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REVIEW

Processing of joint molecule intermediates by structure-selective endonucleases during homologous recombination in eukaryotes

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Abstract Homologous recombination is required for maintaining genomic integrity by functioning in high-fidelity repair of DNA double-strand breaks and other complex lesions, replication fork support, and meiotic chromosome segregation. Joint DNA molecules are key intermediates in recombination and their differential processing determines whether the genetic outcome is a crossover or non-crossover event. The Holliday model of recombination highlights the resolution of four-way DNA joint molecules, termed Holliday junctions, and the bacterial Holliday junction resolvase RuvC set the paradigm for the mechanism of crossover formation. In eukaryotes, much effort has been invested in identifying the eukaryotic equivalent of bacterial RuvC, leading to the discovery of a number of DNA endonucleases, including Mus81–Mms4/EME1, Slx1–Slx4/BTBD12/MUS312, XPF–ERCC1, and Yen1/GEN1. These nucleases exert different selectivity for various DNA joint molecules, including Holliday junctions. Their mutant phenotypes and distinct species-specific characteristics expose a surprisingly complex system of joint molecule processing. In an attempt to reconcile the biochemical and genetic data, we propose that nicked junctions constitute important in vivo recombination intermediates whose processing determines the efficiency and outcome (crossover/non-crossover) of homologous recombination.

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Introduction

Homologous recombination (HR) is a conserved mechanism of high-fidelity DNA repair necessary for maintaining genomic stability. HR is required for accurate chromosome segregation during meiosis and constitutes a key pathway for the repair of DNA double-strand breaks (DSBs), DNA gaps, and interstrand crosslinks. Moreover, HR is required to recover stalled and broken replication forks (reviewed by Li and Heyer 2008). Through the combined efforts of yeast genetics and reconstituted in vitro assays, a detailed mechanistic understanding of recombination has been developed (Fig. 1) (Heyer 2007; Krogh and Symington 2004; Pâques and Haber 1999). In its simplest form, Replication Protein A (RPA) bound to single-stranded DNA (ssDNA) provides the substrate to initiate HR. Mediator proteins assist in the replacement of RPA by the key recombination protein, Rad51. The resulting Rad51–DNA filament performs homology search and DNA strand invasion, the signature reactions of HR. A DNA joint molecule intermediate called the displacement loop (D-loop) provides physical pairing between two otherwise discrete DNA double helices (Fig. 1). Additional DNA junction intermediates, including flaps, nicked or intact Holliday, or double Holliday junctions (HJs), are envisioned to form as a consequence of branch migration, DNA synthesis, or second-end capture (Fig. 1). In order to reconstitute two independent DNA duplex strands, all domains of life have evolved a collection of structure-selective endonucleases which cleave DNA joint molecules with distinct substrate specificity (Table 1). In this review, we start out by briefly elaborating the bacterial RuvC paradigm that guided the search for eukaryotic Holliday junction resolvases, followed by a discussion of the nucleases that were proposed to cleave Holliday junctions in eukaryotes. The biochemical properties of the eukaryotic enzymes and

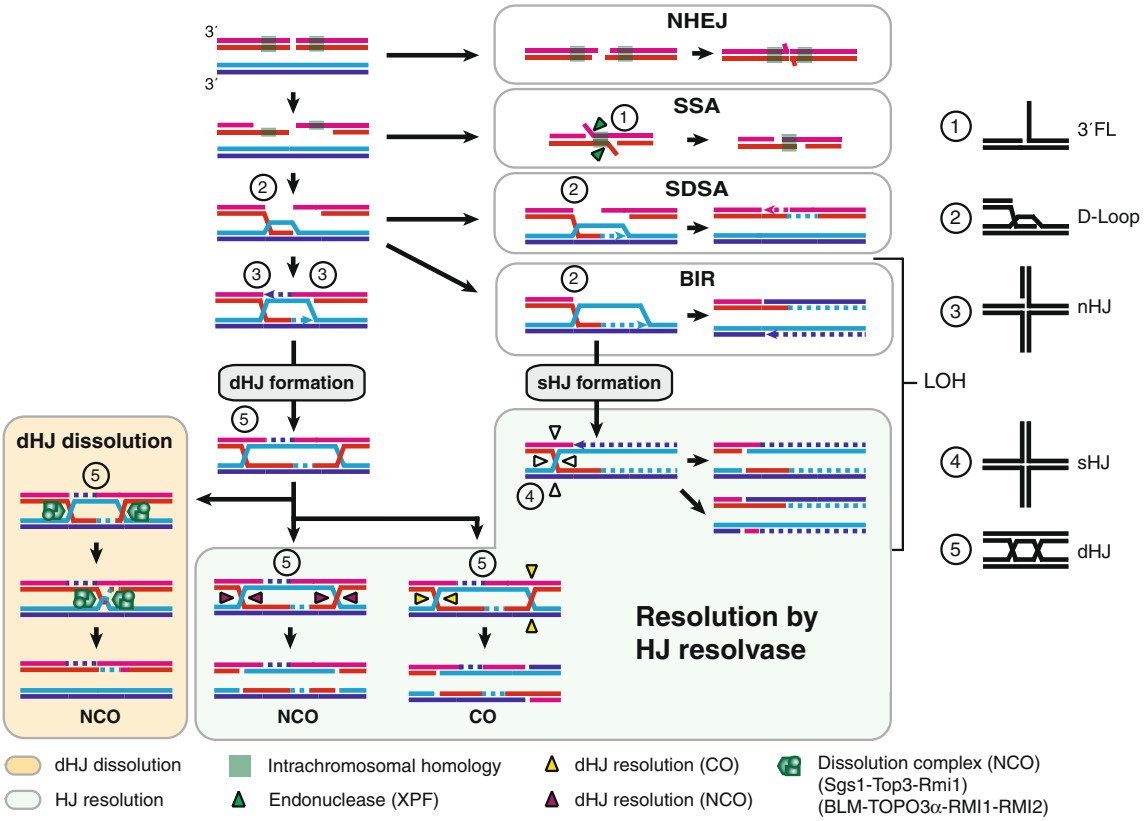

Fig. 1 Multiple DNA repair pathways are employed during double-strand break repair. After DNA DSB formation, the broken ends can be religated using minimal to no nucleotide homology through non-homologous end joining (*NHEJ*). Alternatively, 5′ ends are resected to expose single-stranded DNA favoring alternate routes of DSB repair. The presence of direct DNA sequence repeats may provide homologous regions that can anneal to form a contiguous chromosome in a process called single-strand annealing (*SSA*). Heterologous 3′ flaps are removed by the XPF endonuclease aided by Slx4 and Saw1. Alternatively, Rad51-dependent homology search and strand invasion forms a displacement loop (*D-loop*) to prime DNA synthesis from the 3′-OH end of the broken chromosome on an intact template. Extension of the D-loop and subsequent D-loop disruption and reannealing to the second end repairs the break via synthesis-dependent strand annealing (*SDSA*) resulting in noncrossover (*NCO*) products (Resnick 1976). Formation of an intact replication fork leads to the continuous extension of the D-loop to the end of the chromosome, defining the break-induced replication (*BIR*) pathway and resulting in loss of heterozygosity (*LOH*) (Malkova et al. 1996). The elongated D-loop forms a junction, where branch migration may lead to the formation of a single Holliday junction (*sHJ*). In the event of second-end capture, the displaced strand of the D-loop anneals to the other resected 3′ strand forming first two nicked Holliday junctions (*nHJ*) and after ligation a double Holliday junction (*dHJ*) (Szostak et al. 1983). Double HJs can be dissolved by the combined activities of a DNA motor protein (*S. cerevisiae* Sgs1 or human BLM) and a type IA topoisomerase into NCO products (Wu and Hickson 2003; Cejka et al. 2010) or resolved by coordinated endonuclease cleavage into CO or NCO products (Szostak et al. 1983). Single HJs require resolution by a nuclease and cannot be processed by a dissolution mechanism like dHJs

their in vivo functions, as deduced from genetic analysis, challenge the expectations based on the RuvC paradigm and the classical models involving HJs or double Holliday junctions. Finally, we attempt to integrate the biochemical and genetic data to provide a coherent model, which features a degree of plasticity between different eukaryotic organisms and places the cleavage of nicked joint molecules in a prominent position.

Holliday junctions and the bacterial RuvC paradigm

In a lucid analysis of fungal tetrad data, Robin Holliday proposed a mechanistic model for HR (Fig. 2) containing two major intermediates, heteroduplex DNA and a four-armed DNA junction intermediate, later termed the Holliday junction (Holliday 1964). For an informative discussion of recombination models and their evolution, see Haber (2008). It was envisioned that the coordinated, symmetrical cleavage of HJs across one of two alternative planes, as indicated in Fig. 2, could provide the mechanistic basis for the formation of crossover (CO) and non-crossover (NCO) recombinants. Support for this model came with the identification of proteins in phage and prokaryotes with the enzymatic ability to cleave synthetic HJs in vitro, coined HJ resolvases (Connolly et al. 1991; Iwasaki et al. 1991; Mizuuchi et al. 1982). Biochemical characterization of the *Escherichia coli* HJ resolvase RuvC and its associated proteins RuvA and

Table 1 Structure-selective endonucleases exhibit an array of species-specific differences. Organized by a single identifying subunit, eukaryotic homologs are listed with species designation. Information on their known binding partners, respective endonuclease superfamily, and mechanistic pathway involvement are provided

