INVESTIGATING BIOLOGICAL FUNCTIONS OF THE TUMOR SUPPRESSOR WWOX IN DROSOPHILA MELANOGASTER

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Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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List of publications

Published

O'Keefe, L.V., Lee, C.S., Choo, A. and Richards, R.I., 2015. Tumor suppressor WWOX contributes to the elimination of tumorigenic cells in *Drosophila melanogaster*. PloS one, 10(8), p.e0136356.

Manuscripts in preparation

Lee, C.S., O'Keefe, L.V., Choo, A. and Richards, R.I., 2016. Distinct functional requirements for WWOX in cell death and metabolism. (To be submitted for publication)

Lee, C.S., O'Keefe, L.V., Choo, A. and Richards, R.I., 2016. Regulation of mitochondrial dynamics by WWOX: linking cell metabolism and cell death. (To be submitted for publication)

Abbreviations

°C – degrees Celsius

% – percentage

µm – micrometre

A – adenosine (in context of DNA)

A – alanine (in context of amino acid)

Aβ – amyloid beta

Ack1 – cdc42-associated kinase 1

AGRF – Australian Genome Research Facility

AiA – apoptosis-induced apoptosis

Akt – v-akt murine thymoma viral oncogene homolog/protein kinase B

AP2γ – transcription factor activator protein 2

ATM – ataxia telangiectasia mutated

ATR – ataxia telangiectasia and Rad3 related

Bcl – B-cell lymphoma

BK5 – bovine keratin 5

bp – base pairs

BRCA1 – breast cancer 1, early onset

C – cytosine (in context of DNA)

CD8 – cluster of differentiation 8

cDNA – complementary DNA

CFS – common fragile site(s)

CHK – checkpoint kinase

CIN – chromosomal instability

CNS – central nervous system

CNV – copy number variation

CoVa – cytochrome c oxidase subunit Va

CoVb – cytochrome c oxidase subunit Vb

Cu – copper

da – daughterless

DNA – deoxyribonucleic acid

Drp1 – dynamin-related protein1

DSB – DNA double strand break

Dvl – dishevelled

EGFR – epidermal growth factor receptor

Egr – eiger

en – engrailed

ENU – N-ethyl-N-nitrosourea

ERBB4 – erb-b2 receptor tyrosine kinase 4

ERK – extracellular signal-regulated kinases

ETC – electron transport chain

EV – empty vector

ey – eyeless

F – phenylalanine

FHIT – fragile histidine triad

FLP – flippase

FOR – fragile site FRA16D oxidoreductase

Foxo – forkhead box, sub-group O

FRT – flippase recognition target

G – guanosine (in context of DNA)

GFP – green fluorescent protein

gmr – glass multiple reporter

GSK3β – glycogen synthase kinase 3 beta

HDL-C – high density lipoprotein-cholesterol

hh – hedgehog

hid – head involution defective

HIF1α – hypoxia inducible factor 1α

hnRNP A2/B1 – heterogeneous nuclear ribonucleoproteins A2/B1

hpRNA – hairpin RNA

Hyal-2 – hyaluronidase 2

ICD – intracellular domain

IDH – isocitrate dehydrogenase

ITCH – itchy E3 ubiquitin protein ligase

JNK1 – c-Jun N-terminal kinases 1

kb – kilo base pairs

kDa – kilodalton

K – lysine

LOH – loss of heterozygosity

Mad2 – mitotic arrest deficient 2

MARCM – mosaic analysis with a repressible cell marker

Marf – mitochondrial assembly regulatory factor

Mb – mega base pairs

MEFs – mouse embryonic fibroblasts

MFN 1 – mitofusin 1

MFN 2 – mitofusin 2

MMTV – mouse mammary tumor virus

mRNA – messenger RNA

mTOR – mechanistic target of rapamycin

NAD+ – nicotinamide adenine dinucleotide (oxidised)

NADH – nicotinamide adenine dinucleotide (reduced)

NAD(P)+ – nicotinamide adenine dinucleotide phosphate (oxidised)

NAD(P)H – nicotinamide adenine dinucleotide phosphate (reduced)

ND23 – NADH:ubiquinone reductase 23kD subunit precursor

ND42 – NADH:ubiquinone reductase 42kD subunit precursor

ND75 – NADH:ubiquinone reductase 75kD subunit precursor

NFT – neurofibrillary tangle

NLS – nuclear localization sequence

NMBA – N-nitrosomethylbenzylamine

ns – not significant

Opa1 – optic atrophy 1

ORF – open reading frame

(OXPHOS) – oxidative phosphorylation

P – proline

PEST sequence – proline (P), glutamic acid (E), serine (S), and threonine (T) rich peptide sequence

PCR – polymerase chain reaction

PINK1– PTEN-induced kinase 1

Prp19 – pre-mRNA splicing factor 19

R – arginine

RISC – RNA-induced silencing complex

RNA – ribonucleic acid

RNAi – RNA interference

ROS – reactive oxygen species

SAC – spindle assembly checkpoint

Scrib – scribbled

SDR – short-chain dehydrogenase/reductase

Sima – similar

siRNA – small interfering RNA

SNP – single-nucleotide polymorphism

SOD – superoxide dismutase

$Sod1^{n1}$ –G50S missense mutation in *Sod1*

$Sod1^{n64}$ –G43E missense mutation in *Sod1*

SSB – DNA single strand break

T – thymine (in context of DNA)

T – threonine (in context of amino acid)

TACE – tumor necrosis factor-α converting enzyme

TCA – tricarboxylic acid

TGF-β1 – transforming growth factor β1

Tgo - tango

TIAF 1 – TGF-β1-induced anti-apoptotic factor 1

TNFα – tumor necrosis factor α

TNFR – tumor necrosis factor receptor

TPC6AΔ – trafficking protein particle complex 6A delta

TRADD – tumor necrosis factor receptor type 1-associated DEATH domain protein

Tub – tubulin

U – uracil

UAS – upstream activator sequence

UV – ultraviolet

VDRC – Vienna Drosophila Resource Centre

W – tryptophan

WBP1 – WW domain binding protein 1

WW1 – 1st WW domain of WWOX

WW2 – 2nd WW domain of WWOX

WWOX – WW domain-containing oxidoreductase

$Wwox^1$ – a null allele of *Drosophila Wwox* generated by homologous recombination

$Wwox^2$ – a null allele of *Drosophila Wwox* generated by pBac transposon insertion

Wwox^{trun169} – truncated *Drosophila* Wwox protein that only contains N-terminal 169 amino acid sequences

WWOX-IR – *WWOX* inverted repeat construct

Y – tyrosine

YAP – yes-associated protein

Yki – yorkie

Zn – zinc

Drosophila nomenclature

The *Drosophila* nomenclature used in this thesis is based on conventional notation as stated on the *Drosophila* database, Flybase (www.flybase.org). Genes are shown by italicised text (e.g. *Wwox*) and proteins are shown by non-italicised text (e.g. Wwox).

Abstract

Reduction of *WW domain-containing oxidoreductase* (*WWOX*) expression has been reported for various cancers and in some cases, higher *WWOX* expression has been shown to correlate with better prognosis. Together, these findings support the view that WWOX has some tumor suppressor activity. In addition, genetic variations in *WWOX* have been shown to be associated with distinct metabolic and neurological diseases. However, the mechanism by which altered WWOX enzyme activity contributes to each of these distinct diseases is not clear.

In vitro studies have identified a large number of WWOX binding partners, indicating WWOX may contribute to a wide range of cellular pathways. However, it is unclear which of these pathways are relevant for human disease. In this study, *D. melanogaster* has been used as a model system to investigate cellular functions of the conserved *Wwox* gene product *in vivo*. Wwox has a conserved function in promoting Eiger/TNFα-mediated cell death. Reduced *Wwox* decreases Caspase 3 levels and prevents the elimination of tumorigenic cells *in vivo*, demonstrating that the pro-apoptotic role of Wwox is biologically significant. WWOX protein has multiple sequences that are homologous to known functional motifs. Expression of the N-terminal 169 amino acids (containing WW and cofactor binding domains) is sufficient to promote Eiger/TNFα-mediated cell death, suggesting that this function does not require a complete C-terminal short chain dehydrogenase/reductase (SDR) enzyme region. In contrast, an intact SDR enzyme sequence is required for other WWOX functions, including some of its metabolic roles.

Wwox can also contribute to the cellular dysfunction caused by altering levels of *pre-mRNA splicing factor 19* (*Prp19*), *Yorkie* (*Yki*) and *epidermal growth factor receptor* (*EGFR*). Further investigation revealed that Wwox modifies both Prp19 knockdown and EGFR knockdown phenotypes by promoting caspase-dependent cell death. One possible mechanism by which Wwox contributes to caspase activation is through the regulation of mitochondrial dynamics. Ectopic expression of Wwox can enhance *Drp1*-mediated mitochondrial fission, as well as cause lethality in flies with mitochondrial fusion defects. We hypothesize that dysregulation of mitochondrial dynamics is a common pathogenic pathway in a range of diseases caused by reduced *WWOX* expression. The role of WWOX in this pathway could be a target for therapeutic improvement of prognosis for patients with low *WWOX* levels.

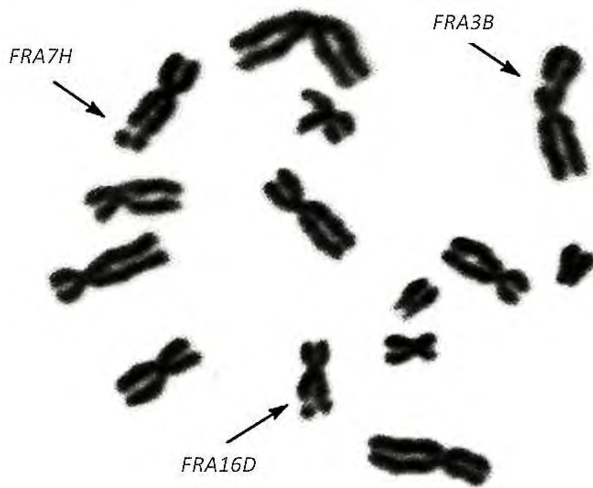
Chapter 1

Introduction

1.1 Common fragile sites

Common chromosomal fragile sites (CFS) are chromosomal regions prone to breakage upon replicative stress *in vitro* (Figure 1.1) (Glover*, et al.* 1984). Although it has been suggested that replication latency is a feature and potential cause of CFS (Le Beau*, et al.* 1998; El Achkar*, et al.* 2005; Palumbo*, et al.* 2010), the initiating mechanism(s) underlying CFS remains unclear. CFS are present in all individuals and can be induced by chemicals that are easily found in the diet and environment, including caffeine (Yunis and Soreng 1984), ethanol (Kuwano and Kajii 1987) and cigarette smoke (Sozzi*, et al.* 1997; Stein*, et al.* 2002). They are of particular interest, as they appear to be sites that are predisposed to DNA instability *in vivo*, which can alter allelic expression of the gene(s) spanning them. Interestingly, the frequency of induced breaks at CFS *in vitro* corresponds to the frequency of DNA instability in various cancer cells *in vivo* (Casper*, et al.* 2002; O'Keefe and Richards 2006), suggesting a relationship between chromosome fragility at CFS and tumorigenesis.

The notion that CFS-associated DNA instability could have a causal role in cancer has been supported by the discovery of genes with tumor suppressor properties spanning *FRA3B* and *FRA16D*, two of the most highly expressed CFS. These genes share a number of attributes. The respective genes, *fragile histidine triad* (*FHIT*) spanning *FRA3B* (Ohta*, et al.* 1996; Druck*, et al.* 1997) and *WW domain-containing oxidoreductase* (*WWOX*) spanning *FRA16D* (Mangelsdorf*, et al.* 2000; Ried*, et al.* 2000; Finnis*, et al.* 2005), are both extremely large (>1Mb). These intergenic fragile site locations are conserved in mouse (Shiraishi*, et al.* 2001; Krummel*, et al.* 2002), suggesting a biologically advantageous relationship. While the benefits of having evolutionarily conserved CFS are unknown, perturbation of *FHIT* and/or *WWOX* expression has been reported in various cancers, such as breast (Guler*, et al.* 2004), prostate (Guo*, et al.* 2000; Qin*, et al.* 2006), gastric (Gemma*, et al.* 1997; Aqeilan*, et al.* 2004a), liver (Yuan*, et al.* 2000; Park*, et al.* 2004) and lung (Sozzi*, et al.* 1997; Fabbri*, et al.* 2005). This suggests that these CFS genes are important for cellular processes. Thus, it is important to understand the cellular functions of these CFS genes, in order to improve the prognosis of cancer patients.



(Figure adapted from Sutherland, *et al.* 1998)

Figure 1.1. Appearance of common fragile sites on metaphase chromosomes after induction with aphidicolin. Three frequently expressed common fragile sites; *FRA3B*, *FRA16D* and *FRA7H* are shown (indicated by arrows).

1.2 WWOX

The human *WWOX* gene spans one of the most highly expressed CFS, *FRA16D*, which is ~260kb in size and located within the 780kb intron 8 of *WWOX* (Figure 1.2). *WWOX* is a very large gene (1.1Mb) with nine exons and eight introns. In contrast, the open reading frame (ORF) of *WWOX* is only 1245bp and encodes a protein of 414 amino acids (Figure 1.2) (Ried, *et al.* 2000). It is named ***WWOX*** because its protein contains two N-terminal **WW** domains (encoded by exons 1-4) and a C-terminal short-chain alcohol dehydrogenase-reductase (SDR) or **OX**idoreductase region (encoded by exons 4-8) (Bednarek, *et al.* 2000). The WWOX protein is evolutionarily conserved across species (Figure 1.3), suggesting that it has important cellular functions.

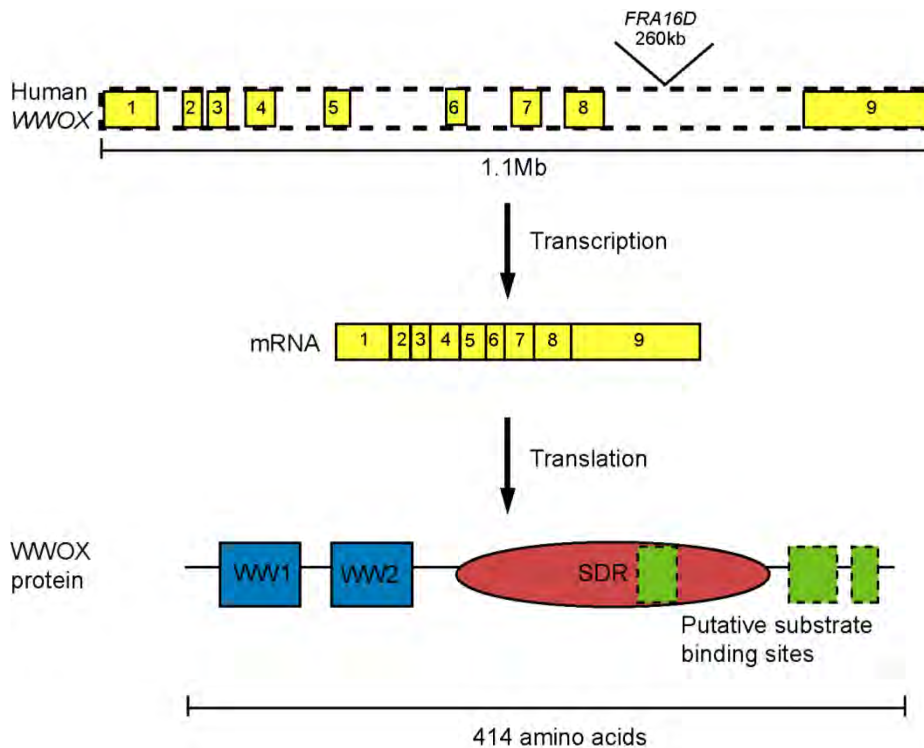
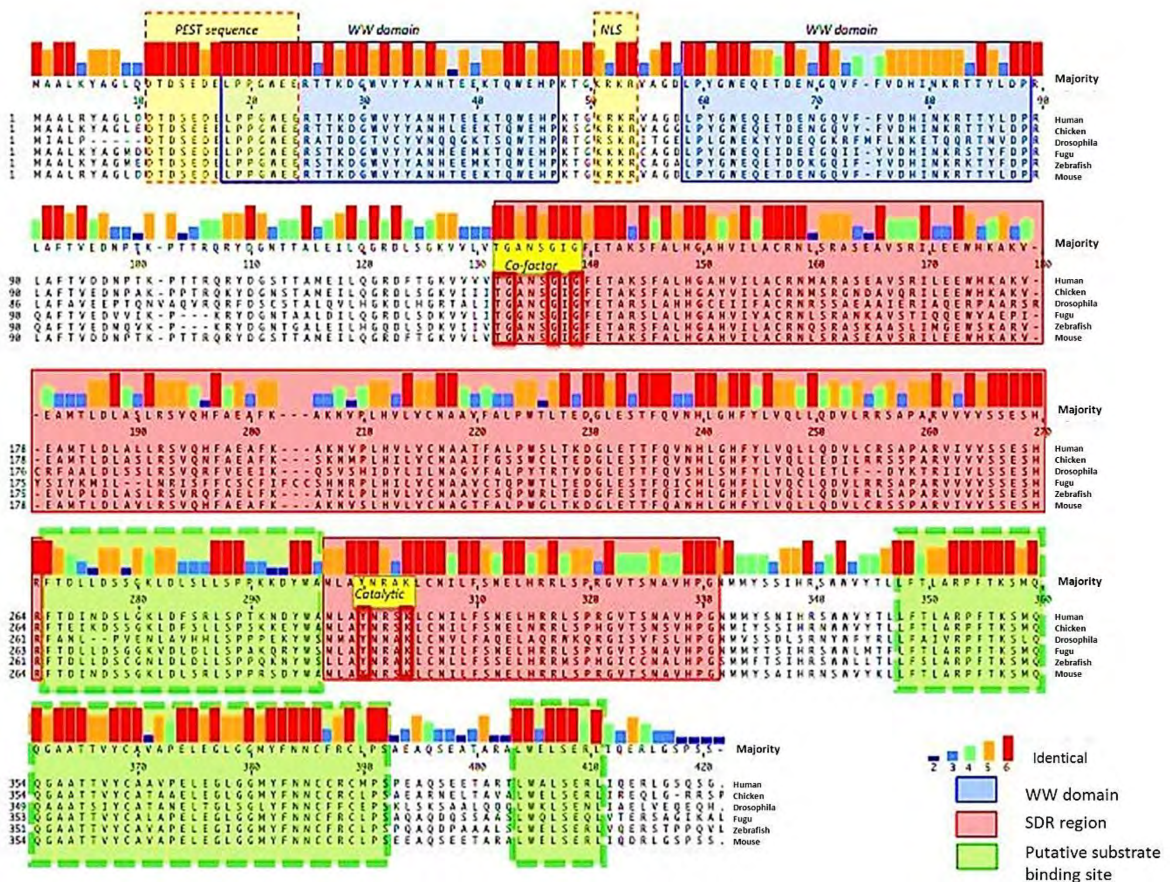


Figure 1.2. Human *WWOX* gene, mRNA and protein. The human *WWOX* gene has nine exons and eight introns with *FRA16D* CFS located within its eighth intron. The size of the *WWOX* gene is 1.1Mb and encodes a protein that is comprised of 414 amino acids. The WWOX protein contains two WW domains (blue boxes), an SDR region (red oval) and the C-terminal putative substrate binding sites (green boxes).

The WW domains of WWOX are characterized by their highly conserved proline and tryptophan residues (Salah*, et al.* 2012). The first WW domain (WW1) of WWOX contains two conserved tryptophan residues, while one of these has been replaced by a tyrosine residue in the second WW domain (WW2) (Bednarek*, et al.* 2000). The WW1 domain of WWOX can bind to proteins that contain a PPxY or LPxY motif and this interaction can be enhanced through phosphorylation of Tyrosine 33 (Y33) (Aqeilan*, et al.* 2004b; Aqeilan*, et al.* 2004c; Aqeilan*, et al.* 2005b). WWOX has also been shown to bind to proteins without these motifs, including p53 (Aqeilan*, et al.* 2004c; Chang*, et al.* 2005), c-Jun N-terminal kinase 1 (JNK1) (Chang*, et al.* 2003) and ΔNp63α (Salah*, et al.* 2013). In contrast, the WW2 domain of WWOX is not able to bind a subset of tested PPxY containing proteins, including SIMPLE (Ludes-Meyers*, et al.* 2004), erb-b2 receptor tyrosine kinase 4 (ErbB4) (Aqeilan*, et al.* 2005b) and WW domain binding protein 1 (WBP1) (McDonald*, et al.* 2012). It has been shown that the WW2 domain can regulate the binding ability of the WW1 domain (McDonald*, et al.*

2012; Schuchardt*, et al.* 2013; Abu-Odeh*, et al.* 2014a), suggesting that WW2 has a different function compared to WW1. In addition, WWOX also contains a proline (P), glutamic acid (E), serine (S), and threonine (T) rich (PEST) region at the N-terminus of WW1, and a nuclear localization signal between the two WW domains (Figure 1.3). PEST sequences provide a signal for proteolytic degradation and are important for the regulation of many proteins involved in metabolism and cell cycle (Rogers*, et al.* 1986; Rechsteiner and Rogers 1996).

The C-terminal SDR region of WWOX contains a TGxxxGxG motif required for NAD(P)(H) co-factor binding and a YxxxK motif for the catalytic function (Figure 1.3). Whilst these are characteristic of the classical SDR family of enzymes, the SDR region of WWOX also has an extended C-terminal sequence, which is the defining property of the extended SDR family of enzymes (Kavanagha*, et al.* 2008; Richards*, et al.* 2015). It has been hypothesized that this C-terminal extension, in addition to residues 265-289 near the catalytic site, are involved in SDR substrate binding (Figure 1.2 and 1.3) (Kavanagha*, et al.* 2008; Abu-Remaileh*, et al.* 2015; Richards*, et al.* 2015). An *in vitro* study has shown that the SDR region of WWOX has dehydrogenase activity for different steroids in the presence of NAD^+ and $NADP^+$ (Sałuda-Gorgul*, et al.* 2011). However, the *in vivo* substrate(s), product(s) and enzymatic function(s) of the SDR region of WWOX have not yet been identified.



(Figure adapted from Richards, *et al.* 2015)

Figure 1.3. Alignment of orthologous WWOX protein sequences from human, chicken, *Drosophila*, fugu, zebrafish and mouse. The consensus sequence is shown at the top of the aligned sequences. All WWOX orthologs contain two WW domains (shaded in blue), an SDR region (shaded in red) and the putative substrate binding site sub-regions (shaded in green). The PEST region, nuclear localization sequence (NLS), cofactor binding site and catalytic site are also shown.

1.3 Tissue distribution and subcellular localization of WWOX

WWOX has variable expression levels in different tissues and organs. Using immunohistochemistry, it has been shown that WWOX is highly expressed in epithelial cells of hormone-regulated organs (Nunez*, et al.* 2006; Aqeilan*, et al.* 2009), neural cells (Nunez*, et al.* 2006), and the lung, skeleton and liver (Aqeilan*, et al.* 2009). In contrast, there is no WWOX expression detected in the lymph node, thymus, spleen, aorta, or adipose, vascular or connective tissues (Nunez*, et al.* 2006).

Although it is clear that WWOX is predominantly found in the cytoplasm (Bednarek, *et al.* 2001; Aqeilan, *et al.* 2004b; Nunez, *et al.* 2005a; O'Keefe, *et al.* 2005; Nunez, *et al.* 2006; Aqeilan, *et al.* 2007c), the subcellular localization of WWOX is still a controversial issue. Chang *et al.* (2001) identified a mitochondrial targeting sequence within the SDR region, which enables the translocation of WWOX to the mitochondria (Chang, *et al.* 2001). Upon apoptotic stimuli, WWOX translocates from the mitochondria to the nucleus (Chang, *et al.* 2001). In contrast, Bednarek *et al.* (2001), Ludes-Meyers *et al.* (2003, 2004) and Nunez *et al.* (2006) have reported that WWOX is mainly localized to the Golgi apparatus (Bednarek, *et al.* 2001; Ludes-Meyers, *et al.* 2003; Ludes-Meyers, *et al.* 2004; Nunez, *et al.* 2006). Although the basis for these discrepancies is not clear, it is possible that WWOX translocates to different organelles (the mitochondria, nucleus or Golgi apparatus) in different cell types or under certain culture conditions to perform various biological functions, such as mediating the proper translocation of its binding partners. Despite different observations for the localization of full length WWOX, Chang *et al.* (2001), Bednarek *et al.* (2001) and Ludes-Meyers *et al.* (2003) have all found that truncated WWOX, which lacks an intact SDR region, is mainly localized in the nucleus, suggesting that the SDR region is important for directing the subcellular localization.

1.4 Diseases caused by reduced *WWOX* levels

WWOX was first proposed to be a tumor suppressor gene, following the observation that its expression is reduced in many cancers (Table 1.1) (Bednarek, *et al.* 2000; Ried, *et al.* 2000; Paige, *et al.* 2001). Recent association studies have also shown that hypomorphic *WWOX* alleles correlate with different metabolic and neurological disorders (Tsai, *et al.* 2010; Wang, *et al.* 2011a; White, *et al.* 2011; Abdel-Salam, *et al.* 2014; Mallaret, *et al.* 2014; Mignot, *et al.* 2014; Ben-Salem, *et al.* 2015; Tabarki, *et al.* 2015; Valduga, *et al.* 2015).

1.4.1 Cancer

Loss of heterozygosity (LOH) and decreased *WWOX* expression have been detected in various cancer cell lines, such as breast (Bednarek, *et al.* 2000), prostate (Qin, *et al.* 2006), liver (Park, *et al.* 2004), gastric (Aqeilan, *et al.* 2004a), lung (Fabbri, *et al.* 2005), ovarian (Paige, *et al.* 2001), colon (Bednarek, *et al.* 2000; Finnis, *et al.* 2005; Alsop, *et al.* 2008) and esophageal carcinomas (Kuroki, *et al.* 2002). By comparing breast cancer cells that originated

from the same tumor at different stages of cancer progression, Finnis *et al.* (2005) showed that both primary and secondary breast cancer cells have the same homozygous deletion breakpoints at *FRA16D*, despite other karyotypic differences, suggesting that loss of WWOX function occurs in the early stages of tumorigenesis. Consistently, Aqeilan *et al.* (2004) also observed that 65% of primary gastric adenocarcinomas have loss of *WWOX* expression (Aqeilan, *et al.* 2004a). This suggests that reduced *WWOX* levels can promote tumorigenesis, and thus it has been proposed to be a tumor suppressor gene (Bednarek, *et al.* 2001; Aqeilan, *et al.* 2004a; Finnis, *et al.* 2005). In contrast, elevated *WWOX* expression has been detected in certain breast and gastric cancer cell lines (Bednarek, *et al.* 2001; Watanabe, *et al.* 2003). These studies however did not screen for mutations in other genes that act downstream of WWOX (Bednarek, *et al.* 2001; Watanabe, *et al.* 2003). While *WWOX* may be up-regulated to induce cell death in these cancer cells, it is possible that these cancer cells have developed other mechanisms to block the pro-apoptotic activity of WWOX (Ghavami, *et al.* 2009).

Loss of WWOX function has also been correlated with more aggressive tumor phenotypes and poor prognosis in a variety of cancer cells (Aqeilan, *et al.* 2004a; Nunez, *et al.* 2005b; Pluciennik, *et al.* 2006; Donati, *et al.* 2007; Lewandowska, *et al.* 2009; Wegman, *et al.* 2013). *In vitro* experiments have shown that decreased *WWOX* levels in cancer cells can increase their chemotherapeutic drug resistance (Guler, *et al.* 2007; Liu, *et al.* 2008; Tsai, *et al.* 2013; Yin, *et al.* 2013). Another study looking at loss of p53 function in treatment-resistant glioblastoma cells (Gjerset, *et al.* 1995; Squatrito, *et al.* 2010) demonstrated that ectopic expression of WWOX is able to kill these cells through a caspase-independent pathway. Collectively, these studies suggest that *WWOX* expression favors cancer cell elimination, and that restoration of WWOX function in cancer cells is a viable therapeutic target.

The function of WWOX as a tumor suppressor is further supported by numerous studies *in vivo*. Over-expression of WWOX in various cancer cells is able to suppress their tumorigenicity in mouse models by inducing cell death (Bednarek, *et al.* 2001; Fabbri, *et al.* 2005; Qin, *et al.* 2006; Iliopoulos, *et al.* 2007; Nakayama, *et al.* 2008; Kurek, *et al.* 2010). It is worth noting that ectopic over-expression of any protein beyond a tolerated threshold may induce non-specific effects (Huang, *et al.* 2000). It would be interesting to test whether ectopic over-expression of WWOX can also induce cell death in normal cells, or if this is unique to cancer cells. WWOX undoubtedly has tumor suppressive function, as Iliopoulos *et*

al. (2007) have demonstrated that reactivation of endogenous *WWOX* in *WWOX*-deficient breast cancer cells suppresses their tumorigenicity *in vivo* (Iliopoulos, *et al.* 2007).

Additionally, it has been demonstrated that a complete knock-out of *Wwox* expression in mice (*Wwox*$^{-/-}$) leads to the development of osteosarcomas (Table 1.2), whilst heterozygous *Wwox* mice (*Wwox*$^{+/-}$) are more susceptible to the development of spontaneous tumors compared to wild-type mice (Aqeilan, *et al.* 2007c; Abdeen, *et al*. 2011). These heterozygous *Wwox* mice also have a higher occurrence of tumorigenesis when challenged with NMBA-induced fore-stomach tumors and ENU-induced lung cancers (Aqeilan, *et al.* 2007b; Aqeilan, *et al.* 2007c). Since *Wwox*$^{-/-}$ mice show postnatal lethality, the effects of complete loss of Wwox function in adult mice cannot be analyzed (Aqeilan, *et al.* 2007c; Aqeilan, *et al.* 2009). In an attempt to explore Wwox function in adult mice, Ferguson *et al.* (2012) conditionally knocked-out *Wwox* through two Cre recombinase approaches (Table 1.2). When *Wwox* was knocked-out in epithelial precursor cells using the bovine keratin 5 promoter driving *Cre* expression (*BK5-Cre*), the mice died prematurely, which was consistent with previous results of Aqeilan *et al.* (2007) (Aqeilan, *et al.* 2007c; Ferguson, *et al.* 2012). However, when they used the mouse mammary tumor virus (MMTV) promoter, which is mainly expressed in the mammary gland epithelium, *Wwox* knock-out mice did not show postnatal death. Intriguingly, neither *Wwox* conditional knock-out mice nor heterozygous *Wwox* mice in their experiments developed tumors. Thus, they claimed that loss of *Wwox* expression in mammary gland epithelium may not have reached the threshold required to initiate tumorigenesis (Ferguson, *et al.* 2012). In addition, there was also no tumor formation in other *Wwox* null mutant models, including rat (Suzuki, *et al.* 2009) and *Drosophila* (O'Keefe, *et al.* 2011). However, all these null models showed altered metabolism (Suzuki, *et al.* 2009; O'Keefe, *et al.* 2011; Ferguson, *et al.* 2012), which is a hallmark of cancer (Hanahan and Weinberg 2011). It is therefore a possibility that WWOX may act to prevent tumorigenesis through its role in the regulation of cellular metabolism.

Table 1.1 Summary of the evidence for *WWOX* as a tumor suppressor gene.

Evidence supporting WWOX as a tumor suppressor	**References**
LOH of *WWOX* is detected in many cancers.	(Bednarek, *et al.* 2000; Ried, *et al.* 2000; Paige, *et al.* 2001; Kuroki, *et al.* 2002; Aqeilan, *et al.* 2004a; Kuroki, *et al.* 2004; Park, *et al.* 2004; Fabbri, *et al.* 2005; Finnis, *et al.* 2005; Qin, *et al.* 2006; Alsop, *et al.* 2008)
Patients with reduced *WWOX* levels have a poorer prognosis.	(Pluciennik, *et al.* 2006; Donati, *et al.* 2007; Wang, *et al.* 2011b; Żelazowski, *et al.* 2011)
Over-expression of *WWOX* in cancer cells is able to suppress their tumorigenicity both *in vitro* and *in vivo*.	(Bednarek, *et al.* 2001; Fabbri, *et al.* 2005; Qin, *et al.* 2006; Iliopoulos, *et al.* 2007; Aderca, *et al.* 2008; Nakayama, *et al.* 2008; Hu, *et al.* 2012; Del Mare and Aqeilan 2015)
Both heterozygous and homozygous *WWOX* mutant mice show a higher incidence in the development of spontaneous and induced tumors.	(Aqeilan, *et al.* 2007c)

Table 1.2 Comparison of different reported *Wwox* mutant mouse models.

Mouse line	***Wwox*** $^{-/-}$	***Wwox*** $^{\Delta/\Delta}$ **(*Ella-Cre Wwox*)**	***Wwox*** $^{gt/gt}$	***Wwox*** $^{\Delta Cre/\Delta Cre}$	***BK5-Cre Wwox***	***MMTV-Cre Wwox***
Method of generating mutation	Targeted disruption	Cre-LoxP system	Gene trap	Cre-LoxP system	Cre-LoxP system	Cre-LoxP system
Expression of Wwox	Complete absence	Absence in bone and major tissues, including liver, lung, skeletal muscle	Hypomorph	Complete absence	Absence in epithelial precursor cells and their derivatives	Absence in mammary gland epithelium expressing cells
Viability	4 weeks	3 weeks	Up to 2 years	3 weeks	Up to 117 days	Up to 1.4 years
Tumor formation	Osteosarcoma s	-	B cell lymphomas	-	-	
Other symptoms	Growth retardation, bone metabolic defects, hypogonadism	Growth retardation, hypoglycemia, defects in bone formation	Hypogonadism	Growth retardation, bone metabolic defects, hypoglycaemia, hypocalcaemia, splenic atrophy, leukopenia and impaired haematopoiesis	Impaired mammary branching morphogenesis	
References	(Aqeilan, *et al.* 2007c; Aqeilan, *et al.* 2009)	(Abdeen, *et al.* 2013)	(Ludes-Meyers , *et al.* 2007)	(Ludes-Meyers, *et al.* 2009)	(Ferguson, *et al.* 2012)	

1.4.1.1 WWOX polymorphisms that have been associated with cancers

Although most studies have shown that WWOX can suppress tumor growth (Bednarek*, et al.* 2001; Fabbri*, et al.* 2005; Qin*, et al.* 2006; Iliopoulos*, et al.* 2007; Nakayama*, et al.* 2008; Kurek*, et al.* 2010), it is classified as a "non-classical" tumor suppressor gene because it does not follow Knudson's "two hit" model. Firstly, loss of one *WWOX* allele is sufficient to cause tumorigenesis (Aqeilan*, et al.* 2007c). Indeed, most cancer cells with *WWOX* gene perturbation still retain one copy of *WWOX*, suggesting that loss of the second *WWOX* allele is not required for cancer formation (Lewandowska*, et al.* 2009). Secondly, LOH of *WWOX* is a common event in multiple types of cancer (Kuroki*, et al.* 2002; Aqeilan*, et al.* 2004a; Park*, et al.* 2004; Fabbri*, et al.* 2005; Finnis*, et al.* 2005; Qin*, et al.* 2006; Alsop*, et al.* 2008). Thirdly, point mutations that cause loss of *WWOX* function are very rare in cancers (Mahajan*, et al.* 2005).

Although somatic point mutations that abolish *WWOX* function are rare, the frequency of single nucleotide polymorphisms (SNPs) is, in fact, relatively high. Some of these SNPs are mis-sense polymorphisms that have been shown to be associated with cancer. For example, it has been shown that the SNP at codon 282 of *WWOX*, which changes proline to alanine (P282A) in the SDR loop (Abu-Remaileh*, et al.* 2015), may predispose carriers to thyroid, breast and gastric cancers (Cancemi*, et al.* 2011; Ekizoglu*, et al.* 2012; Guo*, et al.* 2013). Using SCRATCH Protein Prediction, Cancemi *et al.* (2011) showed that this P282A substitution may change the secondary structure of WWOX and affect its activity (Cancemi, *et al.* 2011). In addition, Ekizoglu *et al.* (2011) detected thirteen other base substitutions in the coding region and twenty-three others in the non-coding region of *WWOX* in breast cancer cells. Interestingly, all base substitutions in the coding region were within exons 4-9, which encodes the SDR domain of WWOX (Ekizoglu*, et al.* 2012).

In the study of Yang *et al.* (2013), they found that loss of copy number variation (CNV)-67048, which is located in intron 5 of *WWOX*, might increase the risk of lung cancer (Yang*, et al.* 2013). Further studies have shown that individuals with loss of this CNV-67048 are also predisposed to gliomas (Yu*, et al.* 2014), epithelial ovarian cancer (Chen*, et al.* 2016), intracranial aneurysm (Fan*, et al.* 2016) and chronic obstructive pulmonary disease (Yang*, et al.* 2015). Loss of CNV-67048 may affect normal *WWOX* splicing and generate a WWOX protein with a truncated SDR region. Together, these association studies suggest that the SDR region is important for the function of WWOX as a tumor suppressor. However,

future studies are required to determine the exact mechanism by which these mutations promote tumorigenesis *in vivo*. In addition to SNP at codon 282 and loss of CNV-67048, there are multiple mechanisms that can also inactivate WWOX activity, such as LOH and hypermethylation (Figure 1.4).

1.4.1.2 Mechanisms of inactivating the tumor suppressor capacity of WWOX

LOH of *WWOX* has been detected in various cancer cells (Kuroki*, et al.* 2002; Aqeilan*, et al.* 2004a; Park*, et al.* 2004; Fabbri*, et al.* 2005; Finnis*, et al.* 2005; Qin*, et al.* 2006; Alsop*, et al.* 2008), and it is one of the mechanisms that reduces WWOX activity. LOH of *WWOX* can be caused by the breakage of *FRA16D* (Figure 1.4), since this region is susceptible to DNA instability upon various environmental stresses (Thavathiru*, et al.* 2005). As the location of *FRA16D* within intron 8 of the *WWOX* gene, LOH not only causes a 50% reduction of *WWOX* expression, but also can produce high levels of alternatively spliced variants (Figure 1.4). In fact, high levels of alternatively spliced transcripts that encode WWOX proteins with a truncated SDR region have been detected in various carcinoma cell lines (Figure 1.5) (Ried*, et al.* 2000; Bednarek*, et al.* 2001; Paige*, et al.* 2001; Driouch*, et al.* 2002; Kuroki*, et al.* 2002; Yendamuri*, et al.* 2003; Aqeilan*, et al.* 2004b). Driouch *et al.* (2002) showed that mRNA corresponding to WWOX isoform 2 (also known as WWOXv6 and FOR III), which contains two WW domains and an incomplete SDR region (Figure 1.5), is present at very low levels in normal breast cells, but is highly expressed in 50% of examined breast tumors (Driouch*, et al.* 2002). This suggests that high levels of aberrant *WWOX* transcripts are associated with tumorigenesis. However, sequencing of breast cancer cell lines with high levels of *WWOX isoform 2* transcripts showed that there is no mutation in the *WWOX* coding region or in its exon-intron junctions, suggesting that *WWOX* alternatively spliced variants can be produced by a normally occurring alternative pre-mRNA splicing event (Driouch*, et al.* 2002). Up-regulation of heterogeneous nuclear ribonucleoparticles (hnRNP) A2/B1 splicing factor has been shown to increase the generation of *WWOX* alternatively spliced mRNA variants lacking exon 6 to 8 (Golan-Gerstl*, et al.* 2011). Interestingly, over-expression of splicing factor hnRNP A2/B1 has also been shown to enhance glioblastoma tumorigenicity and cause poorer prognostic outcomes (Golan-Gerstl*, et al.* 2011), highlighting the possibility that high levels of these splice variants can contribute to tumorigenesis.

Truncated WWOX proteins, encoded by *WWOX* alternatively spliced variants with deletion of exons 5-8, have been detected in human prostate carcinoma cell lines (Mahajan*, et al.* 2005). Mahajan *et al.* (2005) showed that expression of activated Cdc42-associated kinase (Ack1) in prostate adenocarcinoma cell line phosphorylates WWOX at Tyrosine 287 (Y287) and causes its poly-ubiquitination and subsequent degradation. They demonstrated that loss of this Y287 in truncated WWOX protein prevents degradation, and it is therefore more stable compared to full length WWOX (Mahajan*, et al.* 2005). In contrast, Watanabe *et al.* (2003) only detected truncated WWOX proteins in colon carcinoma cell lines after a proteasome inhibitor was added (Watanabe*, et al.* 2003). Thus, they claimed that truncated WWOX proteins were unstable and rapidly degraded by the proteasome. Although the reasons for the differences between these two studies are unclear, both of them have shown that *WWOX* alternatively spliced variants can be translated into proteins and are present in cancer cells.

Many studies have hypothesized that truncated WWOX proteins with an incomplete SDR region could act as dominant negative competitors for WWOX and reduce its activity (Figure 1.4) (Ried*, et al.* 2000; Ludes-Meyers*, et al.* 2003; Mahajan*, et al.* 2005; Bouteille*, et al.* 2009). This is supported by the study of Bouteille *et al.* (2009), who demonstrated that WWOX isoform 2 can reduce the interaction of full length WWOX with Dishevelled-2 (Dvl-2) protein, which consequently represses the inhibitory effect of full length WWOX on the Wnt/β-catenin pathway (Bouteille*, et al.* 2009). This can cause aberrant Wnt/β-catenin pathway activation and lead to tumorigenesis (Klaus and Birchmeier 2008).

Hypermethylation-mediated silencing of *WWOX* has also been shown to reduce *WWOX* expression (Figure 1.4) in breast, lung, prostate, pancreatic, gastric and esophageal squamous cancer cells (Kuroki*, et al.* 2004; Iliopoulos*, et al.* 2005; Qin*, et al.* 2006; Iliopoulos*, et al.* 2007; Wang*, et al.* 2009; Yan*, et al.* 2011; Guo*, et al.* 2013). Treatment with demethylating agent, 5-aza-2'-deoxycytidine has been shown to restore *WWOX* expression in some of these cancer cells (Kuroki*, et al.* 2004; Qin*, et al.* 2006; Iliopoulos*, et al.* 2007) and suppress tumorigenesis *in vivo* (Iliopoulos*, et al.* 2007). This suggests that preventing hypermethylation of *WWOX* can restore its tumor suppressor function. Together, these findings indicate that there are multiple possible mechanisms that can lead to reduced *WWOX* activity.

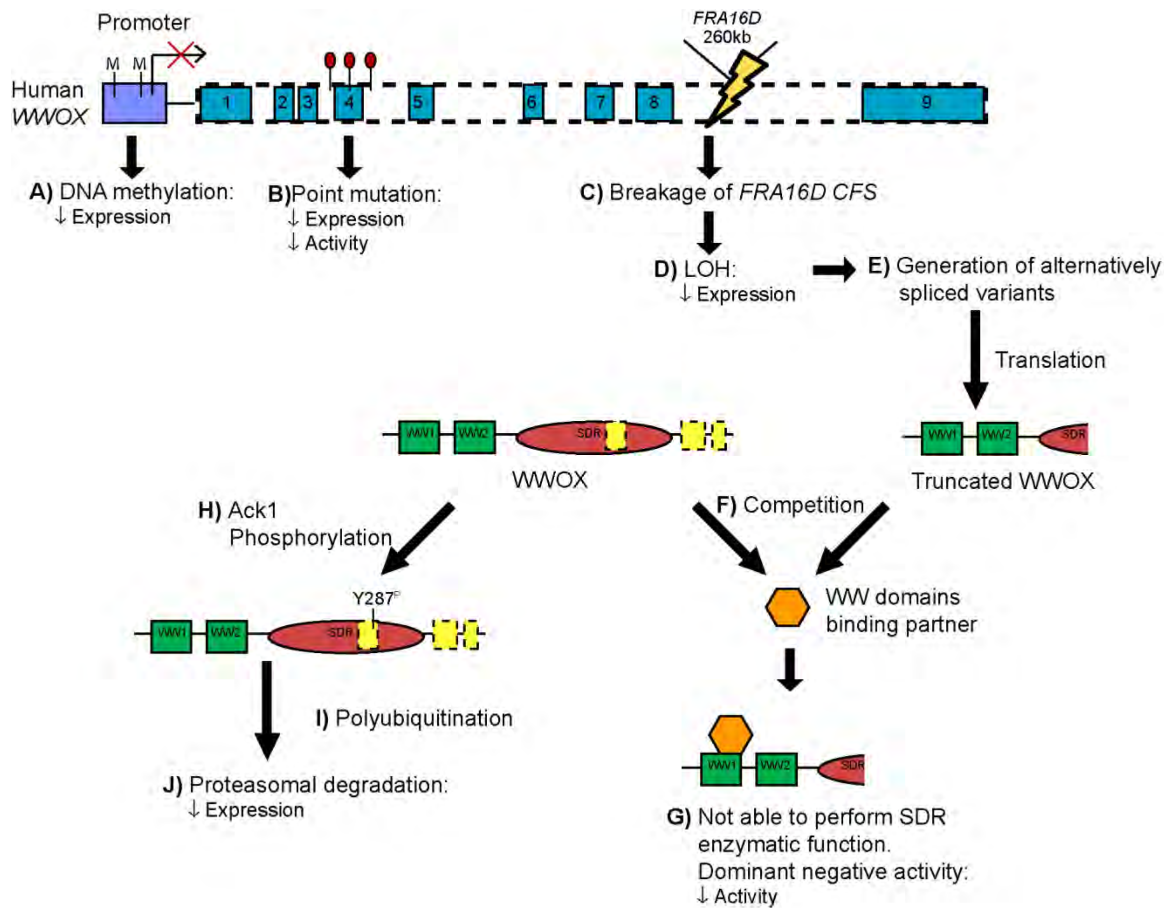


Figure 1.4. Mechanisms that cause reduced WWOX expression and activity. A) Methylation of the *WWOX* promoter prevents transcription and leads to decreased levels of *WWOX*. **B)** Point mutations in the *WWOX* gene can affect either its expression or activity. **C)** Breakage of *FRA16D* CFS causes **D)** LOH of *WWOX*. This can lead to reduction of *WWOX* mRNA levels, as well as **E)** increased levels of *WWOX* alternatively spliced variants encoding truncated WWOX proteins with an incomplete SDR region. **F-G)** These truncated WWOX proteins can compete with WWOX for its binding partners that recognize WW domains, but are not able to perform the enzymatic function(s) of SDR region. Thus, their expression reduces the remaining WWOX activity. **H-J)** Activated Ack1 phosphorylates WWOX at Tyrosine 287 ($Y287^P$), which then leads to polyubiquitination, followed by proteosomal degradation.

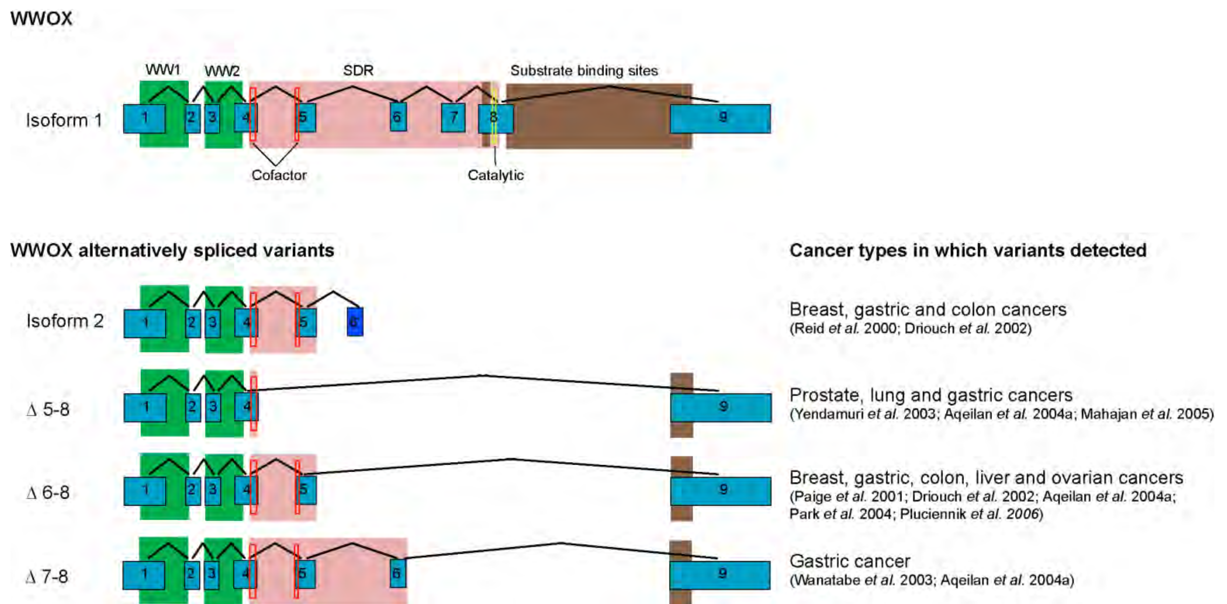


Figure 1.5. The human *WWOX* transcript and its alternatively spliced variants. The human *WWOX* transcript encodes two WW domains (shaded in green), an SDR region (shaded in pink) and putative substrate binding sites (shaded in brown). Cofactor binding (indicated by open red box) and catalytic (indicated by open yellow box) sites are shown. Alternatively spliced variants of *WWOX* encoding truncated WWOX proteins with an incomplete SDR region have been detected in various cancers. Transcripts of WWOX isoform 2 contain five exons and an alternate exon 6 (indicated by dark blue box). Each these alternatively spliced *WWOX* variants do not contain the SDR catalytic site.

1.4.2 Metabolic diseases

WWOX has not only been shown to suppress tumorigenesis, but also has an important role in the regulation of metabolism. Human studies have shown that specific genetic variations in *WWOX* are associated with many different human diseases or disorders that exhibit altered metabolism. These associations include low HDL-C (Lee*, et al.* 2008), Type 2 Diabetes (Tsai*, et al.* 2010; Chang*, et al.* 2012; Sakai*, et al.* 2013), forced vital capacity in lung function (Loth*, et al.* 2014; Xie*, et al.* 2016), hypertension susceptibility (Yang*, et al.* 2012), coronary artery calcification (Polfus*, et al.* 2013), obesity (Wang*, et al.* 2011a) and left ventricular thickness (Vasan*, et al.* 2009). As enzymes of the SDR family are usually involved in various metabolic processes (Kavanagha*, et al.* 2008), it is believed that the SDR region is required for the metabolic functions of WWOX. In support of this, microarray analysis of lymphocytes from a male patient with a disorder of sex development revealed the heterozygous deletion of exons 6-9 within the *WWOX* gene (White*, et al.* 2011). This mutated

WWOX gene encoded a truncated WWOX protein lacking a functional SDR region. This truncated WWOX protein may exert a dominant negative effect by preventing wild-type WWOX activity (Bouteille*, et al.* 2009). As the SDR region of WWOX has dehydrogenase activity for different steroids (Saługa-Gorgul*, et al.* 2011), it suggests that defects in sex-steroid metabolism in this patient could be due to the loss of SDR enzyme activity of WWOX (White*, et al.* 2011).

