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Identification and characterization of a *Drosophila* ortholog of WRN exonuclease that is required to maintain genome integrity

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**Summary**

The premature human aging Werner syndrome (WS) is caused by mutation of the RecQ-family WRN helicase, which is unique in possessing also 3′–5′ exonuclease activity. WS patients show significant genomic instability with elevated cancer incidence. WRN is implicated in restraining illegitimate recombination, especially during DNA replication. Here we identify a *Drosophila* ortholog of the WRN exonuclease encoded by the CG7670 locus. The predicted DmWRNexo protein shows conservation of structural motifs and key catalytic residues with human WRN exonuclease, but entirely lacks a helicase domain. Insertion of a piggyBac element into the 5′ UTR of CG7670 severely reduces gene expression. DmWRNexo mutant flies homozygous for this insertional allele of CG7670 are thus severely hypomorphic; although adults show no morphological abnormalities, females are sterile. Like human WS cells, we show that the DmWRNexo mutant flies are hypersensitive to the topoisomerase I inhibitor camptothecin. Furthermore, these mutant flies show highly elevated rates of mitotic DNA recombination resulting from excessive reciprocal exchange. This study identifies a novel WRN ortholog in flies and demonstrates an important role for WRN exonuclease in maintaining genome stability.

**Key words:** exonuclease; *Drosophila*; genome stability; homologous recombination; Werner syndrome; WRN.

**Introduction**

Werner syndrome (WS) provides a very useful model system for the study of human aging at the molecular level, with patients manifesting many signs of normal aging in an accelerated manner (reviewed in Martin, 1985; Goto, 2001; Cox & Faragher, 2007; Kudlow et al., 2007). The syndrome is caused by mutation of WRN (Yu et al., 1996), a member of the RecQ DNA helicase family. WS patient-derived cells undergo highly premature replicative senescence, with cellular defects including aberrant DNA replication (Pichierri et al., 2001; Rodriguez-Lopez et al., 2002) and hyper-recombination (Salk et al., 1981; Scappaticci et al., 1982; Fukuchi et al., 1985). Hypersensitivity to the DNA-damaging agent 4-nitroquinoline oxide, and the topoisomerase I inhibitor camptothecin (CPT) is characteristic of WS cells (Poot et al., 1999; Prince et al., 1999; Pichierri et al., 2000b). These agents lead to replication fork arrest or collapse, suggesting a function for WRN in DNA replication, which is further supported by its presence at replication foci coincident with RP-A and PCNA (Constantinou et al., 2000; Rodriguez-Lopez et al., 2003) and aberrant replication fork progression in WS fibroblasts (Rodriguez-Lopez et al., 2002). The hyper-recombiant phenotype of human WS cells, suppression of illegitimate recombination in yeast Sgs7 mutants by human WRN (Yamagata et al., 1998), interaction with MRN on replication fork stalling (Franchitto & Pichierri, 2004), the recovery of proliferative capacity after ectopic expression of a Holliday junction resolvase in WS cells (Rodriguez-Lopez et al., 2002), and excessive chromosome breakage at fragile sites in the absence of WRN (Pirzio et al., 2008) all suggest an important role for WRN in homologous recombination after replication fork arrest, either in preventing the formation of homologous recombination intermediates or in their rapid resolution (Dhillon et al., 2007; Rodriguez-Lopez et al., 2007). The importance of WRN in regulating genome stability is highlighted by the high cancer incidence in WS patients, while epigenetic inactivation of WRN is also associated with human cancer (Agrelo et al., 2006).

Identification of the action of WRN in homologous recombination is complicated by the presence of two enzymatic activities within the same protein: the 3′–5′ helicase characteristic of all RecQ family members (reviewed in Bachrati & Hickson, 2003), and a 3′–5′ exonuclease activity (Huang et al., 1998) unique within this family, but which is closely related structurally to the DnaQ exonuclease superfamily (Perry et al., 2006). X-ray crystallographic analysis of the exonuclease domain of human WRN suggests a role in DNA end processing (Perry et al., 2006).
possibly at the stage of strand resection after double-strand breaks, such breaks that occur at collapsed replication forks. This would be consistent with the importance of WRN in DNA recombination. However, the closely related BLM helicase can, at least in vitro, promote dissolution of double Holliday junctions without intrinsic nuclease activity (Wu & Hickson, 2003); determining the relative contributions to homologous recombination of the helicase and exonuclease activities of WRN is therefore important. Moreover, there appears to be a complex interplay between the helicase and exonuclease activities of WRN (Opresko et al., 2001); for example, it is possible that helicase activity may be required to generate a template suitable for cleavage by the nuclease. The distinct roles are difficult to dissect in vertebrate cells since ablation of one activity may affect the other; indeed, point mutation of the helicase is suggested to act in a dominant negative manner (Crabbe et al., 2004).

