Evidence for premature aging in a Drosophila model of Werner syndrome

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A R T I C L E   I N F O

Section Editor: Sean Curran
Keywords:
DNA repair
Werner syndrome
Aging
Tumor
Locomotor function

Werner syndrome (WS) is an autosomal recessive progeroid disease characterized by patients’ early onset of aging, increased risk of cancer and other age-related pathologies. WS is caused by mutations in WRN, a RecQ helicase that has essential roles responding to DNA damage and preventing genomic instability. While human WRN has both an exonuclease and helicase domain, Drosophila WRNexo has high genetic and functional homology to only the exonuclease domain of WRN. Like WRN-deficient human cells, Drosophila WRNexo null mutants (WRNexoΔ) are sensitive to replication stress, demonstrating mechanistic similarities between these two models. Compared to age-matched wild-type controls, WRNexoΔ flies exhibit increased physiological signs of aging, such as shorter lifespans, higher tumor incidence, muscle degeneration, reduced climbing ability, altered behavior, and reduced locomotor activity. Interestingly, these effects are more pronounced in females suggesting sex-specific differences in the role of WRNexo in aging. This and future mechanistic studies will contribute to our knowledge in linking faulty DNA repair mechanisms with the process of aging.

1. Introduction

Werner Syndrome (WS) is an autosomal recessive progeroid disease that affects 1 in 1,000,000 to 1 in 10,000,000 individuals (reviewed in Shamanna et al., 2017). WS is characterized by accelerated aging that usually becomes apparent when patients lack a growth spurt during puberty. WS patients’ expected lifespan is 55 due to increased incidence of heart disease and cancer. Other aging-associated pathologies typical of WS patients include type II diabetes, cataracts, subcutaneous fat loss, osteoporosis, gonadal atrophy and atherosclerosis. Additionally, WS patients may have physical characteristics such as “pinched” face, truncal obesity, and thin limbs, which are indicative of muscle degeneration (reviewed in Yokote et al., 2017).

WS is caused by mutations in WRN, an essential gene for maintaining genomic stability. WRN is a member of the RecQ family of helicases and participates in essential cellular functions such as DNA replication, transcription, recombination, and repair, as well as telomere maintenance (reviewed in Shamanna et al., 2017). Like all RecQ helicases, WRN possesses 3′→5′/5′→3′ ATP-dependent helicase activity. Additionally, WRN is unique in that it has 3′→5′ exonuclease activity (Croteau et al., 2014). To date there are 83 unique WRN mutation variants described, most of which result in a truncation of the 1432-amino acid protein sequence and a loss of the nuclear localization signal (Chun, 2011; Yokote et al., 2017). Fibroblasts from WS patients have increased chromosomal translocations (Au et al., 2008) and an accelerated rate of senescence that is rescued by telomere elongation (Crabbe et al., 2007; Wylie et al., 2000).

Various mouse models have been generated to study WS. However, unlike WS patients, WRN−/− mice are phenotypically normal (Chang et al., 2004; Lebel and Leder, 1998; Lombard et al., 2000). Like WRN−/− mice, WRN helicase-ablated mice (WRNΔhel/Δhel) are phenotypically normal for the first year, but have a shorter median life span overall (Lebel and Leder, 1998). WRNΔhel/Δhel mice also have increased autophagy, inflammatory cytokines, triglycerides, and reactive oxygen species (Lebel and Leder, 1998; Massip et al., 2006), possibly due to mislocalization of the WRN protein to the endoplasmic reticulum (Lombard et al., 2000). To predispose WRN null mice to increased tumor incidence similar to WS patients, researchers combined WRN deficiencies with a deletion of the tumor suppressor gene, p53. WRNΔhel/Δhel p53−/− mice showed rapid tumor growth and high variability of tumor types (Lebel et al., 2001). While WRN−/− p53−/− mice were shorter lived than WRN−/−, they had normal cell proliferation and...
lacked abnormal lesions or tumors (Lombard et al., 2000). Because of WRN’s role in telomere maintenance, WRN+/− mice were created in combination with a null allele of the telomerase RNA component Terc. WRN+/− Terc−/− mice showed premature aging symptoms such as heightened replicative senescence, DNA damage accumulation in cell culture, and increased tumor incidence, making this the most accurate mouse model of WS to date (Chang et al., 2004).

