GENETIC AND BIOCHEMICAL CHARACTERIZATION OF *DROSOPHILA* GEN DURING DNA REPAIR AND RECOMBINATION

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ABSTRACT

Stephanie Patricia Bellendir: Genetic and Biochemical Characterization of *Drosophila* Gen during DNA Repair and Recombination
(Under the direction of Jeff Sekelsky)

Holliday junction (HJ) resolvases maintain genome stability by processing DNA intermediates that arise during DNA repair. While human GEN1 and several orthologs possess HJ resolvase activity *in vitro*, *in vivo* studies indicate that GEN1 (and *S. cerevisiae* Yen1) is secondary to the Mus81–Mms4/Eme1 nuclease. Prior work suggests that this relationship is reversed in *Drosophila*; however, a full characterization of *Gen* has yet to be performed. Here we confirm that Gen is the primary HJ resolvase in *Drosophila* somatic cells and reveal key elements of its biochemistry, including that it preferentially cuts 5' flaps and exists in a monomer-dimer equilibrium. We found that *Gen* mutants are hypersensitive to a variety of DNA damaging agents relative to *mus81* mutants. However, like the human and yeast orthologs, Gen is primarily or exclusively cytoplasmic during interphase. We next purified recombinant Gen and observed robust activity on fixed, mobile, and nicked HJs, as well as on 5' flaps and replication fork-like structures. Our kinetic studies of Gen with 5' flaps and HJs indicate that unlike its orthologs, Gen cuts the 5' flap structure faster than the HJ structure, even at vast excess protein. Unexpectedly, our kinetic data suggests that dimerization accelerates cleavage of the flap. We verified dimerization by atomic force microscopy and gel-shift assays and show that Gen exists in a monomer-dimer equilibrium. In conclusion, we suggest that although HJs represent an important substrate for Gen, flaps and replication fork derivatives may be more relevant than HJs when considering the *in vivo* relationship between Gen and MUS81.

To Jim and Rusty Roo Bodippity Bojangles.
This is for you.

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AFM – atomic force microscopy

BLEO – bleomycin

CPT – camptothecin

CO – crossover

dHJ – double Holliday junction

DSB – double-strand break

DSBR – double-strand break repair

HhH – helix hairpin helix

HJ – Holliday junction

HR – homologous recombination

HU – hydroxyurea

ICL – interstrand crosslink

IR – ionizing radiation

MMS – methyl methanesulfonate

NCO – non-crossover

NDJ – non-disjunction

NH2 – nitrogen mustard

nt – nucleotide

RF – replication fork

SDSA – synthesis-dependent strand annealing

ssDNA – single-stranded DNA

SSE – structure-selective endonuclease

CHAPTER 1

INTRODUCTION

Genome Stability

DNA provides all the information needed for cellular processes; therefore its fidelity is essential for the survival of an organism, and the faithful transmission of the genetic information from one organism to another is essential for the survival of a species. Because damage is encountered through many exogenous and endogenous processes, cells have developed numerous mechanisms to repair our genomes depending on the type of damage encountered. These mechanisms are highly conserved from yeast to humans, and several were the subjects of this year's Nobel Prize in Chemistry. When these DNA repair pathways are not intact, they often lead to cancer predispositions. For example, nucleotide excision repair (NER) removes damage caused by UV light through the actions of the protein XPG. In the absence of XPG, patients develop xeroderma pigmentosum, which is characterized by extreme light-sensitivity and early onset skin cancer. DNA damage can also occur as a result of normal endogenous cellular process, such as during replication, or as the result of exogenous chemicals in the environment. For example, the nitrogen mustard mechlorethamine (HN2) can cause intrastrand and interstrand crosslinks (ICLs) (Wijen et al., 2000). Additionally, hydroxyurea (HU) inhibits ribonucleotide reductase, leading to decreased dNTP pools and fork slowing and stalling (Alvino et al., 2007).

However, double-strand breaks (DSBs) are arguably one of the most detrimental forms of damage, because both complementary strands of DNA are damaged. If left untreated, DSBs can cause genome-scale rearrangements, deletions, and loss of heterozygosity (LOH), which can

ultimately lead to cancer. Surprisingly, despite their toxicity, DSBs are generated deliberately by cells to initiate the process of meiotic recombination. One of the primary mechanisms through which double strand breaks (DSBs) are repaired is homologous recombination (HR).

Double strand break repair (DSBR) model

Our current understanding of DSB repair comes mainly from studies in *S. cerevisiae* meiosis. Like DNA repair, meiotic recombination initiates with a double strand break. In the canonical model (**Fig. 1**) proposed by Szostak *et al.* (reviewed in Kohl and Sekelsky, 2013; Szostak et al., 1983), processing of DSBs begin with the resection of the DNA ends to leave 3' overhangs, which invade a homologous template, forming a dissociation loop (D-loop). The free 3' end then primes synthesis. The other resected end can then anneal to the D-loop, and subsequent synthesis and ligation generates a double HJ (dHJ) intermediate. The orientation of nicks across the junction, produce either crossovers (CO), in which involve the exchange of flanking markers, or non-crossovers (NCO), in which the parental configuration of flanking markers is maintained. Alternatively, two NCO-only pathways were described: synthesis-dependent strand annealing (SDSA), in which the invading strand is dissociated, allowing it to anneal to the other end of the break; and dHJ dissolution, in which two HJs are migrated toward each other and decatenated by a topoisomerase to generate NCOs. One of the most intensely-studied DSBR intermediates is the Holliday junction (HJ), a four-stranded structure that joins two DNA helices.

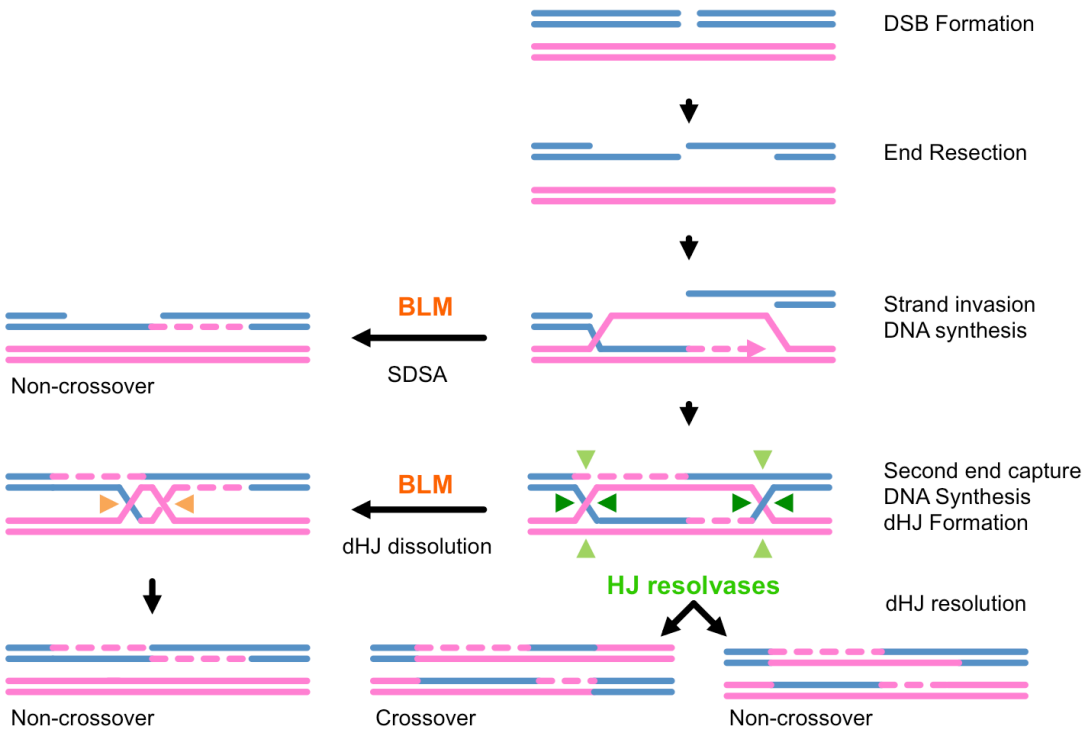

Figure 1. Model for the repair of DSBs via HR. In the Szostak *et al*. model (Szostak et al., 1983), processing of a DSB begins with the resection of the DNA ends to leave 3' overhangs, which invade a homologous template, forming a dissociation loop (D-loop). Further processing of the D-loop generates a double HJ (dHJ) intermediate, which can be resolved to produce either COs and NCOs with equal probability. Alternatively, two NCO-only pathways were described: synthesis-dependent strand annealing (SDSA), in which the invading strand is dissociated; and dHJ dissolution, in which two HJs are decatenated by a topoisomerase. See text for details.

Holliday junction structure

The Holliday junction (HJ) structure was first proposed in 1964 by Robin Holliday to explain recombination products in *Ustilago* meiosis (Holliday, 1964; reviewed in West, 2009). Holliday theorized that these structures were generated by the introduction of two nicks on homologous chromosomes, which would allow single strands on each chromosome to interact with the complementary sequence on the other chromosome. These strand exchanges later became known as Holliday junctions. We now know that Holliday junctions are not formed by symmetric nicks, but are initiated at sites of DSBs. Just a few years after Holliday suggested the

existence of a HJ, they were visualized by electron microscopy during recombination in bacteriophage and *E. coli* (Bell and Byers, 1979; Benbow et al., 1975; Doniger et al., 1973). More recently, HJs were visualized on 2D gels, showing that they are indeed intermediates of meiotic and mitotic recombination (Bzymek et al., 2010; Schwacha and Kleckner, 1995), although mitotic HJs were detected at 10-fold lower levels than meiotic HJs.

In the 1980's, scientists began to generate HJs *in vitro* using the RecA recombinase to generate HJs using two circular double stranded plasmids (Cunningham et al., 1980; West et al., 1983). Soon afterward, formation of a synthetic HJ was achieved by annealing four oligos (Kallenbach et al., 1983); these four-way junctions have been one of the most useful tools for characterizing HJ structure and the enzymes that process them. Surprisingly, depending on the conditions, HJs can adopt several different conformations. In the presence of a divalent ion, free HJs can adopt an antiparallel structure, in which the two DNA helices run antiparallel to each other with the crossing strands adopting a U-shaped conformation (Duckett et al., 1988; reviewed in Lilley, 2000). Alternatively, in the absence of ions, the junction forms an open, symmetric, square-planar formation due to the repulsion of the DNA backbone. Interestingly, the structure-selective enzymes that process HJs, create a conformational change in the DNA junction upon binding, stabilizing its opening to correctly position the DNA in the protein active site (reviewed in Lilley, 2000).

Structure-selective endonucleases

HJ resolvases

Because HJs physically connect the two DNA helices, they must be resolved to allow for chromosome segregation and proper repair. This is done in one of two ways: two HJs can be

migrated toward each other by and dissolved by a helicase and topoisomerase, alternatively two strands of a HJ can be cleaved by an endonuclease. The specific cleavage is achieved by a group of structure-selective endonucleases called Holliday junction resolvases.

Canonical resolvases in prokaryotes

Surprisingly, most HJ resolvases share little to no sequence similarity, but remarkably, they function very similarly (reviewed in West, 2009; White et al., 1997). The first biochemical evidence of HJ resolution came from studies of T4 endonuclease VII and T7 endonuclease I from bacteriophage (de Massy et al., 1987; Mizuuchi et al., 1982). These enzymes recognized specific branched DNA structures, in particular HJs, and cleaved them with a high degree of specificity. *E. coli* RuvABC was the first identified cellular HJ resolvase (Connolly et al., 1991; Connolly and West, 1990; Dunderdale et al., 1994; Iwasaki et al., 1991; Sharples and Lloyd, 1991; Takahagi et al., 1991). Interestingly, T4 endo VII, T7 endo 1, and RuvC share a similar mechanism of action on HJs. They first bind the HJ as a dimer with high specificity, bend the junction DNA into a specific configuration, and finally, introduce nicks symmetrically across the junction onto strands of the same polarity (reviewed in Lilley, 2000). The products of the cleavage reaction can then be ligated without further processing. These HJ resolvases became known as "canonical" HJ resolvases, and subsequent searches to identify eukaryotic HJ resolvases were directed by these criteria. Again, crystallization of these archael resolvases in the late 1990s and early 2000s reemphasized that these proteins share relatively little in terms of amino acid sequence, though some similarities exist at the structural level.

Search for the eukaryotic resolvases

As a result of the independent evolution of the HJ resolvases, it was difficult to identify their functional eukaryotic counterparts. An activity termed ResA, which displayed the properties of a canonical HJ resolvase, was identified from calf thymus tissue (Elborough and West, 1990) and nuclear tissue culture extracts (Constantinou et al., 2001). However, this activity eluded identification until 2008.

MUS81 orthologs

In the meantime, Mus81-Eme1 from *S. pombe*, and later Mus81-Mms4 from *S. cerevisiae*, was the first nuclear HJ resolvase identified in eukaryotes (Boddy et al., 2001; Chen et al., 2001). Both the catalytic Mus81 subunit and its requisite non-nucleolytic *S. pombe* subunit Eme1, or *S. cerevisiae* Mms4, belong to the XPF/ERCC4 family of endonucleases which participate in DNA repair and recombination (reviewed in Schwartz and Heyer, 2011). During meiotic recombination, *S. pombe mus81* mutants exhibit severe meiotic defects, including an 85% decrease in CO formation compared to *wild-type*, as well as increased nondisjunction and spore inviability. Additionally, overexpression of the *E. coli* RusA HJ resolvase rescues the meiotic phenotypes of *mus81* mutants (Boddy et al., 2001). Similarly, *S. cerevisiae mus81* mutants display a modest decrease in meiotic COs (de los Santos et al., 2003; Haber and Heyer, 2001; Smith et al., 2003). These data suggested that Mus81 was the long-awaited eukaryotic HJ resolvase; however, purified *S. pombe, S. cerevisiae*, and human Mus81 only weakly cleaves synthetic HJ substrates *in vitro*, instead favoring nicked HJs and D-loops (Chen et al., 2001; Gaillard et al., 2003; Osman et al., 2003). Further, asymmetric cleavage across the junction of

the intact HJ results in a flapped and a gapped product, which cannot be ligated without further processing, leading investigators to question whether Mus81 is in fact a "canonical" resolvase.

Consistent with doubts that Mus81 was a canonical resolvase, during DNA repair, mutations in *mus81* in most organisms cause sensitivity to DNA damaging agents which block replication forks, including MMS, HN2, and CPT (reviewed in Schwartz and Heyer, 2011). Additionally, loss of Mus81 in mammalian cells increases the number of genome-scale rearrangements and chromosome abnormalities during cell division (Abraham et al., 2003; Dendouga et al., 2005). These data suggested that Mus81 played a role in RF maintenance and restart and perhaps favored other *in vivo* substrates than HJs.

Gen orthologs

In 2008, the ResA activity that had eluded researchers for almost 20 years was finally identified as GEN1. Human GEN1 and its *S. cerevisiae* ortholog, Yen1, were discovered based on their ability to cleave HJs *in vitro* (Ip et al., 2008). For human GEN1, researchers followed intact HJ resolvase activity through extensive fractionation of HeLa cell extracts. The proteins were later renatured, and mass spectrometry (MS) identified a 60 kDa N terminal fragment of an uncharacterized protein. Interestingly, MUS81 was also identified in this screen, which was quite puzzling at the time because MUS81 had shown only weak activity on intact HJs. Similarly difficult was the identification of *S. cerevisiae* Yen1; thousands of proteins from a TAP-tagged library were immunoprecipitated and assayed for nuclease activity (Ip et al., 2008). Remarkably, GEN1 and Yen1 turned out to be orthologs.

GEN1 and Yen1 are members of the FEN-1 family of monomeric flap endonucleases, which play prominent roles during DNA repair and recombination. Both GEN1 and Yen1

contain conserved FEN-1 nuclease domains and a helix-hairpin-helix motif (Ip et al., 2008). The catalytic domain of this protein has been hypothesized to take part in branch migration, and assays using model DNA substrates *in vitro* revealed that GEN1/Yen1 display canonical HJ resolvase activity, preferentially cleaving HJs but also cleaving 5' flaps, RFs, and nHJs with a high degree of specificity (Ip et al., 2008; Rass et al., 2010). Surprisingly, in ensuing genetic studies, *yen1* mutants and siRNA knockdown of GEN1 in HeLa cells exhibited no overt mutant phenotype (Blanco et al., 2010; Ho et al., 2010; Svendsen et al., 2009). Rather, mutations in *yen1* enhanced the DNA damage sensitivity and meiotic phenotypes of *mus81* mutants (Blanco et al., 2010) indicating partial functional redundancy between Mus81 and GEN1/Yen1. Likewise, mutations in *yen1* further decrease the meiotic CO formation defects in *mus81* mutants (Zakharyevich et al., 2012), and spore viability is drastically reduced (Agmon et al., 2011). Interestingly, the CO reduction and the resulting spore inviability are not observed when the mutations in *yen1* are combined with mutations in any other resolvase. Thus, Yen1 and Mus81 function in partially overlapping pathways in *S. cerevisiae*, with Yen1 specifically substituting for Mus81 in its absence. Evidence for an *in vivo* HJ resolution function for GEN1 was suggested from studies utilizing *S. pombe*, which lack an ortholog of GEN1/Yen1. In these studies, ectopic expression of human truncated GEN1 rescued the DNA damage sensitivity and meiotic defects of *S. pombe mus81* mutants to a similar degree as ectopic expression of the *E. coli* RusA resolvase (Lorenz et al., 2010). Thus, the prevailing theory in the field is that *S. cerevisiae* Yen1 and human GEN1 function as a "backup" to Mus81, processing HJ structures missed by Mus81.

Regulation of HJ processing

Processing of HJs are under tight regulation. In somatic *S. cerevisiae* cells, the STR complex (Sgs1-Top3-Rmi1) preferentially processes joint molecules into NCOs. However, in the absence of Sgs1, Mus81 and Yen1 resolve these structures, resulting in an increase in aberrant mitotic COs (Ira et al., 2003). A similar dynamic exists between the BTR complex (BLM-TopIIIa-RMI1-RMI2) in human cells and MUS81 and GEN1 (Wechsler et al., 2011); mutations in *BLM* result in Blooms Syndrome, which is characterized by increased sister chromatid exchange (SCE) and a general increase in cancers. Thus, the BTR/STR complexes are somatic cells' first line of defense in dealing with DNA damage and replication problems, with the HJ resolvases providing a failsafe.

Disruption of HJ regulatory mechanisms results in genome instability. For example, premature activation and nuclear localization of *S. cerevisiae* Yen1 results in sensitivity to the DNA damaging agent MMS and a significant increase in mitotic CO formation at the expense of NCOs and increasing loss of heterozygosity (LOH) (Blanco et al., 2014). Similarly, premature nuclear import of human GEN1 results in significant increases in sister chromatid exchanges (SCEs) (Chan and West, 2014).

Both Mus81 and Yen1/GEN1 are activated in a cell cycle-specific manner reviewed in (Matos and West, 2014). In somatic cells, the activities of Mus81 and Yen1 are low during S phase. Mus81 is kept inactive until G2/M when Cdc5 phosphorylates Mms4 (Gallo-Fernandez et al., 2012; Matos et al., 2011). Mus81 activity peaks at anaphase, and upon cell cycle exit, Mus81 is inactivated by dephosphorylation (Matos et al., 2011). Yen1, on the other hand, is regulated both temporally and spatially. Phosphorylation by Cdk sequesters Yen1 in an inactive state in the cytoplasm (Loog and Morgan, 2005). Upon rapid dephosphorylation by Cdc14, Yen1 is shuttled

into the nucleus and its affinity for DNA increases (Blanco et al., 2014; Kosugi et al., 2009). Human MUS81–EME1 appears to be regulated similarly to yeast Mus81; whereas, human GEN1 is primarily regulated via a functional nuclear export signal (NES), which drives active nuclear exclusion until nuclear envelope breakdown during mitotic entry. At telophase, GEN1 is shuttled back into the cytoplasm via CRM1-mediated nuclear export (Blanco et al., 2014; Chan and West, 2014). In conclusion, the two major pathways are responsible for removing joint molecules during mitotic recombination: the first, mediated by STR/BTR specializes in the removal of HJs through NCO-promoting pathways, such as HJ dissolution; and the second, is mediated by the CO-generating SSEs, with Mus81 activated first and Yen1/GEN1 acting as a "backup." Why GEN1/Yen1, the simplest and most robust canonical HJ resolvase, was relegated to a backup role for Mus81, which has limited activities on intact HJs, presents a paradox.

Studying the Gen paradox in *Drosophila*

We were prompted to conduct this work for several reasons. First, the *Gen* mutation was identified in a screen for DNA repair mutants that exhibited sensitivity to the DNA damaging agents HN2 and MMS (Laurençon et al., 2004); *Gen* mutants were hypersensitive to both. These overt mutant sensitivities indicated that *Drosophila* was the ideal organism in which to study this protein because we would not have to perform our work in a *mus81* mutant background; yet *mus81* is present in *Drosophila*, below.

Furthermore, previous genetic studies suggested that Gen may be the more predominant enzyme in *Drosophila*. *S. cerevisiae*, *mus81 sgs1* double mutants are inviable, but *yen1 sgs1* double mutants are viable (Blanco et al., 2010; Fricke and Brill, 2003; Kaliraman et al., 2001; Mullen et al., 2001). These results are consistent with *S. cerevisiae* Mus81 being the primary

mitotic resolvase with Yen1 acting as a backup. In *Drosophila*, mutations in *mus81* and *Gen* are both synthetically lethal with mutations in *Blm* (the *Drosophila* ortholog of *SGS1*) (Andersen et al., 2011; Trowbridge et al., 2007); yet *Gen Blm* double mutants die much earlier in development than *mus81 Blm* mutants (Andersen et al., 2011; Trowbridge et al., 2007), suggesting that Gen may be the primary HJ resolvase in *Drosophila*.

