

RESEARCH ARTICLE

REV1 coordinates a multi-faceted tolerance response to DNA alkylation damage and prevents chromosome shattering in *Drosophila melanogaster*

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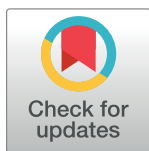
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Abstract

When replication forks encounter damaged DNA, cells utilize damage tolerance mechanisms to allow replication to proceed. These include translesion synthesis at the fork, postreplication gap filling, and template switching via fork reversal or homologous recombination. The extent to which these different damage tolerance mechanisms are utilized depends on cell, tissue, and developmental context-specific cues, the last two of which are poorly understood. To address this gap, we have investigated damage tolerance responses in *Drosophila melanogaster*. We report that tolerance of DNA alkylation damage in rapidly dividing larval tissues depends heavily on translesion synthesis. Furthermore, we show that the REV1 protein plays a multi-faceted role in damage tolerance in *Drosophila*. Larvae lacking REV1 are hypersensitive to methyl methanesulfonate (MMS) and have highly elevated levels of γ -H2Av (*Drosophila* γ -H2AX) foci and chromosome aberrations in MMS-treated tissues. Loss of the REV1 C-terminal domain (CTD), which recruits multiple translesion polymerases to damage sites, sensitizes flies to MMS. In the absence of the REV1 CTD, DNA polymerases eta and zeta become critical for MMS tolerance. In addition, flies lacking REV3, the catalytic subunit of polymerase zeta, require the deoxycytidyl transferase activity of REV1 to tolerate MMS. Together, our results demonstrate that *Drosophila* prioritize the use of multiple translesion polymerases to tolerate alkylation damage and highlight the critical role of REV1 in the coordination of this response to prevent genome instability.

Author summary

Organisms have evolved several ways to continue copying their DNA when it is damaged, grouped into the categories of translesion synthesis and template switching. These damage

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tolerance mechanisms prevent replication forks from collapsing when they encounter DNA damage and prevent catastrophic genome instability and cell death. While the proteins and pathways involved in damage tolerance are beginning to be understood at the single cell level, how they are regulated in multicellular organisms remains an intriguing question. In this study, we investigated the mechanisms by which *Drosophila* tolerate alkylation damage. We discovered that tissues containing rapidly dividing diploid cells favor translesion synthesis over recombination-based mechanisms of damage tolerance, preferentially utilizing different translesion polymerases in a context-dependent manner. Furthermore, we showed that the REV1 protein, best known for its role in recruiting translesion DNA polymerases to damage sites, performs multiple functions during damage tolerance. Together, our results demonstrate that damage tolerance preferences for multicellular organisms may differ from those observed in cultured cells and establish *Drosophila* as a useful model system for studying tolerance mechanisms.

Introduction

Cellular DNA is constantly exposed to both endogenous and exogenous insults, many of which damage the nitrogenous bases. In cells undergoing DNA replication, this base damage can cause replicative polymerases to pause during synthesis, stalling replication forks [1,2]. Prolonged stalling results in disassembly of the replication machinery and, in severe situations, fork collapse, leading to one-ended DNA double-stranded breaks (DSBs). These breaks are known to promote mutagenesis, chromosome translocations, aberrant recombination, and cell death [3,4].

To prevent these genome destabilizing events at stalled replication forks, cells have evolved two sets of DNA damage tolerance (DDT) strategies [5]. The first, called template switching, involves the use of error-free homology-directed mechanisms that stabilize stalled forks and prevent their collapse, while allowing for either lesion repair or bypass. Template switching strategies include homologous recombination (HR)-mediated bypass and fork reversal [6–8]. Both DDT mechanisms are stimulated by PCNA lysine-164 polyubiquitylation, which is catalyzed by the Rad5 E3 ubiquitin ligase in budding yeast and its HLTf and SHPRH counterparts in mammals [9–14].

HR-mediated bypass can occur directly at the fork or post-replicatively at single-stranded gaps following repriming [6,15,16]. In both cases, the RAD51 protein promotes strand invasion and copying from the recently synthesized nascent lagging strand [17]. Fork reversal, also called fork regression, occurs when DNA translocases and helicases anneal the nascent leading and lagging strands at the fork, forming a four-way junction often referred to as a “chicken foot” structure [18–20]. Extension of the leading strand using the newly-synthesized lagging strand allows for bypass of the lesion. The regressed fork can then be acted upon by nucleases and helicases to restart replication [21–26]. The regulation of DNA degradation is critical to the success of this mechanism, as uncontrolled nuclease activity at regressed forks has been shown to be detrimental to genome stability [19,27,28].

A second type of DDT, called translesion synthesis (TLS), occurs by recruitment of specialized TLS polymerases to lesions, enabling damage bypass [29]. TLS can occur ‘on the fly’ at the replication fork, or at single-stranded gaps that result from repriming downstream of the lesion [30]. TLS polymerases that are known to be recruited to sites of damage at stalled replication forks include the Y-family polymerases eta (η), iota (ι), kappa (κ), and Rev1, the B-family polymerase zeta (ζ), and the A-family polymerase theta (θ) [31]. Pol ζ is a multi-component

enzyme composed of the Rev3 catalytic subunit, two subunits of Rev7, and the Pol31 and Pol32 subunits [32–35]. TLS polymerases have larger active sites that can accommodate damaged bases or mismatches formed between lesions and incoming nucleotides and they lack proofreading activity [29]. As a result, TLS polymerases tend to have a lower fidelity than replicative polymerases and are responsible for much of the mutagenesis observed following exposure to DNA damaging agents such as UV and methyl methanesulfonate (MMS) [29,36].

TLS polymerases are recruited to sites of damage in at least two different ways. In budding yeast, RPA-coated ssDNA accumulates at stalled forks and signals for Rad6 and Rad18 to monoubiquitylate PCNA at lysine 164 [9,37,38]. Monoubiquitylated PCNA then recruits TLS polymerases to DNA lesions through interactions with their ubiquitin binding motifs (UBZ in pol η and pol κ , and UBM in pol ι and Rev1) [39]. TLS polymerases can also be recruited to damage sites through interactions with the C-terminal domain (CTD) of Rev1, which uses its BRCT and UBM domains to interact with PCNA [40,41]. These TLS polymerases can replace the stalled replicative polymerase and insert a nucleotide opposite the damaged base, after which the replicative polymerase resumes synthesis. Depending on the nature of the lesion, TLS polymerases may also act sequentially, with one polymerase responsible for the initial insertion and a second, more processive polymerase extending past the lesion [42–44].

While the involvement of Rev1 in TLS polymerase recruitment is well established, several studies have suggested additional roles for Rev1 in DDT. Unlike other DNA polymerases, Rev1 possesses only deoxycytidyl transferase activity, inserting deoxycytosines opposite DNA damaged guanines and abasic sites [45–47]. Rev1 also functions to promote the bypass of G-quadruplexes and other non-B DNA secondary structures during replication [48,49]. Furthermore, Rev1 stabilizes Rad51 filaments to prevent degradation of nascent replication tracts in mammalian cells [50], and associates with Rad5 in budding yeast [51,52].