RNAi depletion of WRN, although highly effective in recapitulating some WS-like phenotypes (Dhillon et al., 2007), eliminates both helicase and exonuclease activities. To study WRN’s role in recombination at the organismal level, we sought to develop a model in which WRN activity may be evaluated at different developmental stages. Although murine models have been described which show some WS-like features on mutation of the WRN helicase alone or with co-mutation of either telomerase or PARP (Lebel, 2002; Lebel et al., 2003; Chang et al., 2004; Massip et al., 2006), the relatively long lifespan and complexity of genetic intervention pose severe limitations on their exploitation. In order to develop a model system more amenable to genetic and biochemical analysis of WRN exonuclease function in vivo, we set out to identify and characterise WRN exonuclease from Drosophila melanogaster.

**Results**

**Identification of Drosophila WRNexo**

We conducted a BLASTP search (Altschul et al., 1997) of the Drosophila melanogaster genome sequence (Release 4.0), using as a probe the sequence of human WRN protein. The Drosophila candidate gene encoding a WRN-like exonuclease is CG7670, with an E-value of $1 \times 10^{-25}$ (Cox et al., 2007, as also noted by Sekelsky et al., 2000). Upon cloning from mRNA and sequencing multiple CG7670 cDNA clones, we found two alleles of CG7670 differing solely by the presence or absence of an AAG codon (lysine) at nucleotide 235, amino acid 79 (GenBank accession numbers EF680279 and EF680280, respectively). Variant 2 (EF680280) lacking lysine 79 was the more commonly occurring clone, encoding a predicted protein of 352 amino acids.

The predicted protein product of the CG7670 locus, which we call DmWRNexo, shares 35% identical and 59% similar amino acids with the exonuclease domain of human WRN over a region of 192 residues (Fig. 1A). Previous crystallographic studies of the human WRN exonuclease domain demonstrated that residues aspartate (D82) and glutamate (E84) within the
nuclease catalytic site are essential for metal ion coordination (Perry et al., 2006). Importantly, these residues are conserved in DmWRNexo (Fig. 1A, asterisks). We have conducted SWISS-MODEL structural predictions (Peitsch, 1996; Schwede et al., 2003) of DmWRNexo from residues 118–312, which suggest that the protein might adopt a very similar configuration to human WRN exonuclease (Perry et al., 2006, PDB accession number 2fbvA, predicted similarity e-value 8.86 × 10^{-26}), with conservation of key alpha helices and beta sheets comprising the nuclease active site (Fig. 1B).

**Hypomorphic allele of CG7670**

To assess the impact of DmWRNexo mutation on flies, we obtained an insertional mutant allele of CG7670, CG7670^e04496, which contains a piggyBac{RB} element (Thibault et al., 2004) inserted within the 5′ UTR (Fig. 2A). Reverse transcription–polymerase chain reaction (RT-PCR) analysis shows that the CG7670^e04496 allele is transcribed at an extremely low level in the homozygous mutant compared with CG7670 expression in heterozygous and wild-type flies (Fig. 2B); no band was detectable in negative controls (data not shown). Interestingly, CG7670^e04496 homozygotes show no gross morphological abnormality, and while the females are sterile (eggs do not hatch), males are fertile. The location of CG7670 on chromosome 3R:14189966.14191859 (Flybase) and its identity with the gene encoding DmWRNexo is consistent with our mitotic recombination deficiency mapping studies (mwh^1 CG7670^e04496/Df(3R)Exel6178 flies display multiple wing hair clones; data not shown).