Indentifying subunit	Protein complex	Endonuclease family	Known in vivo function(s)	References
Rad1	*Sc*Rad1–Rad10	XPF	NER, ICL, SSA	(Cox and Parry 1968; Fishman Lobell et al. 1992)
	*Sp*Rad16–Swi10	XPF	NER, MTS	(Carr et al. 1994; Schmidt et al. 1989)
	*At*RAD1–RAD10	XPF	NER, ICL, SSA	(Gallego et al. 2000; Dubest et al. 2002)
	*Dm*MEI-9–ERCCI	XPF	NER, ICL, Meiosis	(Radford et al. 2005)
	*Hs*XPF(ERCC4)–ERCC1	XPF	NER, ICL	(Biggerstaff et al. 1993; van Vuuren et al. 1993)
Mus81	*Sc*Mus81–Mms4	XPF	HR, RF, Meiosis, ICL	(Interthal and Heyer 2000)
	*Sp*Mus81–Eme1	XPF	HR, RF, Meiosis	(Boddy et al. 2000)
	*At*MUS-EMEA/EMEB	XPF	HR, RF	(Berchowitz et al. 2007)
	*Dm*MUS81–EME1	XPF	HR	(Johnson-Schlitz and Engels 2006; Trowbridge et al. 2007)
	Hs/MmMUS81–EME1	XPF	HR, ICL	(Abraham et al. 2003; Dendouga et al. 2005; Svendsen et al. 2009)
Yen1	*Sc*Yen1	Rad2/XPG	N/D	
	*Dm*GEN	Rad2/XPG	N/D	
	*Ce*GEN-1	Rad2/XPG	DSBR	(Bailly et al. 2010)
	*Hs*GEN1	Rad2/XPG	N/D	
Slx4				
	*Sc*Slx4 complexes			
	Rad1–Rad10–Slx4	XPF	SSA	(Flott et al. 2007)
	Slx1–Slx4	UIY–YIG	rDNA	(Kaliraman and Brill 2002)
	*Sp*Slx4 complexes			
	Slx1–Slx4	UIY–YIG	rDNA	(Coulon et al. 2004)
	*Dm*MUS312 complexes			
	MEI-9–ERCC1?–MUS312	XPF	NER, ICL, Meioses	(Yildiz et al. 2002)
	SLX1–MUS312	UIY–YIG	N/D	
	*Ce*HIM-18 complexes		HR, RF, DSBR, meioses	(Saito et al. 2009)
	XPF–ERCC1?–HIM-18	XPF	N/D	
	SLX1–HIM-18	UIY-YIG	N/D	
	*Hs*BTBD12 complexes			
	XPF–ERCC1?–BTBD12	XPF	N/D	
	MUS81–EME1?–BTBD12	XPF	N/D	
	SLX1–BTBD12	UIY–YIG	HR, ICL, DSBR	(Andersen et al. 2009; Fekairi et al. 2009; Svendsen et al. 2009; Munoz et al. 2009)

N/D not determined, *NER* nucleotide excision repair, *SSA* single-strand annealing, *MTS* mating-type switching, *HR* homologous recombination, *RF* replication fork support, *ICL* interstrand crosslink repair, *rDNA* ribosomal DNA maintenance, *DSBR* double-strand break repair

RuvB sets the paradigm for future resolvases (Bennett et al. 1993; West 1997). RuvB is a DNA motor protein which oligomerizes into a double-hexameric ring on DNA and is tethered to two arms of a HJ through four subunits of RuvA (Fig. 2). This complex drives the migration of HJs to the preferred DNA sequence context for incision by the RuvC dimer. Symmetric cleavage results in perfect nicked duplexes capable of being directly ligated (reviewed by West (1997)). Depending on the cleavage axis, either a CO or NCO event is generated (Fig. 2).

In addition to cleaving HJs, resolvases such as phage T4 endonuclease VII and T7 endonuclease I, as well as bacterial RuvC, have shown activity on a variety of complex DNA lesions and other joint molecules in vitro (Benson and West 1994; Murchie and Lilley 1993; Jensch et al. 1989; Jensch and Kemper 1986). Incision site mapping of the resulting products identifies two symmetrical cuts even in the presence of asymmetric lesions or substrates (Murchie and Lilley 1993; Jensch et al. 1989; Jensch and Kemper 1986; Birkenkamp and Kemper 1995). These observations demonstrate the highly specialized role of these enzymes for the dual, coordinated cleavage of target substrates, unlike the single incision event that occurs for flap endonucleases such as XPF–ERCC1 or XPG

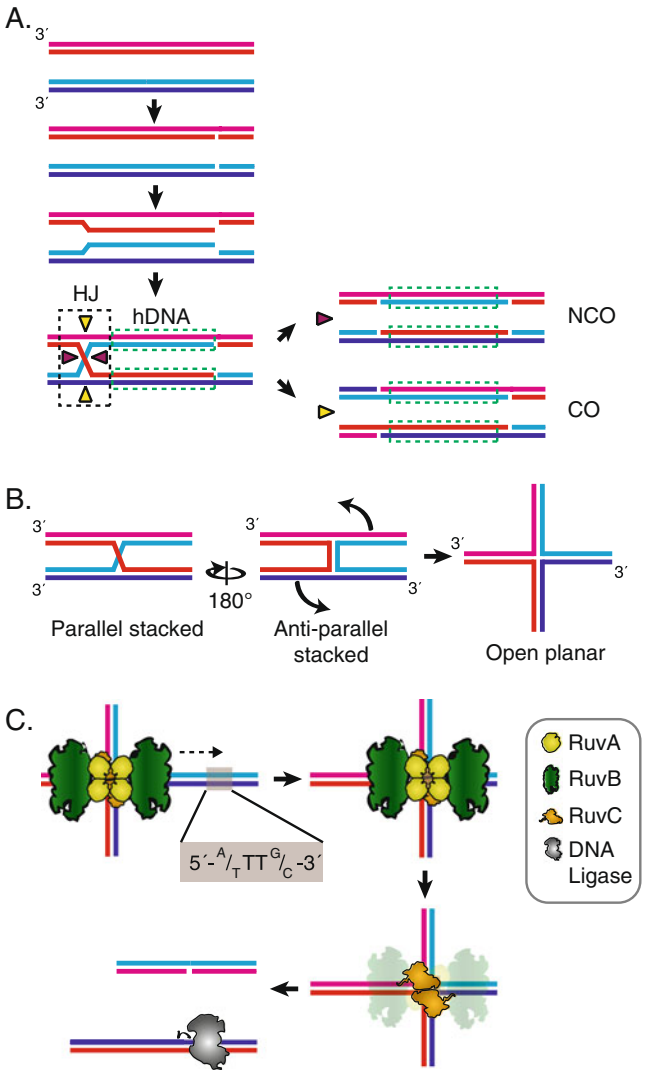

Fig. 2 Holliday model and the RuvC paradigm. **a** The Holliday model involves the nick-stimulated formation of heteroduplex DNA (*hDNA*) and an intact four-way DNA joint intermediate, termed a Holliday junction (*HJ*). The mismatch repair of hDNA leads to gene conversion and the endonucleolytic processing of the junction leads to crossover (*CO*) or non-crossover (*NCO*) products. **b** HJs can exist in a parallel stacked, anti-parallel stacked, or open planar confirmation depending on in vitro buffer conditions and protein binding. **c** *E. coli* resolvase RuvC acts in conjunction with the HJ binding tetramer RuvA and two hexameric rings of the RuvB motor. RuvA binding induces an open planar HJ conformation, ideal for RuvC cleavage. RuvB branch migrates the junction towards the preferred sequence for coordinated RuvC cleavage of alternate strands to form two linear duplexes compatible with direct ligation

(Guzder et al. 1995; Aboussekhra et al. 1995). Resolvases also generally display substrate specificity for HJs over other intact DNA substrates (Benson and West 1994; Whitby and Dixon 1998; Dickie et al. 1987).

As illustrated in Fig. 1, the current HR models are derived from Holliday's original proposal and feature single Holliday junctions (sHJ) or double Holliday junctions (dHJ). The dHJ was introduced as a key intermediate in DSB-initiated HR (Szostak et al. 1983). In an application of the RuvC paradigm, it was envisioned that the alternative, symmetric cleavage of dHJs, as indicated in Fig. 1, could result in CO and NCO products, a mechanism termed dHJ resolution. An alternate mechanism, termed dHJ dissolution, proposes that the two individual junctions of a dHJ can be migrated towards each other to form a hemicatenane which is untangled by a type IA topoisomerase, generating exclusively NCO products (Fig. 1) (Wu and Hickson 2003). Single HJs may form during synthesis-dependent strand annealing (SDSA) or break-induced replication (BIR) by branch migration of the initial D-loop (Fig. 1). However, the paucity of COs during DSB repair in somatic budding yeast cells suggested that SDSA does not involve HJs (Pâques and Haber 1999). The repair of one-sided DSBs by HR to restore a full replication fork, which is formally equivalent to BIR, may result in an sHJ that requires endonucleolytic resolution (see Fig. 1). Importantly, an sHJ is not amenable to the dissolution pathway. Physical analysis in both bacteria (Kobayashi and Ikeda 1983) and yeast confirmed the presence of sHJs (Cromie et al. 2006) and dHJs (Bzymek et al. 2010; Schwacha and Kleckner 1995) as meiotic and mitotic recombination intermediates in vivo. The observed mitotic dHJ levels are tenfold less than in meiotic cells per DSB (Bzymek et al. 2010). However, it is unclear whether this reduction in the steady-state level of mitotic dHJs reflects a real reduction in the proportion of DSBs repaired through this intermediate, less stability of dHJs in mitotic cells than in meiotic cells, or differences in the structure between meiotic and mitotic dHJs that affect the efficiency of the crosslinking procedure (Bzymek et al. 2010).

One of the key challenges has been the identification of enzymes acting on HJs in eukaryotes, and the focus has been to identify eukaryotic HJ resolvases that conform to the RuvC paradigm. This paradigm provided an assay, i.e., cleavage of four-armed DNA structures, and specific parameters such as specificity for HJs over other junctions, symmetric cleavage of HJs, and directly religatable products. With such a robust assay and predictions at hand, the first eukaryotic HJ cleavage activity was identified in the budding yeast *Saccharomyces cerevisiae* and was attributed to the mitochondrial endonuclease, Cce1 (*Sp*Ydc2) (Kleff et al. 1992). Considering the bacterial origin of mitochondria, this finding was gratifying but failed to address the identity of the nuclear eukaryotic enzymes. Further work identified a surprising complexity and plasticity of DNA structure-selective endonucleases in eukaryotes in their ability to cleave a multitude of DNA junctions and joint molecule structures. These findings defy the clarity and attractive simplicity of the RuvC paradigm, and below we discuss these eukaryotic enzymes, their substrate selectivity, and biological functions as deduced from genetic analysis.

