation of dilncRNAs can be detrimental, such as by promoting hyperresection of DSBs (Domingo-Prim et al., 2019), so their regulation is vital for successful repair. So far, these studies have primarily been conducted in mammalian cells, so it will be interesting to test the involvement of IncRNAs in fission yeast HDR.

It would be intriguing to examine in my system the effects of knocking out Dicer, or other RNAi and RNA:DNA hybrid regulators such as RNase H, along with removing other HDR factors. A recent preprint from the Legube group suggests that human BLM promotes BIR over HR when R loops formed by RNA:DNA hybrids are unable to be dissolved (Cohen et al., 2020), so the careful regulation of hybrid formation and dissolution is important for repair outcome. The choice of chromosomal location at which to study DSB repair upon Dicer loss could also influence the results in my system. For instance, Dicer appears to antagonize HR at rDNA and highly transcribed euchromatic genes by release of Pol II from sites of replication to prevent fork stalling (Castel et al., 2014). Studies such as these highlight the importance of cooperation between disparate HDR components in order to foster error-free rather than error-prone repair in a variety of contexts.
In conclusion, there is still much to learn about the varied and sometimes seemingly contradictory roles of RNA in HDR, including Dicer processing during DSB repair in fission yeast. I am excited that Aly Laffitte, a current King Lab graduate student, is following up on this work by pursuing potential roles for Dicer in fission yeast end resection. Her work and that of others in in vivo systems will shed much-needed light on these complex interactions across genome maintenance mechanisms.
Figure 15. Loss of cohesion events are largely dependent on DSB induction and Rad51, and are fewer but longer in dcr1Δ compared to WT. (A) Quantification of loss of cohesion between sister chromatids at donor sequence (two resolvable LacI-GFP foci bound to lacO repeats at mmf1). Frames in which cells were in G2 phase were analyzed for loss of cohesion (for WT, rad51Δ, and dcr1Δ, only DSB cells were included) and assembled as a total percentage across all cells. n.s. = not significant, *p < 0.05, ****p < 0.0001, Kolmogorov-Smirnov test of cumulative distributions (of percentages from individual cells). WT: n=24, 2 lacO at mmf1: n=129, rad51Δ: n=22, dcr1Δ: n=12. (B) Percentages of individual cells with 0, 1, 2, or 3+ loss of cohesion events (one or more consecutive frames with loss of cohesion) during time of Rad52-mCherry loaded at DSB (Δt\text{repair}). Loss of cohesion is that between sister chromatids at donor sequence (two resolvable LacI-GFP foci bound to lacO repeats at mmf1). *p < 0.05, chi square test of observed and expected distributions. WT: n=20, dcr1Δ: n=10. (C) Relative frequency histograms of percentages of G2 frames with loss of cohesion in individual 2 lacO at mmf1 control cells (n = 129), rad51Δ DSB cells (n = 22), WT DSB cells (n = 24), and dcr1Δ DSB cells (n = 12) (≥5 G2 frames per cell).
Figure 16. Early homology search is delayed in dcr1Δ DSB cells, but overall time to repair is not impacted.
(A) Many dcr1Δ DSB cells experience multiple colocalization events during repair, with variability in repair timing as well as number and length of colocalizations (as in WT). Graph of colocalization events of Rad52-mCherry (DSB) and lacI-GFP (donor) foci in representative dcr1Δ DSB cells. Each row represents one individual cell, and each circle represents a time point taken every 5 minutes. Blue circles: time from nuclear division to loss of Rad52-mCherry (relative to Rad52-mCherry loading)
Rad52-mCherry loading. Pink circles: time from Rad52-mCherry loading to unloading for at least 3 consecutive frames. Yellow circles: Colocalization of the DSB (Rad52-mCherry) and donor (LacI-GFP bound to lacO repeats at mmf1) foci. (B) Time from nuclear division to Rad52-mCherry loading in WT and dcr1Δ DSB cells. WT: n = 24, dcr1Δ: n = 15. *p<0.05, Kolmogorov-Smirnov test of cumulative distributions. Error bars represent MSD. (C) Time to first encounter is the difference between the first frame when Rad52-mCherry is visualized and the first colocalization event. WT: n = 23, dcr1Δ: n = 13. p = 0.0748, Kolmogorov-Smirnov test of cumulative distributions. Error bars represent MSD. (D) Percentage of cells in (C) with a colocalization in the first observable frame of Rad52-mCherry loading. (E) Time to repair was measured as the time in minutes from the first appearance of a site-specific DSB (persistent Rad52-mCherry focus) to its disappearance for at least three consecutive frames (5 minute intervals). WT: n = 17, dcr1Δ: n = 10. n.s. = not significant, Kolmogorov-Smirnov test of cumulative distributions. Error bars represent MSD.
Figure 17. DSB cells lacking Dicer are analogous to WT DSB cells in initial positions of and early mobility between DSB and donor.

(A) Distance (d) between DSB and donor at Rad52-mCherry loading was measured as the difference in XYZ between the Rad52-mCherry focus (DSB) and the LacI-GFP focus (donor sequence) in the first frame in which a Rad52-mCherry focus was observable within individual DSB cells. WT: n = 18, dcr1Δ: n = 10. n.s. = not significant, Kolmogorov-Smirnov test of cumulative distributions. Error bars represent MSD.

(B) Difference in 3D distance (Δd) between DSB and donor was measured as in (A) for the first and second frames (5 minute intervals) for each DSB cell. The absolute difference of these two values (|Δd|) was calculated and graphed along with MSD. WT: n = 18, dcr1Δ: n = 10. n.s. = not significant, Kolmogorov-Smirnov test of cumulative distributions.

(C) Difference in 3D distance (Δd) between DSB and donor was measured as in (A) for the first and second frames (5 minute intervals) in which a Rad52-mCherry focus was observable for each DSB cell. The difference of these two values was calculated and graphed along with MSD. Negative values represent a shorter distance between DSB and donor in the second frame relative to the first frame, while positive values represent a longer distance between DSB and donor in the second frame relative to the first frame. WT: n = 18, dcr1Δ: n = 10. n.s. = not significant, Kolmogorov-Smirnov test of cumulative distributions.
**Chromatin remodeling in fission yeast HDR**

Small RNAs produced from loci flanking a DSB may play roles in HDR regulation in various organisms as described above, such as influencing repair pathway choice, kinetics and/or antagonizing or promoting repair factor recruitment. An arguably even more fundamental question is the orchestration of chromatin remodeling required to access the DSB and donor sequence DNA strands in the first place. The lack of correlation in WT cells in my system between mobility of a DSB and timing of repair also prompts additional inquiry into the association between mobility and DSB repair.

*Move along histones, nothing to see here: nucleosome remodeling in DSB repair*

It is well established that the chromatin environment around the DSB changes after its recognition by the DNA damage surveillance machinery in order to facilitate HR, likely involving the recruitment of both repair factors and chromatin remodeling proteins. For efficient resection of the break as well as strand invasion at the donor, these sequences must become accessible to repair proteins through eviction or sliding of histones (Sinha *et al.*, 2009; Gospodinov and Herceg, 2013; Wiest *et al.*, 2017; Clouaire and Legube, 2019). Thus, the actions of chromatin remodelers such as the INO80, SWI/SNF and RSC complexes which are known to promote HR through histone exchange, nucleosome eviction and nucleosome migration (Chai *et al.*, 2005; Shim *et al.*, 2007; Seeber *et al.*, 2013b; Bennett and Peterson, 2015) could influence the persistence and number of encounters I see between these sequences.
The SWI/SNF chromatin remodeling complex is required for HR at a step before strand invasion (Chai et al., 2005). Further, upon recruitment to a DSB by the histone acetyltransferases NuA4 and Gcn5, SWI/SNF promotes ATM and ATR-dependent H2A phosphorylation to form γH2A, a canonical marker of DSBs (Bennett and Peterson, 2015). This in turn is thought to recruit other chromatin remodelers and DSB factors, such as the INO80 chromatin remodeling complex (van Attikum et al., 2004). Importantly, in mammalian cells deletion of ARP8, an actin-related protein subunit of the INO80 complex with ATPase activity, results in defects in Rad51 recruitment and nucleosome turnover at the DSB (Tsukuda et al., 2009a), as well as a decrease in DSB motility (Neumann et al., 2012). Arp8 contributes to efficient DSB repair under DNA damaging conditions in budding yeast (van Attikum et al., 2004; Tsukuda et al., 2009a) and fission yeast (Hogan et al., 2010), and its loss results in increased nucleosome density in fission yeast (Hogan et al., 2010).