Scope of this work

This dissertation describes work I have done to characterize Gen's role in double-strand break repair (DSBR) and Holliday junction resolution during DNA repair and meiosis. To understand how this protein carries out its functions, I took both *in vivo* and *in vitro* approaches. First, I asked what role Gen has in DNA damage repair and what the relationship between MUS81-MMS4 and Gen is in *Drosophila* (Chapter 2). I showed that *Gen* mutants have more severe sensitivities to DNA damaging agents that block replication forks and create DSBs than *mus81* mutants and that this difference from its orthologs is not simply due to Gen's subcellular localization. Next I asked if the sensitivity of *Gen* mutants is due to a defect in processing Holliday junctions using *in vitro* techniques (Chapter 3). I determined that Gen is a flap endonuclease and also a canonical HJ resolvase. Surprisingly, I found that the kinetics with which Gen cleaves these structures is different form its orthologs; Gen cleaves 5' flaps significantly faster than HJs. My kinetics data suggests that this difference is due to the ability of Gen to dimerize on 5' flaps. We confirmed the existence of a monomer-dimer equilibrium by Atomic Force Microscopy (AFM) and EMSAs, and present a model for the mechanism of Gen cleavage on HJs and 5' flaps (Chapter 3 Conclusion). Finally, I describe future avenues of research and preliminary data that might inform it (Chapter 4 and Appendix).

CHAPTER 2

GEN GENETICS AND REGULATION

Introduction

More than 50 years ago, Robin Holliday proposed a four-stranded DNA structure that now bears his name – the Holliday junction (HJ) – as a key intermediate in recombination (Holliday, 1964). He suggested that "at the points where strands exchange partner precise breakage and reunion of non-complementary strands can occur so that there is no deletion or duplication of material." It was more than 25 years before the identification of the first endonuclease with specificity for HJs was identified: *E. coli* RuvC (Connolly et al., 1991). RuvC resolves HJs by making symmetric nicks on non-complementary strands, resulting in nicked duplexes that can be ligated without further processing, as in Holliday's model (Bennett et al., 1993).

It was another ten years before the first good candidate for a eukaryotic nuclear HJ resolvase was identified when Mus81, together with the non-catalytic partner Eme1/Mms4, was shown to cut HJs *in vitro* (Boddy et al., 2001; Chen et al., 2001). The properties of this enzyme are very different than those of RuvC: Recombinant Mus81–Eme1 makes asymmetric nicks on HJs, resulting in one duplex with a flap and one of a gap, which require additional processing before ligation. In addition, Mus81–Eme1 cuts 3' flaps, structures mimicking replication forks, and nicked HJs more efficiently than it cuts intact HJs, raising questions about whether Mus81–Eme1 is a true resolvase (Ciccia et al., 2003; Ehmsen and Heyer, 2008; Oğrünç and Sancar, 2003). *In vivo* studies supported a role in resolution because *S. pombe* Mus81–Eme1 is required

for most meiotic crossovers, and *S. cerevisiae* Mus81–Mms4 is required for a substantial minority of crossovers (Boddy et al., 2001; de los Santos et al., 2003; Smith et al., 2003). Models in which Mus81–Eme1 generates crossovers by cleaving nicked structures offered one possible solution to this apparent paradox (Osman et al., 2003), but electron microscopy studies suggested that meiotic crossovers in *S. pombe* are produced from ligated HJs (Cromie et al., 2006). More recently, *in vitro* experiments demonstrated that HJ resolution can be achieved through collaboration between the human nucleases SLX1, which nicks HJs, and MUS81–EME1, which makes the second nick, in a reaction orchestrated by the SLX4 nuclease scaffolding complex (Castor et al., 2013; Garner et al., 2013; Wyatt et al., 2013). Again, this does not seem to be a complete answer to the paradox, since *S. cerevisiae slx1* mutants do not have reduced meiotic crossovers like *mus81* mutants.

In 2008, Ip *et al.* (Ip et al., 2008) reported that human GEN1 and the *S. cerevisiae* ortholog Yen1 have HJ resolvase activity with properties similar to that of RuvC. At first, it seemed that GEN1/Yen1 could be long sought-after nuclear HJ resolvase; however, genetic studies subsequently found that *yen1* mutants do not have measurable defects in meiotic crossovers and are not hypersensitive to DNA damaging agents (Blanco et al., 2010; Ho et al., 2010; Tay and Wu, 2010). These same studies found that *mus81 yen1* double mutants have more severe phenotypes than *mus81* single mutants. *S. pombe* does not have a GEN1/Yen1 ortholog, but expression of human GEN1 rescues defects caused by loss of Mus81–Eme1 (Lorenz et al., 2010). Together, these results suggested that Yen1 functions primarily as a backup to Mus81.

Studies of the regulation of Mus81 and Yen1 activity throughout the cell cycle provide a mechanism to explain why Yen1 is a backup to Mus81–Mms4. Mus81–Mms4 is not activated until early in mitosis, when the polo-like kinase Cdc5 phosphorylates Mms4 (Gallo-Fernandez et

al., 2012; Matos et al., 2011). Yen1 is kept inactive and cytoplasmic by phosphorylation until anaphase, when it is dephosphorylated by Cdc14 (Blanco et al., 2014; Eissler et al., 2014). Therefore, Mus81–Mms4 is activated prior to Yen1 activation. Human MUS81–EME1 and GEN1 appear to be regulated in a similar fashion (Blanco et al., 2014; Chan and West, 2014).

The first GEN1/Yen1 ortholog to be described was *Drosophila* GEN (Ishikawa et al., 2004). GEN was shown to cleave 5' flaps and model replication forks *in vitro*, but no HJ resolvase activity was detected (Kanai et al., 2007). The first genetic studies of *Gen* reported spontaneous apoptosis and a rough eye phenotype in *mus81 Gen* double mutants, indicating an interaction between GEN and MUS81–MMS4 like that in budding yeast (Andersen et al., 2011). Synthetic lethality between *Gen* and *Blm* was also reported. This is different from *S. cerevisiae*, where *sgs1 yen1* mutants are viable (Blanco et al., 2010). There is synthetic lethality between *mus81* and *sgs1* mutations in *S. cerevisiae* (Fricke and Brill, 2003; Kaliraman et al., 2001), and likewise between *mus81* and *Blm* mutations in *Drosophila* (Trowbridge et al., 2007). Interestingly, *Gen Blm* mutants die much earlier in development than *mus81 Blm* mutants (Andersen et al., 2011; Trowbridge et al., 2007). This observation, combined with the finding that *Drosophila mus81* mutants are not hypersensitive to most DNA damaging agents (Trowbridge et al., 2007), suggested the possibility that GEN plays a more important role in responding to DNA damage than MUS81–MMS4. We tested this hypothesis by comparing sensitivities of *mus81* and *Gen* mutants to a broad range of DNA damaging agents. We found that *Gen* mutants are more sensitive to most agents than *mus81* mutants, and double mutants are even more sensitive, supporting the idea that the functional relationship between GEN and MUS81–MMS4 is reversed in *Drosophila* relative to *S. cerevisiae* and mammals; however, Gen is not required for meiotic recombination. We asked whether the differences in DNA damage

repair are due to protein localization, and found that similar to its orthologs, Gen localized to the cytoplasm of *Drosophila* embryos and S2 cells. Finally, we show that Gen is able to rescue the DNA damage phenotypes of *S. pombe mus81* mutants. Together, these results suggest that Gen is the primary mitotic HJ resolvase in *Drosophila* and that HJs are an *in vivo* substrate; however the genetic differences are not attributable to protein localization.

Results

Gen mutants are more sensitive to DNA damage than *mus81* mutants

To assess the relationship between Gen and MUS81 in *Drosophila*, we examined the sensitivity of single and double mutants to a variety of DNA damaging agents (**Fig. 2**). The agents we used were (a) camptothecin (CPT), a topoisomerase I poison that results in replication-associated DSBs (Liu et al., 2000); (b) methyl methanesulfonate (MMS), an alkylating agent that induces lesions that can block replication forks (Groth et al., 2010); (c) the nitrogen mustard mechlorethamine (HN2), which generates base adducts and interstrand crosslinks (Wijen et al., 2000); (d) hydroxyurea (HU), which inhibits ribonucleotide reductase, leading to decreased dNTP pools and fork slowing and stalling (Alvino et al., 2007); and (e) ionizing radiation (IR), for which the most toxic damage is double-strand breaks (DSBs). At the doses used in our experiments, HN2 was the only agent to which *mus81* single mutants were hypersensitive (**Fig. 2c**). In contrast, *Gen* mutants are hypersensitive to all of the agents tested. Furthermore, *mus81 Gen* double mutants are significantly more hypersensitive to the damaging agents than *Gen* single mutants (**Fig. 2**). The only exceptions were HN2, where *Gen* single mutants were already inviable (**Fig. 2c**), and HU (**Fig. 2d**).

Our results with *mus81* mutants were mostly similar to those of a previous study (Trowbridge et al., 2007) except that we did not detect hypersensitivity (*i.e.*, decreased relative survival) of *mus81* mutants to CPT (**Fig. 2a**) possibly due to a difference in experimental design (see **Materials and Methods**). Additionally, the previous study reported significant hyposensitivity (*i.e.*, greater relative survival) of *mus81* mutants to HU, suggesting that the presence of MUS81 is detrimental to survival in the presence of this drug. In results reported here, the mean relative survival of *mus81* mutants was elevated after treatment with HU, consistent with hyposensitivity, but the difference was not statistically significant (**Fig. 2d**). Regardless, *Gen* mutants do worse after treatment with HU than *mus81* mutants.

In summary, *Gen* mutants are more sensitive to a range of DNA damaging agents than *mus81* mutants, which is the opposite of what has been reported with the yeast and human orthologs. These data suggest that Gen may have important roles in the rescue of blocked or broken replication forks and IR damage and that these roles are partially redundant with MUS81. Specifically, the sensitivity of *Gen* mutants to HU suggests that Gen has at least one function during S phase or in processing structures generated when replication problems arise.

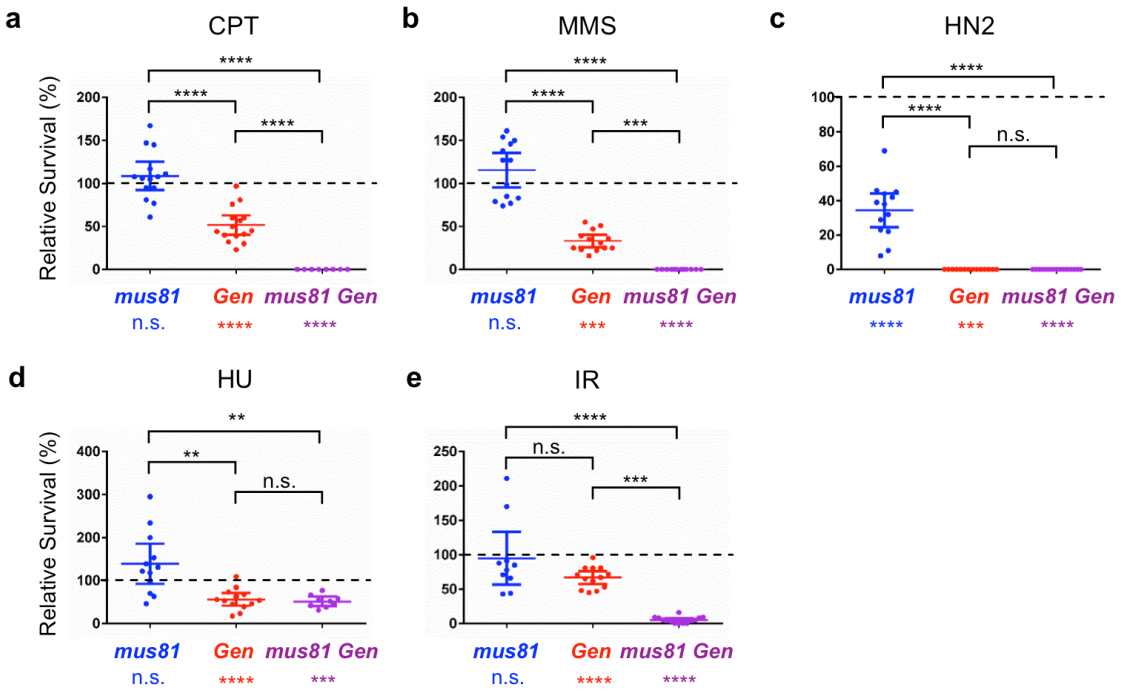

Figure 2. ***Drosophila*** **Gen mutants are more sensitive to DNA damaging agents than** ***mus81*** **mutants.** Graphs show survival of mutants relative to control siblings (see **Materials and Methods**). (**a**) 0.025 mM camptothecin (CPT); (**b**) 0.04% methyl-methane sulfonate (MMS); (**c**) 0.004% nitrogen mustard (HN2); (**d**) 70 mM hydroxyurea (HU); (**e**) 2000 rads ionizing radiation (IR). Each point corresponds to one vial; means and 95% confidence intervals are shown. Dotted lines indicate 100% relative survival (note that Y axes differ between treatments). Paired t-tests between mutant and control individuals were done to evaluate sensitivity of mutants to each treatment; statistical significance of sensitivity is indicated below each genotype. Differences between genotypes were assessed by one-way ANOVA and are indicated above each graph. n.s. = not significant ($p > 0.05$); ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

Gen is not required for meiotic recombination

MUS81 is required for a subset of meiotic crossovers in *S. cerevisiae*, Arabidopsis, and mice, and most or all meiotic crossovers in *S. pombe* (Berchowitz et al., 2007; de los Santos et al., 2003; Holloway et al., 2008; Smith et al., 2003). In yeast, yen1 mutants exhibit WT levels of NDJ and CO; however, *mus81 yen1* mutants fail to produce viable spores. In *Drosophila*, MUS81 is not required for meiosis. We asked whether the relationship between Gen and MUS81 was also flipped in meiotic recombination. Since meiotic COs are important for chromosome

segregation, we first examined Gen's meiotic role by measuring *X* chromosome NDJ in the *Gen* mutant as a readout for meiotic CO defects (**Table 1**). We observed *WT* levels of NDJ in the *Gen* mutant. We next directly measured CO levels in the Gen mutant to determine if there was a subtle decrease in CO levels.

Table 1. *X* chromosome non-disjunction (NDJ) in *Gen* mutants

Allele	Progeny			*X* Nondisjunction (%)
	Normal	Nullo-*X*	Diplo-*X*	
+/*Df*	3,452	1	1	0.11
Gen^{Z4235}/*Df*	1,656	1	0	0.12
Gen^{Z5997}/*Df*	1,290	0	1	0.15

In *Drosophila*, most meiotic crossovers are generated by the presumptive resolvase MEI-9–ERCC1 and the scaffolding protein MUS312 (Radford et al., 2005; Sekelsky et al., 1995; Yıldız et al., 2002). Crossovers are reduced by about 90% in *mei-9* or *mus312* single mutants (Baker and Carpenter, 1972; Green, 1981). Trowbridge *et al*. (2007) found that *mus81 mei-9* mutants were no worse than *mei-9* single mutants. To determine whether Gen produces the crossovers that remain when MEI-9–ERCC1–MUS312 is missing, we measured crossing over in *Gen* and *Gen mus312* mutants. Crossing over in *Gen* single mutants was similar to that in *wild-type* females, and crossing over in *Gen mus312* double mutants were similar to *mus312* single mutants (**Table 2**). These findings suggest that either an unidentified resolvase makes these crossovers or that MUS81 and Gen are redundant for this function. To test this latter hypothesis, we made *mus81 mus312 Gen* triple mutants, which lack all known resolvases. Although most triple mutants died during development, a few did survive to adulthood and were fertile, but none of these carried the marker chromosome that would allow us to score recombination frequency.

As a result of the inviability of the triple mutant with the *net – cn* second chromosome, we will score recombination frequency between two markers, *st* and *e*, on the third chromosome, which also contains the mutations of *Gen* and *mus81*. We are currently building stocks with the appropriate genetic markers. We therefore conclude that Gen is not required for the generation of meiotic COs, even in the absence of the major meiotic resolvase. It will be of great interest to determine whether any COs exist in the *mus81 Gen mus312* triple mutant.

Table 2. Meiotic crossing over in *Gen* and *Gen mus312* mutants

	Genetic distance (MU)						
Genotype	***net−dpp*^{ho}**	***dpp*^{ho}*−dp***	***dp−b***	***b−pr***	**Total**	**% of *WT***	***n***
wild-type[*]	5.1	7.5	27.3	3.5	43.4	100	2320
Gen	2.42	5.21	26.01	9.41	43.05	99	2072
mus312[**]	0	0.28	3.06	0.56	3.9	9	359
mus312 Gen	0.23	0.23	1.15	0.69	2.3	5	433
mus81 mus312 Gen	—	—	—	—	—	—	—

[*] Kuo *et al* (2014)
[**] Yildiz *et al* (2002)

Gen localizes to the cytoplasm of early embryos and S2 cells

Recent work from the West lab has shown that GEN1 is sequestered in the cytoplasm until nuclear membrane breakdown, limiting its activity to cells undergoing mitosis (Chan and West, 2014). A possible explanation for why *Drosophila* Gen predominates over MUS81 comes from a previous report that Gen localizes to the nucleus of 0-3 hr old embryos (Kanai et al., 2007). To confirm this observation using an independent antibody, we generated a polyclonal antibody to a fragment corresponding to Gen residues 236-335. In contrast to the published

results, our immunofluorescence studies detect a protein in *WT* embryos that is largely or exclusively cytoplasmic (**Fig. 3a-b**). We next validated this finding in cultured cells. We expressed Gen carrying a C-terminal hexahistadine (His) tag under control of the $CuSO_4$-inducible metallothionein promoter. In uninduced cells, our antibody detects a cytoplasmic protein that is likely endogenous Gen, with some cells also expressing the tagged Gen (**Fig. 3c-e**). After induction with $CuSO_4$, both anti-Gen and anti-His antibodies detect high levels of a cytoplasmic protein (**Fig. 3f-h**). We conclude that our antibody does detect Gen in cells and that Gen, like Yen1 and GEN1, is primarily or exclusively cytoplasmic during interphase. While we cannot exclude the possibility that a low level of nuclear Gen escaped our detection, our results suggest that the genetic differences between *Drosophila* Gen and its orthologs are not simply due to differences in protein localization.

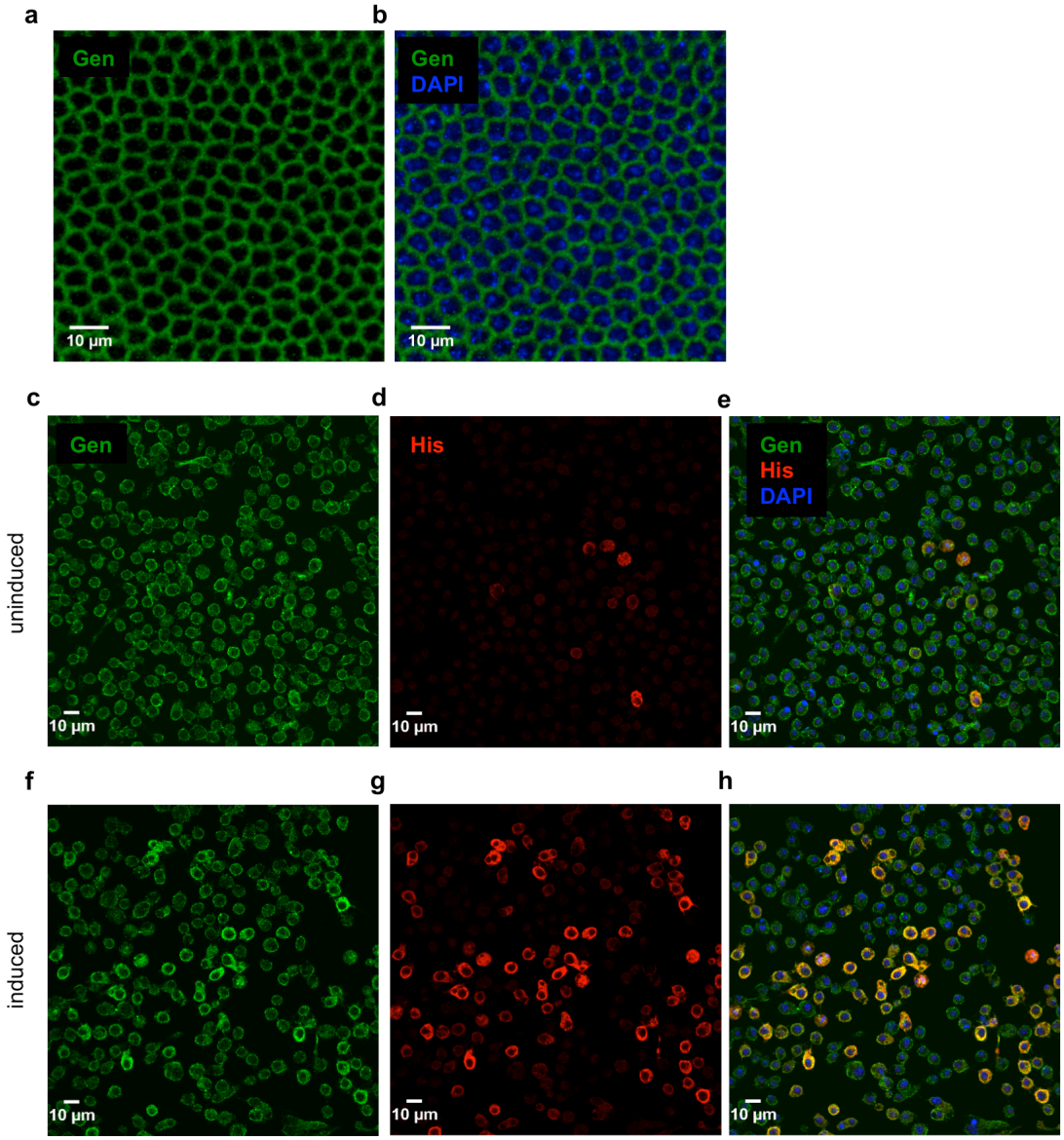

Figure 3. Gen localizes to the cytoplasm in early embryos and cultured *Drosophila* S2 cells. (**a-b**) 2-3 hr old *Drosophila* embryos were stained with DAPI (blue) and antibodies to Gen (green) (**c-h**) Full-length Gen-His was expressed in *Drosophila* S2 cells from the CuSO4-inducible metallothionein promoter. Cells were treated with (**c-e**) or without (**f-h**) CuSO4 for three days, then fixed and stained with DAPI (blue) and antibodies to Gen (green) and to the His tag (red). Both endogenous Gen and overexpressed Gen-His were detected in the cytoplasm but not in the nucleus.