The metabolic role of WWOX is also conserved in mouse. Analysis of *Wwox*-deficient mice showed that their early death was caused by severe metabolic defects. These *Wwox*-deficient mice have abnormal bone homeostasis, morphological defects in the reproductive system and reduced glucose, lipid and calcium levels (Aqeilan*, et al.* 2007c; Aqeilan*, et al.* 2009; Ludes-Meyers*, et al.* 2009; Abu-Remaileh and Aqeilan 2014). Similarly, *Wwox* knock-out rats also show male hypogonadism and lower bone density (Suzuki*, et al.* 2009). Together, these rodent *WWOX* mutants have provided clear evidence that WWOX is an important component in the regulation of metabolism.

1.4.3 Neurological diseases

In addition to a higher risk of tumor development and severe metabolic defects, rodent *WWOX* mutants have also been shown to suffer epileptic seizures (Suzuki*, et al.* 2009; Mallaret*, et al.* 2014). Immuno-histochemical analyses of murine tissues have shown that *WWOX* is widely expressed in various differentiated cells of the developing and adult central nervous system (Chen*, et al.* 2004). This is consistent with findings in normal adult human tissues, which also show that *WWOX* is expressed in a range of neuronal cells (Nunez*, et al.* 2006). Together, these findings support an important role for WWOX in the central nervous system.

Mutations in *WWOX* have also been associated with different human neurological disorders, such as epilepsy, ataxia, mental retardation and neurodegeneration (Table 1.3) (Abdel-Salam*, et al.* 2014; Mallaret*, et al.* 2014; Mignot*, et al.* 2014; Ben-Salem*, et al.* 2015; Tabarki*, et al.* 2015; Valduga*, et al.* 2015). Mallaret *et al.* (2014) uncovered a homozygous *WWOX* mutation changing proline 47 to threonine (P47T) in a family suffering from autosomal recessive ataxia (Mallaret*, et al.* 2014). This mis-sense mutation affects proper WW1 domain folding and abolishes the binding ability of WWOX. They also identified a homozygous *WWOX* mutation, in which glycine 372 was substituted to arginine (G372R), in a different family with ataxia, epilepsy, mental retardation and prominent upper motor disease

(Mallaret*, et al.* 2014). In addition, Alkhateeb *et al.* (2016) identified a homozygous *WWOX* mis-sense mutation, glycine 410 cysteine (G410C), in patients with intellectual disability (Alkhateeb*, et al.* 2016). Both G372R and G410C mutations are located at the C-terminal region of the SDR region of WWOX, suggesting that this region of WWOX is important for normal neurological function. Consistent with these findings, an *in vivo* study in rats showed that deletion of 13bp in the last exon of *Wwox* causes neurological symptoms, male hypogonadism and early lethality (Suzuki*, et al.* 2009). Furthermore, Sze *et al.* (2004) have shown that WWOX has a protective role against Alzheimer's disease (Sze*, et al.* 2004). Formation of neurofibrillary tangles (NFTs), caused by hyperphosphorylation of Tau protein, is a pathological hallmark of Alzheimer's disease (Delacourte and Defossez 1986). WWOX has been shown to prevent phosphorylation of Tau by physically binding with it, and blocking the activity of enzymes that induce its hyperphosphorylation, including JNK1, extracellular signal-regulated kinases (ERK) and glycogen synthase kinase 3 beta (GSK3β) (Sze*, et al.* 2004). WWOX can also prevent aggregation of trafficking protein particle complex 6A delta (TPC6AΔ), TGF-β1-induced anti-apoptotic factor 1 (TIAF1) and amyloid beta (Aβ) proteins, which are all found in the brains of patients with Alzheimer's disease (Chang and Chang 2015). Together, these findings provide evidence that WWOX is important for the regulation of various neurological disorders.

Table 1.3 *WWOX* mutations that have been identified in patients with neurological disorders. "*" symbol indicates a nonsense mutation; "/" symbol separates mutations found in different *WWOX* alleles; "SBD" indicates putative substrate binding domain of WWOX.

WWOX mutations in patients	Domain(s) affected	Neurological diseases/ symptoms	Reference
Homozygous R54*	WW2, SDR and SDB	Growth retardation, microcephaly, epileptic seizures, retinopathy and early death	(Abdel-Salam, *et al.* 2014)
Homozygous P47T	WW1	Spinocerebellar ataxia-12 symptoms	(Mallaret, *et al.* 2014)
Homozygous G372R	SDB		
Homozygous point mutation (NM_016373.3:c.606-1G>A) (Affects splicing event and probably causes skipping of exons 7-9)	SDR and SDB	Growth retardation, microcephaly, refractory seizure, spasticity, and early death	(Tabarki, *et al.* 2015)
Homozygous G410C	SDB	Intellectual disability	(Alkhateeb, *et al.* 2016)
Homozygous exon 5 deletion	SDR and SDB	Growth retardation, microcephaly, epileptic seizures, later developed spasticity and delayed psychomotor development	(Ben-Salem, *et al.* 2015)
Homozygous exons 1-6 deletion	All domains	Encephalopathy, early-onset epileptic, brain anomalies and early death	(Valduga, *et al.* 2015)
Exons 1-5 deletion/exons 6-8 deletion	All domains/ SDR and SDB	Infantile epileptic encephalopathy symptoms	(Mignot, *et al.* 2014)
Exon 6 deletion/W335*	SDR and SDB/SDB		
Frameshift mutation (after the S14)/P47R	All domains/WW1		
Complete deletion/K297*	All domains/SDR and SDB		

1.5 Reported WWOX functions

A wide range of disorders have been reported for patients with low levels of *WWOX*. In order to understand the mechanism(s) for these disorders, it is important to determine WWOX functions and the pathways affected by altering its levels. Targeting these pathways could possibly improve the management of diseases (including cancer, metabolic disorders and neurological diseases) and prognosis in patients with low levels of *WWOX*.

WWOX carries different sequence homology motifs, including WW domains and an SDR region. Since WW domains are known sites of protein-protein interaction, the identity and function of the WWOX binding partners have been investigated as a defining feature of WWOX function (Chang, *et al.* 2001; Aqeilan, *et al.* 2004b; Aqeilan, *et al.* 2004c; Ludes-Meyers, *et al.* 2004; Gaudio, *et al.* 2006; Abu-Odeh, *et al.* 2014a). Abu-Odeh *et al.* (2014) identified 240 binding partners for the WW1 domain of WWOX using mass spectrometry and phage display experiments. These proteins are involved in a range of cellular processes, including transcription, RNA processing, cell death and metabolism (Abu-Odeh, *et al.* 2014a). However, the biological significance of these interactions *in vivo* is still not fully understood.

WWOX has also been shown to regulate the activity of some of its binding partners by sequestering them in the cytoplasm. These binding partners include ErbB4 (Aqeilan, *et al.* 2005b), transcription factor activator protein 2 (AP2γ) (Aqeilan, *et al.* 2004b), p73 (Aqeilan, *et al.* 2004c), c-Jun (Gaudio, *et al.* 2006) and Dvl-2 (Bouteille, *et al.* 2009), which are all important for the regulation of cell survival or proliferation, demonstrating the important role of WWOX in these pathways.

1.5.1 Roles of WWOX in apoptosis

Apoptotic stimuli, such as tumor necrosis factor (TNF), staurosporine, atractyloside and ultraviolet (UV) irradiation, have been shown to induce Y33 phosphorylation of WWOX and activate its pro-apoptotic role (Chang, *et al.* 2001; Chang, *et al.* 2005; Lai, *et al.* 2005). Chang *et al.* (2001) demonstrated that WWOX enhanced TNF-mediated cell death in murine L929 cells by up-regulating *p53* expression and down-regulating *Bcl-2* and *Bcl-xL* levels (Chang, *et al.* 2001). WWOX has been shown to be an essential partner for p53-mediated apoptosis, as WWOX silencing abolishes the apoptotic capacity of p53 (Chang, *et al.* 2001). Conversely, the induction of apoptosis by over-expression of WWOX is not affected by p53

silencing (Chang, *et al.* 2001), suggesting that WWOX is able to induce apoptosis through other pathways (Figure 1.6).

WWOX has been shown to physically interact with p73 and sequester it in the cytoplasm (Aqeilan, *et al.* 2004c; Lin, *et al.* 2013). Although this interaction inhibits the transcription factor activity of p73, levels of apoptosis are increased, suggesting that WWOX can activate cytoplasmic pro-apoptotic activity of p73 (Aqeilan, *et al.* 2004c). In addition, the binding of WWOX to p73 also prevents its degradation by inhibiting the ubiquitination of p73 by itchy E3 ubiquitin protein ligase (ITCH) (Abu-Odeh, *et al.* 2014a). Indeed, WWOX can bind to ITCH directly and is ubiquitinated by it. Lysine 63 (K63)-linked polyubiquitination of WWOX by ITCH enables translocation of WWOX into the nucleus and eventually also enhances p73-mediated cell death (Abu-Odeh, *et al.* 2014a).

WWOX is also involved in cell death induced by transforming growth factor beta 1 (TGF-β1) (Nakayama, *et al.* 2008; Hsu, *et al.* 2009). Binding of TGF-β1 to surface hyaluronidase (Hyal-2) promotes formation of the Hyal-2-WWOX complex. In contrast to the interaction with p73, this complex translocates into the nucleus and increases the transcription of genes driven by Smad, leading to cell death (Nakayama, *et al.* 2008; Hsu, *et al.* 2009). Despite their different localizations, formation of either p73-WWOX or Hyal-2-WWOX complex results in apoptosis (Aqeilan, *et al.* 2004c; Nakayama, *et al.* 2008; Hsu, *et al.* 2009), supporting the pro-apoptotic role of WWOX. Restoration of WWOX expression in leukaemic, lung, cervical and breast carcinoma cell lines has also been shown to induce cell death via the activation of caspase cascades (Iliopoulos, *et al.* 2007; Cui, *et al.* 2013; Qu, *et al.* 2013; Zhang, *et al.* 2014), indicating that Wwox is an upstream effector for caspase signaling.

WWOX apoptotic functions can be inhibited by activated JNK, suggesting that reducing JNK signaling could restore WWOX-mediated apoptosis (Chang, *et al.* 2003; Aderca, *et al.* 2008). In line with this, it has been demonstrated that treating human hepatocellular carcinoma cells with a JNK inhibitor increases WWOX-mediated cell death (Aderca, *et al.* 2008). In turn, WWOX can suppress the transcriptional activity of the downstream effector of JNK, c-Jun, by sequestering it in the cytoplasm (Gaudio, *et al.* 2006). This indicates that there is a form of negative regulation occurring between WWOX and JNK signaling. Interestingly, although transient activation of JNK signaling promotes cell survival, persistent signaling induces cell death (Chen, *et al.* 1996). JNK can induce cell death either by

up-regulating the nuclear expression of pro-apoptotic genes (Dhanasekaran and Reddy 2008), or by inducing the release of cytochrome c from mitochondria (Tournier*, et al.* 2000). Although WWOX suppresses the transcriptional activity of c-Jun, it may enhance JNK-mediated cell death in mitochondria. This is consistent with the findings that over-expression of WWOX can trigger the release of cytochrome c (Cui*, et al.* 2013; Zhang*, et al.* 2014). This collective evidence clearly demonstrates that WWOX is able to induce cell death through multiple mechanisms. In addition to apoptosis, WWOX can also contribute to other important cellular pathways, including DNA damage repair, the Hippo pathway and EGFR signaling.

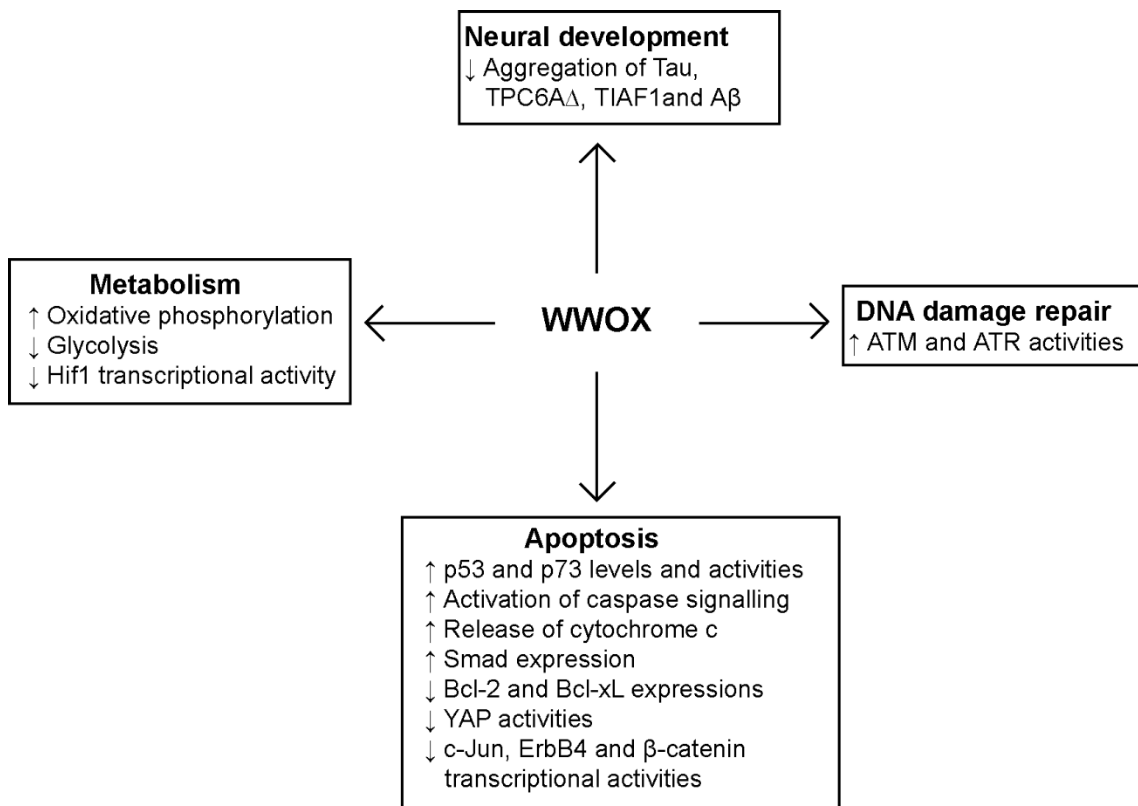


Figure 1.6. Reported WWOX functions in mammals. WWOX can contribute to apoptosis, metabolism, DNA damage repair and neural development through regulating either activity or expression levels of components involved in these processes.

1.5.2 Roles of WWOX in the DNA damage repair

High levels of DNA damage are not merely a characteristic of cancer cells; they are also a mechanism for driving the initiation and progression of cancer. *Ataxia telangiectasia mutated* (ATM) and ataxia telangiectasia and Rad3-related (ATR) are two components that sense and respond to DNA double strand breaks (DSB) (Burma*, et al.* 2001) and single strand breaks (SSB), respectively (Sancar*, et al.* 2004). WWOX has been shown to have a role in the regulation of both ATM and ATR signaling pathways (Abu-Odeh*, et al.* 2014b; Abu-Odeh*, et al.* 2015).

Abu-Odeh *et al.* (2014, 2015) demonstrated that *WWOX* expression is increased upon induction of both DSB and SSB (Abu-Odeh*, et al.* 2014b; Abu-Odeh*, et al.* 2015). Induction of DSB activates ATM, which then leads to the activation of ITCH (Figure 1.7) (Abu-Odeh*, et al.* 2014b). ITCH mediates K63-linked polyubiquitination of WWOX at Lysine 274 (K274) (Abu-Odeh*, et al.* 2014a). Ubiquitination of the K274 residue of WWOX stabilizes it and is important for the translocation of WWOX into the nucleus. In the nucleus, WWOX physically binds with ATM and increases ATM activity in DSB repair, forming a positive feedback loop (Figure 1.7) (Abu-Odeh*, et al.* 2014b).

In addition, reduced *WWOX* expression in human breast cancer MCF7 cells dampens ATR signaling in these cells, suggesting that WWOX can also contribute to the SSB repair (Abu-Odeh*, et al.* 2015). The activation of ATR signaling by WWOX has been shown to be ATM dependent (Abu-Odeh*, et al.* 2015), demonstrating crosstalk between DSB and SSB repair mechanisms (Figure 1.7). These findings show that WWOX can regulate the rate of DNA damage. DNA damage repair normally protects the cell from death (Roos and Kaina 2006). It is likely that WWOX functions in discrete signaling pathways to monitor cellular fitness, and promote the elimination of cells that cannot regain homeostasis. This includes promoting sufficient time for a cell to recover from an insult (such as DNA breakage), or promoting apoptosis when the DNA damage levels are beyond repair. The mechanisms of the cellular response to repair DNA damage and/or shift towards elimination are unknown, but both functions are consistent with tumor suppressor activity for WWOX.

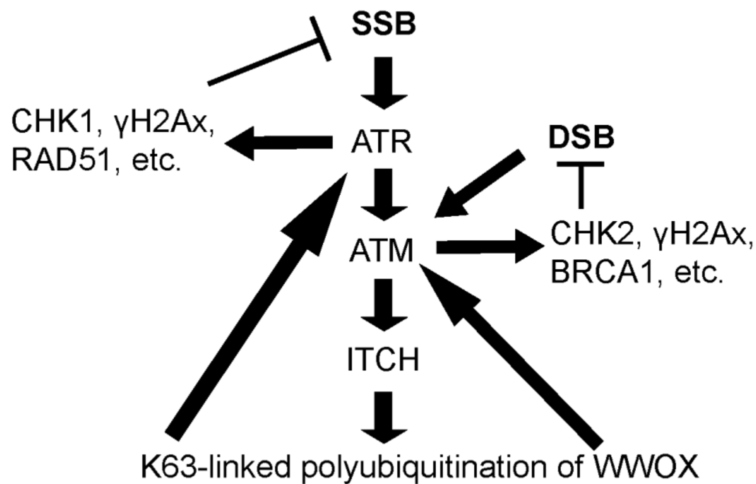


Figure 1.7. Mechanisms by which WWOX contributes to DNA damage repair. Upon single strand breaks (SSB), ATR signaling is activated and induces the activation of components involved in the repair response, including checkpoint kinase (CHK)1, γH2Ax and RAD51. Induction of double strand breaks (DSB) activates ATM signaling, followed by the activation of proteins involved in the repair response, such as CHK2, γH2Ax and breast cancer 1, early onset (BRCA1). ATR can activate ATM, initiating the K63-linked polyubiquitination of WWOX by ITCH. Ubiquitinated WWOX can increase both ATR and ATM signaling, forming a positive feedback loop.

1.5.3 Roles of WWOX in the regulation of Hippo and EGFR pathways

WWOX has also been shown to regulate the Hippo and epidermal growth factor receptor (EGFR) pathways, both of which are involved in similar processes, including the regulation of cell growth, cell differentiation and apoptosis (Bremer, *et al.* 2005; Edgar 2006; Harvey and Tapon 2007; Pan 2007). It is well established that dysregulation of either of these pathways can lead to tumorigenesis (Nicholson, *et al.* 2001; Zhao, *et al.* 2010).

Yes-associated protein (YAP) is the major downstream effector of the Hippo pathway and functions as a transcriptional co-activator to promote transcription of its target genes (Yagi, *et al.* 1999; Komuro, *et al.* 2003). Over-expression of YAP has been shown to contribute to the initiation and progression of various cancer types (Tschaharganeh, *et al.* 2010; Wang, *et al.* 2010; Wang, *et al.* 2012). Similar to WWOX, YAP also possesses two WW domains and interacts with ErbB4 receptor (one of the EGFR family members) via these WW domains (Komuro, *et al.* 2003; Omerovic, *et al.* 2004). Sequential proteolytic cleavage of ErbB4 receptor by tumor necrosis factor α converting enzyme (TACE) and γ-secretase releases a soluble intracellular domain (ICD) fragment (Carpenter 2003). YAP binds with this

ICD fragment and promotes its translocation to the nucleus, where this fragment functions as a transcription factor (Figure 1.8) (Komuro, *et al.* 2003; Omerovic, *et al.* 2004). Translocation of the ICD fragment to the nucleus has been associated with poor prognosis in breast cancer (Junttila, *et al.* 2005). Interestingly, WWOX has the opposite effect to YAP on the ICD fragment. As WWOX has similar tandem WW domains to YAP, it competes with YAP for the binding of ICD fragment (Aqeilan, *et al.* 2005a). Binding of WWOX to the ICD fragment therefore prevents its translocation to the nucleus (Figure 1.8) (Aqeilan, *et al.* 2005a). In addition, expression of WWOX causes ErbB4 to accumulate in the cell membrane without a corresponding increase in the production of the ICD fragment, demonstrating that WWOX can enhance the stability of full length ErbB4 (Aqeilan, *et al.* 2007a). These findings indicate that WWOX can regulate both the YAP/Hippo pathway and EGFR signaling. However, further study is required to confirm the biological significance of WWOX in the regulation of these pathways *in vivo*.

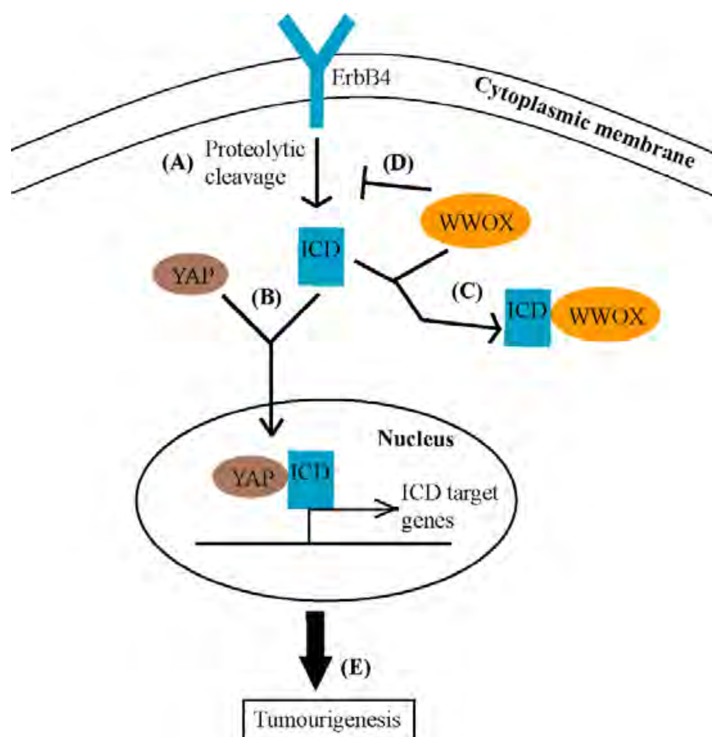


Figure 1.8. Functions of WWOX and YAP on ErbB4 transcriptional activities. (A) The proteolytic cleavage of ErbB4 releases an ICD fragment. **(B)** YAP binds to the ICD fragment, which then promotes its translocation into the nucleus and activates its transcriptional activities. **(C)** WWOX can bind to the ICD fragment and inhibit its translocation. **(D)** WWOX can stabilize full length ErbB4 at the cell membrane. **(E)** Constitutive activation of ErbB4 transcriptional activities can lead to tumorigenesis.

1.6 *Drosophila* model of WWOX

In vitro studies have shown that WWOX can contribute to a wide range of cellular pathways, including apoptosis, metabolism and neuronal development. It is unclear which of these cellular functions of WWOX are biologically significant *in vivo*. In order to investigate WWOX activity *in vivo*, independent *WWOX* mutants have been established in mouse, rat and *Drosophila* (O'Keefe*, et al.* 2005; Aqeilan*, et al.* 2007c; Suzuki*, et al.* 2009; Ferguson*, et al.* 2012). *Wwox* mutant rodents have been shown to have a higher incidence of tumor development, metabolic disorders and epilepsy (Aqeilan*, et al.* 2007c; Aqeilan*, et al.* 2009; Suzuki*, et al.* 2009), which is consistent with diseases reported for patients with low *WWOX* levels. However, the premature lethality in these *Wwox* mutant rodents has prevented further analyses of *in vivo* WWOX functions.

Drosophila has been established as a model to study WWOX functions (O'Keefe*, et al.* 2005). More than 50% of human disease genes have orthologs in *Drosophila*, which allows for dissection of the *in vivo* molecular mechanisms of these diseases (Rubin*, et al.* 2000). In fact, several cancer-related genes were first identified in *Drosophila*, such as *Notch* (Artavanis-Tsakonas*, et al.* 1983) and components of Hippo pathways (Gonzalez 2013).

In 1993, Brand and Perrimon developed the Gal4/UAS system in *Drosophila*, which allows the expression of target genes to be easily manipulated (Figure 1.9) (Brand and Perrimon 1993). GAL4 is a protein identified in the yeast *Saccharomyces cerevisiae* that binds to Upstream Activating Sequence (UAS) and activates the transcription of downstream genes. By introducing a promoter-*gal4* transgene and a construct containing UAS sites upstream of a target gene into the *Drosophila* genome, the expression of the target gene can be altered (Brand and Perrimon 1993). If the UAS-construct contains the complementary DNA (cDNA) sequence or ORF of a target gene, that will result in ectopic expression. In contrast, if the UAS-construct contains an inverted repeat that is complementary to the target mRNA, the expression of the target mRNA will be reduced through post-transcriptional silencing, called RNA interference (RNAi) (Figure 1.9). Gene expression can be altered ubiquitously or in specific tissues depending on the promoter that is used to express the GAL4 protein. Transgenic fly lines are readily available from Bloomington Drosophila Stock Center (BDSC) and Vienna Drosophila Resource Center (VDRC). RNAi fly lines covering 91% of *Drosophila* genome are available in VDRC, which enables genome wide RNAi screens to be performed in *Drosophila* (Sarov*, et al.* 2015).

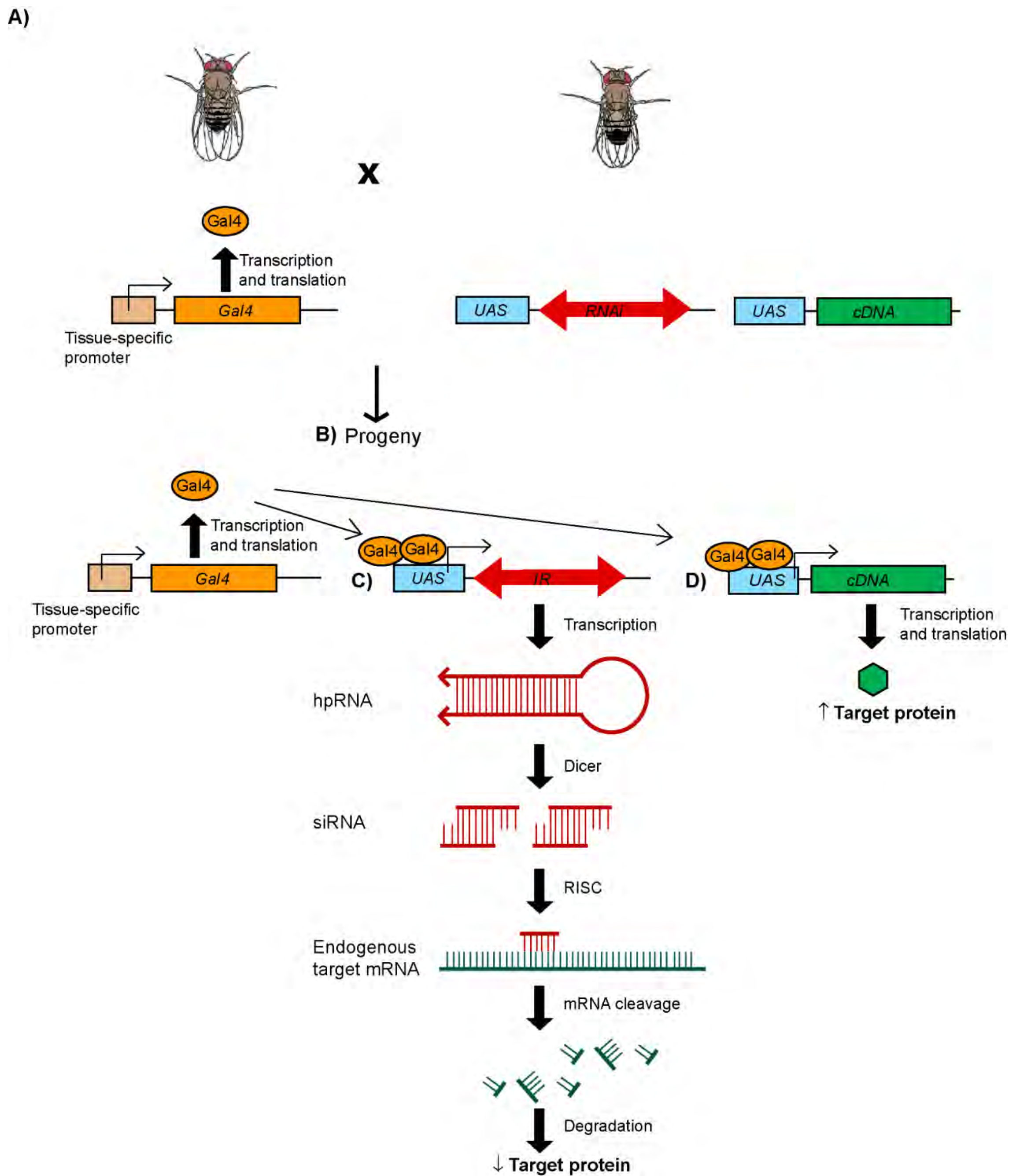


Figure 1.9. Manipulation of gene expression in *Drosophila* by GAL4/UAS system. A) One parent expresses GAL4 protein, but has no target gene to activate. The other parent carries a *UAS-transgene*, but it is not expressed in the absence of the GAL4 protein. **B)** The progeny from their cross, which carry both the *gal4* and *UAS-transgene* constructs express the target transgene in GAL4 expressing tissue. **C)** If the transgene is an inverted repeat construct (IR), its expression will lead to decreased target gene expression. The RNA of this construct forms a hairpin RNA (hpRNA), which is then cleaved by Dicer into small interfering RNA (siRNAs). The siRNA integrates into RNA-induced silencing complex (RISC) and binds to the target mRNA, leading to its degradation. **D)** If the transgene encodes for cDNA of particular gene, its expression leads to an increase of target gene expression.

1.6.1 Conserved metabolic functions of Wwox

The *Drosophila WWOX* ortholog (Wwox) was identified by our laboratory (O'Keefe*, et al.* 2005). It contains six exons that encode conserved WWOX functional domains, including two WW domains, an enzymatic SDR region and putative C-terminal substrate binding sites (Richards*, et al.* 2015). In contrast to rodent models, complete loss of Wwox function in *Drosophila* does not cause any obvious morphological or functional phenotype. We hypothesized that homozygous Wwox mutant flies are able to develop a compensatory mechanism. The compensatory mechanism has also been suggested in both homozygous *Relish*^{E20} mutant *Drosophila* and homozygous *egfl7* mutant zebrafish. While these mutants do not show phenotype, heterozygous *Relish*^{E20} mutation in flies and reduced *egfl7* levels in zebrafish induce sleep defects and vasular defects respectively (Williams *et al.* 2007, Rossi *et al.* 2015), suggesting that there is a compensatory mechanism developed in both of these models. In contrast, heterozygous *Wwox* mutation also does not induce phenotype in flies, and this may because the perturbation has not reached the threshold to induce phenotype in flies.

Although *Wwox* mutant flies do not show any phenotype, microarray and proteomic analyses show that alteration of *Wwox* levels in *Drosophila* significantly changes the levels of many enzymes involved in the tricarboxylic acid (TCA) cycle (O'Keefe*, et al.* 2011), indicating that altered Wwox can modulate oxidative phosphorylation (OXPHOS), a function that is also conserved in mammalian cells. Abu-Remaileh and Aqeilan (2014) demonstrated that WWOX can bind to Hypoxia-inducible factor (HIF) 1α through its WW1 domain and suppress the transcriptional activity of HIF1α in mouse embryonic fibroblasts (MEFs). This prevents up-regulation of the glycolytic genes induced by HIF1α (Abu-Remaileh and Aqeilan 2014). *Wwox*-deficient MEFs have increased glycolysis and decreased OXPHOS (Abu-Remaileh and Aqeilan 2014), suggesting that loss of WWOX function can shift metabolism from OXPHOS to glycolysis, which resembles the "Warburg effect"; a major hallmark of cancer cells (Warburg*, et al.* 1927). In fact, *WWOX* expression is also responsive to the metabolic state of cells (Dayan*, et al.* 2013). *WWOX* levels are up-regulated when there is a metabolic reprogramming of human embroyonic HEK293T cells from a glycolytic state to OXPHOS. Consistently, when these cells are forced to rely on glycolysis, *WWOX* levels are reduced (Dayan*, et al.* 2013). This suggests that metabolic functions of WWOX are conserved between *Drosophila* and mammals and thus have biological significance.

The lack of an obvious phenotype has prevented direct genetic screening for Wwox functions in *Drosophila*. Thus, one of the strategies to identify the biological functions of *Wwox* in *Drosophila* has been to first reduce or completely abolish the gene expression of a particular candidate by RNAi or mutation and then determine any resultant phenotype (Figure 1.10). *Wwox* levels are subsequently altered in this background to examine whether the alteration of *Wwox* levels can modify these candidate gene phenotypes (O'Keefe*, et al.* 2011). Any modification of the phenotype through the alteration of *Wwox* levels suggests that there is functional interaction between Wwox and the candidate gene (Figure 1.10).

Using the strategy stated above, functional interactions between Wwox and isocitrate dehydrogenase (Idh), as well as Cu-Zn superoxide dismutase (Sod1), have been confirmed (O'Keefe*, et al.* 2011). Reduction of *Idh* levels reduces the survival of flies which is worsened by decreasing *Wwox* levels and suppressed by ectopic expression of Wwox. In addition, *Wwox* mutations have also been shown to decrease the survival of *Sod1* mutant flies (O'Keefe*, et al.* 2011). Since Idh and Sod1 can affect the regulation of reactive oxygen species (ROS), the endogenous ROS levels were determined in flies over-expressing Wwox, as well as in *Wwox* mutants. Levels of ROS positively correlated with *Wwox* levels in flies (O'Keefe*, et al.* 2011). Up-regulation of WWOX in HEK293T cells also increased ROS levels (Dayan*, et al.* 2013), demonstrating that the role of WWOX in the regulation of ROS levels is evolutionarily conserved. Decreasing *Wwox* levels would be expected to result in the reduction of the load of ROS in *Sod1* mutant flies and lead to better outcomes, however a reduction of their survival was observed (O'Keefe*, et al.* 2011). It is possible that certain subcellular populations of ROS, rather than overall load of ROS, comprimise the survival of *Sod1* mutant flies (Finkel and Holbrook 2000). In addition, Wwox may titrate hydrogen peroxide, the Sod1 enzymatic end product, in which case the reduction of *Wwox* may further disrupt cellular signaling in *Sod1* mutant flies (Ray*, et al.* 2012) and result in lethality.

In an effort to further elucidate the functions of Wwox in metabolism, genetic screening experiments were performed to identify types of metabolic dysfunction that can be modulated by Wwox (Choo*, et al.* 2015) (Appendix A). The *Drosophila* adult eye was used as a tissue model in these screening experiments (Choo*, et al.* 2015). The eye is a non-essential organ for the survival of *Drosophila* and thus any types of cellular dysfunction, such as those affecting differentiation and/or proliferation can be easily scored without causing overall lethality in the fly (Gonzalez 2013). Different metabolic defects were induced by decreasing the expression of genes involved in TCA cycle and redox homeostasis. Results showed that

altered levels of components of the mitochondrial respiratory complexes disrupts eye patterning, which can be enhanced by reduced *Wwox* levels and suppressed by ectopic expression of Wwox. A mutation in the SDR catalytic active site abolished the suppression of mitochondrial dysfunction by Wwox, suggesting that the SDR enzymatic region of Wwox is involved in the regulation of mitochondrial activity (Choo*, et al.* 2015) (Appendix A).

In addition to metabolic functions, there is also evidence that Wwox has a conserved pro-apoptotic role in *Drosophila*. (Schoenherr*, et al.* 2012). Increased programmed cell death has been shown to protect *Drosophila* against the intestinal infection of *Vibrio cholera* (Berkey*, et al.* 2009). *Wwox* mutants are more susceptible to *V. cholera* infection, supporting that loss of Wwox function decreases the efficiency of programmed cell death in *Drosophila*. In addition, ectopic expression of Head involution defective (Hid) has been shown to promote programmed cell death in *Drosophila* (Grether*, et al.* 1995). Reduced *Wwox* levels can suppress the eye phenotype induced by ectopic expression of Hid (Berkey*, et al.* 2009), further comfirming the *in vivo* pro-apoptotic role of Wwox.

In contrast, reduced *Wwox* levels in cells with spindle checkpoint defects (induced by decreased *mitotic arrest deficient 2* (*Mad2*) levels) promoted cell death (Shaukat*, et al.* 2014). This cell death, however was due to metabolic disruption, as increases in mitochondrial membrane potential and ROS levels were detected in these cells (Shaukat*, et al.* 2014). This effect was reminiscent cell death induced by JNK signaling, in which cells with spindle checkpoint defects were dead when JNK expression was reduced (Wong*, et al.* 2014). Collectively, these studies have shown that both the pro-apoptotic and metabolic functions of WWOX are evolutionarily conserved, and thus they are biologically significant.

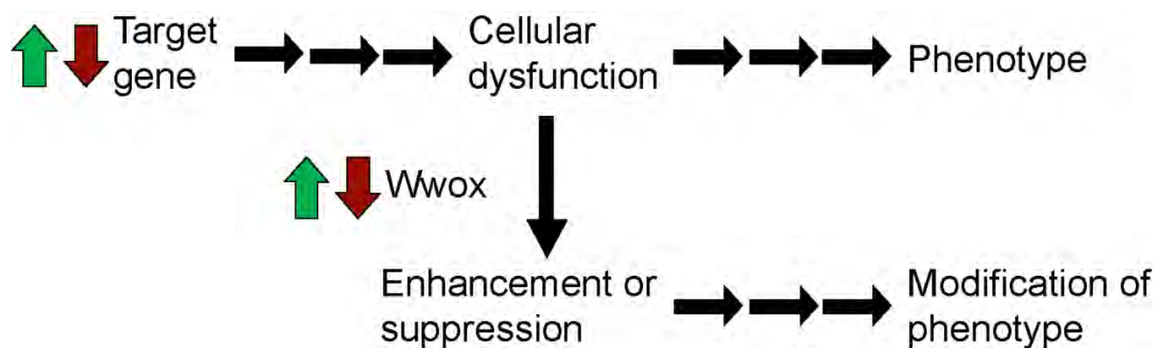


Figure 1.10. Genetic approach used to study Wwox function in *Drosophila*. Cellular dysfunction phenotypes can be induced by altering levels of target genes. Alteration of *Wwox* levels in this background can determine whether Wwox can worsen (enhance) or improve (suppress) this cellular dysfunction. If altered *Wwox* levels reach the threshold to modify the cellular dysfunction phenotype, this suggests that Wwox functionally interacts with the target gene.

1.7 The possible mechanism by which WWOX contributes to metabolic regulation and cell death

Independent *Drosophila* studies have shown that WWOX has conserved functions in both metabolic regulation and cell death (Berkey*, et al.* 2009; O'Keefe*, et al.* 2011; Choo*, et al.* 2015; O'Keefe*, et al.* 2015). However, the underlying molecular mechanism(s) of WWOX in either of these pathways is yet to be defined. One possible mechanism is through the regulation of either mitochondrial fission or fusion, as these processes can contribute to both metabolic regulation and cell death.

Mitochondria exist as part of a highly dynamic network that continuously fuse and divide, in response to different metabolic states and cellular stresses (Abdelwahid*, et al.* 2007; Tondera*, et al.* 2009; van der Bliek 2009; Gomes*, et al.* 2011; Rambold*, et al.* 2011; Thomenius*, et al.* 2011). Defects in mitochondrial dynamics (fission and/or fusion) have been correlated with cancers, metabolic disorders and neurodegenerative diseases (Grandemange*, et al.* 2009; Liesa*, et al.* 2009; de Moura*, et al.* 2010); the same diseases observed in patients with low levels of *WWOX*. This indicates that WWOX may play a role in the regulation of mitochondrial dynamics, which are integral to the same cellular pathways in which WWOX contributes; namely metabolism (Rossignol*, et al.* 2004; Guido*, et al.* 2012; Youle and Van

Der Bliek 2012) and cell death (Abdelwahid*, et al.* 2007; Goyal*, et al.* 2007; Thomenius*, et al.* 2011).

1.7.1 Roles of mitochondrial dynamics in metabolism

The most important function of mitochondria is to generate energy required for different cellular processes, mainly through OXPHOS. Numerous catabolic and anabolic processes occur in the mitochondria, including ETC, TCA cycle and β-oxidation of fatty acid (Kennedy and Lehninger 1949; Fernie*, et al.* 2004). This suggests that the maintenance of mitochondrial functions is critical for metabolic homeostasis.

Mitochondrial functions are tightly regulated by fission and fusion processes. Mitochondria with minor DNA damage can restore their functions by fusing with normal mitochondria (Rapaport*, et al.* 1998; Chen*, et al.* 2010). Mitofusin 1 (MFN1) and mitofusin 2 (MFN2) are essential for mitochondrial fusion. Conditional deletion of both in differentiated skeletal muscle tissue of mice causes muscle atrophy due to severe mitochondrial dysfunction and the depletion of mitochondrial DNA (mtDNA) (Chen*, et al.* 2010). This suggests that mitochondrial fusion is required for the preservation of mtDNA fidelity. Mitochondria that cannot be repaired are eliminated by autophagosomes in a process known as mitophagy (Narendra*, et al.* 2008; Kim and Lemasters 2011). This process prevents the replication of damaged mitochondrial DNA and abolishes any detrimental effects to mitochondrial function. Reduced activity of dynamin-related protein 1 (Drp1), a protein required for mitochondrial outer membrane hydrolysis (Bleazard*, et al.* 1999; Labrousse*, et al.* 1999; Hinshaw 2002), has been shown to block mitophagy (Frank*, et al.* 2012), and results in the accumulation of damaged mitochondria and the activation of an immune response (Zhou*, et al.* 2011). This suggests that mitochondrial fission is crucial for the elimination of dysfunctional mitochondria. In addition, it has also been shown that ectopic expression of Drp1 can suppress bioenergetic defects in PTEN-induced kinase 1 (PINK1) mutant flies by restoring the enzymatic activity of ETC complex I and IV (Liu*, et al.* 2011). This resembles the function of Wwox in the suppression of mitochondrial dysfunction (Choo*, et al.* 2015), supporting the hypothesis that WWOX can contribute to the regulation of mitochondrial dynamics, or more specifically to mitochondrial fission. These findings also demonstrate that mitochondrial dynamics are necessary to sustain a healthy mitochondrial population and maintain metabolic homeostasis.

Mitochondrial morphology not only contributes to the regulation of metabolism, it is also affected by metabolic inputs (Figure 1.11). When cells are grown in galactose medium and forced to rely on OXPHOS, their mitochondria become more fused and condensed (Rossignol*, et al.* 2004). In contrast, induction of mitochondrial fission reprograms the metabolism toward glycolysis (Guido*, et al.* 2012). This suggests that tubular mitochondrial network (due to mitochondrial fusion) favors the production of energy by OXPHOS, while fragmented mitochondria (due to mitochondrial fission) tend to use glycolysis. *WWOX* expression has also been correlated with different metabolic states of cells; it is increased when cells rely on OXPHOS and decreased when glucose is provided solely as a carbon source (glycolysis) (Figure 1.11) (Dayan*, et al.* 2013). In the glycolytic state, more mitochondria are fragmented (Guido*, et al.* 2012) and the cell is sensitized to apoptosis (Frank*, et al.* 2001; Lee*, et al.* 2004; Estaquier and Arnoult 2007). It is possible that the cell down-regulates *WWOX* levels during this state in order to keep mitochondrial fission in check and prevent excess cell death (Figure 1.11).

1.7.2 Role of mitochondrial dynamics in cell death

In addition to a role in metabolism, mitochondria are also integral components of cell death. This is based on studies showing many components released from mitochondria can induce cell death, including cytochrome c (Liu*, et al.* 1996; Zou*, et al.* 1997), apoptosis inducing factor (AIF) (Susin*, et al.* 1996), HtrA Serine Peptidase 2 (HTRA2) (Suzuki*, et al.* 2001), Smac (Du*, et al.* 2000) and Endonuclease G (EndoG) (Li*, et al.* 2001). Among these proteins, cytochrome c has been most intensively studied in mammalian cells (Liu*, et al.* 1996; Luo*, et al.* 1998; Hüttemann*, et al.* 2011). Upon activation of apoptosis, Bax and Bak proteins induce permeabilization of the mitochondrial outer membrane, allowing the release of cytochrome c to activate caspase signaling (Green 2005). Inhibition of mitochondrial fission has been shown to prevent the release of cytochrome c from mitochondria, followed by decreasing caspase activation and cell death (Frank*, et al.* 2001; Lee*, et al.* 2004; Estaquier and Arnoult 2007). This suggests that mitochondrial fission is important for the permeabilization of mitochondria, as well as the cell death pathway (Figure 1.11). In addition, mitochondria become hyperfused in response to different stresses, including exposure of a low dose of apoptotic inducer Actinomycin D (Tondera*, et al.* 2009), starvation (Gomes*, et al.* 2011; Rambold*, et al.* 2011) and autophagy induced by inactivation of mammalian target of

rapamycin (mTOR) (Gomes*, et al.* 2011). This morphological change of mitochondria protects the cell from death (Figure 1.11), optimizes ATP production, as well as prevents mitochondria from undergoing autophagosomal degradation (Tondera*, et al.* 2009; Gomes*, et al.* 2011; Rambold*, et al.* 2011). Increased *WWOX* levels may induce mitochondrial fission and sensitize cells to apoptosis in order to eliminate those that become malignant.

In *Drosophila*, the contribution of cytochrome c to cell death is still a controversial issue. While some studies have shown that cytochrome c can activate caspase cascades in spermatogenesis and is required for the elimination of interommatidial cells in the developing eye (Arama*, et al.* 2006; Mendes*, et al.* 2006), other studies have shown that cytochrome c does not play a role in caspase activation of cell death induced by either UV exposure or ectopic expression of Reaper, Grim or Hid (Zimmermann*, et al.* 2002; Dorstyn*, et al.* 2004; Means*, et al.* 2006; Abdelwahid*, et al.* 2007). Despite these conflicting results regarding the role of cytochrome c in cell death, numerous studies have supported the pro-apoptotic role of mitochondrial fission in *Drosophila*. Inhibition of mitochondrial fission suppresses caspase activation and programmed cell death induced by increasing levels of either *head involution defective* (*hid*) or *reaper* (*rpr*) (Abdelwahid*, et al.* 2007; Goyal*, et al.* 2007; Thomenius*, et al.* 2011). This suggests that the role of mitochondrial fission in promoting cell death is conserved from *Drosophila* to mammals. As this function is similar to that of WWOX, it further supports the hypothesis that WWOX can contribute to the mitochondrial fission (Figure 1.11).

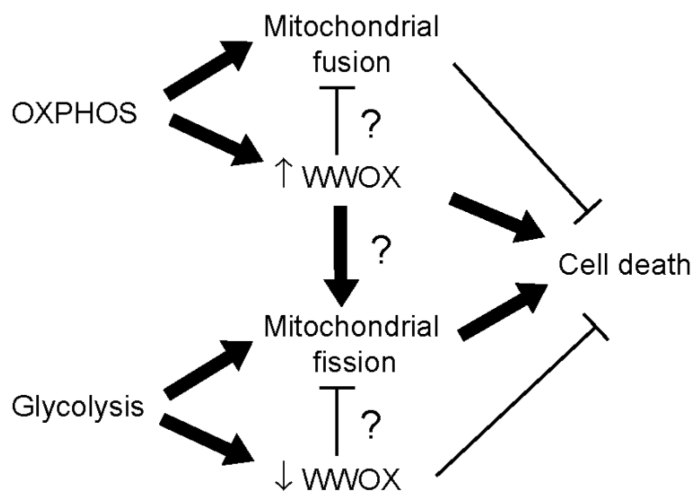


Figure 1.11. Changes in *WWOX* levels and mitochondrial dynamics, in response to different metabolic states, and their contribution to cell death. When cells are forced to use OXPHOS to generate energy, mitochondria fuse to form tubular networks and *WWOX* levels are increased. When cells rely on glycolysis to produce energy, mitochondria show fragmented morphology (due to mitochondrial fission) and *WWOX* levels are reduced. It is hypothesized that WWOX can contribute to mitochondrial fission, where increased *WWOX* levels inhibits mitochondrial fusion or vice versa. Either increased *WWOX* levels or mitochondrial fission can promote cell death.

1.7.3 Proposed WWOX function

Given that WWOX and mitochondrial fission function similarly in the suppression of mitochondrial dysfunction (Liu, *et al.* 2011; Choo, *et al.* 2015) and the enhancement of cell death (Chang, *et al.* 2001; Frank, *et al.* 2001; Lee, *et al.* 2004; Chang, *et al.* 2005; Estaquier and Arnoult 2007), we hypothesize that WWOX performs these functions through its contribution to mitochondrial fission. It is noteworthy that another CFS gene, Parkin (spanning *FRA6E*), has been shown to be involved in the regulation of mitochondrial dynamics (Poole, *et al.* 2008; Buhlman, *et al.* 2014). Loss of *Parkin* in *Drosophila* results in muscle degeneration, mitochondrial dysfunction and abnormal mitochondrial morphology. These defects are suppressed by either increasing *Drp1* levels or decreasing gene expression of mitochondrial fusion promoting factors, including Optic atrophy 1 (Opa1) and the *Drosophila mitofusin* homolog. This demonstrates that phenotypes observed in *Parkin* mutants are due to mitochondrial fission defects, and that Parkin can contribute to mitochondrial fission.

Although both Parkin and WWOX have been shown to have tumor suppressor capacity (Bednarek*, et al.* 2001; Fujiwara*, et al.* 2008), it is unknown whether they also share a common cellular function, such as the promotion of mitochondrial fission. As the functions of mitochondrial fission appear to be conserved in *Drosophila* (Abdelwahid*, et al.* 2007; Goyal*, et al.* 2007; Thomenius*, et al.* 2011), it is a good *in vivo* model to investigate the role of WWOX in this process. Further investigation of conserved Wwox functions in *Drosophila* will enable the determination of those that are biologically significant. This will provide a better understanding of the mechanisms through which low *WWOX* levels contribute to disease progression in patients who suffer from a range of diseases, including cancer, metabolic disorders and neurological diseases. This in turn could improve disease management and patient prognosis.

1.8 Aims of this study:

The first aim of this project is **to determine the role of Wwox in TNFα-mediated cell death** using the *Drosophila* model. It has been shown that Wwox can enhance TNFα-mediated cell death in mouse cell lines *in vitro* (Chang*, et al.* 2001). However, the *in vivo* role of Wwox in this pathway has not been characterized. This study investigates whether Wwox has a conserved function in TNFα-mediated cell death *in vivo*, and clarifies a mechanism of tumor suppression.

The second aim of this project is **to determine the functional requirements of distinct Wwox sequence motifs**. Truncated Wwox protein, that is devoid of most of the SDR region, is ectopically expressed in flies to test whether it retains the ability to carry out previously characterized functions of ectopic full length Wwox. This study allows us to distinguish those Wwox functions that require the SDR enzyme activity, and test whether it is required for metabolic regulation by Wwox.

The third aim of this project is **to identify novel *in vivo* functions for Wwox**. The strategy is to screen for cellular dysfunction phenotypes (induced by altering gene expression of candidate interactors) that can be modified by altered Wwox levels and determine the molecular mechanisms involved in these processes. The hypothesis that Wwox promotes mitochondrial fission as a mechanism for linking cell metabolism and cell death is also investigated.