In agreement with the above, changes in chromatin state are implied in my system by the mobility required for colocalizations and successful long-range homology search, as well as by loss of cohesion at the donor upon DSB induction. This would necessitate the action of one or more chromatin remodeling complexes at the DSB and donor. To better understand the role of chromatin remodeling in my fission yeast DSB system, I deleted Arp8 and asked what impact this would have on colocalizations between DSB and donor (reflecting mobility), loss of cohesion and overall repair efficiency. I chose Arp8 because (as described above) its activity is necessary for aspects of Ino80 (fission yeast
INO80) function most relevant to the DSB response and Ino80 itself has a key role among chromatin remodelers in DDR. Here I report preliminary (i.e. low cell count) data from analysis of this strain.

DSB repair persists in cells lacking Arp8 despite few colocalizations

A hallmark of Arp8 loss in mammalian cells is reduction of chromatin mobility. In a recent preprint collaborating with Simon Mochrie’s lab, our group demonstrated that this is also true in S. pombe when tracking chromatin mobility on very short time scales (<0.01s frame rate for 100 images) (Bailey et al., 2020). Accordingly, in my DSB system on longer time scales (5 minutes between frames) I observe far fewer colocalizations between the DSB and donor sequence in arp8Δ cells relative to WT, rad51Δ and even baseline mmf1 interactions in the 2 lacO at mmf1 strain (Figure 18A-B). This would suggest a drastic effect on the proportion of cells failing to repair, or (more unlikely given the necessity of chromatin remodeling at the donor) that with so few colocalizations with the donor, a single colocalization is more likely to lead to repair, such as in cells lacking Rqh1. However, ~50% of arp8Δ DSB cells are still able to repair within 90 minutes (Figure 19A), and the distribution of repair times are statistically similar to those in WT (Figure 19B). With the reduction in mobility due to loss of Arp8, it may be that homology search is more restricted leading to the observed subtle decrease in repair efficiency. When an encounter is successful it may be more likely to lead to repair, but recombination products could also be more error-prone. A reduction of histone remodeling in the absence...
of Ino80 activity could also influence dissolution of strand invasions by anti-recombinases such as Rqh1 (Hardy et al., 2019).

If loss of cohesion was associated with Ino80-dependent remodeling and/or with colocalizations between the donor and DSB, I would also expect these to be drastically reduced upon Arp8 loss. However, I find that the percentage of G2 frames with loss of cohesion did not appreciably alter in *arp8Δ* DSB cells relative to WT (Figure 20A-B). Since this loss of cohesion is largely dependent on Rad51 and DSB induction (Figure 20A-B), it may reflect the activity of other chromatin remodelers besides Ino80 that are activated upon DSB induction, the activity of site-specific RNAs produced at the DSB interacting with the donor (as discussed above), and/or another as yet unexplored aspect of homology search that is dependent on Rad51.

*Reconsidering mobility’s correlation with DNA damage*

In summary, chromatin mobility is broadly touted as an important component of DSB repair, especially in mammalian systems and when long-range homology search is necessary. However, in fission yeast I found that mobility is not correlated with repair time in WT DSB cells, and cells lacking Arp8 are still able to repair DSBs despite a drastic decrease in mobility. Further work to acquire additional data as well as examine other contributors to chromatin mobility and the nature of repair outcome in my system would shed more light on these surprising results. The global influence of multiple chromatin remodeling complexes on both early and late steps of DSB repair (Tsukuda et al., 2009a; Morrison, 2017), as well as mobility upon DSB formation and cell cycle regulation...
upon replication stress (Morrison et al., 2007; Neumann et al., 2012; Seeber et al., 2013a; Lee et al., 2015c), could render the task of identifying their specific roles in homology search difficult. However, the use of temperature-sensitive and/or separation of function mutants to minimally disturb non-HDR functions could aid in these efforts.

As discussed in the introduction, chromatin mobility can arise from a variety of sources and be induced to varying degrees based on cell cycle as well as the source and nature of DNA damage. There is exciting recent research about other effects on chromatin organization and movement upon DSB induction, such as the intricacies of how topologically-associated domains (TADs) (Ochs et al., 2019) and release of centromeric or telomeric tethers to the nuclear envelope (Strecker et al., 2016; Cho et al., 2017) can restrain or promote DSB recognition and response. Such work suggests chromatin restraints already present in the nucleus so that, while not physically cohesed per se, homologous loci on replicated chromosomes may be limited to diffusing in similar distances from their tethers at chromosome ends and may thus be more likely to interact than a non-homologous sequence at a different distance away from the nearer telomere. It would be interesting to test the effect of chromatin tethering or restraint in my system by introducing a DSB at varying lengths from the centromere or telomere and monitoring repair using the homologue. If this positioning dictated homology search dynamics, when DSBs (and homologue donors) are closer to a telomere and therefore more constrained the breaks would be expected to repair more quickly (with the exception of approaching too
closely the HDR-antagonistic nuclear periphery or heterochromatic, repetitive sub-telomeric sequences). Additionally, this would decouple chromatin mobility \textit{per se} from repair efficiency, which tends to be the case in some DSB induction systems described in Chapter 1, but there is still much to be learned.
Figure 18. arp8Δ DSB cells demonstrate low frequency of colocalization.
(A) Quantification of colocalization of DSB (Rad52-mCherry) and donor sequence (LacI-GFP bound to lacO repeats at mmf1). Frames in which cells were in G2 phase were analyzed for colocalization of DSB and donor (for WT, rad51Δ, and arp8Δ, only DSB cells were included) and assembled as a total percentage across all cells. Colocalization is that of the DSB (Rad52-mCherry) and donor sequence (LacI-GFP bound to lacO repeats at mmf1) (WT, rad51Δ, arp8Δ) or both Chr II homologs in the absence of damage (2 lacO at mmf1). *p < 0.05, **p < 0.001, ***p < 0.0001, Kolmogorov-Smirnov test of cumulative distributions (of percentages from individual cells). WT: n=26, 2 lacO at mmf1: n=129, rad51Δ: n=23, arp8Δ: n = 14. (B) Relative frequency histograms of percentages of G2 frames with colocalization in individual 2 lacO at mmf1 control cells (n = 129), rad51Δ DSB cells (n = 23), WT DSB cells (n = 21), and arp8Δ DSB cells (n = 14) (≥5 G2 frames per cell). Colocalization is for the DSB (Rad52-mCherry) and donor sequence (LacI-GFP bound to lacO repeats at mmf1) (WT, rad51Δ, arp8Δ) or both Chr II homologs in the absence of damage (2 lacO at mmf1).
Figure 19. arp8Δ DSB cells are somewhat less likely to repair within 90 minutes, but repair times are not significantly different from WT. (A) Total percentage of WT (n = 37) and arp8Δ (n = 12) cells with an induced DSB that repair within 90 minutes. (B) Time to repair was measured as the time in minutes from the first appearance of a site-specific DSB (persistent Rad52-mCherry focus) to its disappearance for at least three consecutive frames (5 minute intervals). WT (n = 25), arp8Δ (n = 6) cells. n.s. = not significant, Kolmogorov-Smirnov test of cumulative distributions.
Figure 20. DSB cells lacking Arp8 demonstrate low frequency of loss of cohesion. (A) Quantification of loss of cohesion between sister chromatids at donor sequence (two resolvable LacI-GFP foci bound to lacO repeats at mmf1). Frames in which cells were in G2 phase were analyzed for loss of cohesion (for WT, rad51Δ, and arp8Δ, only DSB cells were included) and assembled as a total percentage across all cells. n.s. = not significant, ****p < 0.0001, Kolmogorov-Smirnov test of cumulative distributions (of percentages from individual cells). WT: n=24, 2 lacO at mmf1: n=129, rad51Δ: n=22, arp8Δ: n=14. (B) Relative frequency histograms of percentages of G2 frames with loss of cohesion in individual 2 lacO at mmf1 control cells (n = 129), rad51Δ DSB cells (n = 23), WT DSB cells (n = 21), and arp8Δ DSB cells (n = 14) (≥5 G2 frames per cell).
**Observing lacO repeats at an alternate site**