*Drosophila* are rapidly becoming a standard metazoan model organism with which to study mechanisms of human disease. *Drosophila* share approximately 75% similarity with human disease-causing genes (Rubin et al., 2000), making them a tractable genetic model for this purpose. Additionally, *Drosophila* have short generation time and life-spans, allowing for quicker manifestation of age-related pathologies. Furthermore, *Drosophila* are genetically malleable, allowing for creation of multiple alleles and conditional mutants that can be used to study physiological processes at different stages of development. To this effect, *Drosophila* models have been generated for neurodegenerative diseases associated with aging such as Parkinson’s, Amyotrophic Lateral Sclerosis (ALS), and Huntington’s (reviewed in Yamaguchi, 2018). Although faulty DNA repair mechanisms often lead to age related diseases, there are relatively few fly models directly linking faulty DNA repair and replication with aging and human disease genes (Garcia et al., 2011; Mounkes et al., 1992; Rimkus et al., 2008; Wu et al., 2008).

The *Drosophila* WRNexo gene shares 35% identity and 59% similarity to exonuclease domain of human WRN (Saunders et al., 2008). Because WRNexo does not contain a helicase domain, we can clearly investigate exonuclease-only roles of the protein, which are more well explored in WS human cell culture and mouse models. Like human WRN, purified WRNexo displays ATP-dependent 3′→5′ exonuclease activity on a variety of DNA substrates, but does not digest blunt ends or abasic sites (Boubriak et al., 2008; Mason et al., 2013). Several hypomorphic WRNexo mutants have been characterized and include phenotypes such as hyperrecombination, female sterility, and sensitivity to the topoisomerase I inhibitor, camptothecin (Boubriak et al., 2008; Mason et al., 2013; Saunders et al., 2008). These phenotypes may be in part attributed to the p-element transposon insertion methodology by which the hypomorphs were created. Second site mutations are commonly generated in these, which may give rise to phenotypes that could be erroneously attributed to the gene of interest (Thomas et al., 2013; Venken and Bellen, 2014). True null WRNexo mutants, WRNexoΔ, are viable and fertile (Bolterstein et al., 2014). Additionally, WRNexoΔ mutants show enhanced embryonic DNA damage and lethality and are sensitive to the DNA replication fork stalling drug, hydroxyurea, suggesting a role for WRNexo in responding to replication stress, especially during early embryogenesis (Bolterstein et al., 2014). However, the physiology of this mutant and the presence of WS-like phenotypes have not yet been evaluated.

Here, we characterize the physiological phenotypes of the WRNexoΔ mutant *Drosophila*. In doing so, we have evaluated WS-like signs of accelerated aging by assessing adult mortality, tumor incidence, body composition, muscular degeneration, and locomotor activity. Together our results present the *Drosophila* WRNexoΔ mutant as a tractable WS model that we can use to better understand mechanisms of aging.

2. Material and methods

2.1. Fly stocks

All fly stocks were maintained on solid cornmeal agar and kept at 25°C under a 12 h:12 h light-dark cycle. WRNexoΔ null mutants were created as described in Bolterstein et al. (2014). For life span and tumor incidence analyses, we used an additional WRNexoΔ stock that was outcrossed four times to w1118 to remove the presence of second site mutations.

For all adult experiments, flies were allowed to mate for 24–48 h following eclosion and then separated by sex under CO₂ anesthetization. For aging experiments, flies were maintained in vials of approximately 20 individuals and transferred to new food every 2–3 days for the duration of aging. For our experimental purposes, “young” flies are 1–4 days old and “old” flies are 28 or 35 days old as noted. For lifespan analysis, 150–200 flies of the same sex and genotype were placed in a population cage that contained a vial of cornmeal agar. Population cages were maintained at 25°C and every 2–3 days dead flies were counted and removed, and food was replaced. A total of 3 biological replicates were performed.

2.2. Histology

Flies selected for pathological analysis were placed in Telly’s fixative (20 parts 70% ethanol, 2 parts 37% formalin, 1 part glacial acetic acid) for at least 48 h prior to sequential immersion in neutral buffered formalin, ethanol and xylene according to standard protocols. Flies were subsequently perfused with wax and embedded in paraffin blocks. They were then sliced into 6 μm thick sections and placed on positively charged glass slides. Slides were baked overnight at 65°C to increase tissue adherence prior to staining with hematoxylin and eosin.

2.3. Larval buoyancy assay

Methods were modified from (Reis et al., 2010). Flies were allowed to lay eggs for 24 h. After 5–6 days, third instar wandering larvae were removed from vials by adding 20% sucrose. Larvae were rinsed in PBS and sets of 30 wandering larvae were transferred to vials containing 4 mL of 8.5% sucrose in PBS. Larvae were agitated and allowed to settle before scoring floating as defined as larvae at the surface of the liquid. A solution of 50% sucrose in PBS was incrementally added and floating larvae scored until all larvae were floating.

