Hypermutability in a *Drosophila* model for multiple endocrine neoplasia type 1

Valeria Busygina\(^1\), Kanya Suphapeetiporn\(^1\), Lorri R. Marek\(^1\), R. Steven Stowers\(^2,\)\(^\dagger\), Tian Xu\(^1\) and Allen E. Bale\(^1,\)\(^*\)

\(^1\)Department of Genetics, Yale University School of Medicine, New Haven, CT 06520-8005, USA and
\(^2\)Department of Molecular and Cellular Physiology, Stanford School of Medicine, Beckman Center, Stanford, CA 94305, USA

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Multiple endocrine neoplasia type I (MEN1) is an autosomal dominant cancer predisposition syndrome, the gene for which encodes a nuclear protein, menin. The biochemical function of this protein has not been completely elucidated, but several studies have shown a role in transcriptional modulation through recruitment of histone deacetylase. The mechanism by which *MEN1* mutations cause tumorigenesis is unknown. The *Drosophila* homolog of *MEN1*, *Mnn1*, encodes a protein 50% identical to human menin. In order to further elucidate the function of *MEN1*, we generated a null allele of this gene in *Drosophila* and showed that homozygous inactivation results in morphologically normal flies that are hypersensitive to ionizing radiation and two DNA cross-linking agents, nitrogen mustard and cisplatinum. The spectrum of agents to which mutant flies are sensitive and analysis of the molecular mechanisms of this sensitivity suggest a defect in nucleotide excision repair. *Drosophila Mnn1* mutants have an elevated rate of both sporadic and DNA damage-induced mutations. In a genetic background heterozygous for *lats*, a *Drosophila* and vertebrate tumor suppressor gene, homozygous inactivation of *Mnn1* enhanced somatic mutation of the second allele of *lats* and formation of multiple primary tumors. Our data indicate that *Mnn1* is a novel member of the class of autosomal dominant cancer genes that function in maintenance of genomic integrity, similar to the *BRCA* and *HNPCC* genes.

INTRODUCTION

Multiple endocrine neoplasia type I (MEN1) is an autosomal dominant cancer predisposition syndrome characterized by parathyroid, pancreatic islet and pituitary tumors as well as other neoplasms such as carcinoids, lipomas, angiofibromas, angiomyolipomas and thyroid tumors (1). The *MEN1* gene was mapped to chromosome 11q13 in 1988 (2) and positionally cloned in 1997 (3,4). The *MEN1* product, menin, is a 610 amino acid nuclear protein with no homology to any known protein (5).

Tumors in MEN1 arise through a two-hit mechanism, with germline mutation in one copy of the gene and somatic loss of the normal allele (2). This genetic mechanism of pathogenesis is typical of classic tumor suppressors such as *RB1*. However, hereditary cancer genes involved in the maintenance of genomic integrity also show allelic loss in tumors. This group of genes includes those that cause hereditary non-polyposis colorectal cancer (6) and the hereditary breast cancer genes, *BRCA1* and *BRCA2* (7–10).

Several lines of evidence suggest that menin might participate in DNA damage sensing or repair. The peripheral blood lymphocytes from MEN1 patients have an elevated frequency of chromosomal abnormalities after exposure to the cross-linking agent, diepoxybutane (DEB) (11). In addition, menin was shown to interact with replication protein A (RPA) (12), a protein important for DNA damage recognition in nucleotide excision repair (NER) (13), as well as with *FANCD2* (14), a gene that underlies the genetic instability syndrome, Fanconi anemia.

At the biochemical level, menin appears to act as a transcriptional co-modulator, regulating the activity of JunD (15,16), Smad3 (17), NF-kB (18) and Pem (19). This function has been shown both in biochemical studies in tissue culture cells and...

Other possible roles for menin are suggested by biochemical interactions with the putative metastasis suppressor, Nm-23 (20,21), intermediate filament proteins GFAP and vimentin (22) and non-muscle myosin II-A heavy chain (23). Menin was also found to associate with telomeres in certain cell types (24) and to inhibit the expression of hTERT, the protein component of telomerase (25).