Chapter 2

Contribution of Wwox in TNFα-mediated cell death

This chapter is based on the article that has been published:

O'Keefe, L.V., Lee, C.S., Choo, A. and Richards, R.I., 2015. Tumor suppressor WWOX contributes to the elimination of tumorigenic cells in Drosophila melanogaster. PloS one, 10(8), p.e0136356.

Statement of Authorship

Title of Paper	Tumor suppressor WWOX contributes to the elimination of tumorigenic cells in *Drosophila melanogaster*
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	O'Keefe, L. V., Lee, C. S., Choo, A., & Richards, R. I. (2015). Tumor suppressor WWOX contributes to the elimination of tumorigenic cells in Drosophila melanogaster. *PloS one*, *10*(8), e0136356.

Principal Author

Name of Principal Author (Candidate)	Louise V. O'Keefe		
Contribution to the Paper	Conceived and designed the experiments, performed experiments (Figure 1, 2D-I, 6, S1, S2 and S4), analysed the data, contributed reagents / materials / analysis tools, writing and revision of the manuscript		
Overall percentage (%)	50		
Signature		Date	11/8/16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author (Candidate)	Cheng Shoou Lee		
Contribution to the Paper	Verified results of Figure 1, performed experiments for Figure 2A-C, 3, 4, 5, S1H and S3, analysed the data, contributed to the writing of manuscript.		
Signature		Date	11/8/16

Name of Co-Author	Amanda Choo		
Contribution to the Paper	Performed experiment for Figure 6 and S4. analysed the data, contributed to writing of manuscript.		
Signature		Date	11/8/16

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<tr><td>Name of Co-Author</td><td colspan="3">Robert I. Richards</td></tr>
<tr><td>Contribution to the Paper</td><td colspan="3">Conceived and designed the experiments, supervised development of work, analysed the data, contributed reagents/materials/analysis tools, contributed to revision of manuscript and is corresponding author.</td></tr>
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RESEARCH ARTICLE

Tumor Suppressor WWOX Contributes to the Elimination of Tumorigenic Cells in *Drosophila melanogaster*

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Abstract

WWOX is a >1Mb gene spanning *FRA16D* Common Chromosomal Fragile Site, a region of DNA instability in cancer. Consequently, altered *WWOX* levels have been observed in a wide variety of cancers. *In vitro* studies have identified a large number and variety of potential roles for WWOX. Although its normal role *in vivo* and functional contribution to cancer have not been fully defined, WWOX does have an integral role in metabolism and can suppress tumor growth. Using *Drosophila melanogaster* as an *in vivo* model system, we find that WWOX is a modulator of TNFα/Egr-mediated cell death. We found that altered levels of WWOX can modify phenotypes generated by low level ectopic expression of TNFα/Egr and this corresponds to altered levels of Caspase 3 activity. These results demonstrate an *in vivo* role for WWOX in promoting cell death. This form of cell death is accompanied by an increase in levels of reactive oxygen species, the regulation of which we have previously shown can also be modified by altered WWOX activity. We now hypothesise that, through regulation of reactive oxygen species, WWOX constitutes a link between alterations in cellular metabolism observed in cancer cells and their ability to evade normal cell death pathways. We have further shown that WWOX activity is required for the efficient removal of tumorigenic cells from a developing epithelial tissue. Together these results provide a molecular basis for the tumor suppressor functions of WWOX and the better prognosis observed in cancer patients with higher levels of WWOX activity. Understanding the conserved cellular pathways to which WWOX contributes provides novel possibilities for the development of therapeutic approaches to restore WWOX function in cancer.

Introduction

Evasion of cell death and altered metabolism are recognized as hallmarks of cancer cells, whilst DNA instability is one of the enabling characteristics [1]. The *FRA16D* Common Chromosomal Fragile Site (CCFS) spanning gene, *WW domain containing oxidoreductase (WWOX)*, participates in each of these phenomena and therefore its perturbation in cancer cells presents

Competing Interests: The authors have declared that no competing interests exist.

multiple possible avenues for contributing to cancer cell biology. CCFS are specific regions of chromosomes that can be induced to break *in vitro* by inhibitors of DNA polymerase and are affected by certain dietary or environmental factors [2–3]. More than 70 common fragile sites have been identified in the human genome and it has been observed that there is a hierarchy of sensitivity *in vitro* that is matched by the frequency with which these sites show *in vivo* DNA instability in various cancers [4]. Of these fragile sites, *FRA3B* and *FRA16D* have been shown to be the most frequent regions of recurrent homozygous deletion in cancer cell lines [5]. CCFS are typically located within extremely large genes (i.e. *FRA3B* in 1.5 Mb *FHIT* gene, *FRA16D* in 1.1 Mb *WWOX* gene), a relationship that is conserved in mice and suggestive of biological significance [4, 6]. DNA instability at these sites, resulting in deletion(s) and / or localised rearrangements, is associated with alterations to CCFS-associated gene expression [7–8].

Altered expression of *WWOX* has been reported for many different cancer cell types (reviewed in [9–11]). In addition, low expression alleles of *WWOX* were found at a higher frequency in patients with lung cancer [12] or glioma [13], consistent with decreased *WWOX* as a predisposing factor for tumorigenesis. *WWOX* hypomorphic mice showed an increased incidence of B-cell lymphoma [14] and mice heterozygous for *WWOX* exhibit higher rates of tumor growth [15], however the tumor cells still express WWOX protein indicating a lack of the typical 'second-hit' somatic mutation that is characteristic of classical tumor suppressors. Thus it appears that a reduced level of WWOX activity is sufficient for contribution to cancer progression. Conversely, ectopically expressed WWOX has been shown to function as a suppressor of tumor growth since restoration of WWOX activity in cancer cells inhibits their growth *in vivo* [16–20]. Correlation of higher WWOX expression with better prognosis has also been reported for various types of cancer including colon, breast and bladder [21–23]. Therefore the pathways that WWOX normally participates in, and the nature of this participation, are of considerable interest for their likely causal and therapeutically targetable contribution to cancer cell biology.

WWOX encodes an enzyme with short-chain dehydrogenase/reductase (SDR) activity in addition to two WW domains that facilitate protein-protein interactions. WWOX has been implicated in a diverse range of cellular pathways and processes in mammalian studies by virtue of its physical and / or functional interactions with other proteins or pathways (reviewed in [24–26]). Whilst various functions for WWOX have been revealed *in vitro*, it is difficult to assess their relative significance and contribution to cancer *in vivo*. A role for WWOX in metabolism has been established through the analysis of knockout models in mouse, rat and *D. melanogaster* [14–15,27–30]. The protein encoded by *WWOX* has been found not only to contribute to cellular metabolism but also is, in turn, regulated by the relative level of glycolysis versus oxidative phosphorylation [31]. WWOX has also been widely reported to play a role in apoptotic pathways, principally through interactions with the tumor suppressor p53 (reviewed in [24–32]). A pro-apoptotic role for WWOX *in vitro* has previously been reported for many different cancer cell types; multiple myeloma [33], colon [34], gall bladder [35], cervical [36], leukaemic [37], glioblastoma [38–39], hepatoma [40], lung [17], pancreatic [18] and squamous epithelia [41]. However the molecular mechanism(s) by which WWOX contributes to cell death pathways *in vivo* has not been determined. The genetically tractable system of *D. melanogaster* is an effective system in which to dissect various aspects of the contribution of WWOX to cellular pathways. Herein we determine the role of WWOX in modulating TNFα-mediated cell death through regulation of Caspase 3 activity. In addition we are able to demonstrate a requirement for WWOX in the elimination of tumorigenic cells, thus supporting a requirement for WWOX function early in the tumorigenic process for the removal of abnormal cells.

Results

Altered WWOX modulates ectopic Egr/TNFα eye phenotypes

Ectopically expressed WWOX has been shown to enhance the *in vitro* cytotoxicity of TNFα in various tissue culture cell lines [42], yet the contribution of WWOX to TNFα-mediated cell death *in vivo* has not been determined. *D. melanogaster* has a single ortholog to TNFα encoded by the *EDA-like cell death trigger* or *Eiger (Egr)* gene [43–44]. Genetic modification analyses have previously revealed a number of metabolic genes that are rate-limiting in their contribution to Egr/TNFα-induced cell death phenotypes in the *D. melanogaster* eye [45]. The *WWOX* gene has been identified as participating in aerobic metabolism in *D. melanogaster* [30] and thus also represents a candidate for contributing to Egr/TNFα-mediated cell death. Ectopic over-expression of a low level expression construct for Egr/TNFα in the eye during its development results in a phenotype characterised by disruption to the precise patterning of repeated ommatidial units on the external surface of the eye as well as a decrease in overall size (Fig 1A and [46]) when compared to a control eye (Fig 2D). This Egr/TNFα phenotype was completely suppressed by RNAi-mediated knockdown of *wengen*, a gene that encodes the *D. melanogaster* TNF receptor (*TNFR*) thus confirming the specificity of the ectopic Egr/TNFα-mediated phenotype (Fig 1B and [47–48]). Introduction of a *WWOX* knockdown construct (WWOX-*IR*$^{\#1}$) resulted in suppression of the Egr/TNFα-mediated rough eye phenotype evident as restoration of ommatidial patterning across the surface of the eye as well as an increase in eye size (Fig 1C and 1D). A similar suppression of eye size was observed with an independent *WWOX* knockdown construct as well as in flies heterozygous for *WWOX* loss-of-function mutant alleles (S1 Fig). This indicates that WWOX can contribute to low level Egr/TNFα-mediated cell death.

Ectopic expression of WWOX alone does not result in any obvious cell death-induced phenotype in the biological context of the *D. melanogaster* eye (S1 Fig). Ectopic over-expression of WWOX cDNA showed enhancement of the Egr/TNFα mediated mild rough eye phenotype evident as further disruption to ommatidial patterning and a significant decrease in eye size (Fig 1E and 1F). A decrease in adult eye size was also observed with ectopic over-expression of an open reading frame (ORF) encoding WWOX although these results were more variable despite comparable levels of expression of WWOX (Fig 1F and S1 Fig). Notably, ectopic expression of WWOX together with Egr/TNFα did not result in any further increase in WWOX levels (S1 Fig). Together these data demonstrate that WWOX contributes to Egr/TNFα-mediated cell death phenotype.

To determine whether the interaction between WWOX and Egr/TNFα was specific we also tested the contribution of WWOX with other inducers of cell death. Given the significant analysis of WWOX function together with p53 in the literature, we also tested for any modification of ectopic p53 phenotypes with altered levels of WWOX. However, we were unable to detect any alteration to the much more severe eye phenotypes generated by ectopic expression of either p53 or Hid (head involution defective), another of the cell death promoting proteins identified in *D. melanogaster* (S2 Fig).

Egr/TNFα-mediated cell death phenotypes are mediated by increased ROS

Reactive oxygen species (ROS) are known to be principle effector molecules of Egr/TNFα-mediated cell death [45]. We were able to confirm this in larval wing discs expressing Egr/TNFα in the posterior region by increased staining for CellRox compared to low levels observed in the anterior control region for each disc (Fig 2A–2C). We also determined whether the Egr/TNFα-mediated rough eye phenotypes can be modified by enzymes known to modify ROS levels. Superoxide dismutase (SOD) activity is required for conversion of superoxide to

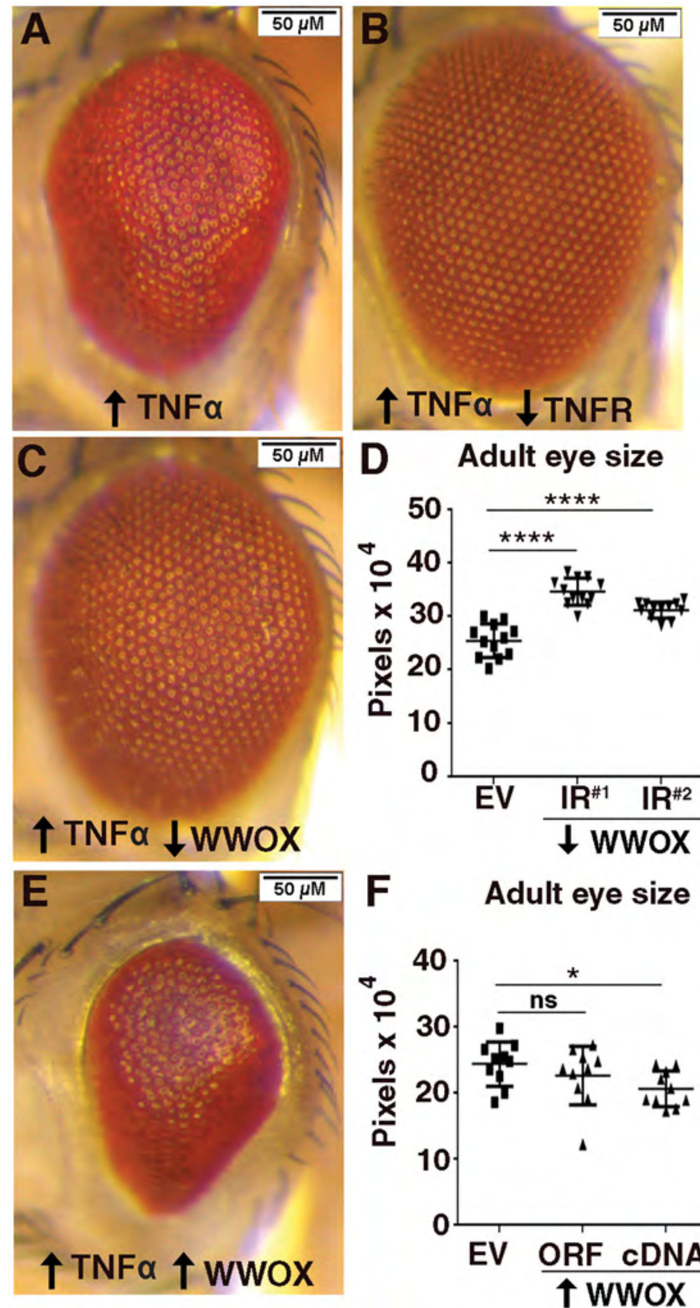

Fig 1. Altered WWOX modifies ectopic Egr/TNFα-mediated eye phenotype. (A) Ectopic expression of Egr/TNFα (GMR>*egr*$^{+W}$>*empty vector (EV)*) results in a decrease in eye size and disruption to ommatidial patterning. **(B)** The ectopic Egr/TNFα phenotype is completely suppressed by decreased levels of TNFR (GMR>*egr*$^{+W}$>*wengen/TNFR-IR*). **(C)** Decreased expression of WWOX by RNAi knockdown (*GMR>egr*$^{+W}$*>WWOX-IR*$^{\#1}$) results in suppression of the rough eye phenotype. **(D)** Quantification of increased eye size with independent WWOX knockdown constructs (*GMR>egr*$^{+W}$*>WWOX-IR*$^{\#1}$ and *GMR>egr*$^{+W}$*>WWOX-IR*$^{\#2}$). **(E)** Increased expression of WWOX by ectopic expression of the WWOX cDNA (GMR>*egr*$^{+W}$>*WWOX-cDNA*) resulted in an enhancement of the Eiger/TNFα phenotype. **(F)** Quantification of decreased eye size with independent ectopic expression constructs for WWOX (GMR>*egr*$^{+W}$>*WWOX-ORF* and GMR>*egr*$^{+W}$>*WWOX-cDNA)*. Significance indicated by **** = p <0.0001, * = p<0.05 and ns = not significant.

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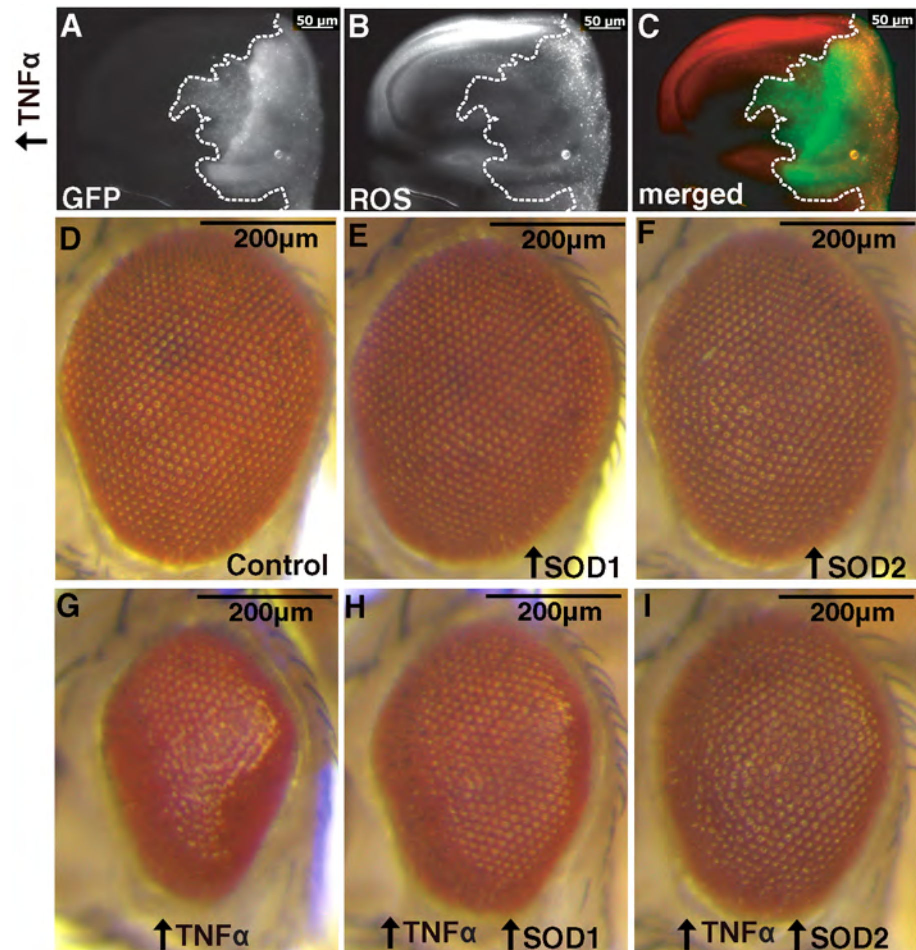

Fig 2. Ectopic expression of Egr/TNFα gives increased ROS and is suppressed by increased SOD activity. (A) Ectopic expression of GFP and Egr/TNFα with *hh-GAL4* in the posterior portion of wing discs of wandering third instar larvae, GFP shows the region of ectopic expression (outlined by dotted line). **(B)** Punctate CellRox staining revealed increased ROS levels at posterior edge of disc. **(C)** Merged image with GFP in green and CellRox in red. **(D)** Control eye phenotype (*GMR>EV*) showing regular ordered arrays of ommatidial units on the surface of the adult eye **(E-F)** Ectopic expression of SOD1 or SOD2 alone has no effect. **(G)**. Ectopic expression of Egr/TNFα in the eye (GMR>egr^{+W}>*EV*) results in a decrease in eye size and disruption to ommatidial patterning. **(H-I)** The ectopic Egr/TNFα phenotype is obviously suppressed by increased levels of SOD1 or SOD2.

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hydrogen peroxide as an intermediary in the detoxification process. There are different SOD enzymes found within the cell; SOD1 (CuZn) is located in cytoplasm whilst SOD2 (Mn) is found in the mitochondria. Ectopic expression of either SOD1 or SOD2 gave no phenotype on their own (Fig 2D–2F) but were able to obviously suppress the Egr/TNFα eye (Fig 2G–2I). This suppression of the Egr/TNFα small eye phenotype was consistently observed in all progeny and supports a role for ROS in these Egr/TNFα-mediated phenotypes.

WWOX remains cytoplasmically localised in response to ectopic Egr/TNFα expression

Nuclear localisation of WWOX has been reported to be necessary for the cell death promoting functions of WWOX in mammalian cells [42]. Although endogenous levels of WWOX are too

low to be detected in *D. melanogaster*, we have previously shown cytoplasmic localization of ectopically expressed WWOX during embryonic development [49]. Here, we also determined the localisation of ectopically expressed WWOX in differentiated cells of the developing eye disc. *GMR-GAL4* was used to ectopically express WWOX in all cells posterior to the morphogenetic furrow. WWOX expression can be visualised in cytoplasmic regions surrounding the DAPI stained clusters of nuclei from photoreceptor cells (S3 Fig). A similar pattern of cytoplasmic WWOX expression was observed in the presence of ectopic Egr/TNFα expression (S3 Fig). Thus we observed no alteration to ectopic WWOX localisation in response to Egr/TNFα *in vivo*. Given the small size and complex organisation of cells in the developing eye disc, the effect of ectopic Egr/TNFα expression on the localisation of WWOX was also determined in cells in the posterior compartment of the wing disc using *hh-GAL4*. Co-expression of GFP allowed for the positive identification of cells in the region of ectopic expression. Ectopic WWOX alone resulted in cytoplasmic staining with WWOX detected in regions surrounding the DAPI stained nuclei throughout the posterior half of the disc (Fig 3A–3D). In the presence of ectopic Egr/TNFα expression, the wing discs are smaller and there is significant disruption to the region of the disc marked by GFP expression (Fig 3E). Closer examination of cells located in the posterior region of the disc showed that ectopic WWOX remains clearly cytoplasmic as staining was observed complementary to the DAPI stained nuclei (Fig 3F–3H). Thus there was no evidence *in vivo* for nuclear localisation of detectable levels of ectopic WWOX in response to Egr/TNFα expression in eye or wing imaginal discs.

Ectopic Egr/TNFα alone promotes apoptosis and disrupts cellular patterning in wing discs

Ectopic expression of Egr/TNFα alone in the posterior region of wing discs resulted in a significant decrease in tissue size and disruption to compartment boundaries as visualised by GFP expression (Fig 4E). In particular there is posterior GFP expression extending into the central wing pouch region of the disc (Fig 4E'). In order to determine the identity of these cells, Cubitis interruptus (Ci) staining was used as a marker of cells specific to the anterior portion of the wing disc. In control discs the region corresponding to Ci staining is complementary to the GFP expression pattern in the posterior region under control of *hh-GAL4* thus defining the boundary of these distinct cell types (Fig 4B). However, in response to ectopic Egr/TNFα expression in the posterior region, there is now a region of Ci positive anterior cells overlapping with the GFP positive posterior cells in the central wing pouch region (Fig 4F and 4F'). Thus ectopic expression of Egr/TNFα has resulted in disruption to normal patterning of the wing disc cells such that there is no longer a clear distinction between cells from the Ci staining portion of the disc (i.e. the wild-type cells from the anterior region) and GFP positive cells from the posterior part of the disc (i.e. cells ectopically expressing Egr/TNFα).

Furthermore, the GFP expression observed in this region of overlap was punctate in appearance suggestive of increased cell death (Fig 4E'). To assess the cell death we examined immunostaining for cleaved Caspase 3 [50]. Whilst negligible levels of Caspase 3 staining were observed in control discs (Fig 4C), increased levels were observed in the central wing pouch region of discs ectopically expressing Egr/TNFα (Fig 4G and 4G'). In addition, Caspase 3 staining was found to extend beyond the GFP region of the wing pouch in two distinct regions (Fig 4G', asterisks). Similar localisation of Caspase 3 staining to these two regions has previously been reported following ectopic expression of Hid or Src64B, together with the apoptosis inhibitor P35 in the posterior region of developing wing discs [51–52]. The extremities of these regions have previously been shown to contain cells undergoing a process of Apoptosis-induced Apoptosis (AiA) with Egr/TNFα shown to be required for the death signal [52]. Closer

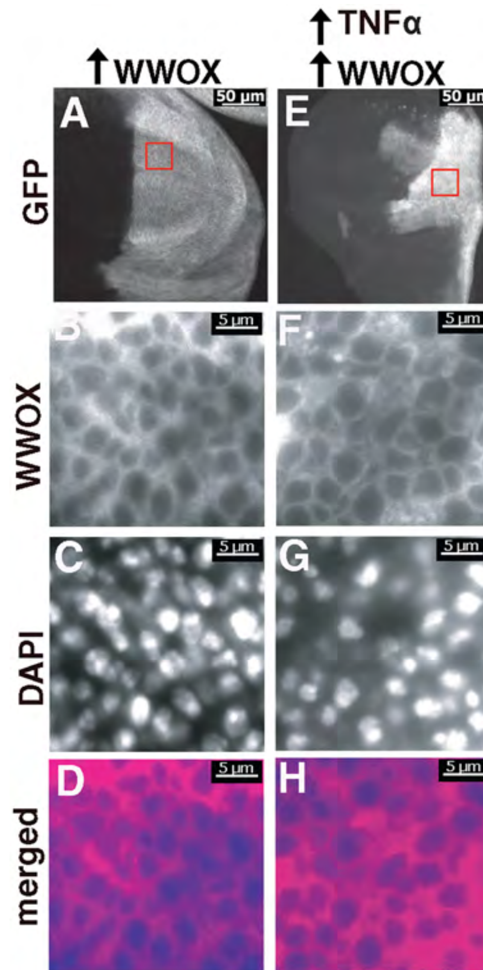

Fig 3. Ectopic Egr/TNFα has no obvious effect on cytoplasmically localized WWOX. (A) Ectopic expression of GFP and WWOX in the posterior portion of wing discs of wandering third instar larvae *(hh>GFP>WWOX)*, GFP shows the region of ectopic expression. **(B)** WWOX staining reveals expression localized to areas complementary to the DAPI stained nuclei shown in **(C)** with the merged image shown in **(D)**. **(E-H)** Ectopic expression of WWOX in the presence of ectopic Egr/TNFα *(hh>GFP>WWOX>egr^{+W})* also results in cytoplasmic localisation of WWOX. Nuclei/DAPI staining shown in blue and WWOX staining in magenta. Boxed regions shown in A and E correspond to regions that are enlarged in B-D and F-H respectively.

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examination of Cell Rox staining (Fig 2B) also revealed increased ROS corresponding to these two distinct regions. These results confirm that over-expression of a low level of Egr/TNFα in the posterior compartment is sufficient to induce ROS and cell death in anterior regions, consistent with a key role of Egr /TNFα as an activating signal for AiA.

WWOX modifies Caspase 3 staining in response to ectopic Egr/TNFα

Since WWOX has been shown to modify adult eye phenotypes resulting from ectopic over-expression of Egr/TNFα, we determined whether WWOX was also able to regulate the increased region of Caspase 3 staining and consequent disruption to the patterning induced by ectopic expression of Egr/TNFα in wing discs. Decreased WWOX activity together with ectopic expression of Egr/TNFα in the posterior portion of the disc resulted in a decrease in the relative area of Caspase 3 staining (Fig 5C, 5D and 5G). Conversely, increased WWOX

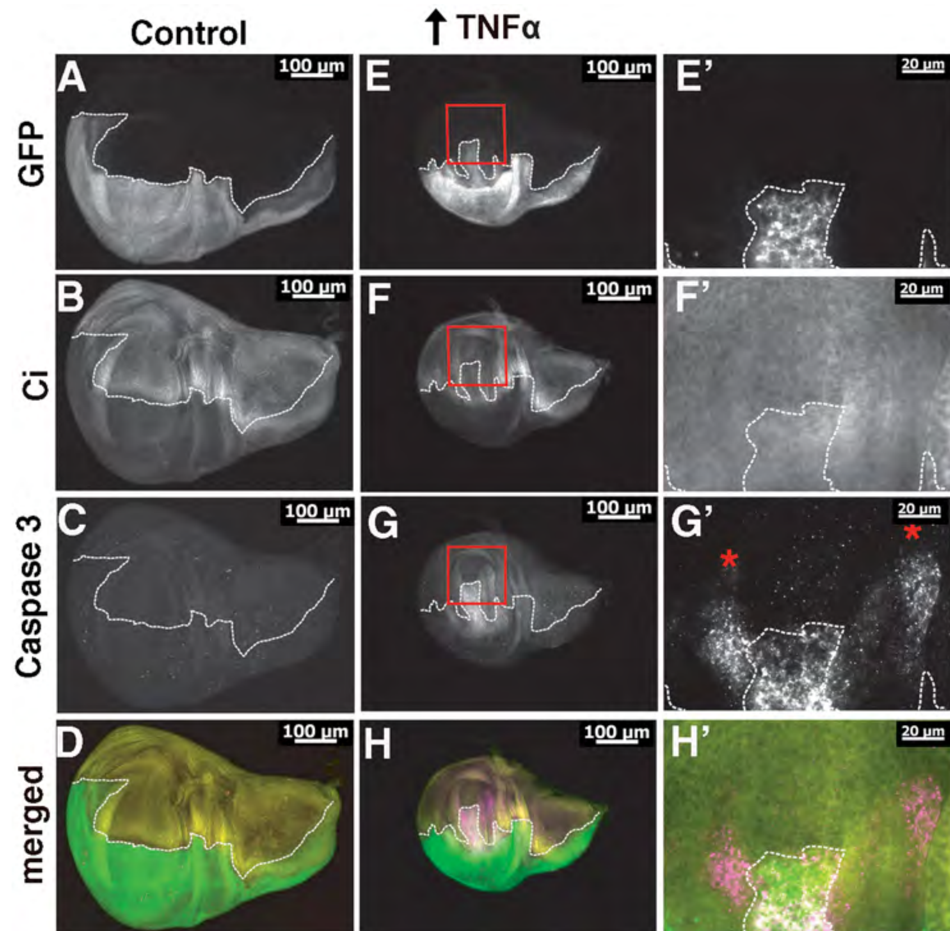

Fig 4. Qualitative effects of ectopic Egr/TNFα and increased apoptosis in the larval imaginal wing disc. (A) Control disc showing ectopic expression in the posterior region of wing discs using *hh-GAL4* visualized by co-expression of GFP. **(B)** Ci staining of control discs show staining of the anterior compartment and is complementary to the region of GFP expression. **(C)** Caspase 3 staining revealed low levels of apoptosis in control imaginal wing discs. **(D)** Merged image where GFP is green, Caspase 3 staining is magenta and Ci is yellow. **(E-E')** Ectopic expression of Eiger/TNFα resulted in a significant decrease in disc size and disruption to the pattern of GFP expression with punctate staining in the central wing pouch region. **(F-F')** Staining of the anterior compartment with Ci reveals expression beyond the boundary and overlapping with the region of GFP expression. **(G-G')** Caspase 3 staining reveals high levels of staining in the central wing pouch region and in two distinct regions extending towards to anterior portion of the disc (indicated with the red asterisks). **(H-H')** Merged image (GFP is green, Caspase 3 is magenta and Ci is yellow). In all images the dotted line outlines the regions of GFP expression corresponding to the posterior region of the discs. Red boxes indicate the regions that are enlarged in E'-H'.

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expression increased the relative area of Caspase 3 staining (Fig 5E–5G). Thus we have shown that WWOX activity is required for, and can contribute to, cell death in Egr/TNFα expressing cells via regulation of Caspase 3 activity. The outcome of this interaction at the interface between wild-type cells in the anterior and the posterior Egr/TNFα expressing cells is suggestive of competitive interactions between these two cell types.

Requirement for WWOX tumor suppressor activity *in vivo*

Competition occurs between cell types that are genetically distinct such that one has a competitive advantage over the other. In order to determine whether decreased WWOX impacts on

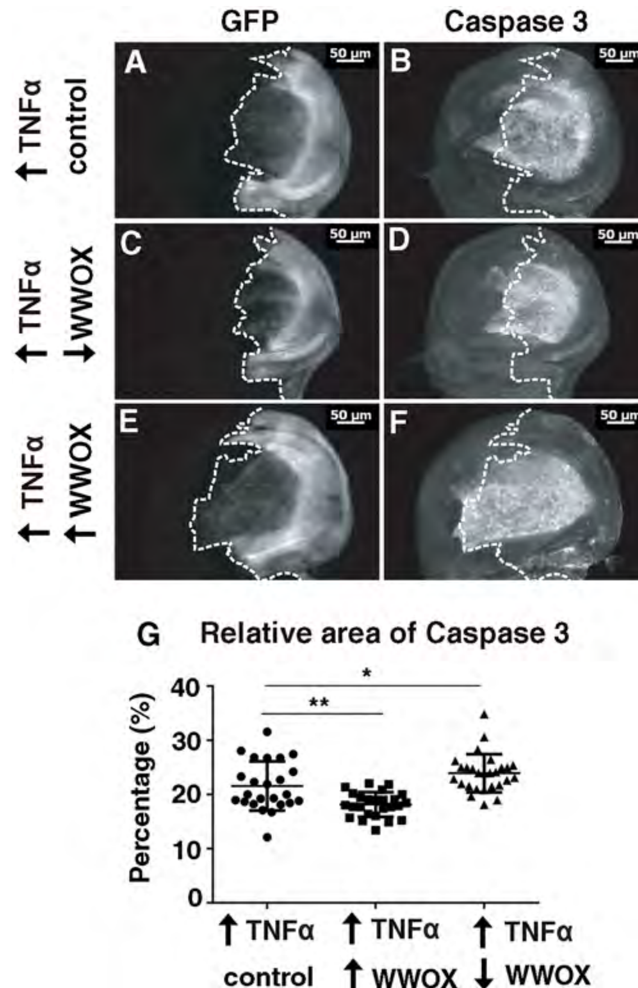

Fig 5. WWOX modifies Caspase3 staining in wing pouch in response to ectopic Egr/TNFα. (A) Ectopic expression of GFP and Eiger/TNFα with *hh-GAL4* in the posterior portion of wing discs of wandering third instar larvae, GFP showing the regions of ectopic expression. **(B)** Caspase 3 staining reveals high levels of apoptosis in the central wing pouch region as well as in two distinct regions extending towards the anterior. **(C)** Decreased WWOX expression results in a decrease in area of GFP expression. **(D)** Decreased WWOX expression results in a decreased region of Caspase 3 staining. **(E)** Increased WWOX expression results in an increase in area of GFP expression. **(F)** Increased WWOX expression results in a increased region of Caspase 3 staining. **(G)** Quantification of the area of Caspase 3 staining as a proportion of the area of the whole disc for individual wing discs of each genotype. Significance indicated by * = p<0.05, ** = p<0.005.

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the ability of tumorigenic cells to compete with non-tumorigenic / normal cells we utilized the well characterised system of mitotic clones of the cell polarity regulator Scribbled (*Scrib)*. Epithelial tissues in *D*. *melanogaster* where all cells are mutant for *Scrib* will overgrow and give rise to tumours [53]. However tumorigenic clones of *Scrib* mutant cells that are surrounded by wild-type cells will be eliminated [46, 54]. Clones of *Scrib* mutant cells generated in this way using the *M*osaic *A*nalysis with a *R*epressible *C*ell *M*arker (MARCM) system are positively labelled with GFP expression [55]. Many cells of the randomly generated mutant clones are eliminated however this process is not complete and some remain and can be visualized by patches of GFP positive cells in developing eye discs (Fig 6A and 6A'). These cells also correspond to regions of disruption to the normal pattern of differentiation as visualised by Elav staining during larval development (Fig 6B, 6B', 6C and 6C'). When WWOX levels were

reduced within these tumorigenic clones, an increase in the proportion of disc area with GFP positive cells was observed despite no change to overall disc size (Fig 6D, 6D', 6G and 6H). These GFP positive cells were also found to correspond to regions disrupted in their differentiation as visualised with Elav (Fig 6E, 6E', 6F and 6F'). Thus a decrease of WWOX within the clones of tumorigenic cells results in a mild but significant increase in their ability to compete, observed as a decrease in their effective elimination during this larval stage.

These tumorigenic *Scrib* clones persist throughout development and differentiation of eye tissue and result in mild adult eye phenotypes characterised by patches of roughness and disruption to ommatidial patterning (Fig 6I). This phenotype is enhanced when WWOX is decreased by RNAi knockdown within cells of the *Scrib* mutant clones where eyes consistently showed a significant decrease in size, as well as an increase in the frequency of black necrotic lesions of increased size on the surface of the adult eye (Fig 6J–6L). This enhanced phenotype also corresponds to an observed decrease in overall viability with flies with decreased WWOX expression in *Scrib* mutant clones showing a survival rate of 31.9% of that expected compared to 74.1% for flies with the *Scrib* mutant clones alone ($^{**}p = 0.0016$). A decrease in adult viability (or increase in pupal lethality) has previously been reported as an indication of reduced elimination of *Scrib* mutant clones in other genetic backgrounds [45–46, 56]. Thus we have demonstrated a cell autonomous contribution from WWOX for the elimination of tumorigenic cells in a whole animal model system. Similar effects on adult eye development were obtained when *Scrib* mutant clones were generated in eye discs where the whole animal had reduced WWOX function (heterozygous for either of two independent alleles of *WWOX*) or where WWOX function is completely removed (trans-heterozygous for independent *WWOX* alleles) (S4 Fig). Together these findings confirm that there is a decrease in the effectiveness of the process whereby tumorigenic *Scrib* mutant clones are eliminated when WWOX activity is reduced either exclusively within cells comprising the mutant clones or when WWOX activity is reduced or completely removed from all cells of the animal. Although mild effects were observed during developmental stages they resulted in more significant outcomes at the end of differentiation.

Discussion

The *WWOX* gene spanning *FRA16D* has previously been shown to have a variety of *in vitro* contributions to known cell death pathways in different mammalian cell lines, however it is unclear how these translate into a role *in vivo*, particularly in relation to the ability of WWOX to act as a tumor suppressor. We have therefore utilized a well-characterized *D. melanogaster* model of cell-cell competition to investigate an *in vivo* role for WWOX in the process of elimination of cancerous cells.

Significantly we determined an *in vivo* contribution by WWOX to the process whereby clones of epithelial cells carrying tumorigenic mutations are eliminated by the surrounding wild-type cells. The outcomes of competitive cell interactions in this way are essential contributing factors to the development of tumors *in vivo* [57]. We report here that reduction, or absence, of WWOX activity specifically in the tumorigenic cells decreased the effectiveness of this elimination process. Although this cell autonomous requirement for WWOX activity resulted in relatively mild effects on GFP expressing mutant cells in the eye imaginal discs, much more striking effects were evident at later stages. Generation of *Scrib* mutant clones in this way is analogous to the accumulation of mutations in cells that can gain a competitive advantage over the surrounding wild-type (non-mutant) cells and ultimately give rise to human cancers. Thus, our results show that endogenous WWOX plays a significant *in vivo* role in the process whereby mutation-bearing cells are eliminated. Together these results represent a plausible mechanism for low WWOX levels contributing to poor prognosis in various cancers [21–23,58].

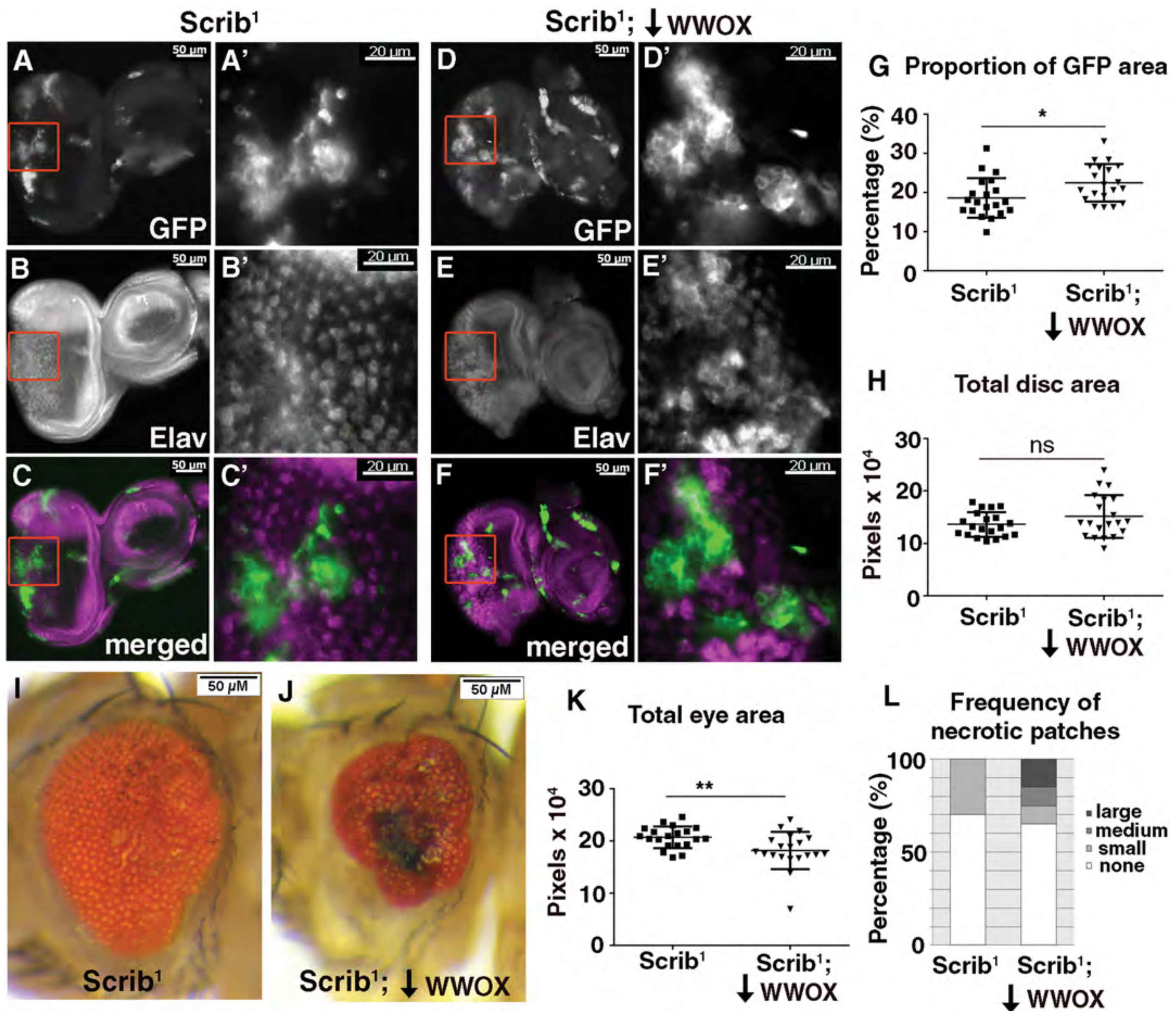

Fig 6. WWOX is required for elimination of *Scribbled (Scrib)* mutant clones. (A-A') Clones of cells mutant for *Scrib* generated in the eye using the MARCM system are positively labelled with GFP. **(B-B')** Elav staining reveals absence of differentiated photoreceptors within a portion of *Scrib* mutant clones. **(C-C')** Merged image showing GFP in green and Elav in magenta. **(D-D')** GFP expressing *Scrib* mutant clones with decreased WWOX expression (*Scrib*^{1}; *WWOX-IR*). **(E-E')** Elav staining reveals absence of differentiated photoreceptors within a portion of the *Scrib*^{1};*WWOX-IR* mutant clones. **(F-F')** Merged image showing GFP in green and Elav in magenta. Red boxes indicate the regions that are enlarged in D'-F'. **(G)** Quantification of the proportion of GFP expressing cells showed a significant increase when WWOX expression was decreased in the *Scrib* mutant clonal tissue compared to the *Scrib* mutant clones alone. **(H)** Quantification of total area of the eye disc containing *Scrib* clones with and without WWOX knockdown revealed no significant difference. **(I)** Clones of cells mutant for *Scrib* generated in the eye using the MARCM system result in a mild adult rough eye phenotype. **(J)** Decreased WWOX expression in the *Scrib* mutant clones gave a stronger phenotype with a decreased eye size, significant disruption to ommatidial patterning and the presence of necrotic lesions. **(K)** Quantification of the overall size of the adult eyes showed a significant decrease when WWOX levels were knocked down in *Scrib* mutant clones. **(L)** Quantification of percentage of adult fly eye showing necrotic spots of different sizes: Small (550–3000 pixels), Medium (3000–5500 pixels) or Large (>5500 pixels). Genotypes used in these experiments: *Scrib*^{1} = *(ey-FLP1*, *UAS-mCD8-GFP;;tub-GAL4 FRT82B tub-GAL80/ FRT82B*, *Scrib*^{1}*)*, *Scrib*^{1}; *WWOX-IR* = *(ey-FLP1*, *UAS-mCD8-GFP; UAS-WWOX-IR#2/+;tub-GAL4 FRT82B tub-GAL80/ FRT82B*, *Scrib*^{1}*)*. Significance indicated by * = p<0.05 and ** = p<0.005, ns = not significant.

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We have also utilised *D. melanogaster* models to dissect the cell death pathways to which WWOX contributes *in vivo*. In mouse L929 cells, an ectopic increase in WWOX was found to enhance TNFα-mediated cell death [42]. Consistent with this observation, altered WWOX levels modulate the phenotype obtained from ectopic expression of Egr/TNFα in the eye of *D. melanogaster*. WWOX was also previously shown to be an essential component of p53-mediated apoptosis in NIH3T3 cells [42], however no impact of altered WWOX levels was observed on the *D. melanogaster* eye phenotype from ectopic expression of *p53*. Similarly, no WWOX-mediated alteration of the *D. melanogaster* eye phenotype from ectopic expression of *hid* was observed herein, although others have reported a mild effect with further reduced WWOX levels on ectopic *hid* expression in the *D. melanogaster* eye [59]. Together these data are consistent with WWOX having a conserved, biologically significant role to play in the cell death mediated by Egr/TNFα.

In vitro nuclear localisation of pro-apoptotic WWOX was reported in L929 cells [42] as well as in MC7 cells in response to DNA damage [60]. However, we found no *in vivo* evidence for nuclear localization of ectopic WWOX in the presence of ectopic TNFα expression, indicating that the tumor suppressive functions may not be at the level of detection or alternatively they may be mediated through cytoplasmic WWOX functions. Conflicting reports appear in the literature for the location of WWOX protein to various cytoplasmically localised organelles including Golgi and mitochondria [16, 49]. Thus the localisation of WWOX may vary in different cell types and in response to different cellular stressors.

We observed no phenotypic effect with ectopic expression of WWOX alone, thus the cell death promoting effects of WWOX may require that WWOX be activated or modified in some way (e.g. phosphorylation) and may only become effective *in vivo* once cells are under some type of stress. Reactive oxygen species (ROS) are known to be principle effector molecules of TNFα-mediated cell death [45]. We have previously shown ectopic expression of WWOX gives high levels of ROS whilst reduced levels of WWOX show decreased ROS in developing *D. melanogaster* larvae [30]. Therefore, a likely mechanism by which WWOX contributes to the Egr/TNFα-mediated cell death pathway is via its regulation of ROS (Fig 7). At least to some extent, this occurs through regulation of the subset of ROS that are also responsive to enzymes of the superoxide dismutase (SOD) family and we have previously shown alterations in isoforms of SOD1 in WWOX mutant flies as well as genetic interactions between WWOX and SOD1 [30]. However the role for WWOX in the regulation of ROS levels may occur in a context dependent manner given the opposing effects reported for altered ROS in response to modified WWOX expression [61–62]. In addition, alterations to ROS levels would occur as a consequence of cancer cells shifting their metabolism from oxidative phosphorylation to a more glycolytic Warburg-based metabolism and we have previously shown that WWOX is both responsive to, and contributes to aerobic metabolism [30–31]. The protein products of other Common Fragile Site-associated genes; *Fragile histidine triad* (*FHIT*) at *FRA3B* and *Parkin* at *FRA6E* have also previously been shown to act as regulators of ROS [63–65]. Thus these genes may act together to maintain genome integrity under conditions of heightened oxidative stress, potentially arising from alterations to cellular aerobic metabolism known to be associated with cancer.

Materials and Methods

Fly lines and crosses

w^{1118}, *UAS-Dmp53* [66], *GMR-GAL4*, *GMR-hid* [67], *hh-GAL4* [68–69], UAS-SOD1 and UAS-SOD2 [70] were provided by Bloomington Stock Centre. *UAS-TNFR-IR* (v9152), *UAS-WWOX-IR*$^{\#1}$ (v22536), and *UAS-WWOX-IR*$^{\#2}$ (v108350) were obtained from Vienna

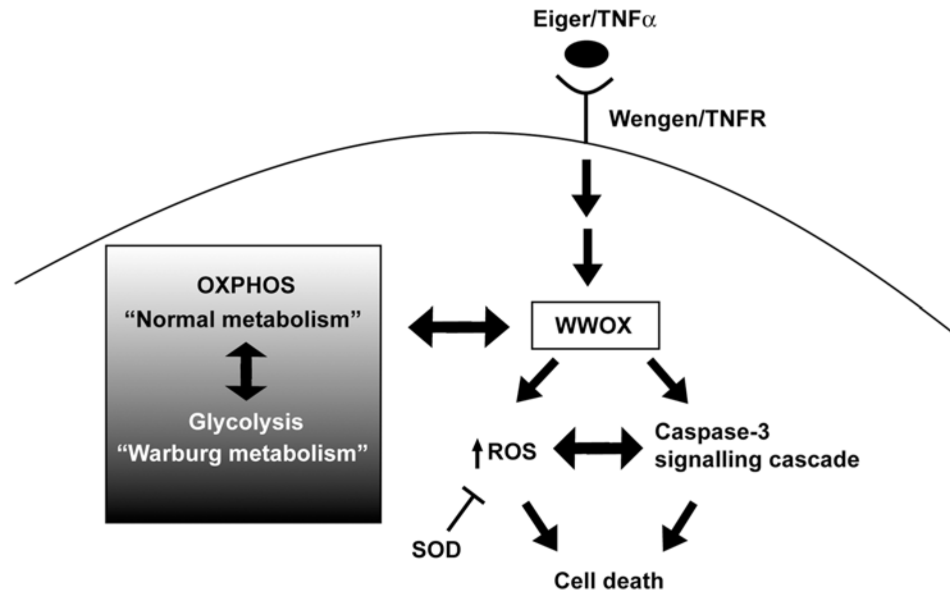

Fig 7. Model for the conserved role of WWOX in TNFα-mediated apoptosis *in vivo* in *D. melanogaster*. The function of WWOX in promoting cell death in response to Egr/TNFα signalling (through Wengen/TNFR) is mediated by ROS and Caspase 3. This can be modulated though expression of SOD enzymes that act to limit cellular levels of ROS and is also responsive to the metabolic status of cells.

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Drosophila RNAi Center. Successful knockdown of WWOX mRNA in each of these lines has previously been shown to be effective by quantitative real-time PCR (30, 61). Ectopic Eiger/ TNFα expression stock (*UAS-egr*$^{+W}$) was kindly provided by Professor Miura [46]. *WWOX*^{1}, *WWOX*^{2}, *UAS-WWOX ORF*$^{\#1}$ and *UAS-WWOX cDNA*$^{\#1}$ have previously been described [49 and 61]. MARCMIII and *FRT82B*, *Scrib*^{1} stocks were kindly provided by Helena Richardson. *D. melanogaster* stocks were maintained on fortified (F1) medium composed of 1% agar, 1% glucose, 6% fresh yeast, 9.3% molasses, 8.4% coarse semolina, 0.9% acid mix and 1.7% tegosept. All crosses were carried out at 25°C unless otherwise stated.

Analyses of Adult Eyes

Photographs of exterior adult female *D. melanogaster* eyes were taken using an Olympus SZX7 microscope fitted with a SZX-AS aperture diaphragm unit. Images were captured using an Olympus ColourView IIIU Soft Imaging System camera and AnalysisRuler image acquisition software. Images prepared using Adobe Photoshop CS version 8.0. The anterior of eye is positioned to the left of all images. For determination of adult eye sizes the outline of ten different randomly selected eye photos were traced using ImageJ and total area (in pixels) for each image was measured. Results for each experiment were graphed as scatterplot and statistical analyses (T-test analyses and One Way ANOVA) performed in GraphPad Prism.