**DmWRNexo mutant flies are hypersensitive to CPT**

Werner syndrome cell lines are sensitive to the topoisomerase I poison CPT (Poot et al., 1999; Pichierri et al., 2000a) which causes replication fork collapse at the bound topoisomerase (Shao et al., 1999); such sensitivity can be partially complemented by expression of a bacterial Holliday junction nuclease (Rodriguez-Lopez et al., 2007), suggesting that WRN acts either to prevent accumulation of Holliday junctions at collapsed forks or to ensure rapid Holliday junction resolution. To test whether *Drosophila* mutant for DmWRNexo are similarly sensitive to CPT, larvae derived from crosses of CG7670^04496 heterozygotes were propagated on medium supplemented with varying concentrations of CPT, or vehicle-only control (0 μM CPT). Emerging flies were scored for heterozygosity or homozygosity of the CG7670^04496 allele. While the heterozygous flies appear to be fully viable at all concentrations of CPT used (Fig. 3), a significant loss of viability of flies homozygous for the CG7670^04496 allele (i.e. those with very low levels of expression of DmWRNexo) was observed even at 0.1 μM CPT, with almost total lethality from 0.2 μM (Fig. 3). Surviving homozygotes displayed roughened eyes, an indicator of cell death, and many died as pharate adults (data not shown), a typical lethal phase for flies exhibiting high levels of cell death. This is consistent with the high levels of apoptosis detected in human Werner syndrome cells exposed to CPT (Poot et al., 1999). Thus, loss of DmWRNexo results in hypersensitivity to CPT.

**Genome instability in DmWRNexo mutant flies**

Since hyper-recombination is a key phenotype of WS patient-derived cell lines (Salk et al., 1981; Scappaticci et al., 1982; Fukuchi et al., 1985), rates of chromosome breakage and/or mitotic recombination in *DmWRNexo* homozygous mutant flies were evaluated using the recessive multiple wing hairs marker (mwh, recombination map position 3-0.7); wing blade cells hemizygous or homozygous for mwh^1 develop tufts of wing hairs instead of single hairs. Note that the adult wing consists of postmitotic cells arising from proliferating cells of the wing imaginal disc, so any recombination giving rise to clones of cells with the mwh phenotype must have occurred during cell proliferation in development.

Wing blades were dissected from flies that were homozygous mutant for *DmWRNexo* but heterozygous for mwh (i.e. w^{+/mwh}.)
mwh\(^1\) CG7670\(^{04249}\)/CG7670\(^{04249}\) and analysed microscopically (Fig. 4A–C). The frequency and size of clones showing multiple wing hairs was determined (Fig. 4D), demonstrating that mwh clones occur at a very high frequency in the DmWRNexo homozygous mutant flies, with an average of over 100 clones per fly. This is in sharp contrast to flies heterozygous for DmWRNexo which show a mean of 0.2 mwh clones per fly (data not shown). Furthermore, some mwh clones in DmWRNexo homozygous mutant wing blades were very large (> 500 cells) (Fig. 4D), while the rare clones observed in heterozygous flies were all single cells (data not shown). In addition to the very high rates of recombination detected in DmWRNexo mutant flies, these data also demonstrate that the CG7670\(^{04249}\) allele is recessive, as reported for patient-derived human WRN mutations (Yu et al., 1996; Moser et al., 1999).

Cells in the wing blade showing the recessive multiple wing hairs phenotype could genetically be either homozygous or hemizygous for the mwh\(^1\) allele. Homozygous mwh\(^1\) cells would result from mitotic recombination via a reciprocal exchange event (Fig. 5A); daughter cells should be euploid without any loss of proliferative fitness. By contrast, chromosome loss or single chromosome/chromatid breakage events (Fig. 5B) would give rise to segmentally aneuploid hemizygous mwh cells, which would be predicted to proliferate more slowly than euploid cells. To distinguish between these possibilities, we have measured the size of each clone in terms of the number of cell cycles since its generation, and plotted the proportion of clones in each size class (on a logarithmic scale) against clone size (Fig. 5C). This type of analysis informs on the nature of the event leading to clone formation as the gradient of the plot reflects the growth rate of the clones (Baker et al., 1978). If clones proliferate at the same rate as surrounding normal cells, the gradient will closely parallel the expectation (that clones of size class \(n\) will be twice as numerous as clones of size class \(n+1\)). Clones that proliferate more slowly, as would be expected for segmental aneuploids, would fit a line of steeper gradient. Our results (‘Observed’, Fig. 5C) indicate that cells comprising wing blade clones in DmWRNexo mutant flies proliferate essentially as...
expected for euploid cells. However, the gradient is slightly shallower than expected for two reasons. First, adjacent smaller clones may have been scored as a single larger clone, and second, the recombination events happening earlier in the lineage of the clone (yielding large clones) depletes the pool of cells from which later events (smaller clones) can occur. The actual frequency of recombination events occurring may also be higher than that observed, since sister chromatid exchange is not scored in this assay. We therefore propose that mitotic recombination is the predominant cause of mwh clones in these flies. Based on mathematical simulations (data not shown), we estimate the recombination frequency on chromosome arm 3L to be at least 0.01 event per cell division. Assuming a similar frequency throughout the genome, this corresponds to an overall frequency of at least 0.05 recombination events per cell division.