XPF endonuclease superfamily

S. cerevisiae Rad1–Rad10/*Drosophila melanogaster* MEI9–ERCC1/human XPF–ERCC1

RAD1 was originally identified in *S. cerevisiae* in a screen for mutants that rendered cells hypersensitive to ultraviolet (UV) radiation (Cox and Parry 1968). Rad1 is the budding yeast homolog of human XPF, representing a ubiquitous class of proteins in eukaryotes, whose primary role appears to be in nucleotide excision repair (NER) (Table 1; Fig. 3) (Ciccia et al. 2008). XPF denotes complementation group F of xeroderma pigmentosum, a human cancer predisposition syndrome caused by the failure to repair UV-induced DNA damage by NER or tolerate such damage by translesion DNA polymerase bypass.

Rad1, as all of its eukaryotic homologs, associates with another protein, Rad10 in *S. cerevisiae*, in a heterodimeric complex (Table 1; Fig. 3). During NER, this complex provides the 5′ incision, whereas another endonuclease, Rad2 (XPG in humans), delivers the 3′ incision to liberate the damage-containing oligonucleotide (Fig. 4) (Aboussekhra et al. 1995; Guzder et al. 1995). In addition to its well-documented role in NER, *S. cerevisiae* Rad1–Rad10 has been known to participate in additional mechanisms of DNA metabolism. Genetic analysis showed that Rad1–Rad10 functions in a form of intrachromosomal recombination termed single-strand annealing (SSA; Fig. 1) (Fishman Lobell and Haber 1992; Aguilera 1995; Ivanov and Haber 1995; Liefshitz et al. 1995; Prado and Aguilera 1995). SSA involving ends with 30 nucleotides or greater 3′-terminal heterologies requires Rad1–Rad10 to remove the heterologous tails that result from strand annealing (Figs. 1 and 4) (Pâques and Haber 1997; Fishman Lobell and Haber 1992; Aguilera 1995; Ivanov and Haber 1995). A similar function of Rad1–Rad10 was identified in microhomology-mediated end-joining (Ahmad et al. 2008; Lee and Lee 2007). Furthermore, genetic analysis indicates that Rad1–Rad10 also participates in the removal of covalent topoisomerase I-DNA complexes (Vance and Wilson 2002). Lastly, Rad1–Rad10 is required for the repair of long heterologies in meiotic heteroduplex DNA (Fig. 4) (Kearney et al. 2001). The involvement of Rad1 in multiple repair and recombination pathways suggests that the complex is under tight regulation and control, perhaps driven by posttranslational modification or context-specific protein interactions. Supporting both possibilities, budding yeast Rad1 has been shown to physically interact also with Slx4 in a phosphorylation-dependent manner (Toh et al. 2010; Lyndaker et al. 2008). Likely acting as a scaffolding protein, Slx4 interaction stimulates Rad1–Rad10 activity on 3′ flap substrates in SSA, but Slx4 has no role in NER (Toh et al. 2010; Lyndaker et al. 2008). Another SSA-specific interactor of Rad1 is Saw1 that, like Slx4, plays a role in maintaining ribosomal DNA integrity (Li et al. 2008), suggesting that SSA is an important pathway to maintain rDNA stability.

The biochemical analysis of *S. cerevisiae* Rad1 has identified a number of potential in vivo joint molecule substrates (Fig. 4). In addition to bubble and flapped substrates, budding yeast Rad1 was reported to also specifically bind and cleave synthetic HJ structures in vitro (Habraken et al. 1994). This activity was observed in the absence of its obligatory partner Rad10 and may be specific to branch-migratable HJs which undergo molecular breathing

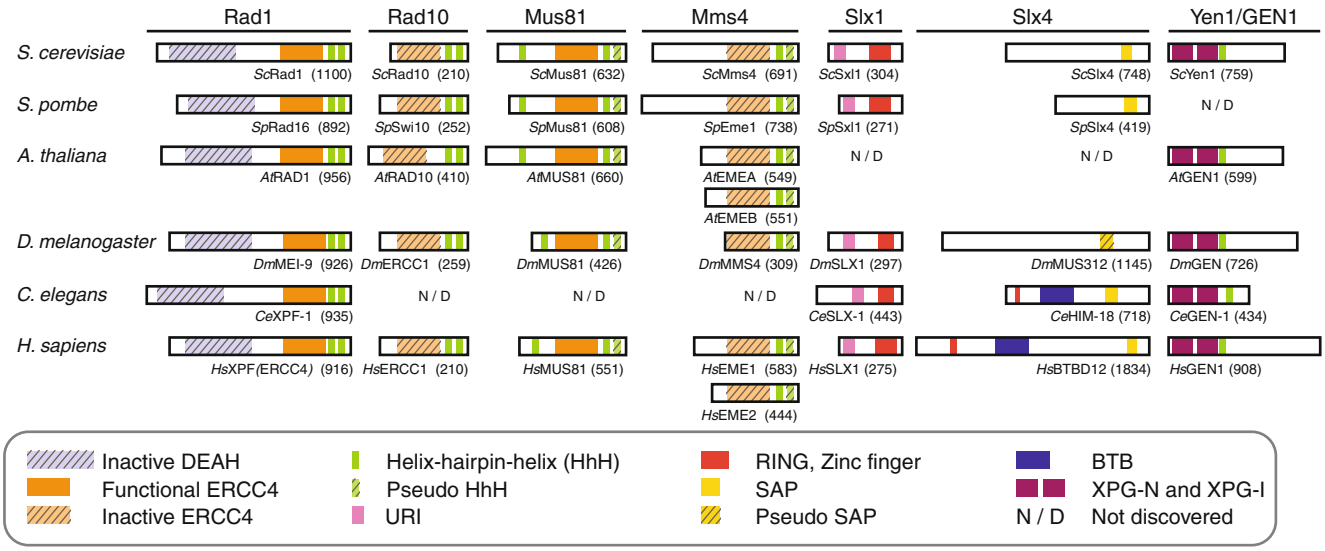

Fig. 3 Domain structure and architecture of eukaryotic structure-selective junction endonucleases. The conserved domains of selected endonucleases were identified using Universal Protein Database (UniProt) and NCBI Conserved Domain Search software. The respective protein names and amino acid lengths are noted below each protein. Domains are identified as described in the *key*. *N/D*, not discovered

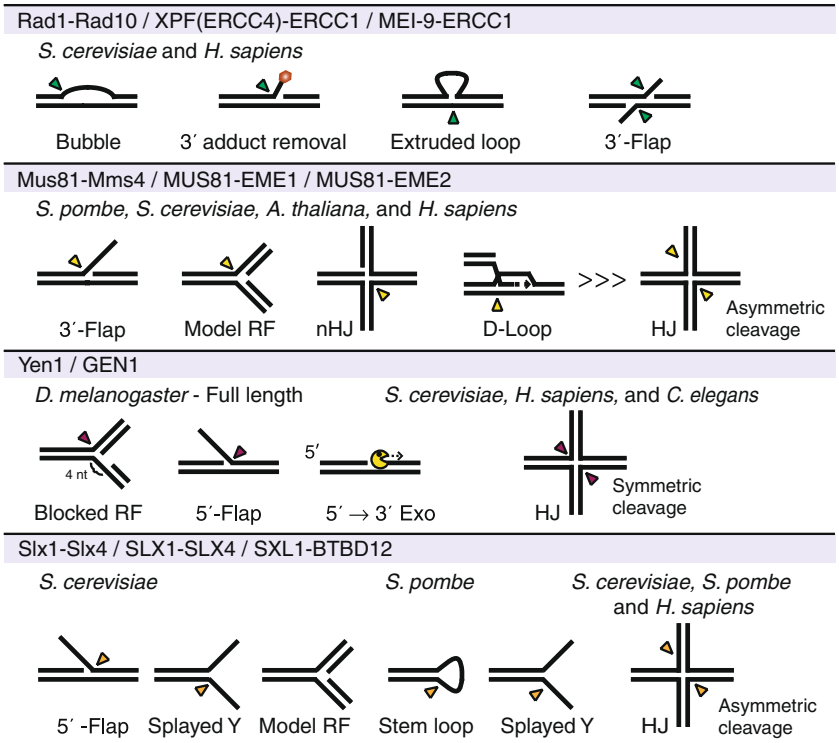

Fig. 4 Substrate specificity of eukaryotic structure-selective junction endonucleases. Mapped incision sites for Rad1 (*green triangles*), Mus81 (*yellow triangles*), Yen1/GEN1 (*purple triangles*), and Slx1–Slx4 (*orange triangles*) are indicated. The incision sites for Slx4 on replication forks have not been mapped and are therefore not represented. *D-Loop*, displacement loop; *nHJ*, nicked Holliday junction; *RF*, replication fork; *NER*, nucleotide excision repair; *SSA*, single-strand annealing; *Model RF*, model replication fork; *Exo*, exonuclease activity

