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MEN1 and FANCD2 mediate distinct mechanisms of DNA crosslink repair

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Abstract

Cells mutant for multiple endocrine neoplasia type I (MEN1) or any of the Fanconi anemia (FA) genes are hypersensitive to the killing effects of crosslinking agents, but the precise roles of these genes in the response to interstrand crosslinks (ICLs) are unknown. To determine if MEN1 and the FA genes function cooperatively in the same repair process or in distinct repair processes, we exploited *Drosophila* genetics to compare the mutation frequency and spectra of MEN1 and FANCD2 mutants and to perform genetic interaction studies. We created a novel *in vivo* reporter system in *Drosophila* based on the *supF* gene and showed that MEN1 mutant flies were extremely prone to single base deletions within a homopolymeric tract. FANCD2 mutants, on the other hand, had a mutation frequency and spectrum similar to wild type using this assay. In contrast to the *supF* results, both MEN1 and FANCD2 mutants were hypermutable using a different assay based on the *lats* tumor suppressor gene. The *lats* assay showed that FANCD2 mutants had a high frequency of large deletions, which the *supF* assay was not able to detect, while large deletions were rare in MEN1 mutants. Genetic interaction studies showed that neither overexpression nor loss of MEN1 modified the ICL sensitivity of FANCD2 mutants. The strikingly different mutation spectra of MEN1 and FANCD2 mutants together with lack of evidence for genetic interaction between these genes indicate MEN1 plays an essential role in ICL repair distinct from the Fanconi anemia genes.

1. Introduction

DNA interstrand crosslinks (ICLs), which covalently join together both strands of a DNA molecule, represent a formidable block to important cellular processes, such as transcription and replication. As a result, ICLs are extremely cytotoxic and mutagenic. Despite the severity of these lesions, very little is known about the mechanism of crosslink repair in higher eukaryotes. What is known about the repair of ICLs is that it appears to involve the coordination of several repair pathways, including nucleotide excision repair (NER), homologous recombination (HR), and translesion synthesis (TLS). Many of the genes and pathways involved in crosslink repair have been identified as a result of studying rare inherited DNA repair disorders.

Fanconi anemia (FA) is one such disorder that has led to the elucidation of an entire sub-pathway involved in ICL repair. FA is a rare genetic disease characterized by genomic instability and a marked increase in cancer risk [1,2]. Cells from individuals with FA show spontaneous chromosomal aberrations and an extreme sensitivity to crosslinking agents, strongly indicating that these genes play a role in the repair of ICLs [1,2]. In mammals, FA is

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genetically heterogenous with 13 complementation groups (A, B, C, D1, D2, E, F, G, I, J, L, M, N). Eight of the FA genes form a nuclear core complex, which following DNA damage, monoubiquitinates FANCD2 [3,4]. This monoubiquitination step results in FANCD2 translocating to nuclear foci, where it co-localizes with other DNA repair proteins, including BRCA1 and Rad51 [5]. Monoubiquitinated FANCD2 has also been shown to interact with BRCA2 (a.k.a. FANCD1) in order to promote BRCA2 loading into chromatin complexes that appear to be required for recombination repair [6]. The fact that monoubiquitinated FANCD2 associates with many proteins involved in the recombination repair pathway suggests that the FA pathway plays a role in the regulation of repair by homologous recombination. While the exact pathogenesis of mutations in Fanconi mutants is still unclear, it seems likely that the Fanconi genes function during the S-phase to coordinate the response to ICLs and possibly other lesions. Although the mutations in Fanconi genes have a milder effect on recombination than mutations in Rad51 and other genes directly involved in the mechanics of recombination, it seems likely that the Fanconi pathway functions by regulating repair processes including homologous recombination and possibly translesion synthesis in such a way that large-scale rearrangements are minimized [27,31,32].

Another gene implicated in ICL repair is the gene responsible for multiple endocrine neoplasia type I (MEN1). MEN1 much more recently has been shown to be involved in DNA repair, and studies suggest that it may have other physiologic functions, including transcriptional regulation, control of cell proliferation, apoptosis regulation, and bone development [7,8]. Evidence supporting the hypothesis that MEN1 functions in DNA repair include the fact that cells mutant for MEN1 are sensitive to crosslinking agents. MEN1-deficient mouse embryonic fibroblasts (MEFs) as well as peripheral blood lymphocytes from MEN1 patients have decreased survival as well as an elevated frequency of chromosomal abnormalities in response to the crosslinking agent, diepoxybutane (DEB) [9,10]. Furthermore, MEN1 mutant flies are specifically hypersensitive to the crosslinking agents, nitrogen mustard and cisplatin, and display a hypermutability phenotype both at baseline and in response to treatment with crosslinking agents [11]. Taken together, these findings suggest that MEN1 also functions in ICL repair, but similar to the FA genes, the precise role of MEN1 during repair remains unknown. Moreover, the relationship between MEN1's role in DNA repair and its other proposed functions remains to be determined.