To further distinguish between chromosome breakage and homologous recombination as the principal mechanism for the elevated frequency of wing blade clones in CG7670mwh homozygotes, we used a second cuticular marker, flare (flr, recombination map position 3–38.8), which causes wing blade hairs to be malformed. Wing blades of flies mutant for DmWRNexo and trans-heterozygous for mwh1 and flr3 (w1118; mwh1 CG7670mwh1/ flr3 CG7670flr3) were examined for mwh clones (arising as a consequence of recombination in the chromosomal interval between mwh and flr) and neighbouring mwh and flr clones (twin spots), resulting from recombination proximal to flr (Fig. 6). If the primary mechanism of genome instability in DmWRNexo mutants is chromosome breakage, mwh flr twin spots should be rare, while if mitotic recombination is the principal cause of wing blade clones, twin spots will be frequent. The observation of mwh flr twin spots at high frequency (Fig. 6E,F) strongly suggests that wing blade clones in the DmWRNexo mutants arise predominantly through mitotic recombination. Because of the high frequency of recombination in CG7670mwh homozygotes, numerical analysis of the twin-spot frequency is difficult and further compounded by sequential repeated recombination events in clone lineages, as inferred from the observation of putative mwh flr double mutant cells (Fig. 6E,F).
We have identified the locus CG7670 as the Drosophila ortholog of human WRN exonuclease. The encoded DmWRNexo protein shows significant structural and sequence similarities to human WRN exonuclease domain, and moreover, a severe hypomorphic mutation of the locus results in both hyper-recombination and CPT hypersensitivity in flies, features characteristic of human WS cells.

Our data demonstrate that loss of DmWRNexo function leads to very high levels of recombination in the developing Drosophila wing (and presumably also in other dividing tissues), consistent with hyper-recombination reported in cells from WS patients (Salk et al., 1981; Scappaticci et al., 1982; Fukuchi et al., 1985). The high frequency of twin spots in wing blade clones seen here strongly supports the assertion that the majority of marked clones in the mutant flies arise as a result of homologous recombination rather than chromosomal breakage. We cannot at this stage rule out the possibility that non-homologous end joining is also aberrant, as is the case in human cells lacking functional WRN (Chen et al., 2003; Otsuki et al., 2007), since such end joining is unlikely to yield scoreable clones in the assays used here.

Mechanistically, it has been difficult to differentiate between the impact of the exonuclease and helicase activities of WRN in vivo since RNAi ablates all activities, while point mutants may act as dominant negatives (Crabbe et al., 2004). By studying a model organism in which the WRN exonuclease activity is encoded on a genomic locus distinct from any putative partner helicase, we can readily ablate the exonuclease activity without the possibility of creating dominant negative complexes. Our data presented here clearly demonstrate the importance of WRN exonuclease in restraining mitotic recombination. Furthermore, it is likely that at least some of the recombination detected in the DmWRNexo mutants occurs as a result of deficiencies in resolving aberrant DNA structures arising during DNA replication. The observed hypersensitivity of CG7670^{e04496} homozygotes to CPT is indicative of a role for DmWRNexo at collapsed replication forks, as predicted from human studies (Pichierri et al., 2001; Rodriguez-Lopez et al., 2002, 2007; Pirzio et al., 2008). Human WRN can regress replication forks in vitro (Machwe et al., 2006); how it supports re-establishment of collapsed forks in vivo is less clear, but our data suggest that the exonuclease activity of WRN may be important in preventing hyper-recombination at this stage. We speculate that DmWRNexo, like human WRN (Saintigny et al., 2002; Dhillon et al., 2007; Rodriguez-Lopez et al., 2007), may act to prevent Holliday junction accumulation at stalled or collapsed replication forks, and that DNA end processing activity of the exonuclease (Perry et al., 2006) may be critical to direct fork re-establishment. Such end processing could result in removal of DNA strands that would otherwise be used in the strand invasion step in homologous recombination. Thus, in cells lacking DmWRNexo, collapsed replication forks (such as at CPT-induced breaks) would persist, and promote Holliday junction formation and homologous recombination.