My work thus far has singularly observed the *mmf1* region as the donor for HDR, but examining other donors would further inform my findings. First, this would aid in confirming the specificity of the encounters I observe (Figure 10) given the inability of my system to assess encounters beyond the diffraction limit of the light microscope (~0.2µm). Second, in Chapter 1 I described studies in which the degree of surrounding homology to a DSB sequence must be considered in order to faithfully recapitulate HDR experimentally. The *lacO* array, as a series of over 200 repeats ectopic to the *mmf1* region, could perhaps deter the homology search by disrupting potential strand invasion sites at the donor. However, in this case I would not expect it to have a large effect, as (1) the array is over 3kb away from the HO cut site on the homologous chromosome (Figure 2), (2) successful strand invasion can occur with 1-2kb of resected DNA (Chung *et al.*, 2010; Jakobsen *et al.*, 2019; Ronato *et al.*, 2020), and (3) the *lacO* array at *mmf1* does not have an appreciable effect on resection rate upstream of homology search (data in rebuttal for (Leland *et al.*, 2018a), available online (doi:10.7554/elife.33402)). Nonetheless, I wanted to confirm whether homology search and repair at *mmf1* without a *lacO* array would proceed similarly to my WT system.

To address these concerns, I mated the haploid *h* strain from my WT assay (MKSP2450) with a haploid strain (MKSP1552, see Table 3) in which a *lacO* array is integrated near the *cut3* gene (Chr II: 1006463-1002489, over 2.4MB upstream of *mmf1*) and acts as a “dummy donor”. The *mmf1* region in this
strain has no \textit{lacO} array integrated, and otherwise I carried out the HO plasmid transformation and DSB induction as described for WT (Methods). I expected the number of encounters between the Rad52-mCherry focus at the HO-induced \textit{mmf1} DSB and the LacI-GFP focus on \textit{lacO} at the non-homologous \textit{cut3} locus to be decreased. Since \textit{mmf1} is available for repair, I also hypothesized that DSBs in this background would exhibit similar repair times and proportion of cells that fail to repair within 90 minutes relative to WT. Alternatively, if the \textit{lacO} array at \textit{mmf1} does impede homology search, I posited that repair may be more efficient in the absence of these ectopic repeats, leading to shorter repair times and fewer cells failing to repair.

Preliminary data from this strain indicates that, as expected, \textit{lacO} at \textit{cut3} cells have significantly fewer encounters per DSB that WT (Figure 21A). I also observe significantly fewer G2 frames with colocalizations than WT, \textit{rad51Δ} and even 2 \textit{lacO} at \textit{mmf1} (Figure 21B), supporting a propensity for greater encounters between homologues even in the absence of DNA damage. However, repair times (Figure 21C) and overall repair efficiency (Figure 21D) of \textit{lacO} at \textit{cut3} DSB cells are not affected by this decrease in encounters as compared to WT (Figure 9A and Figure 21D). These results instill greater confidence in my assay. Further work should first be to repeat these experiments to increase cell numbers for this strain, as well as test additional “dummy donor” sites, which would support these findings being generally applicable to sites non-homologous to the \textit{mmf1} region rather than being unique to \textit{cut3}.
Figure 21. Fewer encounters and G2 colocalization frames in a strain monitoring lacO at cut3 rather than mmf1, but repair timing and efficiency are not impacted. (A) Cells with lacO repeats integrated at cut3 rather than mmf1 have significantly fewer encounters between DSB and donor per cell relative to WT. # of encounters per repair is the number of separate encounters (1+ consecutive frames at 5 min intervals with colocalization) between DSB and donor in individual WT (n = 24) or lacO at cut3 (n = 14) cells. *p < 0.05, Kolmogorov-Smirnov test of cumulative distributions. (B) Colocalizations during G2 are significantly reduced when monitoring a “dummy donor” at cut3 rather than the homologous donor at mmf1. Frames in which cells were in G2 were analyzed for colocalization of DSB and donor (for WT, rad51Δ and lacO at cut3, only cells with persistent, site-specific DSBs were included) and averaged as a total percentage across all cells. Colocalization is that of the DSB (Rad52-mCherry) and donor sequence (LacI-GFP bound to lacO at mmf1) (WT, rad51Δ, lacO at cut3) or both Chr II homologs in the absence of damage (2 lacO at mmf1). *p < 0.05, ****p < 0.0001, Kolmogorov-Smirnov test of cumulative distributions (of percentages from individual cells). WT: n=26, 2 lacO at mmf1: n=129, rad51Δ: n=23, lacO at cut3: n=14. (C) Repair of lacO at cut3 cells is highly efficient and analogous to WT (Figure 9A). Time to repair is the time in minutes from the first appearance of a site-specific DSB (persistent Rad52-mCherry focus) to its disappearance for at least three consecutive frames (5 min intervals, n = 14). Plotted along with MSD. (D) Proportion of DSB cells failing to repair is comparable between WT and lacO at cut3. Total percentage of WT (n = 37) and lacO at cut3 (n = 14) cells with an induced DSB that repaired (within 90 minutes) or did not repair (within 90 minutes).
Efficiency of repair with a homeologous donor

My work described in Chapter 2 has demonstrated that DSB repair by HDR in fission yeast is characterized by a relatively short time to repair and multiple encounters between the DSB and donor sequence. One reason for this efficiency may be the endogenous homology between the sequence surrounding the DSB and the donor sequence on the homologous chromosome (apart from the exogenous drug markers, short HO recognition site and lacO repeats – see Figure 2). As described in the introduction, previous studies of HDR often use ectopic sites or large insertions of non-homologous or homeologous sequence as templates for DSB repair that could decrease the efficiency of homology search. Other studies discussed above ask how much similarity a sequence must have to the original that was broken in order to be favored for repair, and find that even small increases in homeology can drastically decrease nucleoprotein filament targeting and repair efficiency.

Given this, I wondered what effect a homeologous donor would have in my system. Specifically, I asked the following: (1) How efficient is HDR in fission yeast with a homeologous donor? (2) If cells do repair, how long does it take? and (3) Is the rate of encounters between the DSB and donor sequence affected by homeology at the donor?