to form a temporary bubble structure (see Fig. 4) that is readily cleavable by the enzyme (Davies et al. 1995; West 1995). The current DSB repair paradigm predicts the formation and resolution of HJs for proper CO formation, connecting meiotic phenotype with proper HJ resolution. Under this assumption, nucleases observed to have disruptive phenotypes in meiosis are often assumed to play a role in HJ resolution. As a proposed HJ resolvase, Rad1 would be predicted to have a meiotic phenotype. However, there is no genetic evidence in budding or fission yeast for an involvement of Rad1–Rad10 or Rad16-Swi10, respectively (Table 1), in meiotic CO formation ((Dowling et al. 1985); reviewed in Heyer et al. (2003)). Contrary to the situation in budding and fission yeast, in *D. melanogaster*, meiotic COs are largely dependent on the fly XPF homolog, MEI-9 (Sekelsky et al. 1995; Carpenter and Sandler 1974). MEI-9 is undoubtedly the XPF homolog in flies, as mutants in this gene also display the UV and interstrand crosslink sensitivities expected for an NER defect (Table 1) (Radford et al. 2007; Yildiz et al. 2004; Sekelsky et al. 1995). Moreover, these DNA repair defects are identical to the mutant phenotypes of the *ERCC1* homolog (Radford et al. 2005), suggesting that NER is also catalyzed in *D. melanogaster* by the MEI-9–ERCC1 heterodimeric nuclease, as in all other eukaryotes studied to date (Fig. 3; Table 1). However, *ERCC1* mutations cause a significantly milder defect in meiotic CO formation than *MEI-9* mutants (Radford et al. 2005), suggesting that MEI-9 may participate with an alternate subunit for this function. Supporting this conjecture, MEI-9 also interacts with the fly Slx4 homolog, MUS312. Mutations in *MUS312* cause a severe CO defect comparable to mutations in *MEI-9* (Andersen et al. 2009; Yildiz et al. 2002). In both yeast and flies, the XPF homolog has two different non-nuclease interaction partners, Rad10 and Slx4 in yeast and the homologous ERCC1 and MUS312 in flies. However, the functions of these complexes are clearly different as, unlike fly MEI-9 and MUS312, yeast Rad1 and Slx4 have no apparent role in meiotic CO formation ((Dowling et al. 1985; Mullen et al. 2001); reviewed in Heyer et al. (2003)).

A comparison of XPF and XPF protein complexes between yeasts (budding and fission yeast) and *D. melanogaster* reveals a surprising plasticity in their in vivo functions. While in all organisms studied XPF–ERCC1 acts in NER and interstrand crosslink repair, the XPF–ERCC1–SLX4 complex functions in yeast in SSA but not in meiotic CO formation, whereas in flies XPF–ERCC1–SLX4 (MUS312) are responsible for the great majority of meiotic COs. It will be interesting to compare the biochemical characteristics of these protein complexes from yeast and flies to determine if the junction specificity differs between the two species and accounts for the difference in generating meiotic COs.

S. cerevisiae Mus81–Mms4/*Schizosaccharomyces pombe* MUS81–EME1/human MUS81–EME1

Mus81 was identified in two-hybrid screens using the recombination protein Rad54 as bait in *S. cerevisiae*

(Interthal and Heyer 2000) and the damage response kinase Cds1 (*Sc*Rad53, human CHK2) as bait in fission yeast (Boddy et al. 2000). Similar to other XPF family endonucleases, Mus81 forms a heterodimer with a non-nucleolytic subunit: Mms4 in budding yeast or EME1 in fission yeast and humans (Fig. 3; Table 1) (Boddy et al. 2001; Kaliraman et al. 2001; Mullen et al. 2001). Mus81 and Mms4/EME1 are required for the recombination-mediated DNA repair at replication forks, playing a key role in replication fork restart, and in yeast meiotic recombination. In *S. cerevisiae*, *S. pombe*, *Arabidopsis thaliana*, and *D. melanogaster*, Mus81 is essential in the absence of the dHJ dissolution complex, Sgs1–Top3–Rmi1 (Boddy et al. 2000; Mullen et al. 2001; Trowbridge et al. 2007; Hartung et al. 2006). In budding yeast and *Drosophila*, this synthetic lethality was largely suppressed by the loss of *Sc*Rad51 and its homolog, *Dm*SPN-A, providing evidence that the joint molecules processed by Mus81 and Sgs1–Top3–Rmi1 are late HR intermediates, possibly resulting from the repair of ssDNA gaps accumulated during DNA replication (Trowbridge et al. 2007; Fabre et al. 2002; Ii and Brill 2005; Bastin-Shanower et al. 2003). The physical interaction between Mus81 and the key recombination protein Rad54 may suggest that Rad54 targets Mus81 to specific recombination-derived joint molecules (Mimida et al. 2007; Interthal and Heyer 2000). Supporting biochemical evidence in both *S. cerevisiae* and humans showed the Rad54-dependent stimulation of Mus81 activity on a variety of synthetic joint molecules (Matulova et al. 2009; Mazina and Mazin 2008).

Loss of either *MUS81* or *MMS4/EME1* in budding yeast and metazoans significantly increases the number of gross chromosomal rearrangements during normal cellular division (Zhang et al. 2006; Dendouga et al. 2005; Abraham et al. 2003). Specifically in somatic cells, Mus81 appears to be required for HR at stalled or broken replication forks (Heyer 2007; Hollingsworth and Brill 2004; Osman and Whitby 2007). Supporting this idea, most eukaryotic cells with defects in either gene display hypersensitivity to a variety of replication fork stalling agents, including the alkylating agent methyl methanesulfonate (MMS), the ribonucleotide reductase inhibitor hydroxyurea (HU), and topoisomerase I inhibitors, such as camptothecin (CPT) (Boddy et al. 2000; Interthal and Heyer 2000; Deng et al. 2005; Hartung et al. 2006; Berchowitz et al. 2007; Froget et al. 2008; Hanada et al. 2007). Mouse cells defected in *MUS81-EME1* are selectively hypersensitive to interstrand crosslinking (ICL) agents (Abraham et al. 2003; Dendouga et al. 2005), whereas human HeLa cells show only a mild CPT sensitivity upon *MUS81* depletion (Svendsen et al. 2009). Plasmoduction experiments in budding yeast with palindromic plasmids suggested a role of Mus81–Mms4 in the resolution of extruded cruciforms, a substrate that mimics an sHJ (Cote and Lewis 2008). However, it is difficult to determine the exact structure of the cleaved intermediate from the product analysis performed, and it is possible that during DNA replication snap-back structures that contain a nick formed.

In meiotic CO formation, the importance of Mus81 varies greatly from species to species. In budding yeast and mammals, COs are controlled by two main pathways, one dependent on Mus81 and the second requiring the activities of Msh4–Msh5, two proteins with similarity to the MutS class of mismatch repair proteins (Edelmann et al. 1999; Holloway et al. 2008; Abdullah et al. 2004; Khazanehdari and Borts 2000; Ross-Macdonald and Roeder 1994) (Fig. 5). Unlike the Msh4–Msh5 pathway, Mus81-dependent COs do not exhibit CO interference and COs occur randomly across the chromosomes (de los Santos et al. 2001, 2003; Interthal and Heyer 2000; Whitby 2005; Oh et al. 2008). Approximately 35% of meiotic COs in *S. cerevisiae* are dependent on Mus81 (Fig. 5) (de los Santos et al. 2001, 2003; Interthal and Heyer 2000; Oh et al. 2008). However, in *S. pombe*, loss of Mus81 function results in a severe reduction of spore viability (0.1% vs. 80% in wild type) and a dramatic reduction in meiotic COs (Boddy et al. 2000, 2001; Smith et al. 2003). Contrary to fission and budding yeast, mice defective in *MUS81* exhibit rather minor meiotic phenotypes (Holloway et al. 2008; McPherson et al. 2004). Similarly, *Arabidopsis* and fly MUS81 make a moderate to no contribution towards meiotic CO formation (Hartung et al. 2006; Berchowitz et al. 2007; Trowbridge et al. 2007; Johnson-Schlitz and Engels 2006).

It is notable that, while Mus81 plays a significant role in meiotic HR, a DSB-initiated event in budding and fission yeast, Mus81 is not required for DSB survival in mitotic cells (Boddy et al. 2000; Interthal and Heyer 2000; Ho et al. 2010). Neither metazoans nor fungal *mus81*-deficient cells are sensitive to IR or endonuclease-induced DSBs, which require HR for repair. However, using a chromosomal system to detect unselected products of mitotic recombination, Ho et al. (2010) showed a reduction of CO formation following *Sce*-I-induced DSB formation in *mus81* mutants. Without loss of viability, events were channeled in *mus81* mutants to NCO and BIR outcomes (Ho et al. 2010). While these important results implicate Mus81–Mms4 in CO formation in mitotic cells, the nature of the intermediate cleaved by Mus81–Mms4 cannot be determined by these genetic studies.

Mus81-deficient yeast cells are very sensitive to the topoisomerase I inhibitor camptothecin, which is believed to lead to replication-dependent one-sided DSBs that can be recovered by HR similar to BIR as drawn Fig. 1 (Pommier 2006). This might suggest a role of Mus81–Mms4 in one-sided DSB repair, consistent with observations in fission yeast (Roseaulin et al. 2008). However, camptothecin has also been shown to result in topologically stalled forks