Gen rescues the DNA-damage sensitivity of *S. pombe mus81* mutants

As in the budding yeast *S. cerevisiae*, Mus81 in the fission yeast *Schizosaccharomyces pombe* plays an important role in responding to DNA damage (Boddy et al., 2000; Interthal and

Heyer, 2000). *S. pombe* lacks a Yen1 ortholog, but truncated human GEN1 expressed in *S. pombe* rescues phenotypes caused by loss of Mus81 (Lorenz et al., 2010). Consequently, we expressed two forms of *Drosophila* Gen in *S. pombe mus81Δ* mutants (**Fig. 4**): full-length protein and a truncated form (residues 1-518) that is similar to truncated human GEN1 that was expressed in *S. pombe* (Lorenz et al., 2010). Truncated Gen rescues the hypersensitivity of the *mus81Δ* mutant to MMS, CPT, HU, and the radiomimetic drug bleomycin (BLEO), and this rescue is dependent on Gen nuclease activity (**Fig. 4a**). Full-length Gen did not rescue any of these hypersensitivities. Expression of both proteins was confirmed by Western blot (**Fig. 4b**), but it is possible the full-length protein was misfolded or excluded from the nucleus. We conclude that Gen (1-518) is functional *in vivo* in the repair of DNA damage in *mus81Δ* mutants, suggesting that despite their different genetic phenotypes, human GEN1 and *Drosophila* Gen share one or more critical activities.

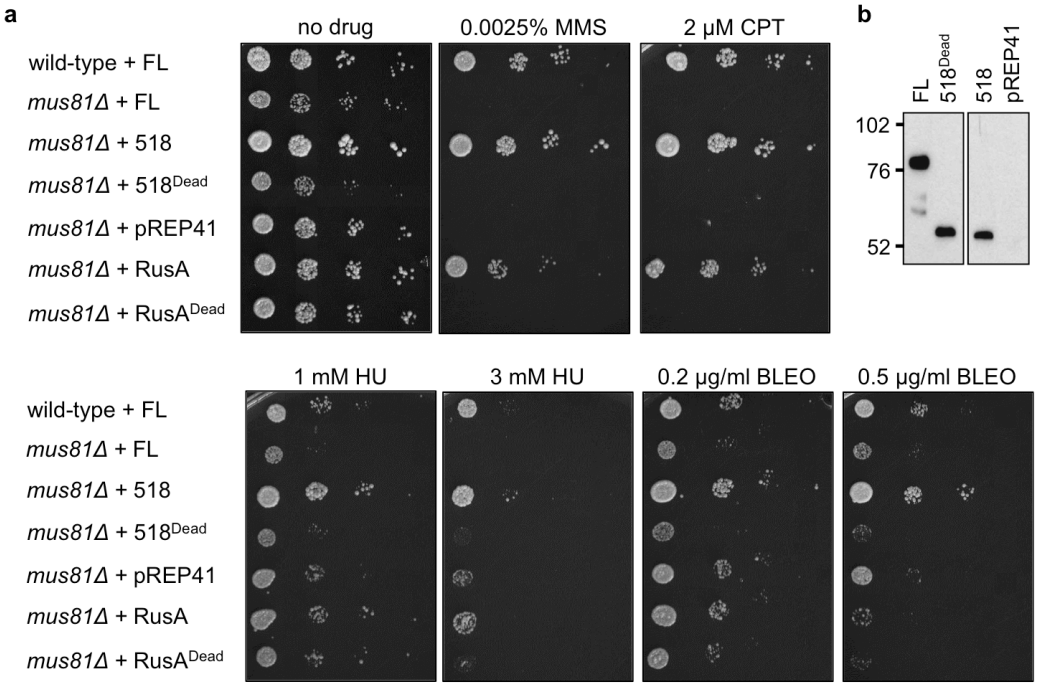

Figure 4. Gen expression rescues the DNA damage sensitivity of *S. pombe mus81* mutants. (**a**) Effect of Gen (1-518) overexpression mimics that of the canonical resolvase RusA. Serial dilutions on EMM2 plates were supplemented with drug. All proteins were expressed from the thiamine-repressible *nmt1* promoter in *mus81Δ* or *WT* strains. Empty pREP41 plasmid was used as a negative control. (**b**) Western blot showing expression of full-length Gen and Gen (1-518) in *S. pombe mus81Δ* mutants. FL = full-length Gen; 518 = 1-518 aa**.**

Conclusion

In conclusion, we showed that the putative Holliday junction resolvase GEN is essential for the repair of damaged DNA that results in broken or damaged replication forks or DNA double-strand breaks. These data directly contrast the mutant phenotypes of *Gen* orthologs, indicating that *Drosophila* is a good organism in which to study this protein. Despite these differences in DNA damage repair phenotype, we found no differences in Gen's absence of a meiotic recombination phenotype or its cytoplasmic localization during interphase. Additionally, we show that *in vivo*, ectopic expression of truncated Gen is able to rescue the DNA damage sensitivities of *mus81* mutants, indicating that *in vivo* it may be a functional HJ resolvase.

Together, these data confirm that Gen is the primary mitotic resolvase in *Drosophila* and is partially genetically redundant with MUS81; however, our data raise questions as to the why the relationship between *Gen* and MUS81 is different in *Drosophila* versus other organisms. To try to identify the origin of these differences, we therefore examined the biochemical properties of Gen, which we discuss in Chapter 3.

Materials and Methods

Drosophila stocks and genetics

All stocks were maintained at 25°C on standard media. The following null mutations were described previously: Gen^{Z5997} (Andersen et al., 2011), which was made hemizygous by putting it over *Df(3L)6103*; and $mus81^{NheI}$ (Trowbridge et al., 2007).

To analyze nondisjunction, virgin females were crossed to *y cv v f* / $T(1:Y)B^{S}$ males. There are four classes of exceptional progeny, which exhibit X chromosome NDJ. These are: Bar-eyed females and WT-eyed males, which are viable; and triplo-X and nullo-Y flies, which are inviable. The total number of exceptional progeny was calculated by multiplying the number of viable exceptional progeny by 2. Percent non-disjunction was calculated by dividing the total number of exceptional progeny by sum of the total number of progeny scored plus the inviable progeny.

To analyze meiotic crossing over, virgin females of various genetic backgrounds were heterozygous for markers on chromosome *2L* (*net* $dpp^{d\text{-}ho}$ *dp Sp b pr cn*) and were crossed to *net* $dpp^{d\text{-}ho}$ *dp b pr cn* tester males, and progeny were scored for 5 days. Sensitivity to DNA damaging agents was determined as in (Yıldız et al., 2002). For HN2, HU, and MMS, 250 µl water containing the agent was added to each vial containing feeding larvae. CPT was dissolved

in DMSO and diluted in 10% ethanol and 0.2% Tween. Control larvae were mock treated with DMSO dissolved in 10% ethanol and 0.2% Tween. For IR, vials with 3rd instar larvae were irradiated with 2000 rads from a 137Cs source (Gammacell GG10). Progeny were scored for five days after eclosion began. Relative survival was calculated as the ratio of mutant to control flies per vial and normalized to the ratio in untreated vials. Statistical analyses were done using GraphPad Prism.

Expression of Gen in *S. pombe* and sensitivity analysis

Strains, RusA plasmids, and pREP41 plasmids are listed in **Table 3**. Transformations were performed using the lithium acetate-based high-efficiency transformation method described in (Okazaki et al., 1990). For spot tests, strains containing plasmids were grown to saturation in EMM2 −Leu dropout media, washed twice with water, diluted to OD600=1, and 10-fold serially diluted to 10-4 cells/ml. 10 µl aliquots from each dilution were spotted onto minimal media plates containing MMS, CPT, HU, or BLEO. Plates were incubated at 32°C for four days before being photographed.

Table 3. Strains and plasmids used in this study.
(courtesy of Dr. Gerry Smith, Fred Hutchinson Cancer Research Center)

Strain	Genotype
GP 2853	*h^+ ura4-D18 leu1-32*
GP 3260	*h^+ mus81::kanMx ura4-D18 leu1-32*
GP 4906	*h^- mus81::kanMx ura4-D18 leu1-32 ade6-52 ura4-aim tps16-23*
GP 4907	*h^+ mus81::kanMx ura4-D18 leu1-32 ade6-3034 arg1-14*
V 1982	*DH5α pREP41 (Amp^r)*
pPC204**	*$RusA^+$*
pPC205**	*$RusA^-$: catalytically inactive mutant (D70N, base 208 G to A)*

**Both have an additional mutation at base 363 A to C. pPC205 has a mutation at base 329 T to C.

Immunofluorescence Microscopy

Polyclonal antibodies were raised to residues 236 to 335 of Gen and affinity-purified by Genomic Antibody Technology (SDIX, Newark, DE). All imaging was done with a laser-scanning confocal microscope (710, Carl Zeiss) and analyzed with ImageJ.

For embryo staining, 2-3 hr old embryos were dechorionated, fixed in equal volumes 7% formaldehyde:heptane, devittelinized, then stained. The primary antibody was rabbit anti-Gen-N (1:1,000), which was visualized with goat anti-rabbit IgG (H+L)-Alexa Fluor 488 (1:500, Life Technologies). DNA was detected by staining with DAPI (1:1000) for 2 min at room temperature.

For *Drosophila* S2 cells, Gen cDNA was cloned into the pMT-V5-HisA vector (Life Technologies), which contains the $CuSO_4$-inducible metallothionein promoter and a C-terminal His tag. The construct was transfected into S2 cells. Cells were plated at 1x106 cells/mL on poly-L-lysine-treated coverslips. Gen-His expression was induced for 3 days before staining. Staining

was performed as in (Lake et al., 2013). The primary antibodies were rabbit anti-Gen-N (1:10,000) and mouse anti-His (1:500). The primary antibodies were visualized with goat anti-rabbit IgG (H+L)-Alexa Fluor 488 (1:10,000) and goat anti-mouse IgG (H+L)-Alexa Fluor 555 (1:10,000, Life Technologies). DNA was detected by staining with DAPI (1:5000, Molecular Probes, Inc.) for 1 min at room temperature.

CHAPTER 3

GEN IS A CANONICAL HJ RESOLVASE BUT PREFERS 5' FLAPS

Introduction

A suite of structure-selective endonucleases (SSEs) has evolved to process branched DNA structures such flaps, bubbles, replication forks, and Holliday junction (HJs), which are four-stranded intermediates in recombination pathways. The first family of such nucleases identified in eukaryotes was initially defined by FEN-1 (flap endonuclease and 5' exonuclease 1) and XPG (xeroderma pigmentosum group G) (Lieber, 1997). These enzymes share conserved nuclease domains related to the 5'-to-3' exonucleases of prokaryotic DNA polymerases, but they have divergent activities and functions. FEN-1 processes Okazaki fragments during replication, whereas XPG nicks the damaged strand at the 3' end of a bubble during nucleotide excision repair. Exo1 (exonuclease 1), an enzyme with numerous repair and recombination functions (Tran et al., 2004), was later found to be a member of this family.

A fourth branch of the FEN-1/XPG family was discovered in *Oryza sativa* (rice) and named *OsSEND-1*, based on the expectation that the protein would be a single-strand DNA endonuclease (Furukawa et al., 2003). Although single-strand endonuclease activity was shown for the *Drosophila* ortholog (DmGen, for XPG endonuclease; hereafter referred to as Gen) (Ishikawa et al., 2004; Kanai et al., 2007), understanding this class of the family was greatly enhanced by the identification of the human and budding yeast orthologs (GEN1 and Yen1, respectively) in searches for enzymes with HJ resolvase activity (Ip et al., 2008).

GEN1 exhibits canonical HJ resolvase activity, making symmetric nicks on non-complementary strands of a HJ thereby yielding nicked duplexes that can be directly ligated (Rass et al., 2010). Surprisingly, genetic studies in *S. cerevisiae* failed to find recombination and repair defects in *yen1* mutants and instead suggested that Yen1 is a backup to another SSE, Mus81 (Blanco et al., 2010; Ho et al., 2010; Tay and Wu, 2010). Mus81 has been implicated in diverse processes across a number of organisms, including replication fork repair and meiotic recombination (reviewed in Schwartz and Heyer, 2011). Although some of these functions can be explained by HJ resolvase activity, in *in vitro* assays, Mus81 (together with its non-catalytic partner Mms4/EME1) cleaves 3' flaps and nicked HJs well but has limited ability to cut intact HJs (Ehmsen and Heyer, 2008; Oğrünç and Sancar, 2003). Why the robust, canonical HJ resolvase Yen1/GEN1 has been relegated to a backup role for Mus81, which has limited activity on intact HJs, presents a paradox.

Studies of the regulation of Mus81–Mms4 and Yen1 help to explain the mechanism for how Mus81–Mms4 acts before Yen1. Both proteins are regulated by cell cycle-dependent phosphorylation and dephosphorylation, such that Mus81–Mms4 is available and active in the nucleus prior to Yen1 (Blanco et al., 2014; Eissler et al., 2014); the human orthologs are regulated similarly (Blanco et al., 2014; Chan and West, 2014). While sequential activation may explain the mechanism for the backup role of GEN1/Yen1, the major unanswered question remains why Yen1/GEN1 is not the predominant HJ resolvase. One partial explanation may be that although MUS81 has fairly poor activity on intact HJs, mammalian MUS81–EME1 can collaborate with the SLX1–SLX4 endonuclease to resolve HJs (Castor et al., 2013; Garner et al., 2013; Wyatt et al., 2013). When both nucleases bind the scaffolding protein SLX4, SLX1 nicks HJs and then MUS81–EME1 completes resolution by making a second nick. However, this is not

a complete answer to the GEN1 paradox, since *S. cerevisiae slx1* mutants do not have reduced meiotic crossovers like *mus81* mutants, and *slx1* mutants have a different phenotype than *mus81* mutants (Fricke and Brill, 2003; Zakharyevich et al., 2012).

To gain insight into these questions, we conducted genetic and biochemical analyses of *Drosophila* Gen. Previous genetic studies suggest that Gen may be the more predominant enzyme in *Drosophila*. In *S. cerevisiae*, *mus81 sgs1* double mutants are inviable, but *yen1 sgs1* double mutants are viable (Blanco et al., 2010; Fricke and Brill, 2003; Kaliraman et al., 2001; Mullen et al., 2001). These results are consistent with *S. cerevisiae* Mus81 being the primary mitotic resolvase with Yen1 acting as a backup. In *Drosophila*, mutations in *mus81* and *Gen* are both synthetically lethal with mutations in *Blm* (the *Drosophila* ortholog of *SGS1*) (Andersen et al., 2011; Trowbridge et al., 2007); yet *Gen Blm* double mutants die much earlier in development than *mus81 Blm* mutants (Andersen et al., 2011; Trowbridge et al., 2007), suggesting that Gen may be the primary HJ resolvase in *Drosophila*.

In Chapter 2, we showed that *Gen* mutants are more sensitive to a broad range of DNA damaging agents than are *mus81* mutants and that like its fungal and human orthologs, Gen is primarily cytoplasmic during interphase. In this chapter, we show that Gen exhibits robust HJ resolving activity, a result that contrasts with a previous report on Gen (Kanai et al., 2007) but is similar to data from fungal and human orthologs (Freeman et al., 2014; Rass et al., 2010). Interestingly, Gen differs from the human and fungal proteins in two significant ways. First, both SEC-MALS and atomic force microscopy (AFM) suggest that Gen exists in a monomer-dimer equilibrium; whereas the human and fungal proteins are monomers and only dimerize by cooperative binding to HJs. Second, *Drosophila* Gen exhibits a significantly higher rate of cleavage for 5’ flaps relative to HJs, even in vast excess enzyme. Our kinetic data coupled with

electrophoretic mobility shift assays (EMSAs) with a 5' flap suggest that dimerization of Gen enhances the cleavage activity of Gen on 5' flaps. Finally, we directly show that a productive dimer-DNA complex and a DNA conformational change are the rate-limiting steps of the HJ cleavage reaction, providing insight into the mechanism of HJ resolution that can likely be extended to all Gen orthologs.

Results and Discussion

The C terminus of Gen is disordered

Truncated versions of human GEN1 and *C. thermophilium* GEN1 were identified and shown to be canonical HJ resolvases (Freeman et al., 2014; Ip et al., 2008; Rass et al., 2010); however, the full-length versions of these proteins were not purified due to the instability of the C terminus. These studies suggested that the disordered C terminus may play important regulatory roles in controlling protein activity. This suggestion is backed up by evidence that the C terminus of EXO1, another member of the FEN-1 family of repair endonucleases to which GEN1 belongs, is also disordered and plays a role in the negative regulation of enzyme activity (Orans et al., 2011). We used to *in silico* tools, Phyre2 and metaPrDOS, to ask whether the C terminus of Gen was disordered (**Fig. 5**). Both programs showed that similar to other orthologs, the N terminal nuclease domain of Gen is highly conserved, whereas the C terminus is highly disordered. We therefore expressed and purified both full-length and truncated (1-518 aa) forms of the protein to assess whether the disordered C terminus played any role in substrate selectivity.

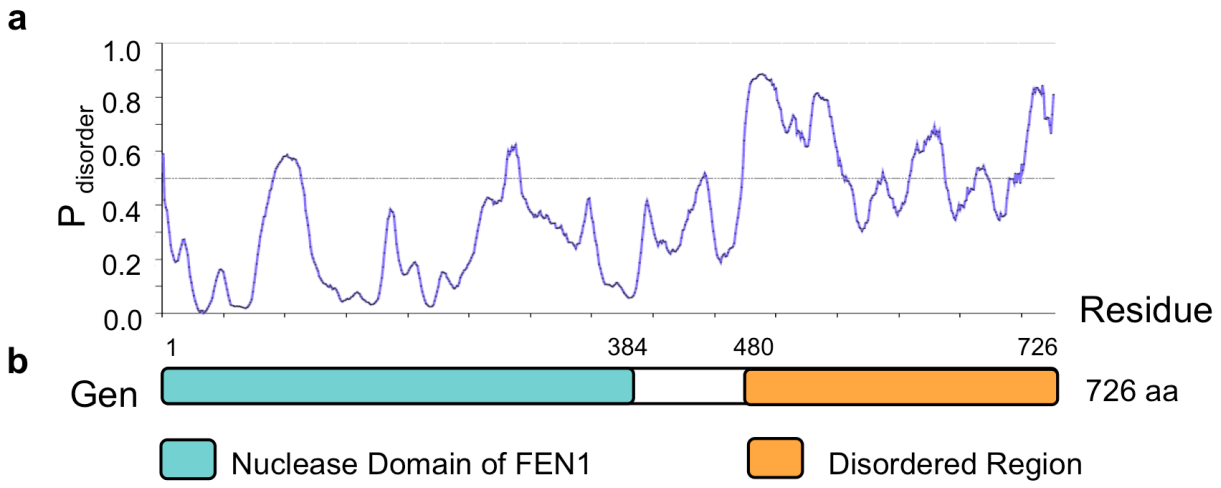

Figure 5. The C terminus of GEN is predicted to be disordered. (**a**) The line graph depicts the disorder tendency (*i.e.* the average probability of intrinsic disorder) predicted by metaPrDOS along the length of GEN. Residues above the line are predicted to be disordered. (**b**) Structural domains were identified by Phyre2. The "Nuclease Domain of FEN1" corresponds to Protein Data Bank fold 1UL1. The "Catalytic core of RAD2 (complex 1)" was also identified by Phyre2 and corresponds to Protein Data Bank fold 4Q0R. The "Disordered Region" indicates a region lacking secondary structure that was predicted by the DISOPRED2 Disorder Prediction program as part of the Phyre2 analysis.

Gen exists in a monomer-dimer equilibrium

Human GEN1, *S. cerevisiae* Yen1, and *C. thermophilium* GEN1 all purify as monomers in solution (Freeman et al., 2014; Ip et al., 2008; Rass et al., 2010). We analyzed Gen (1-518) by SEC-MALS after purification by affinity chromatography and ion exchange chromatography and compared it to a BSA standard (**Fig. 6**). BSA eluted from the column in three distinct peaks, with the monomer (66 kDa) eluting between 14 and 15 min. The truncated Gen (1-518) peak partially overlapped the BSA monomer peak, exhibiting an average molecular weight of 69 kDa (predicted 60 kDa) (**Fig. 6**); however, the peak was skewed, and the sample was slightly polydispersed. These data indicate the presence of a small amount (~15%) of dimers and suggests a monomer-dimer equilibrium. We were unable to accurately quantify the dimer dissociation constant from this experiment but estimate it to be within the low μM range.

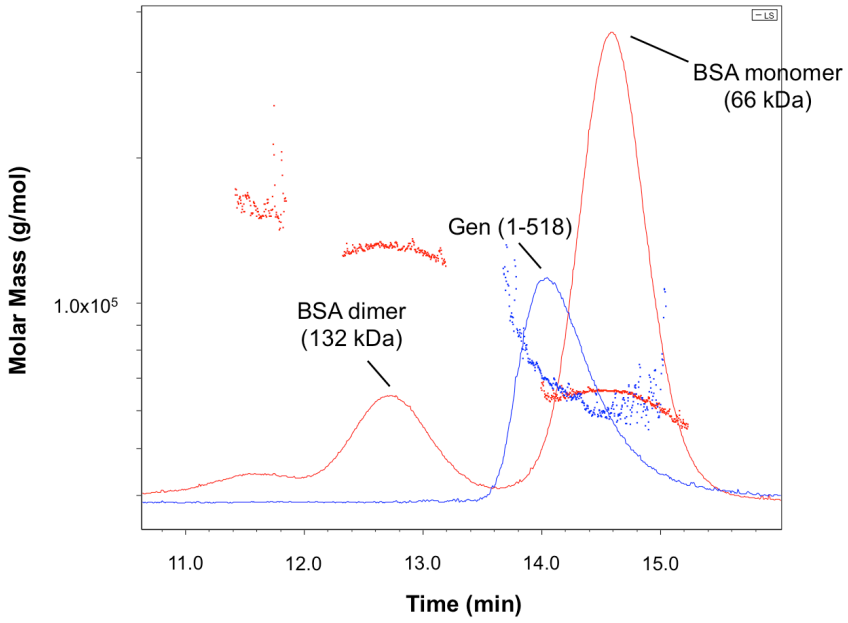

Figure 6. SEC-MALS analysis of Gen shows that both monomers and dimers exist in solution. GEN (1-518)-His is indicated in blue. The BSA standard is indicated in red.