To date, most studies of DDT have focused on unicellular eukaryotes and immortalized cell lines, with a small number of investigations using mouse models lacking REV1 [53–56] and pol η [57]. Here, we have investigated DDT in the context of a genetically tractable multicellular organism, *Drosophila melanogaster*, which possesses TLS polymerases η , ι , ζ , θ , and Rev1, but not pol κ [58]. We find that rapidly dividing diploid tissues in larval *Drosophila*, but not immortalized fly cells growing in culture, rely largely on TLS to tolerate alkylation damage. We also demonstrate that REV1 plays a multi-faceted role in DDT. Cells from *rev1* null mutant flies accumulate double strand breaks and experience chromosome shattering when replicating damaged DNA. While REV1 recruits TLS polymerases via its CTD, in the absence of pol ζ its catalytic activity becomes critically important for DDT. Interestingly, both Pol η and Pol ζ are used during alkylation damage tolerance, with pol η playing an essential role when TLS is impaired by the deletion of the REV1 CTD. Together, our studies establish *Drosophila* as a robust genetic system in which to study DNA damage tolerance strategies in a multicellular organism.

Results

Drosophila rev1 mutants are hypersensitive to damaging agents that stall replication forks

We previously showed that *rev1* mutant larvae are sensitive to ionizing radiation (IR) and fail to develop to adulthood post-irradiation [59]. To determine whether this sensitivity is due to a defect in double-strand break repair or an inability to bypass other types of base damage created by IR [60], we tested *rev1* mutant larvae for their ability to survive exposure to other DNA damaging agents. We created a *rev1* null mutant (*rev1 Δ*) through imprecise excision of a *P*

transposon inserted in the 5' UTR of the gene (S1 Fig). The *rev1Δ* homozygous mutants were mildly sensitive to IR compared to their heterozygous siblings, confirming our previous findings (Fig 1A). However, they were not sensitive to topotecan or bleomycin, both of which are known to create DSBs. *rev1Δ* mutants were also sensitive to both nitrogen mustard, which creates intra- and interstrand crosslinks, and hydroxyurea, which depletes dNTP pools. Strikingly, they were hypersensitive to the DNA alkylating agents methyl methanesulfonate (MMS) and ethyl methanesulfonate (EMS), with fewer than 5% of *rev1Δ* homozygotes surviving doses that did not kill heterozygous larvae. Because DNA crosslinks and alkylation damage can lead to stalled replication forks, these results indicate an important role for REV1 during tolerance of fork-blocking lesions.

In *Saccharomyces cerevisiae* and mammalian cells, mutation of genes involved in homologous recombination (HR) repair, such as *RAD51* or *BRCA2*, results in sensitivity to MMS [61–63]. To determine if this is also true in *Drosophila*, we treated *rad51* and *brca2* null mutants (S1 Fig) with increasing doses of MMS. These mutants are known to be sensitive to both IR and topotecan [64,65]. Surprisingly, we observed no sensitivity to a high concentration of MMS in either mutant (Fig 1B). Thus, although HR is critical for repair of double-strand breaks, it is not the primary pathway used to tolerate alkylation damage in *Drosophila*.

To determine if the *rev1Δ* MMS hypersensitivity is also observed in *Drosophila* cells grown in culture, we used CRISPR-Cas9 genome editing to create a population of S2 cells possessing more than 80% inactivating *REV1* mutations [66] (S2 Fig). Surprisingly, wild-type and *rev1* mutant S2 cell populations showed similar sensitivity to increasing concentrations of MMS (Fig 1C). Resequencing of the *REV1* locus following MMS treatment showed only a slight decrease in the percentage of cells with predicted null mutations in *REV1* (S2 Fig). These data suggest that unlike flies, immortalized *Drosophila* cells do not favor TLS for alkylation damage tolerance.

Loss of REV1 promotes γ -H2AX foci accumulation and chromosome aberrations in MMS-treated larval tissues

Larvae treated with lethal doses of DNA damaging agents often survive early development and die prior to pupal eclosion. This is thought to result from extensive cell death due to DNA double-strand breaks in rapidly dividing imaginal disc tissues, which are precursors to adult structures including wings, eyes, and other appendages. To test whether this could be responsible for the MMS hypersensitivity observed in *rev1Δ* mutants, we dissected wing imaginal discs from homozygous *rev1Δ* third instar larvae and treated them *ex vivo* with MMS for five hours, during which time all cells should replicate their DNA at least once [67] (Fig 2A). We then quantified the number of γ -H2Av foci, which are equivalent to γ -H2AX foci in mammals and indicative of a checkpoint response in response to double-strand breaks or excessive single-stranded DNA (ssDNA) [68]. Strikingly, the number of γ -H2Av foci was more than 9-fold greater in homozygous *rev1Δ* MMS-treated discs compared to heterozygous treated discs (Fig 2B). The *rev1Δ* mutation also increased the number of γ -H2Av foci in untreated discs two-fold, suggesting that REV1 additionally plays a role in genome protection from endogenous damage. Together with the survival data, these results indicate that REV1 protects cells in highly proliferative tissues treated with alkylating agents by preventing the formation of double-strand breaks or accumulating ssDNA that can lead to cell and organismal death.

We wondered whether the increase in breaks and/or ssDNA resulting from loss of REV1 might promote chromosome instability. In *Drosophila*, this instability can be visualized in