That MEN1 and the FA genes share sensitivity to similar DNA damaging agents suggests that these genes function together in the same DNA repair pathway. Furthermore, both MEN1 and FANCD2 function in the IR-inducible S-phase checkpoint [12,13], and one study performed in a mammalian tissue culture system showed that MEN1 and FANCD2 interact in an immunoprecipitation assay [10].

To address whether MEN1 and the FA genes function in the same pathway during ICL repair, we took advantage of the fact that cells mutant for genes functioning in the same pathway have similar mutation spectra. In other words, having a defect in a certain DNA repair pathway results in particular types of mutations that are characteristic for that specific defect. For example, cells mutant for mismatch repair genes have an increase in base substitutions and single base deletions as well as an increase in insertions in homopolymeric tracts as a result of these cells having a defect in repairing replication errors [14]. Thus, by comparing the mutational spectra of MEN1 and FA mutants, we should be able to determine if MEN1 and the FA genes function cooperatively in the same repair process or in distinct repair processes during the repair of ICLs.

In recent years, mutagenesis studies have been conducted using cultured cells or transgenic mice [15,16]. These systems have been invaluable for investigating the mechanisms of mutation induction in many DNA repair deficient models; however, both of these systems have

limitations. Cultured cells do not allow comparative analysis of mutations in various tissues nor do these cells metabolize mutagens in the same way as whole organisms, i.e. many organisms have specialized tissues for detoxifying xenobiotics. Transgenic mice have been useful in studying mutation induction *in vivo*. However, the long generation time and cumbersome genetic techniques make using the mouse model complicated and expensive.

Using *Drosophila melanogaster* as a model system offers an alternative that overcomes many of these challenges in studying mutagenesis. Flies have a short generation time, a plethora of well-defined genetic tools, and a high level of gene and pathway conservation with mammals including those involved in detoxifying xenobiotics [17]. In addition, targeted genetic interaction studies as well as large scale modifier screens can be performed quickly and inexpensively in flies. As a result of these advantages, flies have been used increasingly as a model to study cancer pathogenesis [18]. In particular, studies have shown *Drosophila* to be a valuable tool for understanding the roles of DNA repair genes involved in cancer predisposition. For these reasons, we have adapted for *Drosophila melanogaster* a strategy originally developed in the mammalian system that takes advantage of the supF gene to study mutation induction and repair *in vivo*. Using this novel mutagenesis assay in flies, we analyzed the mutation frequency and spectrum of MEN1 and FANCD2 mutants following treatment with genotoxic agents in order to further characterize the roles of these genes in DNA repair. In addition, genetic interaction between FANCD2 and MEN1 was assessed.

2. Materials and Methods

2.1 Plasmid construction and creation of transgenic animals

The pSP189 vector was used to create a mutagenesis reporter construct in flies [19]. Specifically, the pSP189 vector was digested with SfiI and Eco0109I. Subsequently, the fragment of pSP189, containing the supF gene, ampicillin resistance gene, and the *E. coli* origin of replication, was cloned into the P-element transformation vector pUASp2 at the KpnI and NotI sites using linkers that contain I-SceI sites (KpnISceI: attaccctgttaccctagtag; SfiSceI: tagggataacagggtaataagg; Eco0109ISceI: ggctagggataacagggtaat; NotISceI: ggccattaccctgttacccta). This construct was microinjected into *w1118* embryos by standard procedures (Genetic Services, Inc).

2.2 Fly stocks

The *Drosophila* MEN1 mutant line (*ywMGR; MnnI^{e200}*) as well as flies carrying the FANCD2-RNAi construct were previously described [11,20]. The *lats^{x1}/TM3Sb* stock was obtained from the laboratory of Dr. Tian Xu. Lines *mei-41/FM7a* and *w1118* were obtained from Bloomington Stock Center (<http://flystocks.bio.indiana.edu>).

2.3 *Drosophila* culture conditions and mutagen treatment

All fly stocks and crosses were maintained at room temperature on standard *Drosophila* yeast–cornmeal medium. Mutagens were added directly to the medium as described previously with the following modifications [11]. Briefly, flies were placed in bottles for 48 hours. After two days of egg-laying, the parents were transferred to a new bottle and the progeny were treated with 500µl of each mutagen.