CRISPR-Cas9 editing in fission yeast

To address these questions, I, along with summer student Olivia Sheridan, constructed a series of strains with edited donor sequences using CRISPR-Cas9 recombination (Table 1, Table 2). CRISPR-Cas editing is a set of tools developed
from mechanisms of bacterial immunity to phages (Charpentier and Marraffini, 2014; Hille et al., 2018) and is used to precisely edit sequences of interest in a wide variety of model systems (Doudna and Charpentier, 2014; Xu et al., 2019; Matthews and Vosshall, 2020; Monsur et al., 2020), as well as in biotechnology and clinical applications (Knott and Doudna, 2018; Banan, 2020; Doudna, 2020). It has also been developed for use in fission yeast (Jacobs et al., 2014; Fernandez and Berro, 2016). Along with post-baccalaureate researcher Lily Mirfakhraie, and by utilizing gifts and adapting a protocol from the Berro lab (see Methods), Olivia and I pioneered the use of CRISPR-Cas9 editing in fission yeast in the King lab.

Given the strong response of recombination rates in budding yeast to small variations in homology (described above), we were conservative in our design of mutations in a 41bp region ~220bp away from the locus of HO cassette integration found on the homologous chromosome (Figure 22). MKSP3025, a strain with 2 mutations and therefore 95.2% homology of the affected portion of the donor with the WT sequence (Table 2), was the first successfully CRISPR edited strain produced, so we mated with MSKP2450 to make the diploid and proceeded with the microscopy assay as described in Chapter 2 and Methods.

Homeology decreases encounters but does not affect repair time or efficiency

We hypothesized that if the percentage of complete homology decreases, there might be fewer encounters between the DSB and donor sequence, leading to less repair efficiency and longer times to repair. Consistently, we saw far fewer encounters in MKSP3025 DSB cells than in WT (Figure 23A), and indeed even
fewer than under baseline conditions of chromatin dynamics in the absence of damage in the 2 lacO at mmf1 strain (Figure 7C). However, we also found that the repair rate is analogous to that of WT DSB cells (Figure 23B). Furthermore, there is no significant difference in repair times between these strains (Figure 9A and Figure 23C). Together, these results suggest that repair is still taking place, but (1) an alternate donor is being utilized, (2) infrequent or brief encounters with the homeologous donor that may be missed when monitored at 5 minute time points (Figure 11A-B) are sufficient to promote repair and/or (3) an alternate repair pathway is used that is not dependent on a homologous donor. The lack of repetitive sequence in this region would suggest repair is not occurring through SSA, but an analysis of repair products could provide insight as to whether the donor is still being utilized, just to a lesser degree.

These results are preliminary and there are a number of potentially fruitful directions to follow up with this work in addition to simply acquiring more data with MKSP3025 to confirm the initial trends. During the course of the project, we also produced other CRISPR-edited strains that have not yet been imaged (Table 2). Some of these have off-target mutations that are actually closer to the analogous HO cut site locus on the donor than the ones we originally designed (in which case we were limited by the strongest PAM sites that had less surrounding homology to other regions of the genome), so they may have a greater effect on repair efficiency than more distant base changes. Additional mutations in a given donor sequence may also be required to see changes in repair timing or efficiency. Even if greater homeology were present, the
differences in model system and/or exact repair mechanism utilized could explain discrepancies between our work and previous studies described above.

On a technical level, this protocol was new for our lab and we had lower efficiency of PCRs, transformation and other steps compared with the Berro group. However, this also speaks to the efficiency of fission yeast HDR as a whole, as commonplace laboratory transformations of *S. pombe* with exogenous cassettes or plasmids, even with large flanking regions of homology (200-300bp), are generally much less efficient than in other single-celled organisms (i.e. budding yeast and bacteria). Continued troubleshooting could increase our efficiency in producing many CRISPR-edited mutations at once. For instance, it may help to redesign the donor sequence primers to have more mutations than we hope to obtain in the final sequence. Additionally, the choice of which bases to mutate (other than choosing a unique PAM site) could also affect efficiency - it may be easier to induce mutation at A or T sites since over 85% (6/7) of the mutations we obtained across the four strains (excluding the PAM site) were at such sites.

Finally, more work could be done complementary to my imaging-based assay to elucidate the mechanism by which MKSP3025 cells repair DSBs. For example, the marker loss assay (see Methods and Figure 3B) could be performed to estimate the proportion of DSB cells undergoing HDR, and a strand invasion assay setup such as the one developed by the Heyer group (Piazza et al., 2019) could further elucidate whether strand invasions are occurring at the homeologous donor less frequently than the homologous donor in WT DSB cells.
**Figure 22. Map of primer design for CRISPR editing of donor sequence near HO cut site integration on homologous Chr II.**

Map of primers (P1-4) used in producing donor DNA from Chr II of the h- strain for CRISPR editing (see Methods). P2 and P3 overlap at the 20bp edited sequence, containing various point mutations designed as listed in Table 1 and with outcomes listed in Table 2. Compare with Figure 2A to see that P4 overlaps with site of HO cassette integration. Modified from Figure 2B from a figure by Bryan Leland, approximately to scale.
<table>
<thead>
<tr>
<th>Primer name and description</th>
<th>Sequence (annealing to 3'UTR of <em>mug178</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unedited genomic sequence</td>
<td>ATTTCTCCCGTGAGTCACTTCCGTTTTCTTAAAAACAATAGC</td>
</tr>
<tr>
<td>(100% homology)</td>
<td></td>
</tr>
<tr>
<td>P3a (97.6% homology)</td>
<td>ATTTCTCCCGTGAGTCACTTTGTTTTCTTAAAAACAATAGC</td>
</tr>
<tr>
<td>P3b (95.2% homology)</td>
<td>ATTTCTCCCATGAGTCACTTTGTTTTCTTAAAAACAATAGC</td>
</tr>
<tr>
<td>P3c (90.5% homology)</td>
<td>ATTCCTCCCATGAGTGAATTCTTTCTTAAAAACAATAGC</td>
</tr>
<tr>
<td>P3d (85.7% homology)</td>
<td>ATTCCTGCCATGAGTGACGTCTTAAAAACAATAGC</td>
</tr>
</tbody>
</table>

*Table 1: Primers to amplify donor for CRISPR-Cas9-driven mutations*

Designed point mutations are in red – see Table 2 for actual sequences following transformation. Endogenous PAM sequence is shown in gray in genomic sequence (MKSP3003) and is disrupted in primers. Percent homology is calculated for the selected sequence, not the total sequence between primer and HO cut site (an additional 221bp).
<table>
<thead>
<tr>
<th>Strain ID, primer set used and description</th>
<th>Sequence (Chr II: 3447179-3447032, 3'UTR of <em>mug178</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKSP3003 (unedited)</td>
<td>CAGTAAAACGATTATTTCCTCCCGTGAGTCACTTCGGTTTTCTTA AAACATAGGTTCCTTCTTCTTTCAAGGAATTGTTTTTCAATATTGTTTTT CGTCTGTGTGATAATCGTCTATTATGAAGATCCATCCATTTA CATTATTTTCACTG</td>
</tr>
<tr>
<td>MKSP3025 Primer P3d, on-target (2/6 designed mutations)</td>
<td>CAGTAAAACGATTATTCTCCCTCGTGAGTGACCTCGTTTTCTTA AAACATAGGTTCCTTCTTCTTTCAAGGAATTGTTTTTCAATATTGTTTTT CGTCTGTGTGATAATCGTCTATTATGAAGATCCATCCATTTA CATTATTTTCACTG</td>
</tr>
<tr>
<td>MKSP3032 Primer P3a, on-target (1/1 designed mutations)</td>
<td>CAGTAAAACGATTATTTCCTCCCGTGAGTCACTTCGTAAAACTTA AAACATAGGTTCCTTCTTCTTTCAAGGAATTGTTTTTCAATATTGTTTTT CGTCTGTGTGATAATCGTCTATTATGAAGATCCATCCATTTA CATTATTTTCACTG</td>
</tr>
<tr>
<td>MKSP3033 Primer P3a, off-target (0/1 designed mutations)</td>
<td>CAGTAAAACGATTATTTCCTCCCGTGAGTCACTTCGTAAAACTTA AAACATAGGTTCCTTCTTCTTTCAAGGAATTGTTTTTCAATATTGTTTTT CGTCTGTGTGATAATCGTCTATTATGAAGATCCATCCATTTA CATTATTTTCACTG</td>
</tr>
<tr>
<td>MKSP3034 Primer P3a, off-target (0/1 designed mutations)</td>
<td>CAGTAAAACGATTATTTCCTCCCGTGAGTCACTTCGTAAAACTTA AAACATAGGTTCCTTCTTCTTTCAAGGAATTGTTTTTCAATATTGTTTTT CGTCTGTGTGATAATCGTCTATTATGAAGATCCATCCATTTAC CATTATTTTCACTG</td>
</tr>
</tbody>
</table>

