Chapter 9

Meiotic and Mitotic Recombination: First in Flies

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Chapter Outline

1. INTRODUCTION

1.1 Recombination in *Drosophila*: The First 100 Years

Many species of *Drosophilids* have been adapted for the laboratory since their induction as a model organism over a century ago, but none are as commonly used and widely known as the species we discuss here, *Drosophila melanogaster*. The rediscovery of Gregor Mendel's work around 1900 sparked a sudden and intense interest in the field of genetics and with that came the need for animal models. *Drosophila* proved well suited to the task, requiring little space and simple husbandry. Heredity could be studied at a much faster pace than in plants or mammals due to the short generation time and the vast array of phenotypic markers that obeyed Mendelian rules of inheritance. As a consequence, flies boast an impressive list of firsts in the areas of genome structure and recombination, including the first evidence of meiotic recombination (1911); the first meiotic map (1913); the first use of ionizing radiation to make chromosome breaks (1927); the first physical map of chromosomes (1929); the first evidence of mitotic recombination (1936). Given this list, it is no surprise that important contributions to our understanding of how double-strand breaks (DSBs) are repaired have been made in flies, including, for example, the first model of synthesis-dependent strand annealing (SDSA) (1994).

DSB repair has a dichotic nature in complex organisms: in mitotic cells, recombination can be detrimental, causing loss of heterozygosity and chromosome rearrangements that affect viability; yet in meiosis, recombination is important for accurate chromosome segregation. In both mitosis and meiosis, unrepaired DSBs are deleterious, causing chromosome fragmentation and cell death. Here we discuss the major pathways for DSB repair in mitotic cells: homologous recombination, with an emphasis on SDSA, and end joining; we also explore the regulation of these pathways to promote the

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formation of noncrossover (NCO) products. This chapter also examines the meiosis-specific modifications to DSB-repair pathways that facilitate crossover (CO) formation, including homolog preference and regulation of recombination intermediates, and we describe a novel model for meiotic recombination in *Drosophila*.

1.2 *Drosophila* as a Model Organism: The Basics

D. melanogaster is well suited to a variety of laboratory and experimental conditions. Flies can survive in temperatures ranging from 15°C to 34°C, with the optimal temperature at 25°C (roughly room temperature). Their diet is simple, consisting mainly of sugar and yeast. Flies develop from zygote to sexual maturity in 7–10 days and a single female can lay as many as 3000 eggs in her 45–60-day lifespan [1].

D. melanogaster has four chromosomes that comprise its ~180 Mb genome: a sex chromosome and three autosomes (Fig. 9.1). The sex of a fly is determined by the ratio of *X* to autosomes, not the presence of a *Y* [1]. The *Y* chromosome is predominantly repetitive in sequence, entirely heterochromatic in content, and mostly genetically inert [2–4]. The *X*, *2*, and *3* chromosomes make up the majority of the euchromatin, while chromosome *4* is a mere 4.3 Mb in size and contains only ~100 genes interspersed between regions of heterochromatin [5]. The metacentric chromosomes *2* and *3* are subdivided into left and right arms, designated *2L*, *2R*, *3L*, and *3R*, respectively.

The *Drosophila* nucleus is highly ordered, with centromeres clustering at one pole of the nucleus and most telomeres clustered at the opposing pole [6]. Additionally, homologous chromosomes pair not just during meiosis, but in somatic tissues and the premeiotic germline. Centromere clustering and pairing of homologous chromosomes are independent of one another [7,8].

The ovaries of the female fruit fly are uniquely ordered as well. Each of the two ovaries consists of 12–16 ovarioles and each ovariole has a germarium-containing germline and somatic stem cells at the anterior end. Egg chambers increase in maturity as they migrate toward the posterior end of the ovariole, with a mature egg making up the final chamber [9]. Very early in the development of *Drosophila* as a model organism, researchers observed that meiotic recombination occurred only in female flies. Male flies have an alternate system for the proper segregation of their chromosomes that does not rely on crossing over of homologous chromosomes [10]. Thus, in one model system we have the means to study both meiotic recombination events (female germline) *and* exclusively mitotic recombination events (male germline).

2. MITOTIC RECOMBINATION

DSBs can arise from a variety of exogenous sources such as gamma radiation and chemical mutagens, as well as endogenous sources like collapsed replication forks, making this type of damage a common threat to genome integrity in both

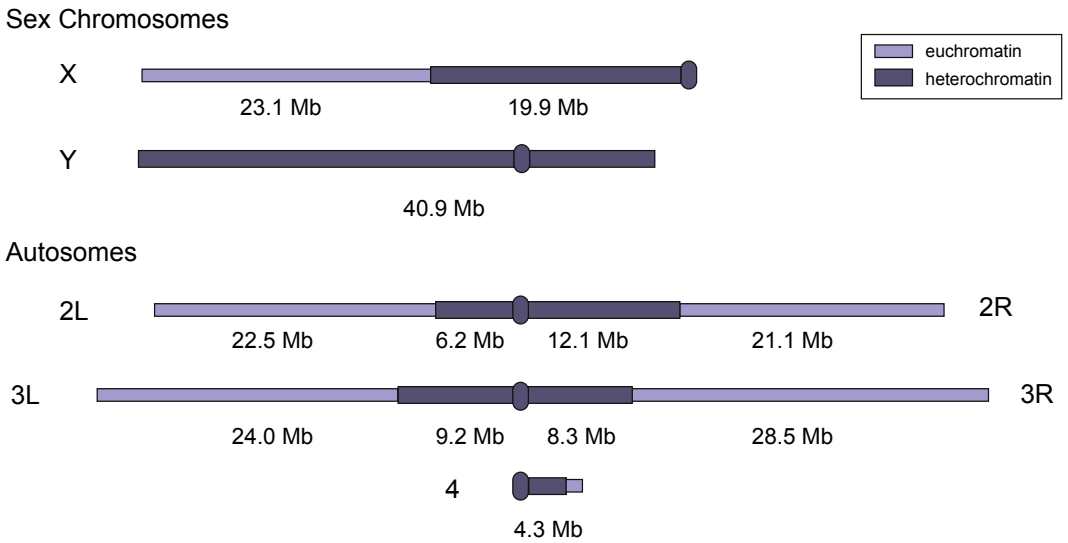

FIGURE 9.1 Basic structure of *D. melanogaster* chromosomes. Schematic representation of the four *Drosophila* chromosomes with approximate length in megabases. Size for *X, 2*, and *3* chromosomes is separated into euchromatin and heterochromatin content. Size for *Y* and *4* chromosomes represents the entire chromosome. Light gray: euchromatin; dark gray: heterochromatin; oval: centromere. Heterochromatin sizes based on cytological evidence as reported in [104]. Euchromatin size from *Drosophila* genome assembly release 6.

mitotically cycling and quiescent cells. Improperly repaired DSBs can lead to mutations and genetic rearrangements; left unrepaired, DSBs cause chromosome fragmentation and cell death. It is critical that mitotic DSBs are properly identified and repaired in such a way that the integrity of the genome is restored and recombination is avoided.