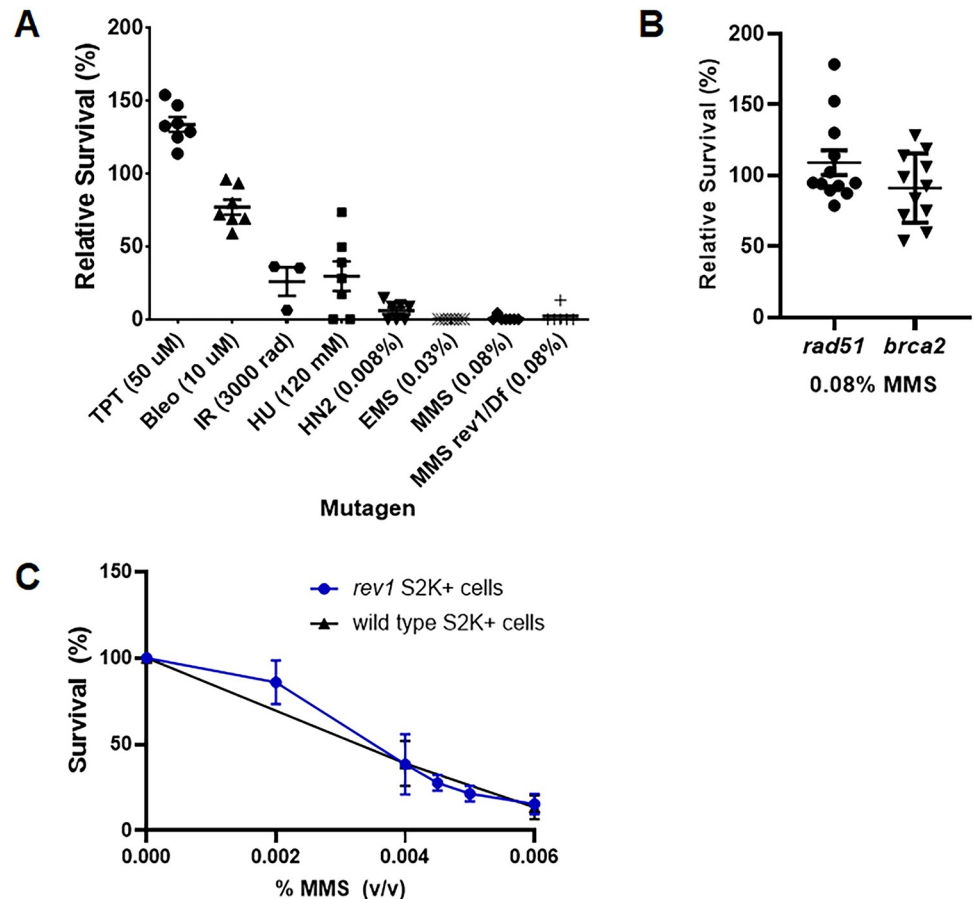


Fig 1. REV1 is vital for tolerance to alkylation damage in *Drosophila melanogaster*, but not S2 cells. (A) Relative survival of homozygous *rev1Δ* mutants to various DNA damaging agents. Heterozygous *rev1Δ* mutants were mated and their larval progeny were treated with indicated concentrations of mutagens or water control in the food. Shown are the percentage of homozygous (or *rev1Δ/Df(3L)BSC798*) progeny surviving to adulthood, relative to the control. Each point represents one set of control and treated vials, with TPT = topotecan, Bleo = bleomycin, IR = ionizing radiation, HU = hydroxyurea, HN2 = nitrogen mustard, EMS = ethyl methanesulfonate, MMS = methyl methanesulfonate. (B) Relative survival of homozygous *rad51* or *brca2* mutant larvae treated with 0.08% MMS. (C) Survival of wild-type or *rev1* mutant S2 cells treated with increasing concentrations of MMS. Shown are mean and SEM for each genotype.

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mitotic spreads from neuroblasts obtained from third instar larval brains. To investigate this question, we dissected brains from wildtype and *rev1Δ* larvae and treated them *ex vivo* for 14 hours with MMS, a period corresponding to approximately two full cell cycles. After obtaining mitotic spreads, we scored them for indicators of chromosome instability, including chromatid breaks and fusions (Fig 3A). While the number of chromatid breaks and chromatid fusions per spread were not significantly different between wild-type and *rev1Δ* flies treated with MMS (Fig 3B and 3C), we did observe a significant increase in a type of catastrophic damage involving chromosome shattering and/or aneuploidy. These events were increased five-fold in *rev1Δ* homozygous neuroblasts treated with MMS, compared to the wild-type control (Figs 3D and S3). In contrast, there was no increase in catastrophic damage in untreated *rev1Δ* mutants. These data, combined with the observed increase in γ -H2Av foci in *rev1Δ* imaginal discs, suggest that upon fork stalling REV1 may prevent the accumulation of DNA breaks and ssDNA that persist into mitosis and lead to genomic catastrophe.

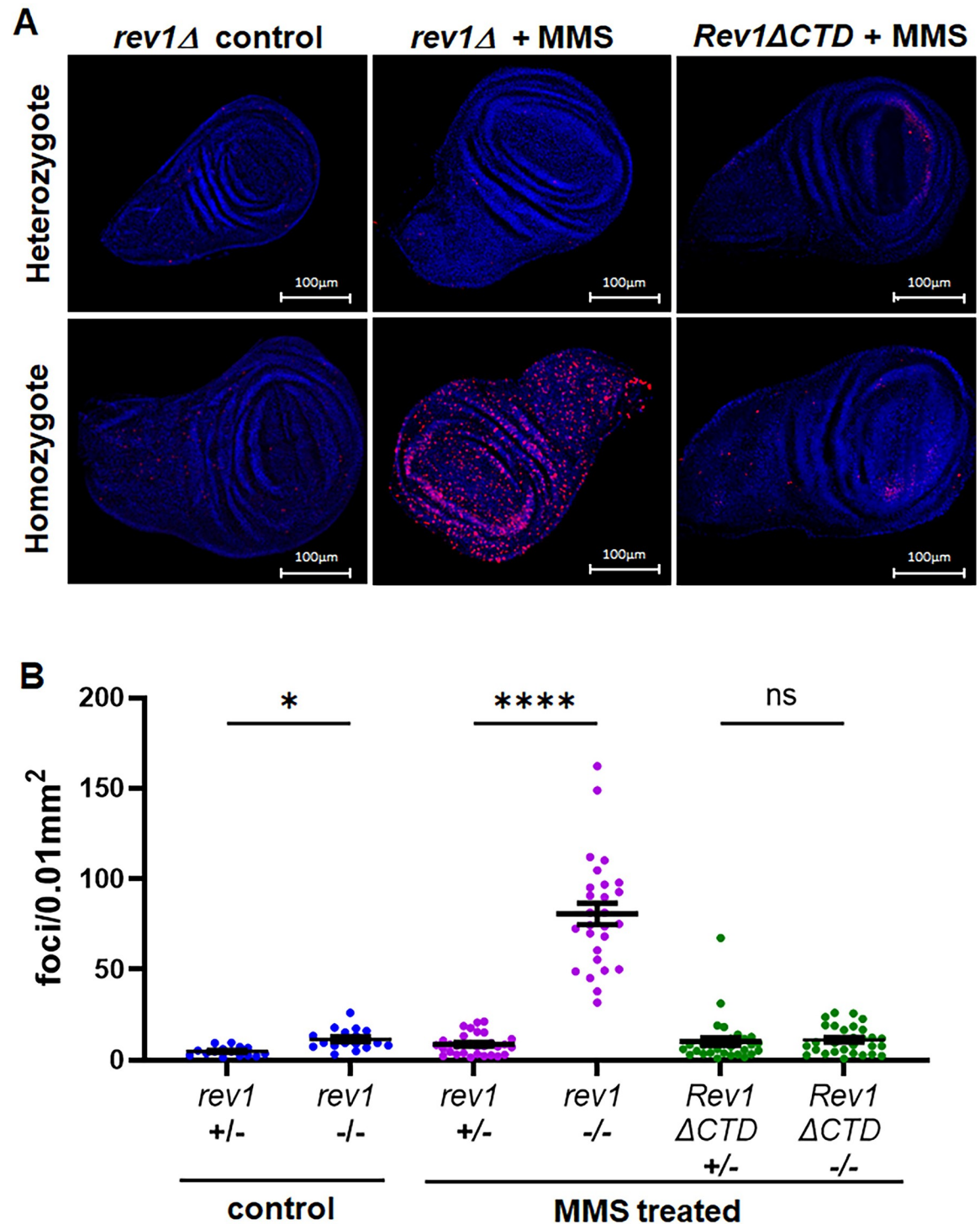


Fig 2. MMS induces DNA double-strand breaks in the absence of REV1. (A) Third instar wing imaginal discs were dissected, treated *ex vivo* for 5 hours with ddH₂O or 0.0025% MMS, and stained with DAPI (blue) and an antibody recognizing γ -H2Av (red). Foci were counted and normalized to wing disc size. (B) Quantification of the number of foci in treated discs. Shown are the mean and SEM for each genotype. Statistical comparisons were done using a Kruskal-Wallis one-way ANOVA with Dunn's multiple comparisons test. N values for control: (*rev1* +/-) = 15, (*rev1* -/-) = 17. N values for MMS treated: (*rev1* +/-) = 31, (*rev1* -/-) = 27, (*Rev1* Δ CTD +/-) = 33, (*Rev1* Δ CTD -/-) = 30. * $p < 0.05$, **** $p < 0.0001$, ns = not significant.