2.4. Negative geotaxis assay

Flies were anesthetized using CO₂ and separated by sex at approximately 20 flies per vial. Young flies were allowed 24–48 h to recover from anesthetization prior to testing; aged flies were tested without anesthetization. Flies were then transferred to an empty vial with markings in 2 cm intervals. Flies were tapped to the bottom of the vial 5–6 times with 1 min of rest in between trials. Experiments were videotaped and later scored for the number of flies to cross the 6 cm mark in 10 s.

2.5. Locomotor activity

Continuous monitoring of locomotor activity was assessed using Drosophila Activity Monitors (DAM2, TriKinetics). Each monitor contained 32 channels and each channel recorded the movements (breaks of an infrared beam in the center of the vial) of a single fly. Flies were anesthetized using CO₂ and individual flies were placed into 5 mm plastic vials (TriKinetics) containing solid media (5% sucrose, 2% agar) and sealed with a small piece of cotton (Pike et al., 2010). Single flies were continually monitored in 1-minute intervals over a 6 day period (experimental days 2–7) using the TriKinetics software. 2–4 biological replicates were performed for each condition tested. Overall activity was calculated in R (R Core Team, 2017) by first calculating the average movements (beam breaks) per minute for each individual fly for each hour of the day (0–23h). These data were then averaged by genotype, sex, and age. Hourly activity was calculated in a similar manner: first, the average activity per minute for each individual fly for each hour of the day was calculated and then these data were averaged by hour, genotype, sex, and age.

2.6. Statistical analysis

Large data sets were assessed for Gaussian distribution using the
D’Agostino & Pearson omnibus normality test; data that did not follow a Gaussian distribution was analyzed using a non-parametric test. Survival curves were compared by the Log-rank Mantel-Cox test and average lifespan, median lifespan, and 90% mortality were analyzed by 2-way ANOVA and multiple comparisons assessed using Tukey’s post-test. Total tumor frequency was assessed by Fisher’s exact test. Larval buoyancy at matched sucrose concentrations was assessed using the Kruskal-Wallis test with Dunn’s post-test for multiple comparisons and by comparisons of area under the curve (AUC) by the Kolmogorov-Smirnov test. Negative geotaxis response was analyzed by 2-way ANOVA and multiple comparisons assessed using Tukey’s post-test. Locomotor activity was analyzed using the Kruskal-Wallis test (indicated by χ² score) with Dunn’s post-test for multiple comparisons and the Kolmogorov-Smirnov test for comparisons between the shape of continuous distributions. Statistical analysis was conducted using GraphPad Prism 6.

3. Results

3.1. WRNexo is required for normal life span

Because early onset of aging is a hallmark symptom of WS, we first measured lifespan of our WRNexoΔ flies. There was a significant reduction in median lifespan in WRNexoΔ mutants in comparison with same sex w1118 controls (Table 1) and a significant difference in survivorship curves for both sexes (Fig. 1, Mantel-Cox log-rank p < 0.0001). Median lifespan for WRNexoΔ was reduced to approximately 56% of w1118 lifespan in females and 81% in males (Table 1). For both sexes, WRNexoΔ survival began to diverge from w1118 at 10 days at which point mutant death rate increased. While w1118 showed no differences in survival between sexes (Table 1), WRNexoΔ females had a shorter median lifespan compared to WRNexoΔ males (p < 0.01 by multiple t-test corrected by the Holm-Sidak method). WRNexoΔ females also exhibited significantly lower average lifespan and 90% mortality compared to w1118 females (Table 1).

3.2. WRNexoΔ mutants exhibit age-related pathologies

Another well-documented pathology of WS patients is high cancer incidence. Therefore, we evaluated aged WRNexoΔ (35 days old) for presence of masses of undifferentiated cells signifying tumors. By this definition, tumors were found in regions associated with highly proliferating cells: the gut and gonads (Fig. 2B, D, F). Transverse midgut sections of age-matched w1118 controls showed epithelial cells with mild to moderate variation in nuclear size and shape, which is a common feature of gut epithelial cells in aging flies. In contrast, WRNexoΔ flies showed small pleomorphic tumor cells that infiltrate the gut wall and form a mass that protrudes into the lumen (Fig. 2A, B). Similarly, the testes of aged WRNexoΔ males contained numerous tumor cells and fewer immature spermatagonia (Fig. 2C, D). We also observed abnormal ovarian follicles in aged WRNexoΔ females while the ovaries were less affected in age-matched controls. The abnormal follicles contained fewer nurse cells and were filled with small, pleomorphic tumor cells whose morphology is reminiscent of germine stem cells (Fig. 2E, F). WRNexoΔ males had a higher overall tumor incidence (p = 0.0029 by Fisher’s exact test), with no tumors detected in the age-matched w1118 controls. While we observed a six-fold increase in percentage of WRNexoΔ females that contained tumors compared to the age-matched w1118 controls, this difference was not statistically significant (p = 0.067) (Fig. 2).