2.4 Isolation of genomic DNA from flies

Approximately 200–300 flies were placed in a falcon tube and frozen on dry ice for 10 minutes. After 10 minutes on dry ice, the falcon tube containing flies was transferred to wet ice and 2.5 mL of a solution containing 100mM Tris (pH 7.5), 600mM NaCl, 100mM EDTA, and 20% sucrose was added to the tube. The flies in solution were ground using a tissue grinder, and

then 2.5 mL of a solution containing 1.67% SDS, 0.4M Tris (pH 9.0), 0.133M EDTA, and 20% sucrose was added. The solutions were mixed and the tube was placed at 65°C for 30 minutes. Following this incubation, 750 µL of 6M potassium acetate was added, after which the tube was placed on ice for 30 minutes. The mixture was spun down for 5 minutes in a centrifuge. The supernatant was transferred to a new tube, and 20 µL of diethylpyrocarbonate (DEPC) was added. The tube was then placed at 65°C for 15 minutes. The mixture was spun again for 5 minutes in a centrifuge and the supernatant was transferred to a new tube. Two volumes of 100% ethanol were added to the supernatant, and then the tube was spun down for 5 minutes to precipitate the DNA. The pellet was washed with 70% ethanol, allowed to dry, and resuspended in TE buffer.

2.5 Measurement of mutation frequency and mutation spectra in flies

Genomic DNA from flies harboring the *supF* gene was isolated and digested with I-SceI. Following digestion, the *supF* construct was gel extracted and re-ligated. The re-ligated construct was electroporated into the *E. coli* strain MBM7070, which carries a nonsense mutation in the β -galactosidase gene, using a BioRad Gene Pulser set at 1.8kV, 25µF, and 200Ω [19]. The electroporated bacteria were allowed to grow for 1hr at 37°C and then were plated on LB plates containing ampicillin, isopropyl β -D-thiogalactoside (IPTG), and 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-gal) as previously described [19,21]. Bacteria with a wild type copy of the *supF* gene suppress the nonsense mutation in the host bacteria β -galactosidase gene, thereby yielding a blue colony, whereas mutations in the *supF* gene yield a white colony. Thus, the mutation frequency was determined by counting the number of white versus blue colonies. Mutants were verified by re-plating and further screened for the presence of the *supF* reporter construct by PCR. All mutant plasmids were purified and sequenced using a primer located upstream of the *supF* gene (F: ggcgacacggaaatgttgaa).

2.6 Analysis of loss of heterozygosity (LOH)

Single nucleotide polymorphisms (SNPs) near the *lats* gene were identified by sequencing regions in and near the *lats* locus. Additional SNPs located on chromosome 3L were identified from the *Drosophila* SNP database (<http://flysnp.imp.ac.at/index.php>; see also [22]). These regions were amplified by PCR in both normal and tumor tissues from the same fly. The normal tissue was first analyzed by restriction enzyme digest to determine if the fly was heterozygous for any of the identified SNPs. Only tumors from flies that were heterozygous at any of those sites were analyzed for the presence of both alleles. Loss of one allele indicated LOH at that locus. Some tumors had digest patterns of both alleles, but one allele was significantly brighter than the other. To rule out contamination from normal tissue, PCR products from these tumors were sequenced. In these instances, loss of one allele was scored when the height of the secondary peak was one third or less of the primary peak.

2.7 Statistical Analysis

The frequencies are expressed as proportion of colonies exhibiting a mutation and the standard errors of these proportions are calculated using a formula based on the binomial distribution. Significance tests for the difference in mutation frequencies at baseline and following treatment were determined by setting confidence intervals for the difference in the proportions. Non-overlapping confidence intervals were considered to be significant. Significance tests for LOH analysis were assessed using the Fisher exact test and significance levels less than 0.05 were considered to be statistically significant.

3. Results

3.1 Creating a mutagenesis assay in flies

As a way to determine the mechanisms of action of DNA repair genes in flies, we created an *in vivo* system to study mutagenesis. We adapted a strategy developed for tissue culture and *in vivo* mouse assays that takes advantage of the bacterial supF gene [15,16]. SupF suppresses the effect of amber mutations by inserting a tyrosine at UAG sites. Thus, when using a bacterial strain that carries a nonsense mutation in the β -galactosidase gene, one can screen for the presence or absence of a functional copy of the supF gene by examining the color of the bacteria grown on X-gal. A functional copy will suppress the nonsense mutation in the β -galactosidase gene, yielding a blue colony, whereas a mutant copy of supF will produce a white colony.

In order to adapt this system for *in vivo* analysis in flies, the supF construct needed two modifications (Figure 1A). To integrate the construct into the fly genome the supF reporter construct was flanked with 5' and 3' P-element arms. In order that the construct could be removed from the genome, the reporter was flanked by I-SceI sites, which are the targets of a yeast endonuclease and do not appear anywhere in the fly genome. Digestion with this enzyme allows one to separate the supF plasmid from genomic DNA. As with the mammalian construct, the reporter construct in flies had an antibiotic resistance gene as well as an *E. coli* replication origin to allow for selection and replication in bacteria.