A Drosophila MEN1 homolog (Mnn1) has been identified that encodes a 751 amino acid protein sharing 46% identity and 56% similarity with human menin (26,27). Despite obvious morphologic differences between vertebrates and mammals, this model organism can be extremely valuable in elucidating human gene function because most key signal transduction pathways are conserved from fruit flies to mammals. Experimental genetic methods are well developed in fruit flies and lend themselves to the study of cancer pathogenesis. We generated the first null mutant allele of Mnn1 in fruit flies and characterized the phenotype of loss of menin function in Drosophila. Our findings support the hypothesis that MEN1 participates in maintenance of genomic integrity and its loss causes cancer by increasing the mutation rate.

RESULTS

Generation of Drosophila Mnn1 mutant

Mnn1, the Drosophila homolog of human MEN1, contains 5 exons with the coding sequence contained in exons 2 through 5 (Fig. 1). A deletion mutant of Mnn1 was created using a Drosophila technology known as ‘male recombination’. Meiotic recombination does not normally occur in the Drosophila male germline. However, if a transposable element (P-element) is mobilized by the expression of transposase in the male germline, it can cause unequal crossing over, resulting in deletions (28). These deletions may extend in either direction from the P-element insertion site, and multiple chromosomal markers are used to monitor the nature of the crossover event and the direction of the deletion.

Two rounds of male recombination were performed beginning with a P-element located in the 3’ end of the milton gene, which lies immediately 5’ to Mnn1 (Fig. 1A). After the first round of male recombination, 5000 progeny were screened and 21 recombinants were selected. Southern blot analysis and PCR showed that one line, P553#15, had a 7 kB deletion extending into the beginning of Mnn1 exon 1 (Fig. 1A). This line, however, still produced Mnn1 mRNA (data not shown), presumably from a cryptic promoter in either the P-element or the surrounding sequence.

After a second round of male recombination, offsprings were screened with a PCR-based method using P-element genomic primers (Fig. 1A). A total of 386 balanced lines were generated at this step, and a single line (Mnn1e200) was isolated based on the length of PCR product it gave (Fig. 1B). Sequencing showed that this line had a deletion extending to the middle of exon 4, which removed ~50% of the Mnn1 coding region. Mnn1 mRNA was undetectable by northern blot analysis (Fig. 1C) in homozygous mutants, indicating that Mnn1e200 is a null allele of Mnn1.

Because Mnn1e200 deletes essential regions of both milton and Mnn1, we maintained in all flies a Milton genomic rescue construct, MGR. This construct rescues the loss of milton function (29). The starting line, P553, bearing MGR to eliminate any effects due to milton inactivation, was used as a control strain.

Loss of Mnn1 leads to reduced viability

Homzygous loss of Mnn1 caused a slight (5–7%), but statistically significant, decrease in viability compared with the control (more than 6000 progeny were screened). Surviving Mnn1e200 homozygous adults were viable and fertile with no apparent morphological abnormalities (data not shown). Despite the fact that Mnn1 is maternally deposited (27), homozygous Mnn1e200 mothers produced normal fertile progeny.
Mnn1 mutants are hypersensitive to ionizing radiation

To investigate the response of Drosophila Mnn1 mutants to DNA damage, we compared their sensitivity to ionizing radiation to that of the wild-type (w1118) strain and the MGR; P553 control strain. We exposed second (72 h after egg deposition) and third (96 h after egg deposition) instar larvae to increasing doses of gamma-irradiation in a 137Cs irradiator and then evaluated their survival to adulthood. Both mutant and wild-type flies were more sensitive to ionizing radiation when exposed as second instar larvae. Mnn1 mutants exhibited a statistically significant decrease in viability at both stages (Fig. 2A and B).

To investigate whether the radiation sensitivity of Mnn1 mutants could be rescued by reintroduction of a wild-type copy of the Mnn1 gene, we generated flies carrying UAS–Mnn1, hs–Gal4 constructs. In this strain, the expression of Mnn1 is activated upon heatshock treatment (37°C, 30 min). Western blot analysis of larval tissue showed that this strain is activated upon heatshock treatment (37°C, 30 min).

