The Drosophila Werner Exonuclease Participates in an Exonuclease-independent Response to Replication Stress

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Running title: WRNexo response to replication stress
Key words: replication restart, Werner Syndrome, double-strand break, DNA replication

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ABSTRACT

Members of the RecQ family of helicases are known for their roles in DNA repair, replication and recombination. Mutations in the human RecQ helicases, WRN and BLM, cause Werner and Bloom Syndrome, which are diseases characterized by genome instability and an increased risk of cancer. While WRN contains both a helicase and exonuclease domain, the *Drosophila melanogaster* homolog, WRNexo, contains only the exonuclease domain. Therefore the Drosophila model system provides a unique opportunity to study the exonuclease functions of WRN separate from the helicase. We created a null allele of WRNexo via imprecise *P*-element excision. The null *WRNexo* mutants are not sensitive to double strand break-inducing reagents, suggesting that the exonuclease does not play a key role in homologous recombination-mediated repair of DSBs. However, *WRNexo* mutant embryos have a reduced hatching frequency and larvae are sensitive to the replication fork-stalling reagent, hydroxyurea (HU), suggesting that WRNexo is important in responding to replication stress. The role of WRNexo in the HU-induced stress response is independent of Rad51. Interestingly, the hatching defect and HU sensitivity of WRNexo mutants do not occur in flies containing an exonuclease-dead copy of WRNexo, suggesting that the role of WRNexo in replication is independent of exonuclease activity. Additionally, *WRNexo* and *Blm* mutants exhibit similar sensitivity to HU and synthetic lethality in combination with mutations in structure-selective endonucleases. We propose that WRNexo and BLM interact to promote fork reversal following replication fork stalling and in their absence regressed forks are restarted through a Rad51-mediated process.

INTRODUCTION

Members of the RecQ family of helicases are known as the "guardians of the genome" due to their roles in DNA replication, repair, and maintenance of genomic integrity. There are five RecQ proteins in humans: RECQ1, RECQ4, RECQ5, BLM and WRN. Mutations in *RECQ4*, *BLM*, or *WRN* cause Rothmund-Thomson Syndrome, Bloom Syndrome and Werner Syndrome (WS), respectively. These autosomal diseases are characterized by high cancer incidence, accelerated aging, and developmental defects (CHU and HICKSON 2009). Most reported mutations in WS patients result in truncation of the 1432-amino acid WRN protein and loss of the nuclear localization signal (CHUN *et al.* 2011). In culture, WS cells exhibit signs of genomic instability including early senescence, a high incidence of chromosomal translocations, and prolonged S phase (SIDOROVA 2008).

Like other RecQ family members, WRN exhibits ATP-dependent 3'-5' DNA helicase activity (GRAY *et al.* 1997). WRN also contains a RecQ C-terminal (RQC) domain and a helicase and ribonuclease D C-terminal (HRDC) domain, which are largely responsible for DNA and protein binding (OPRESKO *et al.* 2002; VON KOBBE *et al.* 2002; VON KOBBE *et al.* 2003). A unique feature of WRN that distinguishes it from other RecQ helicases is its 3'-5' exonuclease activity (KAMATH-LOEB *et al.* 1998). The WRN exonuclease preferentially digests partial double-strand DNA containing a 5' overhang, although it will also digest blunt-end DNA containing a fork, Holliday junction, or D-loop (KAMATH-LOEB *et al.* 1998; ORREN *et al.* 2002; SHEN and LOEB 2000). The exonuclease domain also contains DNA binding sites for replication intermediates, such as forks and 5' overhangs (VON KOBBE *et al.* 2003; XUE *et al.* 2002). Additionally, proteins that can modulate WRN activity have been shown to bind the exonuclease domain including Ku80 and BLM (VON KOBBE *et al.* 2002). Interestingly, DNA and protein binding are not dependent upon the exonuclease or helicase activity of WRN (COMPTON *et al.* 2008; KAMATH-LOEB *et al.* 2012).

WRN has been shown to play an important role in recovery from replication fork stalling. For example, WRN-depleted cells exhibit a greater number of phosphorylated histone 2AX foci following treatment with hydroxyurea (HU), a reagent that causes replication fork stalling (FRANCHITTO *et al.* 2008; MAO *et al.* 2010; MURFUNI *et al.* 2012; OPRESKO *et al.* 2007). Similarly, WS cells exhibit spontaneous Rad51 foci, indicating the presence of double strand breaks (DSB) and their subsequent repair via homologous recombination (RODRIGUEZ-LOPEZ *et al.* 2007; SAKAMOTO *et al.* 2001). Together, these results suggest that WRN may prevent accumulation of DSBs caused by unsuccessful recovery from stalled replication forks.

In *Drosophila melanogaster*, the *WRNexo* gene encodes a protein with 35% identity and 59% similarity to the exonuclease domain of human WRN (SAUNDERS *et al.* 2008). However, *WRNexo* does not contain a helicase domain (Figure 1A). Purified WRNexo exhibits 3'-5' exonuclease activity on single-strand DNA, double-strand DNA with 5' overhangs, and substrates representing replication bubbles. However, WRNexo does not

digest substrates containing blunt ends or abasic sites (BOUBRIAK *et al.* 2009; MASON *et al.* 2013). Activities of WRNexo have been investigated *in vivo* through use of hypomorphic mutants (BOUBRIAK *et al.* 2009; RODRIGUEZ-LOPEZ *et al.* 2007; SAUNDERS *et al.* 2008). One such mutant, *WRNexo*^{e04496}, causes a severe reduction in WRNexo expression resulting from the presence of a piggyBac {RB} transposable element in the 5' UTR of WRNexo. *WRNexo*^{e04496} flies exhibit high sensitivity to the topoisomerase I inhibitor, camptothecin, as well as hyperrecombination. Female *WRN*exo^{e04496} mutants are sterile, but exhibit no other physiological abnormalities (SAUNDERS *et al.* 2008). A second mutant, *WRNexo*^{D229V}, contains a point mutation that ablates exonuclease activity at physiological temperatures (BOUBRIAK *et al.* 2009; MASON *et al.* 2013). Like *WRNexo*^{e04496}, *WRNexo*^{D229V} mutants display hyperrecombination. However, it is important to note that the phenotype of a true null WRNexo allele has yet to be described.

Though much work has been done to delineate the involvement of WRN in responding to replication stress, most hypotheses involve WRN's helicase activity while the role of the exonuclease remains poorly characterized. In this paper, we generate a *WRNexo* null mutant and show that it has defects in recovering from endogenous and exogenous replication stress. Additionally, we explore a role for WRNexo independent of its exonuclease activity and investigate interactions between WRNexo and the DNA repair proteins BLM and Rad51.