After creating transgenic flies by standard methods using injection of the reporter construct into embryos, Southern blot analysis was performed using a probe against the supF gene to verify that the reporter construct was indeed integrated into the fly genome (Figure 1B). We next tested whether or not the reporter construct could be isolated by extracting genomic DNA from flies carrying the supF gene and digesting with I-SceI. Following digestion, the construct was purified by gel extraction, re-ligated, and subsequently electroporated into bacteria carrying an amber mutation in the lacZ gene. The presence of blue colonies (Figure 1C) was proof of principle that this *in vivo* strategy was feasible in flies.

3.2 The supF reporter gene can detect mutations in flies

To establish the validity of this construct for detecting mutations in flies, we measured the mutation frequencies and spectra for wild type flies and *mei-41* mutants. *mei-41* (the *Drosophila* homolog of ATR) is a well-characterized *Drosophila* DNA repair gene, and therefore, these mutants served as a positive control for this assay.

At baseline, wild type flies exhibited a mutation frequency of 0.02% (Figure 2A). To determine the nature of these spontaneous mutants, the supF gene from each white colony was sequenced. The two spontaneous mutants identified were deletion mutants - a single base pair deletion found at the beginning of the gene and a two base pair deletion found at the end of the gene (Figure 2C). These mutations most likely occurred as a result of polymerase slippage since both of these mutations were found in a run of identical bases.

To determine the response of this reporter construct to DNA damaging agents, wild type flies were challenged with nitrogen mustard (HN2), cisplatin (CsPt), acetylaminofluorene (AAF), ionizing radiation (IR), and hydrogen peroxide (H2O2). With the exception of IR, treatment with these agents increased the mutation frequency of wild type flies (Figure 2A). The failure to observe an increase in mutation frequency following treatment with IR may reflect the inability of the supF gene to detect certain mutations. For example, the supF construct is not capable of detecting large deletions or rearrangements due to the fact the supF gene is only 161 bp. Many of the mutations caused by IR are double strand breaks (DSBs), which are commonly repaired by the deletion-generating NHEJ pathway, and the supF reporter gene is probably completely deleted or rendered non-viable by NHEJ repair. Nevertheless, this result

is somewhat surprising given that IR also causes single strand breaks (SSBs) and damaged bases which might be repaired in such a way to create small deletions or single base changes.

To determine the nature of the mutations that this reporter gene can detect, the *supF* gene from the white colonies formed following treatment with the various genotoxic agents was sequenced. Because most of the genotoxic agents used in these experiments form adducts on either one or both strands of a DNA molecule, an increase in base substitutions or small deletions was expected, and in fact the majority of the *supF* mutants analyzed were deletion mutants. These deletions ranged from 1–4 bases and were located throughout the *supF* gene (Figure 4C and data not shown). In addition to deletions, a single base change, A to C, near the 3' end of the *supF* gene, was seen in response to AAF. Because guanosines are the major target of AAF, it is somewhat surprising that we observed an A to C transversion following treatment with this mutagen. However, this mutation was located in a stretch of GC base pairs, any or all of which could have been modified with an AAF adduct. Thus, it is possible that a less stringent bypass polymerase incorporated a C opposite the A during the repair of a modified guanosine in that region or that a mispairing event occurred after polymerase slippage to create the mutation we observed. Regardless of how the mutation was created, that both small deletions and base substitutions were observed in wild type flies indicates that the *supF* reporter gene can detect a range of mutations caused by different genotoxic agents.

With confirmation that this assay is capable of detecting mutations in wild type flies, the applicability of this assay in DNA repair-deficient flies was tested by determining the mutation frequency and spectrum of *mei-41* mutants. Previously, *mei-41* mutants were shown to exhibit a hypermutable phenotype [11]. Consistent with this previous report, *mei-41* mutants exhibited a high mutation frequency at baseline, reaching a 17-fold increase above the baseline frequency of wild type flies (Figure 2B). The majority of these mutations were deletions (Figure 2C), consistent with what has been reported for the ATR homolog, *mec-1*, in yeast [23]. Interestingly, a large number of the single base pair deletions identified from *mei-41* mutants localized to a 4bp region consisting of guanosines at the beginning of the gene, suggesting that this region is a mutational hotspot. This hotspot and others have been observed in other systems, and are dependent on the cell line and tissue of origin [24]. Collectively, these results validate the use of this reporter system to study mutagenesis in DNA repair-deficient flies.