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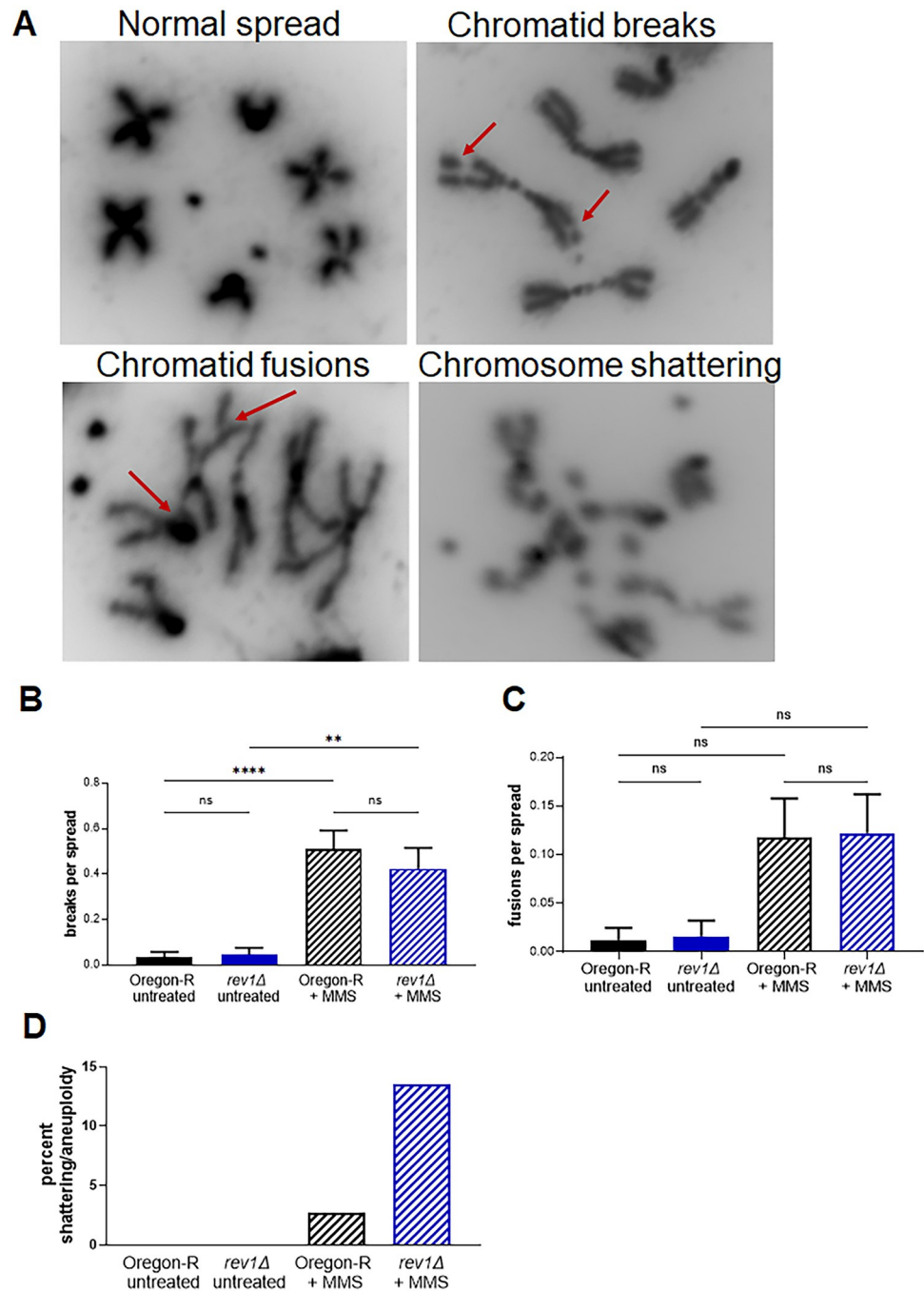


Fig 3. MMS-treated *rev1* neuroblasts have elevated rates of catastrophic chromosome shattering. (A) Representative images of normal and aberrant mitotic spreads. Brains were dissected from wild-type (Oregon-R) and *rev1Δ* third-instar larvae, treated *ex vivo* with ddH₂O or 0.0001% (v/v) MMS for 14 hours, incubated with colchicine for 1.5 hours, and squashed. DAPI-stained mitotic spreads were scored for (B) chromatid breaks, (C) chromatid fusions, and (D) catastrophic events (more than three breaks/chromosome shattering and/or aneuploid spreads). Statistical comparisons were done using a one-way ANOVA with multiple comparisons test. N values for control: (Oregon-R) = 81, (*rev1Δ*^{-/-}) = 62. N values for MMS treated: (Oregon-R) = 110, (*rev1Δ*^{-/-}) = 85.

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Damage tolerance mediated by translesion polymerases eta and zeta is partially independent of the REV1 C-terminal interaction domain

A major role of REV1 in vertebrates is to recruit other translesion polymerases to sites of DNA damage through a physical interaction with its CTD (Fig 4A) [69–73]. This function is conserved in *Drosophila*, where REV1 interacts with TLS polymerases η , ζ , and ι via its CTD [74]. We previously showed that flies with a deletion removing the N-terminal portion of the REV3 catalytic subunit of pol ζ are sensitive to alkylating agents [60]. While we did observe a dose-dependent survival decrease for *rev3 Δ* mutants treated with MMS (Fig 4B), a side-by-side comparison of *rev1 Δ* and *rev3 Δ* mutants showed that *rev1 Δ* mutants are significantly more sensitive to MMS (Fig 4B). This finding differs from observations in *S. cerevisiae*, where *rev1 Δ* and *rev3 Δ* mutants are equally sensitive to alkylating agents [63].

The MMS hypersensitivity of *rev1 Δ* mutants that we observed could be due to the inability of cells to recruit multiple TLS polymerases (in addition to pol ζ) for damage bypass. Alternatively, REV1 might play another role in damage tolerance. To distinguish between these possibilities, we used site-specific integrase mediated repeated targeting (SIRT) [75–77] to generate an allele of *REV1* lacking the portion of the CTD known to interact with pol η , pol ζ , and pol ι [74,78] (Fig 4A). Interestingly, *Rev1 Δ CTD* mutants were less sensitive than *rev1 Δ* mutants but were equally as sensitive to MMS as *rev3 Δ* mutants (Fig 4B). In addition, MMS-treated wing imaginal discs from *Rev1 Δ CTD* homozygous mutants did not show increased γ -H2Av foci when compared to heterozygous mutants (Fig 2). Because *rev1 Δ* MMS-induced damage and lethality is more severe than that of *Rev1 Δ CTD* mutants, we conclude that REV1 has roles in DDT in addition to TLS polymerase recruitment.