Mnn1 mutants displayed hypersensitivity to chemical DNA cross-linking agents

Ionizing radiation causes not only double-strand breaks in DNA but also several other types of DNA damage (30). In order to further refine the spectrum of mutagen sensitivity of Mnn1 mutants, the embryos and first instar larvae from the cross y w MGR;Mnn1e200/CyO × y w MGR;Mnn1e200/CyO were treated with six chemical mutagens with different modes of action (Table 1). Animals were then grown at either room temperature (23–24°C) or 29°C. Use of two different temperatures is standard procedure in investigating mutagen sensitivity in Drosophila; some mutants show increased sensitivity to chemical agents when grown at 29°C (31). The decline in the percentage of homozygous progeny with the increase of mutagen concentration was used as a measure of the sensitivity of Mnn1 mutants to each agent.

Mnn1 mutants showed specific sensitivity to the cross-linking agents nitrogen mustard and cisplatinum (P ≤ 0.005) (Fig. 3). In contrast, the effect of two other cross-linking agents, DEB and mitomycin C, was mild and seen only at the highest mutagen concentration used and only at 29°C (P ≤ 0.05). Other tested agents, MMS, AAF and HU, had no obvious effect on the viability of Mnn1 mutants at the usual doses used in chemical mutagenesis experiments.

Mnn1 mutants have an elevated mutation rate

The fact that Mnn1 mutants are hypersensitive to some types of DNA damage suggests a defect in DNA repair. To investigate this possibility, we evaluated the mutation rate in Mnn1 mutants. Loss of the wild-type copy of the lats gene in lats+/− flies was used to score for the frequency of mutations; lats is a Drosophila and mammalian tumor suppressor. The somatic loss of both lats alleles leads to tumor formation in adult flies (Fig. 4), a phenotype that is easy to score. The development of tumors is an extremely high penetrance phenotype of cells losing both copies of lats, approaching 100% (32).

We evaluated the frequency of mutations of the wild-type lats allele in flies mutant for Mnn1 and in the control line.
Since loss of the wild-type copy of \textit{lats} is an independent event for every cell in the organism, more than one tumor focus can occur in the same animal (\textit{lats}-related tumors in flies do not metastasize and each tumor represents a separate event) (Fig. 4D). The mutation frequency was measured as the number of foci per 100 flies. This assay has been used previously to measure mutagenicity of chemical agents in \textit{Drosophila} (33).

At baseline, prior to any mutagen treatment, \textit{Mnn1} mutants had 2.7 foci/100 flies, a nearly 2-fold increase in the number of mutations compared to the control line (Fig. 5A). After treatment with ionizing radiation, the number of tumor foci per fly increased dramatically in both \textit{Mnn1} mutants and controls but was consistently two to three times higher in \textit{Mnn1} mutants reaching 1.6 tumors per fly at 2 kRad (Fig. 5B). The divergence between mutants and controls was even greater after nitrogen mustard treatment reaching a 7-fold increase in mutation frequency in \textit{Mnn1} mutants (Fig. 5C) at a dose of 0.004%. These data suggest that repair of the type of DNA damage inflicted by nitrogen mustard specifically requires the normal function of menin.

In order to ensure that the observed increased frequency of somatic mutations at the \textit{lats} locus was in fact due to the lack of menin, we rescued the hypermutability phenotype with \textit{UAS–Mnn1}, \textit{hs–Gal4} transgenic constructs as described earlier. Though there was no statistically significant difference between \textit{Mnn1} mutants with or without the rescue construct at baseline (data not shown), nearly complete rescue was observed in flies treated with 200 Rad of radiation (Fig. 5D).