2.1 Mitotic Recombination: A Historical Perspective

Mitotic recombination was first introduced by Curt Stern in 1939 using *Drosophila* as a model organism [11]. While J.T. Patterson had been inducing genomic rearrangements in somatic tissues via X-ray since 1930 [12], it was Stern's elegant and encompassing work that first proved reciprocal genetic exchange between chromosomes occurred outside the germline. From Patterson's work came the knowledge that recombination could be induced in the male germline through X-ray treatment (later determined to cause DSBs), a process still utilized to study DSB repair today. By using the male germline, it is possible to recover not only fully repaired, single mitotic recombination events, but also reciprocal products of a single event in the recombinant progeny of males [13]. Once transmitted to progeny, the event becomes fixed, allowing for molecular analysis from whole flies.

The male germline was effectively used for several decades to study spontaneous and induced mitotic recombination; however, flies lacked a system for generating site-specific DSBs until a powerful tool utilizing transposable elements became available in the 1980s. Evidence of what appeared to be male meiotic recombination in crosses between laboratory females and wild males, but not in the reciprocal cross between wild females and laboratory males, was reported in the 1970s and dubbed "hybrid dysgenesis" [14,15]. In 1982, Rubin, Kidwell, and Bingham showed that hybrid dysgenesis was the result of a transposable element, the P-element, which had been introduced into the wild population after laboratory strains were isolated. Wild populations had developed repression mechanisms to prevent transposition that were passed to the zygote from the mother through silencing the gene on P-elements responsible for transposition: *P-transposase* [16–18]. P-elements were quickly engineered to remove *P-transposase* and contain any sequence of interest. Once integrated into the genome, they are fixed until exposed to an alternate source of P-transposase [19–21]. This discovery revolutionized genome engineering in *Drosophila*, providing fly researchers with a site-specific DSB induction system similar to those in yeast, but with novel attributes. In addition to the genome-editing capabilities of P-elements, DSBs induced by the activity of "tame" P-transposase usually occur in only one sister chromatid, allowing for a more biologically relevant system than previous work with *I-SceI* or HO in yeast, which cut both sisters and required ectopic repair templates.

2.2 Mechanisms of Mitotic Recombination

In *Drosophila*, as in other eukaryotes, DSBs are repaired through either a template-mediated pathway or an end-joining pathway. Template-mediated repair, known as homologous recombination repair (HRR), necessitates access to an undamaged copy of DNA—either a sister chromatid or a homologous chromosome. HRR can have multiple outcomes including both NCO and CO products, but mitotic regulation in *Drosophila* favors NCO formation. End joining (EJ) involves direct ligation of the broken ends, often after processing that can result in small insertions or deletions. *Drosophila* actively uses at least three variations of EJ, depending on the context of the break.

2.3 Initial Response and Pathway Choice

Extensive work in yeast and mammalian cells has established the MRN complex (Mre11–Rad50–Nbs in *Drosophila*) as the DSB sensor for mitotic cells. The MRN complex activates the DNA damage response protein kinase ATM (*Drosophila* tefu), which then phosphorylates many downstream factors to initiate repair, one of which is the histone variant H2AX (*Drosophila* H2AV) [22,23]. The γH2AV signal peaks within 5 minutes of gamma irradiation in flies and provides a scaffold to recruit additional proteins to amplify the repair signal [24].

In yeast and mammalian cells, pathway choice is cell cycle dependent. In G0-G1, phosphorylated 53BP1 binds the broken ends of a DSB to block 5′–3′ resection of the ends preventing HRR. During S–G2, when the genome has been replicated and a sister chromatid is available as a template, BRCA1 is phosphorylated by ATM leading to degradation of 53BP1, freeing the ends for resection [22,23]. Thus, it appears that the choice of HRR or EJ is decided by whether or not resection occurs. Additionally, the role of 53BP1 suggests the default repair mechanism for DSBs is HRR and only by blocking HRR can EJ occur.

It is less clear how pathway choice is made in *Drosophila*. While the core components of the early response are conserved, flies lack many of the regulatory controls such as 53BP1 and BRCA1 (Table 9.1). According to limited studies, pathway choice is somewhat age dependent, with HRR strongly favored in older flies (>2 weeks), while EJ is utilized more

TABLE 9.1 Orthologous Repair Genes in Fly, Human, and Yeast

D. melanogaster	*H. sapiens*	*S. cerevisiae*
Resection		
tosca	*EXO1*	*EXO1*
nbs	*NBS1*	*XRS2*
mre11	*MRE11*	*MRE11*
rad50	*RAD50*	*RAD50*
CG5872	*CtIP*	*SAE2*
CG2990	*DNA2*	*DNA2*
MCMs		
rec	*MCM8*	–
mei-217	*C8ORF45/MCMDC2*	–
mei-218		–
Recombinases		
spn-A	*RAD51*	*RAD51*
–	*RAD52*	*RAD52*
–	*BRCA1*	–
Brca2	*BRCA2*	–
–	*DMC1*	*DMC1*
Helicases and Associated Proteins		
Blm	*BLM*	*SGS1*
Top3α	*TOPO3α*	*TOP3*
–	*RMI1*	*RMI1*
–	*RMI2*	*RMI2*
Fancm	*FANCM*	*MPH1*
Checkpoints		
mei-41	*ATR*	*MEC1*
mus304	*ATRIP*	*DDC2*
tefu	*ATM*	*TEL1*
grp	*CHK1*	*CHK1*
Lok	*CHK2*	*RAD53*
p53	*P53*	–
Nucleases		
mei-9	*XPF*	*RAD1*
Ercc1	*Ercc1*	*RAD10*
Gen	*GEN1*	*YEN1*
mus81	*MUS81*	*MUS81*
mus312	*SLX4/BTBD12*	*SLX4*
hdm	*MEIOB*	–
mms4		

TABLE 9.1 Orthologous Repair Genes in Fly, Human, and Yeast—cont'd

D. melanogaster	*H. sapiens*	*S. cerevisiae*
End Joining		
Irbp	*KU70*	*YKU70*
Ku80	*KU80*	*YKU80*
Lig4	*DNA LIG4*	*DNL4*
–	*XRCC4*	–
–	*XLF*	–
mus308	*DNA pol θ*	–

Comparison of major repair genes in multiple pathways. Although not all inclusive, this table highlights major areas of conservation or divergence. Dashes indicate gene is not present.

in young flies (<2 weeks) [25]. There are two caveats worth noting in this study: (1) the dominant repair pathway was single strand annealing (SSA), a pathway strongly favored by the 157-bp repeats flanking the cut site in the reporter construct; (2) age-dependent pathway choice has only been studied in the male germline and these studies may reveal cell type–specific pathway choice (mature sperm-EJ vs. stem cells-HRR) rather than a true age correlation. Interestingly, tumorigenesis in epithelial cells of older flies correlates with errors in HRR, but not EJ, suggesting adverse effects on fitness with utilization of HRR as flies age [26].