Y-family polymerases can be recruited to sites of damage through interactions with the Rev1 CTD and through interactions of their UBZ (pol η and pol κ) and UBM (pol ι) domains with monoubiquitylated PCNA [79–81]. To determine if TLS polymerases might also have multiple recruitment mechanisms in *Drosophila*, we created flies with *Rev1 Δ CTD* mutations that were also lacking either REV3 or pol η . Both double mutant stocks were more sensitive to a low concentration of MMS than *Rev1 Δ CTD* single mutants (Fig 4C and 4D). Intriguingly, while loss of pol η mildly sensitized flies to MMS, the *Rev1 Δ CTD* pol η double mutant showed extreme MMS hypersensitivity at doses as low as 0.0025% (Fig 4D), suggesting that pol η plays a critical role in alkylation damage tolerance when TLS is compromised by loss of the REV1 CTD.

The deoxycytidyl transferase activity of REV1 becomes important when TLS is compromised

In addition to its CTD, *Drosophila* REV1 contains a BRCT domain, a deoxycytidyl transferase (DTD) domain, and a single ubiquitin binding motif (UBM) (Fig 5A). In mammals, the BRCT domain interacts with PCNA and with 5' phosphorylated primer-template junctions [82–84], while the UBM2 domain associates with ubiquitylated PCNA [85,86]. The DTD catalyzes the insertion of cytosine opposite adducted guanine bases and abasic sites [46,47]. We used SIRT to create inactivating mutations in each of these domains. The *Rev1 Δ BRCT* mutation deletes amino acids 1–121, which corresponds to the entire BRCT domain in mice [56,87]. The *Rev1-DTD* mutant replaces two amino acids in the catalytic domain with alanines (D421A, G422A), a mutation previously shown to abolish deoxycytidyl activity in yeast [88]. Finally, the *Rev1-UBM* mutant changes two conserved residues in the UBM to alanines (L782A, P738A), which impairs the ability of the mouse protein to interact with ubiquitylated PCNA [89]. In all cases, flies with mutations that inactivate each individual domain were not sensitive to MMS (Fig 5B), suggesting that the BRCT, UBM, and DTD domains of REV1 are not required for resistance to MMS-induced damage when TLS is fully functional.

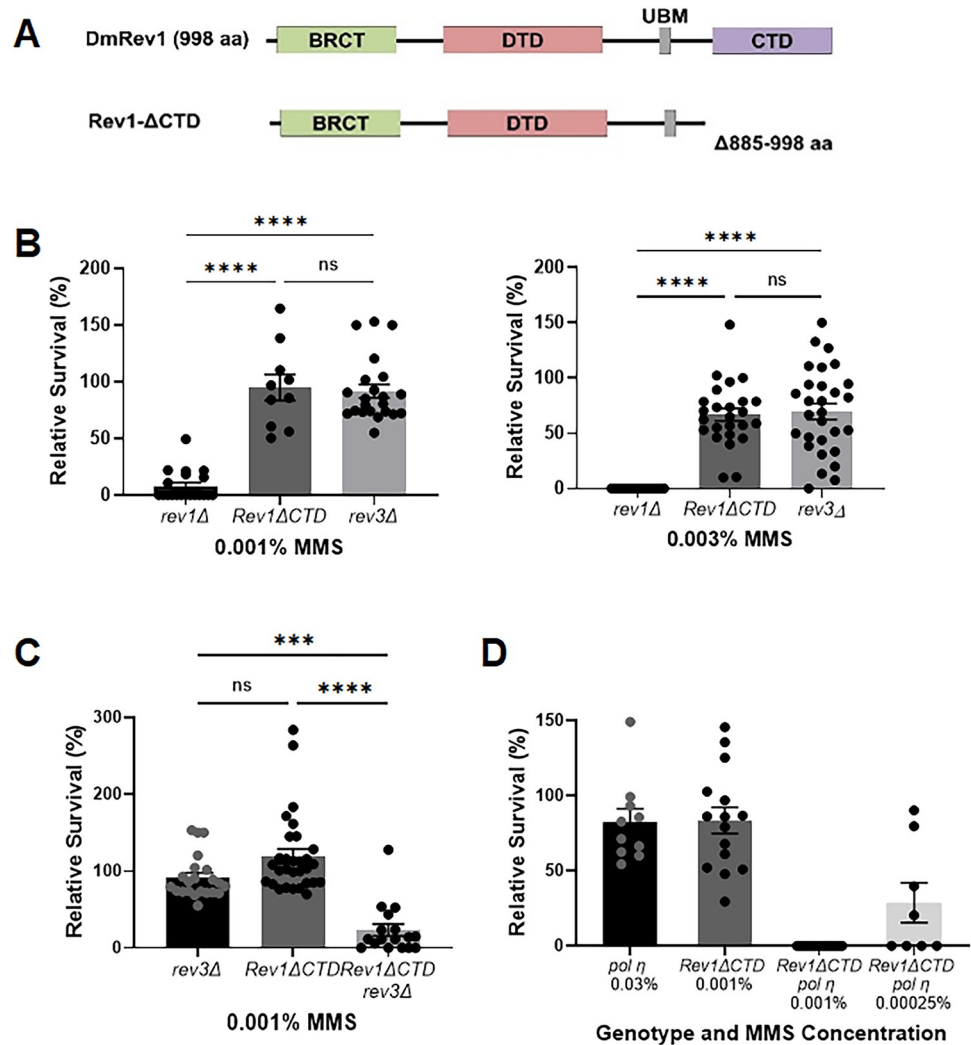


Fig 4. The CTD of REV1 and translesion polymerases η and ζ cooperate to promote MMS-induced damage tolerance. (A) The *Rev1ΔCTD* allele removes the carboxy terminal domain (CTD), which interacts with translesion polymerases η , ζ , and ι in *Drosophila* [74]. (B–D) Relative survival of homozygous DDT mutants to MMS. Heterozygous flies were self-crossed and the resulting larvae were exposed to indicated concentrations of MMS in their food. The percentage of homozygous progeny surviving to adulthood, relative to a water treated control, are indicated. Shown are mean and SEM for each genotype. Statistical comparisons were done using a Kruskal-Wallis one-way ANOVA with Dunn's multiple comparisons test. *** $p < 0.001$, **** $p < 0.0001$, ns = not significant.

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According to the results shown in Fig 4B, the REV3 catalytic subunit of pol ζ is important for TLS bypass of MMS-induced damage. To test whether the other domains of REV1 become necessary for MMS resistance when TLS is compromised, we used genetic crosses to place each REV1 domain-specific mutant in a *rev3* background. *Rev1ΔBRCT rev3* and *Rev1-UBM rev3* double mutants were slightly more sensitive to MMS than *rev3* single mutants (Fig 5C). Interestingly, *Rev1-DTD rev3* double mutants showed a greater increase in sensitivity, equivalent to that observed in the *rev1Δ* null mutant (Fig 5C). Based on these results, we conclude that the BRCT and UBM domains play a less important role than the DTD domain in damage tolerance in the absence of Pol ζ . We also speculate that the hypersensitivity of *rev1Δ* mutants to alkylation damage may largely be due to impaired TLS in the absence of the CTD and simultaneous loss of deoxycytidyl transferase activity.