As a positive control, we monitored the loss of \textit{lats} and subsequent tumor formation in \textit{Drosophila mei-41} mutants. \textit{Mei-41}, the \textit{Drosophila ATR} homolog, is a well-characterized DNA repair gene the loss of which has been shown to lead to hypermutability in \textit{Drosophila} (34). As expected, \textit{mei41; lats +/−} flies showed a higher tumor incidence with a 2-fold increase at baseline and >10-fold increase after treatment with 200 Rad of ionizing radiation compared to control (data not shown).

Dysregulation of proper checkpoint response to DNA damage as well as defects in DNA repair have been shown to result in mutagen sensitivity (31,35). We investigated a possible role for \textit{Mnn1} in the control of cell cycle arrest, apoptosis and DNA repair.

Cell cycle control in response to irradiation in \textit{Drosophila} larval tissue showed no differences between \textit{Mnn1} mutants and control lines. \textit{Mnn1} mutant cells were able to arrest normally at the G2/M transition after irradiation, as determined by staining for the mitotic marker phospho-histone H3 (data not shown). Likewise, the G1/S transition was not halted as evaluated by levels of DNA synthesis measured by BrdU incorporation. Levels of apoptosis in \textit{Mnn1} mutants were indistinguishable from those in wild-type \textit{Drosophila} tissue as assessed by acridine orange staining (data not shown).

To investigate the repair of double strand breaks, we utilized the \textit{sn} allele. This allele was created by tandem insertion of two P-elements into the \textit{singed} (\textit{sn}) locus. Upon the mobilization of these P-elements by transposase, a double strand break is created, resulting in lethality of mutants defective for double strand break repair (31). The viability of \textit{Mnn1} mutants was not affected indicating that loss of \textit{Mnn1} function did not influence double strand break repair in this assay (data not shown).

In a standard cross measuring female germline recombination between the markers \textit{yellow} and \textit{singed}, \textit{Mnn1} mutants appeared to have normal levels of homologous recombination during meiosis (data not shown). There was no increase in meiotic chromosome non-disjunction (data not shown).

**DISCUSSION**

Kinzler and Vogelstein introduced the term ‘gatekeeper’ to describe a class of genes that directly control cellular proliferation and whose dysregulation is necessary to cause neoplastic growth in specific tissues (36). Gatekeepers include many classical tumor suppressors, such as \textit{RB1} and \textit{APC}. Inactivation of both homologs of these genes, often through mutation of one copy and deletion of the second copy, is found both in hereditary forms of cancer and in the great

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Here is the table for sensitivity of \textit{Mnn1} mutant flies to chemical mutagens:

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mode of action (31)</th>
<th>Concentrations</th>
<th>\textit{Mnn1} mutant response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl methanosulphonate (MMS)</td>
<td>Alkylating agent</td>
<td>0.08% and 0.12%</td>
<td>Not sensitive</td>
</tr>
<tr>
<td>Hydroxyurea (HU)</td>
<td>Single strand breaks</td>
<td>40 mM and 80 mM</td>
<td>Not sensitive</td>
</tr>
<tr>
<td>2-Acetylaminofluorene (AAF)</td>
<td>UV mimic</td>
<td>0.05% and 0.1%</td>
<td>Not sensitive</td>
</tr>
<tr>
<td>Nitrogen mustard (HN$_2$)</td>
<td>Cross-linking agent</td>
<td>0.004%</td>
<td>Sensitive at 29°C</td>
</tr>
<tr>
<td>Diepoxybutane (DEB)</td>
<td>Cross-linking agent</td>
<td>0.008%</td>
<td>Sensitive at RT and 29°C</td>
</tr>
<tr>
<td>Mitomycin C (MMC)</td>
<td>Cross-linking agent</td>
<td>0.5 mM, 1 mM, 1.5 mM</td>
<td>Not sensitive</td>
</tr>
<tr>
<td>Cisplatinum (CisPt)</td>
<td>Cross-linking agent</td>
<td>0.5 mM, 1 mM, 2 mM</td>
<td>Sensitive at 29°C but not at RT</td>
</tr>
<tr>
<td>Hydroxyurea (HU)</td>
<td>Single strand breaks</td>
<td>40 mM and 80 mM</td>
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The mutants were considered sensitive to the agent if the proportion of homozygotes versus heterozygotes after treatment with mutagen was significantly skewed ($P \leq 0.05$, goodness of fit test) toward heterozygotes compared to treatment with solvent alone. RT, room temperature.
mone gene promoter region and Drosophila by examining the effects of the loss of its MEN1 in vivo cancer through a role in the maintenance of genomic integrity. The gene functions as a caretaker and leads to genomic instability and promotes mutation 'caretakers' does not directly affect growth control. The loss of caretakers causes genomic instability and promotes mutation of gatekeepers and other genes directly involved in growth control. Mutations in caretakers are not often seen in sporadic cancers. For example, patients with hereditary breast cancer due to BRCA1 or BRCA2 have germline mutations in one copy of the gene and frequent allelic loss of the other copy in tumor tissue, but mutations in the BRCA genes are almost never seen in sporadic breast cancer (7–10). Likewise, a high proportion of colorectal tumors arising in hereditary non-polyposis colorectal cancer (HNPCC) patients have loss of both copies of HNPCC genes, but only a minority of sporadic colorectal cancers arise through this mechanism (6). These genes have often been referred to as tumor suppressors but a more appropriate term for them might be 'mutation suppressors'.