MATERIALS AND METHODS

Fly stocks

A deletion in *WRNexo* was created by imprecise *P*-element excision (ADAMS *et al.* 2003). For the screen, we used *w*^{1118}*; P{EP}G16048*, which contains a *P*-element located 441 base pairs upstream of the *WRNexo* transcription start site (BELLEN *et al.* 2004). The extent of the deletion was determined by Sanger sequencing of a PCR product obtained using the primers: WRN -1240F: 5'-GGCAGTCACTTCCTGCT-3' and 2001R: 5'-GACAACGATCTGCTC AAGCG-3'. The resulting deletion mutant, *WRNexo*$^{\Delta}$, was male sterile, likely due to a second site mutation generated during *P*-element mobilization. The *WRNexo*$^{\Delta}$ stock was backcrossed once to *w*^{1118} to remove the sterility phenotype.

Other mutants used in this study include *Brca2*^{KO}, which completely deletes *Brca2* (KLOVSTAD *et al.* 2008), *Blm*^{N1}, which removes a 2480 bp segment including part of the helicase domain (MCVEY *et al.* 2007) and *Rad51*^{057}, which contains an A148V point mutation in the *Rad51* gene (STAEVA-VIEIRA *et al.* 2003). *WRNexo*^{D229V} was generated through EMS mutagenesis (KOUNDAKJIAN *et al.* 2004). *Gen*^{Z4325}*, mus312*^{D1}*,* and *mus81*^{NheI} were used for analysis of structure-selective endonuclease mutants. *Df(3R)Exel6178,* which deletes 45 genes between cytological units 90F4-91A5, was used to create *WRNexo*$^{\Delta}$ compound heterozygotes. All double mutants were created by standard meiotic recombination and verified by PCR.

Mutagen Sensitivity Assays

Sets of 5-8 heterozygous virgin females and 2-3 (heterozygous or homozygous) males were paired in vials containing standard cornmeal agar medium. Females were allowed to lay eggs for 3 days at 25C before transfer into a second vial to lay for an additional 3 days. The first set of vials was treated with 250 µL camptothecin (dissolved in dimethyl sulfoxide, (DMSO)), bleomycin or hydroxyurea (dissolved in ddH_2O) one day after the transfer of parents. The second set of vials served as the controls and was treated with either 250 µL water (for bleomycin and hydroxyurea experiments) or a matching concentration of DMSO (for camptothecin experiments). Upon eclosion, adults were counted. Relative survival was calculated as (the percentage of viable homozygotes (relative to the total number of viable flies) in mutagen-treated vials) / (the percentage of viable homozygotes in control vials) for each trial. Statistical significance was analyzed using unpaired t tests.

Hatching Frequency Assay

$WRNexo^{\Delta}$, $WRNexo^{\Delta}$ / $WRNexo^{D229V}$, and *w^{1118}* flies were allowed to lay on grape juice agar plus yeast paste for a period of 8-16 hours at 25C. Each independent experiment consisted of 3-4 embryo collection periods for a total of 700-4000 embryos/experiment. After 72 hours, embryos were counted and hatching frequency was determined. Statistical significance was analyzed using unpaired t tests.

Embryo staining

$WRNexo^{\Delta}$ and *w^{1118}* flies were allowed to lay on grape juice agar plus yeast paste for a period of 3-4 hours at 25C. Embryos were then collected, devitillinized, fixed, and stained with a monoclonal antibody specific to γ-H2Av (LAKE *et al.* 2013) at a dilution of 1:3000. Embryos were then exposed to Texas Red®-conjugated rabbit anti-mouse IgG (Abcam) and DAPI at a dilution of 1:1000. Embryos were imaged using a Zeiss Axio Imager M1 microscope with 3D imaging capability and Slide Book software. Embryos used for analysis of nuclear distribution were within the syncytial division period (mitotic cycles 1-13) whereas embryos used for analysis of γ-H2Av were post-cellularization (after cycle 14). Image J was used to calculate the total area of each embryo in pixels. The area of the embryo containing γ-H2Av staining was then calculated to determine the percentage of each embryo in which cells expressed γ-H2Av. Statistical analysis was performed using GraphPad Prism, by combining *$WRNexo^{\Delta}$* and *w^{1118}* images from three separate embryo collection periods, harvested on three consecutive days. Statistical significance was determined using the Mann-Whitney U test.

Life stage-specific synthetic lethality

$Gen1^{Z4325}$ $WRNexo^{\Delta}$ / TM3 P{w[+mC]=ActGFPJMR2, Ser[1] and *$mus312^{D1}$ $WRNexo^{\Delta}$ / TM3 P{w[+mC]=ActGFPJMR2, Ser[1]* heterozygotes were each paired in vials and females were allowed to lay eggs for 2-3 days. The resulting progeny were counted daily from the onset of pupariation to eclosion. Heterozygotes and homozygotes were scored by presence

or absence of GFP respectively. Synthetic lethality was determined at the life stages at which no homozygotes were observed.

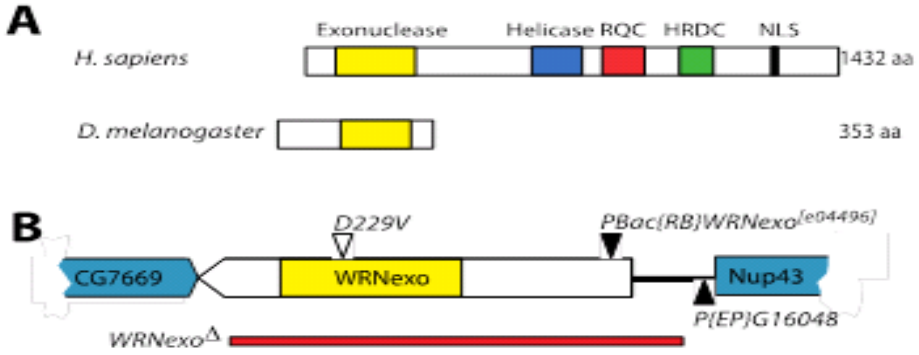
mus81^{NheI}*; WRNexo*$^{\Delta}$ */ TM3 P{w[+mC]=ActGFPJMR2, Ser[1]* heterozygotes were paired in bottles and females were allowed to lay eggs for 3-4 days. The resulting adult progeny were scored daily following eclosion. The percentage of adult homozygotes (survival ratio) was calculated and was compared to the expected survival ratio of 33% homozygotes using the chi-squared test. *mus81* ^{NheI}*; WRNexo*^{D229V} */ TM3 P{w[+mC]=ActGFPJMR2, Ser[1]* were grown and counted in a similar manner and compared to *mus81; WRNexo*$^{\Delta}$ survival ratios using a chi-squared test.