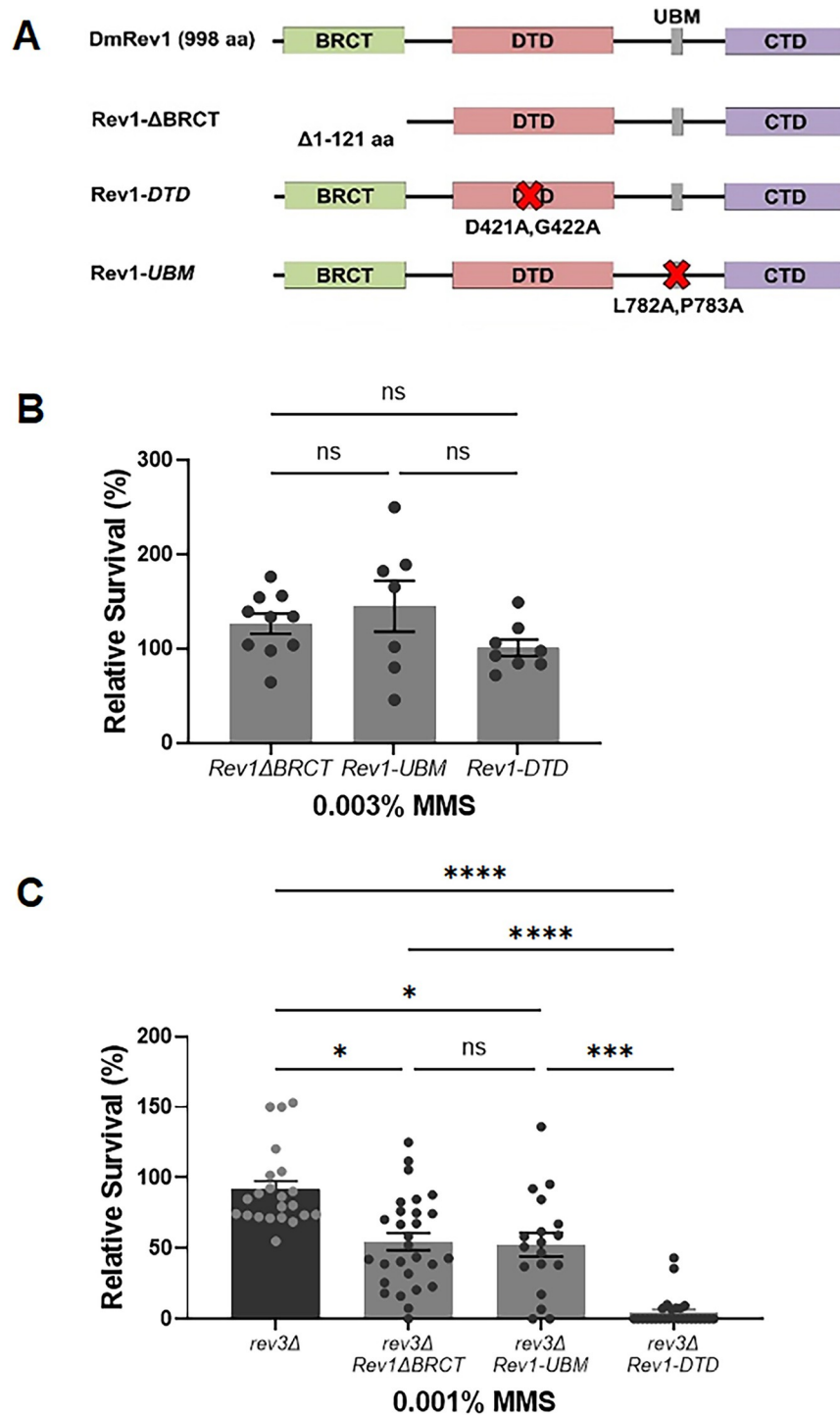


Fig 5. Multiple REV1 domains promote damage tolerance. (A) Domain-specific mutations were created at the endogenous *REV1* locus via SIRT. (B-C) Relative survival of single REV1 domain-specific mutants. Flies heterozygous for the indicated *REV1* domain mutations (B) or homozygous for the *rev3* null mutation and heterozygous for the *REV1* domain-specific mutations (C) were self-crossed and the resulting larvae were exposed to the indicated concentrations of MMS in their food. The percentage of homozygous progeny surviving to adulthood, relative to a water treated control are indicated. Shown are mean and SEM for each genotype. Statistical comparisons were done using a Kruskal-Wallis one-way ANOVA with Dunn's multiple comparisons test. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, ns = not significant.

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Discussion

Here, we have investigated the relative usage of different DNA damage tolerance strategies in *Drosophila*. Contrary to what has been observed in budding yeast and mammalian cells, *rad51* and *brca2* mutants are not sensitive to high concentrations of MMS, implying that homologous-recombination mediated tolerance mechanisms are not a first line of defense in flies. Instead, translesion synthesis, and specifically the REV1 protein, appear to be crucial for damage tolerance. A recent study demonstrated that in budding yeast, Rad52, but not Rad51, acts together with TLS to repair MMS-induced damage in a non-recombinogenic process [90]. Because *Drosophila* do not have a RAD52 ortholog, we think it is unlikely that this mechanism operates in flies.

MMS-treated *rev1Δ* mutants accumulate high levels of double-strand breaks or ssDNA, which activate checkpoint mechanisms and result in γ -H2Av phosphorylation in rapidly dividing larval imaginal disc cells. Additionally, chronic exposure to MMS in *rev1Δ* mutants causes extreme genome instability and chromosome shattering, as we observed in neuroblast mitotic spreads from larval brains. Eventually, this likely leads to extensive cell death, and if compensatory proliferation is unable to restore cell number, causes organismal death prior to adulthood.

These studies highlight the importance of studying DNA damage tolerance responses in multicellular organisms, which may show distinct phenotypes compared to cells growing in culture. This is underscored by our observation that *rev1* mutant S2 cells are not hypersensitive to MMS, in contrast to *rev1Δ* mutant flies. Because REV1 is transcribed at moderate levels in S2 cells [91], it is unlikely that the lack of sensitivity in *rev1* mutant S2 cells can be explained by a lack of protein expression. Instead, we hypothesize that S2 cells may preferentially employ non-TLS mechanisms of damage tolerance, such as template switching. Consistent with this, S2 cells were originally derived from late-stage embryos [92], and it was recently shown that HR is the preferred break repair pathway in older embryos [93].

An interplay of REV1 and TLS polymerases for damage tolerance in *Drosophila*

Comparison of the *rev1* MMS sensitivity to that of various domain-specific mutants suggests that REV1 promotes damage tolerance through multiple mechanisms. While *Rev1-ΔCTD* mutants are mildly sensitive to MMS at concentrations of 0.003%, *rev1Δ* mutants cannot survive exposure to 0.001% MMS. Additionally, we do not observe increased numbers of γ -H2Av foci in *rev1ΔCTD* mutants, which indicates that regions outside of the REV1 CTD prevent the accumulation of checkpoint-activating breaks or ssDNA. This points to an additional role for *Drosophila* REV1 in damage tolerance beyond the recruitment of TLS polymerases. Consistent with this, other groups have shown that REV1 plays multiple roles in the response to UV damage [94,95] and at stalled replication forks [50].

An alternative interpretation of the *rev1Δ* MMS hypersensitivity is that one or more TLS polymerases could be recruited to damaged DNA through interactions outside of the CTD. While this is unlikely to be the case for pol η , yeast two-hybrid analysis has demonstrated interaction of pol ι with a region upstream of the CTD [74]. Thus, it would be interesting to examine whether pol ι is important for damage tolerance in a *Rev1ΔCTD* mutant.