Clonal analyses

Mitotic clones were generated for analyses using the MARCM III system, by crossing *ey-FLP1*, *UAS-mCD8-GFP;;tub-GAL4 FRT82B tub-GAL80/TM6B* flies to those carrying either a *WWOX* mutant allele or *WWOX*^{RNAi} transgene together with *FRT82B*, *Scrib*^{1}. Timed lays were carried out for all eye disc analyses. Third instar wandering larvae were dissected in PBS and fixed with 4% formaldehyde before mounting in glycerol to visualise GFP expression (GFP indicative of

clones and a minimum n = 20 eye discs were analysed per genotype). Significant disruption to eye disc morphology was observed in 13/52 pairs of the *Scrib[1]* clones and 31/50 pairs *Scrib[1]*; *WWOX-IR* clones and these were not included in these analyses. The size of the whole eye disc and area of GFP clones were quantified using Image J. The clonal area was calculated as a percentage of the total size of the eye imaginal disc and the averaged results were graphed as a scatterplot. T-test analyses were performed using GraphPad Prism. For determination of necrotic spots, the area of the black regions on the surface of the adult eyes were measured using ImageJ and divided into/scored as different categories based on size; small (550–3000 pixels), medium (3000–5500 pixels) or large (>5500 pixels). The percentage of eyes in each category was calculated and graphed using Microsoft Excel. For the viability assays, the overall number of adult progeny that eclosed from pupae were scored and the ratio of non TM6B:TM6B progeny were recorded for each cross, as described previously (30). The survival rate is presented as a percentage of the expected ratio of progeny. Statistical analyses were performed using the chi-square test with p = 0.05 as cut off value for significance using GraphPad Prism.

Western blot analyses

30 female adult flies (0–1 day old) per sample were collected and Western blot analyses were performed as previously described (49). Primary antibodies used were anti-C-DmWWOX antibody (1:1000) (49) and mouse monoclonal anti-β-tubulin antibody (1:2000, Sigma). Secondary antibodies used were Anti-Rabbit DyLight 649 antibody (1:2500, Vector Laboratories) and anti-mouse-Cy3antibody (1:200, Jackson Laboratories).

Immunohistochemistry

Wing discs or eye imaginal discs were dissected from late third instar larvae in 1x phosphate buffered saline (PBS) and fixed in 3.7% formaldehyde for 20 minutes. Discs were then washed three times with PBST (1xPBS + 0.3% Triton-X-100) for 20 minutes and blocked with PBSTF (1xPBS containing 5% fetal calf serum) for 90 minutes, followed by incubation of primary antibody overnight at 4°C. Anti-C-DmWWOX antibody (1:100 (52), anti-cleaved Caspase 3 antibody (1:100, Cell Signaling), anti-Elav 9F8A9 (1:10, Developmental Studies Hybridoma Bank) and anti-Ci 2A1 (1:100, Developmental Studies Hybridoma Bank) were used as primary antibodies. Discs were washed with PBST three times for 20 minutes and blocked with PBSTF for 30 minutes, followed by incubation of secondary antibody in the dark at room temperature for 2 hours. Secondary antibodies used were Anti-Rabbit DyLight 649 antibody (1:100, Vector Laboratories) and Anti-Rat rhodamine antibody (1:100). Discs were then washed three times with PBST for 20 minutes before incubation of DAPI (1:1000) for five minutes at room temperature and mounting in 80% glycerol. Relative areas of Caspase 3 staining were quantified in Image J and analysed in GraphPad Prism.

Cell ROS Assay

Reactive oxygen species (ROS) in third instar wing disc were detected using the fluorogenic probe CellRox (Life Technologies) as described previously [70].

Supporting Information

S1 Fig. Altered WWOX modifies ectopic Egr/TNFα-mediated eye phenotype. (A) Ectopic expression of Egr/TNFα (GMR>*egr*$^{+w}$>+) results in a decrease in eye size and disruption to ommatidial patterning. **(B)** Decreased expression of WWOX by RNAi knockdown (*GMR>egr*$^{+w}$*>WWOX-IR*$^{\#2}$) resulted in suppression of the rough eye phenotype. **(C)** Decreased expression of WWOX by heterozygous null allele (*GMR>egr*$^{+w}$*>WWOX*^{1}/+) resulted in

suppression of the rough eye phenotype. **(D)** Decreased expression of WWOX by heterozygous insertion mutation allele (*GMR>egr*$^{+w}$*>WWOX*^{2}/+) resulted in suppression of the rough eye phenotype. **(E)** Increased expression of WWOX (GMR>*egr*$^{+w}$*>WWOX-ORF*) resulted an enhancement of the Egr/TNFα phenotype. **(F)** Quantification of increased eye size with independent heterozygous *WWOX* alleles (*GMR>egr*$^{+w}$*>WWOX*^{1}/+ and *GMR>egr*$^{+w}$*>WWOX*^{2}*/+)*. **(G)** Increased expression of WWOX alone by ectopic expression of the ORF for WWOX (GMR>*WWOX-ORF*) resulted in no effect on development of the adult eye. **(H)** Western blot analysis and **(I)** quantification of the relative levels of WWOX protein expressed in each of the ectopic expression lines compared to a β-Tubulin control. **(J)** Western blot analysis and **(K)** quantification of WWOX protein expressed alone and together with Egr/TNFα compared to a β-Tubulin control.
(TIF)

S2 Fig. Altered WWOX has no effect on ectopic p53 or Hid eye phenotypes. (A) Ectopic expression of Dmp53 in the developing eye *(GMR>DmP53>EV)* at 18°C in the adult eye results in a phenotype characterized by decrease in eye size and significant disruption to ommatidial patterning accompanied by loss of pigment and the presence of small necrotic lesions. **(B)** Decreased expression of WWOX by RNAi *(GMR>DmP53>WWOX-IR*$^{\#1}$*)* resulted in no significant modification. **(C)** Increased expression of WWOX *(GMR>DmP53>WWOX-cDNA)* also resulted in no significant modification. **(D)** Ectopic expression of head involution defective in the adult eye (GMR>*GMR-Hid>EV*) results in a very strong rough eye phenotype with reduction in eye size and almost complete loss of ommatidial structures. **(E)** Decreased expression of WWOX by RNAi *(GMR>GMR-Hid>WWOX-IR*$^{\#1}$*)* resulted in no significant modification. **(F)** Increased expression of WWOX *(GMR>GMR-Hid>WWOX-cDNA)* also resulted in no significant modification.
(TIF)

S3 Fig. Ectopic Egr/TNFα has no effect on the cytoplasmic localization of WWOX in the eye imaginal disc. (A-D) Ectopic expression of WWOX alone with *GMR-gal4* results in WWOX localisation to areas complementary to the DAPI stained nuclei of eye-imaginal discs of wandering third instar larvae. **(E-H)** Ectopic expression of WWOX with *GMR-gal4* in the presence of ectopic Egr/TNFα expression also results in WWOX localisation to areas complementary to the DAPI stained nuclei of eye-imaginal discs of wandering third instar larvae.
(TIF)

S4 Fig. WWOX is required for elimination of *Scribbled (Scrib)* mutant clones in adult eyes. (A) Clones of cells mutant for *Scrib* generated in the eye using the MARCM system result in a mild adult rough eye phenotype. **(B)** Decreased WWOX expression throughout the whole animal (*Scrib*^{1}; *WWOX*^{1}*/+)* resulted in a stronger phenotype with a decreased eye size, significant disruption to ommatidial patterning and the presence of some necrotic lesions. **(C)** Complete absence of WWOX throughout the whole animal (*Scrib*^{1}; *WWOX*^{1}*/ WWOX*^{2}*)* resulted in a phenotype with a decreased eye size, significant disruption to ommatidial patterning and the presence of large necrotic lesions. Genotypes used: *Scrib*^{1} *(ey-FLP1, UAS-mCD8-GFP; +/+;tub-GAL4 FRT82B tub-GAL80/ FRT82B scrib*^{1}*), Scrib*^{1}*;WWOX*$^{-/+}$*(ey-FLP1, UAS-mCD8-GFP; WWOX*^{1}*/+;tub-GAL4 FRT82B tub-GAL80/ FRT82B scrib*^{1}*), Scrib*^{1}*; WWOX*$^{-/-}$ *= (ey-FLP1, UAS-mCD8-GFP; WWOX*^{1}*/ WWOX*^{2}*;tub-GAL4 FRT82B tub-GAL80/ FRT82B scrib*^{1}). Adults carrying *WWOX* mutations were generated by crossing *ey-FLP1, UAS-mCD8-GFP;;tub-GAL4 FRT82B tub-GAL80/TM6B* flies carrying either a *WWOX* mutant allele or *wild-type* second chromosome together with *FRT82B, Scrib*^{1} carrying a *WWOX* mutant allele.
(TIF)

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Author Contributions

Conceived and designed the experiments: LVO CSL AC RIR. Performed the experiments: LVO CSL AC. Analyzed the data: LVO CSL AC RIR. Contributed reagents/materials/analysis tools: LVO CSL AC. Wrote the paper: LVO CSL AC RIR.

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Supplementary Materials

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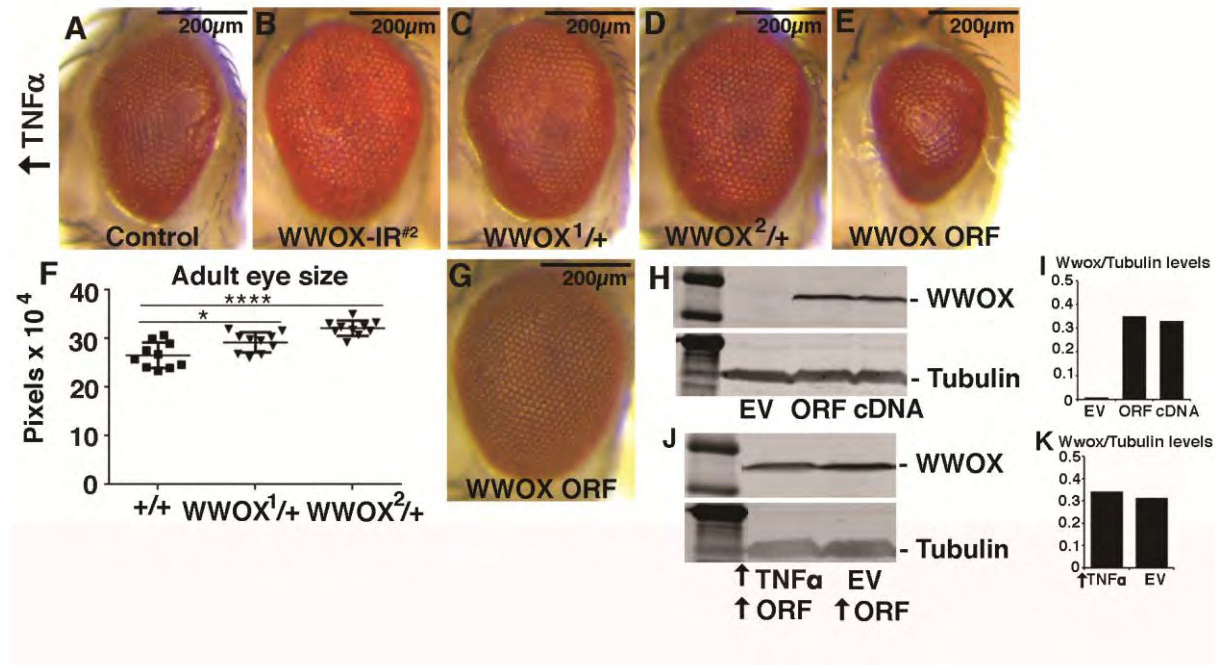
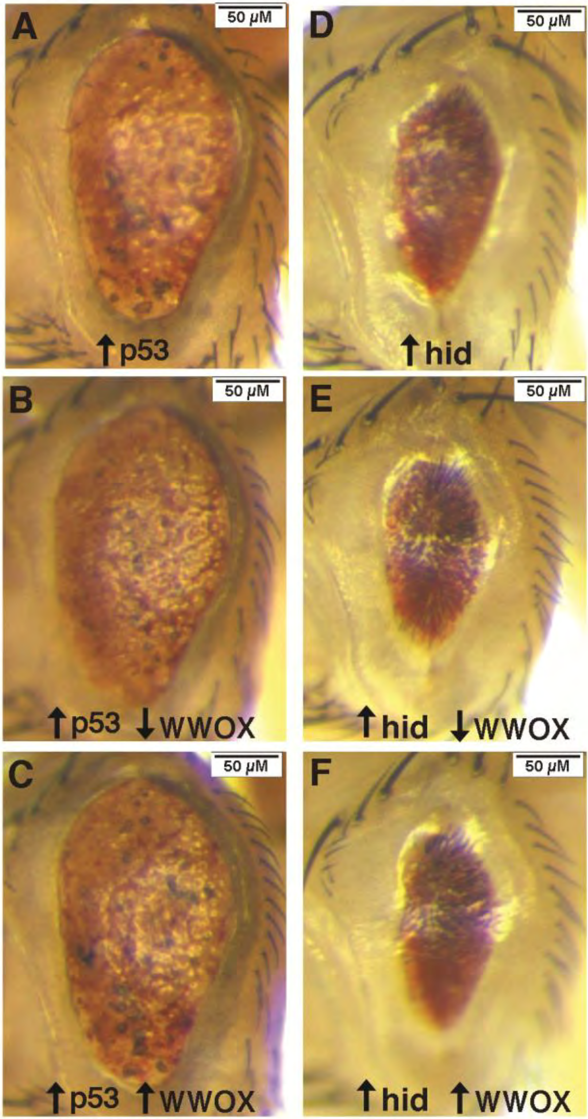
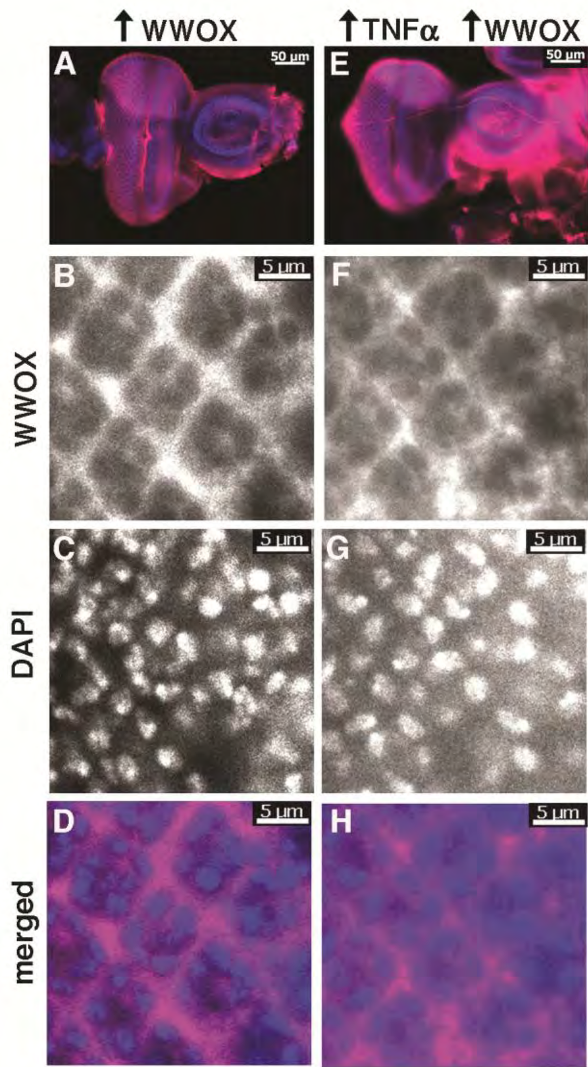


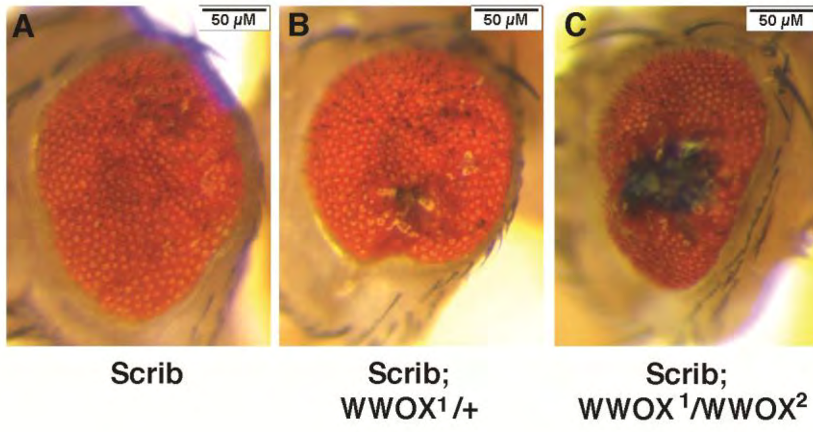
S1 Fig



S2 Fig



S3 Fig



S4 Fig

Chapter 3

Investigation of domains that are required for various Wwox functions in cell death and metabolism

This chapter has been written up as a manuscript for publication:

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Contribution to the Paper	Conceived and designed the experiments, performed all experiments, analysed the data, contributed reagents / materials / analysis tools, writing and revision of the manuscript		
Overall percentage (%)	90		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Conceived and designed the experiments, analysed the data, contributed reagents / materials / analysis tools, contributed to the revision of the manuscript		
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<tr><td>Name of Co-Author</td><td colspan="3">Robert I. Richards</td></tr>
<tr><td>Contribution to the Paper</td><td colspan="3">Conceived and designed the experiments, supervised development of work, analysed the data, contributed reagents/materials/analysis tools, contributed to revision of manuscript and is corresponding author.</td></tr>
<tr><td>Signature</td><td></td><td>Date</td><td>11th August, 2016</td></tr>
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Distinct functional requirements for WWOX in cell death and metabolism

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ABSTRACT

WW domain-containing oxidoreductase (WWOX) was first identified as the gene spanning the *FRA16D* common chromosomal fragile site, a region of DNA instability in cancer. Variations in the WWOX gene have since also been associated with metabolic diseases. The normal biological functions of WWOX are therefore of great interest and these appear to be numerous and diverse. The WWOX protein is conserved throughout evolution, suggesting some fundamental biological roles that likely depend upon its well conserved protein motifs. WWOX has two WW domains that are located N-terminal to a short-chain alcohol dehydrogenase-reductase (SDR) region, which distinguish WWOX from other members of the SDR family of enzymes. WW domains typically bind to other proteins and have therefore been the focus of many efforts to identify the cellular pathways to which WWOX contributes. In this study, we have used expression in *Drosophila* to compare the ability of full length Wwox and a truncated version of Wwox ($Wwox^{trun169}$), which is devoid of a complete SDR region, to act in independent assays. $Wwox^{trun169}$ is able to carry out some of the roles of full length Wwox, namely the enhancement of Eiger/TNFα-mediated cell death and suppression of spindle assembly checkpoint defects. In contrast, the SDR region is required for Wwox functions in mitochondrial dysfunction and oxidative stress defects. These results distinguish pathways in which the SDR region of Wwox is required from those in which is not.

INTRODUCTION

Initial interest in the *WWOX* gene and the protein that it encodes arose from the discovery that it spans the *FRA16D* common chromosomal fragile site (CFS) [1, 2]. CFSs are regions on chromosomes that are predisposed to chromosomal breakage under environmental conditions that lead to replicative stress [3]. They can also be induced by chemicals that are commonly found in the diet and the environment, including caffeine [4], ethanol [5] and cigarette smoke [6, 7].

FRA16D, the CFS found within exon 8 of the *WWOX* gene, is one of the most highly expressed CFSs. WWOX has been classified as a non-classical tumor suppressor because of its atypical characteristics. Firstly, while *FRA16D* associated DNA instability is common, loss-of-function point mutations in *WWOX* are very rare in cancer [2, 8, 9]. Secondly, *WWOX* does not behave according to Knudson's "two hit" model of tumorigenesis [2, 9-12]. On the one hand, reintroduction of WWOX back into WWOX deficient human breast cancer cells severely curtails the establishment and growth of these cancer cells in a nude mouse model [13]. On the other hand, cancers develop in mice with one functional *WWOX* allele, indicating that the complete loss of WWOX function is not a requirement for tumor progression [14]. Indeed human cancer cells with low *WWOX* levels tend to still carry one functional *WWOX* allele, suggesting that haploinsufficiency of WWOX could be adequate for the contribution of WWOX to tumor development [1, 14-16].

In contrast, rare inherited homozygous loss-of-function *WWOX* mutations have been shown to cause severe central nervous system (CNS) pathology including epilepsy, ataxia and mental retardation [17, 18]. Consistent with these observations, both spontaneous rat [19] and mouse gene knock-out [20] *Wwox* mutants also exhibit tonic-clonic seizures. In addition, *Wwox* gene knock-out mice also exhibit growth retardation and early death, along with

hypoglycaemia, hypocalcemia, metabolic acidosis and kidney failure [14, 21], suggesting that WWOX has important metabolic functions. Consistent with these associations, recent functional studies in *Drosophila* also define the Wwox protein as having an integral role in metabolism [22-24]. The amino acid sequence of *Drosophila* Wwox is 49% identical compared to human WWOX with high conservation across functional domains [12, 23, 25]. Although *Wwox* mutant *Drosophila* do not show any obvious morphological or functional phenotype [25], microarray and proteomic analyses have revealed that the alteration of *Wwox* levels in *Drosophila* changes the expression levels of many enzymes involved in metabolic processes, including the tricarboxylic acid (TCA) cycle. This suggests that Wwox has a role in aerobic metabolism [23]. This is further supported by experiments in *Drosophila* demonstrating that Wwox functionally interacts with isocitrate dehydrogenase (Idh), Cu–Zn superoxide dismutase (Sod) [23], subunits of the mitochondrial respiratory chain [22] and mitotic arrest deficient 2 (Mad2) [24]. In addition to its function in metabolism, WWOX has also been shown to contribute to cell death pathways in both mammals and *Drosophila* [26-28].

Although WWOX has an important role in both metabolism and cell death, the domains and molecular mechanisms that are responsible for these functions have not yet been determined. Herein, *Drosophila* was utilized as an *in vivo* animal model to investigate the ability of truncated Wwox, lacking the C-terminal region that includes the catalytic site and potential substrate binding site, to function in a range of independent established functional assays. We have generated and tested *Drosophila* lines ectopically expressing a $Wwox^{trun169}$ transgene that encodes the 169 amino acid N-terminal sequences, including two intact WW domains and a non-functional SDR enzyme region. This N-terminal region of Wwox is able to carry out some, but not all of the functions carried out by normal full length Wwox,

indicating that certain functions of WWOX are independent of a complete SDR enzyme region.

RESULTS

Ectopic expression of C-terminal deleted Wwox in *Drosophila*

In order to investigate the distinct parts of the WWOX protein that are necessary for its different functions, independent *Drosophila* lines expressing an N-terminal *Wwox* (*Wwox^{trun169}*) construct were generated. *Wwox^{trun169}* encodes a 169 amino acid protein with two intact WW domains and an incomplete SDR enzyme region (Figure 1A-B). Wwox^{trun169} lacks the SDR catalytic active site and a potential substrate binding region, but retains an intact NAD(P)(H) co-factor binding site (Figure 1A-B). This truncation of *Drosophila* Wwox corresponds to human truncated WWOX isoforms encoded by alternatively spliced variants that are highly expressed in cancers [2, 13, 29, 30], particularly the WWOX isoform 2 (also known as WWOXv6 or FORIII) encoding transcript (Figure 1A-B). Although steady-state levels of these transcripts are readily detected [2, 29], it is unclear whether these transcripts are translated into *in vivo* proteins, or are subjected to nonsense mediated decay [31, 32].

The molecular weight of full length *Drosophila* Wwox is 47kDa, while the molecular weight of Wwox^{trun169} product is predicted to be 19kDa. A previously generated antibody targeting the N-terminal region of *Drosophila* Wwox protein (anti-N-terminal Wwox antibody) [25] detects both Wwox and Wwox^{trun169}. Using this antibody, a band consistent with the size of full length Wwox was detected in flies ectopically expressing Wwox (Figure S1A). All four independent lines ectopically expressing a single copy of *Wwox^{trun169}* transgene (#1 - #4) also showed a band that was consistent with the expected size of Wwox^{trun169} (Figure

1C), confirming that a truncated Wwox product lacking the C-terminal region is ectopically expressed in flies. The difference in Wwox^{trun169} levels between the four lines most likely reflects different genomic locations and as a consequence, differing expression levels of the inserted transgenes. Ectopic Wwox^{trun169} levels in any of these four lines were much lower relative to ectopic full length Wwox levels (Figure S1B). As levels of ectopically expressed protein depend on the copy number of transgenic constructs, two independent combined lines were generated (#1+2 and #3+4), each carrying two copies of *Wwox*^{trun169} constructs. Each of these lines resulted in increased levels of Wwox^{trun169} (Figure 1C and S1B). No band corresponding to the size of truncated Wwox was detected in lines with either ectopic expression of full length Wwox or empty vector control, indicating that any potential *Drosophila* truncated endogenous Wwox protein is too low in abundance to be detected (Figure 1C).

Cytoplasmic localization of truncated Wwox

Previously, we have shown that ectopically expressed Wwox localizes to the cytoplasm of cells in *Drosophila* embryos, as well as in third instar larval eye and wing imaginal discs [25, 28]. In order to determine whether a truncation of the SDR enzyme region alters the subcellular localization of Wwox, third instar larval wing discs ectopically expressing Wwox^{trun169} were immuno-stained with anti-N-terminal Wwox antibody (Figure 2). The *hedgehog-gal4* promoter was used to express Wwox^{trun169} specifically in cells within the posterior compartment of the imaginal wing disc. This posterior region was identified by co-expressing green fluorescent protein (GFP) protein (Figure 2A and E). Ectopic expression of either Wwox or Wwox^{trun169} showed a cytoplasmic localization, as the staining was complementary to the DAPI-stained nuclei (Figure 2B-D and F-H). This suggests that truncated Wwox has the same localization as full length Wwox and therefore any

distinguishing functions are unlikely to be due to obvious differences in compartmentalization.

Enhancement of Eiger/TNFα-mediated cell death by truncated Wwox^{trun169}

TNFα is an important inflammatory cytokine that can induce both cell death and cell survival, depending upon the activated downstream signaling pathways [33]. Wwox has been shown to contribute to TNFα-mediated cell death in both mammalian cells and *Drosophila* [27, 28]. In the *Drosophila* eye, ectopic expression of the TNFα homolog, Eiger, results in disorganization of ommatidial units and reduced eye size [28] (see also Figure 3Ai and S2A). Reduction of *Wwox* levels suppresses this eye phenotype (restoring the organized arrangement of ommatidial units as well as the overall eye size), while ectopic expression of Wwox shows an enhancement of the phenotype (with a further reduction in the eye size; see also Figure S2B and S2D) [28].

The effect of Wwox^{trun169} on Eiger/TNFα-mediated cell death was tested in the *Drosophila* eye. Eye phenotypes were classified on the basis of severity (Figure 3A i-iii). In this scale, all the flies with ectopic expression of Eiger/TNFα alone showed a consistent eye phenotype that was classified as “mild”. Eyes that were obviously smaller than this were classified as “moderate”, while the phenotype of complete eye loss was classified as “severe”. Using this scale, ectopic expression of one copy of *Wwox* transgene (along with Eiger/TNFα) resulted in a small percentage of eyes showing a moderate phenotype (1.2%) (Figure 3B). Further increasing the ectopically expressed *Wwox* levels (by generating flies carry two copies of *Wwox* transgenes) greatly enhanced the severity of the *eiger*/*TNFα* eye phenotypes with 21.7% of total eyes scored showing a moderate phenotype and 3.3% showing a severe phenotype (Figure 3B). These observations are consistent with the previous finding that increased *Wwox* levels can enhance Eiger/TNFα-mediated cell death [28].

Similar analyses were conducted with ectopic co-expression of a single copy of the Wwox^{trun169} with Eiger/TNFα. Ectopic expression of Wwox^{trun169} also significantly enhanced the severity of the *eiger/TNFα* eye phenotype, with 20.8% of total eyes scored showing a moderate phenotype and 1.4% showing a severe phenotype (Figure 3B). Intriguingly, the enhancement of Eiger/TNFα-mediated cell death by ectopic expression of one copy of *Wwox*^{trun169} transgene was stronger than that observed with one ectopic copy of *Wwox*, and similar to the effect of ectopically expressing two copies of the *Wwox* transgene (Figure 3B). As the ectopic expression levels of one copy of *Wwox*^{trun169} transgene were found to be lower than one copy of *Wwox* transgene (Figure S1B), this suggests that Wwox^{trun169} is more potent than Wwox in the enhancement of Eiger/TNFα-mediated cell death. These results also demonstrate that common domains within both Wwox and Wwox^{trun169} are sufficient for the role of WWOX in enhancing Eiger/TNFα-mediated cell death.

Suppression of spindle assembly checkpoint defects by truncated Wwox

Chromosomal instability is one of the characteristics of most solid tumors and can be induced by reduction of the mitotic arrest deficient 2 (Mad2) proteins, which are involved in Spindle Assembly Checkpoint (SAC) [24, 34]. Reducing levels of *Mad2*, together with the reduction of some functional interactors involved in metabolism (including Wwox) is able to increase cell death, mitochondrial stress, DNA damage and ROS levels in *Drosophila* third instar larval wing discs, and results in adulthood lethality [24, 34]. It was also observed that some of the flies with reduced *Mad2* levels died either at the pupal stage (Figure 4A i) or whilst partially eclosed from their pupal cases (Figure 4A ii). Wwox^{trun169} was ectopically expressed in flies with reduced *Mad2* levels to determine whether it also has an effect on their pupal survival (Figure S3A).

The percentage of flies that were fully eclosed were scored by the number of empty pupal cases (Figure 4A iii). It was found that only 24.4% of flies had successfully undergone pupal eclosion when expression levels of *Mad2* were reduced (Figure 4B and Figure S3B). Reduction of *Wwox* levels in this *Mad2* deficient background further decreased the percentage of flies that had fully eclosed, while ectopic over-expression of Wwox significantly increased this percentage (Figure 4B and Figure S3B). Increased WWOX expression therefore rescues spindle assembly checkpoint defects in *Mad2* deficient flies. Ectopic expression of $Wwox^{trun169}$ with two independent lines (#1+2 and #3+4) also significantly increased the success rate of pupal eclosion (Figure 4B, S3B and S3C). This suggests that $Wwox^{trun169}$ is able to carry out a similar function to that of the Wwox in regards to rescuing the viability of pupae with reduced *Mad2* levels. Similar experiments were performed at a higher temperature (29°C) to increase the efficiency of *Mad2* RNA interference (RNAi) knockdown (Figure S3D) [35]. At this temperature, *Mad2* expression falls below the threshold required for survival, causing complete lethality [24, 34]. Ectopic expression of either Wwox or $Wwox^{trun169}$ can increase the survival of pupae with these further reduced *Mad2* levels (Figure S3D).

In addition to eclosion assays, adult viability was assessed to determine the percentage of *Drosophila* that survived to adulthood. Reduction of *Mad2* levels alone consistently decreased the viability of adult flies, while ectopic expression of either Wwox or $Wwox^{trun169}$ in two independent lines (#1+2 and #3+4) resulted in an increase in the viability of flies with reduced *Mad2* levels (Figure S3E). Although 4.5% of flies eclosed from pupal cases when both *Mad2* and *Wwox* levels were decreased (Figure 4B, S3B), no adult flies were found in this assay (Figure S3E), consistent with our previous report [24]. This suggests that these flies had a very short lifespan and died soon after eclosion.

Together, the results show that the expression of the N-terminal Wwox region is sufficient to suppress the lethal effects of spindle assembly checkpoint defects in flies and is therefore able to carry out the same function as Wwox. This also indicates that the contribution of Wwox in restoring the viability of flies with reduced *Mad2* levels does not require a complete SDR enzyme region.

Ectopic expression of Wwox^{trun169} has a similar effect to reduced *Wwox* levels on eye phenotypes caused by mitochondrial dysfunction

Wwox functionally interacts with six different genes (*ND23*, *ND42*, *ND75*, *CG7580*, *CoVa* and *CoVb*) that encode components of the mitochondrial electron transport chain [22]. Reduction in expression of any of these genes in the *Drosophila* eye (using targeted *UAS-RNAi* knock-down driven by the *eyeless-gal4*) induces mitochondrial dysfunction and causes a range of cellular dysmorphology that can be characterized by loss of cells, overgrowth of eye tissue and defects in differentiation. These eye phenotypes have been classified into three categories based on their severity (Figure 5A i-v). Reduced *Wwox* expression enhances, while over-expression of Wwox suppresses these phenotypes [22].

The effect of the Wwox^{trun169} on the mitochondrial defect-induced eye phenotype was determined. Mitochondrial dysfunction was induced by reducing expression of the Complex I gene, *ND42*. Interestingly, when Wwox^{trun169} was expressed in *ND42* deficient flies, it was observed that a considerable number of flies had very severe eye phenotypes, at a significantly higher percentage than in flies with reduced *ND42* levels alone. This is reminiscent of previously obtained results when *Wwox* levels were decreased. When quantified, ectopic expression of Wwox^{trun169} with two independent lines (#1+2 and #3+4) was found to increase the severity of *ND42* eye phenotypes (Figure 5B, Figure S4A-B). This suggests that Wwox^{trun169} is behaving in the same manner as loss of Wwox function. This

detrimental effect of $Wwox^{trun169}$ on cells with mitochondrial dysfunction was further confirmed in a genetic background of reduced expression of another Complex I gene, *ND75* (Figure S4C). These results suggest that $Wwox^{trun169}$ is interfering with the normal role of Wwox in its suppression of mitochondrial defects.

Ectopic expression of $Wwox^{trun169}$ decreases the survival of $Sod1^{n1}/Sod1^{n64}$ mutant flies

Cu-Zn superoxide dismutase (Sod1) is an enzyme that is involved in anti-oxidant pathways [36]. $Sod1^{n1}$ and $Sod1^{n64}$ mutations are missense mutations in *Sod1* that disrupt dimer contact and the metal ion binding of Sod1 respectively [23, 37]. Flies that are trans-heterozygous for $Sod1^{n1}$ and $Sod1^{n64}$ mutations are viable but have a shorter lifespan compared to wild-type flies. Loss of *Wwox* expression in these trans-heterozygous $Sod1^{n1}/Sod1^{n64}$ flies decreases both their viability and lifespan, indicating a functional interaction between Wwox and Sod1 [23].

Viability assays were carried out to investigate the effect of ectopically expressed $Wwox^{trun169}$ on the survival of trans-heterozygous $Sod1^{n1}/Sod1^{n64}$ flies (Figure S5A). Reduction of *Wwox* expression using RNAi was found to decrease viability in $Sod1^{n1}/Sod1^{n64}$ flies (Figure 6). This is consistent with previous results, where the requirement for Wwox in trans-heterozygous $Sod1^{n1}/Sod1^{n64}$ flies was demonstrated using complete loss-of-function mutations [23]. Whilst ectopic expression of $Wwox^{trun169}$ alone did not affect the viability of wild-type flies (Figure S6), it further decreased the viability of trans-heterozygous $Sod1^{n1}/Sod1^{n64}$ flies (Figure 6 and S5B-C). Therefore, over-expression of $Wwox^{trun169}$ is behaving in a similar manner to a reduction or loss of *Wwox* expression in this assay. This result is consistent with the requirement of the SDR region of Wwox for its functional interaction with Sod1.

DISCUSSION

The normal biological functions of WWOX are of great interest, particularly in regards to how their reduced levels can contribute to cancer. There is increasing evidence for WWOX playing an important role in the regulation of metabolism [14, 22, 23, 38, 39]. WWOX is an integral component of metabolism being both regulated by altered metabolism and as a regulator of metabolism itself [40]. WWOX allelic variation has been shown to be associated with low levels of high-density lipoprotein cholesterol (HDL-C) [41], type 2 diabetes [42-44], coronary artery calcification [45], obesity [46], hypertension susceptibility [47], left ventricular thickness [48] and predisposition to lung cancer [49]. A role for WWOX in metabolism has been conserved through evolution [14, 22, 23, 38, 39], yet the molecular mechanism of WWOX in the regulation of metabolism still remains elusive. It is also unknown whether the role of WWOX in metabolism alone accounts for its function in a diverse array of pathways, including cell death [27].

The WWOX protein has distinct functional sequence motifs. These include two WW domains and a classical-like SDR enzymatic domain. It has been unclear for which of the various functions of WWOX the SDR catalytic region is required. In contrast to the SDR region, it is clear that WW domains of WWOX are involved in protein-protein interactions. Thus, many studies have focused on identifying binding partners of the WW domains of WWOX in an effort to determine WWOX functions by virtue of the identity of the proteins with which it associates [26, 27, 50-52]. Abu-Odeh et al. (2014) have, for example, identified 240 proteins that physically bind to the first WW domain of WWOX using mass spectrometry and phage display experiments. These binding partners are involved in a variety of cellular processes, including transcription, RNA processing, metabolism and signaling pathways [50]. However, the functional significance of these associations *in vivo* or whether the SDR enzyme activity of WWOX is required remains largely unclear.

Herein, *Drosophila* was utilized as a model to investigate the functional requirements for distinct sequence regions of Wwox. Functional assays were used to compare full length Wwox with the N-terminal 169 amino acid form of Wwox, the latter lacking sequences typically important for SDR enzyme activity, including the catalytic region and postulated substrate determining sequences [12]. *Drosophila* lines were generated to stably express a truncated version of Wwox, Wwox^{trun169}, that has two WW domains and the NAD(P)(H) co-factor binding site (Figure 1 A-B). Wwox^{trun169} was found to carry out certain previously characterized functions of full length Wwox, notably the enhancement of Eiger/TNFα-mediated cell death and suppression of spindle assembly checkpoint defects. The pro-apoptotic function of Wwox^{trun169} is consistent with a previous study showing that the first WW domain of WWOX is required for inducing p73 pro-apoptotic function [53]. Thus, it is possible that Wwox^{trun169} contributes to Eiger/TNFα-mediated cell death through interaction with p73. First WW domain of WWOX has also been shown to be required for ATM-mediated DNA damage response [54]. Although it has been shown that reduced *Mad2* levels can induce DNA damage [34], it is not known whether the DNA damage is the cause of lethality or the consequence of spindle assembly checkpoint defects. Future study is required to confirm the conserved Wwox function in DNA damage repair in *Drosophila*, and determine whether the suppression of DNA damage can rescue the lethality of flies with reduced *Mad2* levels.

In contrast, Wwox^{trun169} acted in a contradictory manner to that of Wwox in other previously characterized Wwox assays, which involve either the mitochondrial respiratory chain or anti-oxidant response. In these functions, the behavior of Wwox^{trun169} appears to be consistent with that of a dominant negative competitor of the endogenous Wwox protein. While Wwox^{trun169} encodes an incomplete SDR enzyme region it is still able to carry out some Wwox functions. Wwox^{trun169} includes the NAD(P)(H) co-factor binding site and both WW

protein-binding domains (Figure 1 A-B). These functional domains may be acting via sequestration. SDR enzymes are also noted for their roles in monitoring NAD(P)(H) levels [55, 56]. An effective reduction in free NAD(P)(H) levels by binding to Wwox^{trun169} is therefore a plausible mechanism of action. Similarly since WW domains have roles in protein-protein interactions [57], the normal partner protein(s) for the WW domains of Wwox may be sequestered by binding to the Wwox^{trun169}. These results therefore reveal that there are pathways in which a complete SDR region of WWOX is required, and those in which it is not.

The human *WWOX* gene is very large (>1Mb) with unusually large introns (one exceeding 780kb) and its transcripts are normally subject to alternative splicing. In normal cells the non-full length *WWOX* encoding alternative mRNAs are in low abundance [2]. However cancer cells not only have lower levels of full length *WWOX* transcripts, but typically also have higher levels of *WWOX* transcripts that include both normally occurring and aberrant alternate isoforms [2, 13, 29, 30]. Several of these alternative mRNAs are potentially translated into truncated WWOX isoforms [8, 58]. The Wwox^{trun169} tested in this study is similar to protein isoforms that are encoded by some of these alternatively spliced variants, particularly *WWOX isoform 2* (Figure 1A-B). This transcript is expressed at low levels in normal breast cells, and is highly expressed in about 50% of breast tumors, suggesting the association of this alternatively spliced variant with tumorigenesis [29]. In addition, the mouse *Wwox* gene is also subject to alternative splicing with an analogous RNA transcript (*Wwox-002 ENSMUST00000109107*) [59]. This transcript is able to encode a truncated protein similar to that of human WWOX isoform 2. Despite this conservation through evolution, the contribution, if any, of WWOX isoform 2 to cell biology is unclear, as this protein has been difficult to detect. These alternatively spliced RNAs could be subjected

to high rates of nonsense-mediated decay, although they clearly have sufficient steady-state levels to be readily detected [2, 29].

The majority of *FRA16D*-associated DNA instability detected in cancer cells involves the deletion of sequences encompassing some, or all, of the WWOX gene. Somatic point mutations in cancer samples, while relatively rare, have been detected in the WWOX coding sequence [31]. Of the 53 such WWOX mutations retrieved from *cbioportal* [60, 61] only 7 are located in the N-terminal region that includes the WW domains of WWOX. The remaining 46 are distributed across the SDR enzyme and C-terminal extension (Figure 7), indicating that the WW domains are strikingly under-represented. Given that these mutations indicate which components of WWOX are permissive to cancer cell biology, the functions of WWOX that are mediated by the WW domains are unlikely to be directly responsible. Therefore the roles of WWOX identified herein, including the enhancement of Eiger/TNFα-mediated cell death and the suppression of spindle assembly checkpoint defects, are unlikely to be the pathways by which variation in WWOX contributes to cancer biology. Instead perturbations of metabolic pathways that are dependent upon the SDR enzyme function of WWOX, including its postulated substrate-specificity determinants, are more likely permissive to cancer cell survival.

MATERIALS AND METHODS

Fly lines

All *Drosophila* stocks were maintained in vials containing fortified medium (1% agar, 1% glucose, 6% fresh yeast, 9.3% molasses, 8.4% coarse semolina, 0.9% acid mix, 1.7% tegosept) at either 18°C or 25°C. *da-Gal4, ey-Gal4*, *gmr-GAL4*, *hh-Gal4, Sod1^{n1}/TM3Sb*, *Sod1^{n64}/TM3Sb* and *UAS-GFP* stocks were obtained from the Bloomington stock centre. All *RNAi* lines were obtained from the Vienna Drosophila Research Centre. *UAS-Wwox, UAS-Wwox^{trun169}* and *Empty Vector* constructs were generated and microinjected into *Drosophila* embryos using a standard protocol as previously described [62].

Western blot analyses

Five female adult flies per sample were collected and Western blot analyses were performed as previously described [25].

Immunohistochemistry

Wing discs were dissected from late third instar larvae in 1x phosphate buffered saline (PBS). Immuno-staining and mounting were carried out as previously described [28]. Anti-N-terminal-Wwox antibody (1:100) [25] was used as primary antibody, while anti-rabbit-Dylight 649 (1:200) (Vector Laboratories) was used as secondary antibody. DAPI (1:1000) was incubated for five minutes before the mounting step. Imaging was carried out on the Zeiss Axioplan2 microscope (Carl Zeiss) using an AxioCam MRm camera (Carl Zeiss) and *AxioVision* software.

Viability Assays

Drosophila crosses were set up as shown in Supplementary Figure S3A and S5A and maintained at either 25°C or 29°C. The ratio of non-TM6B:TM6B progeny was recorded once

per day. In the pupal survival assays, all the non-TM6B progeny were scored after 14 days of crosses. Chi-square test analyses were performed using *GraphPad Prism 5* to determine whether there was a significant difference between control and tested samples. $p<0.05$ was used as the cut-off value for significance.

Quantification of eye phenotypes

Adult eye phenotypes caused by either Eiger/TNFα-mediated cell death or mitochondrial dysfunction were grouped into three classes with representative figures shown in Figure 3A and 5A. Eyes of the fly were scored individually. All adult eye images were captured using an Olympus ColourView IIIU Soft Imaging System camera and *AnalysisRuler image acquisition* software. In order to test the effect of ectopically expressed $Wwox^{trun169}$ on Eiger/TNFα-mediated cell death, ten (0-1 day old) adult eye sizes were measured using *ImageJ* software. Chi-square test analyses or Fisher's exact tests were performed using *GraphPad Prism 5* with $p<0.05$ as the cut-off value for significance.

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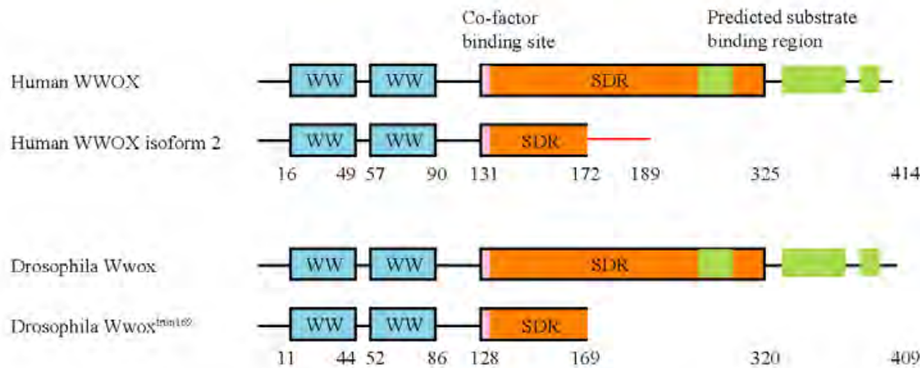
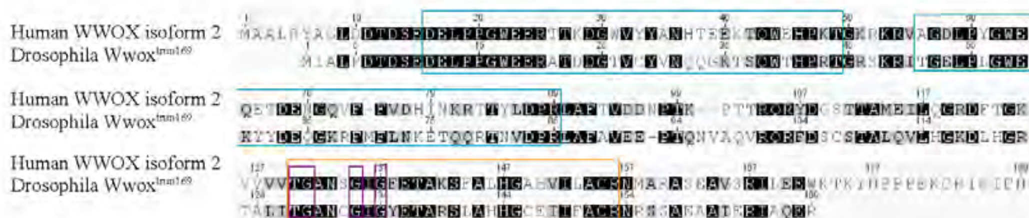
Figures

A)



B)



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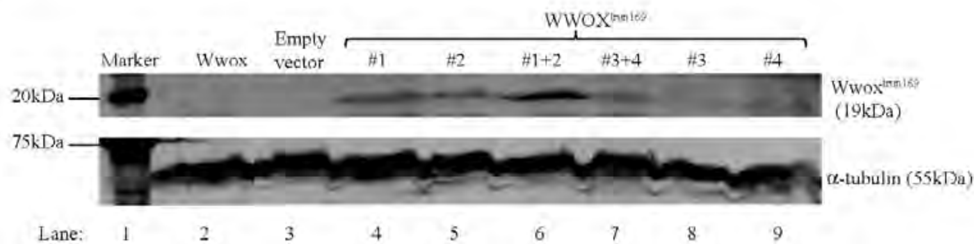

Figure 1. *Drosophila Wwox^{trun169}* construct encodes a *Drosophila* version of truncated Wwox that is similar to human WWOX isoform 2. (A) Human and *Drosophila* WWOX both have two WW domains (blue boxes) and a short-chain dehydrogenase reductase (SDR) enzyme (orange box) with predicted substrate determining regions (green boxes), while human WWOX isoform 2 and *Drosophila* Wwox^{trun169} both contain two WW domains and a truncated SDR. Each of them has a co-factor binding site (pink region) within the SDR sequence. *Drosophila* Wwox^{trun169} is comprised of 169 amino acids, while human WWOX isoform 2 has 189 amino acids. Human WWOX isoform 2 is encoded by five exons and an alternatively spliced exon encoding 17 amino acids at the C-Terminus (red line), which is not found in the full length human WWOX. **(B)** Alignment of *Drosophila* Wwox^{trun169} and human WWOX isoform 2 shows 55.7% similarity with identical amino acids denoted in black and amino acids of similar properties denoted in gray. **(C)** Western blot analysis of six independent lines expressing Wwox^{trun169} (19kDa) (lanes 4-9). Lane 1: Marker; Lane 2: *Drosophila* line ectopically expressing Wwox; Lane 3: *Drosophila* line ectopically expressing empty vector construct (negative control with only endogenous Wwox). The second blot displays the immuno-staining of housekeeping protein, α-tubulin, as a loading control.

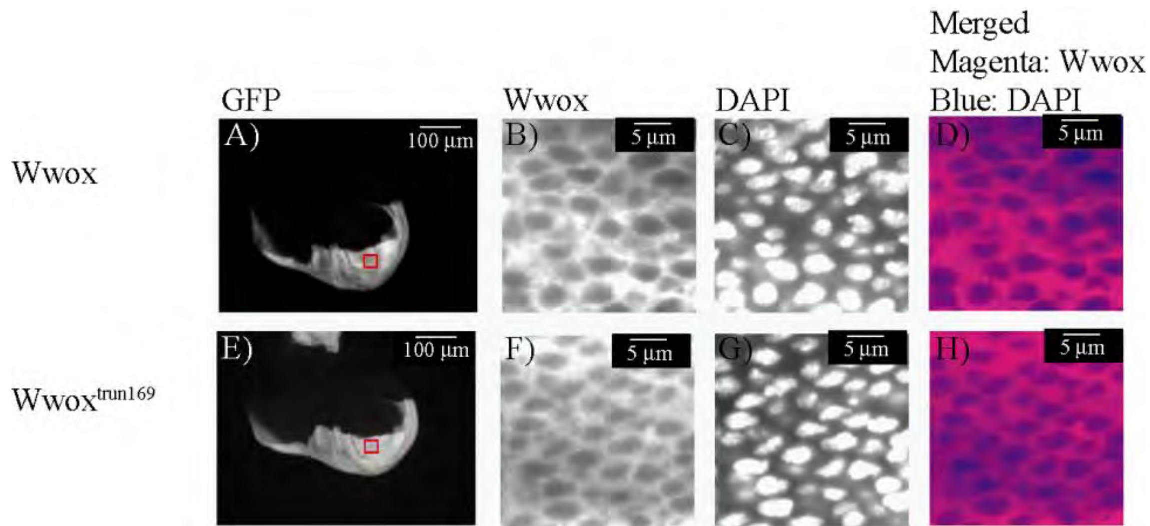

Figure 2. Wwox^{trun169} is localized in the cytoplasm of epithelial cells. Wwox or Wwox^{trun169} were co-expressed with GFP **(A, E)** under *UAS*-control in cells in the posterior compartment of third instar wing discs using the *hedgehog* promoter to drive *gal4* expression. The red boxes **(A, E)** indicate regions of the disc that are enlarged in **(B-D, F-H)**. Immunostaining of Wwox **(B)** and Wwox^{trun169} **(F)** using anti-N-terminal Wwox antibody shows that these proteins are both localized in the regions that are complementary to DAPI stained nuclei **(C, G)**. **(D, H)** Merged images (Wwox in magenta and DAPI in blue).