RESULTS

Characterization of WRNexo null mutants

Previous studies of Drosophila WRNexo were carried out with hypomorphic alleles. Therefore, we used imprecise *P*-element excision of a fly stock containing the *P{EP}G16048* transposable element to generate a WRNexo null mutant, *WRNexo*$^{\Delta}$. *WRNexo*$^{\Delta}$ deletes 426 base pairs upstream of the 5' UTR and 17 base pairs upstream of the 3' UTR. (Figure 1B). Using reverse transcriptase PCR, we showed that this deletion does not affect expression of the upstream gene, *Nup43* (data not shown). *WRNexo*$^{\Delta}$ mutants are homozygous viable, fertile, do not have any observable morphological defects, and eclose at Mendelian ratios (data not shown).

Figure 1: Generation of a WRNexo null mutant. A) Conserved regions of Werner protein in humans and Drosophila. **B)** A 2.5 kilobase deletion (red bar) that removes most of the WRNexo coding sequence was generated through imprecise excision of *P{EP}G16048*. Also shown is the location of *PBac{RB}WRNexo*$^{[e04496]}$ (BOUBRIAK *et al.* 2009; SAUNDERS *et al.* 2008) and the *D229V* exonuclease dead allele (BOUBRIAK *et al.* 2009; MASON *et al.* 2013).



A common phenotype of WS cells is sensitivity to the topoisomerase I inhibitor, camptothecin (CPT), which is due to an inability of these cells to repair double strand breaks (DSB) at affected replication forks (PICHIERRI *et al.* 2001; POOT *et al.* 2001). Similarly, the *WRNexo* hypomorphic fly strain, $WRNexo^{e04496}$, is sensitive to CPT (SAUNDERS *et al.* 2008). To assess CPT sensitivity of $WRNexo^{\Delta}$ mutants, we treated $WRNexo^{\Delta}$ larvae with either CPT or DMSO as a vehicle control. We then calculated relative survival by counting the adult homozygotes eclosed. $WRNexo^{\Delta}$ flies were not sensitive to CPT at doses up to 50 μM (Figure 2A). This result is in contrast to CPT sensitivity observed in flies lacking the homologous recombination gene, *Brca2* (THOMAS *et al.* 2013). We obtained similar results with flies containing a point mutation in *WRNexo* that ablates exonuclease activity, $WRNexo^{D229V}$ (BOUBRIAK *et al.* 2009; MASON *et al.* 2013). Likewise, when flies were treated with topotecan, a structural analog of CPT, no sensitivity was observed (data not shown). These data suggest that WRNexo does not play an important role in the resolution of DSBs caused by topoisomerase I inhibition.

WS cells have also been shown to exhibit a slight sensitivity to ionizing radiation (BOHR *et al.* 2001; POOT *et al.* 2001; YANNONE *et al.* 2001), demonstrating involvement of WRN in DSB repair outside of DNA replication. To investigate the possibility that WRNexo is involved in non-replication-based DSB repair, larvae were treated with the radiomimetic agent, bleomycin. Similar to the results with topoisomerase I inhibitors, $WRNexo^{\Delta}$ flies were not sensitive to bleomycin at doses up to 25 μM (Figure 2B). In contrast, flies with a deletion of *Blm*, a RecQ helicase that is important for homologous recombination, were extremely sensitive to bleomycin. Together, these results suggest that WRNexo is not required in homologous recombination-mediated repair of DSBs.