Tumors in MEN1 patients arise through a two-hit mechanism (2,37,38), but the majority of sporadic tumors of the types seen in this disease arise without MEN1 mutations (39–42). This discrepancy in the rate of mutations in hereditary versus sporadic tumors could indicate that there are alternative pathways of pathogenesis that lead to the tumors seen in MEN1. Parathyroid adenomas, for example, can arise with loss of MEN1 or with translocations between the Parathormone gene promoter region and Cyclin D (43). Alternatively, the low frequency of MEN1 mutations in sporadic tumors may indicate that the gene functions as a caretaker and leads to cancer through a role in the maintenance of genomic integrity.

In the present work, we sought to investigate the function of MEN1 in vivo by examining the effects of the loss of its Drosophila homolog, Mnn1. Mnn1 is not an essential gene in Drosophila, and its inactivation does not lead to lethality, sterility or morphologic abnormalities. These findings provide some evidence against a direct role for Mnn1 in regulation of cell growth and differentiation in Drosophila.

Mnn1 mutant flies showed sensitivity to certain types of DNA damage, and the data are consistent with an elevated mutation rate. Flies without menin function and heterozygous for the lats tumor suppressor gene (lats<sup>+/−</sup>) were exposed to 1 kRad of ionizing radiation, leading to tumors in adults. Tumors of: (A) thorax, (B) eye, (C) leg, are shown (arrows). (D) Multiple tumor foci in one fly.
frequency after exposure to nitrogen mustard suggests that menin participates in repair of the type of damage caused by this agent more specifically. Nitrogen mustard leads to the formation of interstrand cross-links through the alkylation of the N7 position in guanine residues (30). Interestingly, cisplatinum has a mechanism of action similar to that of nitrogen mustard (44), and \(Mnn1\) mutants show considerable sensitivity to cisplatinum as well. On the other hand, both DEB and Mitomycin C, to which \(Mnn1\) mutants are only marginally sensitive, more often affect the N2 position in guanine residues as the site of nucleophilic attack (45,46). It is therefore possible that only certain types of DNA interstrand cross-links require menin for successful resolution. The repair of interstrand cross-links is not fully understood, but it is thought to involve a combination of nucleotide excision repair, homologous recombination, and non-homologous end joining (47). Our analysis showed that \(Mnn1\) mutants were not more sensitive than wild type flies to double strand breaks, suggesting that the mechanisms involved in repair of this type of damage are intact. Furthermore, at least at the level of meiosis, homologous recombination, an important component of double strand break repair (48), was normal in \(Mnn1\) mutants. Taken together, our data suggest that menin might be involved in the pathways that lead to nucleotide excision repair.