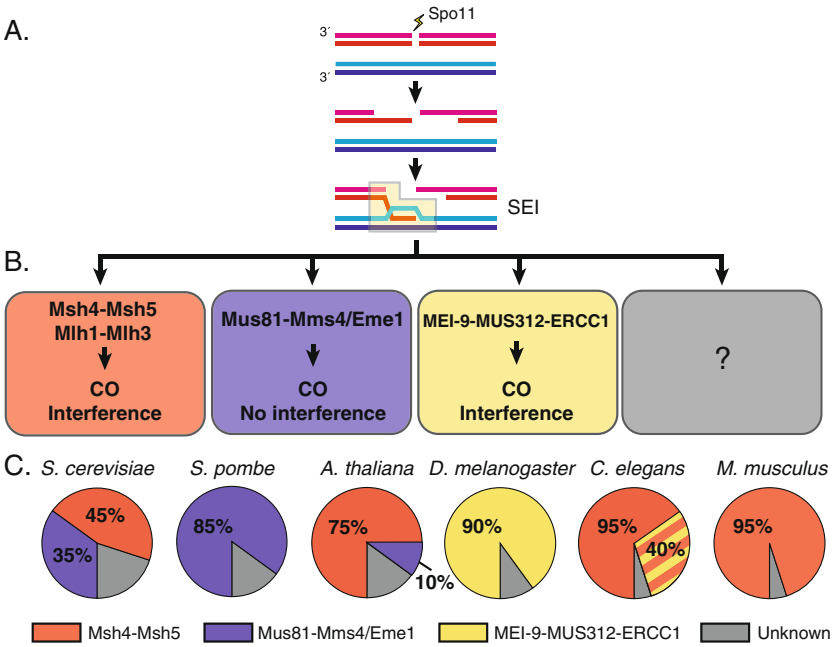

Fig. 5 Meiotic crossover pathways in eukaryotes. **a** In meiosis, double-strand breaks are catalyzed by Spo11. These breaks are processed to form single-stranded 3′ ends capable of strand exchange and D-loop formation. Single-end invasion (*SEI*) events commit the break into a pathway that results in crossover (*CO*) and chiasma formation (Hunter and Kleckner 2001). **b** Eukaryotes have evolved multiple pathways for CO formation as denoted by their genetic definition. Because residual CO formation still remains after the elimination of two or more of these CO pathways, an *empty box* represents CO formation dependent on yet unknown pathways. **c** For each pathway in **b**, CO contribution in percent is organized in *pie graphs* for several eukaryotes. Potential overlapping activities in CO formation between the MSH4–MSH5 and SLX4 are observed in *C. elegans* and are designated as *striped sector*. A subtle role for mouse MUS81 in meiosis has been proposed with histological and cytological data, but no quantification has been made to assess the degree of CO contribution (McDonald and Rothstein 1994; de los Santos et al. 2001, 2003; Argueso et al. 2004; Osman et al. 2003; Berchowitz et al. 2007; Yildiz et al. 2002, 2004; Radford et al. 2005, 2007; Zalevsky et al. 1999; Saito et al. 2009; Edelmann et al. 1999; Holloway et al. 2008)

(Koster et al. 2007), and Mus81–Mms4 might act on these substrates instead of one-sided DSBs (Froget et al. 2008).

Biochemical studies with purified Mus81–Mms4/EME1 complexes from budding and fission yeasts, as well as humans, have generated a wealth of data regarding the substrate preference for these enzymes (Heyer 2007; Hollingsworth and Brill 2004; Osman and Whitby 2007; Ciccia et al. 2008). Difficulties in purifying catalytically active complexes containing the full-length proteins complicate the analysis and required that the experiments were performed with significant excess of protein over substrate under single turnover conditions (Bastin-Shanower et al. 2003; Boddy et al. 2001; Chen et al. 2001; Ciccia et al. 2003; Whitby et al. 2003; Smith et al. 2003; Cote and Lewis 2008; Taylor and McGowan 2008; Constantinou et al. 2002; Gaskell et al. 2007; Doe et al. 2002; Osman et al. 2003; Chang et al. 2008). Classical Michaelis–Menten analysis of catalytically active Mus81–Mms4 under conditions of excess substrate added clarity to the question of in vitro substrate specificity (Ehmsen and Heyer 2008, 2009; Fricke et al. 2005). There is significant congruence in these studies, which showed that Mus81–Mms4/EME1 from all organisms studied greatly prefer substrates that contain a discontinuity or nick adjacent to the branch point of the junction (Fig. 4). This is supported by structural evidence from archaeal XPF family members which share the requirement for a 5′ end near a junction for structural specificity and flexibility of the duplex arms for DNA joint cleavage (Bastin-Shanower et al. 2003; Fricke et al. 2005; Roberts and White 2005a, b; Ehmsen and Heyer 2008, 2009). Also, the available structural information on MUS81–EME1 protein suggests that nicked substrates are favored, as the arms of nicked junctions are flexible to position the incision point into the catalytic site (Chang et al. 2008).

The discordance is in the interpretation of the activity of Mus81–Mms4/EME1 on substrates mimicking HJs (reviewed in Heyer 2007; Hollingsworth and Brill 2004; Osman and Whitby 2007; Ciccia et al. 2008). The activity of Mus81–Mms4/EME1 on HJ substrates with four contiguous strands is very significantly lower than on the preferred nicked substrates. For example, full-length human MUS81–EME1 cleaves model replication forks and 3′ flap structures 75-fold more efficiently than stationary HJs (Ciccia et al. 2003). Furthermore, there is some disagreement about the substrate selectivity in two studies that analyze near-identical N-terminal truncations of both sub-

units in the human MUS81–EME1 complex (Chang et al. 2008; Taylor and McGowan 2008). In one study, no difference to the full-length protein was discovered with no significant cleavage of HJs (Chang et al. 2008). Another study showed cleavage of HJs with tenfold excess of protein and of extruded cruciforms with at least 40-fold excess that was still less efficient than the cleavage of nicked junctions or 3′ flaps (Taylor and McGowan 2008). It will be interesting to understand the reasons for this apparent difference. The budding yeast enzyme also cleaves a variety of joint molecules much better than HJs (Fricke et al. 2005; Ehmsen and Heyer 2008). Note that 3′ flaps are one of the preferred substrates together with replication forks, nicked HJs, and D-loops showing a low nanomolar K_M, whereas HJ cleavage was so inefficient that kinetic parameters could not be determined (Ehmsen and Heyer 2008; Fricke et al. 2005). The observed HJ cleavage was asymmetrical across the junction, producing unligatable nicks along the four-way junction, suggesting the presence of two uncoordinated cleavage events, unlike the paradigmatic RuvC resolvase model (Constantinou et al. 2002; Ehmsen and Heyer 2008; Chen et al. 2001; Boddy et al. 2001; Gaskell et al. 2007; Gaillard et al. 2003; Osman et al. 2003). Mus81 activity on HJs may be subjected to posttranslational control after DNA damage. However, Mus81–Mms4 purified from *S. cerevisiae* after DNA damage induction also exhibited no detectable HJ cleavage (Ehmsen and Heyer 2008). There is also the possibility of an associating factor that modulates substrate specificity, but no candidate has been identified yet genetically or biochemically. Another possible explanation is species-specific differences in enzyme properties, but this has been directly tested and excluded for the budding and fission yeast enzymes (Gaskell et al. 2007).

In summary, Mus81–Mms4/EME1 plays an important role in DNA repair and replication fork support in somatic cells in all organisms studied; however, its contribution to meiotic CO formation displays a considerable variation from organism to organism (Fig. 5). This species-dependent variation mirrors the situation with XPF discussed above, suggesting flexibility in which particular XPF family endonuclease is utilized for meiotic CO formation. There is a wide consensus that Mus81–Mms4/EME1 endonucleases from all organisms studied have strong a preference for nicked junction substrates over classical HJ substrates. However, much of the biological significance of the Mus81 endonuclease, specifically its role in CO formation, has been attributed to its cleavage of HJs, which is low at best. As discussed at the end, this interpretation is largely model-driven (Figs. 1 and 2) and based on the assumption that, in vivo, the majority of recombination intermediates are HJs with four uninterrupted strands (i.e., lacking a nick). Later, we will present an alternative model and revisit some of these key assumptions.

Yen1/GEN1: Rad2/XPG endonuclease superfamily

GEN1 was first identified in rice as *Os*SEND-1 (Furukawa et al. 2003) and later in *Drosophila* as *Dm*GEN (Ishikawa et al. 2004) in a search for novel Rad2 family endonucleases. Named for XPG-like endonuclease, GEN contains both N-terminal and internal conserved Rad2/XPG family nuclease domains with significant homology to the XPG homolog *MUS201* (Fig. 3) (Ishikawa et al. 2004; Kanai et al. 2007). Rad2 (XPG in humans) delivers the 3′ incision to liberate the damage-containing oligonucleotide during NER (Fig. 4). Preliminary biochemical characterization on *E. coli*-expressed *Dm*GEN identified exo- and endonuclease activities on gapped and intact duplex DNA (Ishikawa et al. 2004). A subsequent biochemical analysis of full-length recombinant GEN used an extensive panel of oligonucleotide-based substrates. Similar to its XPG homolog, *Dm*GEN exhibited 5′ flap endonuclease activity and a weak 5′-to-3′ exonuclease activity on nicked duplex substrates (Kanai et al. 2007). However, unlike its Rad2 family counterparts, *Dm*GEN was unable to cleave a bubble structure and instead showed preference towards substrates mimicking stalled replication forks (Kanai et al. 2007). Interestingly, purified full-length *Dm*GEN was unable to cleave intact HJ substrates with almost eightfold molar excess protein over substrate (Kanai et al. 2007). The authors concluded that *Dm*GEN was a flap endonuclease, which constituted a new class of the Rad2/XPG endonuclease family.

In 1985, the identification of RuvC-like HJ cleavage activity in eukaryotic cell extracts initiated a decades-long hunt for the eukaryotic HJ resolvase activity (Elborough and West 1990; Parsons and West 1988; Symington and Kolodner 1985; Constantinou et al. 2001, 2002). The RuvC-like activity in human cells was determined to be dependent on the catalytic activity of the Rad2/XPF endonuclease *Hs*GEN1 (Ip et al. 2008). *Hs*GEN1 was isolated by column fractionation and identified by mass spectroscopy as a 381-amino-acid C-terminal truncation. Concurrent analysis in budding yeast used a TAP fusion library to screen for nucleases capable of cleaving synthetic HJs, identifying both Mus81 and the yeast GEN1 homolog Yen1. Similar to the human *Hs*GEN1 proteolytic fragment, Yen1 exhibited a symmetrical cleavage of synthetic HJs, although it is not clear whether this activity is associated with the full-length protein or a proteolytic fragment (Ip et al. 2008).