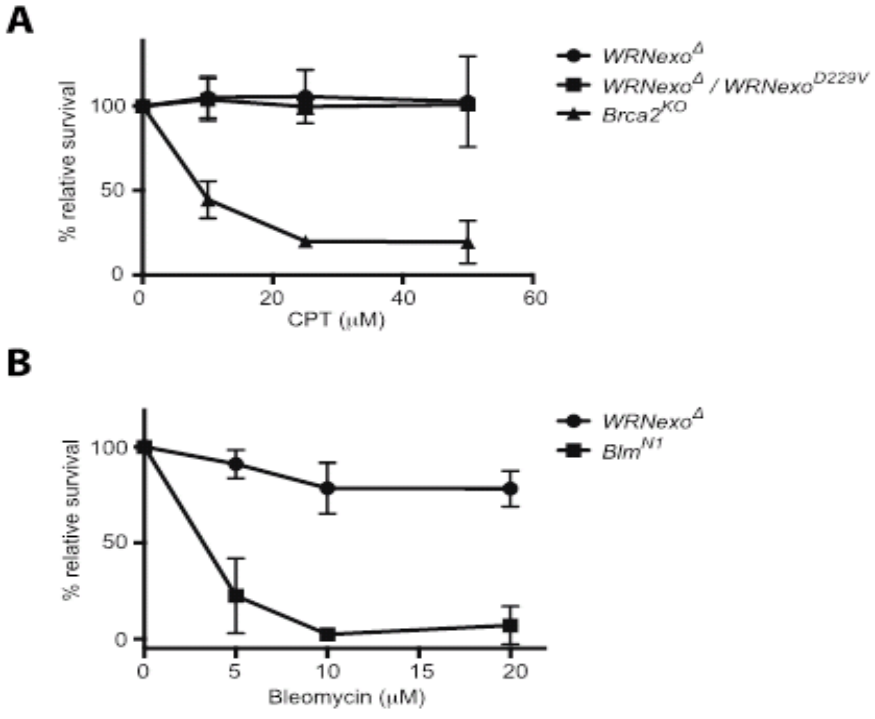


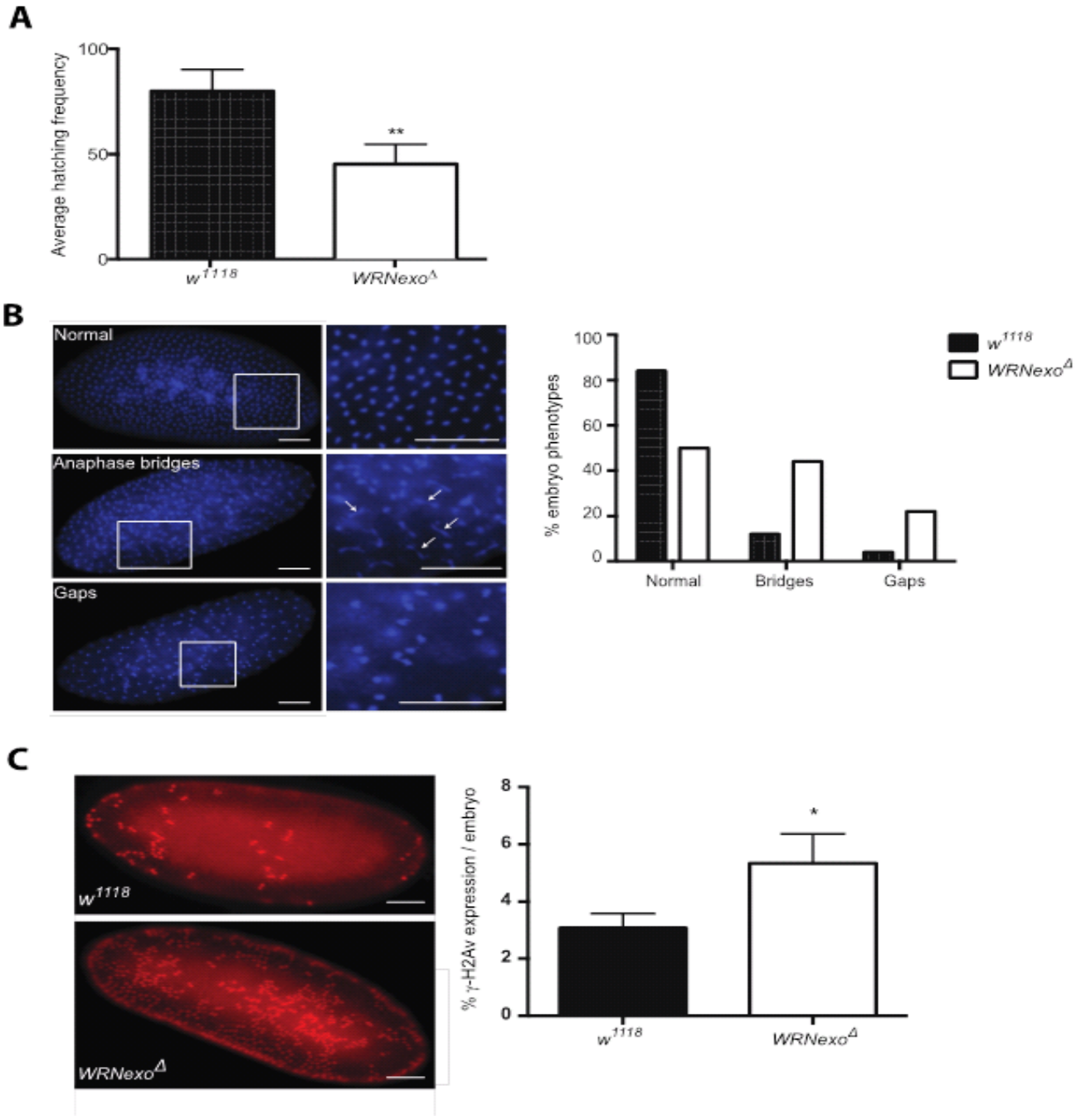
Figure 2: $WRNexo^{\Delta}$ mutants are not sensitive to double-strand break-inducing agents. A) Both $WRNexo^{\Delta}$ and compound heterozygous $WRNexo^{\Delta}$ / $WRNexo^{D229V}$ mutant larvae were exposed to increasing doses of camptothecin (CPT) and adult survival was determined. n = 3 trials for each dose. $Brca2^{KO}$ data were originally reported in (THOMAS *et al.* 2013). **B)** $WRNexo^{\Delta}$ and Blm^{N1} mutant larvae were exposed to increasing doses of bleomycin and adult survival was calculated. n=3

WRNexo is important during early development

WRNexo$^{\Delta}$ mutants exhibit a defect in hatching frequency in which an average of 45% of eggs laid hatch within a 72-hour period compared to 80% of eggs that hatch for *w*^{1118} controls (Figure 3A). We hypothesized that this phenotype arose from defects in syncytial nuclear division, a process in which nuclei divide 13 times prior to cellularization in the first two hours of embryogenesis (FOE 1993). To test this hypothesis, we stained embryos with the fluorescent DNA marker, DAPI, to visualize syncytial nuclei. Embryos undergoing normal syncytial division exhibit an even spatial pattern of nuclei. In contrast, we observed a range of phenotypes in syncytial *WRNexo*$^{\Delta}$ embryos, including a greater incidence of anaphase bridges, which may indicate incomplete replication or chromosome separation at the time of nuclear division (Figure 3B). We also observed cytoplasmic gaps between nuclei in syncytial *WRNexo*$^{\Delta}$ embryos. This phenotype may be due to the embryo's response to the presence of DNA damage, in which nuclei containing incompletely replicated DNA fall into the embryo interior (FOE 1993). Together, these phenotypes are consistent with defects in DNA replication and/or proper chromosomal segregation in the absence of WRNexo.

Many studies have demonstrated an abundance of DSBs in the absence of WRN either during normal cell growth or following treatment with a replication fork-stalling reagent (CHRISTMANN *et al.* 2008; FRANCHITTO *et al.* 2008; LIU *et al.* 2009; MAO *et al.* 2010; MURFUNI *et al.* 2012). To investigate whether the embryonic nuclear defects we observed were due to an accumulation of DSBs, we stained *WRNexo*$^{\Delta}$ embryos for phosphorylated histone 2Av (γ-H2Av). H2Av is homologous to mammalian H2AX (MADIGAN *et al.* 2002) and is considered a marker for the presence of DSBs and replication stress such as stalled forks (DE FERAUDY *et al.* 2010). We observed a greater number of γ-H2Av positive nuclei in *WRNexo*$^{\Delta}$ embryos compared to the *w*^{1118} controls (Figure 3C), indicating that WRNexo may be important for the prevention or repair of DSBs during embryogenesis.

Figure 3: WRNexo prevents DNA replication defects and the accumulation of double-strand breaks during early embryonic development. A) Hatching frequencies were determined for eggs laid by w^{1118} and $WRNexo^{\Delta}$ females. n = 3, at least 700 embryos were counted for each independent experiment. **p <0.01. **B)** DAPI staining of $WRNexo^{\Delta}$ embryos revealed an increased frequency of nuclear division defects, including the presence of anaphase bridges (arrows) and gaps between nuclei. n = 97 (w^{1118}) and 32 ($WRNexo^{\Delta}$). Bar = 100 μm. **C)** Embryos were fixed and stained with an antibody specific for γ-H2Av to determine incidence of double-strand breaks. Image J was used to quantify γ-H2Av staining as a ratio of embryo area and significance determined by Mann-Whitney U test. n = 35 (w^{1118}) and 27 ($WRNexo^{\Delta}$). *p <0.05. Bar = 100 μm.

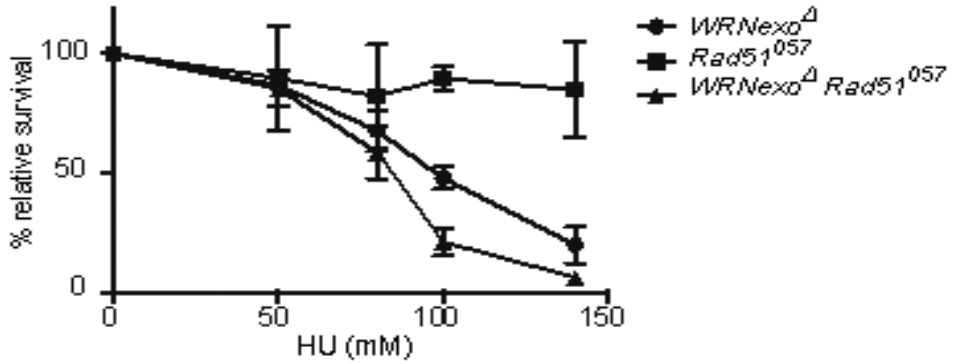


WRNexo is important for the stabilization of stalled replication forks

To further investigate a potential role for WRNexo in a replication stress response, we treated *WRNexo*$^{\Delta}$ larvae with increasing concentrations of the fork-stalling reagent, hydroxyurea (HU). HU induces replication arrest by inhibiting ribonucleotide reductase, leading to localized depletion of dNTPs. *WRNexo*$^{\Delta}$ homozygotes exhibited dose-dependent sensitivity to HU, with only 20% relative survival at 140 mM (Figure 4). Similar HU sensitivity was observed when the *WRNexo*$^{\Delta}$ mutation was combined with a deficiency chromosome, *Df(3R)Exel6178*, which lacks the WRNexo gene (data not shown). These results demonstrate that the HU sensitivity was caused specifically by loss of WRNexo.