Another symptom of accelerated aging in both WS patients and the WRNΔ/TercΔ−/− mouse model is sarcopenia (Chang et al., 2004). Aged WRNexoΔ (35 days old) demonstrate necrosis of indirect flight muscles as shown by complete loss of muscle cell nuclei and muscle fibers exhibiting a frayed appearance compared to well-defined striations in fully functioning muscles (Fig. 3A). This degenerative muscle phenotype occurred in 5.1% and 3.8% of WRNexoΔ males and females respectively compared to 0% of age-matched w1118 controls (Male: w1118 n = 158, WRNexoΔ n = 171; Female: w1118 n = 142, WRNexoΔ n = 239). The degeneration of the indirect flight muscles may indicate lower motor function in the WRNexoΔ flies, which is a common phenotype in aging.

3.3. WRNexoΔ mutants show altered locomotor activity

To address the link between the observed degraded muscle structure in mutants and gross muscle function, we performed negative geotaxis assays. This simple, yet comprehensive assessment measures several behaviors including escape reflex, orientation response, locomotor activity, and climbing ability (reviewed in Iladi et al., 2012). Both WRNexoΔ males and females showed a decline in climbing ability in comparison to age-matched w1118 controls in flies that were 14 days old (Fig. 3B). There was no further climbing decline observed in 28-day old adults in either sex.

We further assessed locomotor activity through continuous monitoring using Drosophila activity monitors (DAMs). Overall locomotor activity levels of young (1–4 day old) and old (28 day old) male and

![Fig. 1. WRNexoΔ mutants have a shortened lifespan. Kaplan-Meyer survival curves for a representative experiment comparing homozygous WRNexoΔ and w1118 flies. Male: w1118 n = 121, WRNexoΔ n = 108; Female: w1118 n = 114, WRNexoΔ n = 145. There is a significant difference in survival curves between WRNexoΔ and w1118 flies of both sexes (Mantel-Cox log-rank p < 0.0001).](image-url)

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**Table 1**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sex</th>
<th>Average lifespan ± SEM</th>
<th>Median lifespan ± SEM</th>
<th>90% mortality ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>w1118</td>
<td>Male</td>
<td>45.2 ± 1.1</td>
<td>48.0 ± 1.2</td>
<td>62.3 ± 1.5</td>
</tr>
<tr>
<td>WRNexoΔ</td>
<td>Male</td>
<td>36.6 ± 2.0</td>
<td>37.7 ± 2.3*</td>
<td>52.7 ± 3.5</td>
</tr>
<tr>
<td>w1118</td>
<td>Female</td>
<td>47.9 ± 1.1</td>
<td>50.0 ± 1.2</td>
<td>64.3 ± 1.8</td>
</tr>
<tr>
<td>WRNexoΔ</td>
<td>Female</td>
<td>28.4 ± 3.1**</td>
<td>28.3 ± 2.0**</td>
<td>38.3 ± 5.0**</td>
</tr>
</tbody>
</table>

Data represent 4 independent experiments each containing 72-191 flies. *p < 0.05, **p < 0.001 compared to same sex w1118 control by two-way ANOVA and Tukey’s post-test.
female $w^{1118}$ and WRNexo$^\Delta$ were monitored over a six-day period (Fig. 4A and B). Within males, mean movements per minute varied significantly across groups ($\chi^2 = 89.17$, $p \leq 2.2e^{-16}$) with both $w^{1118}$ and WRNexo$^\Delta$ flies showing significant reductions in overall activity with age (Fig. 4A; $p \leq 0.003$). Comparisons between young male $w^{1118}$ and WRNexo$^\Delta$ revealed no significant difference in overall activity, while aged male WRNexo$^\Delta$ mutants were significantly less active when compared to the $w^{1118}$ controls ($p \leq 0.0001$). Female mean movements per minute also varied significantly across groups ($\chi^2 = 23.01$, $p \leq 4.03e^{-5}$). However, unlike the males, overall activity in the $w^{1118}$ and WRNexo$^\Delta$ females did not significantly decrease with age (Fig. 4A). Comparisons between young female $w^{1118}$ and WRNexo$^\Delta$ also revealed
Fig. 3. Aged WRNexoΔ mutants exhibit muscle degeneration. A) Example of normal muscle tissue in an aged male (left) compared to an aged WRNexoΔ male exhibiting indirect flight muscle necrosis. B) The loss of climbing capacity is exacerbated at 14 days but attenuated at 28 days in WRNexoΔ. Results were analyzed via two-way ANOVA and means compared by the Tukey post-hoc test. Letters denote statistical categories: $p < 0.001$ between subsequent letters. Error bars represent SEM of the averages of 5 climbing tests for each of 9–36 vials containing approximately 20 flies.
no significant difference in overall activity; however, aged WRNexoΔ mutant females were found to be significantly less active than the w1118 controls (Fig. 4A; p ≤ 0.003). Hourly activity of young and old WRNexoΔ and w1118 flies of both sexes was also quantified across the 24-hour day (Fig. 4B). All groups displayed significant oscillations in daily activity (200.86 ≤ χ^2 ≤ 582.03, p ≤ 2.2e−16, Fig. 4B) with the peaks of activity occurring at approximately the same time as the transitions of the light dark cycle (hours 7 and 19). Aged WRNexoΔ females show a significantly different distribution in activity, with plateaued activity lacking peaks during the day (Fig. 4B, Kolmogorov-Smirnov Test, p ≤ 0.005). Young male and female WRNexoΔ flies show lower activity
levels during the evening activity peak (hour 18) (Fig. 4B).