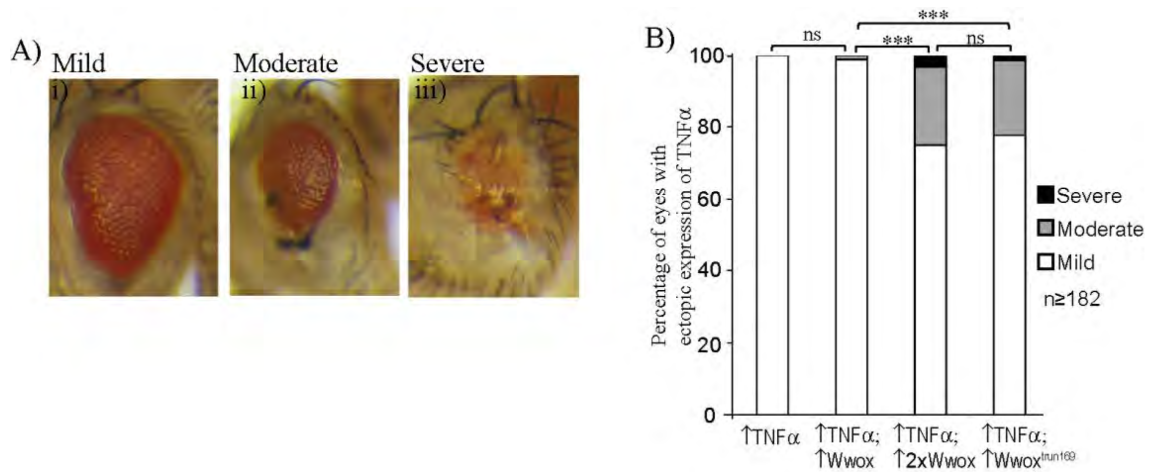

Figure 3. Ectopic expression of Wwox^{trun169} enhances Eiger/TNFα-mediated cell death. (A) Classification of the *eiger/TNFα* phenotypes. (i) Ectopic expression of Eiger/TNFα alone causes disruption to the normal regular arrangement of ommatidial units and a reduction of eye size that is classified as a "mild" phenotype. (ii-iii) Ectopic expression of Wwox or Wwox^{trun169} enhances *eiger/TNFα*-mediated cell death, and causes either (ii) further reduction of the eye size that is classified as a "moderate" phenotype, or (iii) completely loss of eye that is classified as a "severe" phenotype. **(B)** Classification of the *eiger/TNFα* phenotypes into three distinct categories allows for quantitation and shows that ectopic expression of either two copies of Wwox or a copy of Wwox^{trun169} transgene, along with *eiger/TNFα*, significantly increases the proportion of eyes showing moderate and severe *eiger/TNFα* phenotypes. Significance indicated by *** = $p<0.001$ and ns = not significant

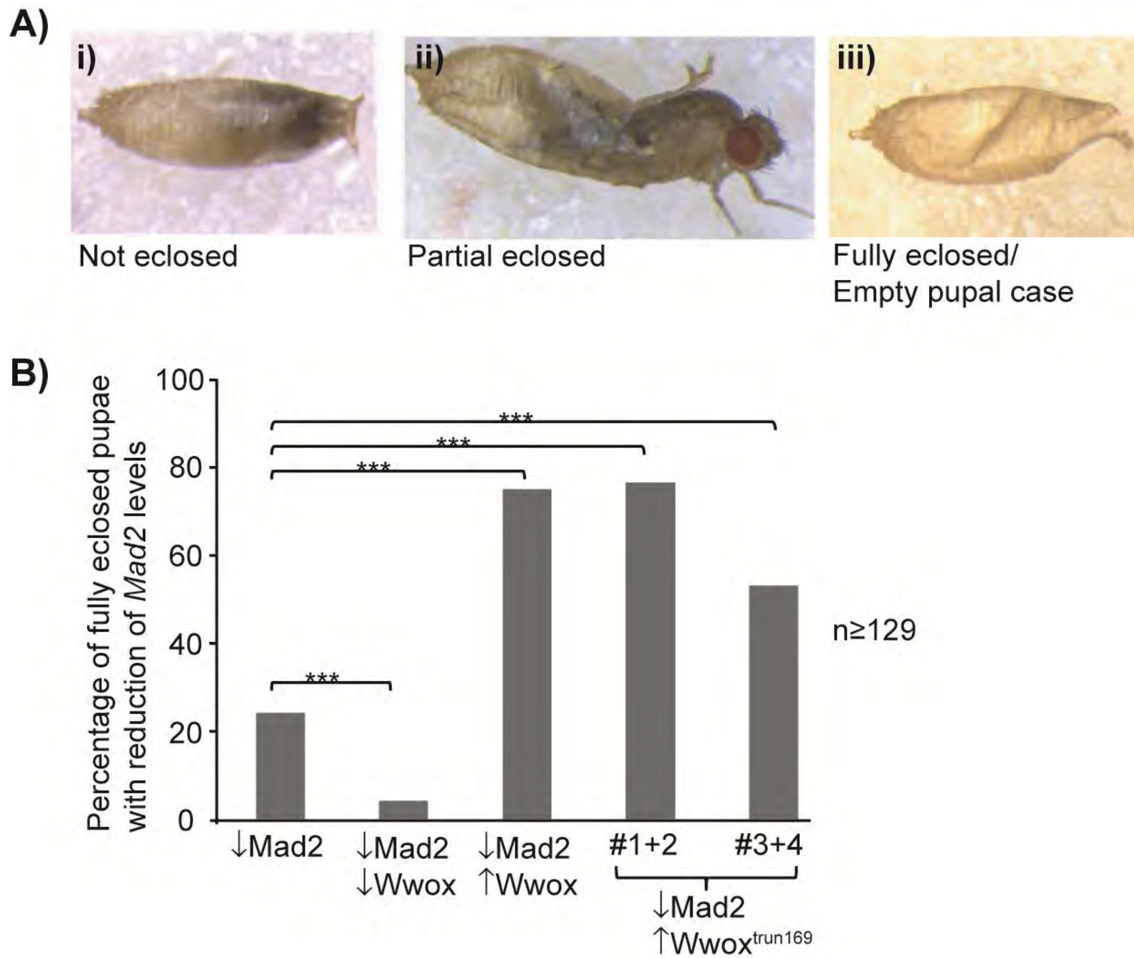

Figure 4. Ectopic expression of Wwox^{trun169} suppresses the spindle assembly checkpoint defects induced by reduction of *Mad2* levels. (A) Pupae with reduced *Mad2* levels are grouped into three classes based on the stages of their death or survival with respect to eclosion: (i) death before eclosion, (ii) death during (partial) eclosion or (iii) successful eclosed. **(B)** In *Drosophila* with reduced *Mad2* levels, co-reduction of *Wwox* levels leads to a decrease in the percentage of flies that are fully eclosed from their pupae, while ectopic over-expression of either Wwox or Wwox^{trun169} increases the percentage of fully eclosed flies. Significance indicated by *** = p<0.001.

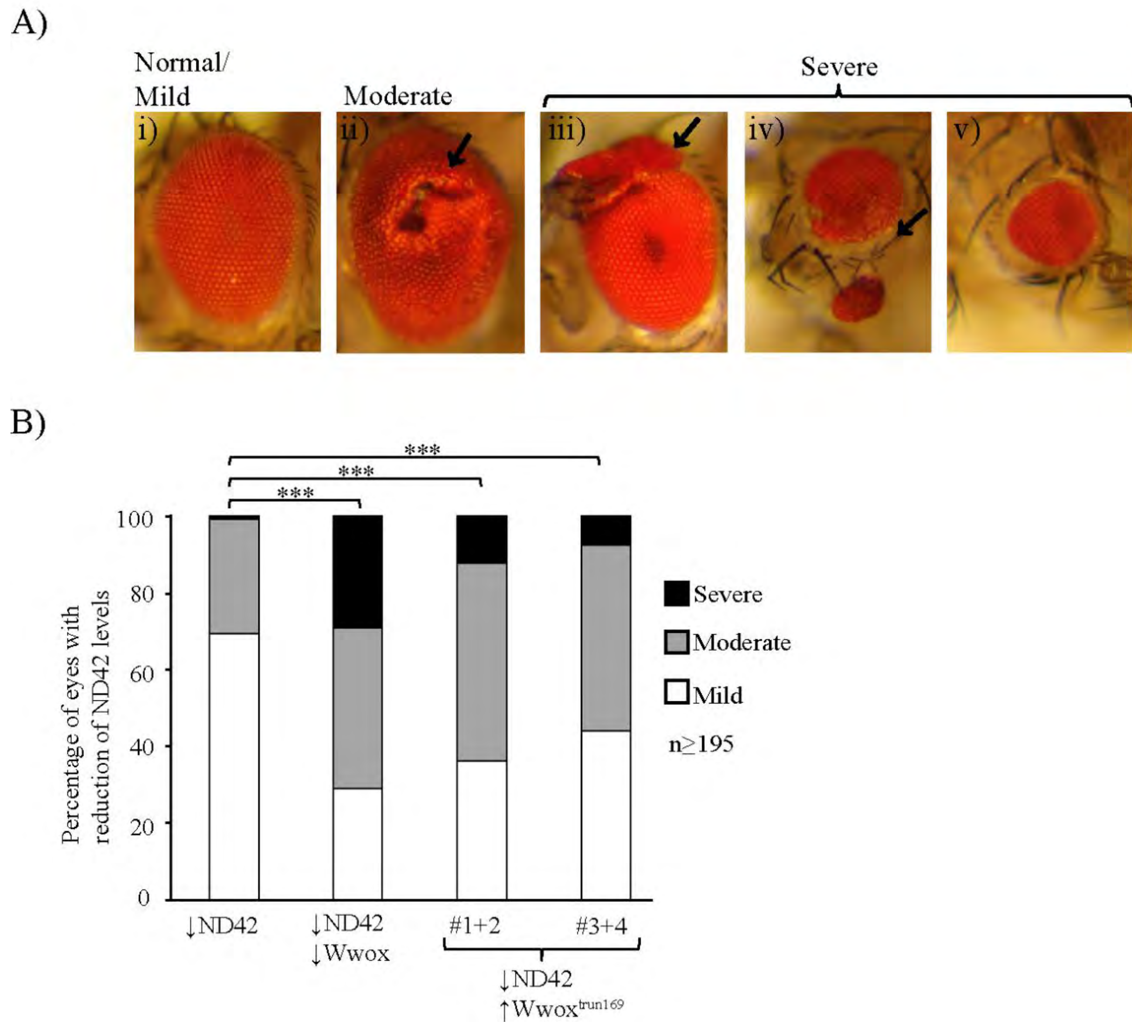

Figure 5. Ectopic expression of Wwox^{trun169} enhances the eye phenotype caused by reduced *ND42* levels. (A) Classification of adult eye phenotypes with mitochondrial defects. (i) Normal eyes or eyes with very mild disruptions to the arrangement of ommatidia are classed as "normal/mild"; (ii) eyes with obvious disruptions to the ommatidia patterning and loss of photoreceptor cells are classed as "moderate"; eyes with (iii) overgrowth of eye tissue, (iv) defects in differentiation and/or (v) small eyes are classed as "severe". **(B)** Either reduction of *Wwox* levels or ectopic expression of Wwox^{trun169} increases the severity of eye phenotypes caused by mitochondrial defects (induced by reduction of *ND42* levels). Significance indicated by *** = $p<0.001$, ** = $0.001<p<0.01$ and * = $0.01<p<0.05$.

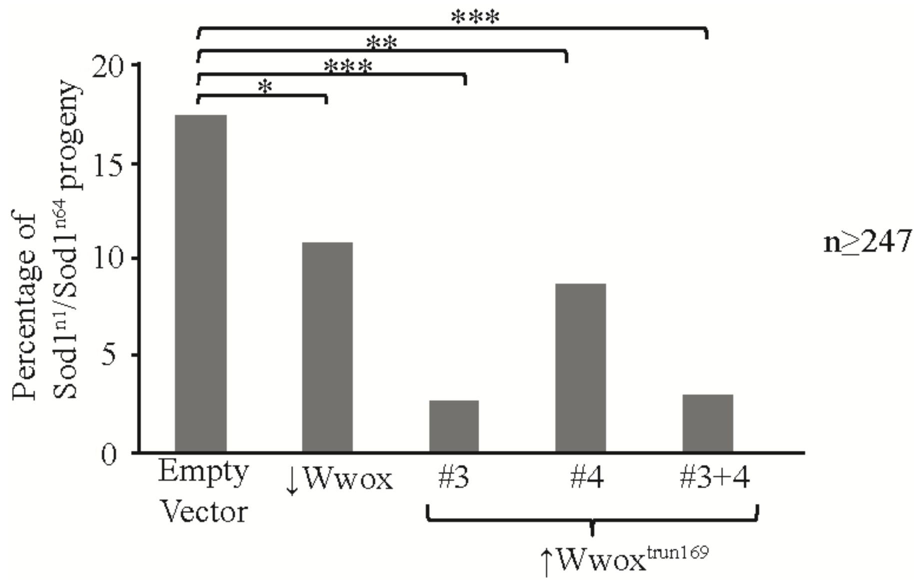

Figure 6. Ectopic expression of Wwox^{trun169} decreases the viability of *Sod1^{n1}/Sod1^{n64}* trans-heterozygous mutants. Either reduction of *Wwox* levels or ectopic expression of Wwox^{trun169} significantly decreases the viability of *Sod1^{n1}/Sod1^{n64}* trans-heterozygous mutants. Significance indicated by *** = $p<0.001$, ** = $0.001<p<0.01$ and * = $0.01<p<0.05$.

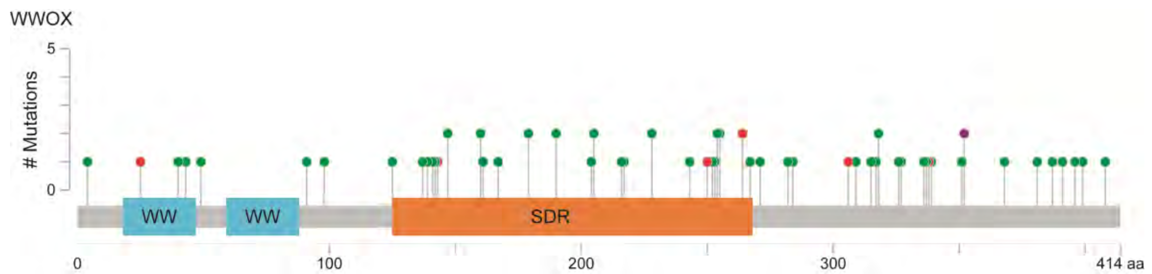

Figure 7. Somatic point mutations of human WWOX that have been detected in various cancers. Data retrieved from *cbioportal* showed that 53 unique mutations are associated with cancers. Y-axis is the frequency of the mutations that have been found at a single position. Green circles represent missense mutations, while red circles show mutations that cause truncated WWOX isoforms, such as nonsense mutations, mutations affecting splicing event and frameshift deletions or insertions. The purple circle indicates an amino acid that has different mutation types.

Supplementary Materials

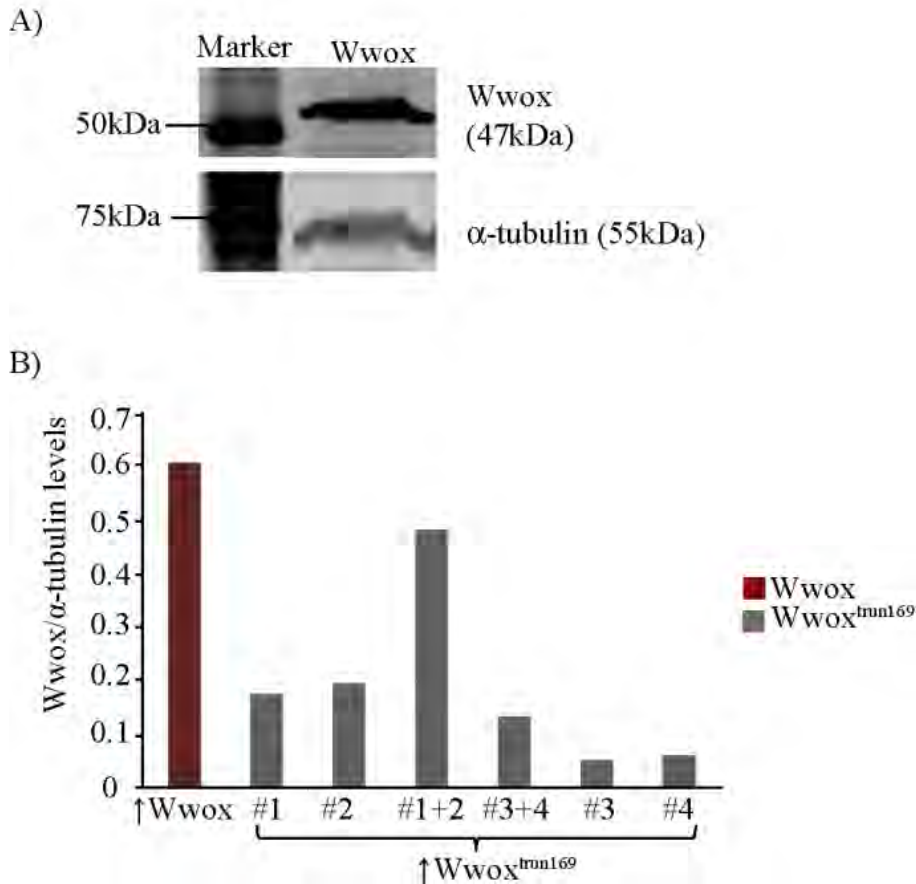

Figure S1. Comparison of ectopically expressed Wwox levels to ectopically expressed Wwox^{trun169} levels in different fly lines. (A) Western blot analysis shows the levels of full length Wwox (47kDa) in fly line ectopically expressing Wwox. The second blot shows the immuno-staining of housekeeping protein control, α-tubulin, as a loading control. **(B)** After being normalized to the α-tubulin housekeeping protein levels, ectopically expressed Wwox levels are higher than the levels of Wwox^{trun169} in all independent lines ectopically expressing Wwox^{trun169}.

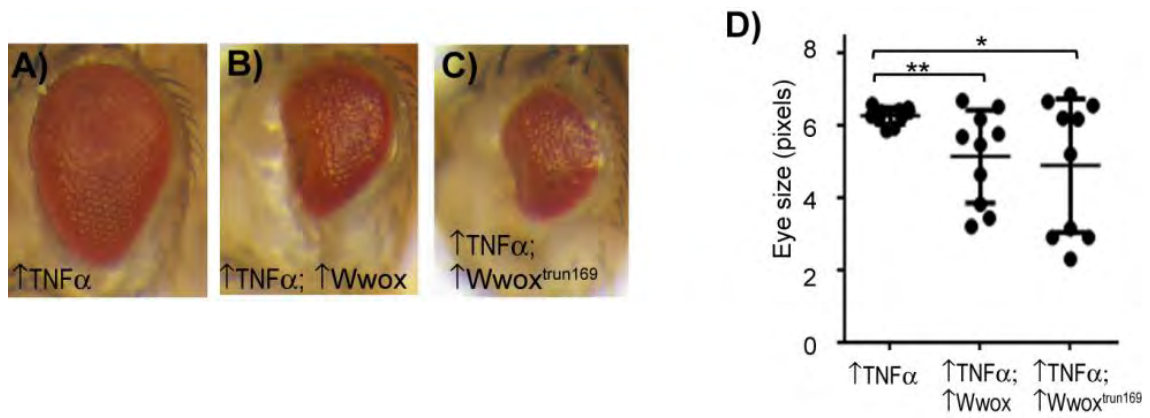

Figure S2. Ectopic expression of Wwox^{trun169} enhances Eiger/TNFα-mediated cell death as determined by eye size. **(A)** Ectopic expression of Eiger/TNFα results in the disarrangement of ommatidial units and a reduction in eye size. **(B-C)** Ectopic expression of either **(B)** Wwox or **(C)** Wwox^{trun169} enhances Eiger/TNFα-mediated cell death characterized by further reduction of eye size. **(D)** Quantification of the size of eyes ectopically expressing Eiger/TNFα with or without different Wwox constructs (Wwox or Wwox^{trun169}). Significance indicated by ** = $0.001<p<0.01$ and * = $0.01<p<0.05$.

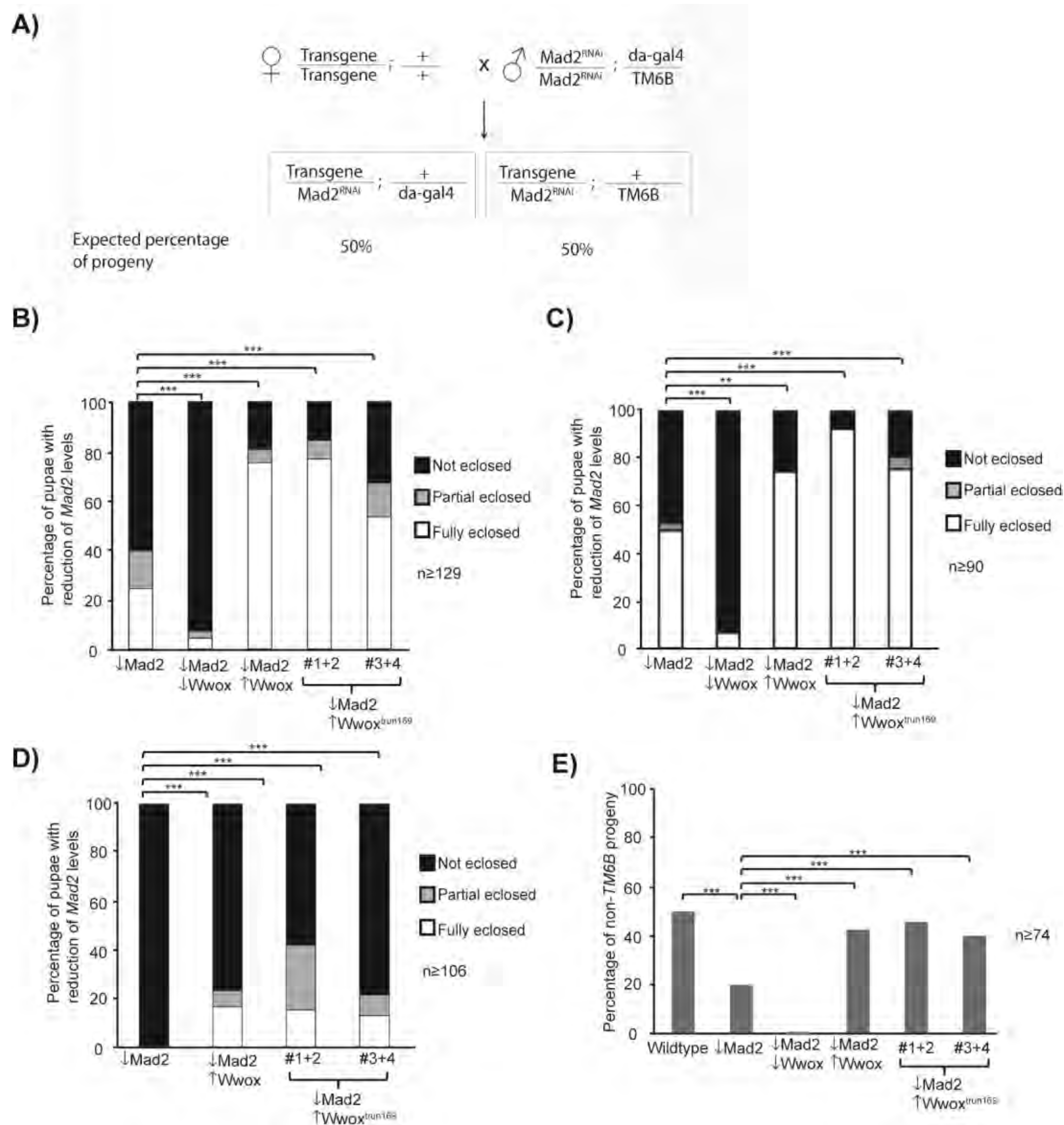

Figure S3. Ectopic expression of Wwox^{trun169} suppresses the spindle assembly checkpoint defects induced by reduction of *Mad2* levels. (A) Genetic crosses were utilized to test the effect of ectopically expressed Wwox^{trun169} on the survival of flies with *Mad2* reduction. ♀ symbol represents virgin female flies, while ♂ symbol represents male flies. "Transgene" represents different tested constructs. **(B)** Reduction of *Wwox* levels decreases the survival of pupae with spindle assembly checkpoint defects, while ectopic expression of either Wwox or Wwox^{trun169} increases their survival. **(C)** Repeat experiment shows a similar effect for altered *Wwox* levels and ectopically expressed Wwox^{trun169} on the survival of pupae with spindle assembly checkpoint defects. **(D)** At 29°C, all progeny with reduced *Mad2* levels die at the pupal stage (do not eclose). Ectopic expression of either Wwox or Wwox^{trun169} increases the survival of pupae with reduced *Mad2* levels. **(E)** Adult viability assay indicates that reduction of *Wwox* levels in flies with reduced *Mad2* levels causes complete lethality, while ectopic expression of either Wwox or Wwox^{trun169} in these flies increases their viability. Significance indicated by *** = $p<0.001$.

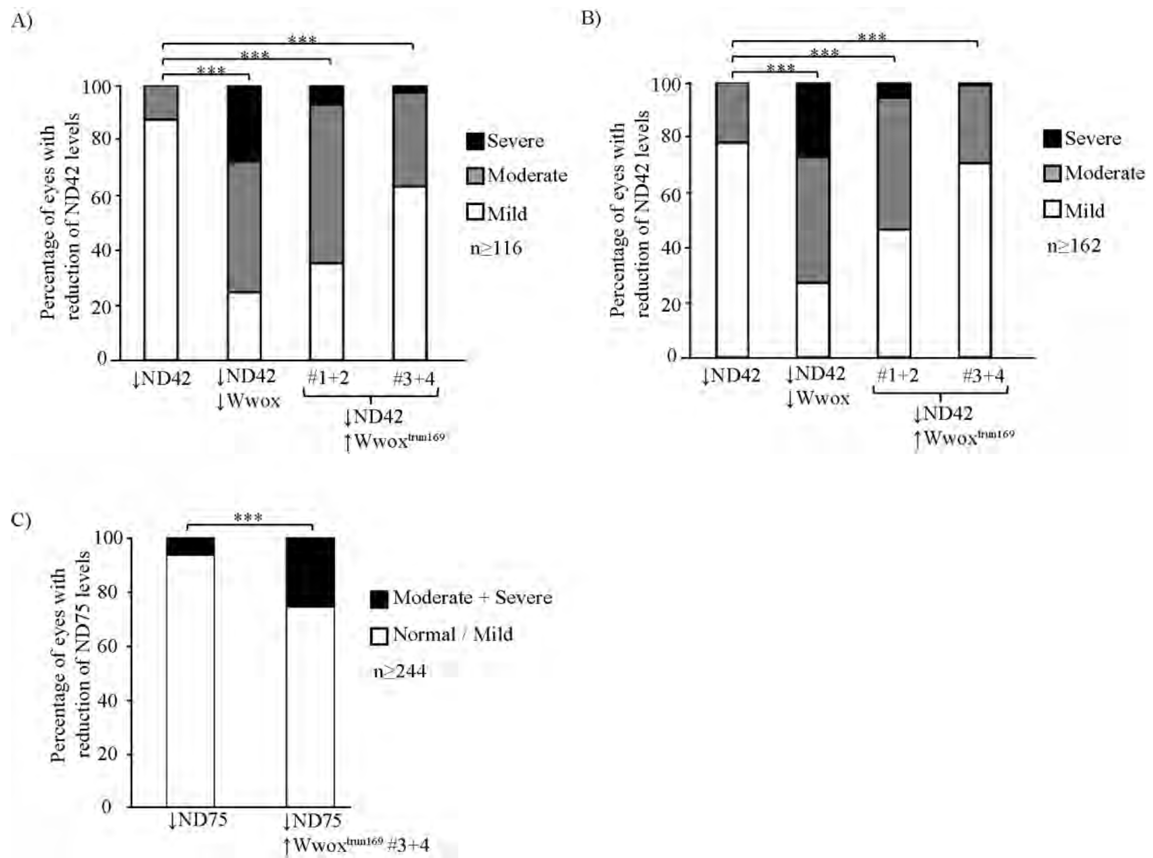

Figure S4. Ectopic expression of Wwox^{trun169} increases the severity of eye phenotypes caused by mitochondrial dysfunction (induced by reduction of either *ND42* or *ND75* levels). (A-B) Repeated experiments of Figure 5B show that either reduction of *Wwox* levels or ectopic expression of Wwox^{trun169} enhances eye phenotypes caused by reduction of *ND42* levels. These results are consistent with that shown in Figure 5B. **(C)** Ectopic expression of Wwox^{trun169} increases the severity of eye phenotypes induced by reduction of *ND75* levels. Significance indicated by *** = p<0.001.

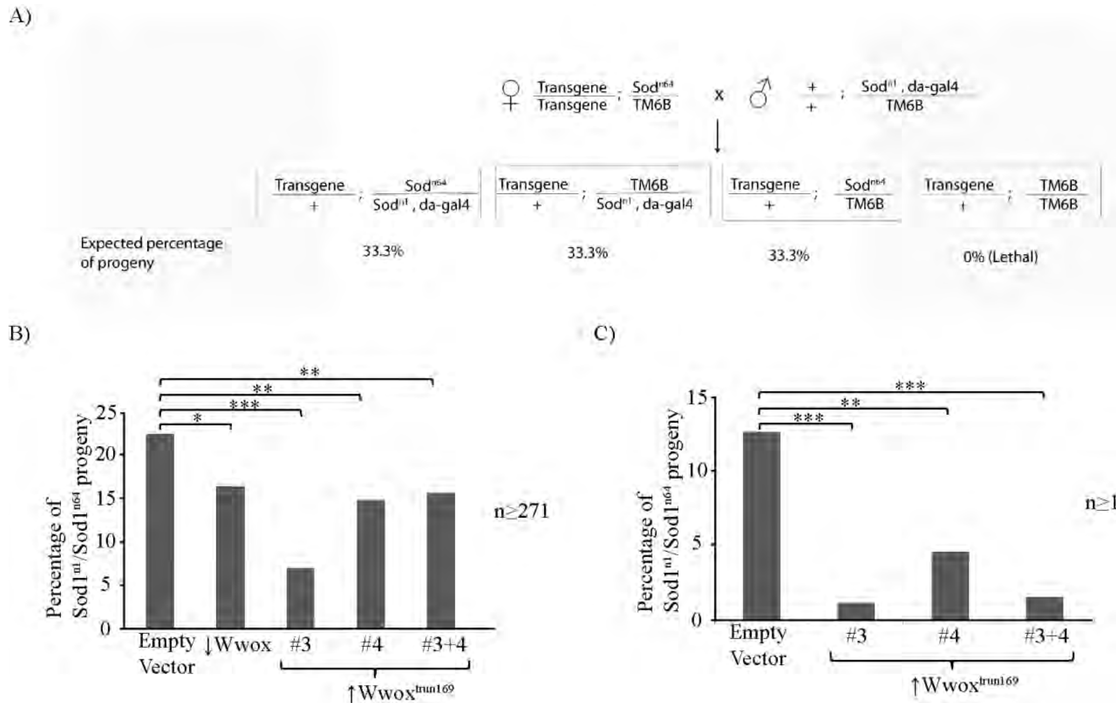

Figure S5. Ectopic expression of Wwox^{trun169} decreases the survival of *Sod1^{n1}*/*Sod1^{n64}* trans-heterozygous mutants. (A) Genetic crosses were utilized to test the effect of ectopically expressed Wwox^{trun169} on the survival of trans-heterozygous *Sod1^{n1}/Sod1^{n64}* flies. ♀ symbol represents virgin female flies, while ♂ symbol represents male flies. “Transgene” represented different constructs that were tested. **(B-C)** Repeated survival assays show similar results to Figure 6. Either reduction of *Wwox* levels or ectopic expression of Wwox^{trun169} significantly decreases the survival of *Sod1^{n1}*/*Sod1^{n64}* trans-heterozygous mutants. Significance indicated by *** = $p<0.001$, ** = $0.001<p<0.01$, * = $0.01<p<0.05$.

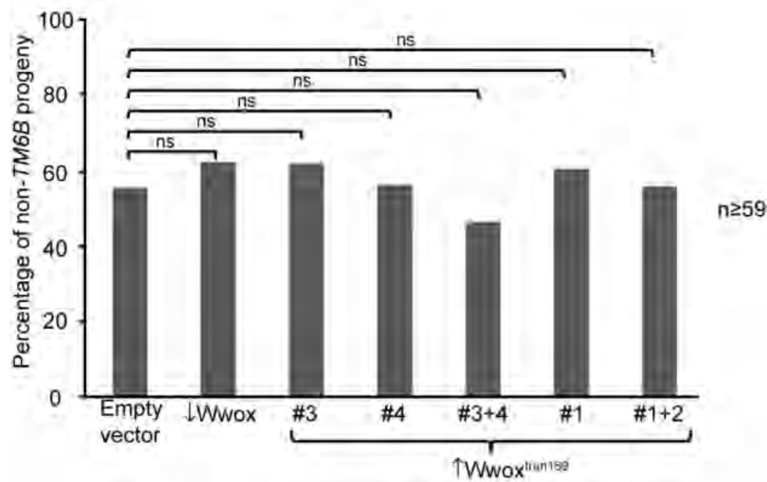

Figure S6. Either reduction of *Wwox* levels or ectopic expression of Wwox^{trun169} alone does not significantly affect the survival of flies. Different *UAS*-constructs (*empty vector*, *Wwox^{RNAi}* and *Wwox^{trun169}*) were ubiquitously expressed using the *daughterless* promoter to drive *gal4* expression. Empty vector is the negative control in this experiment. Chi-square tests show that reduction of *Wwox* levels, as well as ectopic expression of Wwox^{trun169} in all five lines does not significantly alter the survival of flies. Significance indicated by ns = not significant.

Chapter 4

Identification of pathways in which Wwox is involved

This chapter has been written up as a manuscript for publication:

<u>Lee, C.S.</u>, O'Keefe, L.V., Choo, A. and Richards, R.I., 2016. Regulation of mitochondrial dynamics by WWOX: linking cell metabolism and cell death (to be submitted for publication).

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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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<tr><td>Signature</td><td></td><td>Date</td><td>11th August, 2016</td></tr>
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Regulation of mitochondrial dynamics by WWOX: linking cell metabolism and cell death

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ABSTRACT

The *WW domain-containing oxidoreductase* (*WWOX*) gene spans *FRA16D* common chromosomal fragile site, a region that is susceptible to breakage and DNA instability in cancers. Altered WWOX function is associated with tumorigenesis, and can also contribute to distinct metabolic and neurological diseases. However, the consequence of WWOX mutations in each of these pathologies has not been defined. In this study, *Drosophila melanogaster* is used as a model to screen for *in vivo* pathways that are of functional significance for Wwox. We show that altered Wwox can modify functions of pre-mRNA processing factor 19 (Prp19), Yorkie (Yki) and epidermal growth factor receptor (EGFR). Wwox contributes to cell death induced by the reduction of either *Prp19* or *EGFR* levels through the regulation of caspase activity. Further investigation of the mechanism(s) by which Wwox promotes caspase-dependent cell death reveals that Wwox contributes to mitochondrial fission. These results indicate an *in vivo* mechanism by which Wwox links alterations to metabolism and caspase-dependent cell death. This could underlie its ability to act as a suppressor of tumor growth *in vivo*.

INTRODUCTION

Common fragile sites (CFS) are loci on chromosomes that are susceptible to breakage upon replicative stress [1]. They also can be induced by exposure to substances that are commonly found in the diet, including caffeine (Yunis and Soreng 1984) and ethanol (Kuwano and Kajii 1987), as well as in the environment, such as cigarette smoke (Sozzi *et al.* 1997; Stein *et al.* 2002). Currently, more than 200 CFS have been mapped in human lymphocytes [2]. CFS have also been observed in other mammals, such as dog [3], cat [4], pig [5], mouse and rat [6], and, at least on occasion, are located within orthologous genes. This demonstrates the evolutionary conservation of CFS and is suggestive of some biological advantage. Whilst the nature of this advantage is unclear, some genes spanning CFS have been reported to act as tumor suppressors. These include *WWOX*, which spans *FRA16D* [7-9], and *fragile histidine triad* (*FHIT*) which spans *FRA3B* [10, 11]. Reduction or absence of either *WWOX* or *FHIT* expression has been detected in various cancers, such as breast [12], prostate [13, 14], gastric [15, 16], liver [17, 18] and lung [19, 20]. Restoration of either *WWOX* or *FHIT* in tumors can induce cell death and act as a suppressor of tumor growth [19, 21-24].

WWOX protein contains two N-terminal WW domains and a C-terminal short-chain dehydrogenase-reductase (SDR) domain [9, 25]. Although many studies have demonstrated tumor suppressor functions for WWOX [14, 19, 21, 22, 26, 27], the molecular and cellular mechanisms are still not fully understood. In particular, there has been no specific enzymatic function assigned to the SDR region of WWOX, leaving its substrate(s) and product(s) yet to be determined. The WW domains that distinguish WWOX from other SDR proteins have been the focus of previous efforts to determine WWOX function. As WW domains typically bind to other proteins, the identification of potential binding partners has been pursued in an effort to define

the pathways and processes in which WWOX participates. Numerous binding partners have been identified *in vitro* and they are involved in a wide range of biological pathways, including RNA metabolism, signal transduction and chromatin remodeling [28-32]. However, it is unknown which of these binding partners and/or cellular pathways are affected in the diseases caused by low *WWOX* levels. One of the strategies to determine biologically and/or pathologically significant interactions is to characterize the evolutionarily conserved functions of WWOX.

A *Drosophila melanogaster* model has been established to investigate the *in vivo* functions of the conserved *Wwox* gene product. Although *Wwox* mutant flies do not show any obvious phenotype, proteomic analyses have revealed that the levels of many enzymes involved in the tricarboxylic acid (TCA) cycle are altered [33]. In addition, screening experiments were carried out previously to identify cellular abnormalities that could be modified by altered *Wwox* levels. These experiments focused on candidates involved in the TCA cycle and the oxidative stress response. It was found that Wwox was able to modify cellular dysfunction phenotypes caused by genetic alteration of components that comprise the mitochondrial electron transport chain [34]. This is consistent with Wwox being both a regulator of, and regulated by, cellular metabolism [35]. Metabolic roles for WWOX are evolutionarily conserved in mice [36, 37] and rats [38], as well as in humans [35]. In addition, genetic variation in *WWOX* has been associated with many human disorders of metabolism [39-45].

Previous studies have identified many *in vitro* binding partners of WWOX [28-32, 46], as well as proteins whose expression is regulated by WWOX [33]. However, the biological significance of these associations is unclear. Herein, a genetic screen was undertaken *in vivo* to identify candidates whose functional interaction with Wwox is biologically significant. Three candidates were shown to have clear functional interactions with Wwox; they are Prp19, Yki and

EGFR. Further investigation showed that Wwox modulated either *Prp19* knockdown or *EGFR* knockdown phenotypes through the regulation of caspase-dependent cell death. Caspase-dependent cell death was found to be less effective in cells with mitochondrial defects, suggesting that mitochondrial function is important for this process.

Mitochondria are important components in the regulation of both metabolism and cell death. They can change their morphology either by fusion or fission, in response to different environmental stresses and metabolic states [47-52]. When cells rely on oxidative phosphorylation to generate energy, their mitochondria appear fused [53, 54]. In contrast, increased mitochondrial fission can shift metabolism toward glycolysis [55]. Numerous studies have also shown that mitochondrial fission can induce caspase activation [47, 48, 56], suggesting that mitochondrial dynamics also contribute to apoptosis in *Drosophila*. Having previously identified that Wwox can regulate the essential components of oxidative phosphorylation [33, 34] and caspase-dependent cell death [57], we hypothesize that Wwox impacts on metabolism and caspase-dependent cell death through the regulation of mitochondrial dynamics. We therefore investigated the role of Wwox in cells with defects in mitochondrial dynamics. Ectopic expression of Wwox was found to contribute to the dynamin related protein 1 (Drp1)-mediated fission, as well as trigger death in flies with defects in mitochondrial fusion. This provides the first evidence that WWOX mediates mitochondrial fission as a mechanism to integrate cellular metabolism with caspase-dependent cell death.

RESULTS

Cellular abnormalities modified by altered *Wwox* levels

In order to identify cellular abnormalities that can be modified by Wwox, a genetic screen was carried out. As *Wwox* mutant flies do not show any obvious visible phenotype, the expression of each candidate gene was either increased or decreased experimentally to search for a quantifiable phenotype [33, 34, 57]. *Wwox* levels were then altered to test whether *Wwox* could modify the observed phenotype. Genetic modification was targeted to the *Drosophila* eye, as these transgenic flies are usually viable and the resultant phenotypes are readily quantified.

Candidate genes (n=116) for this experiment were selected based on various criteria, including previous reports of their physical interaction with WWOX in biochemical [28, 30, 32, 46, 58] or yeast two-hybrid interaction analyses (unpublished data), as well as proteomic and microarray analyses where their expression was regulated by Wwox in *Drosophila* [33]. These candidates cover a wide range of biological functions, including metabolism, immune response, transcription, cell cycle, cell death and survival (Table S1). Approximately 20% of candidate genes resulted either in an eye phenotype or lethality when their expression was altered; and three of these candidates (Prp19, Yki and EGFR) showed a significant functional interaction with Wwox.

Wwox reduces the impact of low Prp19 activity by mediating programmed cell death

Prp19 has been shown to be involved in a wide range of biological pathways, such as pre-mRNA splicing [59-61], DNA damage repair [62, 63], the regulation of cell death [64-66], cell cycle control [65, 67] and proteolysis [68]. Microarray analyses previously revealed that *Prp19* transcript levels are inversely correlated with *Wwox* levels in flies [33]. Reduction of *Prp19*

levels specifically in the *Drosophila* eye (Figure S1A) resulted in adult eyes with loss of photoreceptor cells (Figure 1A ii-iii), as well as defects in differentiation (Figure 1A iv), indicating that reduced *Prp19* levels alone can affect normal eye development. A small percentage of eyes scored showed a "severe" phenotype when *Prp19* levels were reduced (Figure 1B). Reduction of *Wwox* levels in this *Prp19* deficient background significantly increased the percentage of eyes showing the "severe" phenotype (Figure 1B), demonstrating a functional relationship between Wwox and Prp19. However, the rate-limiting role for WWOX was only evident for its reduction as there was no suppression of the *Prp19* knockdown phenotype by ectopic over-expression of Wwox (Figure 1B).

The functional relationship between Wwox and Prp19 was further investigated in a cellular context using third instar larval wing discs. When GFP was co-expressed to label the region with reduced *Prp19* levels, an obvious disruption to the organization of the disc was observed (Figure 2A v). Wing discs with reduced *Prp19* levels showed an alteration to the normal pattern of expression of the Wingless (Wg) morphogen (Figure 2A vi) [69], suggesting that Prp19 is required for the proper development of wing discs. In addition, high levels of cell death (detected by Caspase 3 levels (Figure 2A vii)) and DNA double strand breaks (detected by γ-H2AX expression (Figure S2A v)) were also detected. Reduction of *Wwox* levels significantly decreased detectable Caspase 3 in the wing pouch (Figure 2B). This is consistent with previous findings that Wwox is a rate limiting factor for the activation of caspase cascades [57]. There was also a trend towards an increase in the intensity of Caspase 3 staining when Wwox was over-expressed, although this increase was not found to be significant (Figure 2B). In contrast, there was no obvious modification to the levels of γ-H2AX staining when *Wwox* levels were altered in

the wing discs with *Prp19* deficiency (Figure S2B), indicating that the contribution of Wwox to Caspase 3 activation is not occurring via DNA damage.

Ectopic expression of Eiger/TNFα suppresses the impact of reduced Prp19 activity

Although a reduction of Wwox resulted in decreased levels of cell death induced by *Prp19* deficiency in wing discs cells, it showed an enhancement of the *Prp19* adult eye phenotype that is characterized by loss of photoreceptor cells. Thus, we hypothesize that blocking cell death in *Prp19* deficient cells during developmental stages could enhance adult phenotypes at the final stages of differentiation. Eiger/TNFα is an extrinsic effector of programmed cell death [70], which is able to induce cell death in wing discs when ectopically expressed [57]. Decreased levels of *eiger/TNFα* resulted in the significant reduction of Caspase 3 activity in *Prp19* deficient wing discs (Figure 3A), demonstrating the involvement of Eiger/TNFα-mediated cell death in cellular abnormalities induced by *Prp19* knockdown. While decreasing *eiger/TNFα* levels in wing discs with *Prp19* deficiency had no effect on adult viability, ectopic expression of Eiger/TNFα significantly increased the number of flies that were fully eclosed (Figure 3B). This is especially notable, given that ectopic expression of Eiger/TNFα alone impaired the viability of wild-type flies (Figure 3B), and suggests that the promotion of cell death in *Prp19* deficient wing discs can improve the survival of flies to adulthood. The impact of altering cell death in the Prp19 deficient eye also was assessed. Consistently, ectopic expression of Eiger/TNFα also suppressed *Prp19* eye defects, while an increase in the percentage of eyes classed as “severe” was observed when *eiger/TNFα* expression was reduced (Figure 3C). A potent inhibitor of apoptosis, the baculovirus protein p35, was also expressed in the *Prp19* deficient background to provide further evidence that cell death is required to suppress the *Prp19* eye phenotype. Ectopic expression of

the caspase inhibitor p35 strongly enhanced *Prp19* eye phenotype (Figure 3D), confirming that inhibition of apoptosis enhances the cellular damage induced by *Prp19* deficiency.

Wwox can suppress the lethality caused by ectopic expression of Yki

Yki is a downstream effector of the Hippo pathway, which plays a role in controlling cell proliferation [71]. Ectopic expression of Yki (using the *ey-gal4* promoter) decreased the survival of adult flies (Figure 4A). Reduction of *Wwox* levels further reduced the survival of these flies, while ectopic expression of *Wwox* resulted in an increase in their survival (Figure 4A), indicating that *Wwox* can suppress the lethality induced by Yki over-expression.

Pupal cases were dissected and it was found that the majority of dead pupae did not have any eyes, and some even showed a “headless” phenotype (Figure 4B). Proper development of the adult head structures requires three types of imaginal discs; eye, labial, and clypeo-labral [72]. Severe disruption in any of these imaginal discs can affect head development. The “headless” phenotype has also been observed previously by Jiao *et al* (2001) following ectopic expression of different transcription factors using the *ey-gal4* promoter [73]. This suggests that reduction of the survival of flies with ectopic expression of Yki is due to defects in head development. Following ectopic expression of Yki, ~47% of progeny did not develop any eyes (Figure 4C), and this percentage was significantly increased by reducing *Wwox* levels (Figure 4C), which was consistent with the survival assays (Figure 4A). This further confirms that Wwox can limit the harm caused by ectopic expression of Yki.

Wwox can contribute to cell death induced by *EGFR* deficiency

EGFR signaling has been shown to activate Yki and trigger positive auto-regulation by inducing transcription of EGFR ligands in *Drosophila* [74-76]. Since WWOX can suppress EGFR activity in human cell lines [46], we determined whether reduction of *EGFR* levels is a possible mechanism by which Wwox rescues the survival of flies with ectopic expression of Yki. 18% of pupae with ectopic expression of Yki successfully underwent eclosion, while 90% of flies with reduced *EGFR* levels alone successfully emerged from pupal cases (Figure 4D). Decreased *EGFR* levels in flies with ectopic expression of Yki increased their eclosion rate (to 38%) (Figure 4D), suggesting that reduced EGFR activity can suppress cellular dysfunction caused by ectopic expression of Yki. In order to determine whether Wwox is also involved in the pathways activated by reduced *EGFR* levels, the interactions between EGFR and Wwox were investigated.

Reduction of EGFR levels alone in the *Drosophila* eye reduced the overall size of eye and caused a rough eye surface (Figure 5A ii and 5B), consistent with previous findings showing that reduced EGFR signaling can cause cell death [77, 78]. Decreased *Wwox* levels suppressed the *EGFR* knockdown eye phenotype, as determined by the significant increase in eye size (Figure 5A iii and 5B). In contrast, ectopic expression of Wwox further reduced eye size (Figure 5A iv and 5C).

To investigate the mode of cell death, the caspase inhibitor p35 was ectopically expressed in the eyes with reduced *EGFR* levels. Ectopic expression of caspase inhibitor p35 significantly increased the *EGFR* knockdown eye size (Figure 6A v and 6B), indicating that cell death in *EGFR* deficient cells is caspase-dependent. As Wwox can contribute to Eiger/TNFα-mediated cell death, we further determined whether caspases mediate cell death following *EGFR*

knockdown. Reduced *eiger/TNFα* levels significantly increased *EGFR* knockdown eye size (Figure 6A vi and 6B), indicating that Eiger/TNFα contributes to this form of cell death. This also demonstrates the crosstalk between EGFR signaling and Eiger/TNFα-mediated cell death.

Wwox can contribute to the regulation of mitochondrial dynamics

Wwox can modify phenotypes induced by altering levels of genes involved in regulating cell death, including *Prp19*, *Yki* and *EGFR*. Eiger/TNFα-mediated cell death is activated in the cellular dysfunction induced by reducing levels of either *Prp19* or *EGFR*. Although it is clear that Wwox can contribute to Eiger/TNFα-mediated cell death through promoting caspase activation [57], the details of this mechanism are still unclear. Interestingly, less cell death occurred in metabolically compromised cells (Figure S3), suggesting that mitochondrial functions are important for Wwox-mediated cell death. We hypothesize that Wwox contributes to caspase-dependent cell death through mitochondrial fission, as this process is involved in caspase activation [47, 48, 56]. Thus, the role of Wwox in the regulation of mitochondrial dynamics was determined.

Similar to wild type control cells (Figure 7A ii), mitochondria in cells with altered *Wwox* levels alone showed a tubular (fused) structure (Figure 7A iii-iv), suggesting that they do not reach any threshold for disruption of mitochondrial morphology. The effect of altered *Wwox* levels was also determined in cells with fragmented mitochondria induced by ectopic expression of Drp1. Drp1 is a member of the dynamin superfamily, which mediates mitochondrial fission by GTP hydrolysis [79-81]. When Drp1 was ectopically over-expressed in wing discs, all cells in the scored region contained mitochondria with the same appearance; they were either all tubular (Figure 7B i-i'), a mixture of tubular and round (Figure 7B ii-ii') or all fragmented (Figure 7B iii-

iii'). This was consistent for 85% of analyzed wing discs, while the remaining 15% of analyzed wing discs have a mixture of cells with different mitochondrial morphology (not all cells have the same mitochondrial morphology) (Table S2). This may because the variety of GAL4 expression between different cells in these wing discs. As mitochondrial fission is important for the induction of cell death [47, 48, 56], we compared the percentage of wing discs, in which all cells in the scored region showed fragmented mitochondria, between different genotypes. 49% of wing discs with ectopic expression of Drp1 showed fragmented mitochondria (all cells in the scored region) (Figure 7C and Table S2), confirming that increased *Drp1* levels can induce mitochondrial fission. Whilst decreased *Wwox* levels were not able to suppress the mitochondrial fission induced by Drp1, ectopic over-expression of both Wwox and Drp1 significantly increased the percentage of wing discs with fragmented mitochondria in the scored region (Figure 7C), suggesting that Wwox can contribute to Drp1-mediated mitochondrial fission.