Proteins purified

Previous *in vitro* experiments using an N-terminal 6xHis-tagged full-length *Drosophila* Gen did not detect activity on HJs (Kanai et al., 2007); however, studies of Gen orthologs with C-terminal tags have demonstrated activity (Freeman et al., 2014; Ip et al., 2008; Rass et al., 2010). Thus, we expressed and purified both N- and C-terminal tagged Gen in full-length and truncated (1-518) forms (**Fig. 7**). The nuclease activity of the N-terminal tagged proteins is weak, but the C-terminal tagged versions of Gen show high activity (**Fig. 8b**), with no evidence of contaminating nuclease activity (**Fig. 8c**).

It was previously reported that the *O. sativa* OsGEN-L ortholog was acutely sensitive to the salt concentration in the nuclease buffer, only showing activity on 5' flaps at KCl concentrations below 100 mM; whereas activity dropped off rapidly at higher KCl

concentrations. HJ cleavage activity was not observed under the same conditions as the 5' flap but only at higher salt concentrations (150 mM). In addition, previous reports with *Drosophila* Gen showed that it preferentially cleaved 5' flaps between 50-75 mM KCl; they did not observe HJ cleavage under these conditions. Importantly, they did not ask whether HJ cleavage was observed at higher concentrations of KCl. We optimized cleavage conditions for *Drosophila* Gen (**Fig. 8a**), and observed robust cleavage of both the 5' flap and HJ0 under all salt concentrations tested.

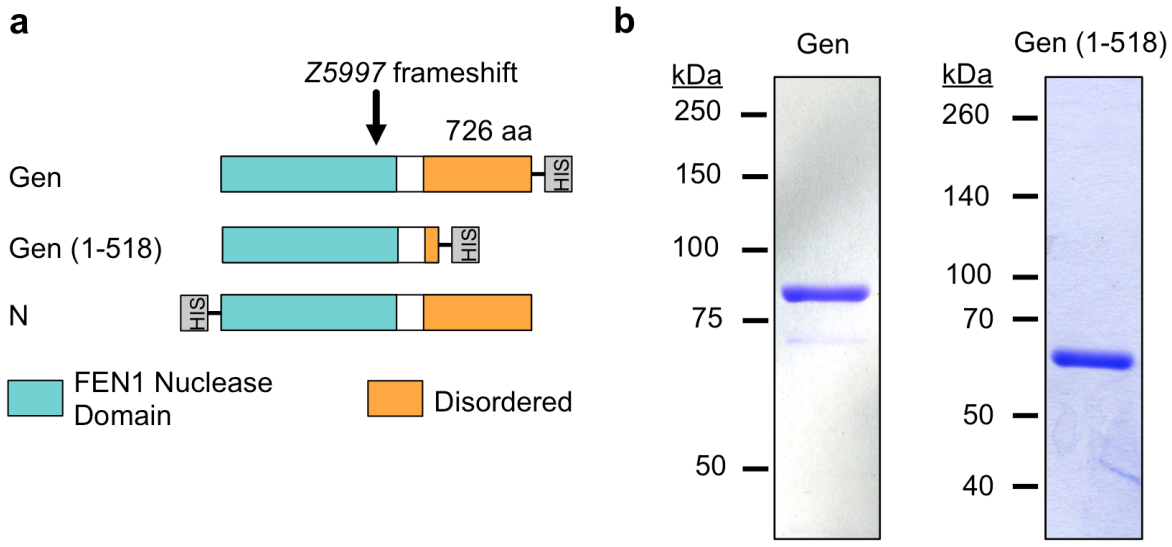

Figure 7. Proteins purified. (**a**) Schematic of recombinant proteins. Domains were determined using Phyre2. The arrow shows the location of the *Z5997* mutation present in *Gen* mutant flies used in **Chapter 2**. (**b**) Coomassie-stained SDS-PAGE gels showing C-terminally His-tagged full-length Gen and truncated Gen (1-518) after purification from *E. coli.*

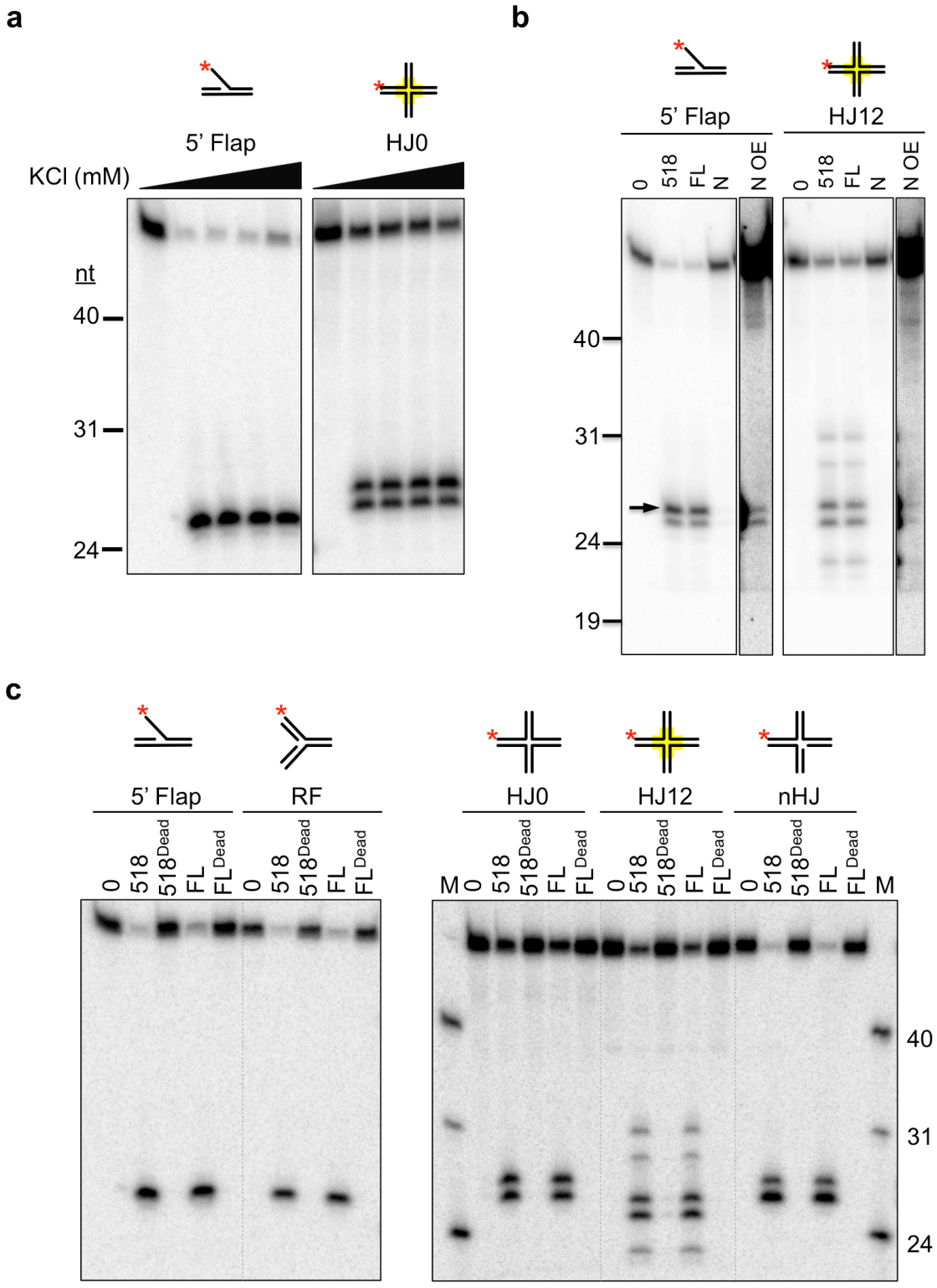

Figure 8. Controls for Gen nuclease assays. (**a**) Gen exhibits nuclease activity in a wide range of salt concentrations. Optimal salt concentration for *in vitro* assays was determined on the 5' flap and HJ0 in fixed endpoint assays with 1 nM radiolabeled DNA and 20 nM truncated Gen (1-518)-His. It was previously reported that the OsGEN-L ortholog was highly sensitive to salt concentration in the reaction

mixture. In that study, the 5' flap was efficiently cleaved at KCl concentrations below 100 mM, and activity dropped off rapidly at higher KCl concentrations. In contrast, HJ cleavage activity was not observed under the same conditions as the 5' flap. Rather, high salt (150 mM) was critical for HJ cleavage. (**b**) The N-terminal His tag interferes with the nuclease activity of Gen. Nuclease assays were done with Gen (FL = full-length; 518 = 1-518; or N = full-length with N-terminal tag). N OE = The gel was overexposed to show residual nuclease activity with N-terminally tagged Gen. Arrow = This band results from extra breathing at the 5' flap ss-dsDNA junction due to an extra A added to the 3' end of Oligo 992 (see **Table 1**). Nuclease assays contained 50 nM protein and 1 nM DNA. (**c**) Substrate cleavage is dependent upon Gen nuclease activity. Nuclease-dead Gen had mutations in two glutamic acid residues in the catalytic domain: E143A and E145A. Assays contained 20 nM protein and 1 nM DNA. All assays were done at 22 for 30 min. were incubated at 22°C for 30 minutes. Products were analyzed by denaturing PAGE.

Gen is a Holliday junction resolvase and a 5' flap endonuclease

To examine substrate specificity, we incubated Gen with radiolabeled DNA substrates (**Fig. 9**). Both full-length and truncated C-terminal His-tagged Gen exhibit robust cleavage of 5' flaps, RFs, and fixed, mobile, and nicked HJs. Similar to other Gen orthologs, denaturing polyacrylamide gel electrophoresis (PAGE) demonstrates that the predominant cut sites are one nucleotide (nt) 3' of the junction branch point on the 5' flap, RF, and immobile HJs (HJ0 and nHJ0) (**Fig. 9**). The secondary cut observed with the HJ0 and nHJ0 are likely due to substrate breathing or weak sequence preference. Finally, we observe multiple cut sites on the mobile HJ12 substrate, which contains a 12 bp homologous core within which the junction can migrate; all cut sites on this structure are within the 12 bp core. We do not detect cleavage of unbranched dsDNA or nicked duplex DNA. We noted weak cleavage of the 3' flap substrate, but the cleavage sites map to a region within the single-stranded flap, suggesting that cleavage is due to secondary structure in this region. Because full-length and truncated Gen exhibited similar substrate specificities and activities (**Fig. 9**), we used the truncated protein, which is more stable, in subsequent experiments.

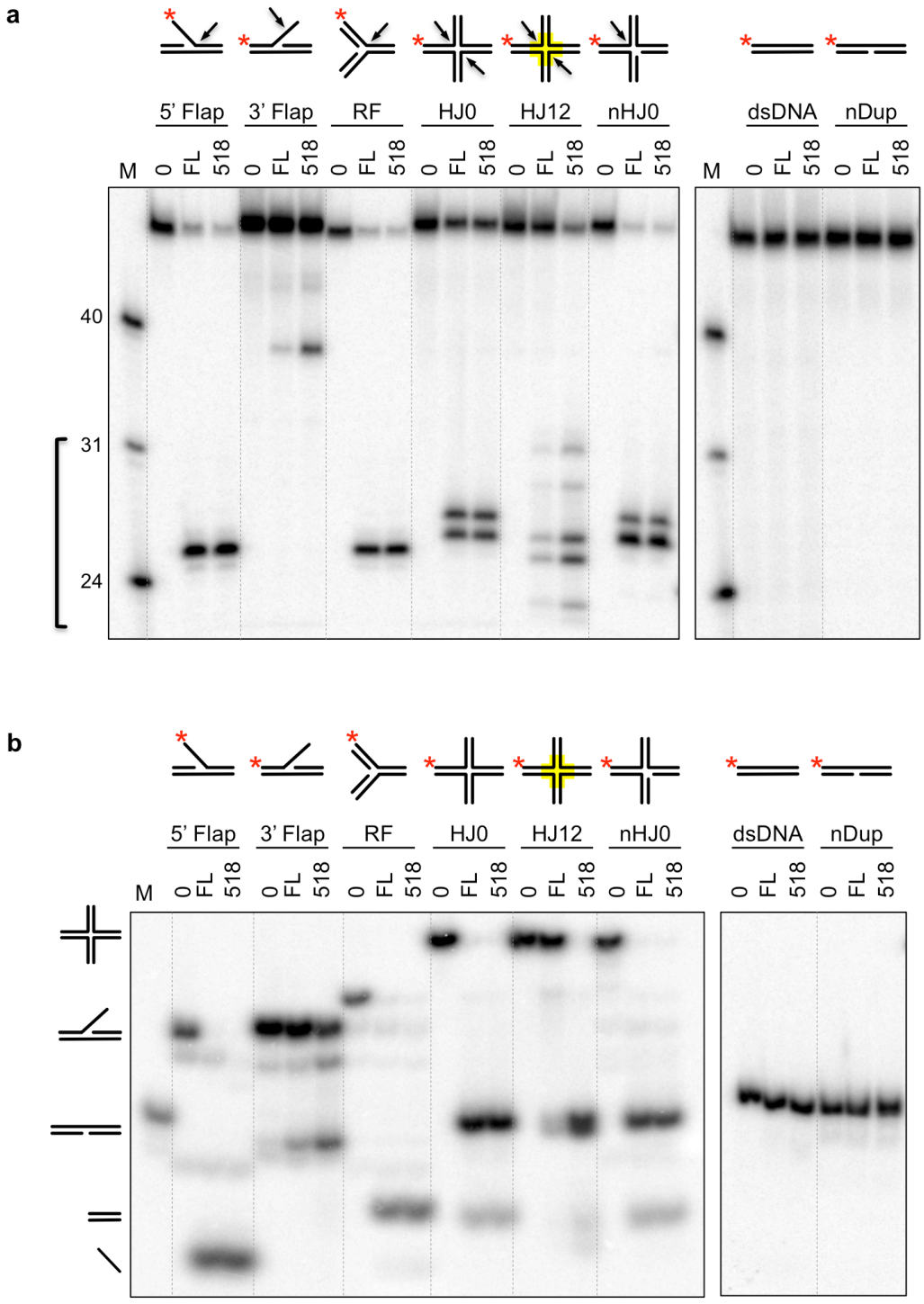

Figure 9 Gen is a Holliday junction resolvase and 5' flap endonuclease. (**a**) Substrates radiolabelled at the 5' end of one strand (asterisks) were incubated with full-length (FL) Gen or truncated (518) Gen. Arrows indicate sites of cleavage determined by denaturing PAGE, shown below. The bracket indicates the expected size range of the cleavage products for the HJ12 substrate. (**b**) The other half of the cleavage reaction was analyzed by native PAGE, which shows that the major products of the HJ and nHJ reactions are nicked duplexes. The major product of the 5' flap reaction is the single-stranded flap.

To map HJ cleavage sites on each strand, we alternately labeled each strand of the immobile HJ0 structure (**Fig. 10**). The major cuts on each strand are located one nt 3' to the junction branch point. As discussed above, on strand A there was a secondary cleavage site two nt 3' to the junction, possibly due to weak sequence preference (**Fig. 9 and 10**). These studies show that *Drosophila* Gen, like its fungal and human orthologs, retains the ability of the FEN-1 nuclease family to cut 5' flaps one nt 3' of the branch point. Additionally, like its orthologs, *Drosophila* Gen is a resolvase that cuts HJs symmetrically.

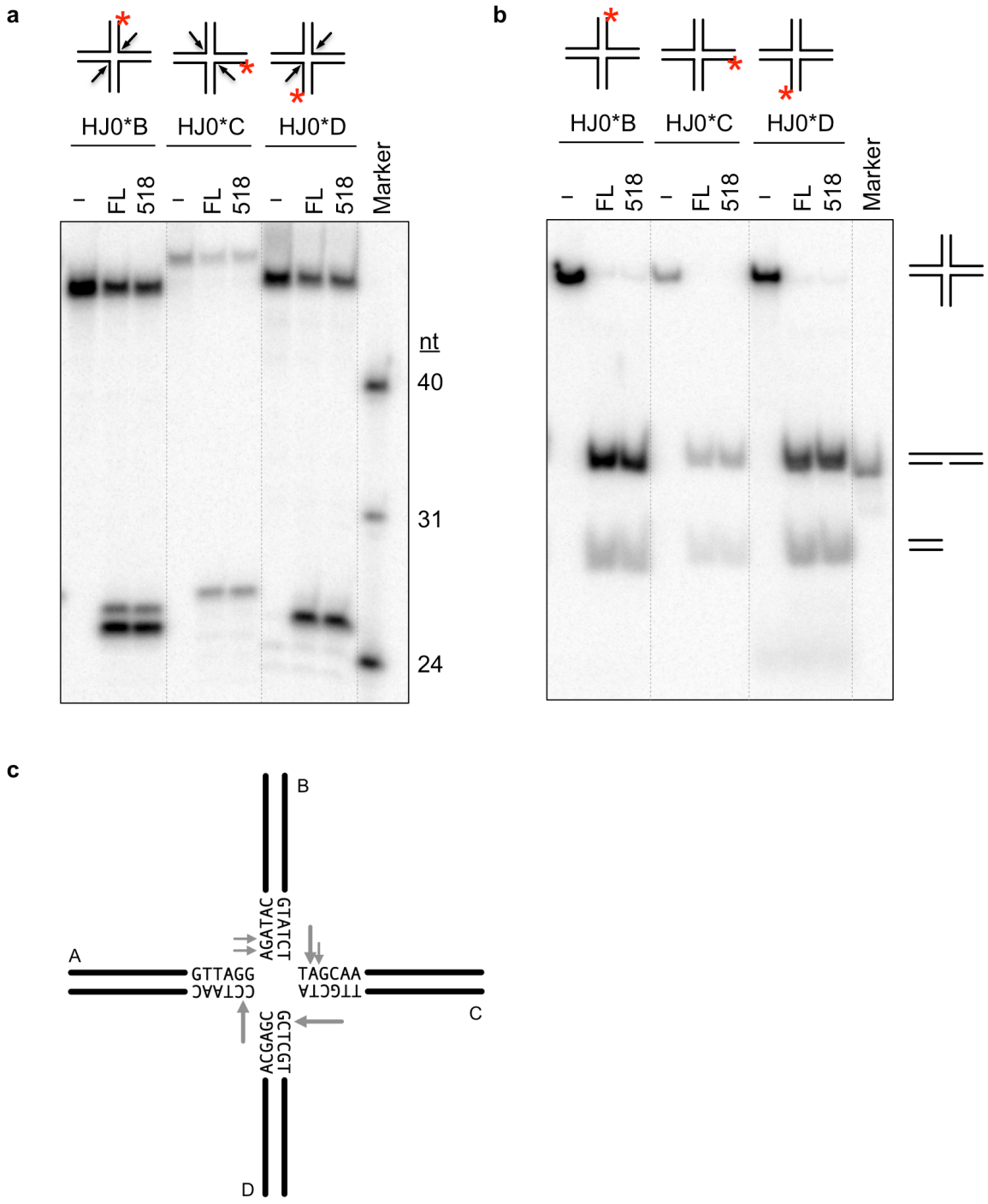

Figure 10. GEN cuts HJs symmetrically to produce two nicked duplexes. The cleavage specificity of GEN for the HJ0 was determined by alternately radiolabeling strand B, C, or D. Denaturing PAGE determined the position of cutting on each strand. (**a**) Denaturing gel (**b**) Native gel (**c**) Summary of cleavage sites identified in (a and b). Longer arrows indicate preferred cleavage sites.

Gen cleaves 5' flaps faster than Holliday junctions

We examined the kinetics of *Drosophila* Gen activity as a function of both substrate and enzyme concentration on the 5' flap and HJ0 in multiple-turnover assays (*i.e.*, excess substrate relative to protein) (**Fig. 11**). Inspection of **Figure 11a** reveals that 5 nM 5' flap is completely cleaved within 1 to 4 minutes (min) using Gen concentrations ranging from 0.5 to 3 nM, with the rate of cleavage increasing with increasing Gen concentration. In contrast, the rates of HJ0 cleavage are ~10-fold slower, with reactions taking 10-30 min to plateau; and only the reaction containing 3 nM Gen goes to completion (**Fig. 11b**). Reactions with the lower concentrations of Gen (0.5 nM to 1 nM) exhibit plateau values that decrease with decreasing Gen concentration, with the plateau value ranging from 20% to 60% of the substrate being cleaved (**Fig. 11b**). Several factors, such as enzyme death and substrate and/or product inhibition can result in less than 100% of the substrate being cleaved; however, the observations that Gen can completely cleave the 5' flap at the same concentrations (**Fig. 11a**) and that only the reaction plateaus of the HJ0 decrease as the substrate:Gen ratio increases (**Fig. 11b**) suggest that the excess HJ0 may be acting as a substrate inhibitor.