Pathway choice does not seem to be affected by chromatin environment in *Drosophila*. It has been proposed that heterochromatin is naturally more resistant to DSBs due to compaction; and when breaks occur, EJ is the preferred repair pathway to avoid illegitimate recombination due to the highly repetitive nature of heterochromatic DNA. Chiolo et al. showed in 2011 that neither of these hypotheses is supported in *Drosophila*: heterochromatin is as susceptible to DSB formation via ionizing radiation as euchromatin, and HRR is still the dominant pathway for repair. *Drosophila* heterochromatin forms a distinct region within the nucleus and using high-resolution microscopy, Chiolo and colleagues were able to show γH2AV within the heterochromatin domain in response to gamma radiation. It was further shown that resection occurred within the heterochromatin domain but the remaining steps of HRR were suspended until the break physically moved to the outer periphery of the heterochromatin and was stripped of the heterochromatin marker HP1a, presumably to reduce compaction and enable repair factors access to the lesion [27]. These data indicate that HRR is the dominant pathway in *Drosophila* regardless of chromatin environment; that EJ and HRR have a dynamic and contextual relationship; and that flies may utilize spatial positioning as a means to regulate repair outcomes.

2.4 Synthesis-Dependent Strand Annealing: A Model Consummated in Flies

SDSA is now the predominant model for DSB repair by HRR [28]. SDSA was first proposed in *Drosophila* to explain repair products arising from P-element-induced mitotic DSBs. Throughout the 1980s, P-elements had been used to generate mutations through a method commonly called "imprecise excision," but probably actually arising from rare imprecise repair events. Sved, Eggleston, and Engels showed that recombination could be induced via P-element in the male germline, that it clustered around the site of a P-element, that the events were premeiotic (and therefore mitotic) in nature, and they could recover reciprocal clusters [19]. Over the next 4 years, Engels' group showed that P-element excision resulted in the formation of a DSB that was repaired via HRR, predominately using the sister chromatid as a template [29,30]. This type of repair requires extensive synthesis to accurately "replace" the missing P-element, and thus the lesion could more accurately be described as a double-strand gap rather than a break. Repair events were dependent on homology between the resected ends and the template as well as highly sensitive to single-base mismatches within the homology, suggesting that a mechanism existed that was capable of finding the precise and correct template for repair anywhere in the genome. Most importantly, they found that these events were rarely associated with COs [31].

Work in yeast suggested that DSBs repaired via HRR formed a joint molecule called a double Holliday junction (dHJ) that can form COs when resolved [32]. This molecule consists of two chromosomes concatenated into a four-stranded structure at two locations and requires endonucleolytic cleavage, either by a type I topoisomerase (coupled with a helicase) or a dsDNA nuclease, to separate the strands. In this model, often called the DSBR model but here referred to as the dHJ

model, CO events would be predicted at a higher rate than those observed by Engels. The popularity of the dHJ model can be attributed, in part, to a difference in experimental method. The assays that definitively show dHJ formation are studies of meiosis—a process biased *toward* CO outcomes (discussed in detail further in this chapter) and gene replacement, which also requires COs. Assays used in other model systems to specifically study mitotic events cut the genome at a variety of locations and relied on a template that was either on the same chromosome separated by few kilobases, or on an ectopic circular plasmid (as discussed in [30,31]). In contrast, Engels' system used P-element-induced DSBs that could repair off of the endogenous sister or homolog.

It was also possible to use tailored templates located at ectopic sites to recover and molecularly analyze repair products. Nassif et al. observed the same fidelity of repair and lack of COs when the template was inserted on a nonhomologous chromosome [33]. Using a variety of template cassette sizes, they were able to recover insertions of up to 8 kb at the repaired locus. Most striking, however, was the preponderance of "conversion-duplication" events that contained sequence from the template followed by sequence from the original P-element. These complex events could only be explained if both the ectopic site and the sister chromatid were used as templates for repair and then annealed at sequence that was complementary, which did not fit the dHJ model. From these conclusions, Nassif et al. combined models from a diverse collection of experiments in bacteria, fungi, and mouse cells to build a model they called SDSA [33]. SDSA was the most parsimonious model to fit the emerging data of the time, though it did not gain wide acceptance in the field until Haber's work in yeast was published 4 years later (his previous work considered homology annealing and strand invasion as two distinct and separate pathways) [34,35].

SDSA is sometimes considered to be a truncated form of the dHJ model because both pathways have the same early steps, which begin with 5′–3′ end resection (Fig. 9.2). The resulting 3′ tail is coated with Spn-A (Rad51 in yeast and humans) to form a stable and flexible filament proficient at finding homology in the dsDNA template. The filament invades