Since stalled replication forks often generate DSBs due to fork collapse, we hypothesized that HU sensitivity in *WRNexo*$^{\Delta}$ mutants could occur because: 1) WRNexo is required for repair of DSBs, or 2) WRNexo prevents DSBs from occurring through the stabilization or restart of stalled replication forks. To distinguish between these possibilities, we measured HU sensitivity in flies that lack Rad51 and are therefore unable to repair DSBs by homologous recombination (HR) (STAEVA-VIEIRA *et al.* 2003). Interestingly, *Rad51*^{057} mutants were not sensitive to HU (Figure 4), suggesting that when WRNexo is present, treatment with HU does not result in the formation of significant numbers of DSBs that require Rad51-mediated HR repair. *WRNexo*$^{\Delta}$ *Rad51*^{057} double mutants were significantly more sensitive to HU than *WRNexo*$^{\Delta}$ single mutants. Thus, HU-induced fork stalling in the absence of WRNexo likely results in the formation of DSBs, at least some of which require Rad51-mediated HR for their repair.

Figure 4: WRNexo functions in a Rad51-independent pathway in replication. *WRNexo*$^{\Delta}$, *Rad51*^{057}, and *WRNexo*$^{\Delta}$ *Rad51*^{057} mutant larvae were exposed to hydroxyurea (HU) and adult survival was determined. n=3-7.



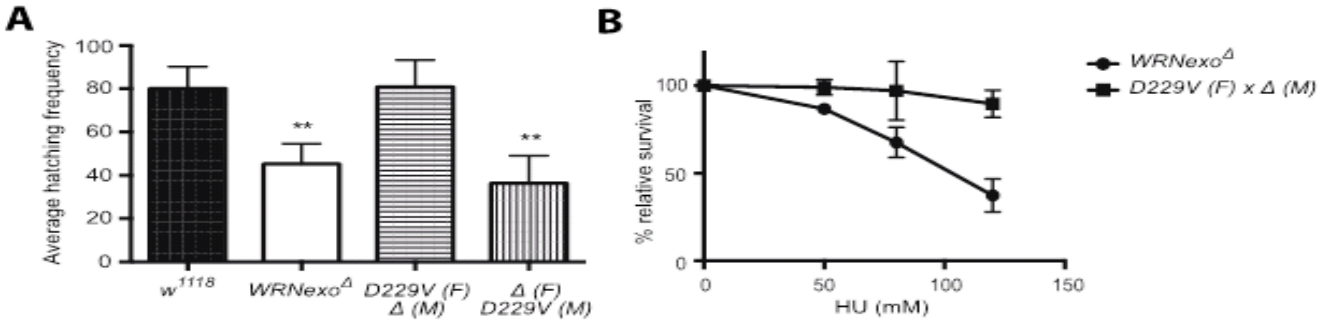
Embryonic defects and HU sensitivity of WRNexo mutants are exonuclease-independent

To determine if the phenotypes observed in *WRNexo*$^{\Delta}$ mutants are due to loss of exonuclease activity, we repeated our experiments with *WRNexo*^{D229V} flies. The *D229V* mutant protein has been well characterized *in vitro* and exhibits no exonuclease activity on WRNexo DNA substrates at physiological conditions (BOUBRIAK *et al.* 2009; MASON *et al.* 2013). To control for the effects of potential second site mutations on the *D229V* chromosome, *WRNexo*^{D229V} / *WRNexo*$^{\Delta}$ compound heterozygotes were used in these experiments.

Unlike *WRNexo*$^{\Delta}$ females, *WRNexo*^{D229V} / *WRNexo*$^{\Delta}$ females did not show a decrease in hatching frequency (Figure 5A). However, embryos laid by *WRNexo*$^{\Delta}$ females that were mated with *WRNexo*^{D229V} / *WRNexo*$^{\Delta}$ males exhibited a reduced hatching frequency similar to *WRNexo*$^{\Delta}$. The normal hatching frequency of embryos laid by *WRNexo*^{D229V} females is likely explained by maternal loading of *D229V* transcript and/or protein into the eggs of *WRNexo*^{D229V} females since in Drosophila, zygotic transcription does not begin until mitotic cycle 13 or approximately two hours into embryogenesis (FOE 1993). Together, these data suggest that the presence of WRNexo protein, but not its exonuclease activity, contributes to normal development during the first two hours of embryogenesis.

Given this unexpected result, we were interested in investigating whether exonuclease-dead WRNexo protein was sufficient to rescue other *WRNexo*$^{\Delta}$ phenotypic defects. Thus, we assessed the sensitivity of *WRNexo*^{D229V} mutants to hydroxyurea (HU). *WRNexo*^{D229V} / *WRNexo*$^{\Delta}$ virgin females were crossed to *WRNexo*$^{\Delta}$ males and the resulting larvae were treated with HU. Surprisingly, *WRNexo*^{D229V} mutants were not sensitive to HU (Figure 5B). This result suggests that exonuclease activity is not important for the role of WRNexo in stabilizing or restarting stalled replication forks.

Figure 5: WRNexo exonuclease activity is not required for normal embryogenesis and hydroxyurea resistance. The D229V point mutation in WRNexo ablates exonuclease activity at physiological conditions (BOUBRIAK *et al.* 2009; MASON *et al.* 2013). **A)** Hatching frequencies were measured for embryos obtained from crosses between *w*^{1118}, *WRNexo*$^{\Delta}$, and *WRNexo*$^{\Delta}$ / *WRNexo*^{D229V} flies as well as crosses between *WRNexo*$^{\Delta}$ females and *WRNexo*$^{\Delta}$ / *WRNexo*^{D229V} males. n = 3, at least 700 embryos were counted for each independent experiment. **B)** *WRNexo*$^{\Delta}$ / *WRNexo*^{D229V} compound heterozygous larvae were treated with HU and adult survival was calculated. n = 3. **p<0.01.