3.3 MEN1 and FANCD2 mutants have different mutation spectra

The *supF* assay gave a mutation frequency in MEN1 mutant flies seven fold higher than the mutation frequency in wild type flies prior to mutagen treatment ($p < 0.01$) (Figure 3A). Following treatment with HN2, the increase in mutation frequency for MEN1 mutants was 5-fold greater than the increase observed in wild type flies treated with the same concentration of HN2 (Figure 3A). The mutation frequency of MEN1 mutants also increased in response to H₂O₂, with an increase that was 5-fold greater than that observed for wild type (Figure 3A). Surprisingly, in contrast to wild type flies, MEN1 mutants exhibited an increased mutation frequency in response to IR (data not shown). Thus, these data indicate that the *supF* construct is capable of reporting mutations induced by IR, and show that MEN1 mutants are prone to repairing IR-induced damage by a process that maintains the integrity of the vector. Consistent with MEN1 mutants maintaining the integrity of the construct, sequencing the *supF* gene from these mutant colonies revealed an increase in single base deletions both at baseline and following treatment with genotoxic agents. Interestingly, all of the single base pair deletions were located within the mutational hotspot identified in *mei-41* mutants (Figure 3B). Unlike *mei-41* mutants, however, mutations other than this single base pair deletion were rarely identified in MEN1 mutants. Only three *supF* mutants analyzed from MEN1 mutants were something other than a single base pair deletion (Figure 3B). These three mutants had 3-bp

deletions, but two of these three mutants were also found within the hotspot zone, suggesting that MEN1 mutants have difficulty repairing mutations that occur within this type of substrate.

In contrast to MEN1 mutants, FANCD2-RNAi mutants displayed a mutation frequency similar to wild type flies at baseline and after exposure to HN2 and H202 (Figure 3A). The few white colonies identified from FANCD2-RNAi mutants were small deletions, ranging from 1–4 deleted bases (Figure 3D).

3.4 Mutation frequency in MEN1 and FANCD2 mutants using the *lats* assay

Lats is a *Drosophila* tumor suppressor gene, which leads to visible growths when both copies of the gene are lost somatically [11,25]. Since the penetrance of these growths, or tumors, is virtually 100% following loss of both copies of the *lats* allele [25], the mutation frequency can be determined by using the number of tumors as a read-out for the number of mutations [11]. All types of mutations can be detected at this locus because any inactivating mutation in the second allele of the *lats* gene results in a tumor. Thus, the *lats* locus provides a comprehensive read-out of mutations. However, a disadvantage of this assay is that it does not offer a simple method for determining mutation spectrum. The *lats* gene is very large, and it not practical to sequence the whole gene from the small amount of DNA that can be extracted from a *Drosophila* tumor. Despite the fact that we could not determine the mutation spectrum using the *lats* assay, we compared the mutation frequency determined by the *lats* assay to the mutation frequency measured by the supF reporter.

MEN1 mutants gave results with the *lats* assay very similar to the results of the supF assay (figure 4; see also [11]). These data suggest that the great majority of mutations caused by the loss of MEN1 are the small deletions shown by the supF assay.

On the other hand, FANCD2-RNAi mutant flies had a mutation frequency 4 times greater than wild type after treatment with 0.004% HN2 (Figure 4; see also [20]). This result differed from the mutation frequency determined by the supF reporter assay, but was similar to the mutation frequency of MEN1 mutants treated with the same concentration of nitrogen mustard. The difference between the results of the supF assay and the *lats* assay may reflect the ability of these reporter assays to detect different mutations. As mentioned above, the *lats* locus provides a comprehensive read-out of mutations since any inactivating mutation, including both large-scale rearrangements and small deletions and base substitutions, leads to tumor formation. The supF reporter gene, however, cannot detect large deletions or rearrangements. Thus, the fact that FANCD2-RNAi mutants exhibited a mutation frequency similar to wild type at the supF locus but had an elevated mutation frequency at the *lats* locus suggests that deficiency of FANCD2 causes an increase in large-scale rearrangements.

3.5 FANCD2-RNAi mutants have an increase in loss of heterozygosity at the *lats* locus

Deletions are one of the most common types of rearrangements in solid tumors and are manifested as loss of heterozygosity for polymorphic loci in the region of the tumor suppressor gene. To evaluate the possibility that FANCD2-RNAi mutants have an increase in large deletions, we tested for loss of heterozygosity (LOH) at SNPs in the region of the *lats* gene in tumors that were induced by treatment with nitrogen mustard. The SNPs used in this analysis were identified by sequencing regions in or near the *lats* gene (Figure 5A). A G/A variant in the third exon of the *lats* gene was differentially digested by BssHII, where only the G variant was cut. Two other SNPs, a C/A variant and a G/A variant, were identified approximately 4kb downstream from the end of the *lats* gene. Similar to the SNP identified within the *lats* gene, both of these SNPs were cut differently by certain restriction enzymes. HaeIII only cut the C allele at the C/A locus, whereas, XhoI only cut the G allele at the G/A locus. Using restriction enzymes, *lats*-related tumors were analyzed for the presence of both alleles. Loss of one allele

indicated LOH at that locus (Figure 5B). Tumors from wild type and MEN1 mutant flies treated with nitrogen mustard were used as controls.