Recombinant *Hs*GEN1(1-527), a fragment similar to the originally identified truncation, readily cleaves 5′ flaps and model replication fork structures (Rass et al. 2010). Contrary to what was found with full-length *Dm*GEN, purified recombinant *Hs*GEN1(1-527) exhibits activity on synthetic HJs in vitro that is higher than on 5′ flaps or model fork structures under single turnover conditions (Ip et al. 2008; Rass et al. 2010). Purification and analysis of

full-length *Hs*GEN1 or the *Drosophila* fragment equivalent to *Hs*GEN1(1-527) have not been reported yet. Characteristics of *Hs*GEN1(1-527) activity on HJs emulated those of the bacterial resolvase RuvC (Ip et al. 2008). Using 15-fold excess protein, cleavage of migratable HJs occurred symmetrically to produce religatable products (Ip et al. 2008). Additional studies found that the *Hs*GEN1(1-527) cleavage across the junction was one nucleotide to the 3′ side of the junction and exhibited an orientation bias similar to RuvC (Rass et al. 2010). Hydrodynamic characterization and in vivo coimmunoprecipitation results suggested that the *Hs*GEN1 fragment is a monomer in solution and oligomerizes on DNA to conduct a coordinated cleavage of four-way junctions (Rass et al. 2010). The observation that only a proteolytic fragment, but not full-length human GEN1, cleaves Holliday junctions is rather puzzling. It has been suggested that post-translational modification may be responsible to unmask the HJ resolvase activity in GEN1 (Ip et al. 2008), but the nature of the modification and mechanisms involved have not been determined yet.

Characterizing the biological function of GEN1/Yen1 will be critical to understanding the significance of their HJ resolvase activity in vivo. Fission yeast *S. pombe* lacks a Yen1/GEN1 homolog, precluding genetic analysis. However, genetic experiments with *yen1* null mutants in budding yeast showed no effect on cell growth, viability, resistance to genotoxic agents, intersister HR at tandem repeats, and meiotic CO formation (Neil Hunter, personal communication) (Johnson et al. 1998; Blanco et al. 2010; Tay and Wu 2010; Ho et al. 2010). Similarly, siRNA knockdown of *GEN1* in HeLa cells exhibits little if any phenotype in response to CPT, MMS, or UV treatment (Svendsen et al. 2009). Furthermore, a mutation in the *Caenorhabditis elegans* homolog, *gen-1*, showed no embryonic lethality or enhanced incidence of XO males, which are sensitive readouts for meiotic chromosome pairing, meiotic HR, or meiotic chromosome segregation defects in worms (Bailly et al. 2010). Hence, *YEN1/GEN1* mutants in three different organisms lack the phenotypes predicted for the major HJ resolvase acting in HR based on the classic model for HJ formation as a major recombination intermediate. Lastly, the genetic analysis of *gen-1* in *C. elegans* suggested a role of GEN-1 in worm DNA damage signaling, as mutants in this gene were isolated in a forward genetic screen for DNA damage response signaling mutants using a complex and indirect visual screen after IR exposure of L4 larvae (Bailly et al. 2010). It is unclear whether GEN-1 is directly involved in signaling or whether pathological DNA intermediates accumulating in these mutants give rise to the observed phenotype, similar to the spindle (*spn*) phenotype in *D. melanogaster* mutants, which encode core components of HR (*spnA* is *Drosophila RAD51*, *spnB* and *spnD* encode Rad51 paralogs) (Ghabrial et al. 1998; Staeva-Vieira et al. 2003).

Given the complexity of structure-selective DNA endonucleases with multiple enzymes potentially competing for the same or similar substrates (see Table 1; Fig. 4), more complex genetic analysis will be required to unravel the in vivo function of Yen1/GEN1. In budding yeast, a *yen1* mutation enhances the phenotype of *mus81* mutant cells for sensitivity to MMS, UV, CPT, or phleomycin (Blanco et al. 2010; Tay and Wu 2010; Ho et al. 2010). Curiously, IR sensitivity even in the *mus81 yen1* double mutant is minimal and only apparent at very high doses of radiation, even though IR-induced DSBs are repaired almost exclusively by HR in yeast (Blanco et al. 2010; Ho et al. 2010). A thorough study of the outcomes (CO, NCO, BIR) of DSB repair in budding yeast *mus81 yen1* single and double mutants exposes a complex system where Mus81 contributes significantly to CO formation after DSB-initiated HR in mitotic cells (Ho et al. 2010). While the *yen1* single mutant had no effect, in a *mus81*-deficient strain the loss of *yen1* essentially eliminated COs (Ho et al. 2010). Interestingly, in the *mus81 yen1* double mutants, events were channeled to BIR, not NCO, without loss of viability (Ho et al. 2010). An analysis of in vivo HJ resolution by Mus81 and Yen1 using a plasmid-based HJ structure transformed into *S. cerevisiae* showed a reduction of substrate cleavage in the *mus81 yen1* double mutant, while the single mutants had no apparent effect on cleavage (Tay and Wu 2010). The residual HJ resolution in the *mus81 yen1* double mutant (~50%) was found to be independent of Rad1 and Slx1 (Tay and Wu 2010). A proportion of cleavage events were attributed to the cleavage of substrates containing two or more nicks, and it is unclear whether the transformed plasmid-based substrate accurately mimics an intact HJ substrate. Since double HJs are considered a key intermediate in meiotic HR, it is also important to extend the characterization of these mutants to meiosis.

An enhanced phenotype in the double mutants compared to the compound single mutants is often interpreted as "redundant activities". However, from evolutionary considerations, completely "redundant" functions are unlikely to exist due to lack of selective pressure i.e., each individual enzyme must have a unique function that provides a selective advantage. Moreover, it is unclear whether the two enzymes, here Yen1 and Mus81–Mms4, truly compete for the same substrate or whether Yen1 cleaves pathological structures that accumulate in *mus81* mutants due to processing of the original Mus81–Mms4 substrate. For example, a nicked HJ may be the in vivo substrate for Mus81–Mms4, but in the *mus81* mutant such a substrate may be ligated and become a substrate for Yen1. The uncertainty about the in vivo substrate of the Mus81–Mms4/EME1 endonuclease further complicates any interpretation about potential Yen1 in vivo substrates. In particular, it is worth pointing out that the relevant structure

for HR in the current model is the dHJ (Fig. 1), but no published data are available for this substrate with RuvC, Mus81–Mms4, or Yen1/GEN1.

The subtle nature of the phenotypes of the *yen1/gen1* mutations brings up a very interesting question. If HJs are the dominating recombination structure, then why is the phenotype of the only eukaryotic enzyme, Yen1, with characteristics of the RuvC resolvase so inconspicuous? The fact that *yen1/gen1* defects cause such a subtle phenotype and are completely absent in *S. pombe* suggests that intact HJs may not be the main recombination intermediate in eukaryotes.

Slx1–Slx4: URI–YIG family endonucleases

Slx1 and Slx4 were identified in *S. cerevisiae* by a productive genetic screen for synthetic lethal mutations with a defect in the Sgs1 helicase (Mullen et al. 2001). Sequence alignment reveals Slx1 to have a conserved UvrC-intron (URI)-endonuclease domain and a C-terminal RING/PHD-type zinc finger domain (Fig. 3) (Fricke and Brill 2003). These characteristics place Slx1 into the URI–YIG family of endonucleases including *E. coli* UvrC (Dunin-Horkawicz et al. 2006). A genetic analysis of *S. cerevisiae slx1* and *slx4* deletion phenotypes identifies these genes to function in the recombination-mediated repair of stalled replication forks (Fricke and Brill 2003; Deng et al. 2005). A biochemical analysis of the Slx1–Slx4 complex demonstrated the cleavage of a variety of DNA junctions in vitro, with preference for Y-splayed, 5′ flaps, and model replication forks (Fricke and Brill 2003). The incision mapping of 5′ flap and model replication forks places phosphodiester cleavage 3′ of the nonhomologous region and adjacent to the branch point, generating ligatable products (Fricke and Brill 2003). Budding yeast Slx1–Slx4 was also tested on HJ substrate, showing some activity towards mobile HJs and very low activity towards fixed HJs. The HJ cleavage was asymmetric across all four arms and the products were not compatible with direct ligation, leading the authors to conclude that HJs are not a physiologically relevant substrate for Slx1–Slx4 (Fricke and Brill 2003).

Interestingly, the very same synthetic lethal screen with *sgs1* also identified Mus81 and Mms4 (Mullen et al. 2001). One of the key arguments to place the Mus81–Mms4 endonuclease in the HR pathway resolving recombination-mediated DNA joint molecules is the suppression of the synthetic lethality of *mus81* (or *mms4)* mutants with an *sgs1* mutation by an HR defect caused by a mutation in *RAD51*, *RAD52*, *RAD54*, *RAD55*, or *RAD57* (Bastin-Shanower et al. 2003; Fabre et al. 2002). This result strongly suggests that HR generates substrates that require processing either by Mus81–Mms4 or the Sgs1–Top3–Rmi1 complex. Importantly, the synthetic lethality between *slx1* or *slx4* with *sgs1* is not suppressed by an HR defect in budding yeast (Fricke and Brill 2003). This indicates that the synthetic lethality of *slx1* (or *slx4*) with *sgs1* is not caused by toxic recombination intermediates. Integrating the in vitro substrate specificity with the genetic data, the Brill laboratory proposed a cogent model for the function of Slx1–Slx4 in replication termination, when two converging replication forks stall (Fricke and Brill 2003). In fact, a specific defect in the replication of the rDNA repeat was uncovered in $sgs1^{ts}$ *slx4* double mutants after a shift to the restrictive temperature (Kaliraman and Brill 2002). The same conclusion was reached in independent studies of the fission yeast Slx1–Slx4 complex (Coulon et al. 2004, 2006). Therefore, genetic and biochemical analyses do not support a role of the Slx1–Slx4 complex in Rad51-mediated recombination in yeasts.