Interestingly, menin has been shown to interact with FANCD2, a protein that plays a central role in repair of DNA cross-links (14). Sensitivity to DNA cross-linking agents is characteristic of cells from Fanconi anemia patients (49). Recent studies showed that monoubiquitination of FANCD2 in response to DNA damage promoted the interaction of FANCD2 and BRCA2 and loading of BRCA2 on...
chromatin in sites of damage (50,51). This interaction, in turn, promotes the assembly of damage-inducible Rad51 foci possibly through conformational changes in BRCA2 that allow it to release Rad51 onto DNA. The monoubiquitination of FANCD2 protein in response to damage caused by DNA cross-linking agents is controlled by ATR (52). It is noteworthy that another menin interactor, RPA, is required for association of ATR and its binding partner, ATRIP, with damaged DNA (53). It is possible that menin assists in bringing together different components of cross-link repair machinery. Alternatively, menin’s association with histone deacetylases (16) such as mSin3A, a subunit of Sin3/Rpd3 HDAC complex (54), suggests that menin may have a more general effect on chromatin remodeling, allowing the repair machinery to access the damaged DNA.

Although Drosophila studies favor a role for Mnn1 as a caretaker gene, data from mouse models of Men1 have been interpreted as indicating a more direct role in growth control. For example, homozygous Men1 knockout mice die during embryogenesis (55,56), suggesting that menin has a role in control of growth or differentiation in the murine embryo. Likewise, tissue-specific gene inactivation in pancreatic islet cells of the mouse leads to widespread hyperplasia and cellular atypia in adults, consistent with a similar role for the gene postnatally. It is possible that menin functions differently in Drosophila and mammals, but several studies have provided evidence that menin plays a role in genomic integrity in humans and mice as well as flies. Targeted disruption of Men1 in mouse embryonic fibroblasts caused moderate sensitivity to DEB and an increase in chromosomal aberrations in response to MMC treatment (14). Similarly, the peripheral blood lymphocytes from MEN1 patients showed increased chromosomal abnormalities after DEB treatment (11). Numerous chromosomal abnormalities were also found in Men1-related pancreatic islet tumors (57).

Many of the apparent discrepancies between mouse and Drosophila may reflect phenotypic differences at the level of the organism that nevertheless have the same biological basis in both organisms at the level of the cell. For example, the embryonic lethal phenotype in mice is due to defects in several organs, but there is little evidence for a cell-autonomous defect in growth control or differentiation. The most important abnormality is in liver organogenesis, and the main cellular phenotype observed in vivo is increased apoptosis in hepatocyte precursors (56). By analogy to BRCA1 and BRCA2, which are known to function in maintenance of genomic integrity, cell death resulting in embryonic lethality can be a manifestation of genomic instability (58). Drosophila embryonic cells may be less sensitive to the effects of menin loss on genomic integrity and less likely to undergo apoptosis.

Tissue-specific gene inactivation indicates that, at the level of individual cells, loss of menin may have little effect on growth or differentiation in juvenile and adult mice. In particular, mice with RIP-driven loss of Men1 had normal pituitary glands and, for the most part, normal pancreatic islet cells at four months of age (59). Frank tumorigenesis was not seen until 6–12 months of age despite early loss of menin, strongly suggesting that additional somatic events were necessary for tumorigenesis. This model is consistent with a function for menin in maintenance of genomic integrity. On the other hand, widespread pancreatic islet hyperplasia was seen in both heterozygotes and tissue specific knockout mice in some studies, suggesting a direct role in growth control (55,60). There may be overlap between the role of the gene in maintenance of genomic integrity and its role in control of cell growth. BRCA1 was initially thought to function by directly inhibiting growth of breast and ovarian epithelial cells (61), a finding that reflects one of the roles of the gene in the broader context of its role in maintenance of genomic integrity.

In conclusion, our work shows a role for the Drosophila homolog of MEN1 in maintenance of genomic integrity and suggests that tumor pathogenesis in the human disease may relate to genomic instability, comparable to that seen in BRCA1-related breast cancer and HNPCC. Generation of a Men1 mutant in Drosophila creates an important resource for the in vivo study of menin’s role in development and carcinogenesis and sets the stage for developing and testing potential cancer therapies for MEN1-related tumors.