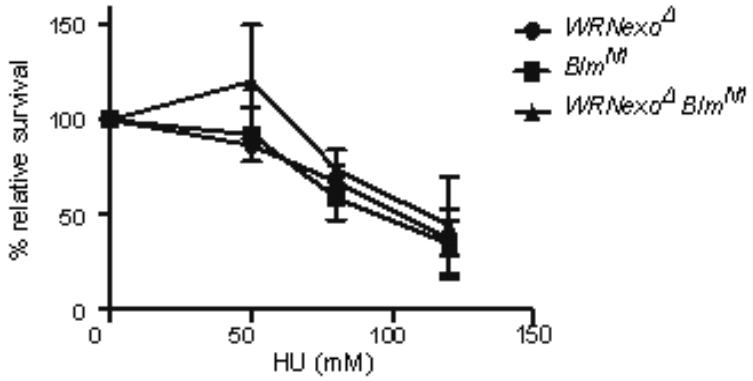


WRNexo may interact with the BLM helicase

In light of our finding that WRNexo exonuclease activity is not important for either normal embryonic development or HU resistance, we speculated that WRNexo may instead recruit another RecQ helicase, such as BLM, to mediate these processes. In humans, both BLM and WRN are important for the resolution of Holliday junctions (MACHWE *et al.* 2011) and have been found to co-localize and physically interact with each other in cell culture (VON KOBBE *et al.* 2002). In Drosophila, BLM is encoded by the *mus309* gene and is highly homologous to human BLM. Like *WRNexo*$^{\Delta}$ mutants, Drosophila *Blm* mutants have nuclear defects during embryogenesis, suggesting that BLM is involved in similar developmental processes (MCVEY *et al.* 2007). However, HU sensitivity of *Blm* mutants has not been reported.

We assessed HU sensitivity in *Blm*^{N1} and *WRNexo*$^{\Delta}$ *Blm*^{N1} mutants to discern if BLM shares a role with WRNexo in recovery from fork stalling. *Blm*^{N1} and *WRNexo*$^{\Delta}$ *Blm*^{N1} mutants exhibited similar sensitivity as *WRNexo*$^{\Delta}$ mutants (Figure 6), suggesting that BLM and WRNexo work in the same pathway following HU-induced replication fork stalling.

Figure 6: WRNexo and Blm have an epistatic relationship in response to hydroxyurea-induced replication stress. *WRNexo*$^{\Delta}$, *Blm*^{N1}, and *WRNexo*$^{\Delta}$ *Blm*^{N1} mutant larvae were exposed to hydroxyurea (HU) and adult survival was calculated. n=3



Stalled replication forks may form Holliday junctions and intermediates, such as four-way junctions that are cleaved by structure-selective endonucleases (SSEs) such as MUS81, MUS312 (SLX4), and GEN. It is thought that in the absence of BLM, cleavage of these structures by SSEs promotes mitotic crossovers (ANDERSEN *et al.* 2011). In Drosophila, BLM and SSEs comprise two alternative mechanisms for an essential cellular function, as flies that lack both BLM and a single SSE exhibit developmental stage-specific synthetic lethality. *mus81; Blm* mutants arrest as pharate adults, while *Blm mus312* mutants die as pupae and *Blm Gen* mutants do not progress past the first instar larval stage (ANDERSEN *et al.* 2011).

To determine if WRNexo is required in the absence of SSEs, we created double mutants and monitored their developmental progression. We observed that *WRNexo*$^{\Delta}$ *SSE* double mutants also display synthetic lethality, but die at later developmental stages than *Blm SSE* mutants (Figure S1). *mus81*^{NheI}*; WRNexo*$^{\Delta}$ survived to the adult stage; however, homozygotes eclosed at frequencies lower than predicted by Mendelian ratios and demonstrated poor survival (Figure S2). *mus312*^{D1} *WRNexo*$^{\Delta}$ mutants survived until the

pharate adult stage, while *Gen*^{Z4325} *WRNexo*$^{\Delta}$ mutants arrested as pupae. Our results are consistent with the observation that *Blm Gen* mutants have the most deleterious phenotype of all of the *Blm SSE* mutant combinations (ANDERSEN *et al.* 2011). Because loss of either BLM or WRNexo results in synthetic lethality in the absence of SSEs, it is likely that these two proteins share a common role in stabilizing or resolving replication intermediates that arise during development.

We were interested to see whether the exonuclease activity of WRNexo is important to prevent the lethality observed in *WRNexo*$^{\Delta}$ *SSE* double mutants. Thus, we created a *mus81*^{NheI}*; WRNexo*^{D229V} mutant, which like *mus81*^{NheI}*; WRNexo*$^{\Delta}$, survived to adulthood. However, in contrast to *mus81*^{NheI}*; WRNexo*$^{\Delta}$ mutants*, mus81*^{NheI}*; WRNexo*^{D229V} homozygotes are healthy and eclose at significantly higher ratios ($\chi^2 = 251$, $p < 0.001$, Figure S2). This result suggests that in absence of SSEs, the presence of WRNexo, but not its exonuclease activity, is required to produce phenotypically normal adults.

DISCUSSION

The WRN protein is critically important for the maintenance of genome stability, due to its multiple roles in DNA replication, repair, and the prevention of aberrant recombination. However, most published WRN studies have focused on potential roles of its helicase domain. We took advantage of Drosophila's highly conserved exonuclease domain, allowing us to study the role of its exonuclease activity independently from that of the helicase domain. Here, we have demonstrated that Drosophila WRNexo is important for recovery following both endogenous and exogenous replication stress. Importantly, its role is independent of Rad51-mediated homologous recombination repair. Our results also show that the critical role of WRNexo during replication stress does not depend on exonuclease activity, suggesting that it acts as part of a larger protein complex to respond to stalled or collapsed replication forks. Because *WRNexo*$^{\Delta}$ and *Blm* mutants have similar phenotypes, we speculate that the two RecQ orthologs may constitute or be critical members of this complex.

An important role for WRNexo during early embryogenesis

We have identified a requirement for WRNexo during early embryogenesis as shown by the presence of anaphase bridges and gaps in nuclear distribution in *WRNexo*$^{\Delta}$ embryos. Drosophila embryos go through 13 syncytial nuclear divisions prior to cellularization of the blastoderm, which takes place in the first two hours after fertilization (FOE 1993). This rapid replication may result in fork arrests, which if not restarted, could contribute to improper chromosomal segregation, and/or improper nuclear division. These defects can manifest as anaphase bridges and uneven nuclear distribution. It is possible that *WRNexo*$^{\Delta}$ embryos are unable to rapidly process stalled replication forks, resulting in slowed replication that does not allow for proper nuclear division and embryonic development. Human WS cells exhibit a

prolonged S phase, indicating slower replication or inhibition of the S phase checkpoints (CHENG *et al.* 2007). More specifically, it has been proposed that WRN is required for promoting DNA elongation following replication fork restart, resulting in shorter nascent DNA tracts in cells lacking functional WRN (RODRIGUEZ-LOPEZ *et al.* 2002; SIDOROVA *et al.* 2008).