Although the REV1 single domain mutants lacking either BRCT or UBM function are not themselves sensitive to MMS, both showed increased sensitivity to MMS when combined with the loss of the catalytic domain of pol ζ . The BRCT domain has been shown to bind to PCNA for TLS-related functions [82,83]. In addition, in both yeast and mammals the REV1 BRCT domain contains an N-terminal α -helix that can bind to ssDNA, helping recruit it to damage sites [84,96]. It is possible that the recruitment of REV1 to sites of damage is hindered but not completely abolished without the BRCT domain, due to interactions with ubiquitinated

PCNA through the REV1 UBM domain. In humans and yeast, the UBM2 domain of REV1 is responsible for binding with monoubiquitinated PCNA [85,86]. The similarity in sensitivities between the *Rev1ΔBRCT rev3* and *Rev1-UBM rev3* mutants could correlate with the overlapping functions of these domains in recruiting REV1 to sites of damage. In their absence, Y-family TLS polymerases would be recruited to lesions less effectively, which could result in additive MMS sensitivity when pol ζ is defective.

Interestingly, a greater synergism was seen with the *Rev1-DTD rev3* double mutant. In many contexts, the catalytic activity of REV1 is dispensable. However, the DTD domain can insert cytosine opposite damaged guanines and abasic sites [46,47,97]. In *Drosophila* without pol ζ, there seems to be a critical role for REV1 deoxycytidyl transferase activity, even when other TLS polymerases are available. It is currently unclear why the DTD domain becomes so important in the absence of pol ζ. One possibility is that the absence of both REV3 and REV1 deoxycytidyl transferase activity results in the processing of misincorporations at O6-methyl-guanine by mismatch repair, leading to nicks and breaks [98]. Pol ζ is known to be an extender following insertion of a nucleotide opposite a damaged base by Y-family polymerases, but it can also bypass abasic sites on its own [99]. Given the mild sensitivity of pol η mutants, it will be interesting to see if the DTD domain is also critical in the absence of pol η. If so, it may be that *Drosophila* REV1 DTD, pol ζ, and pol η have unique but partially overlapping abilities to insert nucleotides opposite different MMS-induced lesions.

A TLS-centric model for damage tolerance in *Drosophila*

One of the interesting results from this study involves the relative MMS resistance of *Rev1ΔCTD* single mutants compared to the hypersensitivity of *Rev1ΔCTD rev3* and *Rev1ΔCTD pol η* double mutants. In many organisms, PrimPol plays a critical role in repriming DNA synthesis downstream of damage [100]. While *Drosophila* lacks an obvious PrimPol ortholog, repriming could also be accomplished through the action of primase/pol α, producing ssDNA gaps that would need to be filled. In chicken DT40 cells, ‘on the fly’ translesion synthesis, which occurs directly at the replication fork, requires the REV1 CTD but not PCNA ubiquitylation [41]. However, post-replicative filling of single-stranded gaps does require PCNA ubiquitylation in DT40 cells. If a similar scenario exists in *Drosophila*, in the *Rev1ΔCTD* mutant polymerases η and ζ may be compromised in their TLS role at the fork but could still perform their gap-filling functions after fork passage (S4 Fig). Loss of either polymerase in a *Rev1ΔCTD* background would compromise TLS bypass both at the fork and during postreplication repair, resulting in enhanced MMS sensitivity. In a REV1 competent background, translesion synthesis bypass at the fork would still be available and could be carried out by polymerases with overlapping abilities, explaining why *pol η* and *pol ζ* mutants are only mildly sensitive to MMS. Validation of this model in *Drosophila* will require experiments in a genetic background in which gap filling by TLS polymerases is compromised, as might occur in a PCNA K164R mutant that is unable to be ubiquitylated.

We have shown that the BRCT, UBM, DTD, and CTD domains of *Drosophila* REV1 all play roles in DNA damage tolerance, with their relative importance dependent upon the availability of other tolerance mechanisms and TLS polymerases. Due to the extreme sensitivity of *rev1Δ* mutants, it is possible that other REV1 protein regions are also important. For example, REV1 could be important for stabilizing regressed forks, recruitment of proteins important for fork reversal or template switching, protection of regressed forks from cleavage by structure-specific endonucleases, and/or prevention of hyper-resection. We are currently investigating these possibilities.

Notably, these studies may have relevance to cancer research, as mutagenic TLS is strongly implicated in carcinogenesis, tumor progression, and chemotherapeutic resistance [101–103].

Pertinent to this study, suppression of REV1 is known to inhibit both cisplatin- and cyclophosphamide-induced mutagenesis, which sensitizes tumors to traditional therapeutics and suppresses the development of tumor chemoresistance [104]. A novel small molecule, JH-RE-06, induces REV1 dimerization and inhibits TLS, making it attractive as a potential therapeutic [105,106]. As we have shown that TLS is a vital damage tolerance mechanism in *Drosophila*, we propose this model system may be useful for studying strategies employed by tumor cells exposed to fork-stalling agents and inhibitors of these processes.

Materials and methods

Drosophila husbandry and stocks

Flies were raised on standard cornmeal agar at 25°C on a 12 hr:12 hr light/dark cycle. The *rev1Δ* null allele was generated by an imprecise excision screen using *P[49]Rev1[G18538]* (Bloomington Stock #28417) with the *P* element inserted 55 bp of the transcription start site for *REV1*. The imprecise excision deletes 4531 bp downstream of the *P* element (the entire *REV1* gene), leaving behind 951 bp of the *P* element at the deletion site. The *pol η*¹² and *rev3*^{3B} knockout alleles were generated previously in the lab through imprecise *P*-element excision [60]. The *rad51* (encoded by *spn-A*) mutants were compound heterozygotes of *spn-A*^{093A} and *spn-A*⁰⁵⁷ [107]. The *brca2*^{KO} null allele replaces the entire coding sequence of the *BRCA2* gene with the *mini-white* gene [108]. The *Df(3L)BSC798* deficiency removes 49 genes from cytological region 61C1-61C8, including the *REV1* gene [109]. We used FlyBase (release FB2024_02) to find information on alleles, stocks, and gene expression.

Generation of *REV1* mutant S2 cells

Plasmid pLib6.4 containing a sgRNA targeting the first exon of *REV1* was transfected into PT5 cells derived from S2R+ cell line [66] with DOTAP Liposomal Transfection Reagent (Roche). Puromycin selection was initiated on day 3. After repeated passaging for 30 days, cells were centrifuged and genomic DNA was extracted. PCR using primers flanking the Cas9 targeting site was conducted and purified PCR product was subjected to Sanger sequencing, followed by ICE analysis (Synthego) to quantify the percentage of indels within the *REV1* gene.

S2 cell MMS treatment and quantification of survival

Wild-type or *rev1* mutant PT5 cells were grown in TNM-FH media (Sigma) with 10% FBS and antibiotic-antimycotic solution (Gibco). Cells were plated on day 0 in 96-well clear bottom cell culture plates at 5 X10⁴ cells/well and treated on day 3 with control media or media containing MMS at various concentrations. On day 7, the ATP-lite Cell Viability Assay (Revvity Health Sciences) was used to determine relative survival at each dosage. Each assay was repeated with at least 3 biological replicates.