MATERIALS AND METHODS

Drosophila culture conditions

Unless otherwise indicated all fly stocks and crosses were maintained at room temperature on standard Drosophila yeast-cornmeal medium.

Drosophila stocks

Line y w MGR; Sco/CyO was generated by Dr S. Stowers. Lines w;FRT82A latX1/TM3Sh and w;al dp b pr c px sp/ CyO were obtained from the laboratory of Dr T. Xu. Line P553/CyO was available from Berkeley Drosophila Genome Project (http://www.fruitfly.org). Lines Δ2-3Sh/TM6B–, DF(2L)J–H(al-SM5–sp, Adv/CyO–, hs–Gal4/TM3Sh, mei-41/FM7a and w1118 were obtained from Bloomington Stock Center (http://flystocks.bio.indiana.edu). The description of all the alleles can be found at FlyBase: a database of the Drosophila genome (http://www.flybase.org).

Generation of Mnn1 mutant allele

The Mnn1 mutant allele was generated by two rounds of male recombination (28). Briefly, flies with genotype y w;P553/CyO were crossed to w;al dp b pr c px sp/ CyO, Δ2-3Sh/TM6B–, where P553 is a P-element insertion located 8 kb’ to the Mnn1 start codon. P553/Δ2-3Sh/+ males were then mated with +/al-SM5–sp females. From the progeny of this cross, P553sp/Δ2-3Sh–sp males were selected as potential recombinants and were individually mated with y w;Adv/CyO females to generate balanced stocks. A total of 21 potential recombinant lines were generated. These were further analyzed by Southern blotting using full length Mnn1 cDNA as a probe. The borders of the deletion in P553#15 line were analyzed by sequencing the PCR products generated by the following primer pairs: PLAC1 (cacaacaggtcetgctcaat–)–122328F (atgcctccacttacacta) for the left end of the deletion and PRY4 (caatcatactgctgctca)–131352R (tcgcatccctctcactc) for the right end. PCR was performed using AmpliTaq DNA polymerase (Perkin Elmer) with
standard PCR conditions. The sequencing was done by BigDye terminator method on 377 ABI Sequencer.

In the second round P553#15/CyO males were crossed to 8-3Sb/TM6B females. The sons from this cross with genotype P553#15/+:8-3Sb/+ were individually mated with y w Ady/CyO females to establish balanced lines. A total of 386 independent y w P553/CyO balanced lines were generated and screened by PCR with primer pairs PLAC1–155744F (taacatgaatgggattagg) and PRY4–2082R (ctattgtacctctcactt) to find lines in which the change in P-element position occurred. Long-range PCR (primer pairs PRY4/2082R) Taq Extender (Invitrogen) was added to the PCR reaction and the following PCR cycle was used: 97°C, 2 min; 35 cycles of 96°C, 30 s; 60°C, 1 min; 72°C, 2 min; 72°C, 5 min.

For northern blot, 20 μg of total RNA from adult females was used. The RNA was extracted using RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. Full length Mnn1 cDNA was used as a probe.

All recessive markers used in the first round of male recombination were recombined out of the chromosome carrying the Mnn1e200 allele.

Generation of UAS-Mnn1, hs-Gal4 transgenic strain

The UAS-Mnn1 strain was generated by cloning Drosophila Mnn1 cDNA into the P-element transformation vector pUASP (62). Germline transformation was performed by standard procedures (63) using w1118 as a host strain. UAS–Mnn1 and hs–Gal4 transgenes were placed on the same chromosome by homologous recombination.

Immunostaining and acridine orange staining

Wing and eye imaginal discs were dissected from third instar larvae before and 1 h after 2 kRads of ionizing radiation and fixed in 4% paraformaldehyde in PBS. For G2/M checkpoint, larvae before and 1 h after 2 kRads of ionizing radiation and Wing and eye imaginal discs were dissected from third instar larvae for immunostaining and acridine orange staining procedures (63) using w1118 as a host strain. UAS–Mnn1 and hs–Gal4 transgenes were placed on the same chromosome by homologous recombination.