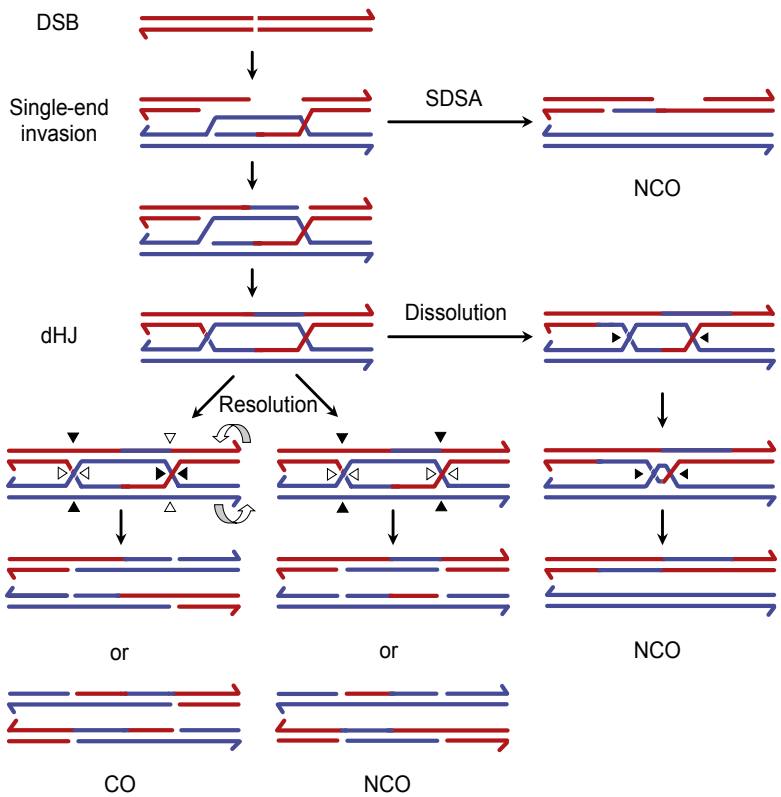

FIGURE 9.2 Homologous recombination repair (HHR) model. HRR begins with a DSB that is resected to form 3′ tails that invade a dsDNA template to form a D-loop (single end invasion). SDSA occurs when the D-loop is dismantled and the complementary ends anneal, followed by gap filling to yield an NCO. If the D-loop is not dismantled, second-end capture occurs and primes synthesis to yield a ligated dHJ product that can be disentangled through migration and decantenation (dissolution) to yield an NCO. Alternatively, the ligated dHJ can be cleaved through unbiased endonucleolytic cleavage of the HJs to form CO products in either orientation (*open arrowheads* versus *black arrowheads*) or NCO products in either orientation (*open arrowheads* versus *black arrowheads*). *Figure adapted from Crown KN, McMahan S, Sekelsky J. Eliminating both canonical and short-patch mismatch repair in* Drosophila melanogaster *suggests a new meiotic recombination model. PLoS Genet September 2014;10(9):e1004583.*

the duplex template, displacing the nontemplate strand (strand exchange) and facilitating extension of the invading strand via synthesis; this forms a structure called a D-loop. It is at this point that the two HRR pathways diverge: in SDSA, the D-loop is dismantled, freeing the newly synthesized end to find its complement from the opposite side of the break. In *Drosophila* P-element assays, Blm helicase is necessary to dismantle the D-loop [20]. If complementarity is not found between the nascent strand and the processed end at the other side of the break, reinvasion of the template occurs. This process of invasion, dismantling, complementarity search, and reinvasion occurs until annealing is achieved or the cycle is terminated and the ends are joined [36–38].

These findings are corroborated using other reporter systems as well. The activity of *I-Sce*I produces a DSB with 4 nt complementary overhangs, in contrast to the P-element system which generates 17 nt overhangs that are not complementary. Preston et al. found that a variation of SDSA that does not require synthesis, SSA, was strongly preferred over the dHJ model when *I-Sce*I was used [25]. In fact, EJ and SSA worked in a compensatory fashion to facilitate repair while dHJ events were exceedingly rare, suggesting that SDSA is the dominant form of HRR and not simply an aborted version of the dHJ model [25].

2.5 End Joining in *Drosophila*

Drosophila utilize multiple forms of end joining to repair DSBs. Canonical end joining, called nonhomologous end joining (NHEJ), involves ligation of DSB ends without synthesis. In vertebrates, the Ku70/Ku80 heterodimer binds the ends of a DSB and recruits DNA-PKcs. Autophosphorylation of DNA-PKcs activates the complex, recruiting accessory factors to process damaged nucleotides and single-base overhangs. Lastly, the ligation complex consisting of Lig4 and XRCC4 (LIG4, XRCC4, and XLF) ligates the ends of the break [39]. Because it is untemplated and does not rely on complementary overhangs or resection, it is thought to be more error prone (though much faster) than other pathways.

In P-element systems, end joining is observed when strand exchange is prevented via *spn-A* mutations. These events are independent of Lig4 and rely on microhomology, and are thus categorized as microhomology-mediated end joining (MMEJ) [40,41]. Assays using zinc finger nucleases also provide evidence for end joining in both *wild-type* and *lig4* mutants. Lig4-independent events were not microhomology mediated, suggesting that a third type of end joining, alternative end joining (alt-EJ), is also possible [42]. *Drosophila*, like other invertebrates, lack the key regulator of canonical NHEJ, DNA-PKcs, yet they retain orthologs of Ku70/Ku80, Lig4, and XRCC4, suggesting that canonical NHEJ is still utilized, though how it is regulated or how ends are processed remains unknown.

Work with *mus308* (PolQ in humans) suggests MMEJ or alt-EJ is used regularly by *Drosophila* to repair DSBs. Chan, Yu, and McVey showed that MMEJ was *mus308* dependent [43]. They also showed that *mus308* mutations are synergistic with mutations in *spn-A*, suggesting that mus308-mediated MMEJ is a compensatory response to inactivated HRR. In contrast, *lig4 spn-A* double mutants had no viability, fertility, or morphological defects, indicating that NHEJ is dispensable in the absence of HRR. They further showed that MMEJ occurred in wild-type backgrounds and increased in frequency in *lig4*-deficient backgrounds [43,44]. Collectively, these data indicate that *Drosophila* actively utilize multiple forms of EJ to repair DSBs and that MMEJ can compensate for both HRR and NHEJ, perhaps providing an alternative to DNA-PKcs-mediated end processing. This role for PolQ in *lig4*-independent EJ was corroborated in mammals [45–47].

2.6 Mitotic COs and the dHJ Model

The data presented thus far points to SDSA being strongly favored in DSB repair events, with some form of EJ providing a back-up mechanism; however, mitotic COs are observed in certain genetic backgrounds, such as *Blm* mutants, suggesting that the dHJ model is still a valid and utilized pathway for repair in *Drosophila* [13]. dHJ formation occurs when the second resected end of a DSB anneals to the D-loop and begins synthesis (Fig. 9.2). This process is thought to occur sequentially, with strand invasion occurring first to open the D-loop, followed by synthesis and ligation of the nascent end to the opposing 5′ strand without dismantling of the D-loop. Once ligated, the second resected end is "captured" by the single-stranded D-loop. As synthesis continues, the nascent strand eventually meets the opposing side and ligates to the remaining 5′ end to form a concatenated joint molecule—the dHJ. dHJs are toxic structures that prevent proper segregation during mitosis and block transcription; it is imperative that the chromosomes are separated accurately, preferably without exchange of genetic information in the form of COs. There are two possible mechanisms for disentanglement: dissolution via migration and decatenation or resolution via endonucleolytic cleavage.

Migration and decatenation is carried out by the BTR complex in humans (BLM, TOPO3α, RMI1/2) and the STR complex in yeast (Sgs1, Top3α, Rmi1) (Table 9.1) [48,49]. BLM helicase migrates the junctions toward each other and TOPO3α

(a type I topoisomerase) decatenates the strands through nicking and religating one strand of the dsDNA. The RMI proteins are thought to provide stability to the complex as well as facilitate decatenation through coordination with TOPO3α. Mitotic COs are elevated in *Blm* mutant flies, suggesting that the function of the complex is conserved in *Drosophila* [13]. Interestingly, flies do not have orthologs to the RMI proteins; the C-terminal region of Top3α has a large insertion that may play a similar role but this hypothesis has not been tested [50].