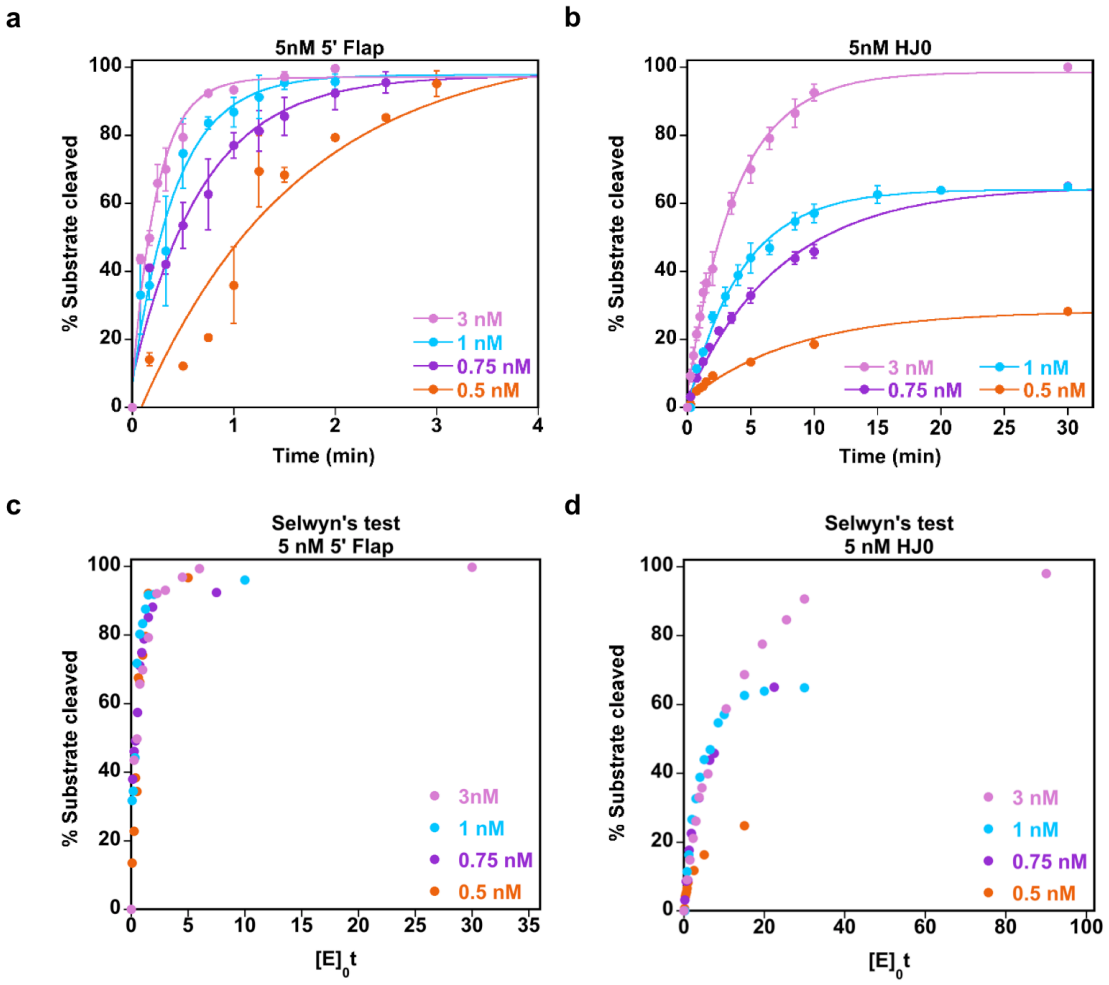

Figure 11 Gen cleaves 5' flaps faster than HJ0s under conditions of excess substrate. (**a-b**) Time courses of Gen progression under conditions of excess substrate: (**a**) 5' flap (**b**) HJ0. For each time course, aliquots were taken at various time points (note that the time scales differ in each panel). The intensity of each cleavage product was quantified by ImageQuant, and the data were normalized to the expected amount of detectable product (see **Materials and Methods**). Each dot represents the mean of three experiments, except in (**a**), which is the mean of two experiments. Error bars indicate standard error of the mean. (**c**) Selwyn tests to examine nuclease progression curves in (**a-b**). The percentage of substrate cleaved was plotted against the initial enzyme concentration multiplied by time for various enzyme concentrations to examine perturbations to the nuclease reaction. Overlapping curves indicate that there were no perturbations to the reaction (for example, enzyme death or substrate and/or product inhibition).

We used the Selwyn test to determine whether there were any perturbations to our nuclease reactions, such as enzyme instability, or substrate or product inhibition (**Fig. 11c and d**). If the shapes of the nuclease progress curves are due solely to the reactants, they will overlap

when different enzyme concentrations are multiplied by time and plotted as function of percent substrate cleaved. For the 5' flap reactions, the progress curves with different concentrations of protein overlapped, indicating that progress is governed solely by the reactants (**Fig. 11c**). However, curves for the HJ0 reactions did not overlap, but fell into three distinct groups (**Fig. 11d**). The progressive decrease in protein activity with increasing substrate is indicative of substrate inhibition. Human GEN1, which is a monomer in solution but must dimerize on an HJ for cleavage, shows similar inhibition of cleavage when the HJ substrate is in excess of GEN1 (Rass et al., 2010). In this scenario, excess substrate molecules bind GEN1 monomers, thereby reducing the concentration of dimer available to cleave the HJ. Given that *Drosophila* Gen is in a monomer-dimer equilibrium (**Fig. 6**), a likely source of the reduced plateau levels is substrate inhibition. In addition, these data, along with the observation that the HJ0 is always cut on both sides (**Fig. 9 and 10**), indicate that dimerization is required for HJ cleavage but not for 5' flap cleavage, similar to the Gen orthologs (Freeman et al., 2014; Rass et al., 2010)

Human GEN1 cleaves 5' flaps more rapidly than HJs when the enzyme is limiting, perhaps due to substrate inhibition, but it cleaves HJs more rapidly when there is excess enzyme present (Rass et al., 2010). Thus, we conducted experiments with a large excess of protein relative to substrate (**Fig. 12**). Under these conditions, reactions reached completion within 20 seconds to 3 min. Notably, even at 200 nM Gen, the rate of 5' flap cleavage is ~7-fold higher than the rate of HJ0 cleavage (**Fig. 12b**).

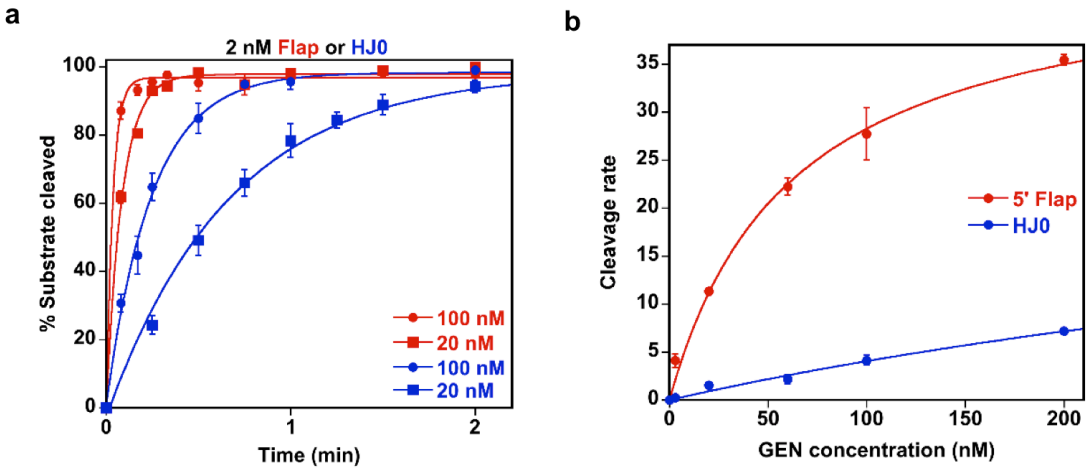

Figure 12 Gen cleaves 5' flaps faster than HJs under conditions of excess protein. (**a**) Time courses of Gen progression on 5' flaps and HJ0s under conditions of excess protein. For each time course, aliquots were taken at various time points (note that the time scales differ in each panel). The intensity of each cleavage product was quantified by ImageQuant, and the data were normalized to the expected amount of detectable product (see **Materials and Methods**). Each dot represents the mean of three experiments. (**b**) Each individual replicate from (**Fig. 11 and 12**) was fit to a single exponential curve to obtain the cleavage rate, and mean cleavage rates were plotted as a function of protein concentration. Note that the first point (3 nM protein) was performed with 5 nM 5' flap or HJ0, whereas the rest of the experiments (20, 60, 100, and 200 nM protein) were performed with 2 nM DNA. The 5' flap and HJ0 data from these and additional experiments (not shown) were fit to a hyperbolic binding curve given by the equation $y=m_1*x/(m_2+x)$, where m_1 = maximum rate at saturating protein concentrations and m_2 = the equilibrium dissociation constant. With the 5' flap, m_2 = 62.12 nM, and with the HJ0, m_2 = 656.46 nM.

Because the cleavage rates of both the HJ0 and 5' flap increased with increasing concentration, we plotted the cleavage rates as a function of excess Gen concentration (**Fig. 12b, Table 4**). The cleavage rates fit well to hyperbolic binding curves for both the 5' flap and HJ0 (**Fig. 12b**). The $K_{1/2}$ determined from the fit of the HJ0 is ~660 nM, which suggests that the binding of Gen to the HJ0 is weak. This result is similar to that seen for human GEN1 cleavage of a cruciform structure in plasmid DNA, where the rate of cleavage continued to increase up to 3 μM GEN1 (Rass et al., 2010). Interestingly, the $K_{1/2}$ for 5' flap cleavage is ~60 nM. The significance of these results in understanding the mechanism of cleavage of the HJ0 and 5' flaps is discussed in the following sections.

Table 4. Summary of cleavage rates from all kinetics experiments.

Substrate	DNA (nM)	Gen (nM)	Average Rate	SE
5' Flap	5	0.5	0.70	0.01
	5	0.75	1.43	0.29
	5	1	2.52	0.77
	5	3	4.11	0.71
	2	20	11.56	0.32
	2	60	22.24	0.88
	2	100	29.01	2.88
	2	200	35.44	0.56
HJ0	5	0.5	0.12	0.01
	5	0.75	0.13	0.01
	5	1	0.23	0.03
	5	3	0.26	0.02
	2	20	1.53	0.17
	2	60	2.16	0.50
	2	100	4.12	0.51
	2	200	7.17	0.14

The rate-limiting step of HJ0 cleavage is assembly of a productive dimer complex on the substrate

The apparent weak binding affinity of Gen for the HJ0 determined from kinetics (**Fig. 12b**) contrasts with data on the *C. thermophilium* ortholog of Gen, *Ct*GEN1, which exhibits tight (~10 nM) cooperative binding of a dimer to an HJ (Freeman et al., 2014). The weak $K_{1/2}$ could

be a result of weak binding of the dimer to the HJ0, or it could represent a pre-equilibrium step if the rate-limiting step is a conformational change after binding. In the experiments described above (**Fig. 11 and 12**), the DNA, Gen, and Mg^{++} are added simultaneously, so it is not possible to determine whether the rate-limiting step is after binding. To assess the possibility that the rate-limiting step is a conformational change after binding, we pre-incubated Gen with both the 5' flap and HJ0 in the absence of Mg^{++}, allowing time for the dimer to assemble on the DNA, and then initiated cleavage by the addition of Mg^{++} (**Fig. 13**). If conformational change/assembly of the dimer on the substrate is rate-limiting, then cleavage will be significantly more rapid in the pre-incubation experiment than in the simultaneous addition experiments. We were unable to determine the rate-limiting step with the 5' flap because residual Mg^{++} in the DNA buffer was sufficient to promote robust cleavage (**Fig. 13b**), but in pre-incubation experiments with HJ0 we observed a burst of cleavage before the first time point (5 seconds) followed by a slow rate of cleavage similar to that seen in the simultaneous addition experiment (**Fig. 13a**). This observation strongly suggests that given sufficient time, Gen can cooperatively assemble into a productive complex on the HJ0. This suggestion is supported by data on the Gen ortholog *Ct*GEN1, which binds cooperatively to HJs with a high affinity (~10 nM). We used a Gen concentration of 3 nM, corresponding to 1.5 nM dimer. If all the Gen were pre-bound as dimers to the HJ0 and poised to undergo rapid cleavage, we would expect burst heights of 30% for 5 nM HJ0 and 15% for 10 nM HJ0. We observed burst heights of approximately 25% and 10% (**Fig. 13a**), suggesting that the majority of Gen is bound in a productive dimer complex prior to the addition of Mg^{++}. These results strongly suggest that the rate-limiting step is a conformational change after productive assembly of the dimer on the HJ.

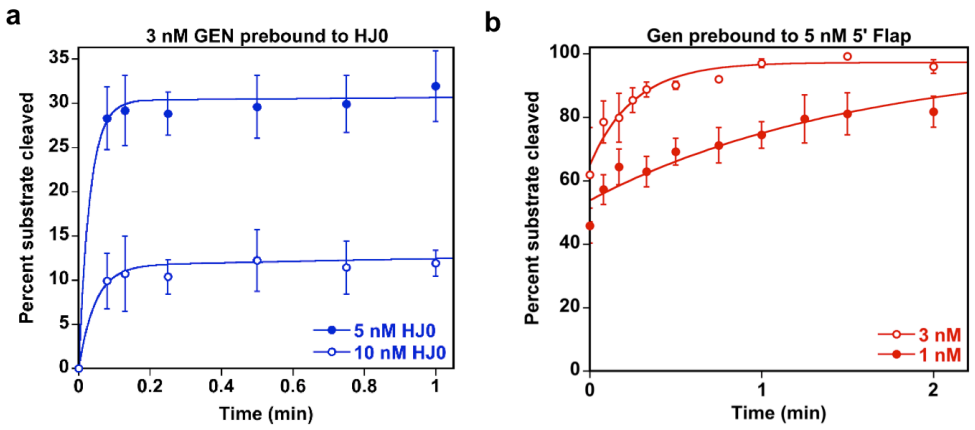

Figure 13 The rate-limiting step of the HJ0 reaction is formation of a productive dimer-DNA complex. (**a**) To determine whether the rate-limiting step of the HJ0 reaction is binding and/or a conformational change, we pre-incubated 3 nM Gen with 5 or 10 nM HJ0 before starting the time course experiment with Mg^{++}_2. (**b**) To determine whether the rate-limiting step for the 5' flap reaction was formation of a productive complex, we pre-incubated 1 or 3 nM Gen (1-518)-His with 5 nM 5' flap before starting the reaction with Mg^{++}. Unfortunately, contaminating divalent ion in the DNA storage buffer was sufficient to initiate cleavage and turnover before addition of Mg^{++} (indicated by 45-60% of the substrate cleaved at time 0) and prevented us from determining the rate-limiting step.

In conclusion, the pre-incubation experiments indicate tight binding of a Gen dimer to the HJ0 with only a small amount of substrate inhibition (**Fig. 13a**); whereas, the simultaneous addition experiments yield a very weak $K_{1/2}$ (660 nM) and exhibit significant substrate inhibition, suggesting a monomer of Gen binds tightly to the HJ0 (**Fig. 11 and 12**). Taken together, these data lead us to suggest a model (see **Fig. 14** and **Chapter 3 Conclusion**) in which a monomer binds tightly to the HJ0 followed by a second monomer binding to form a nonspecific dimer on the HJ0 with a weak binding affinity. Next, this nonspecific dimer-HJ0 complex undergoes a conformational change to a productive complex followed by rapid cleavage. For human GEN1, a conformational change triggered by formation of a dimer on HJs was proposed to explain the observation that human GEN1 does not nick HJs, but always cuts both strands (Rass et al., 2010). Other HJ resolvases, including the RuvC and T4endo7 resolvases, which are obligate dimers, have been shown to select for HJs in a specific

conformation and then alter the conformation prior to cleavage (Fogg and Lilley, 2000; Pohler et al., 1996). Our results are consistent with the idea that a prerequisite for the Gen dimer to bind tightly to the HJ0 is that the DNA has to be in the proper conformation. Consequently, if the HJ0 is not in a conformation that presents a proper dimer interface, it could promote the dissociation of one monomer of the dimer.

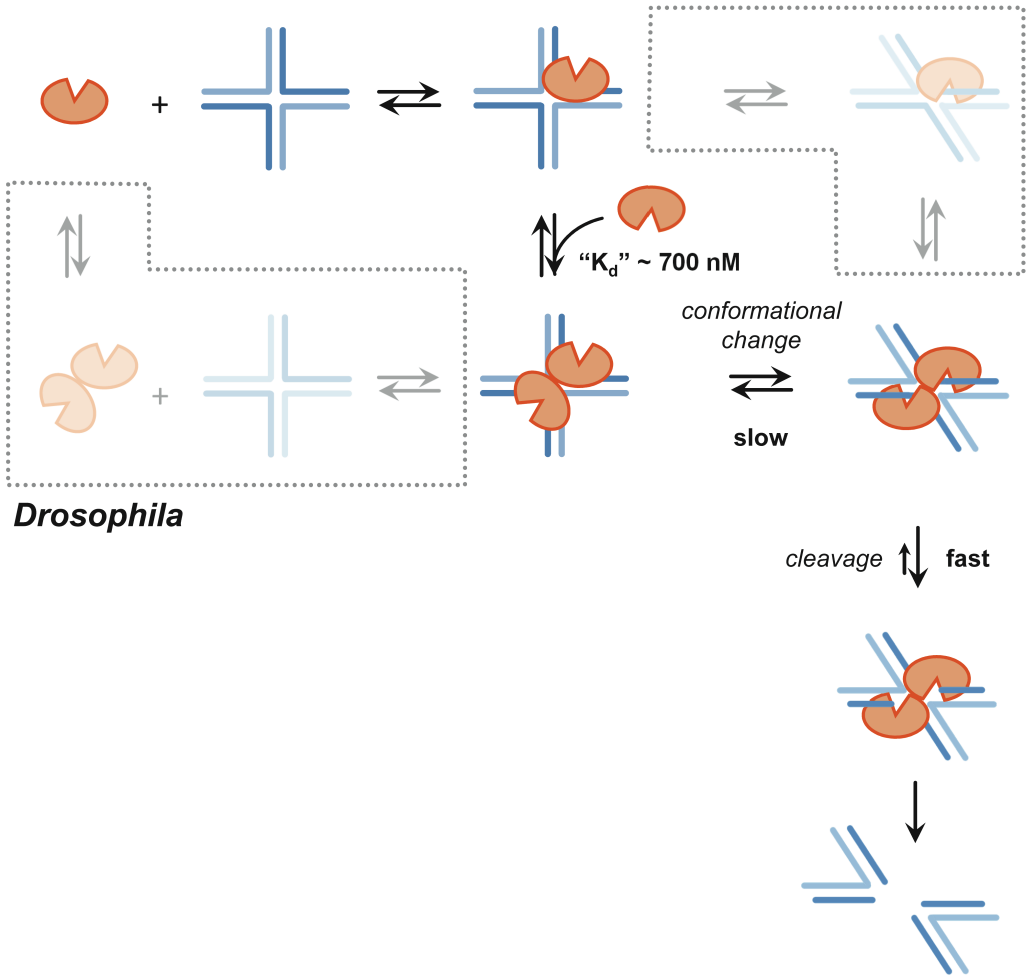

Figure 14. Model of *Drosophila* Gen function on HJs. (See text for more details.) A Gen monomer binds the HJ, followed by weak, non-specific binding of a second monomer. Formation of a productive dimer complex, exhibiting the correct DNA conformation required to position opposite DNA strands in the Gen active sites, is slow. Once a productive dimer-HJ0 complex is formed, the dimer cooperatively nicks across the junction. (*bottom left box*) It is unlikely that a pre-formed dimer will encounter a HJ exhibiting the proper conformation required for cleavage. If the dimer-DNA complex is not productive,

one monomer likely dissociates from the HJ, allowing other Gen proteins access to the junction. (*top right box*) It is also possible that a DNA conformational change occurs prior to the second monomer binding; however, given our observation that production of a productive dimer-DNA complex is the rate-limiting step, this is unlikely to represent a main pathway.

Dimerization of Gen on 5' flaps stimulates its cleavage activity

In contrast to human and fungal GEN1 (Freeman et al., 2014; Rass et al., 2010), *Drosophila* Gen cleaves the 5' flap more rapidly than the HJ0 at all concentrations tested, and the rate of flap cleavage increases with increasing protein concentration with a $K_{1/2}$ ~60 nM (**Fig. 12b**). Consistent with our SEC-MALS result that shows that, unlike human and fungal GEN1 (Freeman et al., 2014; Rass et al., 2010), *Drosophila* Gen exists in a monomer-dimer equilibrium (**Fig. 6**), we wondered whether the increasing cleavage rate of the 5' flap with increasing Gen concentration resulted from dimerization of Gen on the 5' flap. To examine this possibility, we used electrophoretic mobility shift assays (EMSAs) to monitor binding to the 5' flap and atomic force microscopy (AFM) to directly observe the oligomerization state of the protein. We conducted the EMSAs in the presence of EDTA to chelate any contaminating $MgCl_2$ to prevent substrate cleavage. In addition, we stabilized the complexes by crosslinking with glutaraldehyde using a gentle vapor-diffusion method (Fadouloglou et al., 2008) prior to running the complexes on the gel because uncrosslinked complexes dissociated during electrophoresis, resulting in band smearing. As expected, two shifted bands appear in a concentration-dependent manner on the HJ0 (**Fig. 15a**), consistent with a monomer and dimer binding to the HJ0 and suggesting the successive recruitment of two monomers of Gen to the HJ0. Notably, we also observe two shifted bands on the 5' flap (**Fig. 15a**), even at concentrations as low as 7.5 nM Gen, suggesting the formation of a dimer of Gen on the 5' flap. Interestingly, the concentration dependence of dimer binding to the 5' flap in the EMSAs (**Fig. 15a**) mimics the concentration dependence of

cleavage on the flap determined from the kinetics assays (**Fig. 11 and 12**). Performing the EMSAs in the absence of EDTA results in complete cutting of the 5' flap at all concentrations of protein, and no shifted bands are observed (**Fig. 15b**). These results indicate that glutaraldehyde crosslinking does not lead to accumulation of non-specific protein-DNA complexes and therefore that the bands observed with the 5' flap are the result of specific interactions of a monomer and dimer of Gen interacting with the 5' flap.