Homologous recombination, double strand break repair and chromosome disjunction

To evaluate homologous recombination levels two markers on the X-chromosome (sn and y) were used. w sn/y w MGR;Mnn1e200 females were crossed to w males, and the percentage of recombinant male progeny (containing both sn and y or no sn and no y on the same chromosome) was evaluated and compared to the predicted value (64). Double strand break repair was assessed as described (31). Chromosome disjunction was evaluated as described (65).

Radiation treatment

A total of 150 females and 100 males were placed in each bottle with Drosophila culture medium and synchronized in a 12 h light/dark incubator for 4 days. The flies were then transferred to fresh bottles and eggs were collected for two 4 h periods. Larvae were washed out of the media with PBS 72 h (second instar stage) or 96 h (third instar stage) after egg deposition (AED), placed into 15 mm petri dishes, irradiated with 0, 1, 2 or 2.5 kRad in 137Cs irradiator, and returned to the fresh food vials. The survival to adulthood was evaluated as a percentage survival compared to 0 kRad dose. A total of 100 larvae were picked for each dose/each genotype and each experiment was repeated five times. The survival numbers represent averages of five repeats.

Chemical mutagens treatment

Six mutagens were used in this experiment (Table 1). All chemicals were purchased from Sigma. Cisplatinum was a generous gift from the laboratory of Dr Peter Glazer (Yale University). The mutagens were diluted in ddH2O with the addition of AAF, which was diluted in 0.6% DMSO in 70% EtOH. The experiment was performed as described previously (31). Five males and five females were placed in the vial for 48 h, after which parents were transferred to the next vial and the progeny (embryos and first instar larvae) were treated with 250 μl of mutagen or with solvent alone. The concentrations of mutagens used in this work were based on the data on sensitivity of various Drosophila DNA repair mutants to these agents (66–68). Mnn1e200 homozygous females and Mnn1e200 heterozygous males were used in the experiment. The treated progeny were grown at room temperature (23–24°C) or at 29°C. Upon hatching, the survival was measured as a ratio of the number of either homozygous or heterozygous adults to the total number of flies. At least five vials were set up for each mutagen concentration and genotype.

Determination of the frequency of loss of wild-type Lats allele

The following crosses were set up:

1. y w MGR;Mnn1e200 x y w MGR;Mnn1e200;latsX1/TM3Sb
2. yw MGR;P553 x y w MGR;P553;latsX1/TM3Sb
3. yw MGR;Mnn1e200;UAS-Mnn1 hs–Gal4/TM3Sb x y w MGR;Mnn1e200;latsX1/TM3Sb
4. mei-41/FM7a;latsX1/TM3Sb x mei-41

For radiation treatment, 100 females and 50 males were placed in bottles with fresh Drosophila medium. The flies were allowed to lay eggs for 24 h, and the parents were then moved to another bottle. After 24 h, the progeny were collected and treated with 0.2, 1 or 2 kRad of ionizing radiation as described earlier. Progeny from crosses 3 and 4 were treated only with 0.2 kRad. The nitrogen mustard treatment
(only crosses 1 and 2) was performed exactly as described earlier. For baseline mutation levels, the progeny of all the crosses were left untreated.

Upon hatching, the adults with genotypes: y w Mgr;Mnn1 e200, lats X1/+; y w MGR;P553; lats X1/+; y w MGR;Mnn1 e200, UAS– Mnn1 hs– Gal4; lats X1 and mei-41; lats X1/+ were examined for tumor formation. The mutation frequency was estimated as the number of tumor foci per 100 flies of a given genotype. Each experiment was repeated at least three times.

Statistical analysis

Student’s t-test (69) was used to analyze the data from radiation treatment and mutation rate experiments. Chemical mutagenesis results were analyzed by goodness of fit test (69).

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