The presence of mitotic COs in *Blm* mutants, rather than an increase in lethality, suggests unbiased resolution of dHJs by structure-specific endonucleases called resolvases. Andersen et al. showed that *Blm* mutations are lethal when combined with mutations in the genes *mus81*, *mus312*, or *Gen* (MUS81, SLX4, GEN1, respectively, in humans), all of which encode subunits of putative HJ resolvases [51]. The synthetic lethality of the double mutants could be partially rescued by mutating *spn-A*, (in the case of *mus81 Blm* double mutant, fully rescued) suggesting that the phenotype was strand invasion dependent, and therefore related to a toxic HRR product [51]. The absence of mitotic COs in flies with wild-type *Blm*, combined with the viability of single endonuclease mutants, indicate that the primary pathway for disentangling dHJs is Blm-mediated dissolution with endonuclease cleavage serving as a back-up mechanism.

3. MEIOTIC RECOMBINATION

It is clear that somatic cells have a complex system with multiple interacting pathways to prevent dHJ formation and COs during DSB repair. Yet in germ cells undergoing meiosis, crossing over of genetic material between homologous chromosomes is required for proper segregation of chromosomes, suggesting that a completely separate regulatory network exists to promote dHJ formation and crossing over during meiotic recombination. Much of the research investigating the genetic basis of meiotic recombination began using *Drosophila* as a model organism.

3.1 Meiotic Recombination: A Historical Perspective

Drosophila researchers have been making pioneering discoveries in the field of meiotic recombination for over a century. In 1910, Thomas Hunt Morgan was the first to report meiotic recombination when he observed progeny that could arise only from maternal crossing over between the homologous sex chromosomes [52]. Following the discovery of meiotic recombination, Morgan hypothesized that genes are arranged linearly along chromosomes [53]. In 1913, Morgan's student Alfred Sturtevant reasoned that if Morgan's linear arrangement hypothesis is correct, he could determine the relative location of genes by measuring CO frequency [54]. By mapping six genes in a linear arrangement, Sturtevant did in fact prove Morgan's hypothesis to be true, and as a consequence, Sturtevant was the first to build a meiotic map. In this landmark study, Sturtevant also observed that the occurrence of one CO reduces the formation of a nearby CO, a phenomenon referred to as CO interference. Although CO interference was first observed over a century ago, the mechanism in which interference acts is still largely unknown.

In 1930, Theodosius Dobzhansky used chromosomal translocations induced by X-rays to construct a cytological map of *D. melanogaster* chromosome *2*. During this study, he noticed that there was a discrepancy between cytological distance (ie, physical distance) and genetic distance through the observation that genes in the middle of the chromosome arm undergo more recombination than the genes at the ends of the arm [55]. George Beadle performed a similar experiment using CO rates from translocations of chromosome *3* in 1932. Beadle's data indicated that the spindle fiber attachment region (now referred to as the centromere) impedes crossing over in adjacent regions on the chromosome. This reduction in COs occurred even when genomic regions located in the middle of the arm were experimentally placed adjacent to the centromere via translocations [56]. This phenomenon is now referred to as the centromere effect and has been observed in fungi, plants, and vertebrates.

By the early 1960s, much about the process of meiosis had been described through studies from *Drosophila*, maize, and fungi; however, surprisingly little was known regarding meiotic regulation. It was understood that recombination during meiosis is important for proper separation of chromosomes (meiotic disjunction), so Larry Sandler and colleagues screened natural *Drosophila* populations to find mutations that increased meiotic nondisjunction (improper separation of homologous chromosomes) [57]. Fifteen naturally occurring mutations that affected disjunction in one or both sexes were recovered. Baker and Carpenter performed a second screen, this time inducing mutations of the *X* chromosome via ethylmethane sulfonate (EMS) and uncovered additional novel meiotic mutants [58]. Together, these screens provided the scientific community with valuable resources still being used today; most importantly, the subsequent analysis of these mutants revealed new principles surrounding the mechanisms of meiotic recombination.

3.2 Mechanisms of Meiotic Recombination

Meiotic recombination is initiated by the formation of programmed DSBs, which are resected to yield 3′ DNA overhangs that invade the homologous chromosome, giving rise to a D-loop structure (Fig. 9.2). Similar to mitotic recombination, after synthesis the D-loop can either be unwound through SDSA to generate a NCO or can be stabilized so it can mature into a dHJ or other joint molecules. Unlike in mitotic recombination, joint molecules are preferentially resolved to form COs, which are vital for proper meiotic disjunction [59,60]. Because the meiotic recombination pathway utilizes many of the same repair proteins used during mitotic DSB repair, meiotic recombination has long been thought to have evolved from mitotic pathways [60–63]. Nonetheless, the fundamental purpose of these two processes are distinct: the outcome of mitotic recombination is complete and error-free repair of DSBs, while the primary goal of meiotic recombination is to carefully form stable COs between two homologs to ensure proper bipolar orientation of homologous chromosomes at the meiotic spindle [64]. For CO formation between homologs to occur during meiotic recombination, several meiosis-specific modifications to the somatic DSB-repair program have to transpire, as discussed later [60,62,63].

3.3 Initiation of Recombination

DSBs occur at a much higher frequency during meiosis when compared to the somatic cell cycle [65]. This increase is required to ensure that sufficient amounts of meiotic COs are formed to achieve proper segregation of homologous chromosomes. Accordingly, an important feature of meiotic recombination is deliberate and controlled DSB formation to initiate the repair process. In most, if not all, sexually reproducing organisms, Spo11, a type II-like topoisomerase conserved throughout eukaryotes, is the nuclease responsible for creating these meiosis-specific DSBs [66,67]. In most organisms, including yeast and mouse, Spo11 is not only responsible for creating meiotic DSBs, but it also initiates recombination by promoting repair through interactions with the MRN complex [68,69]. The MRN complex, along with Exo1, is responsible for resection of the break, marking the beginning of the repair process.

3.4 Preference of Homolog as Repair Template

In contrast to mitotically dividing cells that use the sister chromatid, cells undergoing meiotic recombination use an intact homologous chromatid as a repair template. This preference ensures CO formation between homologs, which prevents nondisjunction and promotes genetic diversity [70]. Invasion of a homologous duplex is promoted by DNA strand-exchange proteins of the RecA family. In most eukaryotes, there are two RecA homologs that aid in strand exchange during meiotic recombination, Rad51 and Dmc1 [71]. *RAD51* and *DMC1* diverged during the separation of the prokaryotic and eukaryotic kingdoms. While Rad51 participates in both mitotic and meiotic recombination, Dmc1 is meiosis specific, suggesting its function is to promote recombination preferentially between homologs [72]. The *DMC1* gene is found in most eukaryotes, including *Saccharomyces cerevisiae*, plants, mice, and humans. Interestingly, all Dipteran insects, including *Drosophila*, are missing DMC1, and it appears to have been lost independently in other clades, including fission yeast and some nematodes [73]. One explanation for this loss of *DMC1* in Dipteran insects may be the timing of formation of the synaptonemal complex (SC) in *Drosophila*, as discussed below.