3.4. **WRNexo is required for normal body weight and body composition**

**WS patients experience physiological abnormalities such as smaller size and subcutaneous fat loss. To measure potential fat loss in **WRNexo**, we used the larval buoyancy assay, which has been shown to accurately correlate with body fat percentage (Reis et al., 2010). A smaller percentage of **WRNexo** larvae float in 8.5–12.5% sucrose compared to w**1118** controls, indicating lower fat composition (Fig. 5A). This conclusion was confirmed by assessing the area under the curve (AUC) for percentage floating larvae across all sucrose concentrations tested (Fig. 5B).** While there was no significant difference in the dry weights of **WRNexo** larvae, young female **WRNexo** flies are significantly smaller than **w1118** controls (Table 2). There was no difference in body size in **WRNexo** males.

4. **Discussion**

Because of flies’ genetic similarity to humans, short generation time, and our ability to control their environment, researchers have capitalized on the fly model system to better understand the process of aging. In Drosophila, lifespan has been shown to be heavily influenced by multiple factors including genetic background, environment, diet, and sex (reviewed in Iliadi et al., 2012). Drosophila have also been used to model progeroid and degenerative diseases that result in shortened life span including Parkinson’s disease, Type II diabetes, ALS, and cancer (Yamaguchi, 2018). Perhaps most relevant to this study, Drosophila have also been shown to be effective in studying DNA replication and repair-associated diseases such as Bloom syndrome, Rothman-Thomson syndrome, ataxia telangiectasia, and Xeroderma pigmentosum (Garcia et al., 2011; Mounkes et al., 1992; Rimkus et al., 2008; Wu et al., 2008). Though Drosophila **WRNexo** lacks the RecQ helicase domain found in human WRN, its mechanistic functions in DNA replication and repair are similar (Bolsterstein et al., 2014; Boubriak et al., 2008; Saunders et al., 2008). This similarity in functionality may be possible due to **WRNexo**’s genetic association with Blm, another RecQ helicase that is critical in DNA replication and repair (Bolsterstein et al., 2014). While **WRNexo**’s biochemical and genetic functions have been studied in flies (Bolsterstein et al., 2014; Boubriak et al., 2008; Saunders et al., 2008), this is the first characterization of WS-like phenotypes in **WRNexo** mutant populations.

Unlike WS patient etiology, the accelerated aging phenotypes observed in **WRNexo** mutants are manifested only in females. **WRN**Δ**hel**−/− mice also display sex-specific phenotypes, where researchers have observed greater levels of blood glucose, triglycerides, and insulin resistance in mutant females (Massip et al., 2006). Likewise, constitutive overexpression of **WRNexo** in Drosophila causes increased lifespan in females, but decreased lifespan in males (Shaposhnikov et al., 2015). It is common to see sex-specific differences in Drosophila lifespan, which may in part be attributed to differential reproductive costs between males and females (reviewed in Iliadi et al., 2012). Mating has been shown to have a differential effect on the sexes: while females show lower mean longevity than males, males show increased early mortality (Pletcher, 1996). However, because we did not observe a difference in average lifespan between w**1118** males and females, female-specific costs of mating are unlikely to be a large causative factor in lifespan decrease in **WRNexo** mutants. Differential gene expression may also influence our sex-specific observations. **WRNexo** is more highly expressed in female adult flies, with the highest expression shown in the ovaries (Chintapalli et al., 2007). Because of **WRNexo**’s function in DNA replication during early embryogenesis, the high ovarian expression is likely due to maternal loading of the **WRNexo** protein into eggs (Bolsterstein et al., 2014). However, an alternate explanation could be that **WRNexo** is required for normal ovarian function and protection against accelerated aging.