Surprisingly, ubiquitous expression of Drp1 had no effect on the survival of flies, and there was no change when *Wwox* levels were altered in this background (Figure 7D). Thus, an alternative approach was used to induce increased levels of mitochondrial fission by reducing levels of proteins required for mitochondrial fusion. Mitochondrial assembly regulatory factor (Marf) and Optic atrophy 1 (Opa1) are essential proteins for mitochondrial outer and inner membrane fusion, respectively [82]. Reduction of *Marf* levels ubiquitously also had no effect on survival, and there was no modification when *Wwox* levels were reduced (Figure 7E). In contrast, ectopic expression of Wwox in these flies resulted in lethality (Figure 7E), suggesting that the enhancement of mitochondrial fission by increased *Wwox* levels in these flies reached a threshold to cause death. Reduction of *Opa1* levels alone was, however, sufficient to decrease survival, and this was suppressed by reducing *Wwox* levels (Figure 7F). Ectopic expression of Wwox was not

able to further decrease the survival of flies with decreased *Opa1* levels (Figure 7F), demonstrating that other mitochondrial fusion proteins, such as Marf, can prevent further enhancement of mitochondrial fission. Together, the results herein indicate a novel function for Wwox in mediating mitochondrial fission.

DISCUSSION

WWOX was first identified for its chromosomal location in relation to DNA instability in cancer [25], and as the gene that spans the *FRA16D* CFS [9]. Subsequently, it was found that WWOX has the ability to suppress tumor growth [21], and that lower levels of *WWOX* are associated with cancers [7, 9, 14, 15, 17, 19, 25, 83-86], as well as different metabolic diseases [39, 41, 43, 87-89] and neurological disorders [90, 91]. Although many WWOX binding partners have been identified [28], the molecular mechanisms for the diseases caused by reduced *WWOX* levels are not fully understood. Herein, *Drosophila* was utilized as an *in vivo* model to screen for candidate proteins identified by various associations with Wwox for their ability to participate with Wwox in common pathways *in vivo*. Using this experimental approach, Prp19, Yki and EGFR were found to participate in common pathways with Wwox *in vivo*.

Reduction of *Prp19* levels, a novel interactor of Wwox, induced DNA damage and led to cell death in *Drosophila*, suggesting that the functions of Prp19 in the regulations of DNA damage repair and cell death are conserved in *Drosophila*. The cell death induced by reduced *Prp19* levels involved Eiger/TNFα secretion and caspase activation. Reduction of *Wwox* levels decreased caspase-dependent cell death induced by *Prp19* deficiency, but resulted in the enhancement of *Prp19* eye phenotype. Ectopic expression of Eiger/TNFα in *Prp19* deficient cells during developmental stages resulted in the improvement of adult phenotypes, suggesting that the

induction of cell death is required to eliminate *Prp19* deficient cells. This is consistent with a model in which WWOX could function to drive the elimination of cells with DNA instability. DNA damage is a common feature in most cancers, and has been detected in early human lesions, suggesting that it is a cause for tumorigenesis [92]. Activation of apoptosis is important for the elimination of DNA damaged cells, which prevents the occurrence of cancer [92, 93]. As reduced *WWOX* expression allows the survival of DNA damaged cells, it increases the risk of these cells progressing to malignancy, indicating a mechanism by which *WWOX* deficiency contributes to tumorigenesis. It has also been shown that down-regulation of *FHIT* CFS gene prevents cells from DNA damaged-induced killing [94], suggesting that both *WWOX* and *FHIT* CFS genes share a similar function.

The role of Wwox in cell death was further confirmed by its functional interactions with positive regulators of anti-apoptotic genes, Yki and EGFR. Wwox not only suppressed the lethality induced by ectopic expression of Yki, but also enhanced cell death induced by decreased *EGFR* levels. In contrast to *Drosophila*, humans have four EGFR family members, including EGFR (also known as ErbB1), ErbB2, ErbB3 and ErbB4. Functional interactions between WWOX, YAP (human homolog for Yki) and ErbB4 have previously been shown in mammalian cells [46]. YAP can bind with the ErbB4 cytoplasmic fragment and promote its translocation to the nucleus, as well as activate its transcriptional activity. Translocation of the ErbB4 fragment to the nucleus has been correlated with poor prognosis in cancer cells [95]. WWOX can compete with YAP for binding the ErbB4 fragment and inhibit ErbB4 activity by sequestering it in cytoplasm [46]. Although the role of WWOX in the regulation of ErbB1 activity in mammalian cell remains to be determined, the identification of Yki and EGFR as WWOX functional

interactors in *Drosophila* suggests that the contribution of WWOX in the regulation of both EGFR and Hippo pathways, at least, are conserved throughout evolution.

The role of WWOX in apoptosis, or more specifically in caspase activation, is also conserved in mammalian cell lines [22, 96-98], suggesting that the role of Wwox in this pathway is biologically significant. While there is lower caspase activation when WWOX is reduced, it is possible that other caspase-independent cell death pathways, such as necrosis, become activated. This is supported by the finding that ectopic expression of TNFα can induce both apoptosis and necrosis in murine L929 fibrosarcoma cells, where inhibition of caspase activity results in the increase of necrosis [99]. Upon necrosis, damage-associated molecular patterns (DAMPs) are released, leading to inflammation and the induction of cell death in neighboring cells [100]. Thus, WWOX may have an indirect role in the suppression of necrosis, and limit cell death to damaged cells through caspase activation. We have also shown previously that Wwox has conserved functions in the regulation of metabolism [33-35]. Together, studies in *Drosophila* reveal that Wwox is an integral component in the regulation of caspase-dependent cell death and metabolism.

Mitochondria are dynamic organelles that continuously fuse and divide to control and respond to metabolism [53, 54]. Not only are they involved in the regulation of metabolism, but they also participate in cell death. It has been shown that mitochondrial fission can contribute to the activation of caspase signaling in *Drosophila* [47, 48, 56]. This function is independent of the release of cytochrome c, which is a well-known cell death effector [47]. As mitochondrial fission/fusion can regulate these important pathways, we hypothesize that Wwox exhibits pro-apoptotic function during metabolic stress by inducing mitochondrial fission. Ectopic expression of Wwox enhanced Drp1-mediated mitochondrial fission and was lethal to flies with defects in

mitochondrial fusion. This suggests that Wwox can contribute to cell death by modulating mitochondrial dynamics.

WWOX can physically activate the mechanistic target of rapamycin (mTOR) signaling [101]. Activated mTOR allows the translocation of Drp1 to mitochondria by inhibiting the phosphorylation of Ser63 of Drp1. This, in turn, promotes mitochondrial fission [51]. Thus, WWOX may contribute to mitochondrial fission through the activation of mTOR signaling (Figure 8). In addition, high levels of ROS have been shown to induce mitochondrial fission [102-104]. We have shown previously that ectopic expression of Wwox can increase ROS levels, while a reduction of ROS levels has been observed in *Wwox* mutants [33]. Thus, WWOX may also facilitate mitochondrial fission through its regulation of ROS levels (Figure 8). Activation of Yki/YAP has been shown to cause mitochondrial fusion in *Drosophila* and also in human cells through up-regulating mitochondrial fusion genes, including *Opa1* and *Marf* [105]. Interestingly, activation of Yki reduces ROS levels, which has the same effect as loss of Wwox [33, 105]. Therefore, it is also possible that WWOX inhibits Yki/YAP transcriptional activity on mitochondrial fusion genes and results in the promotion of mitochondrial fission (Figure 8), as well as the increase of ROS levels. Induction of mitochondrial fission promotes the clearance of damaged mitochondria through mitophagy and, in the case where mitophagic clearance cannot be completed, it activates caspase signaling to promote cell death (Figure 8).

It is noteworthy that another common fragile site gene, *Parkin* (spanning FRA6E), also has a role in promoting mitochondrial fission [106-108]. Drp1 is required for the mitochondrial fission induced by Parkin [108], demonstrating that both Parkin and WWOX require the same functional interactor to contribute to mitochondrial fission. The molecular mechanism of Parkin-mediated mitochondrial fission is still unknown. It has been hypothesized that Parkin can either

ubiquitinate mitochondrial fission proteins to facilitate their translocation to mitochondria, or decrease mitochondrial fusion proteins through degradative ubiquitination [107]. WWOX has also been shown to be involved in the ubiquitination pathway by competing with itchy E3 ubiquitin protein ligase (ITCH) for its substrates, such as ΔNP63α and p73, and inhibits their degradation [28, 109]. Thus, the regulation of ubiquitination state of mitochondrial fission/fusion proteins may be one of the mechanisms by which WWOX contributes to mitochondrial fission.

Dysfunction in mitochondrial dynamics is associated with different neurological disorders. Mutations in mitochondrial fusion genes, such as *Opa1* and *MFN2* (human homolog for *Marf*) have been shown to cause dominant optic atrophy and Charcot-Marie-Tooth disease type 2a respectively [110-112]. In addition, it has also been reported that a newborn girl carrying a dominant negative *Drp1* allele showed severe neurological symptoms, including optic atrophy, microcephaly and abnormal brain development, and died at age 37 days [113]. This indicates that alterations in mitochondrial dynamics strongly impact neuronal functions. Loss of WWOX function is also associated with neurological disorders, including epilepsy, ataxia, mental retardation, intellectual disability and optic atrophy [90, 91, 114-118], suggesting that WWOX could play an important role in preventing neurological disease symptoms. Here, we have shown that WWOX can contribute to mitochondrial fission, which is consistent with a protective role in preventing neurological disorders. Reduced *WWOX* levels decrease the efficiency of mitochondrial fission, resulting in an increase of damaged mitochondria and reduced levels of caspase-dependent cell death. As a result, there is an increase of cells with mitochondrial dysfunction, which is a common cause for cancers, different metabolic disorders and neurological diseases [119-121].

Mitochondrial transfer between cells is becoming of major interest. Cancer cells with impaired mitochondria can obtain functional mitochondria from normal cells, resulting in the potential for increased resistance against chemotherapy [122]. In contrast, metastatic cells can lose their metastatic ability by swapping their mitochondrial DNA (mtDNA) with non-metastatic cells, and vice versa [123]. In addition, neurons also exchange their mitochondria with astrocytes, in order to dispose the damaged mitochondria and replace them with functional mitochondria [124, 125]. Disruption of this process results in the worsening of neurological outcomes [124]. Together, this evidence demonstrates the importance of maintaining mtDNA fidelity by mitochondrial transfer. Mitochondrial fission is required for the segregation of mitochondria from the network, and thus important for the mitochondrial transfer between cells. The roles of WWOX in the maintenance of mitochondrial function [34] and dynamics highlight the possible mechanism by which it suppresses tumorigenesis and neurological diseases.

Materials and methods

Maintenance of fly lines and crosses

UAS-yki and *UAS-eiger*^{weak} lines were kindly provided by Kieran Harvey and Professor Miura respectively. The *UAS-Wwox* line was generated as previously described [57, 126]. All the gal4 driver lines and the remaining over-expression lines were obtained from Bloomington. RNAi lines were obtained from either Bloomington *Drosophila* Stock Center (Indiana University, USA) or Vienna *Drosophila* Resource Centre (VDRC, Vienna, Austria). All *Drosophila* stocks were maintained in vials containing fortified medium (1% agar, 1% glucose, 6% fresh yeast, 9.3% molasses, 8.4% coarse semolina, 0.9% acid mix, 1.7% tegosept) at either 18°c or 25°C.

Genetic screening experiment

Gene expression of either candidates or *Wwox* was altered in the developing *Drosophila* eye using the *eyeless (ey)-gal4* promoter in conjunction with UAS constructs [127]. All crosses for genetic screens experiment were set up at 25°C.

Promoters used to alter *Prp19*, *Yki* and *EGFR* levels

All the functional assays of *Prp19* were done by altering gene expression specifically either in the *Drosophila* developing eye using the *ey-gal4* or in wing pouch using the *MS1096-gal4*. All the functional assays of *Yki* were done by altering gene expression using the *ey-gal4*. *EGFR* eye phenotype was induced by reducing *EGFR* levels using the *Glass multiple reporter* (*GMR*)-gal4 promoter, which expresses specifically in all cells posterior to the morphogenetic furrow of eye. Crosses used to test the effect of either reduced *Prp19* levels, ectopic expression of Yki or decreased *EGFR* levels in eye were done at 18°C. Crosses used to test the effect of reduced *Prp19* levels in wing disc were set up at 25°C.

Analyses of mitochondrial morphology

All crosses that were set up to test for mitochondrial morphology were done at 29°C. Gene expression was altered in the posterior cells of wing discs using the *engrailed-gal4* and mitochondria were labelled by co-expression of GFP protein containing a mitochondrial import signal (mito-GFP; generated by fusing the N-terminal 31 amino acids of human cytochrome c oxidase subunit VIII to GFP [128, 129]). The mitochondrial morphology of peripodial cells at the posterior-anterior boundary of the wing pouch was analyzed for each wing disc. The percentage of wing discs, in which all cells in the scored region showed fragmented mitochondria (Figure 7B

iii-iii'), was compared between each genotype (Figure 7C). Chi-square test analyses were performed using *GraphPad Prism 5* with $p<0.05$ as the cut-off value for significance.

Quantitative real-time (qPCR) assays

RNA extraction and purification, as well as qPCR assays, were carried out as described previously [33]. qPCR primer pairs used:

Prp19: CTTCGACCAAAGCGGAACCTA / CCGTGTGGTCGTTGAATACCTT

Drp1: GAAAAGAGGCGGCAGATATGC / TTGCTCCAATCGCTGACCA

Microscopy and imaging

Adult eye images were captured using an Olympus ColourView IIIU Soft Imaging System camera and *AnalysisRuler image acquisition* software. Images for immuno-staining and mitochondrial morphology were captured using the Zeiss Axioplan2 microscope (Carl Zeiss) with an AxioCam MRm camera (Carl Zeiss) and *AxioVision* software.

Immuno-staining

Immuno-staining of late third instar larval wing discs were performed as described previously [57]. Primary antibodies included anti-cleaved Caspase 3 antibody (1:100, Cell signalling), anti-Wg antibody (1:100, Developmental Studies Hybridoma Bank) and anti-γ-H2AX antibody (1:400, Rockland, Limerick, PA, USA). Secondary antibodies were Anti-rabbit DyLight 649 antibody (1:100, Vector Laboratories) and anti-mouse Cy3 antibody (1:100, Jackson ImmunoResearch Laboratories).

Quantification of antibody intensity

All images of wing discs with Caspase 3 staining were captured with a fixed exposure time. The mean intensity of Caspase 3 within the wing pouch region was determined using AxioVision software and this value was then deducted with the Caspase 3 background signal measured from the notum of wing disc. The area of wing disc and its γ-H2AX staining were measured using *ImageJ* software. Statistical analyses were performed using two-tailed T-test with $p \leq 0.05$ as a cut-off value for significance in *GraphPad Pris*m.

Quantification of eye phenotype

Eye phenotypes induced by reduced *Prp19* levels were categorized into three classes with representative figures shown in Figure 1A. Each eye of the fly was scored individually. Chi-square tests were performed using *GraphPad Prism* with $p \leq 0.05$ as the cut-off value for significance. In order to determine the size of adult eyes with reduced *EGFR* levels, ten random adult eye images (0-1 day old) were measured using Image J software. Statistical analyses were performed using two-tailed T-test in *GraphPad Prism*. $p \leq 0.05$ was used as the cut-off value for significance.

Viability assays

Viability assays of flies with ectopic expression of Yki were maintained at 18°C. Viability assays of flies with either reduced *Prp19* levels or defects in mitochondrial dynamics were maintained at 25°C. The expression of genes involved in mitochondrial dynamics was ubiquitously altered using the *daughterless* (*da*)-*gal4*. The ratio of non-TM6B:TM6B progeny was recorded for adult viability assays. In the pupal viability assays, the ratio of eclosed:dead pupae was recorded after either 14 days of development at 25°C or 30 days of development at 18°C. Two-tail T-tests were

performed for adult viability assays, while Fisher's exact tests were performed for pupal viability assays using *GraphPad Prism* with $p \leq 0.05$ as the cut-off value for significance.

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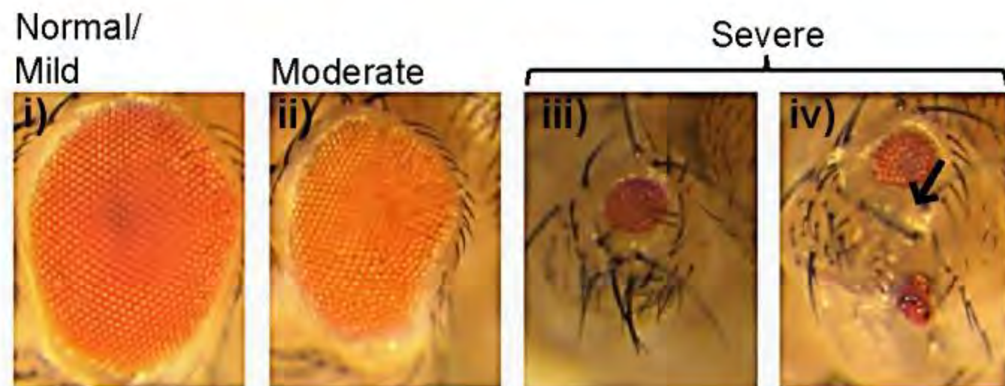
Figures

A)



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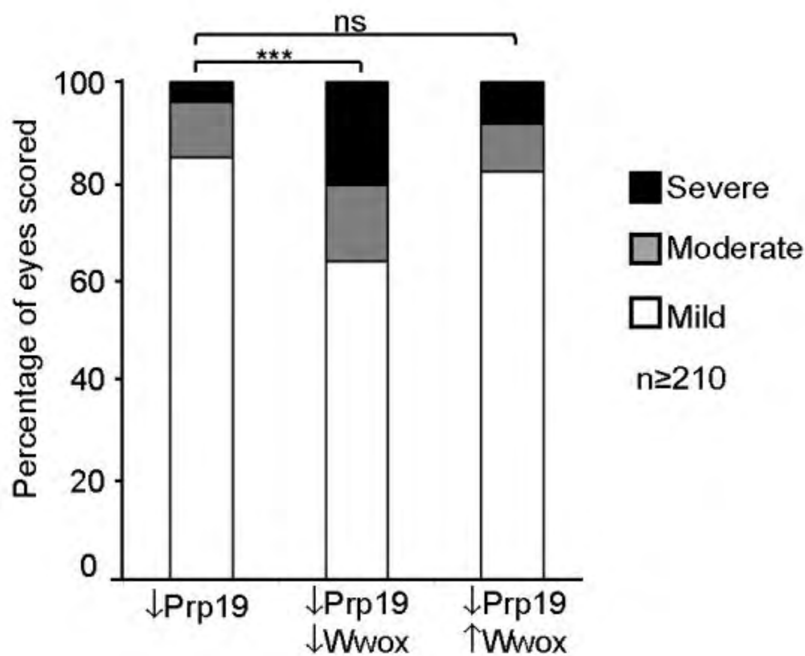
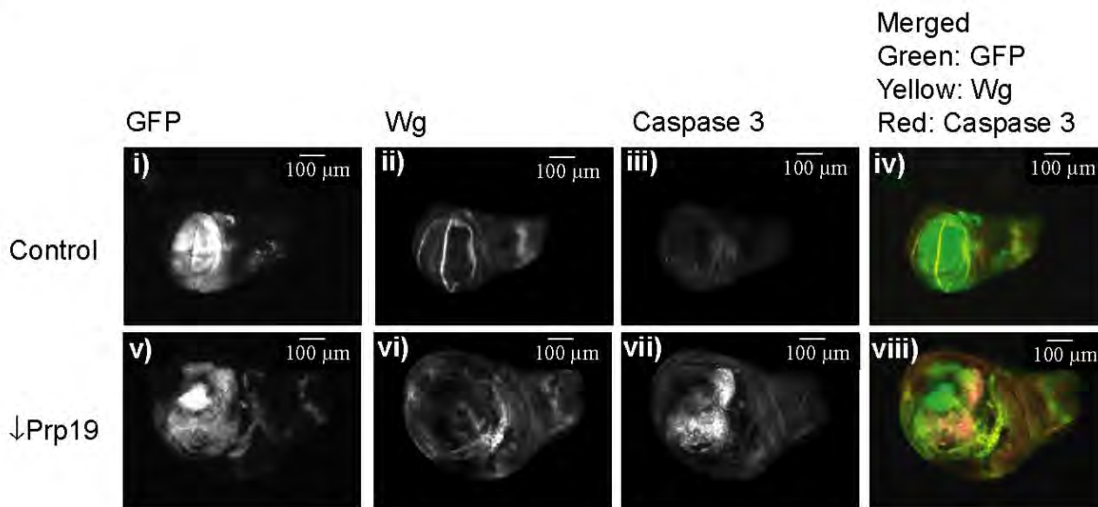


Figure 1. Reduction of *Wwox* levels enhances cellular dysfunction caused by decreased *Prp19* levels. A) Decreased expression of Prp19 results in a range of phenotypes that can be classified into three groups: **i)** eyes with no obvious disruption are classified as "normal/mild" group; **ii)** eyes with loss of photoreceptor cells are classified as "moderate"; **iii)** small eyes and **iv)** eyes with defect in differentiation (as pointed to by arrow) are classified as "severe". **B)** Reduced Wwox expression in the eye with decreased *Prp19* levels significantly increases the percentage of eyes showing the severe phenotype. There is no obvious modification to the phenotype when Wwox was ectopically expressed in the eye with reduced *Prp19* levels. Significance indicated by ns = not significant and *** = $p<0.0001$.

A)



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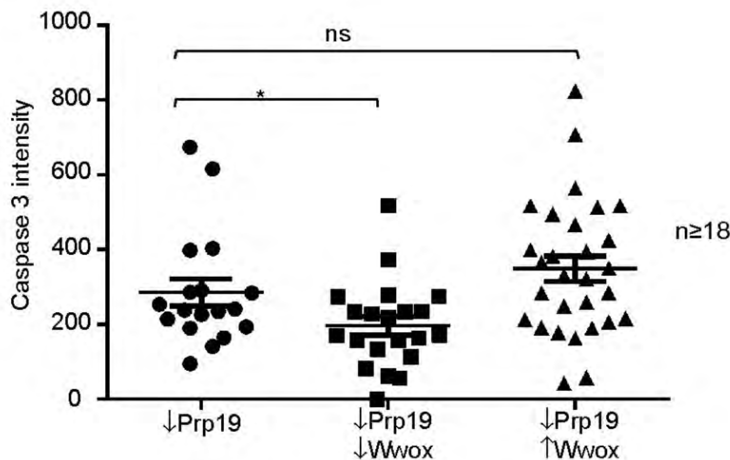


Figure 2. Reduced *Wwox* expression decreases cell death induced by decreased *Prp19* expression. A) i) Control disc showing the expression pattern of *MS1096-gal4* as visualized by co-expression of GFP. **ii)** Control disc shows Wg staining in cells surrounding the wing pouch, cells at dorsal-ventral boundary and some cells at dorsal region. **iii)** Caspase 3 staining shows that control disc has low levels of cell death. **iv)** Merged image of control disc with GFP shown in green, Wg shown in yellow and Caspase 3 shown in red. Wing disc with reduction of *Prp19* levels shows altered **v)** GFP and **vi)** Wg expression patterns, as well as **vii)** high levels of Caspase 3. **viii)** Merged image of wing disc with decreased *Prp19* levels. **B)** Quantification of the Caspase 3 intensity for individual wing discs with different genotypes reveals that reduced *Wwox* expression decreases Caspase 3 levels induced by *Prp19* deficiency. Significance indicated by ns = not significant and * = $0.01<p<0.05$.

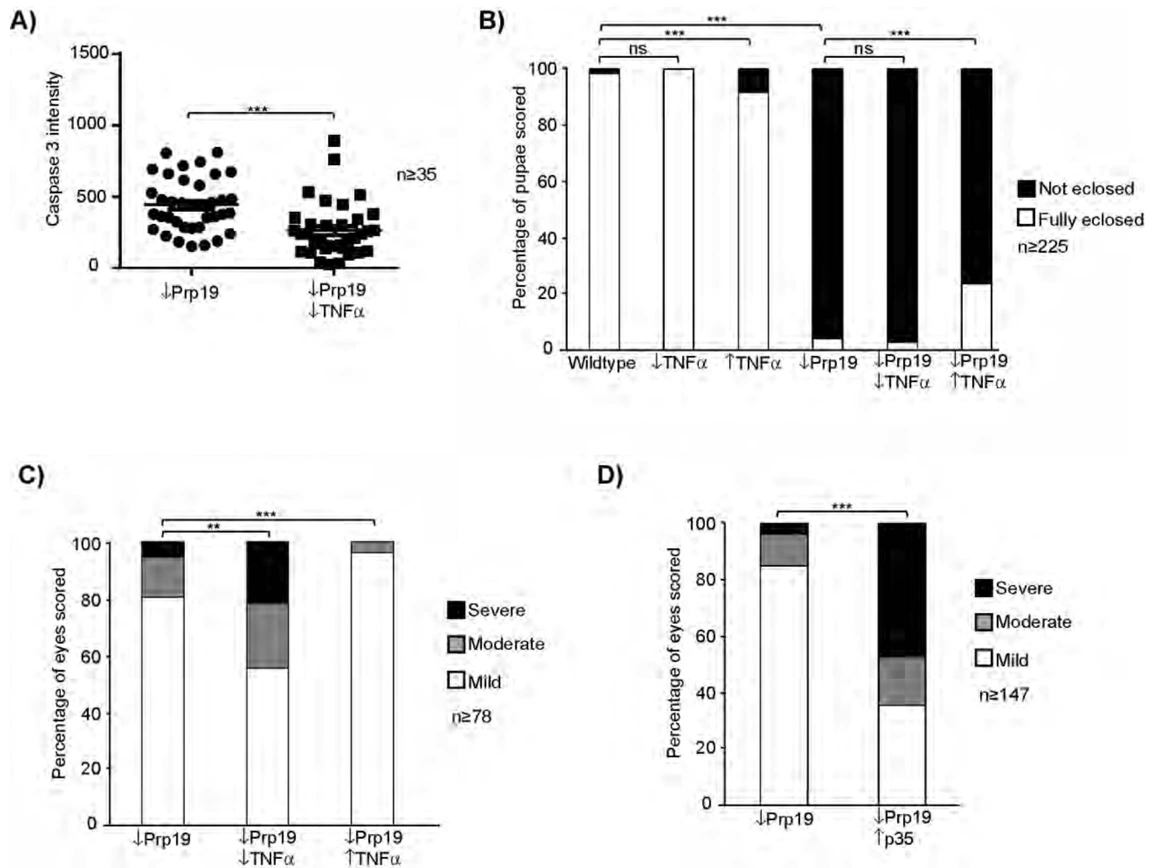


Figure 3. Increased cell death suppresses the lethality and eye phenotype caused by reduced *Prp19* levels. A) Quantification of Caspase 3 intensity for each individual wing disc shows that the reduction of *eiger/TNFα* levels decreases Caspase 3 levels induced by *Prp19* deficiency. **B)** Pupal survival assay reveals that increased *eiger/TNFα* levels significantly increase the eclosion rate of pupae with reduced *Prp19* levels in the wing disc. **C)** Reduced *eiger/TNFα* levels enhance the severity of eye phenotypes induced by reducing *Prp19* levels, while ectopic expression Eiger/TNFα suppresses these eye phenotypes. **D)** Ectopic expression of p35 in eyes with reduced *Prp19* levels significantly increases the severity of eye phenotypes. Significance indicated by ns = not significant, ** = $0.0001<p<0.01$ and ***= $p<0.0001$.

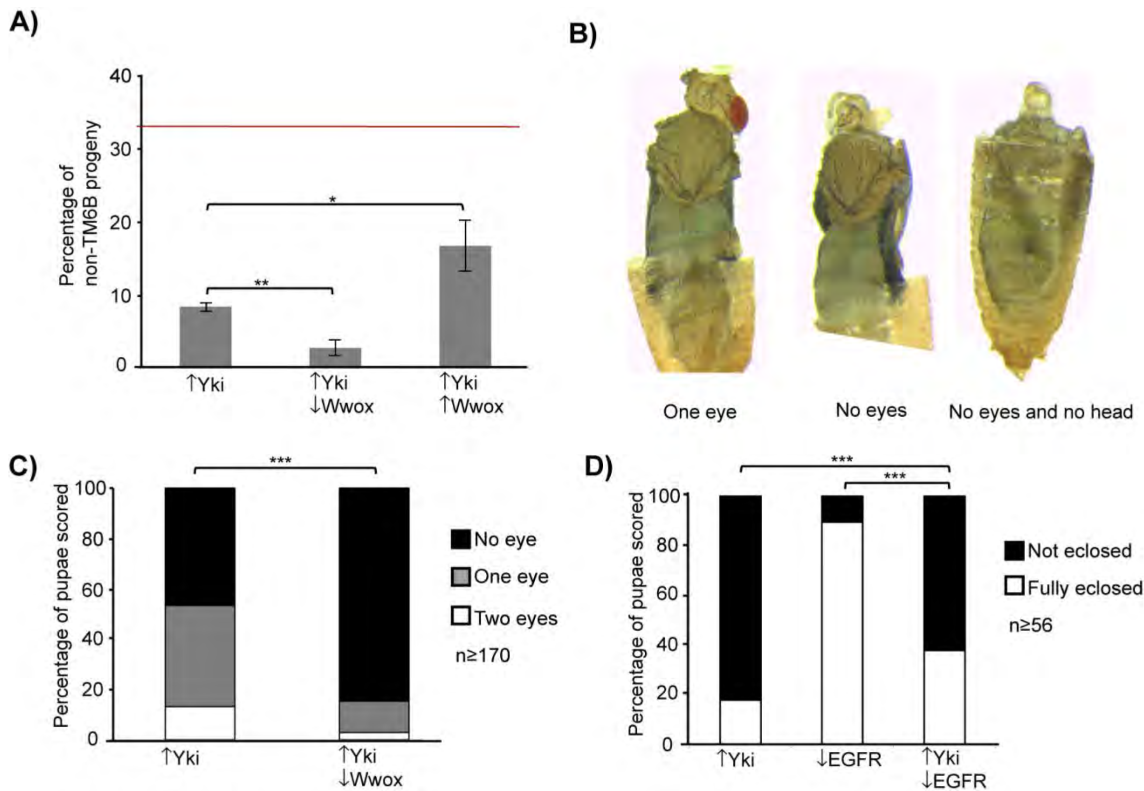
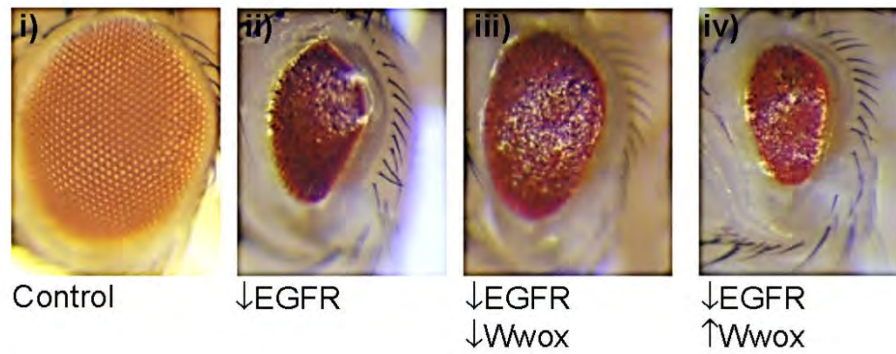


Figure 4. Either altered *Wwox* levels or reduced *EGFR* levels can modify the survival of flies with ectopically expressed Yki. A) Ectopic expression of Yki decreases the survival of flies. Reduced *Wwox* expression further decreases the survival of these flies, while ectopic expression of Wwox results in a suppression of this lethality effect. The expected percentage of progeny based on Mendelian laws of inheritance is labelled by the red line. **B)** Ectopic expression of Yki results in either loss of eye(s) or headless phenotype. **C)** Reduced *Wwox* expression in pupae with ectopically expressed Yki significantly increases the percentage of pupae with loss of both eyes. **D)** Reduced *EGFR* expression increases the eclosion rate of pupae with ectopic expression of Yki. Significance indicated by * = $0.01<p<0.05$, ** = $0.0001>p>0.01$ and ***= $p<0.0001$.

A)



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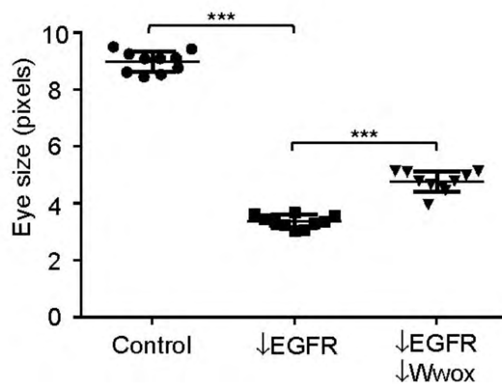


C)

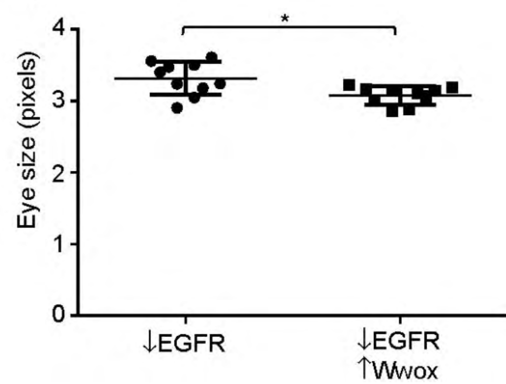
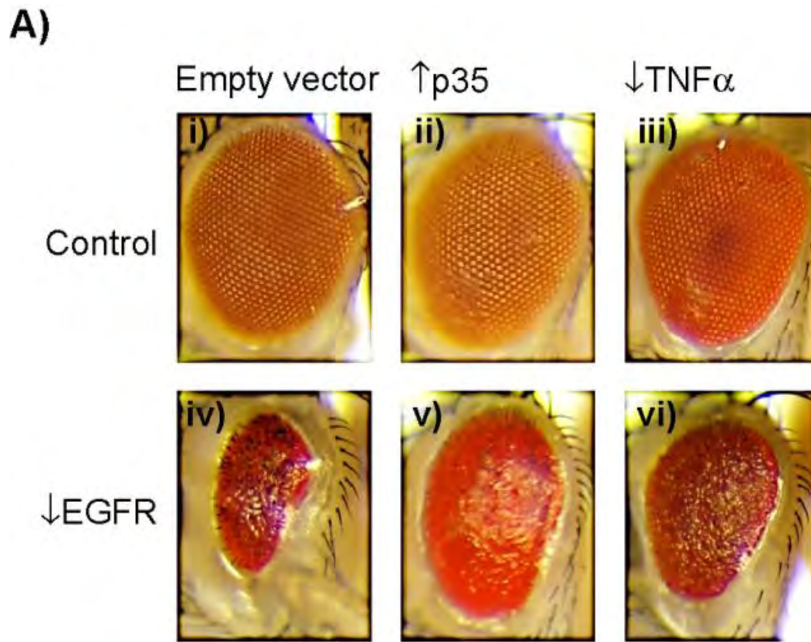


Figure 5. Altered *Wwox* levels modify eye phenotype induced by decreased *EGFR* levels. A) i) The normal control eye shows an organized ommatidia. **ii)** Reduction of *EGFR* levels decreases the eye size and disrupts ommatidial pattern in the eye. **iii)** Reduced *Wwox* levels suppress the eye phenotype induced by decreased *EGFR* levels as indicated by the increase of eye size. **iv)** Ectopic expression of Wwox enhances the *EGFR* knockdown eye phenotype, as shown by a further reduction of eye size. **B)** Quantification of eye size shows that reduced *EGFR* expression significantly decreases the eye size. Reduced *Wwox* levels significantly increase *EGFR* knockdown eye size. **C)** Quantification of eye size with reduced *EGFR* levels shows a significant reduction of eye size by ectopic expression of Wwox. Significance indicated by * = $0.01<p<0.05$ and ***= $p<0.0001$.



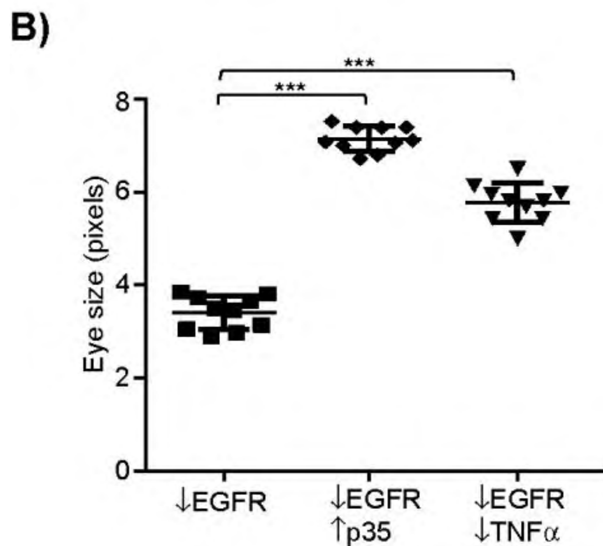
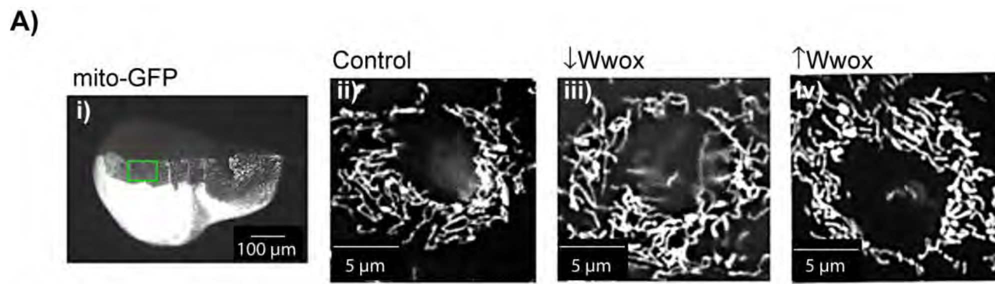
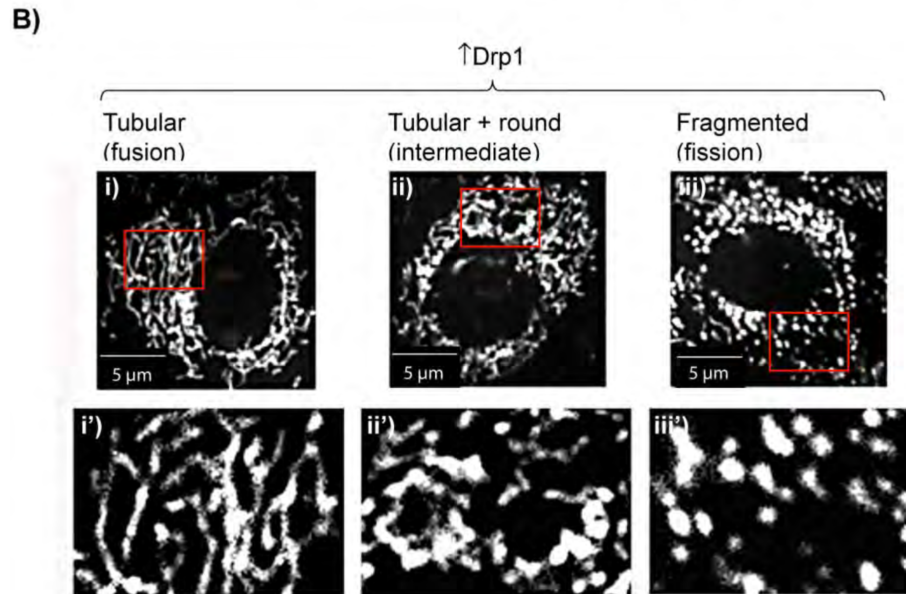
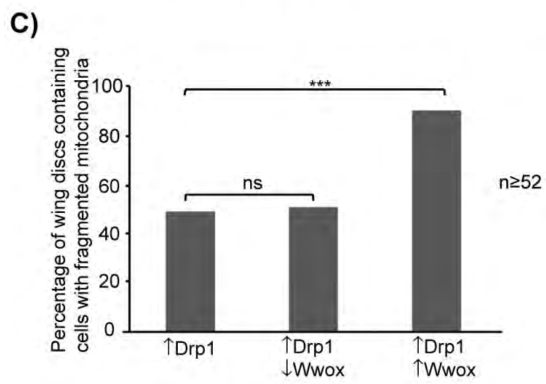
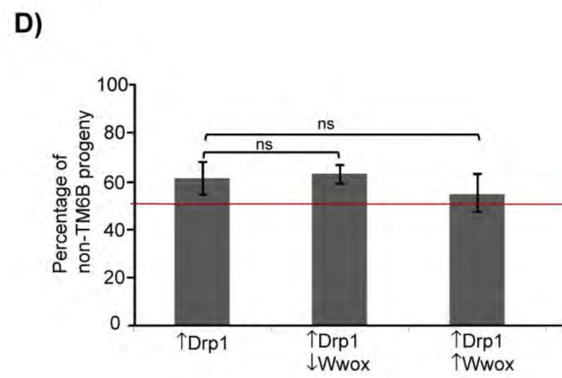


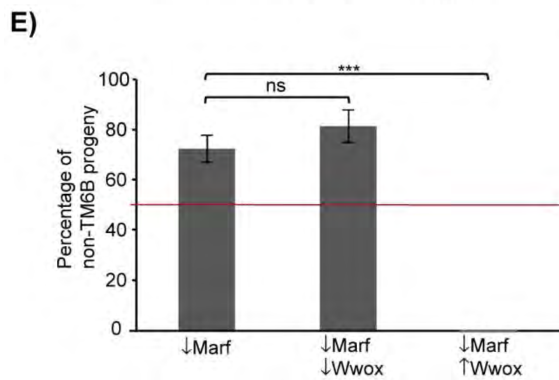
Figure 6. Either inhibition of caspase signaling or reduction of *eiger/TNFα* levels suppresses *EGFR* knockdown eye phenotype. A) i) The control empty vector eye has a regular arrangement of ommatidia. There is no obvious eye phenotype when either **ii)** p35 was ectopically expressed or **iii)** *eiger/TNFα* expression was reduced. i**v)** The *EGFR* knockdown eye phenotype is suppressed by either **v)** ectopic expression of p35 or **vi)** reduction of *eiger/TNFα* levels. **B)** Quantification of *EGFR* knockdown eye size shows that either ectopic expression of p35 or decrease of *eiger/TNFα* levels significantly increases the eye size. Significance indicated by ***= p<0.0001.


A)
mito-GFP
i)
100 µm
Control
ii)
5 µm
↓Wwox
iii)
5 µm
↑Wwox
iv)
5 µm


B)
↑Drp1
Tubular
(fusion)
Tubular + round
(intermediate)
Fragmented
(fission)
i)
ii)
iii)
5 µm
i')
ii')
iii')


C)
Percentage of wing discs containing cells with fragmented mitochondria
***
ns
n≥52
↑Drp1
↑Drp1 ↓Wwox
↑Drp1 ↑Wwox


D)
Percentage of non-TM6B progeny
ns
ns
↑Drp1
↑Drp1 ↓Wwox
↑Drp1 ↑Wwox


E)
Percentage of non-TM6B progeny
***
ns
↓Marf
↓Marf ↓Wwox
↓Marf ↑Wwox

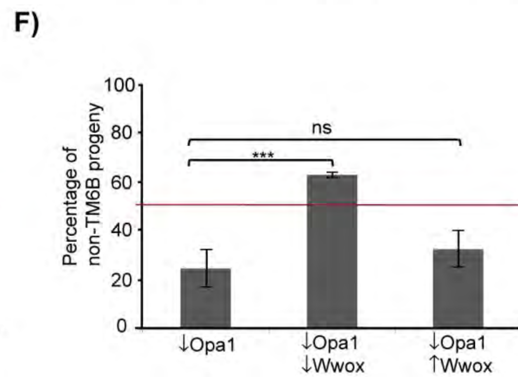

F)
Percentage of non-TM6B progeny
ns
***
↓Opa1
↓Opa1 ↓Wwox
↓Opa1 ↑Wwox

Figure 7. Wwox can contribute to the mitochondrial fission. A) i) Gene expression was altered in the posterior region of the wing disc as labelled by co-expression of mito-GFP. The green box indicates the region of mitochondrial morphology scored for each wing disc. **ii)** Mitochondria in control cells show a tubular structure. Either **iii)** reduced *Wwox* levels or **iv)** ectopic expression of Wwox does not alter the mitochondrial morphology. **B)** Cells with ectopically expressed Drp1 results in either **i-i')** tubular, **ii-ii')** a mixture of tubular and round or **iii-iii')** fragmented mitochondria. The red boxes in **i-iii** show regions that are enlarged in **i'-iii'**. **C)** Ectopic expression of Wwox together with Drp1 significantly increases the percentage of wing discs containing cells with fragmented mitochondria. **D)** There is no obvious modification to the survival of flies with ectopic expression of Drp1 when *Wwox* levels were altered. **E)** Ectopic expression of Wwox causes lethality to the flies with reduced *Marf* levels. **F)** Reduced *Wwox* levels increase the survival of flies with reduced *Opa1* levels. The expected percentage of progeny based on Mendelian laws of inheritance is labelled by the red line. Significance indicated by ns = not significant and ***= $p<0.0001$.

Figure 8. Model for the *in vivo* mechanisms by which Wwox contributes to mitochondrial fission, cell death and mitophagy. Reduction of either *Prp19* or *EGFR* levels induces Eiger/TNFα-mediated cell death. Eiger/TNFα induces cell death through the activation of caspase cascades and the increase of ROS levels. Increased levels of either ROS or *Drp1* can induce mitochondrial fission. Reduction of mitochondrial fusion proteins, including Marf and Opa1, also leads to mitochondrial fission. Wwox can contribute to mitochondrial fission by increasing ROS levels, activating mTOR or suppressing transcriptional activation of *Marf* and *Opa1* by Yki. Activation of mTOR can promote the translocation of Drp1 into mitochondria, resulting in mitochondrial fission. Mitochondrial fission is important for the activation of caspase signaling, cell death and mitophagy.

Supplementary Materials

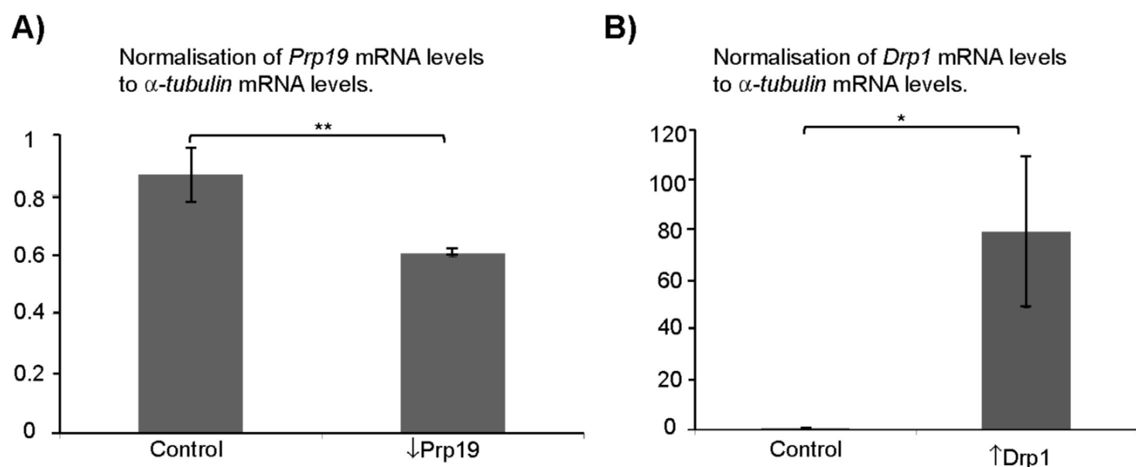
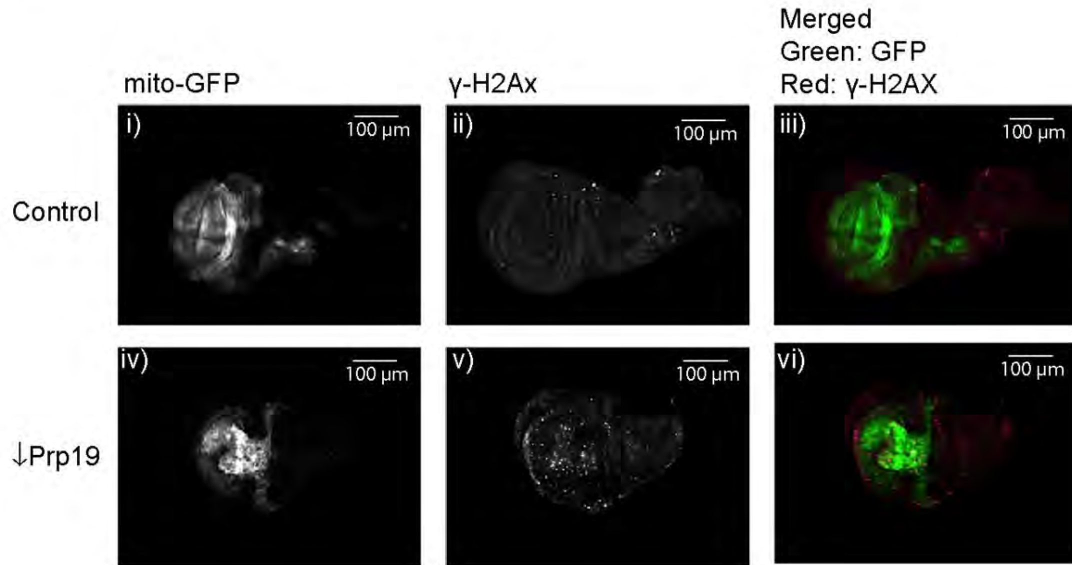


Figure S1. *Prp19* and *Drp1* mRNA levels were measured by quantitative PCR (qPCR). A) qPCR assays verify the reduction of *Prp19* mRNA levels in *Prp19* knockdown flies. **B)** qPCR assays show the increase of *Drp1* levels in flies with ectopically expressed Drp1. Significance indicated by * = $0.01<p<0.05$ and ** = $0.0001<p<0.01$.

A)



B)

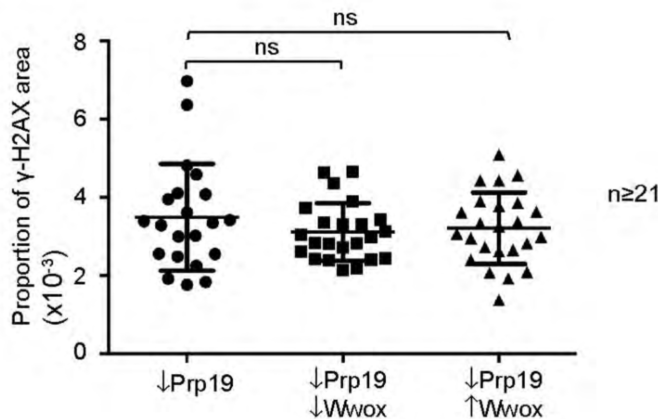


Figure S2. Altered *Wwox* levels are not able to modify DNA damage levels induced by decreased *Prp19* levels. A) i) Ectopic expression of mito-GFP labeled the area where the *MS1096-GAL4* was expressed in control disc. **ii)** The control disc shows low levels of DNA damage as indicated by γ-H2AX staining. **iii)** Merged image of the control disc with GFP shown in green and γ-H2AX shown in red. Reduction of *Prp19* levels **iv)** decreases the GFP area and **v)** induces high levels of γ-H2AX. Merged image (GFP in green and γ-H2AX in red) of wing disc with reduced *Prp19* levels. **B)** Quantification of γ-H2AX area in individual wing discs shows that altered *Wwox* levels do not significantly modify DNA damage levels induced by reduced *Prp19* levels. Significance indicated by ns = not significant.