The SC is a tripartite proteinaceous structure that connects paired homologous chromosomes along the length of their axes to provide an environment suitable for successful recombination during meiosis. Although the true function of the SC is unknown, it was initially thought to aid in the pairing of homologs before recombination could begin. However, this initial hypothesis was refuted when Spo11-dependent DSBs were shown to appear before formation of the SC during recombination in yeast, plants, and mammals, indicating that SC formation is not a prerequirement for recombination in these organisms [74].

Surprisingly, it was later found that in *Drosophila*, the SC is formed before the occurrence of DSBs, and in fact, normal levels of Spo-11 DSBs are dependent on the proper formation of the SC [75]. The only other organism known to exhibit this reversal of SC formation and DSB appearance in meiosis is the nematode *Caenorhabditis elegans* [76], which also lacks the *DMC1*. This being said, the structure of the SC may provide enough restraint on the chromosomes to ensure invasion of the homolog rather than the sister, negating the need for Dmc1 in *Drosophila* and *C. elegans*, whereas later formation of the SC in yeast, plants, and mammals necessitates a specialized strand invasion protein to facilitate homolog preference [73]. This hypothesis is supported by the finding that Ord, a *Drosophila* sister chromatid cohesin protein that promotes proper assembly of the SC, also promotes homolog bias during meiotic recombination [77]. The contrast between the *Drosophila* and the yeast/mammal recombination initiation suggests that mechanisms for homolog preference are not necessarily equivalent across model organisms; however, the fact that each species has a mechanism for it reinforces the importance of recombination between homologs in meiosis.

3.5 Promoting CO Formation: Pro-CO Complexes

Formation of COs is necessary to achieve proper chromosomal disjunction in meiosis I, but there are more DSBs than COs; surplus DSBs are repaired into NCOs. In *S. cerevisiae*, most NCOs are formed earlier than COs via SDSA, and are dependent on Sgs1 [78]. To promote COs, specialized proteins antagonize the activity of Sgs1 [79], and in most organisms, these specialized pro-CO proteins are MSH4 and MSH5, the subunits of MutSγ [80–83]. Interestingly, neither MSH4 nor MSH5 have roles in gene conversion or mismatch repair (MMR), but without either, CO formation is severely reduced, implicating them in the maturation of CO products [81,82]. Through biochemical studies, it has been shown that MSH4 and MSH5 form a heterodimer that preferentially binds to dHJs to form a sliding clamp, presumably to stabilize and protect recombination intermediates from disassembly by helicases, thereby promoting the dHJ pathway and CO formation [84]. The use of MSH4–5 as a pro-CO complex in meiotic recombination is highly conserved, yet it is absent in *Drosophila* [85]. In fact, a meiosis-specific pro-CO complex in *Drosophila* was not identified until 2012 by Kohl et al. in a landmark study [85].

Kohl studied three *Drosophila* genes, *mei-218*, *mei-217*, and *rec*, whose functions at the time were unknown. The gene *mei-218* was first discovered in the Baker and Carpenter screen in 1972 [58], while *mei-217* was discovered by Liu and McKim decades later [86]. *mei-217* and *mei-218* are transcribed as a dicistronic message and mutations in these genes result in 80–90% reduction of COs [85,86]. Studies suggest that female mutants for *mei-218* may fail to produce recombination intermediates, yet these mutants do not show a significant change in NCOs. Together, these observations suggest that the formation of CO-fated recombination intermediates is impeded when *mei-217* and *mei-218* are disrupted [87,88].

The *rec* gene was discovered in 1984 by Rhoda Grell through an EMS screen for temperature-sensitive meiotic mutants [89]. Interestingly, *rec* mutants display the exact phenotype of *mei-217* and *mei-218* mutants, such that REC is required for a majority of COs yet does not affect NCO formation. REC was shown to be the *Drosophila* ortholog of MCM8 and has no apparent role outside of meiosis [90]. Although MEI-217 and MEI-218 have no obvious sequence similarities, Kohl showed through structural analysis that these two proteins are predicted to fold like MCM proteins and have apparently evolved from an ancestral MCM-like protein [85]. Further, Kohl showed that MEI-217 interacts with both REC and MEI-218, together forming a complex referred to as the mei-MCM complex. In budding yeast, the CO defect in *msh4* mutants in *S. cerevisiae* is suppressed by eliminating Sgs1, suggesting that Msh4–5 promotes CO formation by antagonizing Sgs1 [79]. Paralleling this result, the removal of Blm in *Drosophila* suppresses the CO defects seen in *mei-MCM* mutants [85]. This observation indicates that the mei-MCM complex functionally replaces Msh4–5 in *Drosophila*, and more importantly, suggests that the general strategy of promoting CO formation in meiotic recombination may be universal to all sexually reproducing organisms.

3.6 Promoting CO Formation: Meiotic Resolvases

Somatic cells utilize resolvases as a last resort for dHJ resolution; this can still result in NCO formation through unbiased cleavage. In meiosis, recombination intermediates need to be resolved with a bias toward CO products, requiring a specialized set of resolvases. In *S. cerevisiae*, the primary meiotic resolvase is MLH1–3, the MutLγ heterodimer [91]. In *mlh1* or *mlh3* mutants, joint molecules are formed normally, but COs are severely reduced [92]. In humans, as well as in mice, MLH1–3 has also been implicated as the major meiotic resolvase [93]. In contrast, the primary meiotic resolvase in *Drosophila* is a complex containing MEI-9, MUS312, ERCC1, and HDM.

The gene *mei-9* was also discovered by Baker and Carpenter [58]. Females mutant for *mei-9* show a 90% reduction in COs but NCOs are not reduced [58,87]. The protein encoded from *mei-9* is an ortholog of the *S. cerevisiae* nucleotide excision repair protein Rad1 and the human structure-specific endonuclease XPF [94,95]. Females mutant for the gene *mus312* show meiotic phenotypes similar to that of *mei-9* mutants, with the formation of COs being reduced by 90% of *wild type* [95,96]. Through a yeast two-hybrid screen, it was shown that the proteins MUS312 and MEI-9 physically interact, and this interaction is required for formation of meiotic COs. Interestingly, MUS312 is shown to participate in interstrand crosslink repair, but not in nucleotide excision repair, while its meiotic binding partner, MEI-9, participates in both processes [95,97].