Mutations in *SLX4* display significantly enhanced and novel phenotypes compared to defects in its partner *SLX1* (Deng et al. 2005; Fricke and Brill 2003; Flott et al. 2007). Slx4 binds Rad1 in a manner that is mutually exclusive with Slx1 and stimulates the 5′ flap endonuclease activity of Rad1–Rad10 to facilitate the cleavage of nonhomologous tails during SSA (Fig. 1; Table 1) (Toh et al. 2010; Flott et al. 2007). The nature of this interaction is specific to SSA and has no involvement in the Slx4-dependent recovery of stalled replication forks after MMS damage (Flott and Rouse 2005; Li et al. 2008) (recently reviewed in Lyndaker and Alani (2009)). Analyses of human and *Drosophila* Slx4 homologs, BTBD12 and MUS312, respectively, have revealed the conservation of this interaction in other eukaryotes (Table 1) (Andersen et al. 2009; Fekairi et al. 2009; Munoz et al. 2009; Svendsen et al. 2009; Toh et al. 2010). Budding yeast Slx4 also binds the BRCT-containing protein, Rtt107, and is responsible for Mec1-dependent Rtt107 phosphorylation in response to alkylation damage (Roberts et al. 2006).

Regulation of Slx4 by posttranslational modification may facilitate these context-specific protein interactions. Slx4 is the target of DNA damage-induced phosphorylation by the Mec1/Tel1 kinases in response to IR, MMS, CPT, HU, and 4-nitroquinoline oxide (Flott and Rouse 2005). Mutation of seven putative Mec1 phosphorylation sites on Slx4 abolishes its interaction with Dpb11 causing sensitivity to replication stress induced by the alkylating agent MMS but not HU or CPT (Ohouo et al. 2010). This suggests that, upon MMS-induced replication stress, Mec1 phosphorylation induces the assembly of a complex between Dpb11, the Slx4–Slx1 nuclease complex, and the Slx4-associated scaffold protein Rtt107, which perform yet-to-be-determined functions in replication fork recovery (Ohouo et al. 2010).

Significant progress was made by the discoveries of the mammalian, worm, and *Drosophila* Slx4 homologs, BTBD12, HIM-18, and MUS312, respectively, which share with Slx4 a conserved C-terminal region (SAP or pseudo-SAP domain in Fig. 3) that may act as a DNA docking site used for substrate specificity (Andersen et al. 2009; Fekairi et al. 2009; Munoz et al. 2009; Svendsen et al. 2009; Saito et al. 2009). Mammalian BTBD12 and *C. elegans* HIM-18 share a conserved coiled–coil domain, as well as a Broad-complex, Tramtrack, Bric-a-brac (BTB) domain that has been speculated to mediate protein interactions (Fig. 3) (Stogios et al. 2005). As seen in budding yeast, mammalian and *Drosophila* SLX4 was observed to bind multiple endonuclease partners by coimmunoprecipitation and proteomic approaches using ectopically expressed proteins (Fekairi et al. 2009; Munoz et al. 2009; Svendsen et al. 2009) (Table 1). Some of these partners were homologous to known budding yeast Slx4-associating proteins including yeast Slx1 and Rad1, corresponding to XPF and MEI-9 in human and *Drosophila*, respectively (Svendsen et al. 2009). Several novel Slx4 interactions not previously found in yeast were also observed, including MUS81, mismatch repair proteins, and telomere-associated proteins (Svendsen et al. 2009).

Mammalian BTBD12 (SLX4) complexes were reported to cleave a variety of DNA junction substrates including 5′ flaps and model replication forks, similar to the budding yeast enzyme (Fig. 4) (Fekairi et al. 2009; Munoz et al. 2009; Svendsen et al. 2009; Fricke and Brill 2003). Immunoprecipitated BTBD12 complexes were also reported to cleave HJs, similar to the low level of HJ cleavage observed with budding yeast Slx1–Slx4 (Fekairi et al. 2009; Munoz et al. 2009; Svendsen et al. 2009; Fricke and Brill 2003). Several HJ substrates were analyzed for cleavage and incision mapping, showing that BTBD12 and associating proteins have divergent incision patterns, and only 25% of resolved products are religatable (Fekairi et al. 2009; Munoz et al. 2009; Svendsen et al. 2009). These enzymatic characteristics are similar to what was observed in yeast and are inconsistent with the RuvC paradigm of symmetrical cleavage and robust catalytic HJ cleavage activity. The biochemical analysis of the mammalian BTBD12 complexes and associated endonuclease activities (Fekairi et al. 2009; Munoz et al. 2009; Svendsen et al. 2009) is still rather incomplete and hampered by the unavailability of catalytically active enzyme preparations, requiring the addition of excess protein over substrate and making potential contaminations a pertinent issue. In addition, the effect of different cations (Mn^{++} versus Mg^{++}) on catalytic efficiency or substrate selectivity has not been fully evaluated, but significant differences in cleavage efficiency have been noted (Fekairi et al. 2009). Moreover, the biochemical analysis of immunoprecipitated BTBD12 is complicated by its association with three different nuclease subunits, SLX1, XPF, and MUS81. It is presently unclear whether these interactions all occur simultaneously or are mutually exclusive. Hence, it is difficult to assess the significance of the reported cleavage of HJs by mammalian BTBD12-containing complexes.

The identification of *Drosophila* MUS312 as a Slx4 homolog is of particular importance as this gene is genetically well characterized and required for 90–95% of meiotic COs in flies (Fig. 5) (Yildiz et al. 2002; Green 1981). MUS312 functions together with MEI-9 (XPF) in meiotic CO formation but has no role in the NER function of MEI-9. Moreover, it appears that MUS312 has a more crucial function and acts independently of MEI-9 in interstrand crosslink repair. Similar to the situation in yeast, a defect in *MUS312* is synthetically lethal with a mutation in *MUS309*, encoding the fly BLM helicase (budding yeast Sgs1), as is a defect in the fly *MUS81* homolog (Trowbridge et al. 2007; Andersen et al. 2009). Importantly, and again in congruence with genetic findings in yeast, the synthetic lethality of *mus81 mus309* double mutant is suppressed by an HR defect caused by a *spnA* mutation affecting the fly Rad51 homolog, while the synthetic lethality of the *mus312 mus309* double mutant is not suppressed by *spnA* (Trowbridge et al. 2007; Andersen et al. 2009). This suggests that, like in yeast, the synthetic lethality between *mus312* (*slx4*) and *mus309* (*BLM*/*sgs1*) is not caused by toxic recombination intermediates.

Metazoan SLX4 not only acts during meiosis but also in somatic cells where it contributes to interstrand crosslink repair and general recombination-mediated repair. Knockdown of human BTBD12 or SLX1 causes a sensitivity to interstrand crosslinking agents in HeLa (Andersen et al. 2009; Svendsen et al. 2009; Fekairi et al. 2009), HEK293 (Munoz et al. 2009), and U2OS cells (Andersen et al. 2009), supporting a role of this protein in interstrand crosslink repair, consistent with its initial discovery and subsequent analysis in flies (Andersen et al. 2009; Yildiz et al. 2002; Green 1981). The reduced expression of BTBD12 and SLX1 also reduced DSB-initiated recombination in a GFP reporter assay in U2OS cells (Munoz et al. 2009). It is unclear whether this reduction could be a reflection of a cell proliferation defect that was observed when the expression of these genes was knocked down HeLa cells (Andersen et al. 2009). Contrary to what would be expected from a general HR defect, U2OS cells depleted for BTBD12 or SLX1 are not sensitive to IR (Svendsen et al. 2009). However, BTBD12 and SLX1 knockdown in HEK293 cells does cause IR sensitivity (Munoz et al. 2009). These disparate results may be the result of using different cell lines or different degrees of knockdown and suggest that caution is required before concluding that BTBD12–SLX1 is involved in HR in somatic human cells.

In conclusion, the in vivo function of Slx1–Slx4 shows considerable plasticity between species and context-specific situations. In flies, *MUS312* controls the majority of meiotic COs; in budding and fission yeast, however, *slx4* or *slx1* mutants do not affect spore viability, strongly suggesting that meiotic HR does not require Slx1–Slx4. Knockdown studies in flies and mammals show a role of SLX4 in interstrand crosslink repair, a process that involves HR. However, the synthetic lethality observed between Slx4 and the Sgs1 helicase is not rescued by an HR defect in either budding yeast or *Drosophila*, which suggests that the related functions between Slx4 and the Sgs1 complex occur independently of recombination-mediated joint molecule resolution but possibly in replication termination. It will be of great interest to characterize the *Drosophila* meiotic MUS312-containing nuclease complexes and reconstitute the different human BTBD12 complexes in vitro.

A unified model for all eukaryotes appears unlikely

The complexity of DNA structure-selective endonucleases involved in DNA repair, replication, and recombination is surprising, suggesting that the various enzymes address different junction types or similar junctions occurring in different compartments (nucleus, nucleolus, mitochondria?) during different times during the cell cycle or in the different functional contexts of DNA replication, DNA repair, or meiotic recombination. For example, it is perplexing that fission and budding yeast mutants in the Mus81–Mms4/Eme1 complex are not more sensitive to DSBs in somatic cells but show defects in meiotic recombination (in fission yeast, an essentially absolute defect), a DSB-initiated HR event. It could be argued that somatic DSB repair proceeds always via SDSA, a pathway in which Mus81–Mms4/Eme1 may not play a role, while meiotic DSB repair involves dHJs. However, recently, dHJs were discovered as intermediates in DSB repair in vegetatively growing (somatic) budding yeast cells (Bzymek et al. 2010) and provide evidence against this line of thought. In addition, the present analysis documents significant plasticity between the specific functions of individual nucleases in different organisms. The prime example is XPF, which plays no role in meiotic CO formation in budding or fission yeast, but is essential for this process in flies. These documented differences prevent the proposal of an all-encompassing model on the specific functions of these enzymes that would hold true for all eukaryotes. Furthermore, the overlapping substrate specificity of the nucleases as determined by biochemical experiments does not allow unambiguous assignment and requires substrate targeting or compartmentalization in vivo. Lastly, despite the power of yeast genetics, the interpretation of double or multiple mutant data is difficult as every mutant creates a pathological state and a function of a protein under these conditions may not necessarily reflect such a function in wild-type cells.