*Table 2: Resulting CRISPR-Cas9-driven mutations at donor*
Genomic sequences of original strain (MKSP3003) and CRISPR-Cas9 edited strains (MKSP3025, 3032, 3033, 3034). Actual point mutations are in red (as opposed to designed mutations, see Table 1). Endogenous PAM sequence is shown in gray in original genomic sequence, while grayed nucleotides in edited sequences are unmutated but were designed to change in the primer set. See Table 3 for full strain genotypes.
Figure 23. Introduction of homeology at the donor sequence promotes fewer frames of colocalization between the DSB and donor during G2, but does not alter efficiency or timing of repair.

Strain construction and data acquisition for this figure was conducted with Olivia Sheridan; CRISPR-Cas9 protocol assistance by Lily Mirfakhraie.

(A) Quantification of colocalization of DSB (Rad52-mCherry) and donor sequence (LacI-GFP bound to lacO repeats at mmf1 or cut3). Frames in which cells were in G2 phase were analyzed for colocalization of DSB and donor (for WT, rad51Δ, and 3025, only DSB cells were included that had a minimum of 5 G2 DSB frames) and assembled as a total percentage across all cells. Colocalization is that of the DSB (Rad52-mCherry) and donor sequence (LacI-GFP bound to lacO repeats at mmf1) (WT, rad51Δ, 3025) or both Chr II homologs in the absence of damage (2 lacO at mmf1). *p < 0.05, **p < 0.01, ****p < 0.0001, Kolmogorov-Smirnov test of cumulative distributions (of percentages from individual cells).

(B) Total percentage of WT (n = 37) and MKSP3025 (n = 9) cells with an induced DSB that repaired (within 90 minutes) or did not repair (within 90 minutes).

(C) Time to repair in MKSP3025 DSB cells was measured as the time in minutes from the first appearance of a site-specific DSB (persistent rad52-mCherry focus) to its disappearance for at least three consecutive frames (5 minute intervals, n = 9), and plotted along with MSD.
Matchmaker, matchmaker, make me a (mis)match: Mlh1 in S. pombe HDR

In discovering that fission yeast DSB cells have a propensity for multiple encounters between the DSB and donor during HDR, I considered what factors could be responsible for the dissolution of strand invasions contributing to this phenomenon. This led me to investigate anti-recombinases, such as Rqh1 (as described above) and MutL homologue 1 (Mlh1), a strongly conserved component of the mismatch repair (MMR) pathway.

Briefly, MMR targets incorrectly incorporated nucleotides during replication for excision and replacement. Eukaryotes have several key heterodimers that function in MMR, orthologs of MutS, MutL and/or MutH in bacteria. For instance, in budding yeast MutSα and MutSβ recognize base-base mismatches and initiate excisions, followed by recruitment of MutLα/β (Marti et al., 2003; Spies and Fishel, 2015; Chakraborty and Alani, 2016; Tham et al., 2016). MMR also utilizes other DDR proteins such as Exo1, PCNA and RPA to facilitate degradation and incision of the strand containing the mismatch (Amin et al., 2001; Genschel and Modrich, 2003; Li, 2008), followed by synthesis and ligation to restore homology.

In fission yeast, MutLα (Mlh1-Pms1) is required to prevent frameshift mutations, of which Pms1 is largely dispensable for this function (Marti et al., 2003). In humans, MutLα (MLH1-PMS2) is required to prevent mismatches and indels in heteroduplex DNA (Li and Modrich, 1995). MLH1 is also the most prominent of a number of MMR proteins that when mutated results in Lynch syndrome, characterized by increased risk of various cancers, in particular hereditary non-
polyposis colorectal cancer (Bronner et al., 1994; Papadopoulos et al., 1994; Germano et al., 2018; Tamura et al., 2019).

Because it is important to mediate MMR during replication and/or before completion of DSB repair so that any mis-incorporated bases are readily identified and removed, MMR proteins are antagonistic to recombination, in particular by blocking heteroduplex extension at a homeologous donor (Datta et al., 1996; Sugawara et al., 2004). Therefore, in the absence of these proteins strand invasions are more likely to lead to synthesis and ligation but with less proofreading to ensure homology.

There is relatively very little research on Mlh1 in S. pombe compared to that in S. cerevisiae, but based on MMR protein activity during HDR in other organisms, I hypothesized that Mlh1 could act to dissolve strand invasion structures in fission yeast in a similar manner to Rqh1. Correspondingly, I expected that upon loss of Mlh1, trends of repair timing and efficiency, as well as colocalization number and/or longevity, might reflect those of Rqh1 null cells.

Instead, the rate of colocalizations between DSB and donor in mlh1Δ were slightly lower but not statistically different from those in WT DSB cells (Figure 24A-B). Apart from outliers there may be statistical significance, but even so the trend is distinct from that in rqh1Δ DSB cells (Figure 13C), suggesting a different mechanism for Mlh1 activity or lack thereof in fission yeast HDR. Loss of cohesion between the DSB and donor was markedly reduced (Figure 25A-B), approximating that in rad51Δ cells (Figure 25A-B), perhaps indicating issues with prolonged strand invasions. However, I also found that a greater proportion of
mhl1Δ cells than WT had more than one Rad52 focus and/or were cell cycle arrested (not shown), suggesting higher levels of nonspecific DNA damage and precluding the ability to judge repair times and efficiency for site-specific DSBs. These obstacles unfortunately hinder me from formulating meaningful conclusions from this data about the nature of HDR repair in fission yeast lacking Mlh1.

In future, imaging and assessing more cells to be able to weed out those with nonspecific damage may provide the additional needed specificity. Alternately, a separation of function mutation could be used to disconnect Mlh1’s meiotic crossing-over (CO) functions from mitotic MMR and DNA damage response functions, such as in temperature-sensitive budding yeast MLH1 mutants (Argueso et al., 2002), to restrict Mlh1 incapacitation to a short time prior to and following DSB induction. This could permit detection of HDR-specific functions (if any) of Mlh1 in my system if (1) the non-specific damage is a result of aberrant MMR processing in Mlh1’s absence (which the MMR-capable mutant could ameliorate at the permissive temperature) and/or (2) the non-specific damage was a holdover from mhl1Δ cells going through MMR-impaired meiosis when mating for diploids. Lastly, other MMR proteins in fission yeast may have a stronger effect on heteroduplex rejection, for instance the MutS proteins Msh2, Msh3 or Msh6 which have varying roles in MMR and HDR and act earlier in the MMR pathway (Mansour et al., 2001; Tornier et al., 2001; Marti et al., 2003; Villahermosa et al., 2017). However, in looking at Mlh1 I was hoping to avoid some of the greater genomic instability that can be associated with loss of MutS.
proteins relative to MutL proteins (Spell and Jinks-Robertson, 2003; Sugawara et al., 2004). Other common HDR anti-recombinases could also be studied, some of the most prominent of which are the nucleases Mus81 and Yen1, but both have difficulties in fission yeast. The former renders strains very sick when it is knocked out, probably due to its myriad crucial functions for resolution of DNA damage in *S. pombe* (Cullen et al., 2007; Farah et al., 2009; Zhao et al., 2018). In contrast to Rqh1, loss of Mus81 in fission yeast results in fewer crossovers in both meiotic and mitotic DSB resolution (Osman et al., 2003; Hope et al., 2007), so nonetheless it would be fascinating to determine whether in my system homology search is also more efficient with shorter repair times in *mus81Δ* cells as in *rqh1Δ*, but without the decrease in cells repairing within 90 minutes if there is not an increase in non-homologous strand invasions, and/or whether the sequence-level outcome varies between these two knockout strains even if the dynamics and timing are similar. The latter (Yen1) so far does not have an *S. pombe* ortholog (Ip et al., 2008; Munoz-Galvan et al., 2012; Wyatt and West, 2014).