Even if *WRNexo*$^{\Delta}$ embryos do successfully complete the syncytial divisions, accumulation of DNA damage may hinder further embryonic development. We observed a greater percentage of γ-H2Av positive nuclei in *WRNexo*$^{\Delta}$ embryos, which can be interpreted as a higher incidence of double-strand breaks (DSBs), However, it is important to note that the presence of γ-H2Av may not exclusively indicate DSBs, but may also be a signal for replication stress and stalled replication forks (DE FERAUDY *et al.* 2010). Our finding is consistent with studies in which elevated levels of endogenous DSBs were observed in WS and WRN-depleted cells (FRANCHITTO *et al.* 2008; MAO *et al.* 2010; OPRESKO *et al.* 2007; PICHIERRI *et al.* 2001; SZEKELY *et al.* 2005; VON KOBBE *et al.* 2004).

WRNexo demonstrates a Rad51-independent role in promoting recovery of stalled replication forks

In addition to phenotypic defects caused by endogenous replication stress, *WRNexo*$^{\Delta}$ mutants are sensitive to the fork-stalling reagent, hydroxyurea (HU). Following HU treatment, the stalled replication fork can either collapse, forming a DSB, or undergo regression, forming an intermediate four-way junction or “chicken foot” structure. Fork restart can occur through reversal of the regressed fork or by cleavage of the Holliday junction by endonucleases followed by homologous recombination (HR)-mediated repair (OSBORN *et al.* 2002). In order to delineate how WRNexo may contribute to stalled fork recovery, we tested HU sensitivity of flies in both *WRNexo*$^{\Delta}$ and *Rad51*^{057} mutant backgrounds. *WRNexo*$^{\Delta}$ single mutants were sensitive to HU, whereas *Rad51*^{057} larvae lacking Rad51 were resistant to HU. Since Rad51, and therefore HR repair, is not required for HU resistance, it is likely that when WRNexo is present, our treatment protocol does not induce DSBs. Meanwhile, we observed high HU sensitivity in *WRNexo*$^{\Delta}$ *Rad51*^{057} double mutants, suggesting that WRNexo and Rad51 operate in separate pathways in response to HU-induced replication fork stalling. We propose that in the presence of WRNexo, regressed replication forks undergo reversal and subsequent recovery and restart (Figure 7). When WRNexo is absent, this reversal process is impaired and the regressed forks can be cleaved by endonucleases. The resultant DSBs can be repaired by HR in a WRNexo-independent manner. This model is supported by evidence that WRN prevents the occurrence of DSBs and subsequent recruitment of Rad51 in human cells (FRANCHITTO *et al.* 2008; PICHIERRI *et al.* 2011).

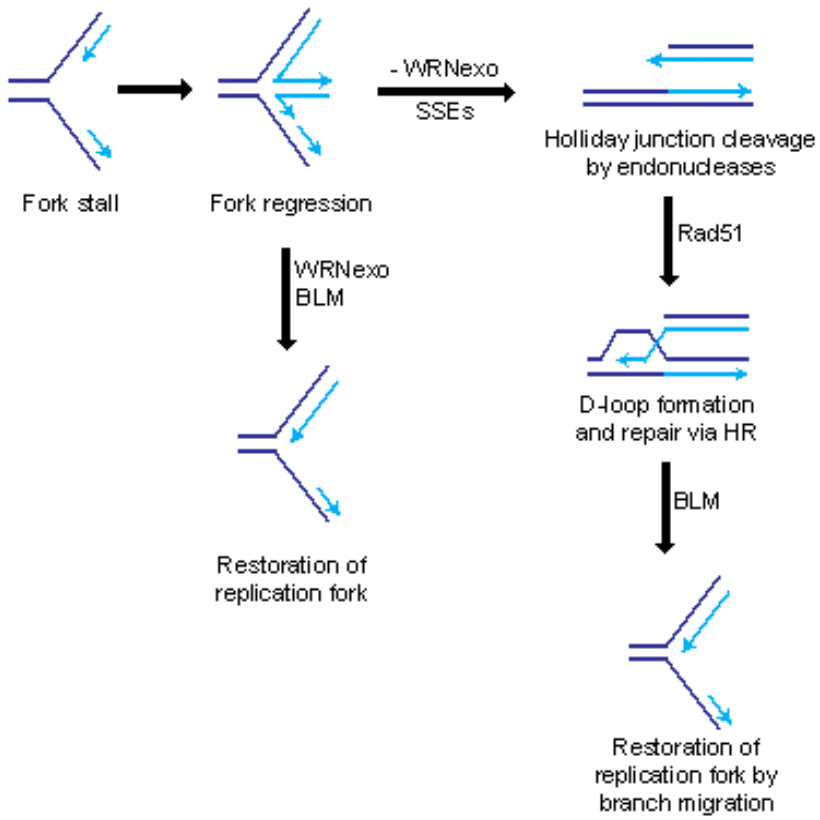


Figure 7: A model for the role of WRNexo in recovery from replication fork stalling. Stalled replication forks can undergo regression forming an intermediate "chicken foot" structure. Fork restart can occur through WRNexo-mediated reversal of the regressed fork, possibly through recruitment of BLM helicase. In the absence of WRNexo, the four-way junctions are cleaved by endonucleases and repaired by HR in a Rad51-dependent manner.

Our finding that WRNexo operates in a Rad51-independent manner in response to HU-induced replication stress contrasts with several lines of evidence supporting a role for WRN in HR. Co-localization of WRN and Rad51 has been observed in human cell culture, though no direct interaction between the proteins has been observed (SAKAMOTO *et al.* 2001). Likewise, Sidorova *et al.* observed an epistatic relationship between WRN- and Rad51-depleted cells in response to HU treatment, suggesting that these proteins collaborate at stalled forks (SIDOROVA *et al.* 2013). We hypothesize that in humans, the role of WRN in HR is helicase-mediated, which further supports the use of Drosophila as a model to delineate exonuclease-specific functions of WRN.

Our data demonstrating insensitivity of *WRNexo*$^{\Delta}$ to the topoisomerase I inhibitors, camptothecin and topotecan, as well as the radiomimetic agent, bleomycin, further support our hypothesis that WRNexo is not involved in HR repair of DSBs. Both camptothecin and topotecan cause replication-dependent DNA breaks that are usually repaired by HR. Camptothecin sensitivity is a hallmark phenotype of WS cells, likely due to lack of WRN helicase activity. Since WRNexo lacks a helicase domain, a different helicase may be involved in responding to camptothecin-induced damage in Drosophila. Although the *WRNexo*^{e04496} hypomorphic mutant is sensitive to camptothecin (SAUNDERS *et al.* 2008), other observed phenotypic differences between *CG7670*^{e04496} and *WRNexo*$^{\Delta}$, such as female sterility, lead us to postulate that *CG7670*^{e04496} may contain one or more second site mutations that could be responsible for these phenotypes.