High cancer incidence has been widely reported in human WS patients (reviewed in Yokote et al., 2017). This etiology may be explained by the Somatic Mutation Theory of Aging, which posits that accumulation of mutations in somatic cells drive formation of tumors during the process of aging (reviewed in Kennedy et al., 2012). Interestingly, high tumor incidence has been inconsistently reported in WS mouse models. While embryonic stem cells from **WRN**Δ−/− mice show higher mutation frequencies, a gross tumor phenotype was not observed in either **WRN**Δ−/− mice or mice containing deletions in both **WRN** and the tumor suppressor gene, p53 **WRN**Δ−/− p53−/− (Lebel and Leder, 1998; Lombard et al., 2000). The lack of a tumor phenotype in WS mouse models could be attributed to several differences in **WRN** function between humans and mice. While **WRN** protein is localized to the

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**Table 2**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Larvae</th>
<th>Adult male</th>
<th>Adult female</th>
</tr>
</thead>
<tbody>
<tr>
<td>w<strong>1118</strong></td>
<td>4.2 ± 0.5</td>
<td>1.7 ± 0.3</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td><strong>WRNexo</strong>Δ</td>
<td>3.5 ± 0.8</td>
<td>1.5 ± 0.3</td>
<td>2.0 ± 0.5</td>
</tr>
</tbody>
</table>

n = 10 groups of 10 individuals per sex/genotype.

* p < 0.05 compared to same sex w**1118** control by Student’s t-test.
nucleolus in humans, its murine expression is diffuse across the nucleoplasm (Marcinjak et al., 1998). A possible functional redundancy of WRN may also be present in the mouse (Lombard et al., 2000). Perhaps the most impactful difference between mice and humans is their differences in telomere maintenance: mice have long telomeres and constitutively active telomerase, which may negate the effect of a lack of WRN (Kipling and Cooke, 1990). Similarly, premature senescence and chromosomal abnormalities were prevented in WS cells expressing telomerase (Crabbe et al., 2007; Wyllie et al., 2000). Therefore, a telomerase-deficient WS mouse model was developed (WRN<sup>−/−</sup> Terc<sup>−/−</sup>), which was shown to have accumulation of DNA damage, chromosomal instability, and higher tumor incidence (Chang et al., 2004). Because telomeres are maintained by a transposon-based mechanism in Drosophila that is different from human and mouse models (Celniker et al., 2006), it is unlikely that WRN<sub>exo</sub> is involved in this capacity.

When we remove the possibility that the high tumor incidence in WRN<sub>exo</sub> flies may indicate improper telomere maintenance, the more likely cause of our observed phenotypes is deficiencies in DNA repair and replication. We observed significantly higher tumor incidence in aged WRN<sub>exo</sub> males, but not females, compared to age-matched controls. This result was surprising given shortened lifespans of WRN<sub>exo</sub> females and it is possible that younger WRN<sub>exo</sub> females have tumors, but die before our 28-day collection. Tumors were observed in gut and germline tissues where WRN<sub>exo</sub> expression is highest in the adult fly (Chintapalli et al., 2007). Cells in the gut, ovaries, and testes are highly proliferative, exacerbating the mutation frequency caused by replicative errors. Because WRN<sub>exo</sub> is required for normal replication (Bolsterlein et al., 2014), replication defects may occur at a higher frequency in these cell populations, leading to tumor-forming mutations. Exacerbating this effect, aged Drosophila have reduced ability to repair double-strand breaks with age as was demonstrated through elevated γ-H2A in 29-day-old spermatagonia (Delabaere et al., 2017). Importantly, we are the first to report a sex-dependent difference in tumor incidence in a WS model as sex was not a factor investigated in WRN-depleted mice (Chang et al., 2004; Lebel and Leder, 1998; Lombard et al., 2000).