In conclusion, the anti-recombinase activity of Mlh1 as described previously in budding yeast may also play a role in fission yeast HDR. If so, the effect is subtle as regards colocalizations, though non-specific damage precludes developing a strong hypothesis from my data.
Figure 24. DSB cells lacking Mlh1 have largely reduced frequency of colocalizations, but with outliers show overall proportion of G2 colocalization frames similar to WT. 

(A) Quantification of colocalization of DSB (Rad52-mCherry) and donor sequence (LacI-GFP bound to lacO repeats at mmf1). Frames in which cells were in G2 phase were analyzed for colocalization of DSB and donor (for WT, rad51Δ, and mlh1Δ, only DSB cells were included that had a minimum of 5 G2 DSB frames) and assembled as a total percentage across all cells. Colocalization is that of the DSB (Rad52-mCherry) and donor sequence (LacI-GFP bound to lacO repeats at mmf1) (WT, rad51Δ, mlh1Δ) or both Chr II homologs in the absence of damage (2 lacO at mmf1). *p < 0.05, **p < 0.01, ****p < 0.0001, Kolmogorov-Smirnov test of cumulative distributions (of percentages from individual cells). 

(B) Relative frequency histograms of percentages of G2 frames with colocalization in individual 2 lacO at mmf1 control cells (n = 129), rad51Δ DSB cells (n = 23), WT DSB cells (n = 21) and mlh1Δ DSB cells (n = 26) (≥5 G2 frames per cell). Colocalization is for the DSB (Rad52-mCherry) and donor sequence (LacI-GFP bound to lacO repeats at mmf1) (WT rad51Δ, mlh1Δ) or both Chr II homologs in the absence of damage (2 lacO at mmf1).
Figure 25. *mlh1Δ* DSB cells exhibit lower overall G2 frames and relative proportion of G2 frames with loss of cohesion than WT DSB cells.

(A) Quantification of loss of cohesion between sister chromatids at donor sequence (two resolvable LacI-GFP foci bound to lacO repeats at *mmf1*). Frames in which cells were in G2 phase were analyzed for loss of cohesion (for WT, *rad51Δ*, and *mlh1Δ*, only DSB cells were included) and assembled as a total percentage across all cells. n.s. = not significant, 

****p < 0.0001, Kolmogorov-Smirnov test of cumulative distributions (of percentages from individual cells).

WT: n=24, 2 lacO at *mmf1*: n=129, *rad51Δ*: n=22, *mlh1Δ*: n=26. (B) Relative frequency histograms of percentages of G2 frames with loss of cohesion in individual 2 lacO at *mmf1* control cells (n = 129), *rad51Δ* DSB cells (n = 23), WT DSB cells (n = 21), and *mlh1Δ* DSB cells (n = 26) (≥5 G2 frames per cell).
Chapter 4: Discussion

Some of the commentary in this chapter is adapted from my manuscript in revision: Vines AJ, Cox KL, Leland BA and King MC, MBoC 2020. “Homology-directed repair involves multiple strand invasion cycles in fission yeast.”

Goin’ fission: Lessons from live cell dynamics of HDR in S. pombe

Taken together, my data indicate a highly efficient homology search in fission yeast. Surprisingly, I observe not one but multiple site-specific and Rad51-dependent colocalization events between the DSB and donor prior to successful repair. This suggests that (1) the first successful homology search event is not always followed by repair and/or (2) multiple strand invasion events contribute to repair, likely by SDSA. These exciting results have pivotal implications for HDR, which (as described herein or above in Chapters 2 and 3) find support in recent literature as well as raise additional points of uncertainty and provide cause for further work.

Efficiency of repair in fission yeast

The relatively quick repair of many site-specific DSBs in my system (Figure 9A) seems to belie longer repair times reported in other organisms (discussed above). However, hints as to the efficiency of fission yeast HDR can be found in such traits as (1) their faster rate of initial resection (Yan et al., 2019) relative to budding yeast (Zhu et al., 2008b) potentially allowing for earlier homology search, and (2) timely (within ~1hr) resumption of replication following replication fork collapse and ensuing recombination (Nguyen et al., 2015). It also follows from previous work that repair with an endogenous homologous donor as
in my system is more efficient than repair at an ectopic site in other systems, as discussed above.

**Support for and against multiple encounters during HDR**

Historically in the HDR field, it was assumed that a single encounter between the DSB and donor was sufficient to allow strand invasion, synthesis and resolution of the break, and this assumption is still commonly made when interpreting DSB assay results (Szostak *et al.*, 1983; Haber, 1995; Kanaar *et al.*, 1998; Helleday, 2003; Jasin and Rothstein, 2013; Renkawitz *et al.*, 2014; Chapman *et al.*, 2017). Additionally, my data support the hypothesis that one encounter is the minimum required for DSB repair in certain WT fission yeast cells as well as many of the *rqh1Δ* cells going on to repair.

However, heteroduplex regulation by Rqh1 and other anti-recombinases as well as the common mechanism of SDSA encompassing a limited time of nucleoprotein interaction with its target sequence suggest that, in some cases, repeated strand invasions are both feasible and likely before the completion of repair to prevent aberration crossing-over events. Further, repeated strand invasions to probe for longer stretches of homology could serve as a kinetic proofreading mechanism complementary to Rad51 homology recognition (Hopefield, 1974; Piazza and Heyer, 2019).

Multiple encounters between a DSB and a homologous sequence during homology search have also been invoked previously in several contexts. During mating type switching in budding yeast, fluorescently labelled DSB and donor loci interacted repeatedly in individual cells (Houston and Broach, 2006). Both the
Symington and Heyer groups also describe evidence for multiple strand invasions in *S. cerevisiae* mitotic repair through the use of multiple templates in translocations, either sequentially or concurrently (Smith *et al.*, 2007; Piazza *et al.*, 2017). However, multiple strand invasions are not restricted to repair utilizing homeologous templates; they have also been invoked in *Drosophila*, which relies heavily on SDSA for DSB repair (Adams *et al.*, 2003; McVey *et al.*, 2004). Additionally, during meiotic prophase in fission yeast, homologous loci have been observed to associate and dissociate repeatedly (Ding *et al.*, 2004), so it is not a great stretch that similar mechanisms might be in place during interphasic or mitotic DSBs.

In summary, my research is uniquely the first evidence of multiple encounters between the DSB and donor during S/G2 HDR in fission yeast, and provides another example of the regulated mobilization of and interactions between homologous loci to promote both efficient and reliable DSB repair.

*Call for future work*

In Chapter 3, following my presentation of data regarding specific HDR proteins in my microscopy assay, I described proposals of experiments to clarify or confirm those particular findings. Here I emphasize further steps that I would be most interested to continue given recent findings from other groups and that I feel would best advance the WT results as well as my work more broadly.