Loss of heterozygosity analysis did not reveal any statistical difference between wild type and MEN1 mutant tumors. Of the 31 *lats*-related tumors analyzed from wild type flies, LOH was found in 7 tumors (22.6%). Similarly, 4 out of 21 (19%) MEN1 tumors exhibited allelic loss. In contrast to wild type and MEN1 mutant tumors, 6 out of 6 *lats*-related tumors from FANCD2-RNAi mutants were found to have LOH ($p < 0.001$ compared to WT and MEN1 mutants) (Figure 5C). This high frequency of allelic loss at the *lats* locus in FANCD2-RNAi mutants is consistent with these mutants having large deletions and supports the hypothesis that the difference observed in mutation frequencies in the supF assay versus the *lats* assay is due to the inability of supF gene to detect all types of mutations.

There are alternative explanations for the observed LOH in FANCD2-RNAi mutants. For example, the possibility remains that the LOH occurred as result of whole chromosome loss (nondisjunction). To determine if the entire third chromosome was lost in *lats*-related tumors that arose in FANCD2-RNAi mutants, SNPs on the other arm of the third chromosome were analyzed for LOH. All *lats*-related tumors from FANCD2-RNAi mutants retained heterozygosity at this additional locus (Figure 5D). These results indicate that the observed LOH in the *lats* gene was not a result of whole chromosome loss.

Because gene dosage was not evaluated due to the minute amounts of DNA available in *lats* tumors, our studies do not distinguish between deletion of a portion of chromosome 3 resulting in hemizygosity for *lats* versus LOH through a mechanism involving somatic recombination resulting in homozygosity at the *lats* locus.

3.6 Genetic interaction studies between MEN1 and FANCD2

Flies expressing the FANCD2-RNAi construct under the *ey*-GAL4 promoter are viable but deficient for FANCD2 in eye cells. Treatment of these flies with crosslinking agents causes a rough eye phenotype [20], which is the typical result of massive apoptosis in response to genotoxic agents during eye development [26]. Neither overexpression nor loss of MEN1 results in a rough eye phenotype in response to cross linking agents, indicating that this gene does not lie immediately upstream or downstream of FANCD2 in a linear pathway. Nevertheless, MEN1 could function in a more complex network with FANCD2, augmenting its function or serving a role in some but not all aspects of FANCD2 function. As such, many modifier genes detected in *Drosophila* screens do not produce a phenotype on their own but suppress or enhance the phenotype created by mutations in another gene.

To further analyze the relationship between the role of MEN1 and FANCD2 function in the repair of crosslinks, we performed genetic interaction studies to determine if overexpression of MEN1 or loss of MEN1 could change the eye phenotype observed in FANCD2-RNAi mutants following DNA damage. We generated flies that had both the MEN1 overexpression construct (UAS-MEN1) and the FANCD2-RNAi construct driven by *ey*-GAL4 as well as flies that were homozygous mutants for MEN1 and had FANCD2 knocked down in the eye (using *ey*-GAL4). Following treatment with various concentrations of cisplatin, these flies were scored for an eye phenotype. Neither overexpression nor loss of MEN1 changed the eye phenotype observed in FANCD2-RNAi mutants alone (Figure 6). Based on the fact that mutation of MEN1 alone does not cause an abnormal eye phenotype in response to DNA damage, additive or epistatic relationships cannot be evaluated based solely on these genetic interaction studies. However, these results are consistent with MEN1 and FANCD2 functioning in distinct repair processes.

4. Discussion

The identification of cancer predisposition genes that are required for the repair of DNA interstrand crosslinks has added an exciting new layer of interest in studies addressing the regulation of DNA repair. Genes involved in both Fanconi anemia and MEN1 have been implicated in human cancers and have also been shown to play a critical, yet unknown, role in crosslink repair. The similarities in cellular phenotypes caused by mutations in the FA and MEN1 genes, which include sensitivity to the killing effects of crosslinking agents, hypermutability, chromosome aberrations, and in the case of FANCD2 and MEN1, an S-phase checkpoint arrest defect following ionizing radiation [1,9–13], raise the possibility that MEN1 and the FA genes participate in the same cellular process that leads to ICL repair. In order to help clarify the relationship between the Fanconi pathway and MEN1, we evaluated the mutation spectrum of MEN1 and FANCD2 mutant flies and performed genetic interaction studies. By performing a side-by-side comparison of the behavior of MEN1 and FANCD2 mutant flies in these various DNA repair assays, we demonstrate that MEN1 and the FA genes regulate distinct mechanisms of ICL repair.