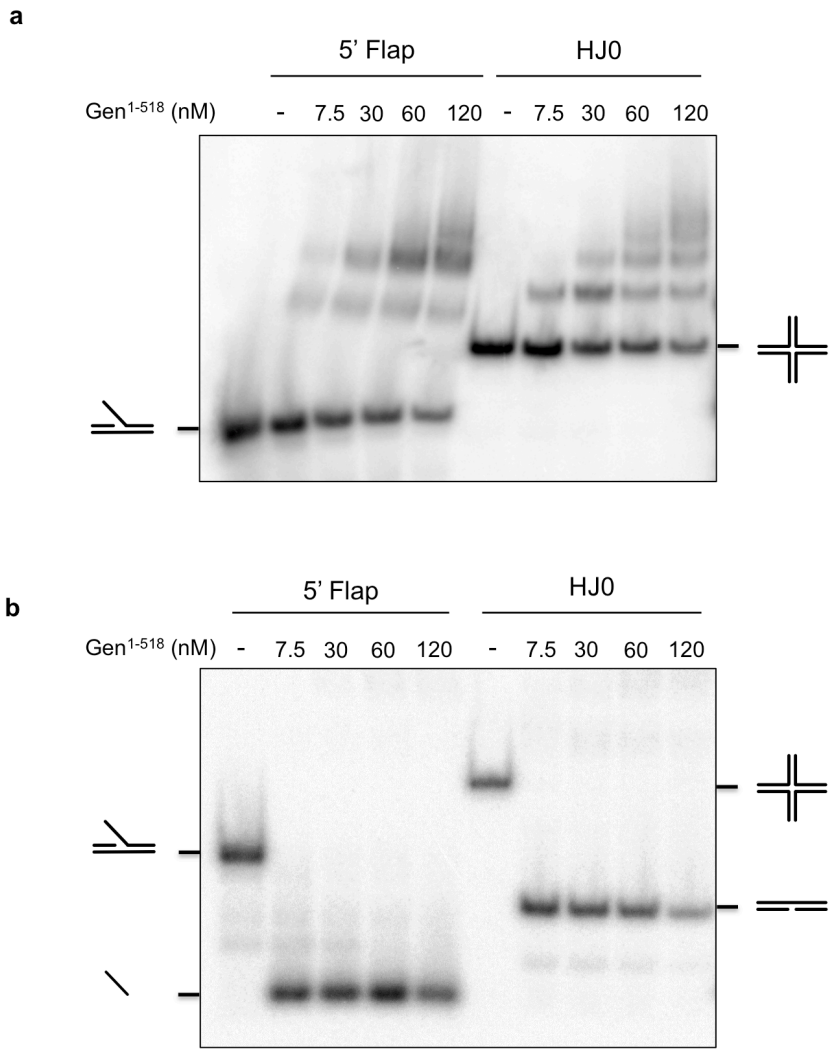

Figure 15. Gen dimerizes on the 5' flap and HJ0. (**a**) EMSA analysis of Gen with 5' flap and HJ0. Truncated Gen was incubated with 5 nM radiolabeled DNA in the presence of EDTA. Complexes were crosslinked by vapor diffusion with a mixture of glutaraldehyde and HCl before analysis on a 4% native

polyacrylamide gel. (**b**) In the absence of EDTA, no shifts are observed on the 5' flap or HJ0, suggesting that complexes formed in (**a**) are not an artifact of glutaraldehyde crosslinking.

To garner additional evidence for the dimerization of Gen, we used AFM (**Fig. 16**) to analyze Gen at concentrations similar to those used in our kinetics experiments (**Fig. 11 and 12**). Previous studies show that there is a linear relationship between the molecular mass of a protein and its observed volume in AFM images, which allows oligomerization state and the association constants of the protein-protein complexes to be determined (Ratcliff and Erie, 2001; Yang et al., 2003). At 20 nM and 37 nM Gen, we observe two major populations of peak volumes: one consistent with the volume of a Gen monomer and the other consistent with the volume of a dimer of Gen (**Fig. 16**). From these studies, we estimated the protein dissociation constant to be within the 60 nM to the μM range. Quantitative assessment is not possible because of overcrowding on the sample surface at concentrations closer to the predicted dissociation constant (**Fig. 16c**). These results further support the existence of a monomer-dimer equilibrium in solution and our suggestion that dimerization of Gen on a 5' flap enhances its cleavage activity. Although a Gen dimer is not required for 5' flap cleavage, we hypothesize that the dimer cleaves the flap more rapidly than the monomer by introducing additional constraints to the conformation of the DNA (possibly to the single stranded flap), thereby promoting a productive cleavage complex (see **Fig. 17** and **Chapter 3 Conclusion**).

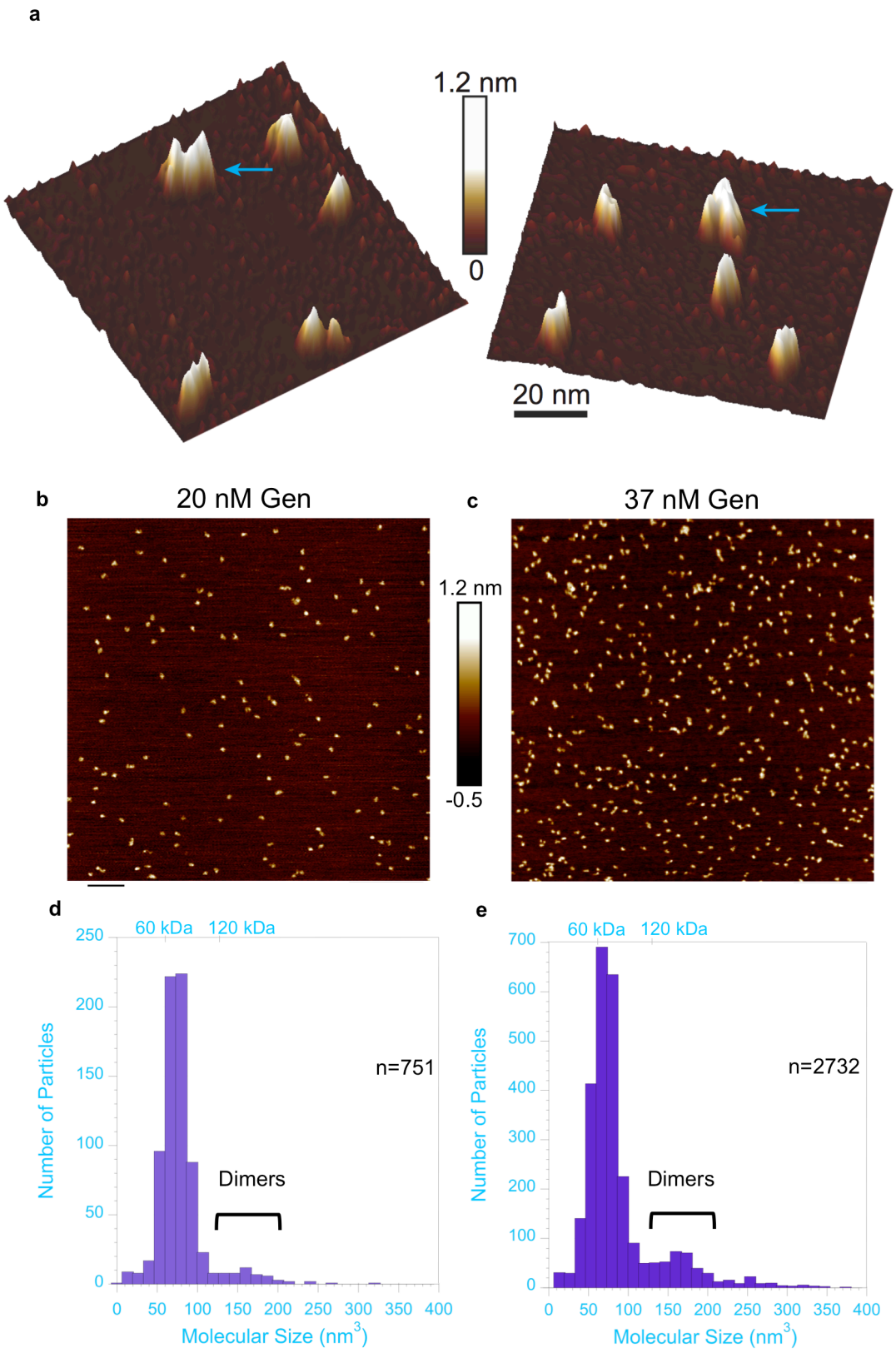

Figure 16. Direct visualization of Gen monomers and dimers by AFM. (**a**) Topographical AFM images of truncated Gen showing monomers and dimers. 20 nM truncated Gen was deposited onto naked

mica and imaged with tapping mode AFM in air. The gradient bar represents 0-1.2 nm height above the mica surface. Yellow arrows denote Gen dimers. (**b-c**) Representative 1x1 μm AFM images of truncated Gen at (**a**) 20 nM and (**b**) 37 nM. Bar represents 100 nm. (**d-e**) AFM volume analysis of particles in (**b, c**), respectively. Particles from at least 7 images for each concentration were analyzed for volume (nm^3). Resulting volumes were binned in 30 bins and graphed as a histogram. The volume calculated is directly proportional to molecular size. The predicted molecular mass of proteins from the AFM-derived volume was based on Equation 1 in **Materials and Methods**. Note that observed volumes are slightly larger than predicted volumes of Gen based upon molecular size. Brackets indicate volumes representing dimers.

Conclusion

In this chapter, we show that *Drosophila* Gen, a unique member of the Class IV monomeric FEN-1/XPG endonucleases, is a key SSE during the repair of DNA damage. Gen exhibits the characteristic substrate preferences of its yeast and human orthologs; namely, it cleaves 5' flaps and RFs with a high degree of specificity and is a *bona fide* HJ resolvase. Notably, we show that Gen displays dramatically altered nuclease kinetics on these substrates relative to other orthologs that have been studied, cleaving 5' flaps substantially faster than HJs *in vitro*. Further, this enhanced rate is due to the ability of the protein to dimerize in solution and on the 5' flap substrate. Finally, while it has been suggested that a conformational change is the rate-limiting step to cleavage on an HJ, our studies provide direct evidence that a conformational change occurs after the second monomer of Gen binds to the HJ0. Together, these data allow us to propose two models regarding Gen's mechanism of action on the HJ and 5' flap (**Fig. 14 and 17**, respectively).

We propose that a monomer binds tightly to the HJ0 (**Fig. 14**) followed by a second monomer binding with a weak affinity ("K_d" ~ 700 nM), forming a nonspecific dimer on the HJ0. Formation of a productive dimer-HJ0 complex exhibiting the correct dimerization interface, coupled with a DNA conformational change to position opposing DNA strands in the Gen active sites, is slow. Once a productive dimer-HJ0 complex is formed, the dimer rapidly nicks the two

opposing strands of the junction in a cooperative and symmetric manner, yielding two nicked duplexes. It is possible that a Gen dimer can form in solution prior to binding the HJ (**Fig. 14**, *bottom left box*); however, our results indicate that the HJ0 must be in the proper conformation for the Gen dimer to bind tightly. Given the affinity of the monomer for DNA, the HJ0 may promote the dissociation of one monomer of the non-productive dimer-DNA, allowing other Gen proteins access to the junction. It is also possible that a DNA conformational change occurs prior to a second Gen monomer binding the HJ (**Fig. 14**, *top right box*). Our observation that the production of a productive dimer-DNA complex is the rate-limiting step, coupled with observations that the human GEN1 monomer does not nick the HJ (Rass et al., 2010), indicates that this is unlikely to represent a major mechanistic pathway.

The mechanism of action of Gen on the 5' flap is similar to its orthologs in that a Gen monomer can bind the 5' flap, which undergoes a conformational change to position the DNA in the Gen active site thereby allowing the protein to rapidly cleave the flap strand one nt 3' of the junction branch point (**Fig. 17**, *top*). Our studies elucidate a *Drosophila*-specific pathway (**Fig. 17**, *bottom*). Gen can dimerize in solution, and a dimer can bind the 5' flap (**Fig. 17**, (*i*)). Alternatively, two monomers can sequentially bind the 5' flap with a predicted "K_d" ~ 60 nM (**Fig. 17**, (*ii*)). We hypothesize that the additional DNA binding sites provided by the second monomer help to constrain the DNA, facilitating the DNA conformational change that positions the flap strand in the active site of the other Gen monomer. Cleavage of this 5' flap-dimer complex is much faster than cleavage by the 5' flap-monomer complex.

In light of our biochemical data, this work reveals two fundamental differences between *Drosophila* Gen and other orthologs. First, although *Drosophila* Gen is a *bona fide* HJ resolvase, the difference in the *in vivo* function of Gen from its orthologs may be due to the enhanced

action of the protein on flaps and replication fork intermediates as opposed to HJs. Second, the ability of Gen to dimerize in solution and on the 5' flap suggests that this function underlies the increased activity on these substrates. The implications of these apparent differences in substrate preference may be that while Gen still plays a role in the resolution of HJs *in vivo*, its main role may reside outside of HJ cleavage. We speculate that other DNA substrates may also represent relevant repair intermediates for human GEN1 and yeast Yen1.

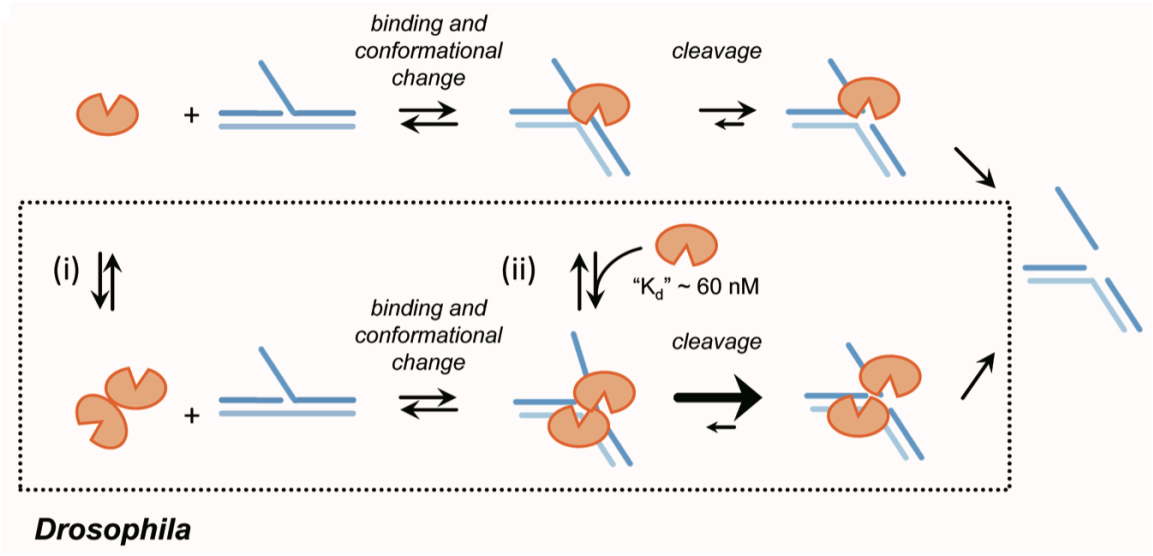

Figure 17. Model of *Drosophila* Gen function on 5' flaps. (See text for more details.) (*top*) Gen monomer binds the 5' flap, triggering a DNA conformational change and rapid cleavage of the flap strand one nt 3' of the junction branch point. (*bottom*) A *Drosophila*-specific pathway is depicted in the box. (i) A pre-formed dimer can bind the 5' flap. (ii) Alternatively, two monomers can subsequently bind the 5' flap with a predicted "K_d" ~ 60 nM. The additional DNA contacts provided by the second monomer facilitate 5' flap cleavage.

Materials and Methods

Purification of full-length and truncated Gen (1-518) from *E. coli*

Drosophila Gen cDNA was codon-optimized by GenScript. Full-length Gen (1-726 aa) and truncated Gen (1-518 aa) were cloned into the *NdeI* and *XhoI* sites of pET21b (Novagen),

which carries a C-terminal hexahistidine tag. The nuclease-dead mutations E143A E145A, previously described by (Kanai et al., 2007), were made by QuikChange site-directed mutagenesis (Agilent Technologies). Gen-His was expressed in RDK cells (Richard D. Kolodner, courtesy of Dr. Steve Matson (University of North Carolina at Chapel Hill)) with 0.4 mM IPTG, and Gen (1-518)-His was expressed in Rosetta II pLysS (Novagen) with 1.0 mM IPTG. All proteins were expressed at 18°C for 18 hrs. The Gen (1-518)-His and Gen (1-518)^{Dead}-His pellets were lysed in NiA buffer (20 mM KH_2PO_4 pH 7.0, 100 mM ammonium acetate, 1 mM TCEP, 0.02% sodium azide, 500 mM NaCl, 50 mM imidazole), sonicated, pelleted, and the clarified supernatant was loaded onto a 5 mL HisTrap HP column (GE Healthcare Life Sciences) and eluted with NiB (20 mM KH_2PO_4 pH 7.0, 100 mM ammonium acetate, 1 mM TCEP, 0.02% sodium azide, 500 mM NaCl, 500 mM imidazole). Peak fractions were diluted in NiA minus salt to 50 mM NaCl and loaded onto a 6 mL Resource S column (GE Healthcare Life Sciences) pre-equilibrated with MonoSA (20 mM KH_2PO_4 pH 7.0, 100 mM ammonium acetate, 1 mM TCEP, 0.02% sodium azide, 50 mM NaCl) and gradient eluted with MonoSB (20 mM KH_2PO_4 pH 7.0, 100 mM ammonium acetate, 1 mM TCEP, 0.02% sodium azide, 1 M NaCl). Peak fractions were concentrated to 5 mL and loaded onto a Superdex S200 column (GE Healthcare Life Sciences) and eluted with S200 buffer (50 mM HEPES pH 7.0, 400 mM NaCl, 100 mM ammonium acetate, 1 mM TCEP, 0.02% sodium azide). Full-length Gen-His and Gen^{Dead}-His were purified over HisTrap and S200 columns. Following elution from the S200 column, all proteins were analyzed by dynamic light scattering and SDS-PAGE.

Size Exclusion Chromatography and Multiangle Light Scattering (SEC-MALS)

Gen (1-518)-His protein was dialyzed into high salt buffer (25 mM HEPES pH 7.5, 100 mM sodium acetate, 10 mM magnesium acetate, 5% glycerol, 1 mM DTT) before injection onto a Superdex 200 column (GE Healthcare Life Sciences) connected to a Wyatt DAWN HELEOS-II light scattering instrument and a Wyatt Optilab T-rEX refractometer. The Wyatt Astra V software package (Wyatt Technology Corp.) was used to determine the molar mass of the sample.

Nuclease assays

Synthetic DNA substrates were prepared by annealing oligonucleotides shown in **Table 5**. Oligos 888, 891, 892, 893, 894, 895, 897, and 992 were described previously in (Kaliraman et al., 2001), and oligos 940 and 994 were modified from (Kaliraman et al., 2001). Substrates were prepared as previously described (Wright et al., 2011). Briefly, one oligonucleotide was 5' end-labeled using T4 polynucleotide kinase and γ-^{32}P ATP. Substrates were annealed in annealing buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 5 mM DTT), PAGE-purified, and quantified by A_{260}.

For nuclease assays, Gen was incubated with the ^{32}P-labeled structures in a 10 µL reaction mixture containing 50 mM Tris pH 8, 100 µg/ml BSA, 1 mM DTT, 10% glycerol, 50 mM KCl, and 5 mM $MgCl_2$ at RT. For fixed end-point assays, unless otherwise indicated, 20 nM protein was incubated with 1 nM substrate. The reaction was stopped in an equal volume of formamide loading dye (85% formamide, 50 mM EDTA, 1% bromophenol blue, 1% xylene cyanol), heated at 95°C for 5 min, and a fraction was loaded onto a polyacrylamide gel. After

running, gels were dried and imaged on a Typhoon Trio+ (GE Healthcare Life Sciences). Bands were quantified using ImageQuant (GE HealthCare Life Sciences).

For time point analysis, 1 μL aliquots were removed and quenched in 2.5 mg/ml Proteinase K, 2.5% SDS, and 125 mM EDTA. The amounts of protein and substrate used in the kinetics assays are given in the figure legend. To determine the percentage substrate cleaved, the amount of product was calculated as a fraction of the total radioactivity per lane. For the HJ0, only half the cleavage products (those in which the labeled strand is cut) can be detected. For the 5' flap, a fraction of the substrate was unproductive or degraded. To account for this, the data were normalized to the expected amount of detectable product. The cleavage rate was determined by fitting the full reaction curves to a single-exponential function using KaleidaGraph software (Synergy, Reading, PA).

DNA-Binding Assays

Gen (1-518)-His was incubated in a 10 μL reaction with 5 nM ^{32}P-labeled DNA in binding buffer (10 mM HEPES pH 7.5, 100 μg/ml BSA, 1 mM DTT, 5% glycerol, 60 mM KCl) containing either 5 mM EDTA or 5 mM $MgCl_2$. Incubation occurred at RT for 30 min. Samples were then cross-linked as described in (Fadouloglou et al., 2008). Briefly, each sample was placed onto a siliconized coverslip, which was then inverted over 1 well of a 24-well tissue culture plate containing a 40 μL mixture of 25% (v/v) glutaraldehyde acidified with 1 μL 5 N HCl, and sealed with vacuum grease. After 10 min incubation at RT, reactions were immediately analyzed by 4% neutral PAGE at 4°C.