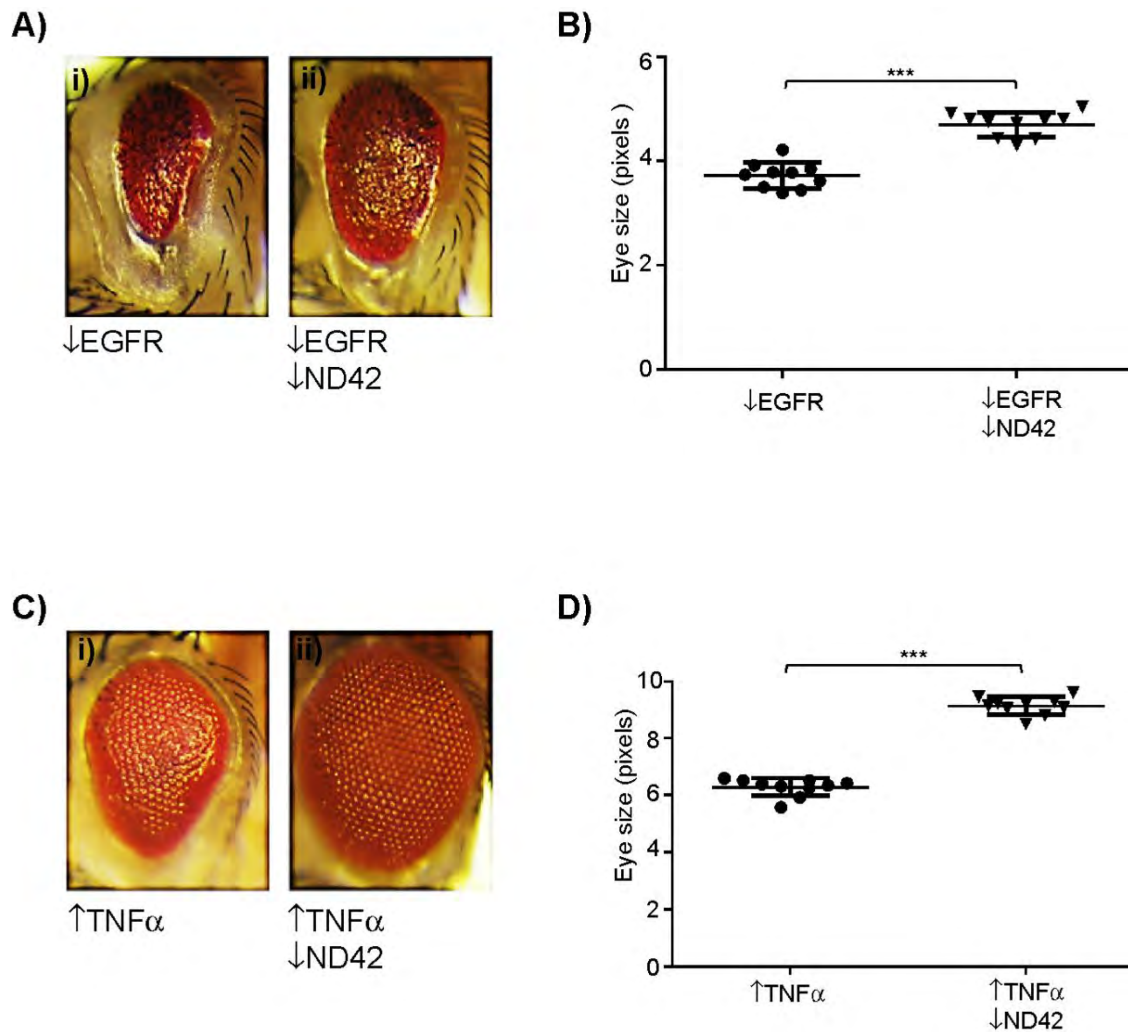


Figure S3. Mitochondrial dysfunction suppresses the eye phenotype induced by either reduction of *EGFR* levels or ectopic expression of Eiger/TNFα. A) i) The eye phenotype induced by reduction of *EGFR* is suppressed by **ii)** reduction of *ND42* levels as indicated by **B)** a significant increase of eye size shown in the graph. **C) i)** Ectopic expression of Eiger/TNFα causes a rough and small eye. **ii)** Reduction of *ND42* levels suppresses the ectopic Eiger/TNFα eye phenotype. **D)** Quantification of eye size shows that reduced *ND42* expression significantly increases the size of eyes with ectopic expression of Eiger/TNFα. Significance indicated by ***= $p<0.0001$.

Table S1. Summary of the screening experiment testing for candidates that have genetic interaction with Wwox

	Gene targeted		Knockdown (RNAi) or over-expression (UAS) line		Eye phenotype caused by eye-specific altered gene expression	
	Annotation Symbol	Name	Line	Type	Altered gene expression of candidate alone	Modification by altered Wwox levels
Metabolism						
1	CG10638		v102914	RNAi	No phenotype	No phenotype
			v31306	RNAi	No phenotype	No phenotype
2	CG32101		v41786	RNAi	No phenotype	No phenotype
3	CG10924	Phosphoenol pyruvate carboxykinase	v107092	RNAi	No phenotype	No phenotype
			v13929	RNAi	No phenotype	No phenotype
4	CG3481	Alcohol dehydrogenase	v50970	RNAi	No phenotype	No phenotype
5	CG5590		v109314	RNAi	Overgrowth of cells at the bottom of eye	No obvious modification
			v42020	RNAi	Disarrangement of ommatidia	No obvious modification
6	CG7470	Glutamate 5-kinase	v101476	RNAi	No phenotype	No phenotype
			v38953	RNAi	No phenotype	No phenotype
7	CG17285	Fat body protein1	v37881	RNAi	No phenotype	No phenotype
8	CG10120	Malic enzyme	v104016	RNAi	No phenotype	No phenotype
9	CG2952	Poly Phenol Oxidase 3	v50737	RNAi	No phenotype	No phenotype
10	CG31075		v101809	RNAi	No phenotype	No phenotype
11	CG3972	Cytochrome P450-4g1	v30205	RNAi	No phenotype	No phenotype
			v30207	RNAi	No phenotype	No phenotype
			v102864	RNAi	No phenotype	No phenotype
12	CG9042	Glycerol-3-phosphate dehydrogenase	v105359	RNAi	No phenotype	No phenotype
			v29015	RNAi	No phenotype	No phenotype
			v29013	RNAi	No phenotype	No phenotype

13	CG3425	Type III alcohol dehydrogenase	v105128	RNAi	No phenotype	No phenotype
14	CG7430	Pyruvate dehydrogenase E3 subunit	v106126	RNAi	No phenotype	No phenotype
15	CG7998	Mitochondrial malate dehydrogenase	v101551	RNAi	No phenotype	No phenotype
			v22654	RNAi	No phenotype	No phenotype
16	CG6816	Cytochrome P450-18a1	v5601	RNAi	No phenotype	No phenotype
17	CG5026		v34915	RNAi	No phenotype	No phenotype
18	CG7899	Acid phosphatase 1	v3579	RNAi	No phenotype	No phenotype
19	CG7176	Isocitrate dehydrogenase	v42916	RNAi	No phenotype	No phenotype
20	CG6439	Isocitrate dehydrogenase	v14443	RNAi	No phenotype	No phenotype
			v100822	RNAi	No phenotype	No phenotype
21	CG7755		v25544	RNAi	No phenotype	No phenotype
22	CG12233	Lethal (1) G0156	v106091	RNAi	No phenotype	No phenotype
23	CG3483		v101958	RNAi	No phenotype	No phenotype
24	CG4600	Yippee interacting protein 2	v26562	RNAi	No phenotype	No phenotype
25	CG6821	Larval serum protein 1 gamma	v50108	RNAi	No phenotype	No phenotype
26	CG7070	Pyruvate kinase	v38129	RNAi	No phenotype	No phenotype
			BL35218	RNAi	No phenotype	No phenotype
27	CG3200	Rhythmically expressed gene 2	v107185	RNAi	No phenotype	No phenotype
28	CG15912		v14125	RNAi	No phenotype	No phenotype
			v100777	RNAi	No phenotype	No phenotype
29	CG3105	PAS kinase	v107025	RNAi	No phenotype	No phenotype
30	CG15771		v106331	RNAi	No phenotype	No phenotype
31	CG5565		v100396	RNAi	No phenotype	No phenotype
			v27463	RNAi	No phenotype	No phenotype

32	CG31673		v101618	RNAi	No phenotype	No phenotype
			v25796	RNAi	No phenotype	No phenotype
33	CG5028		v103834	RNAi	No phenotype	No phenotype
34	CG8893	Glyceraldehyde 3 phosphate dehydrogenase 2	BL26302	RNAi	No phenotype	No phenotype
35	CG1411	Collapsin Response Mediator Protein	BL53354	RNAi	No phenotype	No phenotype
36	CG40411	Poly-(ADP-ribose) polymerase	BL35792	RNAi	No phenotype	No phenotype
37	CG6213	Vacuolar H^+ ATPase 13kD subunit	BL38233	RNAi	No phenotype	No phenotype
38	CG8318	Neurofibromatosis Factor 1	v35877	RNAi	No phenotype	No phenotype
			v109637	RNAi	No phenotype	No phenotype
39	CG9620	Neuronally altered carbohydrate		UAS	Disruption in eye patterning	No obvious modification
Oxidative response						
40	CG30404	Transport and Golgi organization 11	v29385	RNAi	No phenotype	No phenotype
41	CG11987	Tango	BL9583	UAS	Disruption in eye patterning	No obvious modification
42	CG7951	Similar	v106187	RNAi	No phenotype	No phenotype
43	CG11793	Superoxide dismutase 1	v31552	RNAi	No phenotype	No phenotype
44	CG8905	Superoxide dismutase 2	v42162	RNAi	No phenotype	No phenotype
45	CG6871	Catalase	v103591	RNAi	No phenotype	No phenotype
46	CG11919	Peroxin 6	v32429	RNAi	No phenotype	No phenotype
Transcription, transcriptional modification and translation						
47	CG1034	Bicoid	v12743	RNAi	No phenotype	No phenotype
48	CG1849	Runt	v3025	RNAi	No phenotype	No phenotype
49	CG15455	RunxB	v30539	RNAi	Lethal	No modification

50	CG13425	Heterogeneous nuclear ribonucleoprotein K	v2912	RNAi	No phenotype	No phenotype
51	CG3018	Lesswright	BL31396	RNAi	Disruption in eye patterning	No obvious modification
52	CG7958	Tonalli	BL29372	RNAi	No phenotype	No phenotype
53	CG13298		BL36619	RNAi	No phenotype	No phenotype
54	CG11266	Caper	BL44431	RNAi	No phenotype	No phenotype
55	CG12135	c12.1	BL34952		Induced a range of eye phenotypes with different severity	No obvious modification
56	CG5519	Pre-mRNA splicing factor 19	v22146	RNAi	No phenotype	No phenotype
			v22147	RNAi	Disruption in eye patterning	Enhanced by reduced Wwox levels; no modification by increased Wwox levels.
			v108575	RNAi	Induced a range of eye phenotypes with different severity	No modification by reduced Wwox levels; suppression by increased Wwox levels.
			v41438	RNAi	Induced a range of eye phenotypes with different severity	Enhanced by reduced Wwox levels; no modification by increased Wwox levels.
Protein folding response and degradation						
57	CG2852		v102376	RNAi	No phenotype	No phenotype
			v15069	RNAi	No phenotype	No phenotype
58	CG7235	Hsp60C	v47705	RNAi	No phenotype	No phenotype
			v47706	RNAi	No phenotype	No phenotype
			v47708	RNAi	No phenotype	No phenotype
59	CG14526		v47138	RNAi	No phenotype	No phenotype
			v39838	RNAi	No phenotype	No phenotype
60	CG11912		v104242	RNAi	No phenotype	No phenotype
61	CG11459		v16573	RNAi	No phenotype	No phenotype
			v102522	RNAi	No phenotype	No phenotype
62	CG11912		v28314	RNAi	No phenotype	No phenotype

Immune response						
63	CG2993		v33605	RNAi	No phenotype	No phenotype
64	CG12359	Ulp1	BL31624	RNAi	No phenotype	No phenotype
65	CG12276	Aos1	BL36074	RNAi	Disruption in eye patterning	No obvious modification
66	CG7528	Ubiquitin activating enzyme 2	BL35806	RNAi	Disruption in eye patterning	No obvious modification
67	CG5576	Immune deficiency	BL38933	RNAi	No phenotype	No phenotype
Maintenance of cell cycle or organelle structure						
68	CG17462		v19799	RNAi	No phenotype	No phenotype
69	CG9506	Slow as molasses	v24049	RNAi	No phenotype	No phenotype
70	CG4965	Twine	v46064	RNAi	No phenotype	No phenotype
71	CG6533	Chorion Protein 16	v19870	RNAi	No phenotype	No phenotype
72	CG32025	Formin homology 2 domain containing ortholog	v34032	RNAi	No phenotype	No phenotype
73	CG5014	VAMP-associated protein of 33kDa ortholog A	v100809	RNAi	No phenotype	No phenotype
74	CG33113	Reticulon-like1	v110545	RNAi	No phenotype	No phenotype
75	CG8895	Reticulon-like1	v7866	RNAi	No phenotype	No phenotype
76	CG1725	Disc large 1	v41134	RNAi	Disruption in eye patterning	No obvious modification
77	CG44128	Src oncogene at 42A	BL6410	UAS	Lethal	No obvious modification
			v100708	RNAi	Disruption in eye patterning	No obvious modification
78	CG7524	Src oncogene at 64B	BL8477	UAS	Lethal	No obvious modification
			BL30517	RNAi	No phenotype	No phenotype
79	CG10701	Moesin	37917	RNAi	No phenotype	No phenotype
80	CG8068	Suppressor of variegation 2-10	BL35956	RNAi	Disruption in eye patterning	No obvious modification
81	CG10719	Brain tumor	BL34646	RNAi	Disruption in eye patterning	No obvious modification

Cell fate decisions						
82	CG14992	Activated Cdc42 kinase	v39857	RNAi	No phenotype	No phenotype
				UAS	No phenotype	No phenotype
83	CG43398	Scribbled	v27424	RNAi	Disruption in eye patterning	No obvious modification
84	CG10961	TNF-receptor-associated factor 6	v110266	RNAi	No phenotype	No phenotype
			v16125	RNAi	No phenotype	No phenotype
85	CG3048	TNF-receptor-associated factor 4	v110766	RNAi	No phenotype	No phenotype
86	CG10873	p53	v38238	RNAi	No phenotype	No phenotype
87	CG5483	Leucine-rich repeat kinase	BL32457	RNAi	No phenotype	No phenotype
88	CG12919	Eiger		UAS-eiger	No phenotype	No phenotype
				UAS-regg	No phenotype	No phenotype
89	CG9375	Ras oncogene at 85D		UAS	Lethal	No obvious modification
90	CG17051	Dodo	v25210	RNAi	No phenotype	No phenotype
91	CG10079	Epidermal growth factor receptor	v107130	RNAi	Disruption in eye patterning	Suppressed by reduced Wwox levels; enhanced by increased Wwox levels.
92	CG15793	Downstream of raf1	BL33639	RNAi	Disruption in eye patterning	No obvious modification
93	CG10334	Spitz	v103817	RNAi	No phenotype	No phenotype
94	CG12559	Rolled	v109108	RNAi	No phenotype	No phenotype
95	CG4005	Yorkie	v104523	RNAi	Disruption in eye patterning	Inconsistent modification by altered *Wwox* levels
				UAS	Disruption in eye patterning	No obvious modification
96	CG12072	Warts	BL34064	RNAi	No phenotype	No phenotype
			BL41899	RNAi	Disruption in eye patterning	No obvious modification
			v106174	RNAi	Disruption in eye patterning	No obvious modification

97	CG11228	Hippo	BL33614	RNAi	No phenotype	No phenotype
			BL35176	RNAi	Disruption in eye patterning	No obvious modification
			v104169	RNAi	Disruption in eye patterning	No obvious modification
98	CG8544	Scalloped	BL35481	RNAi	No phenotype	No phenotype
99	CG33193	Salvador	BL32965	RNAi	No phenotype	No phenotype
			v101323	RNAi	No phenotype	No phenotype
Histone modification						
100	CG6170	Histone deacetylase 6	BL31053	RNAi	No phenotype	No phenotype
101	CG2128	Histone deacetylase 3	BL31633	RNAi	No phenotype	No phenotype
102	CG1770	Histone deacetylase 4	BL28549	RNAi	No phenotype	No phenotype
103	CG4107	Gcn5 ortholog	BL33981	RNAi	No phenotype	No phenotype
104	CG7471	Histone deacetylase 1	BL32241	UAS	No phenotype	No phenotype
			BL31616	RNAi	Disruption in eye patterning	No obvious modification
105	CG5229	Chameau	BL27027	RNAi	No phenotype	No phenotype
106	CG9696	Domino	BL31054	RNAi	No phenotype	No phenotype
Regulation of mitochondrial dynamics						
107	CG3210	Dynamin related protein 1	BL51647	UAS	No phenotype	No phenotype
			v44156	RNAi	No phenotype	No phenotype
			v44155	RNAi	No phenotype	No phenotype
108	CG8479	Optic atrophy 1 ortholog	v106290	RNAi	Disruption in eye patterning	No obvious modification
			BL32358	RNAi	Disruption in eye patterning	No obvious modification
109	CG3869	Mitochondrial assembly regulatory factor	105261	RNAi	No phenotype	No phenotype
			BL31157	RNAi	No phenotype	No phenotype
Unknown function						
110	CG30152		v105959	RNAi	No phenotype	No phenotype
			v38848	RNAi	No phenotype	No phenotype
111	CG7384		v7667	RNAi	No phenotype	No phenotype
112	CG4404		v48183	RNAi	No phenotype	No phenotype
113	CG7730		v47572	RNAi	No phenotype	No phenotype
114	CG4999	Tetraspanin 66E	v37572	RNAi	No phenotype	No phenotype

115	CG11377		v107205	RNAi	No phenotype	No phenotype
116	CG33129		v107365	RNAi	No phenotype	No phenotype
			v27554	RNAi	No phenotype	No phenotype

Table S2 Percentage of wing discs that are classified based on the mitochondrial morphology of their cells.

	All cells have tubular mitochondria	**All cells have a mixture of tubular and round mitochondria**	**All cells have fragmented mitochondria**	**Have a mixture of cells with different mitochondrial morphology**
↓Drp1	19%	17%	49%	15%
↓Drp1 + ↓Wwox	26%	17%	51%	6%
↓Drp1 + ↑Wwox	0%	4%	90%	6%

Chapter 5

Discussion and Conclusion

5.1 Summary of results

Reduced *WWOX* expression correlates with poor prognosis in many cancers (Aqeilan*, et al.* 2004a; Guler*, et al.* 2004; Park*, et al.* 2004; Fabbri*, et al.* 2005). In order to develop therapies that are able to compensate for reduced Wwox, it is important to understand the cellular mechanism(s) by which WWOX can act to suppress cancer cell growth. Studies *in vitro* have identified many WWOX binding partners, which are involved in a wide range of biological pathways (Chang*, et al.* 2001; Aqeilan*, et al.* 2004b; Aqeilan*, et al.* 2005b; Gaudio*, et al.* 2006; Bouteille*, et al.* 2009; Abu-Odeh*, et al.* 2014a). However, it is unknown which of these interactions are biologically significant and which could be required to slow cancer progression *in vivo*. The aims of this project include the investigation of Wwox function in Eiger/TNFα-mediated cell death, the determination of motifs required for discrete Wwox functions and the identification of novel cellular pathways in which Wwox can contribute *in vivo*. All identified functional interactors of Wwox in these studies are summarized in Table 5.1.

Table 5.1 Types of the cellular dysfunctions that can be modified by Wwox. "-" symbol indicates that no obvious modification was detected.

Sensitized background	**Model**	**↓ Wwox**	**↑ Wwox**
Induced cell death	↑ Eiger/TNFα	Suppress	Enhance
Induced tumorigenic cells	*Scrib* mutations	Enhance	-
Induced DNA damage	↓ Prp19	Enhance	-
Induced cell death	↓ EGFR	Suppress	Enhance
Induced defects in differentiation	↑ Yki	Enhance	Suppress
Induced mitochondrial fission	↑ Drp1	-	Enhance
Induced mitochondrial fission	↓ Marf	-	Enhance
Induced mitochondrial fission	↓ Opa1	Suppress	-

In addition, functions of a truncated Wwox variant, which contains two WW domains but lacks a complete SDR domain, were also tested in this study. Truncated Wwox can carry out some full length Wwox functions, namely enhancement of Eiger/TNFα-mediated cell death and suppression of spindle assembly checkpoint defects. In contrast, truncated Wwox mimicked the effect of reduced Wwox activity with respect to its functions in mitochondrial dysfunction or oxidative stress defects. This is consistent with truncated Wwox behaving as a dominant negative competitor, and suggests that SDR region is required for these processes.

5.2 Contribution of this project to WWOX-related studies

This study has shown that Wwox contributes to caspase activation induced by either ectopic expression of Eiger/TNFα, reduced *Prp19* levels or decreased *EGFR* levels. In addition, Wwox can also inhibit the detrimental effects induced by either ectopic expression of Yki or *Scrib* mutations. All these components are important for the regulation of cell survival, and their dysregulation can cause tumorigenesis (Nicholson, *et al.* 2001; Balkwill 2006; Pan 2010; Fang, *et al.* 2013). Results herein suggest that Wwox can suppress the impacts caused by altered levels of either *Scrib*, *Prp19*, *Yki* or *EGFR* by promoting the elimination of these cells. This uncovers the potential mechanism of how reduced *Wwox* levels could increase the risk of tumorigenesis.

In addition, Wwox can contribute to the regulation of mitochondrial dynamics, which is responsive to both metabolism and cell death. Ectopic expression of Wwox increases *Drp1*-mediated mitochondrial fission, uncovering a novel mechanism by which Wwox can contribute to caspase-dependent cell death. Mitochondrial fission segregates damaged mitochondria from the network, so that they can be phagocytosed and degraded by the autophagosome in a process called mitophagy. Another CFS gene, *Parkin* (spanning *FRA6E*), also plays a role in mitochondrial fission (Poole, *et al.* 2008; Buhlman, *et al.* 2014). Parkin is recruited to impaired mitochondria by Pink1 (Narendra, *et al.* 2008; Vives-Bauza, *et al.* 2010), and ubiquitinates several mitochondrial proteins, including MFN 1 and MFN 2 (Gegg, *et al.* 2010). This promotes fragmentation and marks the segregated mitochondria for mitophagic degradation (Gegg, *et al.* 2010; Ziviani, *et al.* 2010). This suggests that Wwox can also contribute to mitophagy by promoting mitochondrial fission, which is consistent with the role of Wwox in the suppression of mitochondrial dysfunction (Choo, *et al.* 2015).

Loss of WWOX function is associated with many neurological disorders, but the underlying mechanism in these cases is still unknown (Abdel-Salam*, et al.* 2014; Mallaret*, et al.* 2014; Mignot*, et al.* 2014; Ben-Salem*, et al.* 2015; Tabarki*, et al.* 2015; Valduga*, et al.* 2015). Defects in mitochondrial fission have also been reported in neurological conditions (Alexander*, et al.* 2000; Delettre*, et al.* 2000; Züchner*, et al.* 2004; Waterham*, et al.* 2007), indicating the possibility that mutations in WWOX contribute to neurological disorders through impaired capacity for the regulation of mitochondrial dynamics. Defects in mitochondrial fission decrease the efficiency of mitophagy and caspase-dependent cell death, resulting in the increase of cells with mitochondrial dysfunction (Goyal*, et al.* 2007; Youle and Van Der Bliek 2012). Damaged mitochondria can release mtDNA and formyl peptides to the extracellular matrix, and are recognized as mitochondrial-derived DAMPs. These molecules activate toll-like receptors (TLRs) in neighboring cells, and trigger innate immune system (Sun*, et al.* 2016). Activation of innate immunity is a hallmark for different neurological diseases, including Alzheimer's disease, Huntington's disease, Lou Gehrig's disease and Parkinson's disease (Heneka*, et al.* 2014; Richards*, et al.* 2016). It induces cell death not only in the damaged neural cells, but also in the neighboring cells, leading to a more extensive loss of neurons (Richards*, et al.* 2016). This innate surveillance-mediated cell death has also been hypothesized to be a common pathogenic pathway responsible for different neurological diseases (Richards*, et al.* 2016). WWOX can mediate mitochondrial fission to promote either mitophagy to remove damaged mitochondria, or caspase-dependent cell death when the number of impaired mitochondria excesses a tolerated threshold. This would regulate the activation of innate surveillance-mediated cell death that leads to neurological diseases. As defects in mitochondrial dynamics are also a known cause of different cancers and metabolic disorders (Grandemange*, et al.* 2009; Liesa*, et al.* 2009; de Moura*, et al.* 2010), we hypothesize that it is a potential common casual pathway in a spectrum of diseases that show altered *WWOX* expression. This new knowledge could contribute to the development and/or improvement of therapeutics that can restore WWOX function in these patients.

This work has also shown that some functions of WWOX do not require SDR enzyme activity. The truncated Wwox protein tested in this study is analogous to products of WWOX alternatively spliced variants that are highly expressed in cancer cells, particularly *WWOX isoform 2*. Results from this study show that if *WWOX isoform 2* can be translated into a truncated WWOX protein, it could potentially exhibit dominant negative activity in some metabolic pathways. It is worth noting that most cancer cells still retain one wild-type copy of

WWOX, suggesting that perturbation of just one allele of the *WWOX* gene is sufficient to contribute to tumorigenesis (Bednarek*, et al.* 2000; Ried*, et al.* 2000; Alsop*, et al.* 2008; Lewandowska*, et al.* 2009). As truncated WWOX isoforms, as well as lower levels of WWOX, have been detected together in cancer cells (Mahajan*, et al.* 2005), it is possible that cancer cells are affected by either the haploinsufficiency of WWOX*,* the dominant negative activity of truncated WWOX isoforms, or both.

One of the possible mechanisms that can lead to decreased *WWOX* expression and/or increased *WWOX* alternatively spliced variants is through breakage at *FRA16D* (Figure 1.4). This raises the question as to why a chromosomal fragile site such as *FRA16D,* that is susceptible to environmental stress, is located within a tumor suppressor gene. This relationship is conserved during evolution, suggesting the biological significance of large genes spanning fragile sites. Moderate mitochondrial fission enables the induction of mitophagy to clear up damaged mitochondria (Frank*, et al.* 2012), and allows the cell to swap their damaged mitochondria with neighboring normal cell (Ishikawa*, et al.* 2008). Reducing *WWOX* levels to less than 50% may prevent excessive fission of mitochondria that triggers caspase-dependent cell death. This could provide an opportunity to re-establish metabolic homeostasis and develop adaptive resistance to acute stressors in cells. We therefore hypothesize that breakage of *FRA16D* under environmental stress is intended to reduce WWOX activity, which would otherwise induce a potentially lethal cascade of cell death. Although this CFS breakage allows cells to cope with stressful conditions, it may leave residual mitochondrial dysfunction due to incomplete clearance of damaged mitochondria (Liu*, et al.* 2011; Zhou*, et al.* 2011; Choo*, et al.* 2015). Given that carcinogenesis is a stepwise process, these damaged cells are more susceptible to become cancer cells when they receive another deleterious "hit" by mutations in other genes. Breakage of *FRA16D* can also lead to the production of dominant negative truncated WWOX, which sensitizes cells to carcinogenesis by reducing full length WWOX functions. When these cells become cancerous, both mitophagy and mitochondrial transfer can be hijacked to promote survival and proliferation. Mitophagy has been shown to help the survival of cancer cells in hypoxic and poor nutrient environment (Lisanti*, et al.* 2010). In addition, cancer cells with defective mitochondria can also acquire functional mitochondria from adjacent normal cells to increase their energy production, which favors their proliferation and invasion (Caicedo*, et al.* 2015). Thus, it is possible that most cancer cells retain one functional *WWOX* allele (Lewandowska*, et al.* 2009), in order to have moderate levels of fissional mitochondria to promote mitophagy

and mitochondrial transfer. It has been shown that *Drp1* levels are upregulated in several cancer cell lines, including MDA-MB 231 breast and A549 lung cancer cell lines (Rehman *et al.* 2012, Zhao *et al.* 2013). *Drp1* levels are also higher in invasive breast cancer cell lines (MDA-MB 231 and MDA-MB 436) compared to non-metastatic cancer cell line (MCF-7) (Zhao *et al.* 2013), suggesting that mitochondrial fission has been hijacked in these cancer cells. Interestingly, *WWOX* expression is abolished in A549 lung cancer cell line (Fabbri *et al.* 2005), and it is 2.5 fold increased in MDA-MB 231 breast cancer cell line compared to MCF7 breast cancer cell line (Bednarek *et al.* 2001), demonstrating that *WWOX* levels are indeed inversely correlated with the expression of *Drp1* in these cancer cells. This supports the hypothesis that cancer cells down-regulate *Wwox* levels, in order to inhibit the mitochondrial fission. Together, this study has provided novel insight into the evolutionarily conserved relationship of the *FRA16D* CFS within the *WWOX* tumor suppressor gene.

5.3 Future directions

This study has shown that Wwox can contribute to caspase-dependent cell death induced by various apoptotic factors. Contribution of Wwox to *Drp1*-mediated mitochondrial fission is one possible mechanism responsible for this function. There may be other mechanisms of cell death regulated by Wwox, such as activating the apoptotic functions of its other binding partners, including p53 and p73 (Aqeilan*, et al.* 2004c). Similarly, Wwox might also suppress mitochondrial dysfunction through the regulation of Hif1α activity (Dayan*, et al.* 2013; Abu-Remaileh and Aqeilan 2014; Choo*, et al.* 2015). Further understanding of the mechanism of WWOX in the regulation of mitochondrial dynamics, and motifs required for this function, could potentially link these (and other) WWOX functions.

The SDR enzyme region of Wwox is required for some of the metabolic functions of Wwox, but neither the substrate(s) nor the product(s) are known. It has been hypothesized that the loop region within SDR domain (residues 265-285), as well as its extended C-terminal sequence, are substrate binding sites (Kavanagha*, et al.* 2008; Abu-Remaileh*, et al.* 2015; Richards*, et al.* 2015). Future studies could mutate these regions, in order to undertake high throughput screens for molecules that bind wild-type, but not mutant WWOX protein. This would allow for the identification of candidate substrates and products for WWOX enzymatic function. The SDR enzyme activity is necessary for the role of Wwox in suppressing mitochondrial dysfunction (Choo*, et al.* 2015). As such, either reduction of the substrate or

compensation for the product of WWOX activity could be a way to improve prognosis in cancer patients with low *WWOX* levels. Candidate drugs could be tested using Wwox functional assays that have been established in *Drosophila* (Stilwell, *et al.* 2006; Pandey and Nichols 2011).

5.4 Conclusion

Since the discovery of *WWOX* as the *FRA16D* CFS gene (Bednarek*, et al.* 2000; Ried*, et al.* 2000), many studies have focused on its reported role as a tumor suppressor. These studies have shown that lower levels of *WWOX* are associated with tumorigenesis and cause poorer prognosis to cancer patients (Aqeilan*, et al.* 2004a; Guler*, et al.* 2004; Park*, et al.* 2004; Fabbri*, et al.* 2005). In recent years, evidence has emerged that lower *WWOX* levels are correlated with different metabolic diseases (Lee*, et al.* 2008; Vasan*, et al.* 2009; Wang*, et al.* 2011a; Chang*, et al.* 2012; Yang*, et al.* 2012; Polfus*, et al.* 2013) and neurological disorders (Abdel-Salam*, et al.* 2014; Mallaret*, et al.* 2014). The cellular functions of WWOX are still not fully understood and it is unknown whether there are common pathways affected in the distinct diseases that are caused by lower *WWOX* levels.

Drosophila has been established as a model to investigate *in vivo* pathways to which Wwox can contribute. Results in this study have shown that Wwox can contribute to caspase activation in cells with various sensitized backgrounds, namely those induced by either ectopic expression of Eiger/TNFα, reduced *Prp19* levels or decreased *EGFR* levels. Wwox also plays a role in the suppression of cellular dysfunction induced by either ectopic expression of Yki, *Scrib* mutations or mitochondrial dysfunction (Appendix A) (Choo*, et al.* 2015). We hypothesize that the regulation of mitochondrial dynamics is a mechanism that underpins WWOX pro-apoptotic and metabolic functions. This study has also shown that the SDR enzyme region of Wwox is important for some metabolic functions *in vivo*. This project has therefore identified novel functions for WWOX and revealed those that are dependent on SDR enzyme region.

Appendices

Appendix A

Tumor suppressor WWOX moderates the mitochondrial respiratory complex

Choo, A., O'Keefe, L.V., Lee, C.S., Gregory, S.L., Shaukat, Z., Colella, A., Lee, K., Denton, D. and Richards, R.I., 2015. Tumor suppressor WWOX moderates the mitochondrial respiratory complex. Genes, Chromosomes and Cancer, 54(12), pp.745-761.

Candidate is involved in the following publication. Some contents of this publication have been mentioned in Chapter 1. It is included for reference and to provide a broader background for this work.

Statement of Authorship

Title of Paper	Tumor suppressor WWOX moderates the mitochondrial respiratory complex		
Publication Status	☑ Published	☐ Accepted for Publication	
	☐ Submitted for Publication	☐ Unpublished and Unsubmitted w ork w ritten in manuscript style	
Publication Details	Choo, A., O'Keefe, L.V., **Lee, C.S.**, Gregory, S.L., Shaukat, Z., Colella, A., Lee, K., Denton, D. and Richards, R.I., 2015. Tumor suppressor WWOX moderates the mitochondrial respiratory complex. *Genes, Chromosomes and Cancer*, *54*(12), pp.745-761.		

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Contribution to the Paper	Conceived and designed the experiments, performed experiments, analysed the data, contributed reagents / materials / analysis tools, writing and revision of the manuscript		
Overall percentage (%)	85%		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	8/12/14

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Tumor Suppressor WWOX Moderates the Mitochondrial Respiratory Complex

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Fragile site *FRA16D* exhibits DNA instability in cancer, resulting in diminished levels of protein from the *WWOX* gene that spans it. WWOX suppresses tumor growth by an undefined mechanism. WWOX participates in pathways involving aerobic metabolism and reactive oxygen species. WWOX comprises two WW domains as well as a short-chain dehydrogenase/reductase enzyme. Herein is described an *in vivo* genetic analysis in *Drosophila melanogaster* to identify functional interactions between WWOX and metabolic pathways. Altered WWOX levels modulate variable cellular outgrowths caused by genetic deficiencies of components of the mitochondrial respiratory complexes. This modulation requires the enzyme active site of WWOX, and the defective respiratory complex-induced cellular outgrowths are mediated by reactive oxygen species, dependent upon the Akt pathway and sensitive to levels of autophagy and hypoxia-inducible factor. WWOX is known to contribute to homeostasis by regulating the balance between oxidative phosphorylation and glycolysis. Reduction of WWOX levels results in diminished ability to respond to metabolic perturbation of normal cell growth. Thus, the ability of WWOX to facilitate escape from mitochondrial damage-induced glycolysis (Warburg effect) is, therefore, a plausible mechanism for its tumor suppressor activity. © 2015 Wiley Periodicals, Inc.

INTRODUCTION

Common fragile sites (CFS) are specific regions of chromosomes found in all individuals that have been observed to correspond to regions of DNA instability in various types of cancers (Richards, 2001). More than 200 different fragile sites have been induced to appear in human cells *in vitro*, and among them is the *FRA16D* CFS on chromosome 16 (Mrasek et al., 2010). Several CFS, including *FRA16D* have been identified as frequent sites of small homozygous deletions in cancer, consistent with a correlation between chromosomal fragility observed *in vitro* and *in vivo* DNA instability in cancer (Bignell et al., 2010). Numerous CFS are located within genes, leading to the suggestion that CFS-mediated perturbation of the function of these genes could have a role in cancer cell biology (Gao and Smith, 2014).

The *FRA16D* CFS is located within the *WW domain containing oxidoreductase* (*WWOX*) gene (Bednarek et al., 2000; Ried et al., 2000). Phylogenetic analyses have revealed that a WWOX ortholog is found in organisms as evolutionarily distinct as humans and sea sponge, indicating that WWOX likely performs a unique and primary biological function (Richards et al., 2015). Reduced levels of full-length WWOX have been reported in multiple types of cancers (Paige et al., 2001; Lewandowska et al., 2009; Gardenswartz and Aqeilan, 2014; Schrock and Huebner, 2014) and correlate with poorer prognosis (Pluciennik et al., 2006; Zelazowski et al., 2011). Evidence of individuals with low WWOX levels being more predisposed to developing lung cancers and gliomas also support a role for WWOX in tumorigenesis (Yang et al., 2013; Yu et al., 2014). Aberrant WWOX transcripts are often observed in tumors with reduced levels of full-length WWOX (Paige et al., 2001; Driouch et al., 2002; Ishii et al., 2003; Mahajan et al., 2005; Pluciennik et al., 2006). These transcripts mostly lack part, if not all, of the exons that encode its short-chain dehydrogenase/reductase (SDR) enzyme, with several of these transcripts shown to

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be translated into truncated protein products that lack the complete SDR enzyme (Ishii et al., 2003; Mahajan et al., 2005). These findings support the conclusion that loss of the SDR enzymatic activity of WWOX has a significant contribution to tumorigenesis. In efforts to define the pathway(s) in which WWOX participates, many studies have been directed at identifying protein-binding partners of the WW domains of WWOX. Many such partners and candidate pathways have been identified; however, the manner in which the SDR enzyme of WWOX contributes to such pathways is unclear as the SDR enzyme function has not been defined. Neither the enzymatic product of WWOX nor its endogenous substrate(s) have been identified.

WWOX has been shown to have tumor suppressor activity (Bednarek et al., 2001), although it does not seem to act as a highly penetrant classical tumor suppressor (Aldaz et al., 2014). Low levels of WWOX, rather than complete absence of WWOX, are often observed in tumors, indicating that the tumor suppressor function of WWOX does not fit Knudson's two hit hypothesis of tumorigenesis. An increase in incidence of tumor formation was reported in a WWOX knockout mouse model (Aqeilan et al., 2007). However, as of yet, there are no reports of neoplasia in a different null mutant mouse model (Ludes-Meyers et al., 2009; Ferguson et al., 2012), spontaneous null mutant rats (Suzuki et al., 2009), *Drosophila* null mutants (O'Keefe et al., 2005), or humans with nonsense mutations of WWOX resulting in epilepsy and mental retardation (Abdel-Salam et al., 2014; Mallaret et al., 2014). The mutant rodents, however, were all found to have metabolic disorders, including bone metabolic disorders, impaired steroidogenesis, and metabolic acidosis (Aqeilan et al., 2008, 2009; Ludes-Meyers et al., 2009; Suzuki et al., 2009). A metabolic role for WWOX is also supported by genome-wide association studies that showed associations between WWOX and triglyceride and high density-lipoprotein cholesterol levels in humans (Saez et al., 2010; Iatan et al., 2014).

Evidence of WWOX having a contribution to metabolism has also been reported in *Drosophila melanogaster* (O'Keefe et al., 2011). Although loss of WWOX has no obvious effect on the viability and lifespan of *Drosophila*, microarray and proteomic analyses of flies with altered levels of WWOX revealed qualitative and quantitative alterations in various metabolic mRNAs and/or proteins, particularly those involved in oxidative phosphorylation. Genetic analyses confirmed that WWOX has an important role in aerobic metabolism, through contributions to biological processes that require isocitrate dehydrogenase as well as superoxide dismutase (SOD). Alterations in WWOX levels also resulted in altered endogenous levels of reactive oxygen species (ROS). A recent study in WWOX knockout mouse embryonic fibroblasts has provided further support for WWOX having a role in aerobic metabolism, with loss of WWOX promoting aerobic glycolysis through a physical interaction between its WW domain and the hypoxia-inducible factor (HIF)-1α (Abu-Remaileh and Aqeilan, 2014). In addition, experiments in human cells have demonstrated that the WWOX gene responds to external factors that affect the metabolic state of cells (Dayan et al., 2013). Increased WWOX mRNA was observed under conditions that promote oxidative phosphorylation, whereas decreased levels were seen when cells are in a glycolytic and hypoxic state. Hence, it is not only the function of WWOX but also its regulation that is intrinsically integrated with the metabolic state of cells. Together, those results define WWOX as a major participant in pathways regulating metabolism and oxidative stress.

Altered metabolism is now recognized as one of the hallmarks of cancer cells (Hanahan and Weinberg, 2011) and is the focus of therapeutic targets for cancer treatment (Porporato et al., 2011). The molecular basis for this has not been defined; however, mutations identified in tumors include those affecting the tricarboxylic acid (TCA) cycle as well as the mitochondrial respiratory chain (Carew and Huang, 2002; Kroemer and Pouyssegur, 2008; Wallace, 2012). The identification of WWOX having a role in aerobic metabolism suggests that loss of WWOX is a contributor to the metabolic reprogramming of cells that accompanies tumorigenesis. Herein, we describe a genetic study aimed at detecting novel functional interactions between WWOX and components of metabolic pathways. Through this study, we have identified a novel functional contribution for WWOX in a mitochondrial-mediated pathway that is dependent upon its SDR enzyme function.

MATERIALS AND METHODS

Drosophila Husbandry

Drosophila stocks were maintained on fortified medium (1% agar, 1% glucose, 6% fresh yeast, 9.3% molasses, 8.4% coarse semolina, 0.9% acid mix, and 1.7% tegosept). Crosses were carried out

at 25°C. The w^{1118}, ey-GAL4, UAS-SOD1, UAS-SOD2, UAS-Catalase, and UAS-Foxo stocks were obtained from Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN). The WWOX lines have previously been described (O'Keefe et al., 2005, 2011). RNAi lines were obtained from Bloomington and the Vienna *Drosophila* Resource Centre (VDRC, Vienna, Austria).

Site-Directed Mutagenesis of WWOX

In vitro site-directed mutagenesis was used to introduce the different amino acid substitutions into the *Drosophila* WWOX open reading frame in pENTR/D-TOPO vector (Life Technologies, Pleasanton, CA). Constructs carrying the desired mutation were subcloned into the *Drosophila* transformation vector (Gateway pTW-attB, obtained from the *Drosophila* Genomics Resource Centre, Indiana University) by LR clonase recombination according to manufacturer's guidelines (Life Technologies). Constructs were then microinjected into the ZH-68E line (FBst0328401) by standard methods to obtain germline transformants. The constructs were inserted into the same site in the *Drosophila* genome to minimize insertional effects and to ensure that any observed differences are due to the particular mutation. Mutations in the transformed *Drosophila* lines were verified using sequence analyses conducted by Australian Genome Research Facility Ltd. (AGRF), Adelaide, Australia.

Primers used for the mutagenesis reactions were as follows (5′ → 3′):

Y29R: GACGGCACCGTTTGCCGTGTGAA CCAGCAGGGA;

W58F: CGAA TTACCCTTGGGCTTCGA GAAGTACTACG;

T127A: CACCGCGCTGATAGC GGGCGCAA ATTG;

Y288F: GGAGCATGATGGCCTTCAA CAAT GCCAAGC; and

P277A: GCATCACCTTTCGGCGCCGCCGG AGAAATAC

Western Blot Analyses

Western blot analyses were performed on adult flies ($n = 5$ per sample) as previously described (O'Keefe et al., 2011), with the following modifications: secondary antibody Anti-Rabbit DyLight 649 (1:2,500, Vector Laboratories, Burlingame, CA), mouse monoclonal anti-α-tubulin (1:2,000, Sigma, St. Louis, MO), and mouse anti-Cy3 (1:200, Jackson Laboratories, West Grove, PA).

Antibody Staining

Eye imaginal discs were dissected from late third instar larvae in 1× phosphate-buffered saline (PBS) and fixed in 4% formaldehyde for 20 min. Three 10-min washes were performed in PBST (1× PBS + 0.3% Triton-X-100). Discs were incubated with PBST containing 5% fetal calf serum for 1 h before incubation with primary antibody overnight at 4°C, followed by three 20-min washes with PBST and 2 h incubation with second antibody at room temperature in the dark before another round of three 20-min washes with PBST. Primary antibodies used were mouse anti-Elav-9F8A9 (1:10, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), rabbit anti-phospho-histone H3 (S10) (1:1,500, Cell Signaling, Danvers, MA), and rabbit anti-phospho-Akt (Ser473) (1:100, Cell Signaling). Secondary antibodies were anti-mouse Cy3 (1:200, Jackson Laboratories) and anti-rabbit-Dylight 649 (1:200, Vector Laboratories). Discs were mounted in 80% glycerol, and fluorescence was visualized using a Zeiss Axioplan 2 microscope. Images were compiled using Axiovision (Carl Zeiss) and Adobe Photoshop software.

Detection of ROS

CellROX® Deep Red Reagent (Life Technologies) was used to detect presence of ROS in live cells as a measure of oxidative stress. Eye imaginal discs were dissected from wandering third instar larvae in D22 media (insect culture media, pH6.8) and incubated in 5 μm CellROX® in D22 media for 15 min (in the dark at room temperature). The discs were then subjected to two quick washes with 1× PBS, fixed with 3.7% formaldehyde for 5 min, and then mounted in 80% glycerol for fluorescence visualization. The absorption/emission maxima of the dye is ~644/665 nm. Images were taken using the 20× PlanApo objective.

Light Microscopy

Photographs of exterior adult *Drosophila* eyes were taken using an Olympus SZX7 microscope fitted with a SZX-AS aperture diaphragm unit. Images were captured using an Olympus ColourView IIIU Soft Imaging System camera and AnalysisRuler image acquisition software. Images were prepared using Adobe Photoshop CS version 8.0. Anterior of the eye is positioned on the right of all images.

Phenotypic Quantification

Adult eyes were grouped into different phenotypic categories depending on analysis. Representative

images of each phenotypic category are presented in Supporting Information Figure S1. Eyes were scored from multiple independent crosses under identical conditions, and counts were pooled to obtain the final tally per genotype, with a minimum of 120 eyes scored per genotype. In the analyses performed to determine the effect of reduced WWOX levels, the adult eyes were grouped into three different categories based on the severity of the phenotype: "mild/normal"—normal or mild disorganization in the patterning of the ommatidia; "moderate"—disruption in the patterning of the ommatidia with the loss of photoreceptor cells; and "severe"—significant disruption in the gross structure and development of the eye (e.g., tissue outgrowth, presence of ectopic structures in the eye, or significant loss of cells resulting in decreased eye size). Chi-square test analyses were performed to compare the differences in the three categories. Analyses were also performed using Fisher's exact test to compare two categories—"severe" vs. combined "moderate + mild/normal" in order to determine whether reduced WWOX levels specifically affect the proportion of severe phenotypes. In the analyses performed to determine the suppressive effect of increased WWOX levels, eyes with any significant tissue disruption ("phenotype") were compared with eyes with no considerable tissue disruption ("no phenotype"), and Fisher's exact test analyses were performed to determine significance. Phenotypic scoring of the different WWOX over-expression mutant lines was performed in a blinded manner. For the larval phenotype, eye discs were examined for the presence or absence of an outgrowth with a minimum of 50 eye discs scored per genotype. Statistical significance was determined using Fisher's exact test, with $P = 0.05$ as the cut-off value for significance. All statistical analyses were performed using GraphPad Prism. Percentages of eyes or eye discs in each category were plotted for visual representation using Microsoft Excel. Appropriate controls were used in all experiments: an empty vector (EV) control was used for experiments with over-expression of genes, whereas a nonspecific RNAi (lacZ^{RNAi}) control was used for the experiments with RNAi lines to account for any effects due to nonspecific activation of the RNAi machinery.

Quantitative Real-Time polymerase chain reaction Assay

The quantitative polymerase chain reaction assays were performed using the protocols previously described by O'Keefe et al. (2011). For the assay done with *Drosophila* heads, 50 adult heads were used per sample, whereas for the assay with whole flies, five adult flies were used per sample. For all assays, three biological replicates were used for each genotype and triplicate reactions were performed for each biological replicate. mRNA levels were normalized against the *Drosophila* house-keeping gene ribosomal protein 49 (rp49). *t*-test analyses were performed using Microsoft Excel to determine significance, with $P = 0.05$ as the cut-off value for significance.

The quantitative real-time polymerase chain reaction primer pairs used are as follows: rp49—ATCGATATGCTAAGCTGTCGCAC/TGTCGATACCCTTGGGCTTG; WWOX—ATTGTGCTGTCATCCGAGTCG/ATTCTCCACGGGCAGGTTG; and ND42—GCTGAAGCCGCATTTGGTTAT/TCGCTGAACACCTTTGACTGC.

RESULTS

Screening Metabolic Pathways for Visible Cellular Dysfunction in *Drosophila*

Our previous study in *Drosophila* has shown that WWOX has an impact on metabolic pathways such as the TCA cycle and redox homeostasis (O'Keefe et al., 2011). To gain a better understanding of the role of WWOX in these metabolic pathways, an *in vivo* RNAi screen was conducted to identify novel functional interactions between WWOX and genes involved specifically in the TCA cycle and related oxidative processes. As altered WWOX expression does not result in any visible phenotype in *Drosophila* and a phenotype is required for the genetic dissection of pathway interactions, the approach taken was to induce cellular dysfunction (which would result in a phenotype) by targeting metabolic genes and to test whether WWOX is able to modify those phenotypes. Modification of the phenotype by WWOX would indicate that WWOX is able to functionally interact with those metabolic genes and would identify the types of cellular dysfunction that WWOX is able to affect.

Drosophila is a good model for such genetic interaction assays because of the capability of expressing multiple transgenes using the binary GAL4-UAS system (Brand and Perrimon, 1993) and the availability of various RNAi lines to reduce expression of candidate genes (Dietzl et al., 2007). This analysis was performed in the *Drosophila* eye, which has long been used as a model system to dissect biological pathways. RNAi constructs targeting metabolic genes were

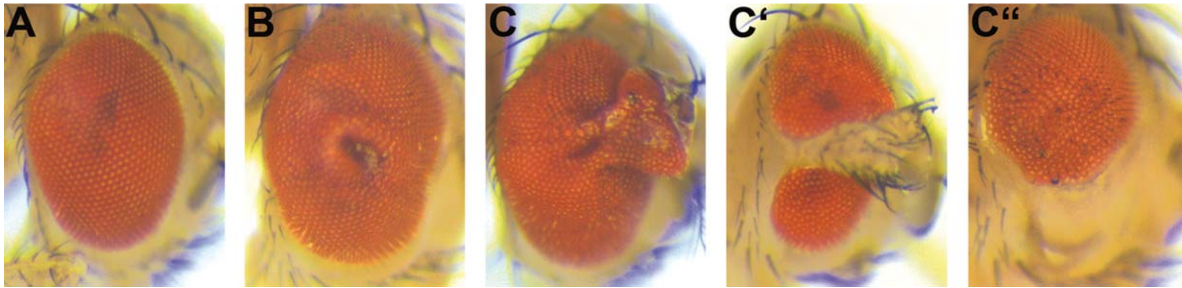

Figure 1. Adult eye phenotypes indicative of cellular dysfunction caused by reduced expression of mitochondrial respiratory complex genes. Decreased expression of ND23, ND42, ND75, CG7580, CoVa, and CoVb all results in a range of phenotypes: (A) mild or no disruption to the patterning of ommatidia; (B) absence of photoreceptor cells within the eye field forming a cavity in the eye; or phenotypes indicative of defects in development, which include (C) outgrowth of the eye tissue, (C′) presence of ectopic structures within the eye field, and (C″) significant loss of cells resulting in an overall decrease in eye size. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

expressed in the developing eye using the ey-GAL4 promoter together with binary GAL4-UAS system. Out of the 56 genes tested, reduction in expression of 10 genes resulted in a visible disruption to eye morphology, indicative of cellular dysfunction (Supporting Information Table S1).