To supplement the marker loss assay which provides information of omission about sequence changes at the DSB, future studies using my induction system could include performing single-cell sequencing on fission yeast cells.
upon increasing intervals of time incubated with uracil (inducing HO endonuclease cutting, see Methods). This would provide repair outcome information (besides the binary repaired/unrepaired categories) that my assay does not supply in itself. It would also ameliorate the setback inherent to population-based sequencing of often losing the contextual and temporal information about the variety of repair events within individual cells (which is a boon of my imaging system).

Additionally, repair proteins are regulated by a wide variety of posttranslational modifications. Notably, in recent literature the ubiquitin-like SUMO modification has been shown to regulate many HDR proteins, including promoting Rad51 recruitment by RPA and antagonizing the DNA binding of Rad52 (Altmannova et al., 2010; Dou et al., 2011; Maréchal and Zou, 2015; Bonner et al., 2016), and work in our lab is ongoing to further understand its contribution to fission yeast HDR at the nuclear periphery. In my system, it would be interesting to examine the effects of Rqh1 modification on its ability to repress strand invasions. For instance, human BLM exhibits phosphorylation, ubiquitination and SUMOylation at different stages of HDR (Böhm and Bernstein, 2014), and there is evidence that phosphorylation of budding yeast Sgs1 regulates its helicase activity (Grigaitis et al., 2020), thus influencing the propensity for crossovers in DSB resolution.

Further study is also needed to fully define the relationship between genome organization and HDR efficiency and outcome. I find that the initial position of the DSB relative to the donor sequence had no bearing on overall
repair duration, although DSBs that began closer to the donor sequence experienced a first colocalization more efficiently. Although a similar lack of correlation was recently described in a trans repair reporter assay for budding yeast NHEJ (Sunder and Wilson, 2019), studies of ectopic HDR have documented a correlation of initial position with repair efficiency in budding yeast (Agmon et al., 2013; Lee et al., 2015a). While this could reflect inherent differences between model organisms, I also note that these studies leverage a relatively short homologous cassette inserted at ectopic sites rather than the homologous chromosome employed here. Moreover, my observations suggest that, although dependent on homology search, repair efficiency in this system is primarily dictated by the number of strand invasion events. One possibility is that while D-loop dissolution promotes SDSA, it also limits the extent of synthesis from a single homology search event. Thus, multiple stand invasion cycles may be necessary for the extent of synthesis required to span the initial DSB, thereby supporting subsequent strand annealing and repair.

Epilogue: The elegance and intricacy of DNA repair pathways

Despite the many outstanding questions concerning the regulation of DDR, it can be appreciated that cells are able to withstand the bulk of naturally acquired DNA damage due to the inherent appropriate mechanisms at play. Most, if not all, organisms studied to date have options through which to employ a more conservative or more daring path to repair a DSB (retain homology to the broken sequence, or diverge by annealing the broken sequence on itself or repairing with a homeologous sequence). The regulation of this choice allows for
genetic preservation of vital information such as the recipe for an essential protein, or for genetic plasticity in the case of meiotic reproduction or adaptation to a new environment. Though there appears to be redundancy in some repair mechanisms, upon closer inspection there are frequently precise ramifications following the activities of specific repair factors. One of numerous examples, a recent study from the Sung group and others demonstrates this by describing the tailored regulation of DSB end resection based on distinct surrounding DNA lesions (Daley et al., 2020), highlighting the importance of carefully managing simultaneous diverse manifestations of DNA damage.

How are these intertwined relationships complex enough that they take decades upon decades of research to begin unraveling to a discernable degree, yet simple enough that there are orthologs of many repair proteins across kingdoms of life, aiding in the aforementioned research? Why do some proteins in DDR multitask so well across different repair pathways? How might these have evolved in tandem with so many other biological molecules into these functional pathways preserving genome integrity, not to mention numerous more proteins in pathways maintaining the remainder of cellular homeostasis? Will we scientists, rather like helicases, at some distant time hence fully unwind the secrets of the genetic code? Or will we, at the pinnacle of solving one puzzle, then always discover a dozen more to be deciphered? The natural world seems strangely equipped to both confound and inspire humankind, even (perhaps especially) the brightest among us.
Materials and Methods

Yeast culture, strain construction and DSB induction

The strains used in these studies are listed in Table 3. *S. pombe* were grown, maintained, and crossed using standard procedures and media (Moreno *et al.*, 1991). Gene replacements were made by gene replacement with various MX6-based drug resistance genes (Bähler *et al.*, 1998; Hentges *et al.*, 2005). In one haploid *h*-strain, the 10.3 kb LacO array was inserted between Mmf1 and Apl1 on the right arm of chromosome II (Chr II: 3,442,981) using a modified two-step integration procedure that first creates a site-specific DSB to increase targeting efficiency of linearized plasmid pSR10_ura4_10.3kb (Rohner *et al.*, 2008; Leland *et al.*, 2018a). In another haploid *mat2*-102 strain (competent to make a stable diploid when mated with an *h*-strain), a modified MX6-based hygromycin-resistance cassette containing the HO cut site was inserted between Apl1 and Mug178 on chromosome II (Chr II: 3,446,249). This insertion is 3.2 kb distal to the site of LacO insertion in the *h*-strain. DSB induction using the Purg1lox-HO system was performed as previously described (Leland and King, 2014; Leland *et al.*, 2018a).

DSB induction using Purg1lox-HO

The uracil-responsive Purg1lox expression system was used, with slight modifications, to induce HO endonuclease expression and create site-specific DSBs at the HO cut site (Watt *et al.*, 2008; Watson *et al.*, 2011). A fresh integration of the HO gene at the endogenous *urg1* locus was performed for each experiment in order to reduce long-term instability at the HO cut site or the
development of HO resistance, presumably due to insertion/deletion events caused by basal expression levels of HO. The pAW8ENdel-HO plasmid (a gift from Tony Carr) was transformed into *S. pombe*, which were then plated onto EMM-leu+thi-ura plates (-leucine: plasmid selection; +thiamine: Pnmt1-Cre repression; -uracil: Purg1lox-HO repression). After 4–6 days of growth at 30°C, 20–60 individual colonies were combined to obtain a reproducible plasmid copy number across the population. Cre-mediated HO gene exchange at the endogenous Urg1 locus (urg1::RMCEkanMX6) was induced by overnight culture in EMM-thi-ura+ade+NPG media (-thiamine: expression of Cre from pAW8ENdel-HO; -uracil: Purg1lox-HO repression; +0.25 mg/mL adenine: reduce autofluorescence; +0.1 mM n-Propyl Gallate (NPG): reduce photobleaching in microscopy experiments, prepared fresh). The following day, site-specific DSBs were induced in log-phase cultures by the addition of 0.50 mg/mL uracil. This induction strategy resulted in ~15% of cells making a DSB within ~2 hr (Figure 3C).

**Microscopy**

All images were acquired on a DeltaVision widefield microscope (Applied Precision/GE) using a 1.2 NA 100x objective (Olympus), solid-state illumination, and an Evolve 512 EMCCD camera (Photometrics). Slides were prepared ~10-20 min after adding 0.50 mg/ml uracil to log-phase cultures to induce HO endonuclease expression and DSB formation. Cells were mounted on 1.2% agar pads (EMM +0.50 mg/mL uracil, +2.5 mg/ml adenine, +0.1 mM freshly prepared NPG) and sealed with VALAP (1:1:1 vaseline:lanolin:paraffin). Image acquisition
began between 40 and 80 min after uracil addition. Imaging parameters for microscopy assay data acquisition were as follows. Transmitted light: 35% transmittance, 0.015 s exposure; mCherry: 32% power, 0.08 s exposure; GFP: 10% power, 0.05 s exposure. At each time point (every 5 min for 2.5-4 hr), 25 Z-sections were acquired at 0.26mm spacing (16 Z-sections were acquired at 0.42mm spacing to mitigate photobleaching in some samples).