WRNexo's role in recovering from replication stress is exonuclease-independent

Human WRN exonuclease acts at stalled replication forks, specifically by degrading the leading strand of four-way junctions produced by regression of stalled forks (MACHWE *et al.* 2011). Therefore, we had originally assumed that the defects observed in our *WRNexo*$^{\Delta}$ mutants were due to lack of exonuclease activity. Surprisingly, we found that eggs laid by *WRNexo*^{D229V} females had normal hatching frequencies and *WRNexo*^{D229V} mutant larvae were not sensitive to HU.

The biochemical properties of the *D229V* mutation have been characterized extensively *in vitro* (BOUBRIAK *et al.* 2009; MASON *et al.* 2013). The aspartate at amino acid position 229 is not located within the putative active site of WRNexo. Instead, the *D229V* mutation is thought to alter the surface structure of the protein, compromising the ability of WRNexo to bind DNA and guide it to the active site (MASON *et al.* 2013). Under physiological conditions, WRNexo containing the *D229V* mutation exhibits no exonuclease activity on its preferred substrates: single-strand DNA and double-strand DNA containing a 5' overhang (BOUBRIAK *et al.* 2009). Furthermore, the *D229V* mutation is non-processive, limiting digestion to a single nucleotide (MASON *et al.* 2013). Because *D229V* ablates exonuclease activity at physiological conditions, it is unlikely that *WRNexo*^{D229V} mutants possess exonuclease activity that would result in normal phenotypes. In support of this, *WRNexo*^{D229V} flies exhibit elevated mitotic recombination, suggesting that WRNexo exonuclease activity is required to prevent aberrant HR and excessive recombination (BOUBRIAK *et al.* 2009).

Since the exonuclease activity of WRNexo is not required for a proper response to endogenous and exogenous replication stress, we hypothesize that WRNexo may instead act as a scaffold for other DNA repair proteins. Human WRN has been shown to physically bind to several proteins within the exonuclease domain, including Ku80 (LI and COMAI 2000) and BLM (VON KOBBE *et al.* 2002). Furthermore, it has been suggested that WRN recruits DNA processing proteins to DNA damage sites due to its ability to bind both proteins and replication intermediates (KAMATH-LOEB *et al.* 2012). Therefore, there is a strong possibility that WRNexo binds similar repair proteins in Drosophila.

WRNexo may interact with BLM at stalled replication forks

We showed that *WRNexo*$^{\Delta}$, *Blm*^{N1}, and *WRNexo*$^{\Delta}$ *Blm*^{N1} double mutants exhibit similar sensitivity to HU. This epistatic relationship suggests that WRNexo and BLM interact following replication stress and may promote reversal of the regressed replication fork (Figure 7). Mao *et al.* also discovered an epistatic relationship between WRN and BLM in which co-depletion of these proteins suppressed proliferation in cell culture to the same degree as BLM-depleted cells (MAO *et al.* 2010). Similarly, WRN and BLM are both required for fork progression following HU treatment as shown by cell cycle delay when both proteins were depleted (SIDOROVA *et al.* 2013). This result demonstrates the ability of WRN and BLM to partially substitute for each other in responding to stalled replication forks, likely due to their shared helicase function. Since WRNexo does not contain a helicase, our results suggest a

novel interaction between WRNexo and BLM in recovery of stalled replication forks in Drosophila.

We have also shown that mutants in both WRNexo and the structure-selective endonuclease genes *mus312*^{D1} and *Gen*^{Z4325} are synthetically lethal at different developmental stages. Synthetic lethality was also observed in flies mutant in *Blm* and either *mus81*, *mus312* or *Gen*, but at earlier development time points than observed in *WRNexo*$^{\Delta}$ mutants (ANDERSEN *et al.* 2011). These results suggest that WRNexo and BLM may have a shared role in development. We hypothesize that WRNexo and BLM are important for an efficient response to replication-related problems that arise during various stages in development. In the absence of WRNexo and BLM, stalled replication forks cannot be restarted and instead, replication intermediates are processed by SSEs (Figure 7). If SSEs are also unavailable, improper chromosome segregation and cell death occur.

Although *WRNexo* and *Blm* mutants exhibit similar phenotypes in response to HU treatment and loss of SSEs, it is unlikely that deletion of WRNexo results in destabilization of BLM and a reduction in its activity. We have observed strong sensitivity of *Blm*^{N1} mutants to the DSB-inducing reagent, bleomycin, a phenotype not shared by *WRNexo*$^{\Delta}$ mutants (Figure 2B). This result demonstrates that BLM is involved in repair pathways independent of WRNexo and suggest that BLM protein is stably expressed in *WRNexo*$^{\Delta}$ mutants.

We propose that in Drosophila, BLM may serve as a "partner helicase" with WRNexo to carry out functions similar to WRN in human cells. This hypothesis is supported by evidence that WRN physically interacts with BLM in human cells and more importantly, binds BLM within its exonuclease domain (VON KOBBE *et al.* 2002). We showed that the exonuclease activity of WRNexo is not important in recovery from replication stress using *WRNexo*^{D229V} mutants. However, because the *D229V* mutation has been postulated to affect DNA binding (Mason et al, 2013), it is possible that any residual exonuclease activity in this mutant may be enhanced through an interaction with BLM. This seems unlikely, given that in humans, WRN and BLM have different substrate preferences (KAMATH-LOEB *et al.* 2012; VON KOBBE *et al.* 2003), and the exonuclease activity of WRN is inhibited when bound to BLM (VON KOBBE *et al.* 2003). Therefore, our data are most consistent with a scenario in which WRNexo recruits BLM to stalled replication forks where BLM can act to unwind replication intermediates to promote fork progression (Figure 7). WRNexo and BLM may also work together to prevent DSBs from occurring through alternate processing of replication intermediates. This alternate processing can result in unscheduled recombination events and elevated mitotic recombination, which has been described in both *Blm* and *WRNexo* mutants (MCVEY *et al.* 2007; SAUNDERS *et al.* 2008).

In summary, our findings support a novel, exonuclease-independent role for WRNexo in recovering from both endogenous and exogenous replication stress in Drosophila. To date, many investigations have attributed WRN's involvement in replication processes to its helicase activity. Therefore, our findings suggest that further investigation of exonuclease-specific functions of WRN is warranted.

Acknowledgements: We thank Jeff Sekelsky for his generous donation of fly stocks and the γ-H2Av antibody and Adam Thomas for use of his data describing CPT sensitivity in *Brca2*^{KO} mutants. We also thank Bloomington stock center for mutant fly stocks. This work was funded by the National Institute of General Medical Sciences IRACDA program (K12GM074869) and by NSF grant MCB0643253. Additionally, our undergraduate researchers were supported by the NSF Research Experience for Undergraduates program (DBI1263030).

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