Atomic Force Microscopy

50 μM Gen (1-518)-His was diluted to 2 μM in storage buffer (50 mM HEPES pH 7.0, 400 mM NaCl, 100 mM ammonium acetate, 1 mM TCEP, 10% glycerol) and then to 20 and 37 nM in high salt buffer (25 mM HEPES pH 7.5, 100 mM sodium acetate, 10 mM magnesium acetate, 5% glycerol, 1 mM DTT) and 20 μL was immediately deposited onto freshly-cleaved mica. The mica surface was then immediately washed with water, and a stream of nitrogen gas was used to dry the surface. Images were acquired with a Nanoscope III 3A atomic force microscope (Veeco, Santa Barbara, CA) in tapping mode with a resolution of 512×512 pixels at a scan rate of 1.97 Hz and over a 1×1 μm scan size. AFM tips were from NanoSensors (Neuchatel, Switzerland) with a spring constant between 21 and 98 N/m and resonance frequencies between 146 and 236 kHz. AFM images for the samples were consistent over two depositions and multiple tips (at least two for each deposition). Poor images resulting from blunted tips were excluded from analysis. At least seven representative images of each sample were 2nd order plane-fitted and flattened, and three-dimensional images were generated using NanoScope Analysis version 1.53r1 (Bruker Instruments). Volume analysis of protein peaks was conducted with Image SXM 195-1 (Steve Barrett, University of Liverpool, UK) as described in (Ratcliff and Erie, 2001). Volumes corresponding to protein aggregates were excluded from analysis. Volume plots were generated using KaleidaGraph 4.1.3 (Synergy, Reading, PA). Protein molecular mass was converted into predicted AFM volume using the following equation:

(1) $V = 1.2 * M - 14.7$

where V is AFM volume in nm^3, and M is molecular mass in kDa (Yang et al., 2003).

Table 5. Oligos used in this study

Oligo ID	Length (nt)	Sequence	Structure (* = labeled strand)
888	49	GACGCTGCCGAATTCTGGCGTTAGGAGATACCGA TAAGCTTCGGCTTAA	5'Flap, RF, HJ0*, nHJ*, dsDNA*, nDup*, 3'Flap*
891	49	ATCGATGTCTCTAGACAGCACGAGCCCTAACGCC AGAATTCGGCAGCGT	5'Flap*, RF*, HJ0, nHJ, 3'Flap
892	49	GACGCTGCCGAATTCTGGCTTGCTAGGACATCTTT GCCCACGTTGACCC	HJ12*
893	50	TGGGTCAACGTGGGCAAAGATGTCCTAGCAATGT AATCGTCTATGACGTT	HJ12
894	51	CAACGTCATAGACGATTACATTGCTAGGACATGC TGTCTAGAGACTATCGA	HJ12
895	50	ATCGATAGTCTCTAGACAGCATGTCCTAGCAAGC CAGAATTCGGCAGCGT	HJ12
897	49	GGGTCAACGTGGGCAAAGATGTCCTAGCAAGCCA GAATTCGGCAGCGTC	
940	24	CCTAACGCCAGAATTCGGCAGCGT	nDup
992	24	TTAAGCCGAAGCTTATCGGTATCT	5'Flap, RF, nDup
994	25	GCTCGTGCTGTCTAGAGACATCGAT	RF, nHJ, 3'Flap
888 rc	49	TTAAGCCGAAGCTTATCGGTATCTCCTAACGCCA GAATTCGGCAGCGTC	dsDNA
993	26	CAACGTCATAGACGATTACATTGCTA	nHJ
990	49	TTAAGCCGAAGCTTATCGGTATCTTAGCAATGTA ATCGTCTATGACGTT	HJ0, nHJ
991	51	CAACGTCATAGACGATTACATTGCTAGCTCGTGC TGTCTAGAGACATCGAT	HJ0

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

The goal of this dissertation is to genetically and biochemically characterize the role of the *Drosophila* Gen structure-selective endonuclease in genome maintenance via DNA repair and recombination. In the broadest sense, this work is important because the maintenance of the genetic material is crucial for the propagation of a species. Therefore, understand the interplay between the functions of various SSEs and how they are regulated to prevent aberrant DNA cleavage is of great importance. This dissertation more specifically addresses the paradox of why the simple canonical eukaryotic Holliday junction resolvase, Yen1/GEN1, which robustly cleaves HJs *in vitro*, is a backup to Mus81, which cleaves intact HJs very poorly.

Highlighted Findings

We were prompted to conduct this work because previous genetic studies suggested that Gen may be the more predominant enzyme in *Drosophila*. In *S. cerevisiae*, *mus81 sgs1* double mutants are inviable, but *yen1 sgs1* double mutants are viable (Blanco et al., 2010; Fricke and Brill, 2003; Kaliraman et al., 2001; Mullen et al., 2001). These results are consistent with *S. cerevisiae* Mus81 being the primary mitotic resolvase with Yen1 acting as a backup. In *Drosophila*, mutations in *mus81* and *Gen* are both synthetically lethal with mutations in *Blm* (the *Drosophila* ortholog of *SGS1*) (Andersen et al., 2011; Trowbridge et al., 2007); yet *Gen Blm* double mutants die much earlier in development than *mus81 Blm* mutants (Andersen et al., 2011; Trowbridge et al., 2007), suggesting that Gen may be the primary HJ resolvase in *Drosophila*.

By comparing the sensitivities of *Gen* mutants to *mus81* mutants, we found that contrary to other organisms, *Gen* mutants were significantly more sensitive than *mus81* mutants to DNA damaging agents that result in replication fork breakage, stalling, and double strand breaks (Chapter 2). As in other organisms, we observed partial functional redundancy between Gen and MUS81. These findings confirmed that Gen is indeed a primary mitotic resolvase.

We initially hypothesized that this flip in importance was due to an enhanced ability of Gen to cleave HJs relative to its orthologs. We envisioned that some sort of regulatory difference, such as constitutive nuclear localization, constitutive activation, or a change in substrate specificity underlies the differences between *Drosophila* Gen and its orthologs. However, our immunofluorescence microscopy studies revealed that the majority of Gen protein was localized to the cytoplasm in interphase cells (Chapter 2). These data were particularly interesting because they ruled out that a simple change in protein localization produced the differences observed genetic differences and prompted us to explore differences in the biochemical activity of Gen.

Surprisingly we found that Gen exhibited similar substrate specificities as GEN1 and Yen1, cleaving flaps, RFs, and HJs 1 nt 3' to the junction branch point (Chapter 3). Unexpectedly, Gen displayed dramatically different kinetics, with the 5' flap being cleaved substantially faster than the HJ. Additionally, the enhanced cleavage rate is due to the acquired ability of the protein to dimerize in solution and on the 5' flap. Finally, while it has been suggested that a conformational change is the rate-limiting step to cleavage on an HJ, our studies provide direct evidence that a conformational change occurs after the second monomer of Gen binds to the HJ0, a mechanism which is likely shared by all *Drosophila* orthologs. Together, these data allowed us to propose two models regarding Gen's mechanism of action on the HJ and

5’ flap (**Fig. 14 and 17**, respectively), which are discussed in detail in the **Chapter 3 Conclusion**.

In light of our biochemical data, this work reveals two fundamental differences between *Drosophila* Gen and other orthologs. First, although *Drosophila* Gen is a *bona fide* HJ resolvase, the difference in the *in vivo* function of Gen from its orthologs may be due to the enhanced action of the protein on flaps and replication fork intermediates as opposed to HJs. Second, the ability of Gen to dimerize in solution and on the 5’ flap suggests that this function underlies the increased activity on these substrates. The implications of these apparent differences in substrate preference may be that while Gen still plays a role in the resolution of HJs *in vivo*, its main role may reside outside of HJ cleavage. We speculate that other DNA substrates may also represent relevant repair intermediates for human GEN1 and yeast Yen1. Given that human GEN1 similarly cleaves and turns over 5’ flaps with exceedingly fast kinetics, we feel that the importance of our findings extend beyond *Drosophila*.

Future Directions

While this dissertation answered an important question regarding why Gen is a predominant SSE in *Drosophila*, it raised several interesting questions, which can be addressed in future studies. One of the first and most interesting questions raised by both our genetic and biochemical analyses is what is the role of Gen at replication forks? Most mitotic COs arise during replication fork repair. In *E. coli*, a connection was made between the structure specific RuvABC complex, which migrates and cleaves HJs, and DSB induction at stalled and broken replication forks (Seigneur et al., 1998). In mammalian ES cells, Mus81 was shown to generate DSBs at stalled replication forks, promote fork recovery, and prevent chromosomal aberrations

(Hanada et al., 2007). Finally, yeast Sgs1 (BLM) was shown to promote template-switching to bypass lesions.

Evidence for a role for Gen in replication fork repair is based on the synthetic lethality observed in *Gen Blm* double mutants (Andersen et al., 2011). These double mutants exhibit a very severe phenotype, dying as first instar larvae; however, this synthetic lethality is partially rescued in *Gen* Blm^{N2} double mutants, in which replication fork reversal is still intact. Thus, Gen may be required to help process stalled and blocked replication forks in the absence of BLM. This role is also supported by the hypersensitivity of *Gen* single mutants to DNA damaging agents that block and stall replication forks, including MMS, HN2, CPT, and HU (Chapter 2).

Interestingly, partial rescue of lethality is also seen in *spn-A Gen Blm* triple mutants. *spn-A* encodes the *Drosophila* ortholog of RAD51. Recent evidence suggests a role for RAD51 in protecting single stranded DNA regions exposed during fork regression (Branzei and Foiani, 2010; Bugreev et al., 2011), facilitating template switching and synthesis (Sengupta et al., 2003; Yoon et al., 2004). Thus, it seems that GEN may act on two different structures during replication fork repair: (1) GEN may cleave structures not reliant on SPN-A (i.e. stalled forks), and (2) GEN may be required to cleave structures stabilized by SPN-A (i.e. fully regressed forks). In addition to assess the formation of DSBs in cells depleted for Gen and BLM, *in vitro* studies addressing the kinetics with which Gen cleaves RFs in comparison to HJs and 5' flaps will shed some light on the specific structures it cleaves during the reestablishment of replication.

A second question of great interest is how is Gen regulated? There is substantial evidence that other members of the FEN1 family of flap endonucleases are tightly regulated to restrict inappropriate nuclease activity. FEN-1, for example, is regulated by several different

mechanisms, including multiple types of PTMs (Zheng et al., 2011) that modulate its subcellular localization, its ability to interact with PCNA, its stability, and its nuclease activity (reviewed in (Finger et al., 2012). Additionally, it is hypothesized that the disordered C terminus becomes structured in the presence of its protein binding partners (Guo et al., 2008a), exposing multiple protein interaction domains.

These data raise questions about the roles of post-translational modifications, subcellular localization, and disordered regions within Gen. Phosphorylation sites are located along the length of the human GEN1 and yeast Yen1 proteins. In the case of Yen1, phosphorylation of these sites sequesters the protein in an inactive state in the cytoplasm. Dephosphorylation of key phosphoresidues increase Yen1's affinity for DNA and signal its import into the nucleus (Blanco et al., 2014; Eissler et al., 2014). Consistent with Yen1's inactivation and cytoplasmic sequestration upon phosphorylation, FEN1 family members are negatively regulated by phosphorylation. Phosphorylation of human Exo1 decreases its ability to promote DNA resection and triggers its proteosomal degradation (El-Shemerly et al., 2005; Morin et al., 2008). In contrast, phosphorylation of FEN-1 does not change its affinity for DNA, but affects localization, degradation, nuclease activity, and ability to interact with PCNA (reviewed in (Finger et al., 2012; Guo et al., 2008b). We putatively identified several phosphorylation sites using the CDK1 conserved consensus sequence (S/T)-P-X-(K/R) (**Fig. 19**). These sites appear in both the conserved nuclease domain and the unstructured C terminus. It will be very interesting to IP Gen and perform mass spectrometry to identify additional types of PTMs. Mutation of the identified phosphoresidues and subsequent biochemical assays to assess activation, as well as staining to assess localization, can provide valuable information about the regulation of Gen.

Another aspect of Gen regulation may be the role of the disordered C terminus. The C terminus of EXO1 contains an autoinhibitory domain, which physically interacts with the N terminal nuclease domain to inactivate it (Orans et al., 2011). When the mismatch repair protein MutSα detects a mismatch, it subsequently binds the C terminus of EXO1, releasing the nuclease domain, stimulating DNA excision. Though we previously hypothesized that the C terminus may modulate substrate preference by controlling whether or not the protein could dimerize to cleave HJs, we found no difference in the substrate specificities of full-length and truncated Gen. An alternative hypothesis is that the unstructured C terminus may be the site of protein interaction domains. In support of this, it is thought that the FEN-1 C terminus is disordered in the absence of its protein partners (Guo et al., 2008a). Additionally, disorder exists within the helical arch, or clamp, which forms one side of the active site. Notably, only upon substrate binding, does this region become ordered (reviewed in Orans et al., 2011). Similarly, the helical arch of hEXO1 is a mobile domain that is able to adopt different conformations (Orans et al., 2011). Previous research on FEN1 showed that interactions with the C terminus help to recruit the protein to DNA (Guo et al., 2008a). An exciting question to answer is whether Gen interacts with protein partners in this region. Similar to the experiments with PTMs, if Gen has any protein partners, they can be identified after IP and MS.

A final avenue of regulatory research to explore is whether Gen translocates to the nucleus at different stages of the cell cycle and/or in response to certain types of damage. To this end, I have preliminary data showing that after IR, Gen localization did not substantially change to the nucleus (**Fig. 18**) (Appendix). These data are consistent with the continued nuclear localization of human GEN1 after damage (Chan and West, 2014); however, it will be of great interest to identify Gen's subcellular localization in response to damage after the removal of the

Blm helicase, which preferentially processes DNA damage to prevent aberrant mitotic recombination.

Thirdly, how does Gen localize to sites of damage? While conducting a Phyre2 analysis to identify domains within Gen, the analysis identified a putative chromodomain, predicted to be similar in structure to the CBX7 human chromodomain, within the central region of the protein (**Fig. 20 and 21**). Chromodomains bind directly to methylated H3. The human polycomb CBX7 chromodomain interacts with the H3K27me2 modification through 3 aromatic cage residues (Yap et al., 2010). Only one of these residues is conserved, but it is possible that the Gen putative chromodomain interacts with different PTMs. Chromatin modifications play important roles during DNA repair. For example, a key modification at DSBs is the phosphorylation of histone H2A/H2AX in yeast and mammals (reviewed in van Attikum and Gasser, 2005). Thus, it is tempting to speculate that the putative chromodomain directs Gen to sites of damage. It will be very interesting to mutate these residues or delete the chromodomain and examine the sensitivity of flies to various DNA damaging agents or the ability of the ectopically-expressed protein to rescue the DNA damage sensitivities of the *S. pombe mus81* mutant.

Alternatively, Gen may be recruited to sites of damage via interaction with poly(ADP-ribose) (PAR). PAR synthesis by PARP1 is an immediate response to DNA damage, including single-strand breaks and double-strand breaks. PAR synthesis on histones adjacent to the site of damage facilitates recruitment of DNA damage repair proteins (Kim et al., 2005; Luo and Kraus, 2012). A recent report demonstrated that PAR binding to the N terminal nuclear PIN domain of human EXO1 facilitated its localization within 30 sec to sites of damage induced by microirradiation (Zhang et al., 2015). Interestingly, the purified PIN domain, located within the conserved nuclease domain, of human GEN1 pulled-down with PAR (Zhang et al., 2015),

suggesting that PAR may mediate the fast recruitment of GEN to sites of damage. *Drosophila* also has a functional PARP1 ortholog (Miwa et al., 1999), and it will be very interesting to determine whether Gen recruitment to its substrates during DNA repair is also dependent upon PAR.

Finally, the biochemical results presented in Chapter 3 raise interesting questions about the Gen dimer. We hypothesize that the additional constraints conferred by the DNA binding sites of the second Gen monomer facilitate the correct positioning of the flap strand in the active site of the other Gen monomer. Therefore, it would be exciting to perform crystallography studies to determine which regions of the flap are contacted by the second monomer.

Also of interest is the identity of Gen's dimerization interface. Unfortunately, we were not lucky enough to crystallize Gen. As an alternative, we tried predicting *Drosophila*-specific regions that may be involved in the dimerization interface of Gen (**Fig. 23**). Additionally, a human FEN-1 dimer (PDB:1B43) was previously crystallized (Hosfield et al., 1998). Presumably, the dimer interface in the crystal is similar to the one exhibited by Gen. Based on the interface of the crystal and additional modeling studies, we could create deletions to determine whether the two proteins interact using FRET.

In conclusion, through this dissertation, I have provided insight into the flip in usage between *Drosophila* and other organisms of the SSE Gen. I have shown that although Gen is the predominant mitotic HJ resolvase in *Drosophila*, it is more active on 5' flaps due to its ability to dimerize on the flap. These data suggest that DNA substrates such as 5' flaps and RFs may also represent biological substrates for other Gen orthologs. Additionally these data will inform future experiments to further elucidate the relationships and regulation of SSEs in DNA repair and recombination.

APPENDIX

PRELIMINARY STUDIES OF THE REGULATION OF DROSOPHLA GEN

Introduction

The tight regulation of structure-selective endonucleases is crucial for preventing genome instability. In mitotic cells, it is thought that human BTR (BLM-TOPIIIα-RMI1-RMI2) and yeast STR (Sgs1-Top3-Rmi1) preferentially dissolves HJ intermediates to promote NCO formation; whereas Mus81 and Yen1/GEN1 activity is limited to the later stages of mitosis to prevent aberrant CO formation (Matos and West, 2014). This sequential activation is evidenced by cells from Blooms syndrome patients, which exhibit a high frequency of sister chromatid exchanges (SCEs) that arise through the actions of the HJ resolvases (Wechsler et al., 2011).

Additionally, recent studies have shown confirmed the importance of limiting Yen1 and GEN1 activity to anaphase of mitosis. Premature activation and nuclear localization of *S. cerevisiae* Yen1 results in sensitivity to the DNA damaging agent MMS and a significant increase in mitotic CO formation at the expense of NCOs and increasing loss of heterozygosity (LOH) (Blanco et al., 2014). Further, premature nuclear Yen1 can suppress the synthetic lethality of *sgs1 mus81* double mutants and rescue the DNA damage sensitivities of *sgs1* and *mus81* single mutants, suggesting that misregulated Yen1 cleaves DNA intermediates that are normally processed by the STR complex and Mus81 (Blanco et al., 2014). Similarly, premature nuclear import of human GEN1 results in significant increases in SCEs. Additionally, precocious nuclear GEN1 partially rescues the inviability of cells lacking both BLM and MUS81 but increases the number of chromatid breaks in these cells (Chan and West, 2014). To gain further insight into the regulation of *Drosophila* Gen, we asked how damage affects the subcellular localization of

Gen, and whether post translational modifications or protein domains may influence its activity and localization.

Results and Discussion

Preliminary attempts to localize Gen after DNA damage

In Chapter 2, we showed that *Drosophila* Gen is predominantly cytoplasmic in interphase cells; however, many repair proteins localize to the nucleus after damage. To determine whether ionizing radiation, which causes DSBs, affects the subcellular localization of *Drosophila* Gen, we expressed full-length Gen-His from the $CuSO_4$-inducible metallothionein promoter in cultured *Drosophila* S2 cells prior to inducing damage. After recovery, we stained the cells with antibodies to γH2AV, which is phosphorylated at the site of DSBs (Talbert and Henikoff, 2010), and Gen. Our preliminary data shows that Gen remains predominantly cytoplasmic even after the induction of DSBs by IR (**Fig. 18**). We can rule out neither the possibility that a fraction of Gen is nuclear prior to damage nor that a small portion of Gen that escapes our detection localizes to the nucleus after the induction of damage. These data are consistent with data showing that human GEN1 is mainly cytoplasmic in interphase and remains mainly cytoplasmic after DNA damage (Chan and West, 2014; Matos et al., 2011). However, given that NCO formation is favored in somatic cells, it will be interesting to determine the subcellular localization of Gen after damage in cells depleted of *Drosophila* Blm.

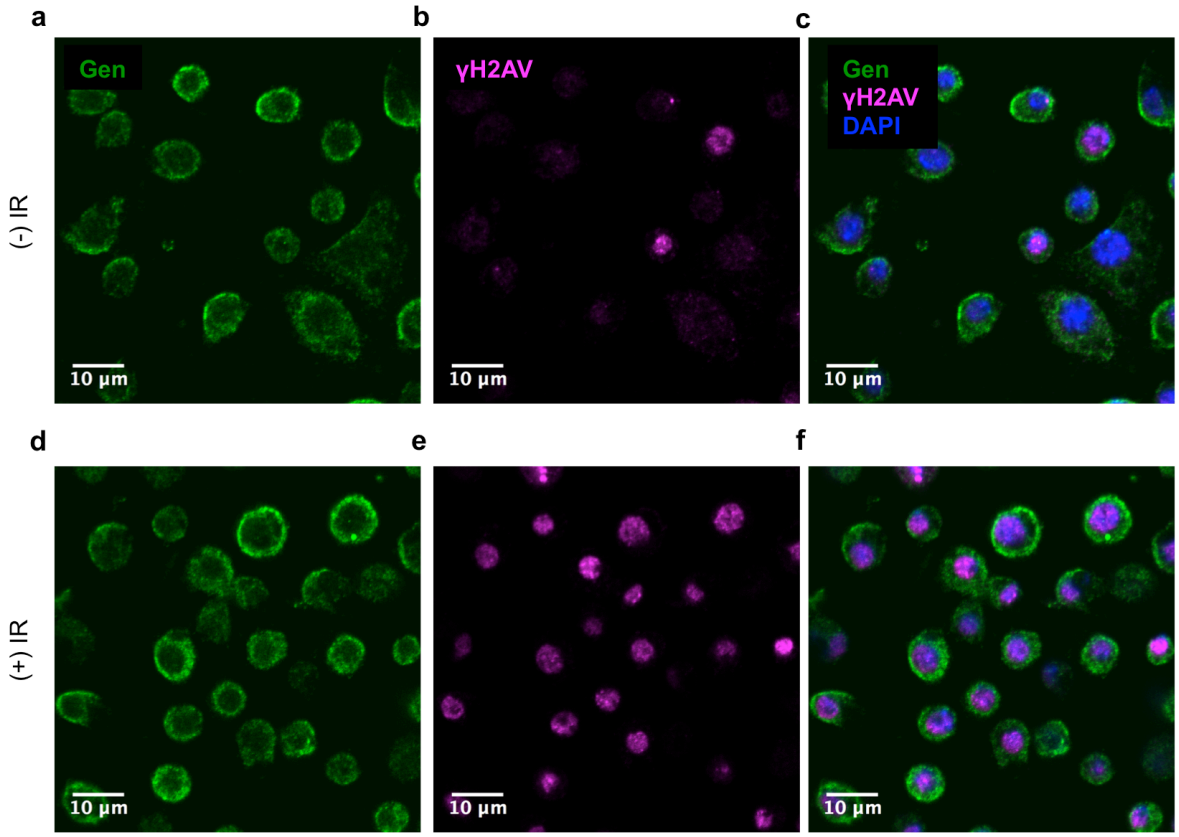

Figure 18. The majority of Gen remains localized to the cytoplasm in cultured *Drosophila* S2 cells after damage with ionizing radiation (IR). Full-length Gen-His was expressed in *Drosophila* S2 cells from the $CuSO_4$-inducible metallothionein promoter. Cells were irradiated with 2500 rads (**d-f**) and compared to unirradiated controls (**a-c**). After a 15 min recovery, cells were fixed and stained with DAPI (blue) and antibodies to Gen (green) and to γH2AV (magenta).