The *Drosophila* supF assay developed for the current study uses a reporter gene small in size, thus allowing for rapid sequencing of the reporter to identify the site(s) of damage. This assay is therefore particularly suited for determining the nature as well as frequency of mutations. Indeed, this assay allowed us to confirm the hypermutable behavior of *Drosophila* MEN1 mutants as well as identify the precise type of DNA sequence that is particularly subject to mutation in MEN1-deficient flies. This assay is limited, however, with regard to detecting large-scale rearrangements, as evidenced by the fact that deficiency of Fanconi proteins in *Drosophila* resulted in seemingly different mutation frequencies when the *lats* and the supF assays were used. Analysis of the nature of these mutations revealed that the difference in mutation frequencies observed in the FANCD2-RNAi mutants resulted from differing abilities of the reporter systems to detect large-scale mutations, such as large deletions and rearrangements. These data provide conclusive evidence that the mutation rates of FANCD2 mutant flies are high, but that assays biased toward detection of point mutations miss the types of mutations that are frequent in Fanconi mutants (i.e. large deletions). Our findings help explain conflicting data from previously published reports showing either hypomutability or hypermutability depending on the assay used [27–30].

In contrast to FANCD2 mutants, MEN1 mutants were prone to single base deletions within a homopolymeric tract as assessed by the supF reporter construct. These findings indicate that MEN1 normally functions in a set of repair processes different from the Fanconi genes. The data from the *lats* assay do not exclude the possibility that menin also participates in a pathway that prevents large deletions, possibly the Fanconi pathway. That deficiency of menin leads to a mutation frequency many times higher than wild type but that the relative proportion of mutations manifested as LOH is the same as wild type suggests that the absolute rate of large deletions is elevated in menin mutants. Nevertheless, the mutation spectra indicate that MEN1 and FANCD2 have at least some distinct functions in repair, and the lack of genetic interaction suggests that they are not functioning together at all in relation to ICL sensitivity. This model raises the obvious question about the relevance of the interaction observed between MEN1 and FANCD2 in immunoprecipitation assays [10]. Because these immunoprecipitation assays were performed in MEFs, these findings may reflect differences between the mammalian and *Drosophila* repair systems. However, this interaction could also reflect the fact that both of these genes seem to have two distinct functions in the damage response. In addition to having repair functions, both MEN1 and FANCD2 have been implicated in an IR-induced S-phase checkpoint. Thus, it is possible that these genes function together in the S-phase checkpoint, but not in the repair of interstrand crosslinks.

So, what is MEN1's role in crosslink repair? The preponderance of small deletions located within a run of 4 identical bases could reflect a deficiency in mismatch repair or a relative excess of the error-prone translesion synthesis pathway. That tumors from both MEN1 patients and MEN1 mutant mice do not display microsatellite instability (a phenotype observed in 90% of tumors with mismatch repair defects) strongly argues against MEN1 functioning in mismatch repair [33,34]. If, in fact, MEN1 mutants have a relative excess of translesion synthesis, the normal role of menin could be to regulate error-prone polymerases. This model would be consistent with the growing evidence for an essential role of translesion synthesis in ICL repair [28]. Alternatively, menin may be part of a novel, high-fidelity ICL repair pathway that is replaced by a translesion synthesis mechanism in MEN1 mutants.

In addition to being able to discern MEN1's role in repair, the novel *in vivo* supF assay described in this study could have broad applications in functional studies of DNA repair genes and in mutagenesis assays for xenobiotics. For example, numerous DNA-repair related genes with unknown function have been identified in *Drosophila* screens for mutagen-sensitive (*mus*) strains [35]. Analyzing the mutation spectra of these *mus* strains and comparing them with the spectra of known DNA repair mutants will provide insight into the role that these DNA repair genes play in the maintenance of genomic integrity. Furthermore, this mutagenesis assay can be used to study the effects of potentially mutagenic or carcinogenic compounds. Similar to the Ames test, in which genetic alterations of bacteria serve as the assay system [36], the *Drosophila* supF reporter can provide a direct measure of the genetic toxicity of various chemicals. The fly assay has an advantage over the Ames test in that it allows for the study of compounds in a multicellular organism that bears a specialized system for metabolizing xenobiotics. This mutagenesis assay also has the potential to identify the genes that are involved in the metabolism of such chemicals. For example, a genetic screen can be performed to identify novel mutations that render flies especially sensitive or resistant to the mutagenic effects of genotoxic compounds. Identification of such genes may provide greater insight into the molecular chain of events involved in mutagenesis as well as provide possible therapeutic targets for reducing the carcinogenic potential of various molecules. Taken together with the power of *Drosophila* genetics, this mutagenesis assay has the capability of identifying and dissecting complex biological pathways involved in both DNA repair and mutagen metabolism.

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Non-standard abbreviations

FA, Fanconi anemia; MEN1, multiple endocrine neoplasia type I; ICLs, interstrand crosslinks.

Figure 1.