Other DNA repair deficiencies have been shown to cause heightened tumorigenesis in Drosophila. For example, Garcia et al. (2011) reported higher tumor incidence in flies lacking Blm, another RecQ helicase (Garcia et al., 2011). At 35 days-old, Blm males possessed predominantly gut tumors while bhm females possessed tumors in both the gut and ovaries (Garcia et al., 2011), demonstrating that tumors derived in both WRN<sub>exo</sub> and bhm aged adults are derived from epithelial cells. This epithelial tumor origin is similar to Bloom syndrome patients who most commonly show carcinomas, but in contrast with WS patients display more mesenchymal cell-derived sarcomas (reviewed in Chu and Hickson, 2009). This difference in tumor tissue specificity between human patients and flies may be attributed to the different roles of the WRN<sub>exo</sub> and Blm proteins in each species. Drosophila WRN<sub>exo</sub> lacks the RecQ helicase domain, but because of its epistatic relationship with Blm in DNA replication (Bolsterlein et al., 2014), these proteins may more closely associate with each other in Drosophila leading to similar tumor manifestations.

Sarcopenia, or loss of muscle mass is a classic sign of age-associated senescence that is present in both invertebrates and mammals (reviewed in Iliadi et al., 2012). Muscular deterioration has been observed in both WS patients and WRN<sup>−/−</sup> Terc<sup>−/−</sup> mice (Chang et al., 2004; Yamaga et al., 2017). In Drosophila, indirect flight muscle structure and function have been shown to degrade with age (Das et al., 2015; Ferguson et al., 2005; Groteveld et al., 2005). Similarly, researchers have observed an age-associated decline in flight ability, where 50 day-old flies show no flight capacity (Miller et al., 2008). This decline of flight ability has been correlated with an age-related change in myofilbr structure in which the myofilbr heads move closer to the filaments resulting in greater muscle fiber stiffness, tension, and more power output. Further muscular deterioration in old flies (56 days) was linked to deterioration of mitochondria in the muscle fibers, suggesting that the age-related structural changes in muscle fiber structure may be compensatory reactions to low levels of available ATP (Miller et al., 2008).

A decline in locomotor activity is one consequence of muscular deterioration and is easily measured in Drosophila (Iliadi et al., 2012; Iliadi and Boullianne, 2010). To capture different aspects of behavioral changes, we measured fly locomotor activity using two complementary methods: negative geotaxis and continuous monitoring using Drosophila activity monitors (DAM). It has been found that aged and short-lived strains of Drosophila show lower climbing ability in a strain and sex-dependent manner (Fernández et al., 1999; Le Bourg, 1987; Niveditha et al., 2017; Rhodenizer et al., 2009), which was supported by our data showing decreased negative geotaxis responses at 14 days in both sexes of WRN<sub>exo</sub> compared to w<sup>1118</sup>. It is unlikely that flight muscle deterioration influenced these results as flight does not contribute to negative geotaxis performance (Rhodenizer et al., 2009). Our result that 28 day-old WRN<sub>exo</sub> males have higher climbing ability may suggest selective mortality in the negative geotaxis assay in which the weakest individuals are excluded from analysis. In contrast to the negative geotaxis data, DAM data showed an age-related decline in overall activity in males only. While this result was unexpected, it is not unusual as aging has been shown to influence locomotor activity in a strain- and sex-dependent manner and in some instances, female activity has been reported to increase with age (Fernández et al., 1999; Le Bourg, 1987; Rhodenizer et al., 2009). These sex-dependent differences may be attributed to a shift in behaviors that occur as flies age, favoring stationary activities that are not detectable by our methods, such as preening (Carey et al., 2006). It is also possible that because of the shortened median lifespan in WRN<sub>exo</sub> females (27 days), at 28 days we may be capturing a less vulnerable population with increased activity. However, our measurements of locomotor activity using DAM also showed lower overall activity levels in both male and female aged WRN<sub>exo</sub> compared to their w<sup>1118</sup> counterparts, demonstrating that a WRN<sub>exo</sub> deletion has a negative impact on locomotor activity.

The circadian system synchronizes daily physiological functions so that organisms can optimally respond to predictable changes in environment. Characteristic diurnal patterns in Drosophila show peaks in locomotor activity around transitions of the light/dark cycle (reviewed in Allada and Chung, 2010). Using DAM to monitor hourly levels of locomotor activity, we found that w<sup>1118</sup> exhibited the expected strong activity peaks at light transition times independent of sex or age. Young WRN<sub>exo</sub> of both sexes also exhibited activity peaks associated with light transitions, however hourly analysis shows reduced activity in both WRN<sub>exo</sub> males and females during the evening activity peak (hour 18).