Endogenous *REV1* mutant generation

Endogenous *REV1* domain mutants were generated through site-specific integrase mediated repeated targeting (SIRT) [75–77]. The *Rev1ΔBRCT* allele deletes the first 121 amino acids of *REV1*. *Rev1-DTD* is a double D421A, G422A mutation within the catalytic domain of *REV1*. *Rev1-UBM* is a double L782A, P783A mutation within the conserved region of the UBM domain. The *Rev1ΔCTD* allele deletes the last 113 amino acids (885–998) of *REV1*. All mutations were validated prior to each experiment by amplicon PCR and Sanger sequencing.

Mutagen sensitivity assays

Mutagen sensitivity assays were conducted as described in [110]. Heterozygous mutant males and females were mated by placing them in a vial for three days, then placed into another set of vials for three more days before being removed. The first set of vials were treated with 250 μ L of mutagen diluted in ddH₂O, one day after the parental flies were removed (treatment vials). The second set of vials were treated with 250 μ L of ddH₂O one day after the parental flies were removed (vehicle control vials). The number of homozygous and heterozygous eclosed flies were counted in control and treated vials. The relative survival for each vial was calculated as the percent of homozygotes relative to total number of flies in the treated vials divided by the percent of homozygotes relative to total number of flies in the control vials.

Imaginal disc culture and immunofluorescence

Third instar wing imaginal discs were dissected and cultured for 5 hours at 25°C in 20% fetal bovine serum (FBS), 0.7% sodium chloride, 0.1% dimethyl sulfoxide (DMSO) and 0.0025% MMS. Following 5 hours of culture, 90% of wing imaginal disc cells have entered S-phase [111]. Discs were washed twice with cold phosphate buffered saline with 0.1% Tween 20 (1xPBST), fixed with formaldehyde, and incubated overnight at 4°C in 1:500 anti- γ H2Av antibody (Rockland Inc) in 5% bovine serum albumin (BSA) in 1xPBS containing 0.3% Triton X-100. Discs were washed 4X for 5 minutes with PBST and incubated for 2 hours at room temperature in 1:1000 goat anti-Rabbit IgG Rhodamine Red conjugated antibody and 500 μ g/mL DAPI in 1xPBS + 5% BSA. Discs were washed and mounted in Vectashield on microscope slides [67]. γ -H2Av foci were imaged at 20x magnification using a Zeiss Z-stacking microscope and with filter sets compatible with DAPI and Rhodamine. Discs were imaged multiple times along the Z-axis, processed by deconvolution, and compressed into one image by extended depth of field algorithms. The area of the disc and number of foci per disc were calculated using ImageJ.

Mitotic chromatid spreads

Incubation of third instar larval brains were modified from Gatti and colleagues [112]. Third instar larval brains were dissected and cultured in 20% FBS, 0.7% sodium chloride, 0.1% DMSO and 0.001% MMS for 14 hours at 25°C. Colchicine was added to a concentration of 50 μ M and the discs were cultured for an additional 1.5 hours. Larval brains were swollen by incubating for 10 minutes in 0.5% sodium citrate, fixed for 20 seconds in acetic acid, methanol, and pico-pure H₂O (5.5:5.5:1), and placed into a drop of 45% acetic acid on siliconized coverslips. Poly-L-lysine coated slides were placed onto the coverslip and pressure was gently applied for 10 seconds. Complete spreading of mitotic chromatids was achieved by squishing the coverslip and slide using a clamp. Slides and coverslips were then frozen for 15 minutes at -80°C. Coverslips were removed and slides placed into -20°C ethanol for 20 minutes. Slides were removed from the ethanol and dried vertically at room temperature or overnight at 4°C. Slides were rehydrated in 2xSCC for 5 minutes at room temperature. Slides were then incubated for 5 minutes in 2xSCC with 200 μ g/mL DAPI for 5 minutes. Slides were washed twice for 10 seconds with 2xSCC, dried at room temperature, and then mounted using Vectashield. Mitotic chromatid spreads were imaged at 100x magnification using a Zeiss (Thornwood, NY) Axio Imager M1 microscope with a DAPI filter set and Slide Book software. Scoring of chromosome aberrations was conducted with blinding, with each spread scored by 2 individuals.

Supporting information

S1 Fig. Mutants used in this study. (A) The *rev1Δ* mutant was created through imprecise excision of a *P* transposable element inserted in the 5' UTR of *REV1*. The resulting deletion removes 4531 bp downstream of the *P* element (the entire coding sequence of the *REV1* gene), while retaining 951 bp of the *P* element 5' end. White regions indicate the protein coding region, while shaded regions indicate the untranslated regions. Numbers indicate nucleotide position from the start of transcription. (B) The *spn-A*⁰⁵⁷ allele is a V205A missense mutation that has been shown to act as a null allele [107]. The *spn-A*⁰⁹³ allele is a Q70stop nonsense mutation that also behaves as a null allele [107]. Both mutations were created through EMS mutagenesis. Mutation positions are indicated with vertical lines. Numbers indicate amino acid positions. (C) The *brca2*^{KO} allele was created through ends-out homologous recombination and replaces the entire coding sequence of *BRCA2* with the *mini-white* gene [108]. (D) The *pol eta*¹² and *mus205*^{3B} (*rev3*^{3B}) mutants were described in [59]. These mutations were created through imprecise excision of a *P* element. Both mutations are large deletions that result in frameshifts and premature stop codons and are predicted to create null alleles. (PDF)

S2 Fig. (Extension to Fig 1C). The frequency of *rev1* mutant S2 cells does not change after treatment with MMS. (A) Cas9-expressing S2R+ cells [66] were transfected with pLib6.4 containing a sgRNA targeting a sequence in the first exon of *REV1* and the cells were passaged for 30 days under puromycin selection. Genomic DNA was extracted from the transfected cells, PCR was used to amplify the region flanking the Cas9 cut site, the PCR product was Sanger sequenced, and knockout efficiency was analyzed using ICE analysis (Synthego). The inferred sequences present in the edited population and their relative proportions are indicated in the contribution column. The cut site is represented by a black vertical dotted line. By ICE analysis, 95% of the cells possess indels and 83% of the cells have indels that are predicted to create null mutations in *REV1*. (B) The cell population analyzed in (A) was treated with 0.004% MMS (v/v) for 4 days. Cells were washed and grown in fresh media for 1 day, after which genomic DNA was isolated and subjected to ICE analysis as above. Post MMS treatment, 86% of the cells have indel mutations, of which 71% are predicted to create null mutations. (PDF)

S3 Fig. (Extension to Fig 3). Examples of aberrant *rev1Δ* mitotic spreads. Brains were dissected from *rev1* mutant third-instar larvae and squashed according to [112]. Representative images of catastrophic events involving multiple breaks, chromosome shattering, and aneuploidy are shown. (PDF)

S4 Fig. A model for DNA damage tolerance preferences in *Drosophila*. Translesion synthesis (TLS) is favored in rapidly dividing cells in imaginal discs and neuroblasts, as indicated by the darker arrows. *REV1* may recruit multiple TLS polymerases to the fork for immediate bypass during replication. Alternatively, PCNA-Ub may recruit Pol η and Pol ζ to single-strand gaps behind the fork to carry out post-replication repair. When TLS is compromised, either in *REVΔCTD* flies or flies lacking one or more TLS polymerases, RAD51-mediated fork regression or template switching can compensate. (PDF)

S1 Data. Primary data used to generate graphs and charts in Figs 1–5. (XLSX)

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