The product of the *Ercc1* gene physically interacts with MEI-9, as initially shown via yeast two hybrid, and is required for the role of MEI-9 in nucleotide excision repair [98]. Its role was implicated in the generation of meiotic COs with MEI-9 and MUS312 when Radford et al. demonstrated that all three proteins physically interact, and that ERCC1 is required for a subset of meiotic COs [99]. Lastly, the gene *hdm* encodes HDM, a protein that contains three OB fold domains, which are often associated with single-stranded DNA-binding capabilities. HDM physically interacts with MEI-9 and ERCC1 and is also required for a subset of meiotic COs [100].

In *Drosophila* meiotic recombination, as in other organisms, meiosis-specific features enable sufficient number of COs between homologs; yet the details of meiotic recombination seem to be vastly different in *Drosophila* as compared to other models: DSBs are primarily dependent on the formation of the SC, presumably negating the requirement for DMC1; the

mei-MCM complex functionally replaces MSH4–5; and the meiotic nuclease complex is MEI-9, MUS312, ERCC1, and HDM. Together, these differences in proteins raise the question: *Is the dHJ model, elucidated primarily in yeast, applicable to meiotic recombination in Drosophila?*

3.7 Meiotic Recombination in *Drosophila*: Double-End Engagement Model

The dHJ model of meiotic recombination was largely elucidated in *S. cerevisiae* using recombination hotspots, which are loci with a high frequency of recombination. By molecularly manipulating hotspots, yeast geneticists could recover recombination intermediates for molecular analysis [101,102]. High conservation of meiotic proteins has led to an assumption that the dHJ model is also conserved across species; however, the model had never been directly tested in a metazoan because of an inability to reproduce a system for physical analysis of recombination intermediates like that in yeast. In 2014, Crown et al. used molecular analysis of *Drosophila* heteroduplex DNA (hDNA) to provide the first evidence suggesting that some features of the dHJ model differ *Drosophila*; instead, they proposed that unligated dHJs give rise to both COs and a substantial fraction of NCOs [59].

During recombination, strand invasion and subsequent synthesis create recombination intermediates that contain hDNA, in which each strand of the duplex is derived from a different parental chromosome (Fig. 9.3). hDNA is repaired by MMR machinery to yield NCOs and COs without mismatches. By inactivating MMR machinery, hDNA generated during recombination can be preserved in these products. The hDNA tracts can then be molecularly analyzed to determine the orientation of the hDNA tracts, and through this, the structure of the recombination intermediate can be inferred.

In an attempt to recover and analyze hDNA in CO and NCO products in *Drosophila*, Radford et al. eliminated the canonical MMR machinery by mutating Msh6 [103]. Surprisingly, the hDNA recovered was not continuous, meaning that some patches of hDNA were repaired and some were not, even in the same recombination event. From these data, Radford proposed the noncontinuous hDNA tracts resulted from a short-patch MMR system that was able to repair some mismatches in hDNA in concert with the canonical MMR machinery, and this short-patch MMR may include NER proteins, as shown in fission yeast. In 2014, Crown tested this hypothesis by inactivating both MMR and NER pathways through mutations in *Msh6* and *Xpc*, respectively, and found that all hDNA were preserved [59].

According to the dHJ model, NCOs are formed primarily through SDSA. hDNA tracts by SDSA are predicted to be in *cis*-orientation, meaning all of the markers from the donor are on one strand of the product (Fig. 9.2); however, Crown et al. found that only half of the NCO synthesis tracts were associated with *cis*-hDNA. Surprisingly, the other half of NCO tracts

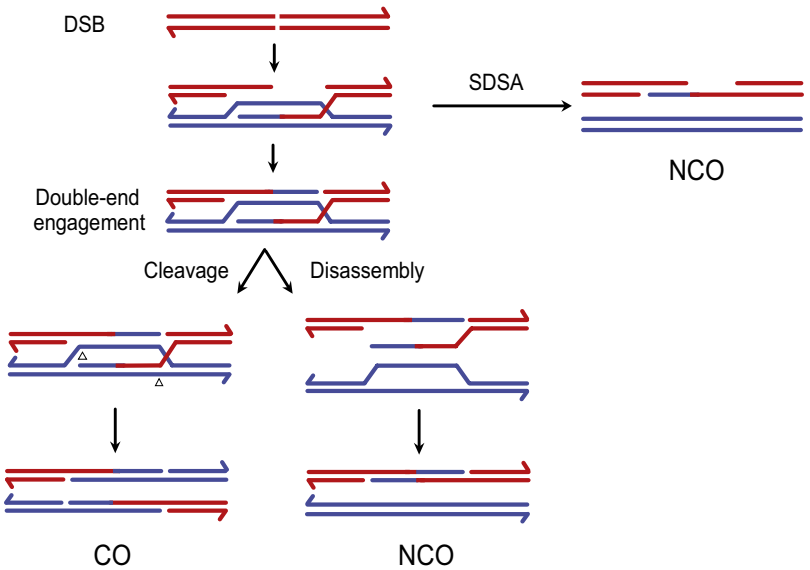

FIGURE 9.3 **Double-end engagement (DEE) model.** In *Drosophila* meiosis, a Spo11-generated DSB is resected and one 3′ tail invades the homologous chromosome to form a D-loop. If the D-loop is dismantled, an NCO product is formed through SDSA. If the D-loop is protected, both 3′ tails anneal to the same template and prime synthesis to form an unligated dHJ, termed DEE. The DEE can be processed by disassembly through migration of the single ligated HJ to yield an NCO or through biased cleavage (at open *arrowheads*) by meiotic resolvases to generate a CO. *Figure adapted from Crown KN, McMahan S, Sekelsky J. Eliminating both canonical and short-patch mismatch repair in* Drosophila melanogaster *suggests a new meiotic recombination model. PLoS Genet September 2014;10(9):e1004583.*

had two adjacent tracts of hDNA in *trans*-orientation, meaning the markers from the donor are on both strands of the product, which is not predicted by the SDSA model. Additionally, the dHJ model predicts that COs are formed by the resolution of dHJ in either of two orientations, both of which are equally likely (Fig. 9.2). However, the COs that were recovered with hDNA only appeared in one orientation. Based on these data, Crown proposed a new model with an unligated dHJ as an intermediate as opposed to the fully ligated dHJ model.

Together, these results suggest a novel and unified model for CO and NCO formation in *Drosophila*, referred to as the double-end engagement (DEE) model. In this model, up to half of all NCOs may arise from SDSA, giving rise to NCO products associated with hDNA in *cis*-orientation. The intermediates that do not undergo SDSA are processed into an unligated dHJ, referred to as a DEE intermediate, which can either be cleaved by MEI-9 to form a CO or can be disassembled by a helicase, such as Blm, to form an NCO. It is possible that the SDSA-mediated NCOs are early events similar to NCO formation in yeast, while nicked dHJ-mediated NCOs represent later recombination events, which may suggest a fine-tuning mechanism to ensure that proper number of COs per meiosis exists in *Drosophila*. Additionally, the DEE intermediate represents a joint molecule that is both simple to resolve and regulate.