The *supF* construct can be integrated into the fly genome and efficiently separated from the genomic DNA. (A) Schematic representation of the *Drosophila* *supF* reporter construct. The *supF* gene was flanked by 5' and 3' P-element arms to allow the construct to insert into the fly genome and by two I-SceI sites to allow the construct to be separated from the genomic DNA. For selection and replication in bacteria, the construct contained an ampicillin resistance gene and an *E. coli* origin of replication. The *white* gene was used as a visible marker to identify flies that carry an integrated construct. (B) Southern blot analysis of genomic DNA from wild type (WT) and *supF*-carrying adult flies. DNA was digested with I-SceI and hybridized with a probe against the *supF* gene to verify that the reporter construct was integrated into the fly genome. (C) Representative plate illustrating the presence of blue colonies following electroporation of the *supF* construct into MBM7070 bacteria.

Figure 2.

Mutation spectrum of the *supF* gene in wild type and *mei-41* mutant flies. (A) Graphical representation of the mutation frequencies of wild type flies at baseline and following treatment with various mutagens (HN2 - nitrogen mustard; CsPt - cisplatinum; AAF - acetylaminofluorene; IR - ionizing radiation; H2O2 - hydrogen peroxide). (B) Graphical representation of the mutation frequency of *mei-41* mutants at baseline compared to wild type. (C) Distribution of mutations in the *supF* gene identified from wild type and *mei-41* mutant flies at baseline. Deletions are indicated by ■. A bracket () denotes the deletion of multiple bases.

Figure 3.

Mutation spectrum of the *supF* gene in MEN1 and FANCD2-RNAi mutants. (A) Graphical representation of the mutation frequencies of MEN1 and FANCD2-RNAi mutants compared to wild type at baseline and following treatment with nitrogen mustard or hydrogen peroxide. MEN1 mutants exhibited a hypermutable phenotype at baseline and in response to treatment. No difference between the mutation frequencies of FANCD2-RNAi mutants and wild type flies was observed. (B, C, D) Distribution of mutations in the *supF* gene identified from WT, MEN1 mutants, and FANCD2-RNAi mutants at baseline and following treatment with nitrogen mustard. Deletions are indicated by ■. A bracket () denotes the deletion of multiple bases.

Figure 4.

MEN1 and FANCD2-RNAi mutants are hypermutable at the *lats* locus. The mutation frequency in MEN1 and FANCD2-RNAi mutants was measured at baseline and following various doses of nitrogen mustard in both the *lats* assay and the *supF* assay. (A) A dramatic increase in the number of eye tumors was observed in flies with FANCD2 knocked down in the eye (FAD2-RNAi/*ey*-GAL4) following nitrogen mustard treatment as compared to control flies not expressing the RNAi construct (FAD2-RNAi/*CyO*) in the *lats* assay. (B) Similarly, homozygous MEN1 mutant flies were observed to have a dramatic increase in the number of total tumors in the *lats* assay compared to the number of total tumors observed in the starting line, p553, which was used as a control. (C) FANCD2-RNAi mutants (FAD2-RNAi/*actin*-GAL4) did not have a mutation frequency that different from wild type flies in the *supF* assay. (D) In contrast to FANCD2 mutants, MEN1 mutant flies had a marked increase in mutation frequency in the *supF* assay compared to wild type flies both at baseline and following nitrogen mustard treatment.

Figure 5.

FANCD2-RNAi mutants have a high frequency of LOH at the *lats* locus. (A) Schematic representation of the region around the *lats* gene showing the relative positions of SNPs and genes on chromosome 3. (B) Representative sequence from one tumor that displayed LOH (top) and one tumor that retained heterozygosity (bottom) for a SNP located 4kb downstream from the end of the *lats* gene. Arrow indicates position of the SNP. (C) Agarose gel showing PCR products from *lats*-related tumors taken from the thorax of FANCD2-RNAi mutants (FAD2-RNAi/*pnr*-GAL4) digested with HaeIII. This enzyme cuts the C allele of the C/A variant located 4kb downstream from the end of the *lats* gene. All digested tumor samples from the FANCD2-RNAi mutants show loss of the bottom two bands compared to the heterozygous control, indicating that the C allele was lost in these tumor samples. The faint bands observed in these lanes were due to contamination from normal tissue, which in these knockdown flies were heterozygous for this SNP (data not shown). (D) Agarose gel showing PCR products from *lats*-related tumors taken from the thorax of FANCD2-RNAi mutants (FAD2-RNAi/*pnr*-GAL4) digested with NsiI. This enzyme cuts the T allele of the T/A variant located on chromosome 3L. All digested tumor samples from FANCD2-RNAi mutants retain heterozygosity at this distance locus.

Figure 6.

Genetic interaction studies between MEN1 and FANCD2. FANCD2-RNAi mutants exhibit a rough eye phenotype in response to crosslinking agents. Flies that had both the MEN1 overexpression construct (UAS-MEN1) and the FANCD2-RNAi construct driven by *ey*-GAL4 as well as flies that were homozygous mutants for MEN1 and had FANCD2 knocked down in the eye were treated with various concentrations of cisplatin. Neither overexpression nor loss of MEN1 changed the proportion of FANCD2 mutants with a rough eye phenotype.