Single Holliday and double Holliday junctions: rethinking the paradigm

Ever since the original model by Robin Holliday, the identification of a nuclease capable of symmetric cleavage of HJs to result in CO and NCO products has become a holy grail in the HR field. Bacterial RuvC served as a guide for a search of the equivalent enzyme in eukaryotes. However, the absence of a clear phenotype for mutants in Yen1/GEN1 as well as the abundance of DNA junction-selective nucleases cause significant hesitation to accept the traditional HJ-based model using the RuvC paradigm (Figs. 1 and 2). Currently, the paradigm describes sHJs and dHJs as containing four uninterrupted strands and acting as the major recombination intermediate to direct CO and NCO products (Fig. 1). This expectation is model-driven, and evidence for the presence of intact HJs and dHJs does not exclude the possibility of nicked substrates.

Seminal experiments in the Kleckner laboratory established dHJs as key intermediates for meiotic CO formation (Schwacha and Kleckner 1994, 1995, 1997). Psoralen-crosslinked DNA from meiotic time courses was analyzed by 2D gel electrophoresis and demonstrated the existence of dHJs. Component strand analysis of dHJs by denaturing gel electrophoresis identified full-length, uninterrupted strands in the predicted non-recombinant configuration. While this analysis demonstrated the presence of four uninterrupted strands in the population of dHJs analyzed, it does not demonstrate that every dHJ contains four interrupted strands. Figure 6a illustrates how a population of dHJs, where each individual dHJ has two nicks, can provide an evidence for the existence of four uninterrupted strands in the molecular analysis of the entire dHJ population. dHJs have also been identified as an intermediate in somatic DSB repair in budding yeast (Bzymek et al. 2010), and similar caveats apply there. Analogous arguments can be made for sHJs, which were postulated to be CO intermediates in fission yeast (Cromie et al. 2006).

Maybe the paradigm that HJs, single or double, comprise four uninterrupted strands is too narrow? For Mus81–Mms4/Eme1 and Yen1, there is sufficient biochemical and genetic data to start speculating on a solution on the following conundrums. Problem #1: the only eukaryotic nuclear protein (in fact, a proteolytic fragment) that cleaves HJs with RuvC-like specificity and symmetry has no

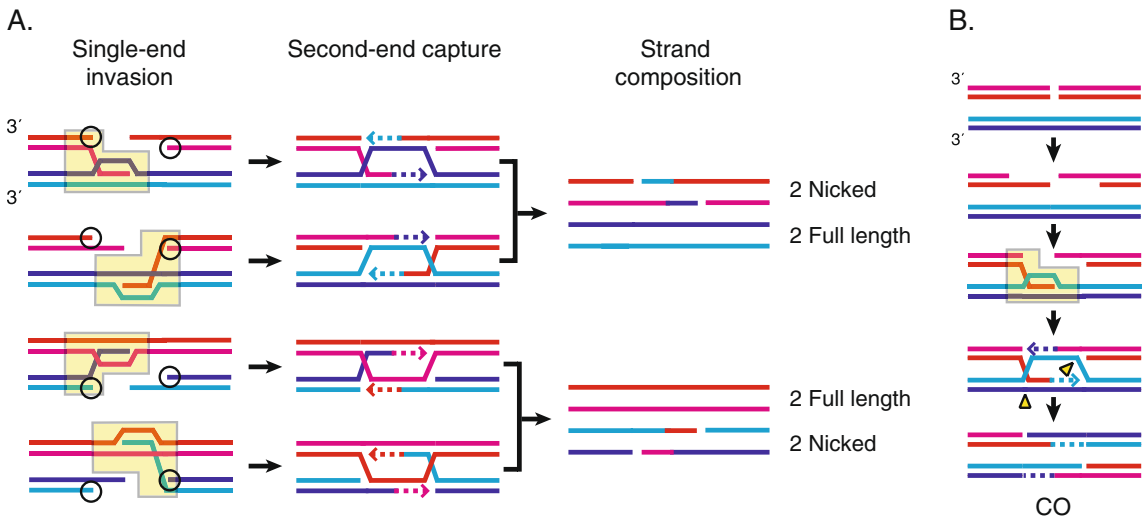

Fig. 6 Crossover formation by cleavage of nicked junctions. **a** Four types of displacement loops are predicted from single-end invasion events initiated by either the left or right DSB ends of the maternal or paternal homolog. *Yellow boxes* designate the single-end invasion intermediates and *circles* indicate the two 5′ ends available to direct subsequent cleavage. The location of the 5′ end will be largely affected by the rate of end resection. From these initial invasion events, second-end capture forms a mixed population of nicked double HJs. The resulting strand compositions of the products are shown. Note that in the population each full-length component strand is represented. **b** Model for crossover formation via concomitant or sequential cleavage of nicked joint molecules. Repair of meiotic DSBs undergo end-processing, single-end invasion, and second-end capture to form joint molecules comprised of two nicked Holliday junctions. Cleavage of both nicked HJs by a structure-selective endonuclease (*yellow triangles*) results in only crossovers due to the directionality imposed by the available nicks

apparent function as a single mutant in HR in budding yeast and mammalian cells and is entirely lacking in fission yeast. Problem #2: Mus81–Mms4/Eme1 control most, if not all, meiotic COs in fission yeast and a significant fraction of COs in budding yeast, yet these enzymes display little and, in some studies, no activity towards intact HJs in vitro but readily cleave nicked junctions in all instances studied. Moreover, the analysis of recombination junctions in budding yeast *mms4* cells demonstrated a decrease in the levels of dHJs and single-end invasions (de los Santos et al. 2003). This suggests that Mus81–Mms4 is not involved in processing these intermediates in *S. cerevisiae* and implies that the crosslinking procedure that discovered dHJs and single-end invasions is unable to identify the Mus81–Mms4 in vivo substrates. In fission yeast, HJs accumulate in *mus81* mutants (Cromie et al. 2006; Gaillard et al. 2003), but it is unclear (1) whether HJs are the substrate for Mus81 or a precursor joint molecule, such as a D-loop, and (2) if these HJs contain a nick or consist of four uninterrupted strands.

How can the processing of nicked junctions lead to COs? Figure 1 indicates a number of DNA junction molecules that may occur during HR, and Fig. 6b illustrates a potential pathway where the sequential cleavage of nicked junctions leads to CO formation based on previous proposals (Heyer et al. 2003; Osman et al. 2003; Whitby 2005). Importantly, this sequence of junction processing always leads to a CO outcome. A genetic analysis led Cromie and Leach (2000) to propose that nicks could provide the asymmetry required for the differential loading of the endonuclease and contribute to a bias of CO or NCO products depending on its orientation. A related proposal on nick-instructed resolution has been made before (Gilbertson and Stahl 1996), and a recent model (Stahl and Foss 2010) posits that the position of a nick directs the mismatch repair machinery to heteroduplex regions at meiotic DSB.

Mus81–Mms4/EME1 strongly prefers nicked junction substrates over classic HJs, and cleavage of nicked substrates would also provide a rationale for the recombination-dependent lethality of the *mus81 sgs1* double mutant. Absence of Mus81–Mms4 activity may lead to the ligation of these nicked junctions, generating a different substrate in *mus81* (or *mms4*) mutants. Nicked dHJs formed during replication fork support, DSB, or gap repair may thus become intact dHJs with four uninterrupted strands, a substrate for dissolution by the Sgs1–Top3–Rmi3 complex. Likewise, the absence of Mus81 could allow for nicked joint molecules to progress into intact HJs, where the function of nucleases capable of cleaving this substrate such as Yen1 may become critical. This scenario would explain why *yen1* single mutants lack a phenotype and exacerbate the phenotype of *mus81* cells. Possibly, only the *mus81* mutant condition, but not wild type, provides a substrate (HJ) that can be targeted by a HJ resolvase. Likewise, Yen1 and Sgs1–Top3–Rmi1 may need to address sHJs and dHJs, respectively, that arise as consequences of ligation in wild-type cells

and are processed very late in the cell cycle leading to anaphase bridges (Chan et al. 2007, 2009).

Conclusion and outlook

A significant progress over the recent years has produced almost a surfeit in candidates for junction nucleases in eukaryotic HR. Only Yen1/GEN1 displays the biochemical hallmarks of the bacterial RuvC resolvase, whereas the XPF family and the URI–YIG family nucleases appear to show preference for nicked substrates as compared to the intact HJ. There is an important mechanistic distinction between classical Holliday junctions (single or double HJs) with four uninterrupted component strands and nicked junction resolution. An appreciation of this difference rationalizes the need for multiple endonucleases that target different junctions in varying cellular contexts (replication, recombination, meiosis) and possibly in different nuclear compartments. The sheer number of nicked-junction nucleases and the absence of meiotic and mitotic phenotypes in Yen1/GEN1 mutants highlight the importance for processing nicked recombination intermediates.

Intriguingly, not all players have been analyzed yet, and evidence for additional HJ endonucleases candidates is available. Mlh3, part of the Mlh1–Mlh3 complex required for meiotic CO formation in the Msh4–Msh5 pathway (Fig. 5) (Hunter and Borts 1997; Wang et al. 1999; Argueso et al. 2004), contains the same putative endonuclease motif as that of human PMS2 and yeast Pms1 (Kadyrov et al. 2006, 2007; Nishant et al. 2008). Potential catalytically defective mutants in the putative endonuclease domain of Mlh3 exhibit the same meiosis and CO defect of *mlh3* null mutants, opening the possibility that Mlh3 might be an endonuclease active in the Msh4–Msh5 CO pathway (Nishant et al. 2008). It will be of great interest to understand the mechanism of CO formation in the Msh4–Msh5 pathway. Moreover, the recent discovery of FAN1, a structure-selective endonuclease associated with the FANC pathway of interstrand crosslink repair (Kratz et al. 2010; Liu et al. 2010; MacKay et al. 2010; Smogorzewska et al. 2010), adds yet another candidate endonuclease to the mix.

The importance of DNA junction endonucleases in DNA replication, DNA repair, and recombination is well established. The plasticity in the use of individual related nucleases in distinct functional contexts in various organisms provides an interesting puzzle to solve. Given the progress made over the last 10 years, the prospect of elucidating the individual roles of these nucleases is good.

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