Regardless, COs and NCOs arising from the same intermediate sharply contrast the dHJ model. It remains unclear if the structural difference in joint molecules explains the difference in meiotic resolvases between *Drosophila* and other eukaryotes, or if unligated dHJs are more common in metazoans than previously suspected. Pathways responsible for short-patch MMR have not been determined in other organisms, so it is not yet possible to do the type of analysis that Crown et al. did [59]. Likewise, there is not yet biochemical data on substrate preferences for the MEI-9 complex used in flies or for the MutLγ complex from fungi, plants, and mammals.

4. *DROSOPHILA*: THE NEXT 100 YEARS

A wealth of insight into both mitotic and meiotic recombination has been found using the unique traits of *Drosophila* as a model organism, yet much remains unknown. The field of *Drosophila* EJ is still in its infancy and the interplay of NHEJ, MMEJ, and alt-EJ—both the individual pathway regulation and the mechanisms by which they compensate for each other-is a field ripe for discovery. Likewise, mechanisms of intermediate steps in HRR are imperfectly understood. While SDSA is the dominant mechanism of DSB repair in mitotically dividing cells, it is still unclear how complementarity is found and annealing facilitated during the final stages. In the absence of complementarity, how is the choice made to reinvade versus capture the second end to form a dHJ? Are there mitotic dHJ agonists that prevent D-loop dissociation or is second end capture a stochastic event? Is the choice dependent on physical restraints within the highly ordered nucleus?

With regard to meiotic recombination, *Drosophila* is both intriguingly different and astonishingly similar to other model systems. Certain events must occur for successful meiosis in all sexually reproducing organisms: CO formation, homolog bias, and controlled joint molecule resolution; nonetheless, the mechanisms by which those events occur can vary widely between organisms, both through temporal alterations and divergence or outright replacement of meiosis-specific proteins. A benefit to this is that each system can enhance our overall understanding of the universal mechanisms governing meiotic recombination; still, many uncertainties remain. One major unanswered question is: *How are COs regulated?*

The development of sophisticated tools such as the CRISPR/Cas9 system and ultra-resolution microscopy, combined with further engineering of established assays and the versatile fly genome, may provide fine-tuned tools with which to ask these nuanced questions. Through continued study in *Drosophila*, we have the opportunity to examine universal properties of mitotic and meiotic recombination that affect all complex organisms.

GLOSSARY

Alternative end joining End joining that is independent of Lig4 and does not rely on microhomology.
Autophosphorylation The ability of a kinase to phosphorylate a residue within itself.
Autosome A chromosome that is not a sex chromosome.
***cis*-hDNA** A region of hDNA that contains all of the markers from the donor on one strand of the recombination product.
Concatenate DNA that is linked together and must be nicked or cut to disentangle.
Crossover Reciprocal exchange of genetic material between chromosomes.
Decatenate To disentangle concatenated or linked DNA.
Disjunction The proper segregation of homologous chromosomes.
D-loop A three-stranded DNA structure formed when a 3′ single strand of DNA invades a duplex template, displacing one strand.
Donor strand A single-strand of DNA involved in recombination that is used as a template during synthesis.
Double Holliday junction A recombination intermediate containing two Holliday junctions.
Double-strand break A break in both strands of a duplex DNA molecule.

End joining Generic term for ligation of the ends of a double-strand break.
Endogenous Originating from within.
Endonuclease An enzyme that cuts DNA between two bases.
Euchromatin Decompacted chromatin that often contains actively transcribed genes.
Exogenous Originating from the exterior environment.
Gene conversion Change of DNA sequence on one chromosome to the donor sequence (typically the same locus on the homologous chromosome).
Germ cells Egg and sperm cells.
Heterochromatin Densely compacted chromatin that contains silenced genes and repetitive sequences.
Heteroduplex DNA A region of double-strand DNA where each strand of the duplex is derived from a different parental chromosome and originates from recombination.
Holliday junction A four-stranded DNA structure in which the strands swap pairing partners.
Homologous chromosomes A pair of chromosomes that contains a maternal chromosome and a paternal chromosome.
Homologous recombination repair The process of DSB repair that uses an intact duplex DNA template to restore genetic information to the broken chromosome.
Hotspot A locus with a high frequency of meiotic recombination.
Hybrid dysgenesis Mating between strains that produces unidirectional lethality; in the context of P-elements, progeny from wild males and laboratory females are inviable or sterile, whereas the reciprocal cross yields viable progeny.
Meiosis Specialized type of reductive cell division.
Microhomology-mediated end joining End joining of a DSB that is Lig4 independent and relies on small homologies (4–8 nt) of the broken ends for ligation and repair.
Mitosis Nonreductive cell division.
Noncrossover Nonreciprocal exchange of genetic material between chromosomes; most noncrossovers are detected as gene conversion.
Nondisjunction Missegregation of homologous chromosomes.
Nonhomologous end joining Canonical end joining that is Lig4 dependent.
P-element A DNA (cut-and-paste) transposable element in *Drosophila* that requires a source of transposase for excision.
Progeny Descendants from a mating.
Recombination The rearrangement of genetic material due to DNA repair.
Resection Enzymatic activity that removes bases from one strand of duplex DNA in a 5′ to 3′ direction to yield 3′ ssDNA tails.
Resolvase An enzyme that cuts Holliday junctions or similar recombination intermediates.
Single-strand annealing A form of DSB repair that utilizes direct annealing of complementary resected ends without template invasion or synthesis.
Somatic cells Nonreproductive cells.
Synthesis-dependent strand annealing A type of DSB repair that utilizes a template and synthesis but does not utilize a double Holliday junction intermediate.
Transposable element A piece of DNA with the capacity to excise and/or integrate into the genome of its host; can be autonomous or require enzymatic activity from a different locus for mobility.
***trans*-hDNA** A region of hDNA that contains the markers from the donor on both strands of the recombination product.

LIST OF ACRONYMS AND ABBREVIATIONS

alt-EJ Alternative end joining
CO Crossover
dHJ Double Holliday junction
DSB Double-strand break
dsDNA Double-stranded DNA
EJ End joining
hDNA Heteroduplex DNA
HRR Homologous recombination repair
kb Kilobase
Mb Megabase
MMEJ Microhomology-mediated end joining
MMR Mismatch repair
NCO Non-crossover
NHEJ Nonhomologous end joining
nt Nucleotide
SC Synaptonemal complex
SDSA Synthesis-dependent strand annealing
SSA Single-strand annealing
ssDNA Single-stranded DNA

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