This study demonstrates the strength of using a genetically amenable model system for analysis of genes associated with genomic instability and human aging, even though the adult is largely postmitotic; absence of DmWRNexo function through fly development manifests as a hyper-recombinant phenotype in the mature adult. This raises the exciting possibility of using the short-lived fruit fly as model system for analysis and experimental modulation of WRN function in vivo.
Experimental procedures

Bioinformatics

BLAST searches (Altschul et al., 1997) were conducted against Release 4.0 of the *Drosophila melanogaster* genome sequence, and reciprocally against the human protein RefSeq database. BioEdit was used to generate the alignments following processing with Clustal W. Structural predictions were carried out using SWISS-MODEL (Peitsch, 1996; Schwede et al., 2003) based on the structure of human WRN exonuclease domain (Perry et al., 2006).

DNA and RNA analysis

Total RNA was extracted from flies using RNeasy spin columns (Qiagen, Crawley, West Sussex, UK) and quantitated using a Qubit fluorometer (Invitrogen, Paisley, UK). For analysis of transcript levels, one-step RT-PCR (Qiagen) was carried out with gene-specific primers (CG7670 Exon1–2: 5′-ATGAAGTTCCTCCAAAGGAGG-3′; CG7670 Exon1–2 R: 5′-GATGGCCTGATACATTAGTT-3′; actin 5C F: 5′-CACCCTGTACGTTGGACT-3′, actin 5C R: 5′-GGACT CGTCGTACTCCTGCT-3′) using 0.5 μg total RNA. Products were analysed on 0.9% agarose gels stained with ethidium bromide, against a 100-bp ladder (Roche, Burgess Hill, West Sussex, UK).

To clone CG7670, cDNA was prepared from freshly isolated RNA from female flies (TM6B/TM3, wild type for CG7670) using Omniscript reverse transcriptase (Qiagen) and random hexamer primers (Operon, Cologne, Germany) or oligo-dt(16) primers (Applied Biosystems, Warrington, UK). The cDNA was PCR-amplified using pfx50 proofreading DNA polymerase (Invitrogen) and primers Forward F1A (CGGGTTATGGAAAAATATT-TAACAAAATGGCC) and Reverse R-2 A (AGCTTACAGAGTCACCTCGTTGATCTTGG), to yield a blunt-end PCR product which was cloned into TOPO vector (Zero Blunt®TOPO® PCR Cloning Kit, Invitrogen). DNA sequencing was performed in-house by Geneservice on an ABI 3730xl DNA Analyser.

Fly stocks

Fly stocks were obtained from the Bloomington *Drosophila* Stock Center (http://flystocks.bio.indiana.edu/), and were maintained on a standard oatmeal, yeast, molasses and agar medium. Wing blades were dissected from flies stored in 70% ethanol, mounted in Gary’s Magic Mountant (Lawrence et al., 1986) and analysed by brightfield microscopy.

Camptothecin sensitivity studies

Fly medium containing 60 g L⁻¹ each of dextrose and yeast, 3% w/v nipagin and 3% v/v propionic acid was supplemented with CPT in a 5% ethanol/5% Tween-20 solution to achieve final CPT concentrations of 0–0.8 μM in vials containing 10 mL fly food (Cunhe et al., 2002). Heterozygous CG7670<sup>CG7670e04496/TM6B</sup> flies were crossed and eggs were seeded into 4–5 vials per dose at ~200 eggs/vial, according to Clancy & Kennington (2001) and allowed to develop at 18 °C. Surviving heterozygous and homozygous adult flies were scored.

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