Because many physiological functions are governed by circadian rhythms, it is unsurprising that disruptions in daily circadian patterns are associated with aging and disease. To this point, the strength of Drosophila diurnal activity peaks and consistency of their rhythms has been shown to weaken with age (Driver et al., 2004; Koh et al., 2006; Rakshit et al., 2012). Furthermore, researchers have described sex-specific effects on Drosophila aging, lifespan, and sleep patterns caused by mutations in circadian regulatory genes (reviewed in Iliadi and Boullianne, 2010). Our data showed altered diurnal activity in WRN<sub>exo</sub> females only: while hourly activity was lower in aged WRN<sub>exo</sub> males compared to w<sup>1118</sup>, the transition period activity peaks were still evident. However, light transition-associated activity peaks were absent in aged WRN<sub>exo</sub> females with relatively constant daytime activity levels. Expression of WRN<sub>exo</sub> in females remains comparatively high as flies age (Graveley et al., 2010) and therefore the requirement of WRN<sub>exo</sub> in maintaining normal activity levels and behavior may be greater in older flies.

The connection between aging, circadian rhythms, and tumorigenesis may be partially explained through interactions between clock
regulatory genes and genes that regulate cell cycle and DNA repair (Dakup et al., 2018; Krishnan et al., 2008; Miki et al., 2012, 2013; Oklejewicz et al., 2008; Pregueiro et al., 2006). Specifically, the clock regulator, PER2, is directly regulated by p53 (Miki et al., 2013), a tumor suppressor that is commonly mutated in human cancers (Hollstein et al., 2016). Similarly, DNA damage induced by ionizing radiation has been shown to cause phase shifts in circadian rhythms through interactions through involvement of the ATM/ATR DNA damage signaling pathway (Oklejewicz et al., 2008). Studies have shown that mutations in circadian regulatory genes ablate circadian oscillations in expression of DNA damage response genes in the presence of cisplatin in mice (Dakup et al., 2018) and oxidative stress in Drosophila (Krishnan et al., 2008). On a global level, the interactions between the cell cycle and circadian oscillations have been suggested to explain the higher rates of cancer in shift workers (Feillet et al., 2015). WRN’s DNA repair and replication functions may interact with processes controlling circadian rhythms. However, additional experiments are required to thoroughly address the relationship between WS and altered circadian rhythms.

Together, our findings may suggest that increased DNA damage and/or replication stress during early development may negatively impact larval growth and subsequent development into an adult. WS patients generally have repressed growth during puberty, which leads to short stature and subcutaneous fat loss as adults, as well as thin limbs as a sign of muscular degeneration (reviewed in Yokote et al., 2017). Likewise, WRN / Terc −/− mice show lower body weight and a reduction in adipose tissue (Chang et al., 2004). We observed changes in body composition in WRNexo, as marked by lower larval body fat and smaller size of young adult females. While larvae tended toward lower weight, the difference was not statistically significant. Reduced body size alone is unlikely to influence lifespan as body size has been shown to inversely correlate with lifespan (Khazaeli et al., 2005). Because larval growth and development is a determining factor of adult body size (reviewed in Yongmei Xi, 2015), reduced body fat in WS cells has shown differentially regulates circadian oscillations and genes that regulate cell cycle and DNA repair (Cervera et al., 2014). Similarly, treatment with vitamin C has been shown to rescue aging-related pathologies in WRN-deficient mice (Aumailley et al., 2015a; Massip et al., 2010). On a molecular level, WRN protein has been associated with the base excision repair pathway, which is the mechanism of removing 8oxo-G DNA lesions caused by oxidative stress (Das et al., 2007; Harrigan et al., 2006, 2007). Specifically, WRN helicase has been shown to unwind substrates for long-patch base excision repair (BER) (Harrigan et al., 2003). While long-patch BER is likely the preferential BER mechanism in flies (Sekelsky, 2017), WRNexo alone is likely insufficient to aid in this pathway as it does not contain a helicase domain. Instead, WRNexo may recruit a “partner” helicase (e.g. Blm) to participate in the long-patch BER mechanism. Therefore, it is possible that in our model, WRNexo protects against damage caused by oxidative stress that contributes to our observed accelerated aging phenotypes.

In conclusion, we have shown that Drosophila can be used to model accelerated aging phenotypes similar to those seen in WS patients. Because the causative factors of aging are so numerous, more research needs to be done to determine how WRNexo promotes longevity. By using model systems of degenerative diseases, we can learn more about the mechanisms behind normal aging.

Acknowledgements

Research reported in this publication was supported, in part by the National Science Foundation (MCB0643253), the U.S. Department of Education Title III Award (P031C110157), Northeastern Illinois University, the National Institutes of Health’s National Institute of General Medical Sciences IRACDA program (K12GM074869), and the ChicagoCHEC Partnership funded through the National Cancer Institute (U54CA202995, U54CA202997, and U54CA203000). The authors would also like to thank Mary Kimble for her proofreading and all of the Bolterstein Lab undergraduate trainees for their help with experiments and in maintaining the fly stocks.

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