Image analysis

For the microscopy assay of interhomologue repair, data were acquired for each cell cycle individually, including time of nuclear division, time of cytokinesis, frames in which Rad52-mCherry focus was visible and frames in which Rad52-mCherry focus colocalized with the LacI-GFP focus at the diffraction limit (in the case of the 2 lacO at mmf1 strain (Figure 7A-B), colocalizations between both LacI-GFP foci were recorded instead). Time to repair was measured as the time in minutes from the first appearance of a site-specific DSB (persistent rad52-mCherry focus) to its disappearance for at least three consecutive frames. Only site-specific DSBs (defined as Rad52-mCherry focus persistence for at least 4 frames that began in late S or early G2 phase) were considered, since spontaneous DSB events can occur within the genome especially in G1 and early S phase (see Figure 4). Fields were analyzed manually, using the same contrast settings throughout for consistency.

Images from representative cells for some strains (Figure 5A-B, Figure 6A-D, Figure 7B, Figure 8A, Figure 12A-B) were prepared using ImageJ macros to automate merge and montage image creation using the same gate size
(height and width), while allowing for manual selection of the Z plane and centering on the nucleus. For visual clarity, the contrast of some images was adjusted according to the histogram using Levels sampling functions of Adobe Photoshop (2018) to set the darkest pixel as black and the brightest pixel as white. Merged images are either max projection or single planes with Rad52-mCherry in focus for visual clarity. Distance between Z slices for each frame is the distance in Z between the Z slice containing the center of the LacI-GFP focus and the Z slice containing the center of the Rad52-mCherry focus (or the center of the nucleus (denoted by middle Z slice of diffuse Rad52-mCherry signal) in frames with no Rad52-mCherry focus).

Data were plotted and analyzed using GraphPad Prism 7.01 as described below, unless otherwise noted in figure legends. Percentages of G2 frames with colocalization from individual cells were analyzed using the Kolmogorov-Smirnov test of cumulative distributions, relative frequency histograms and cumulative frequency histograms. Linear regressions and chi square were calculated using default Prism settings. Dotted lines on linear regression graphs represent 95% confidence intervals. The number of encounters per repair were analyzed using the Kolmogorov-Smirnov test of cumulative distributions. Error bars represent mean and standard deviation (MSD).

**Marker loss assay**

To examine repair outcome of the DSB in my system results based on sequence changes resulting from different repair pathways at the HO cut site, I performed a marker loss assay to assess the proportion of induced cells in which
the MX6-based drug resistance gene (Bähler et al., 1998; Hentges et al., 2005) was lost due to use of the donor sequence during HDR. DSB induction was performed on WT diploid \textit{S. pombe} cells as described above. At 2 hours following induction in log phase (growth for 2 hours in EMM-ura+ade+NPG with uracil added), cells were resuspended in EMM-ura media and plated to YE5S at 1:1000 (n=3), 1:2000 (n=3) and 1:5000 (n=2) dilutions. After 24 hours, YE5S plates were replica plated to YE5S+Kanomycin (at HO cut site – lost when the DSB is repaired using the homologous donor) and YE5S+Hygromycin (at \textit{urg1::RMCE} – lost when the pAW8ENdel-HO plasmid is flipped in via Cre recombination prior to induction). Colonies were counted with a Bio-Rad Molecular Imager VersaDoc (total colony count between 50 and ~160 cells per YE5S plate). Percentage of cells from each YE5S plate that had repaired by interhomologue HDR was calculated as ($\%$Kan sensitive colonies/$\%$Hyg sensitive colonies)*100. Data along with MSD were plotted using GraphPad Prism 7.01.

\textit{CRISPR-Cas9 editing of donor sequence}

The protocol used to edit the donor sequence at \textit{mug178} near \textit{mmf1} in certain strains was modified from one used by the Berro group (Fernandez and Berro, 2016 and correspondance via Lily Mirfakhraie). Briefly, guide RNAs were designed using PAM sequences at \textit{mug178} near \textit{mmf1} (at indicated distances from placement of HO cut site on the homologous chromosome; Table 1) and with homology to the pJB106 plasmid (a gift from the Berro lab) containing Cas9. These guide RNAs were amplified off of pJB106 and annealed through several
rounds of PCR resulting in a ~725bp dsDNA product. To create a donor sequence for repair, several series of four primers were designed to produce megaprimers and then the final ~480bp donor sequence (containing a 23bp region of possible homeology) over several rounds of PCR. The final sequence serves to “kill” the Cas9 recognition site as well as provide one of several altered donor sequences. MKSP3003 (fex1/2Δ) was transformed using a standard LiAc protocol (adapted from Moreno et al., 1991) with ~1µg of the annealed guide RNAs, ~10µl of the altered donor sequence PCR product and 100ng of pJB166 digested with CspC1, a gift from the Berro lab (Addgene plasmid #86998). Yeast were plated to YE5S + 1mM NaF and grown at 32°C for a minimum of 2 days until colonies formed. Colonies were re-streaked to fresh YE5S + 1mM NaF plates and grown at 32°C for a minimum of 2 days until colonies formed again. Colonies were screened using checking PCR and sequenced to confirm actual point mutations made (Table 2) before mating with MKSP2450 for use in the DSB microscopy assay (see above).
Table 3: S. pombe strains used in these studies
Strains containing PD\text{Is}1-GFP-La\text{cl}-N\text{LS} were modified from Shimada et al., 2003. Strains containing the RMCE Pur\text{g}1\text{lox} expression system were modified from Watson et al., 2011. Strains containing LacO integrations were modified from strains with original integrations by Bryan Leland as described in Leland and King, 2014.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Complete Genotype</th>
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<tr>
<td>MKSP1552</td>
<td>WT homology search assay with lacO repeats at cut3 (not at mmf1 donor) (mate with MKSP2450 to make diploid)</td>
<td>h- leu1-32 ura4-D18 his7+::PD\text{Is}1-GFP-La\text{cl}-N\text{LS} cut3-477::lacO- cut3+ urg1::RMCE-hphMX6</td>
<td>King Lab</td>
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<td>MKSP2230</td>
<td>WT diploid with lacO repeats on both Chr II copies (control: no HO integration/DSB formation)</td>
<td>mat2-102/h- leu1-32/leu1-32 ura4-D18/ura4-D18 his7+::PD\text{Is}1-GFP-La\text{cl}- N\text{LS}/ his7+::PD\text{Is}1-GFP-La\text{cl}- N\text{LS} ChrII:3442981::Ura4-10.3kbLacO/ ChrII:3442981::Ura4-10.3kbLacO ChrII:3446249::HOcs-hphMX6/WT Rad52-m\text{Cherry}::natMX6/Rad52-m\text{Cherry}::natMX6 urg1::RMCE-kanMX6/URG1</td>
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<td>MKSP2450</td>
<td>WT homology search assay (mated with MKSP2489 to make diploid) and marker loss assay (mated with MKSP3038 to make diploid)</td>
<td>mat2-102 leu1-32 ura4-D18 Rad52-m\text{Cherry}::natMX6 ChrII:3446249::HOcs-kanMX6</td>
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<td>MKSP2578</td>
<td>arp8Δ homology search assay (mated with MKSP2583 to make diploid)</td>
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<td>Rad52-mCherry::natMX6 ChrII:3446249::HOcs-kanMX6 rqh1::hphMX6</td>
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