Preliminary attempts to identify post-translational modifications

For *S. cerevisiae* Yen1, cell cycle-dependent phosphorylation and dephosphorylation by Cdk and Cdc14, respectively, exert two independent levels of control upon the protein, modulating its affinity for DNA and determining its subcellular localization (Blanco et al., 2014; Eissler et al., 2014). Human GEN1 is also phosphorylated and dephosphorylated in a cell-cycle dependent manner, though the role of these modifications is still unclear (Chan and West, 2014). To determine whether post-translational modifications affect *Drosophila* Gen's activity and localization, we planned to take two approaches to identify putative phosphorylation sites:

immunoprecipitation (IP) followed by mass spectrometry (MS) and mapping of putative CDK1 consensus sites. After mutation of identified phosphorylation sites, we hoped to perform functional assays and assess the subcellular localization of the protein. We were unable to IP Gen from whole flies, embryos, ovaries, or S2 cells with the anti-Gen-N antibody; however, using the conserved CDK1 consensus sequence [S/T*]-P or [S/T*]-P-x-[K/R] (Holt et al., 2009), where * is the site of phosphorylation and x represents any amino acid, we manually identified 6 putative CDK1 sites in Gen, 5 of which were conserved across all 12 *Drosophila* species (**Fig. 19**). Before functional studies are undertaken, future attempts to confirm these putative CDK1 sites by IP and MS will benefit from tagging Gen on the C terminus to facilitate pulling it down.

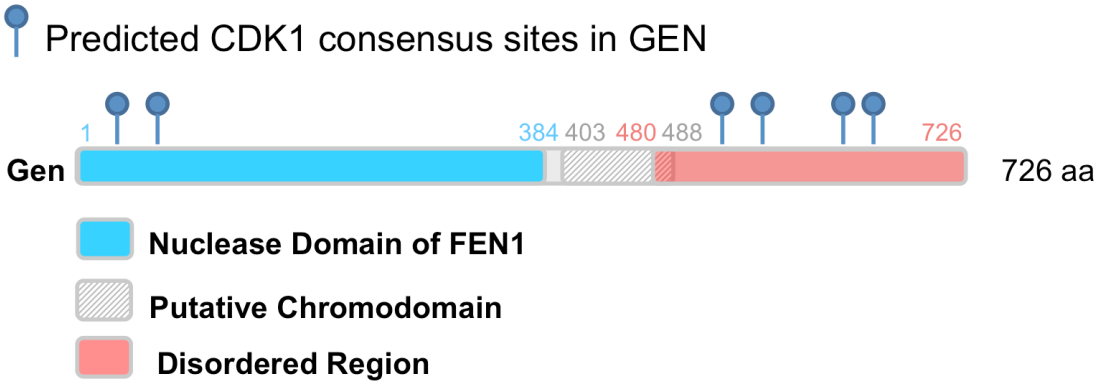

Figure 19. Predicted CDK1 consensus sites. Putative CDK1 sites were mapped manually using the conserved consensus sequence.

Putative regulatory regions

Cdk phosphorylation holds Yen1 inactive in the cytoplasm until anaphase when dephosphorylation of several phosphoresidues, including one that overlaps a nuclear localization signal (NLS) (Kosugi et al., 2009), restores NLS functionality, driving nuclear relocalization (Blanco et al., 2014). Human GEN1, on the other hand, is primarily regulated via a functional nuclear export signal (NES), which drives active nuclear exclusion until nuclear envelope

breakdown during mitotic entry. At telophase, GEN1 is shuttled back into the cytoplasm via CRM1-mediated nuclear export (Chan and West, 2014). We asked whether *Drosophila* Gen contains a functional NLS or NES (**Fig. 20**). We used the NetNES 1.1 Server, which predicts nuclear export signals in eukaryotic proteins using a database of previously-characterized nuclear export signals, we identified a putative NES in the disordered C terminus of *Drosophila* Gen. The putative NES was well-conserved within the 12 *Drosophila* species. Although this sequence was not well conserved in human GEN1, it aligned to a region in the human sequence that was only 35 aa away from the functional NES. Both PSORTII and NLStradamus identified a putative NLS within the same region of Gen; however, the putative NLS was only conserved between 6 of the 12 *Drosophila* species. Further, the Yen1 NLS does not align well to its orthologs; interestingly, it is located within the disordered C terminus of Yen1. Future studies will help to identify the importance of these regions for Gen regulation.

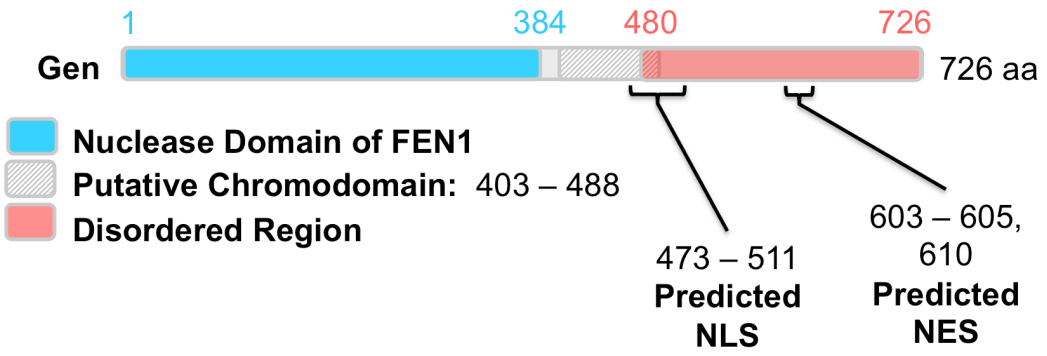

Figure 20. Model of Gen. The nuclease domain and putative chromodomain were predicted by Phyre[2]. The disordered region was predicted by metaPrDOS. The putative NLS predicted by PSORT II and NLStradamus. The putative NES predicted by NetNES.

Finally, the Phyre2 analysis, which compared Gen's predicted secondary structure to a database of previously-crystallized proteins, identified a putative chromodomain within the central region of Gen, predicted to be similar in structure to the CBX7 human chromodomain

(**Fig. 20 and 21**). The putative domain was also identified in the *Arabidopsis* GEN1 and GEN2 paralogs and the human GEN1 ortholog; however, it was absent from both *S. cerevisiae* Yen1 and *C. elegans* GEN1. The CBX7 chromodomain contains three aromatic cage residues (**Fig. 21**, residues highlighted in red) that interact with the H3K27me2 modification (Yap et al., 2010). Only one of these residues is conserved in *Drosophila*, suggesting that the Gen putative chromodomain does not interaction with H3K27me2; however, it is possible that it interacts with a different PTM. Using peptide arrays containing different combinations of histone modifications, we tried to identify interacting residues in collaboration with Dr. Brian Strahl's lab. Unfortunately, the Gen chromodomain did not bind any residues on the array. Future studies can still address the role of this putative domain via mutation of the conserved W420 followed by ectopic expression in *S. pombe* to assess its ability to localize to and rescue the DNA damage sensitivity of the *mus81* mutant.

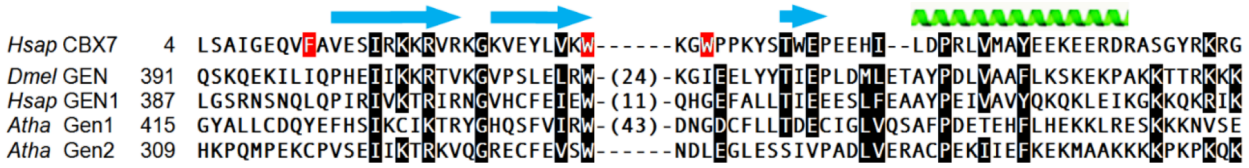

Figure 21. Putative chromodomain identified by Phyre2. Alignment of predicted chromodomains from *Drosophila* Gen, human GEN1, and Arabidopsis GEN1 paralogs aligned to the human CBX7 chromodomain based on predicted structure. The solved structure is at the top: blue arrow = beta sheet; green helix = alpha helix. CBX7 residues in red form the aromatic cage that binds histone H3K27^{Me2}. Numbers in parentheses are the number of residues in the expanded loop.

FEN-1 family of monomeric 5' flap endonucleases

How are Gen's cleavage specificities determined? The FEN-1 family of structure-selective endonucleases encompasses four classes of proteins, which have diverse functions in DNA repair and recombination. Understanding the mechanisms and regulation of the FEN-1

family members can inform our studies of *Drosophila* Gen. The FEN-1 family consists of monomeric 5' flap endonucleases. Crystal structures of FEN-1 and EXO1 bound to DNA reveal that these proteins recognize their substrates by inducing a 100° bend in the DNA, which can only be accommodated if the substrate contains a nick or a gap (Finger et al., 2012; Grasby et al., 2012; Tsutakawa et al., 2011). Additionally, a helical cap sterically prevents double-stranded substrates and DNA that does not have a free 5' end from entering. XPG and GEN1, however, cleave substrates that do not have a free single-stranded 5' end. Namely, XPG makes the incision 3' of the bubble structure formed during NER, and GEN1 cleaves HJs symmetrically. How do these proteins recognize and specifically cleave diverse substrates? Particularly, how do the active sites of XPG and GEN1 accommodate dsDNA?

Examination of the alignments of the FEN-1 family members and of the crystal structures of FEN-1 and EXO1 revealed several key differences in the domain structures of these proteins, summarized in **Figure 22** (Finger et al., 2012; Grasby et al., 2012; Tsutakawa et al., 2011). Interestingly, while some features that participate in the active site cleavage, such the a helical gateway and a hydrophobic wedge, are conserved; the helical cap, which limits the active site to single-strand DNA, is not present in XPG or GEN1 (**Fig. 22 and 23**). Further, XPG was shown to have a flexible linker between the conserved N and I nuclease domains, which is required for efficient processing of bubble structures (Hohl et al., 2007). It will be extremely interesting to crystallize the nuclease domain of Gen to identify which key differences are involved in the evolution of this HJ resolvase.

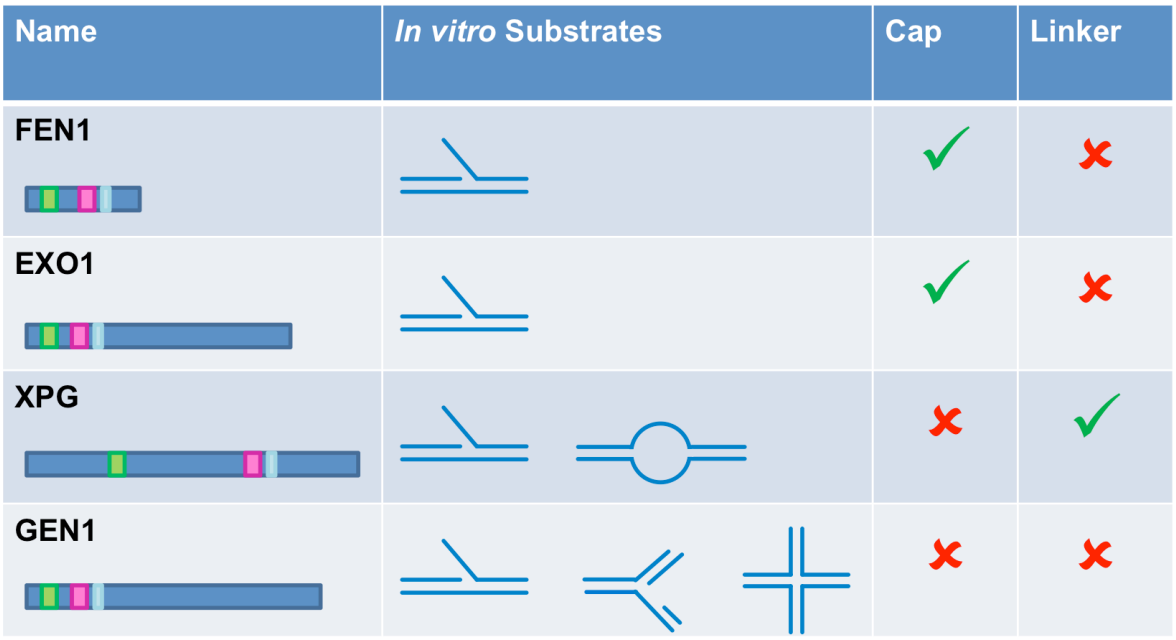

Figure 22. Summary of human FEN-1 family substrates and conserved active site features. N and I nuclease domains (green and pink, respectively); helix-hairpin-helix domain (light blue).

Determination of the dimerization interface

Human GEN1 and yeast Yen1 are monomers in solution, but dimerize in order to cleave HJs. In Chapter 3, we presented SEC-MALS, AFM, and gel shift data showing that *Drosophila* Gen is unique among its orthologs because it exists in a monomer-dimer equilibrium and dimerizes on not only the HJ but also the 5' flap. Interestingly, our kinetics data suggested that the ability to dimerize on the 5' flap enhances its cleavage rate. We were interested in predicting *Drosophila*-specific regions that may be involved in the dimerization interface of Gen. Shown in **Figure 23** is an alignment of *Drosophila* Gen and its orthologs in several different species.

Additionally, a human FEN-1 dimer (PDB:1B43) was previously crystallized (Hosfield et al., 1998). Although the dimer was an artifact of the buffers used for crystallization, the dimer interface in the crystal is likely similar to the one exhibited by Gen. Modeling studies of Yen1 and GEN1 predict that they exhibit a similar structure to human FEN-1 (Svendsen and Harper,

2010). Any future studies of Gen's dimerization interface will benefit greatly from the crystal structure of the FEN-1 dimer.

Conclusion

In conclusion, understanding the regulation of *Drosophila* Gen is of great interest given the evolution of two separate mechanisms to regulate Yen1/GEN1 in lower and higher eukaryotes. To this end, we initiated several genetic and biochemical studies to further understand the regulation of *Drosophila* Gen. We have preliminary data showing that Gen does not significantly change its subcellular localization after the induction of DSBs by IR, a result that is similar to previous studies with GEN1. It will be interesting to determine the localization of the protein after damage that induces problems during replication and in cells depleted of Blm helicase. Although the anti-Gen-N antibody was invaluable for these IF studies and for blotting for over-expressed protein, we have unfortunately determined that it is not suitable for our immunoprecipitation studies. Nevertheless, we were able to use *in silico* programs to identify putative regulatory regions, such as an NLS, NES, putative chromodomain, and CDK1 phosphorylation sites. Finally, our alignments suggest several *Drosophila*-specific regions that may contribute to Gen dimerization. Hopefully, these data will inform future studies.

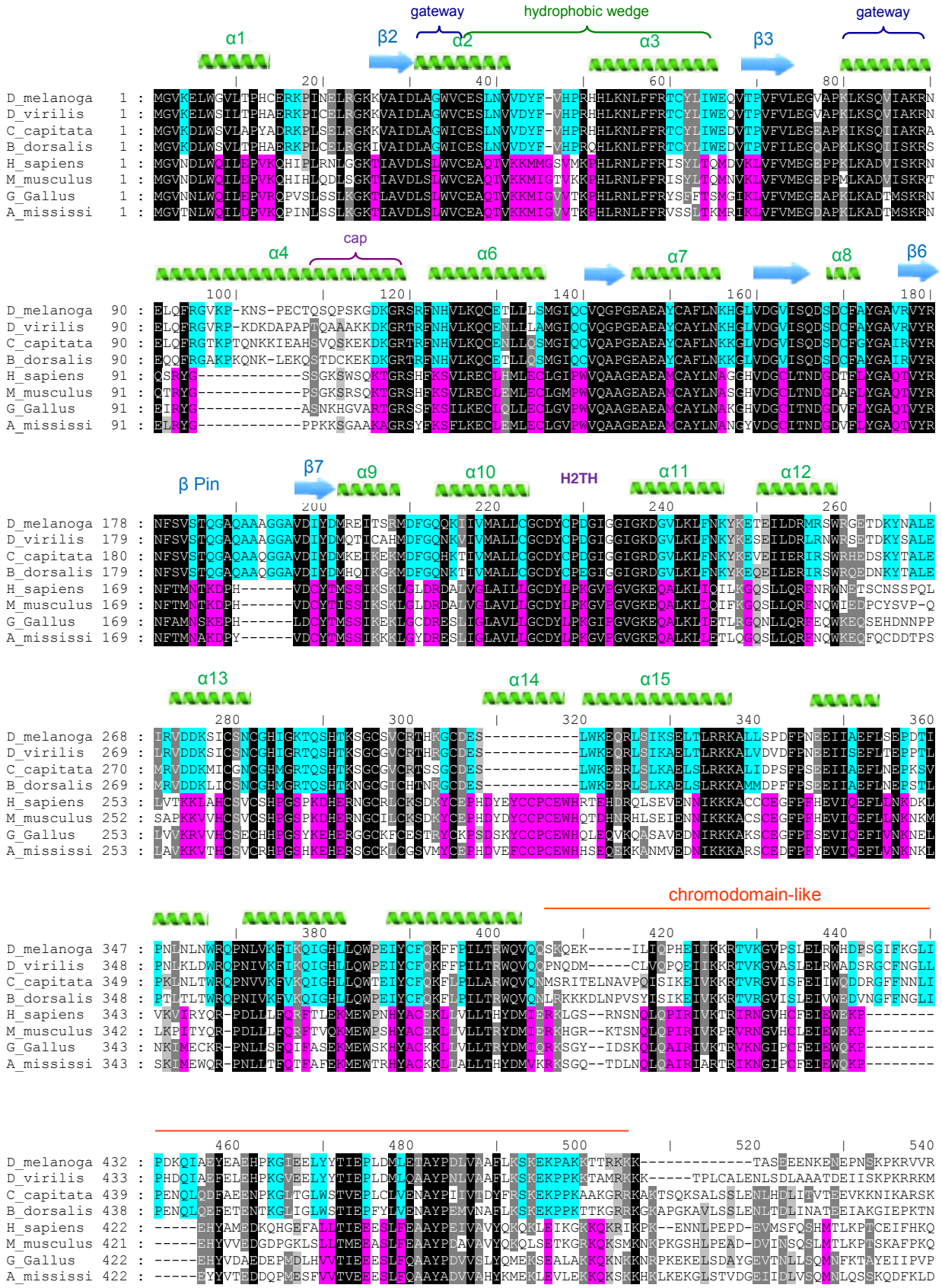
gateway
hydrophobic wedge
gateway
α1
β2
α2
α3
β3
cap
α4
α6
α7
α8
β6
β Pin
β7
α9
α10
H2TH
α11
α12
α13
α14
α15
chromodomain-like
D_melanoga
D_virilis
C_capitata
B_dorsalis
H_sapiens
M_musculus
G_Gallus
A_mississi

Figure 23. Sequence alignment between Gen and orthologs to try to determine the dimerization interface.

Materials and Methods

Protein sequence analysis

The amino acid sequence of Gen was downloaded from FlyBase (flybase.org). We analyzed both full-length Gen (1-726 aa) and truncated Gen (1-518 aa) sequences. We used Phyre2 (www.sbg.bio.ic.ad.uk/phyre2/html/page.cgi?id=index) to generate protein secondary structure prediction, protein domain prediction, and protein disorder prediction. We confirmed disorder prediction using metaPrDOS (prdos.hgc.jp/cgi-bin/meta/top.cgi). To predict the presence of a NLS, we used PSORT II (psort.hgc.jp/form2.html) and NLStradamus (www.moseslab.csb.utoronto.ca/NLStradamus/). We used the NetNES 1.1 Server (www.cbs.dtu.dk/services/NetNES/) to identify a putative NES. For multiple sequence alignments of Gen orthologs, we downloaded the amino acid sequences from NCBI, and used Clustal to perform the analysis.

Immunofluorescence Microscopy

Gen cDNA was cloned into the pMT-V5-HisA vector (Life Technologies), which contains the $CuSO_4$-inducible metallothionein promoter and a C-terminal His tag. The construct was transfected into S2 cells, and Gen-His expression was induced for 3 days. Then cells were plated at $3x10^6$ cells/mL on poly-L-lysine-treated coverslips, allowed to attach for 1 hr, irradiated at 2500 rads from a ^{137}Cs source (Gammacell GG10), and allowed to recover for 15 min. Staining was performed as in (Lake et al., 2013). The primary antibodies were rabbit anti-Gen-N (1:10,000) and mouse anti-γH2AV (1:10,000). The primary antibodies were visualized with goat

anti-rabbit IgG (H+L)-Alexa Fluor 488 (1:10,000) and goat anti-mouse IgG (H+L)-Alexa Fluor 555 (1:10,000, Life Technologies). DNA was detected by staining with DAPI (1:2500, Molecular Probes, Inc.) for 1 min at room temperature. Imaging was done with a laser-scanning confocal microscope (710, Carl Zeiss) and analyzed with ImageJ.

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