Reduced Expression of Mitochondrial Respiratory Genes Result in Significant Cellular Dysfunction

Out of the 10 genes that, when reduced in expression, produced a characteristic eye phenotype, altered WWOX activity was able to modulate the cellular dysfunction caused by six of those genes (Supporting Information Table S1). The six genes (*ND23*, *ND42*, *ND75*, *CG7580*, *CoVa*, and *CoVb*) all encode components of the mitochondrial respiratory chain. Although some flies displayed mild or no disruption in eye tissue (Fig. 1A), a substantial percentage of flies exhibited a phenotype where there is loss of cells, resulting in a cavity in the surface of the adult eye (Fig. 1B). More severe eye phenotypes were observed in a small percentage of flies (Figs. 1C and 1C″), where there was outgrowth of eye tissue (Fig. 1C), presence of ectopic cuticle structures within the eye field (Fig. 1C′), or substantial loss of cells resulting in an overall decrease in eye size (Fig. 1C″). These phenotypes indicate that there is significant cellular dysfunction caused by decreased expression of mitochondrial respiratory complex genes. Targeting of ND42 and CoVa by different RNAi lines has previously been shown to result in defects in mitochondrial activity in *Drosophila* eye discs (Ambrus et al., 2013), and we have also previously shown that the use of our RNAi line to target ND42 results in significant loss of mitochondrial membrane potential (Shaukat et al., 2014). The production of the same phenotypes by independent RNAi targeting of six different genes of the mitochondrial respiratory complexes strongly support that the phenotypes are caused by loss of mitochondrial respiratory chain function.

Loss of WWOX Exacerbates Cellular Dysfunction Caused by Mitochondrial Defects

A phenotype scoring system was established to determine whether altered WWOX levels could significantly modulate the cellular dysfunction produced by reduced expression of these mitochondrial respiratory complex genes. Eyes were scored individually for a phenotype and grouped into different categories according to severity (mild/normal, moderate, and severe or phenotype and no phenotype; Supporting Information Fig. S1A). Reduced WWOX expression was found to enhance the mitochondrial-mediated cellular dysfunction, with significantly higher proportions of adult eyes exhibiting the severe phenotypes (tissue outgrowth, presence of ectopic structures, or reduced eye size). This was observed when WWOX expression was reduced by RNAi targeting, together with decreased expression of any one of the six mitochondrial respiratory complex genes (Fig. 2A and Supporting Information Figs. S1B and S1C) ($P \leq 0.01$, Supporting Information Table S2). Independent verification was obtained using a different RNAi line targeting WWOX as well as with loss of one functional copy of the *WWOX* gene in a heterozygous WWOX loss-of-function mutant (*WWOX*^{1}/+), together with decreased expression of the three different Complex I genes (Supporting Information Fig. S2 and Supporting Information Table S2). These results indicate that reduced WWOX expression contributes to the

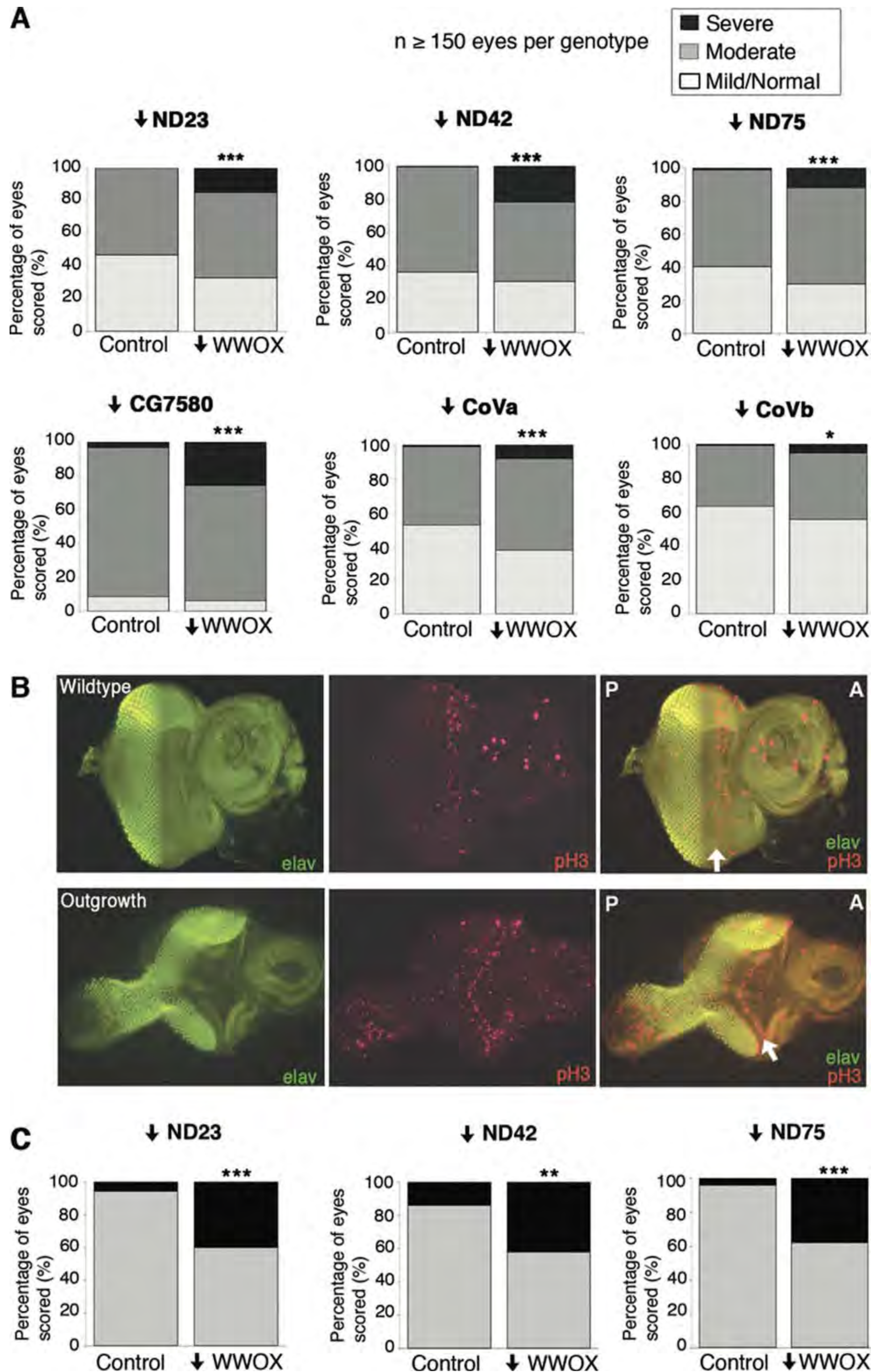

Figure 2. Decreased WWOX expression exacerbates the cellular dysfunction caused by reduced expression of mitochondrial respiratory complex genes. (A) Reduced WWOX levels result in higher proportions of adult eye phenotypes indicative of cellular dysfunction. Adult eyes were scored based on severity of phenotype (severe, moderate, and mild/normal). $^{*}P < 0.01$ and $^{***}P \leq 0.001$, compared with respective controls, determined by χ^2 test. *P*-values and further statistical analyses are presented in Supporting Information Table S2. $n \geq 150$ eyes per genotype. (B) Outgrowth phenotype in larval eye discs. The protrusion is characterized as an increased region of differentiated cells (neuronal cells stained with anti-elav, green) behind the morphogenetic furrow (MF, indicated by white arrow). Differentiation occurs in a sequential manner from posterior (P) to anterior (A) of the disc with mitotic cells (stained with anti-phospho-histone H3 (pH3), red) normally observed anterior of the MF. In discs with the outgrowth phenotype, mitotic cells are observed posterior of the MF at the tip of the protrusion. (C) Reduced WWOX levels result in higher proportions of larval eye discs with the outgrowth phenotype. $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ compared with control determined by Fisher's exact test (Supporting Information Table S2). $n \geq 50$ discs per genotype. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

dysfunction caused by loss of these mitochondrial respiratory complex genes.

Similar enhancement of cellular dysfunction by decreased WWOX levels was observed in third instar larval eye imaginal discs, which are the developmental precursors to the *Drosophila* adult eye (Figs. 2B and 2C). An outgrowth phenotype, characterized as a protrusion from the eye disc, was seen in a small proportion of larvae when mitochondrial dysfunction was induced and is indicative of defects in cellular differentiation and proliferation (Fig. 2B). The abnormal protrusion appears to be an extension of the eye tissue and is comprised of an increased region of both differentiated cells (detected by elav, a marker of differentiated neurons) and mitotic cells that are abnormally distributed (as stained by anti-phospho-histone H3, Fig. 2B). In the larval eye disc, differentiation of eye cells occurs in a sequential manner from the posterior to the anterior side of the disc, following the movement of the morphogenetic furrow (MF). Mitotic cells are usually observed anterior of the MF where cells are yet to differentiate. In the eye discs with the outgrowth phenotype, mitotic cells were observed posterior of the MF at the distal tip of the protrusion, suggesting that there is proliferation of cells driving the outgrowth of tissue. A significantly higher proportion of this outgrowth phenotype was observed in larvae with reduced expression of both WWOX and the Complex I gene (Fig. 2C). These data further support the conclusion that reduced WWOX expression contributes to mitochondrial-mediated cellular dysfunction.

Increased WWOX Levels Suppress the Tissue Disruption Caused by Mitochondrial Defects

Given that decreased WWOX is able to exacerbate the tissue disruption caused by mitochondrial respiratory complex defects, *WWOX* cDNA was over-expressed together with reduced expression of the mitochondrial respiratory complex genes to determine whether increased WWOX is able to suppress the phenotype. The phenotypes at the adult stage were examined to ascertain whether increasing WWOX levels had any significant lasting effect throughout cellular development. The proportions of adult eyes displaying any considerable tissue disruption were compared with the proportions of normal eyes. It was observed that for the three Complex I genes and two Complex IV genes, increased WWOX expression resulted in significantly higher proportions of normal eyes and lower proportions of eyes with any considerable tissue disruption (Fig. 3A) ($P < 0.001$, Supporting Information Table S3). This demonstrates that increased WWOX expression is able to suppress some of the cellular dysfunction caused by mitochondrial defects. In the case of the highly penetrant Complex III gene phenotype, however, increased WWOX expression was unable to significantly rescue the tissue disruption, suggesting that there may be a particular threshold of cellular dysfunction that increased WWOX is unable to overcome. Nevertheless, the data altogether illustrates that WWOX is involved in the maintenance of cellular homeostasis in response to mitochondrial defects, with low WWOX levels resulting in further perturbation of cellular dysfunction, whereas elevation of WWOX levels is able to restore homeostasis in some cells, thus leading to a suppression of the phenotypes (Fig. 3B).

The SDR Catalytic Active Site of WWOX Is Required for Its Functional Interaction with the Mitochondrial Complex Genes

The WWOX protein contains distinct functional elements that are well conserved across species. These conserved regions include two WW domains and essential components of an SDR enzyme. The WW domains have been shown to physically interact with various proteins with a PPxY and LPxY motif (Aqeilan et al., 2004; Hu et al., 2004; Abu-Odeh et al., 2014a), and phosphorylation of the first tyrosine residue (Tyr33) in the first WW domain is required for WWOX-mediated apoptosis in the nucleus (Chang et al., 2003). Not much is currently known about the endogenous function of the SDR enzyme. It is classified as a SDR enzyme based on amino acid sequence homology, which includes the presence of a putative NAD(H) or NADP(H) co-factor binding motif (TGxxxGxG) as well as a catalytic active site motif (YxxxK), characteristic of the classical SDR family of enzymes.

To determine which functional elements of WWOX are essential for the observed modulation of cellular dysfunction caused by mitochondrial defects, mutations were generated in amino acids that have previously been shown or are predicted to be critical for WWOX function (Figs. 4A and 4B and Supporting Information Table S4). The mutagenesis targeting was based on studies that have demonstrated that such mutations are able to abolish or decrease the activity of WWOX or other similar proteins (Oppermann et al., 1997;

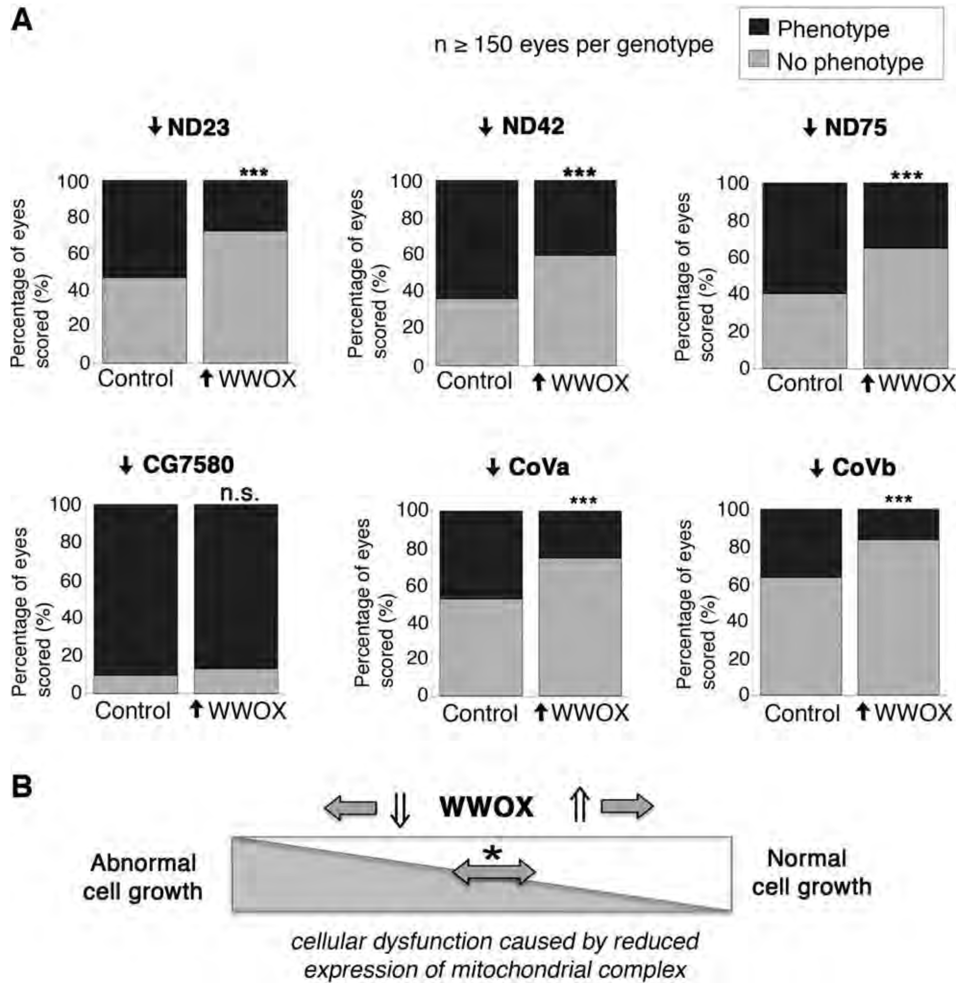

Figure 3. Increased WWOX expression rescues some of the cellular dysfunction caused by reduced expression of mitochondrial respiratory complex genes. (A) Over-expression of WWOX cDNA results in higher proportion of normal eyes when expressed together with decreased levels of ND23, ND42, ND75, CoVa, and CoVb. Eyes scored based on the presence or absence of a phenotype. $***P \leq 0.001$ compared with respective controls determined by Fisher's exact test (Supporting Information Table S3), n.s. indicates nonsignificance. $n \geq 150$ eyes per genotype. (B) Model for the moderation of mitochondrial complexes by WWOX. Endogenous WWOX is both a regulator of metabolism and responsive to changes in metabolism. It normally acts over a range of endogenous levels (*) in the maintenance of cellular homeostasis. Reduction in WWOX levels below this range limits its ability to contribute to the maintenance of homeostasis, resulting in an increased frequency of the cell growth phenotype (due to mitochondrial dysfunction). Elevation in WWOX levels above this range, on the other hand, restores homeostasis and rescues cells from the cellular dysfunction produced by deficiencies in the mitochondrial complexes.

Nakajin et al., 1998; Koepf et al., 1999; Filling et al., 2002; Aqeilan et al., 2004; Cancemi et al., 2011). Transgenes containing these specific mutations were expressed in *Drosophila* to test for their ability to suppress the tissue disruption caused by decreased expression of a Complex I gene, *ND23*.

Comparable levels of WWOX protein were observed in *Drosophila* ubiquitously expressing the wildtype and mutant forms of WWOX except for the mutant form that contains a mutation in the cofactor binding site in the SDR region (T127A) (Fig. 4C), which suggests that the mutation results in a significant reduction in stability of the WWOX protein. The same mutation in another SDR enzyme has previously been shown to alter cofactor binding preference and abolish some (but not all) of its enzymatic activity (Oppermann et al., 1997; Filling et al., 2002). It was thought that this threonine residue in the cofactor-binding motif does not bind to the cofactor itself but is involved in stabilizing the framework for cofactor positioning. Hence, it is possible that although this mutation may not necessarily abolish all enzymatic SDR activity, it is able to affect protein folding and, thus, overall stability of the WWOX protein, resulting in lower levels of protein being produced.

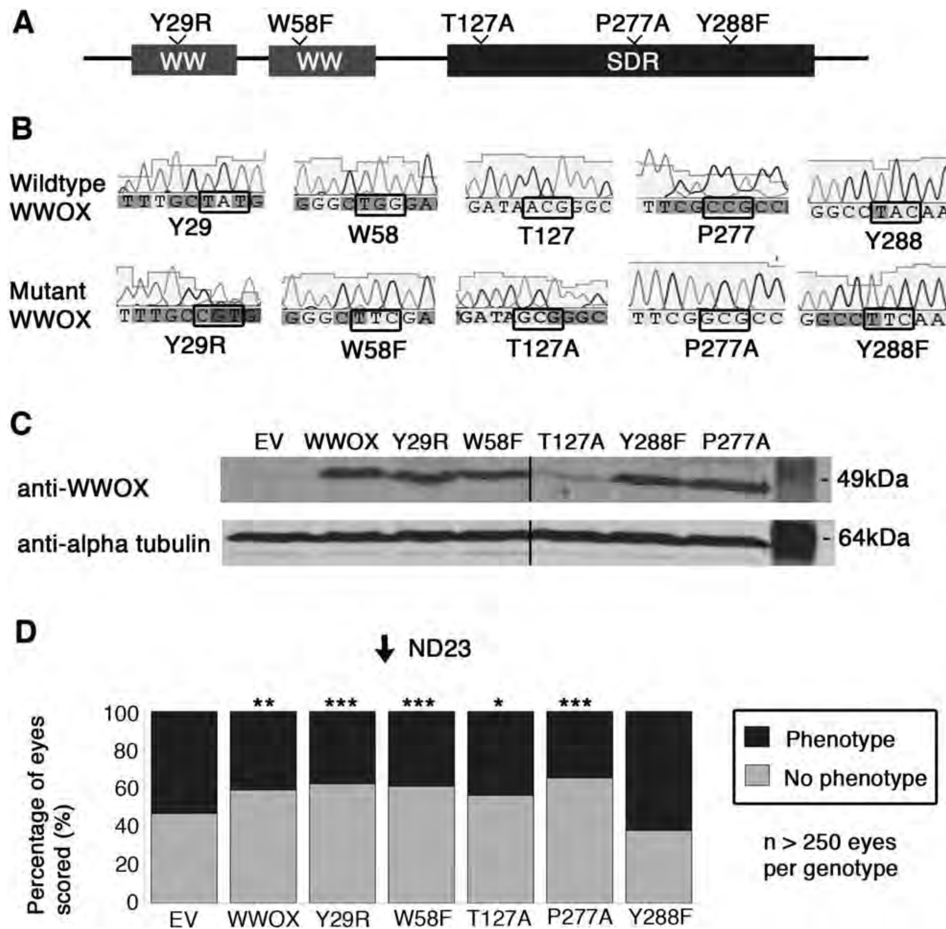

Figure 4. Catalytic active site in the SDR region of WWOX required in its suppression of tissue disruption caused by mitochondrial defects. (A) Critical amino acid residues mutated in the different functional elements of WWOX. (B) Sequencing results of nucleotide substitutions giving rise to the different amino acid mutations. (C) Comparable levels of WWOX protein detected by Western blot analysis in WWOX mutant lines compared with the wildtype control (WWOX), except for lower levels observed in the cofactor binding mutant (T127A). Alpha tubulin used as a loading control. Only endogenous WWOX is present in the empty vector (EV) control, which was too low to be detected in this analysis. A lane was removed from the gel image between the W58F and T127A samples during processing of the image (as indicated with the black line delineating the boundary between the samples); however, this does not affect the data that are presented. (D) Different WWOX mutant lines tested for their ability to suppress the tissue disruption caused by decreased ND23 expression. Proportion of eyes with presence or absence of phenotype compared with EV control. $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$, indicative of suppression compared with EV control determined by Fisher's exact test. *P*-values and further statistical analyses are presented in Supporting Information Table S5. $n \geq 250$ eyes per genotype.

As previously seen, over-expression of the wildtype WWOX protein was able to suppress the tissue disruption caused by decreased expression of ND23 as demonstrated by the higher proportion of eyes displaying no considerable tissue disruption. Similar suppression was evident with over-expression of four mutant forms of WWOX (Y29R, W58F, T127A, and P277A), indicating that these specific amino acid residues are not required for this suppressive function of WWOX. The Y288F mutant, however, was unable to rescue the tissue disruption (Fig. 4D and Supporting Information Table S5); rather the disruption seems to be slightly stronger in this mutant. The lack of suppression due to this Y288F mutation was also observed when tested with two other Complex I genes, ND42 and ND75 (Supporting Information Fig. S3 and Supporting Information Table S6). The Y288F mutant contains a mutation in the catalytic active site in the SDR region—such a mutation has previously been shown to completely abolish enzymatic activity of other SDR proteins (Nakajin et al., 1998; Filling et al., 2002). Comparable levels of protein were observed for the wildtype and Y288F mutant form of WWOX (Fig. 4C), indicating that the lack of suppression by the Y288F mutant was not due to insufficient WWOX protein being expressed. Thus, this demonstrates that the SDR enzymatic activity of WWOX is required for its cellular response to mitochondrial defects.

Reactive Oxygen Species Are Effectors of the Mitochondrial-Mediated Cellular Dysfunction

Mitochondrial respiratory complex dysfunction has been shown to promote tumor progression through the production of high levels of ROS (Sharma et al., 2011). We have previously shown that decreased expression of ND42, a component of the mitochondrial respiratory Complex I, in the *Drosophila* wing disc produces an increased level of ROS that is detectable by CellRox® staining (Shaukat et al., 2014). Similar CellRox® stains were performed on the larval eye discs with the mitochondrial-induced outgrowth phenotype. Punctate staining corresponding to presence of ROS was detected in the outgrowth region of the eye discs (Fig. 5A), which demonstrates that the mitochondrial dysfunction can result in increased levels of ROS. Over-expression of antioxidant enzymes catalase, SOD1, or SOD2 was able to significantly reduce the proportion of larval eye discs with the outgrowth phenotype (Fig. 5B and Supporting Information Table S7). Conversely, decreased expression of SOD1 or SOD2 significantly increased the proportion of the outgrowth phenotype (Fig. 5C and Supporting Information Table S7). These results indicate that ROS generated by mitochondrial dysfunction are effectors of the outgrowth phenotype.

Requirement of Akt and Foxo in the Mechanism of Cellular Dysfunction Caused by WWOX-Associated Mitochondrial Defects

Because mitochondrial complex deficiencies have been associated with tumorigenesis, the mitochondrial dysfunction-mediated cellular dysmorphology is of particular interest. Other genes that are known to be commonly involved in tumorigenesis were tested to identify potential common pathways involved in this model. Several recent studies, in particular, have shown that inhibition of the mitochondrial respiratory complex results in increased phosphorylation of the *v-AKT* murine thymoma viral oncogene (AKT) and subsequently activation of AKT-mediated signaling pathways, leading to malignancy (Pelicano et al., 2006; Sharma et al., 2011; Santidrian et al., 2013). Aberrant AKT activation has been reported in numerous cancers and has been described as one of the most common molecular changes in tumor cells (Bellacosa et al., 2005). Furthermore, WWOX has been shown to regulate AKT activation (Hu et al., 2014). Although there are three known AKT proteins in humans, there is only one identified AKT ortholog (Akt) in *Drosophila*. Detectable staining using a monoclonal antibody specific for phosphorylated Akt (pAkt) was observed in the positive control Dp110-overexpressing discs around individual ommatidia (Fig. 6A, second row panels) compared with the weaker staining in wildtype eye discs corresponding to low levels of endogenous pAkt (Fig. 6A, first row panels). pAkt staining was also detectable in the mitochondrial dysfunction discs (Fig. 6A, third row panels), suggesting that there is elevated Akt activation in the eye discs that have mitochondrial dysfunction. Reduction of Akt levels using a RNAi line, which has previously been shown to strongly reduce Akt protein levels as well as pAkt function (Ye et al., 2012), significantly suppressed the outgrowth phenotype caused by decreased expression of ND42, with a lower proportion of eye discs exhibiting the phenotype compared with the corresponding control (Fig. 6B and Supporting Information Table S7). This result identifies Akt signaling as the pathway that mediates the outgrowth phenotype.

A well-known target of AKT signaling is the forkhead box O (FOXO) transcription factor (Jünger et al., 2003; Kramer et al., 2003; Greer and Brunet, 2005; Calnan and Brunet, 2008), which can function as a tumor suppressor by upregulating various target genes involved in detoxification of ROS, cell cycle arrest, DNA damage repair as well as autophagy (Calnan and Brunet, 2008). Activated AKT signaling results in phosphorylation of FOXO in response to cellular stimuli, such as oxidative stress (Vurusaner et al., 2012), and causes it to be retained in the cytoplasm instead of translocating into the nucleus to activate transcription of its target genes, thus inhibiting its tumor suppressive function. AKT-mediated inhibition of FOXO activity has been reported in various cancers and has been thought to be a contributing factor to tumor progression (Greer and Brunet, 2005). *Drosophila* has only one FOXO gene, which contains conserved Akt phosphorylation sites and has been shown to be a critical target of *Drosophila* Akt (Jünger et al., 2003; Kramer et al., 2003; Puig et al., 2003). Overexpression of Foxo in larval eye discs with decreased levels of ND42 was able to suppress the larval eye disc outgrowth phenotype, with a lower proportion of eye discs displaying that phenotype (Fig. 6C and Supporting Information Table S7). Thus, it seems that Foxo activity is able to reduce or prevent the mitochondrial defect-mediated cellular dysfunction, demonstrating that Foxo has either a protective or compensatory role in cells with mitochondrial dysfunction.

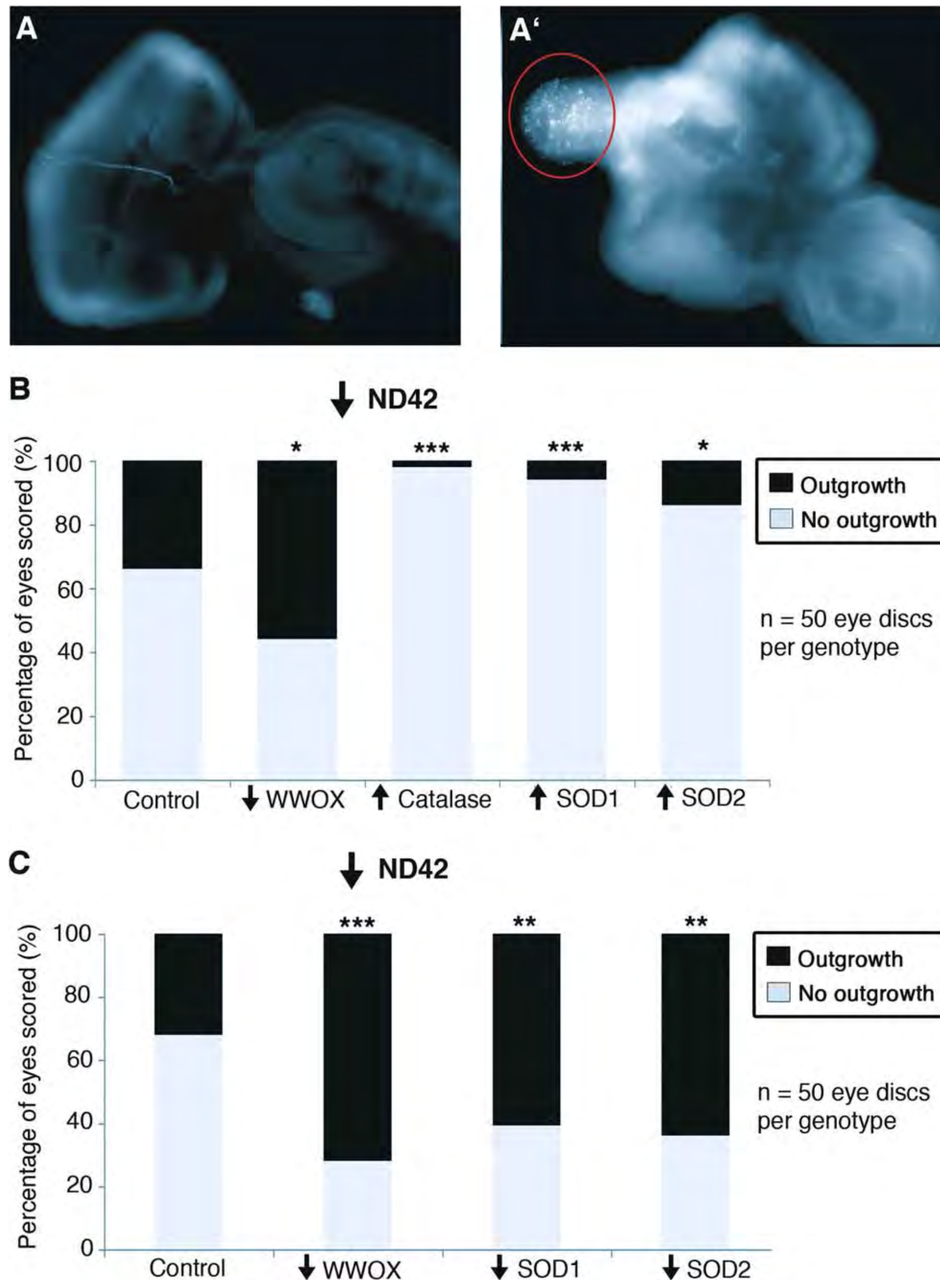

Figure 5. Reactive oxygen species (ROS) are effectors of the larval eye disc outgrowth phenotype. (A) Presence of ROS detected by CellRox® staining in the outgrowth region of the affected larval eye discs. Punctuate staining is observed in the outgrowth region of affected discs (A′) but not in wildtype discs (A). (B, C) Changes in the levels of antioxidant enzymes modify the proportion of the outgrowth phenotype caused by decreased expression of ND42. A recombinant ey, $ND42^{RNAi}$ fly line that has a stronger phenotype was used for the modification analyses. The $WWOX^{RNAi}$ line was used as a positive control for the experiment. $*P < 0.01$, $**P < 0.05$, and $***P \leq 0.001$ compared with respective controls determined by Fisher's exact test. Exact values are presented in Supporting Information Table S7. $n = 50$ discs per genotype for both experiments. Expression of the different antioxidant enzyme lines by themselves has no effect on the morphology of the eye disc. (B) Overexpression of antioxidant enzymes, such as catalase, SOD1, and SOD2, result in significantly lower proportion of the outgrowth phenotype compared with the control (which has overexpression of an empty vector, EV). (C) Decreased expression of SOD1 and SOD2 result in significantly higher proportion of the outgrowth compared with the control (which is expressing a nonspecific RNAi, $lacZ^{RNAi}$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Participation of HIF-1∝ and Autophagy in the Mitochondrial-Mediated Cellular Dysfunction

Recent studies have shown a reciprocal regulatory relationship between the HIF-1∝ and WWOX (Abu-Remaileh and Aqeilan, 2014); hence, it was of interest to examine whether HIF-1∝ has any contribution to the larval eye disc outgrowth phenotype. HIF-1∝ has been shown to have contrasting

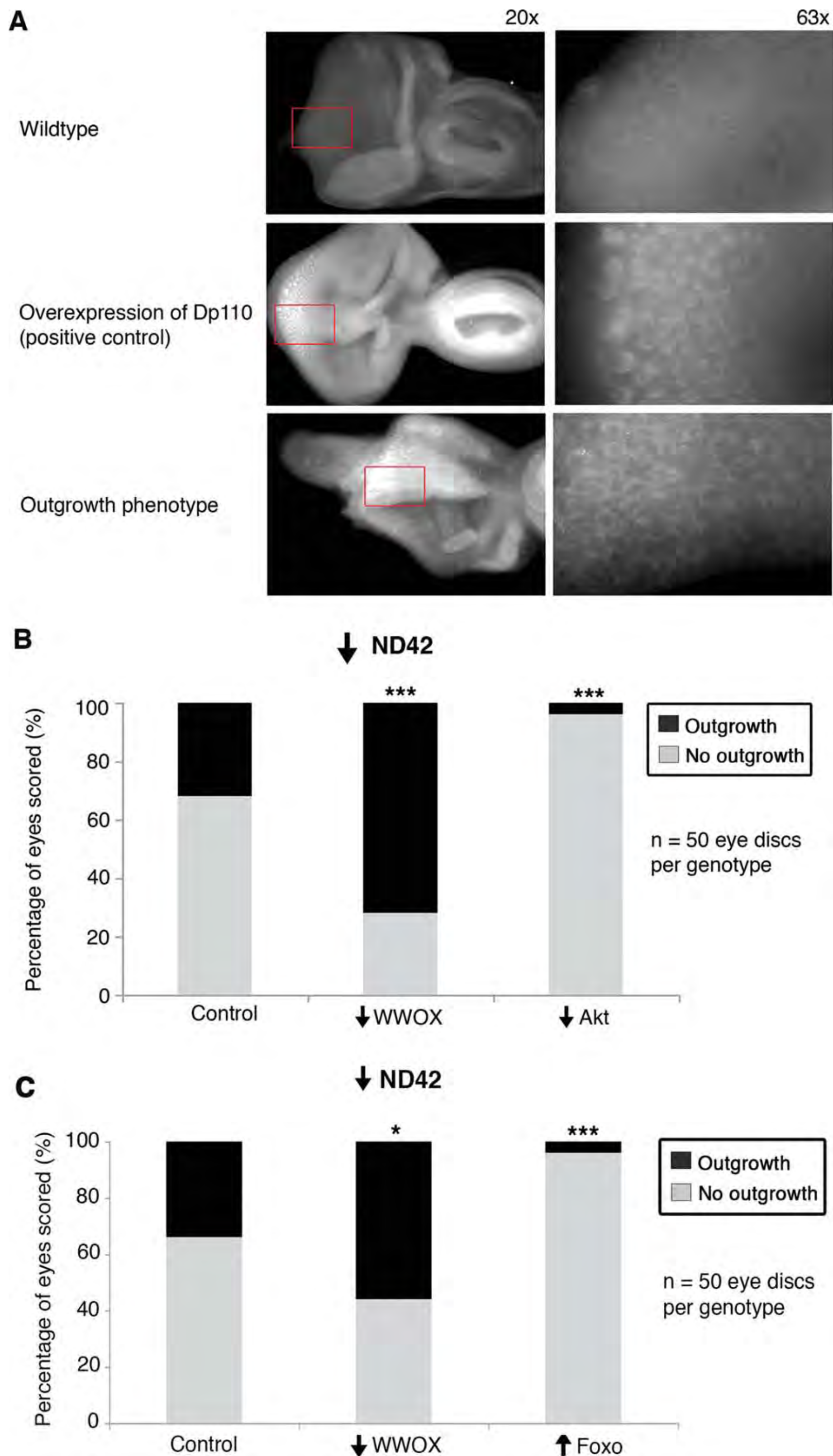

Figure 6. Requirement for Akt and Foxo in the larval eye disc outgrowth phenotype induced by mitochondrial dysfunction. (A) Detection of increased pAkt staining in third instar larval eye imaginal discs. Low levels of pAkt staining was present in the wildtype eye disc, although stronger pAkt staining was observed in discs with the outgrowth phenotype. Eye discs with ectopic expression of *Drosophila phosphoinositide 3-kinase* (Dp110) driven by the ey-GAL4 promoter were used as a positive control for pAkt staining. Panels on the right were taken at 20× PlanApo objective, whereas panels on the left were taken using the 63× PlanApo objective. The 63× images were taken of the area highlighted with the red boxes in the 20× images. (B) Decreased Akt expression by RNAi targeting results in suppression of the outgrowth phenotype, with significantly lower proportion of eye discs showing the phenotype (4%) compared with the lacZ^{RNAi} control (34%) (***$P = 0.0004$). $n = 50$ eyes per genotype. (C) Increased expression of Foxo is able to suppress the mitochondrial-mediated larval eye disc outgrowth phenotype, with a lower proportion of the phenotype (4%) observed compared with the EV control (34%) (***$P = 0.0002$). $n = 50$ eyes per genotype. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

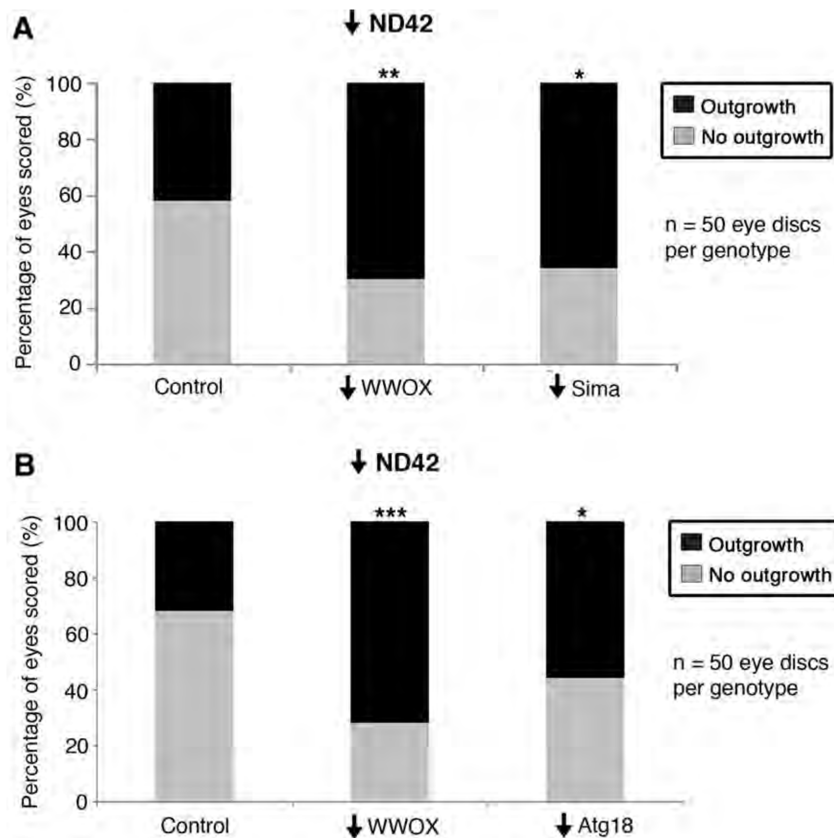

Figure 7. The mitochondrial-mediated cellular outgrowth is affected by Sima/HIF-1α levels as well as autophagy. (A) Decreased expression of Sima/HIF-1α by RNAi targeting results in higher proportion of the outgrowth phenotype (66%) compared with the $lacZ^{RNAi}$ control (42%) (*$P = 0.0268$). The result obtained with decreased Sima is parallel with that obtained with decreased Wwox. $n = 50$ eyes per genotype. (B) Decreasing expression of an autophagy gene, *Atg18*, results in enhancement of the cellular dysfunction caused by mitochondrial defects. A significantly higher proportion of larval eye disc outgrowth phenotype (56%) was observed compared with the $lacZ^{RNAi}$ control (32%), *$P = 0.0262$. $n = 50$ eye discs per genotype.

roles in tumorigenesis. Although most studies are indicative of activated HIF-1∝ having a protumorigenic function (Semenza, 2002), there are some studies that suggest that HIF-1∝ is able to negatively regulate tumor growth through induction of apoptosis and/or autophagy (Sowter et al., 2001; Rankin and Giaccia, 2008; Chiavarina et al., 2010). Indeed, the *Drosophila* ortholog of HIF-1∝, Similar (Sima), has been shown to be a negative regulator of cell growth (Romero et al., 2007). Decreased expression of Sima/HIF-1∝ was found to result in a significant increase in the proportion of the outgrowth phenotype (Fig. 7A and Supporting Information Table S7), consistent with its role as a negative regulator of cell growth. The result obtained with decreased Sima/HIF-1∝ expression was parallel to that with decreased expression of WWOX, suggesting that the regulatory relationship between WWOX and HIF-1∝ in this context could be a control mechanism to cope with the perturbation of cellular homeostasis (Dayan et al., 2013).

Sima/HIF-1∝ has been shown to have an effect on autophagy in *Drosophila*, with RNAi targeting of Sima resulting in the inhibition of compensatory autophagy (Löw et al., 2013). Autophagy was previously found to be a protective mechanism for restricting metastatic growth caused by mitochondrial Complex I defects in cancer cells (Santidrian et al., 2013); thus, it is possible that autophagy could also be a normal cellular mechanism used to restrict the larval eye disc outgrowth caused by mitochondrial defects in this model. In addition, *Drosophila* Foxo has also been demonstrated to be required for maintaining expression of an essential autophagy gene, *Atg18* (Omata et al., 2014). Thus, expression of Atg18 was decreased by RNAi targeting to determine any contribution to the mitochondrial defect phenotype. Decreased Atg18 expression resulted in an enhancement of the larval eye disc outgrowth phenotype, with higher proportions of the phenotype compared with the corresponding control (Fig. 7B and Supporting

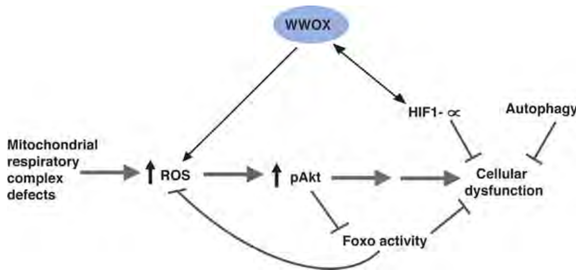

Figure 8. Mechanism for the contribution of WWOX to cellular homeostasis. Deficiencies in the mitochondrial respiratory complexes results in cellular dysfunction through increased production of ROS and activation of the Akt signaling pathway, with the effects moderated by Foxo and HIF-1∝ levels as well as autophagy. WWOX has been shown to maintain cellular homeostasis caused by such mitochondrial respiratory complex deficiencies. It is proposed that WWOX acts to moderate excesses in metabolism and facilitate the transition between glycolysis and oxidative phosphorylation through regulation of ROS and reciprocal interactions with HIF-1∝. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Information Table S7), indicating that the normal level of autophagy is able to restrict the cellular dysfunction caused by mitochondrial defects.

Genetic dissection herein, of the molecular pathway by which mitochondrial dysfunction leads to cellular dysmorphology, has revealed a mechanism that is not only sensitive to the level of WWOX enzyme activity but also mediated by ROS and AKT and influenced by the levels of HIF-1∝ and autophagy (Fig. 8).

DISCUSSION

The study herein utilized *D. melanogaster* to genetically dissect pathways in which WWOX participates. In previous studies, WWOX has been shown to have a role in metabolism, and therefore, an RNAi screen was performed to identify those components of metabolism that give rise to a phenotype, which could then be modified by altering levels of WWOX to below and/or above endogenous levels. WWOX has a functional relationship with *IDH*, a component of the TCA cycle and *SOD1*, a regulator of ROS; therefore, genes involved in the TCA cycle and oxidative processes emanating from the TCA cycle were examined to identify other novel functional interactions. Out of the 56 genes tested in the developing eye of *Drosophila*, 10 were found to result in cellular dysmorphology. WWOX was able to modulate the cellular dysmorphology seen with six of these genes, all of which are components of the mitochondrial respiratory chain. Reduction in endogenous levels of WWOX exacerbated the cellular dysfunction, resulting in a higher proportion of eyes displaying severe dysmorphology phenotypes in adults and outgrowth of tissue in the larval eye imaginal discs. These severe adult eye dysmorphology phenotypes (which include tissue outgrowths, presence of ectopic structures, and reduced eye size) as well as the larval eye disc outgrowth are evidence of defects in growth control, differentiation, and survival, all of which are processes that have been associated with tumorigenesis. This demonstrates that loss of WWOX together with these mitochondrial complex genes contributes to cellular properties often exhibited by cancer cells.

Defects in mitochondrial respiratory complexes have been reported in cancer, with these defects shown to promote the tumorigenic potential of cancer cells (Santidrian et al., 2013). One of the major effects of mitochondrial dysfunction is the production of high ROS levels, which promote tumor progression and metastasis (Sharma et al., 2011; Taddei et al., 2012). Indeed, the larval eye disc outgrowth phenotype produced by defects in mitochondrial function was shown to correlate with high levels of ROS. WWOX has been found to have an effect on ROS levels, although the resultant effect appears to be context dependent (O'Keefe et al., 2011; Shaukat et al., 2014). We have previously shown that decreasing WWOX levels in cells with chromosomal instability results in high levels of ROS and DNA damage (Shaukat et al., 2014); hence, there is a precedent for loss of WWOX contributing to cellular dysfunction that is mediated by high ROS levels. Loss of WWOX by itself has now also been associated with impaired DNA damage response, potentially driving genomic instability (Abu-Odeh et al., 2014b, Aqeilan et al., 2014); thus, it is likely that this is through the modulation of ROS levels.

Significantly, increased WWOX expression above endogenous levels was able to suppress the tissue disruption caused by Complex I and IV genes. This indicates that increased WWOX levels are able to provide a more favorable outcome for cells that have mitochondrial dysfunction. Evidence pointing toward reduced mitochondrial respiration has recently been shown in WWOX-deficient mice (Abu-Remaileh and Aqeilan, 2014), suggesting that WWOX may be able to affect mitochondrial function. There are also other studies that observed up-regulation of WWOX expression in damaged mitochondria of mice with retinal defects (Chen et al., 2005) and that increasing WWOX expression can induce mitochondrial-mediated apoptosis in various cancer cells (Qin et al., 2006; Iliopoulos et al., 2007; Zhang et al., 2012; Cui et al., 2013). These

studies are consistent with a requirement for mitochondrial WWOX function. The observation that increasing WWOX above endogenous levels is able to suppress the disruption caused by mitochondrial defects in this model indicates that WWOX is able to moderate the effect of mitochondrial dysfunction in cells.

Structure–function analyses were carried out to identify the amino acid residues required for the role of WWOX in moderating mitochondrial dysfunction. The ability of WWOX to suppress the tissue disruption caused by mitochondrial defects was abolished when a mutation was introduced specifically into the active site of its SDR enzyme. Such mutations have been shown to result in complete abolishment of enzymatic activity of SDR enzymes (Nakajin et al., 1998; Filling et al., 2002). This demonstrates that the modulation of mitochondrial-mediated cellular dysfunction by WWOX is dependent upon its ability to carry out its SDR enzymatic activity. Indeed, WWOX has been shown to be able to translocate into the mitochondria under stress conditions, and a mitochondrial localization signal has been identified in its SDR region (Chang et al., 2001). Although the exact enzymatic reaction(s) catalyzed by the SDR region of WWOX is currently unknown, SDR enzymes have been reported to have essential roles in metabolism and cellular NAD(P)(H) redox sensor systems (Kavanagh et al., 2008). Changes in the levels of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) in WWOX-deficient mice with reduced mitochondrial respiration have been reported in a recent study (Abu-Remaileh and Aqeilan, 2014).

The results described herein report a novel role for the SDR region of WWOX in maintaining cellular homeostasis, specifically in response to mitochondrial dysfunction. This has physiological relevance for human diseases, both in cancer and also the neuronal dysfunction observed in patients with WWOX mutations, as mitochondrial dysfunction and the resultant oxidative stress have been shown to have pathological contributions to tumorigenesis (Sharma et al., 2011; Ma et al., 2013; Santidrian et al., 2013) and neurodegeneration (Koutnikova et al., 1997; Zeviani and Carelli, 2007). In cancer, mitochondrial dysfunction has been proposed to have a role in tumor development, progression and metastasis, with emerging evidence that deficiencies in mitochondrial respiratory complexes are able to promote tumor progression and metastasis (Sharma et al., 2011; Ma et al., 2013; Santidrian et al., 2013). Genetic analyses of the molecular pathways involved in the cellular dysmorphology caused by mitochondrial dysfunction have identified ROS, AKT, HIF-1α, and autophagy as key players in this model, with Sima/HIF-1α and autophagy appearing to act in feedback mechanisms to restrict the cellular damage. Altered levels or activity of these key players are noted to be characteristic of cancer cells. Indeed, dysfunction of mitochondrial respiratory Complex I has been found to result in high levels of ROS and promote tumorigenesis through AKT activation in human cell lines (Sharma et al., 2011). WWOX has previously been shown to block activation of AKT in cancer cell lines through an unknown mechanism (Hu et al., 2014); thus, it is likely that reduced WWOX levels lead to AKT activation and contribute to the cellular dysmorphology possibly through modulation of specific ROS levels and/or their compartmentalization. Reduced levels of WWOX, rather than complete absence, are often observed in tumors and contribute to both cancer initiation and progression. The identified role for WWOX in moderating cellular homeostasis in a pathway mediated by ROS and AKT thus provides a mechanism for how reduced WWOX levels can contribute to tumor growth.

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Supplementary Material

Tumour Suppressor WWOX Moderates the Mitochondrial Respiratory Complex

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Inventory of Supplementary Information

Table S1 summarises the overall results from the RNAi screen of metabolic genes

Figure S1 shows representative images of the different phenotypic categories for the adult eye phenotypes and the qPCR results confirming successful RNAi targeting for WWOX and ND42 (related to Figure 1).

Figure S2 shows independent verification that reduced WWOX expression exacerbates the cellular dysfunction caused by mitochondrial defects (related to Figure 2).

Table S2 provides statistical analyses supporting that reduced WWOX expression exacerbates the cellular dysfunction (related to Figure 2 and Figure S2).

Table S3 provides statistical analyses supporting that increased WWOX expression suppresses the cellular dysfunction (related to Figure 3).

Table S4 provides details regarding the mutations generated in the different domains of WWOX (related to Figure 4).

Table S5 provides statistical analyses supporting that the SDR active site of WWOX is required for its suppression of cellular dysfunction (related to Figure 4)

Figure S3 shows additional verification that the SDR catalytic active site of WWOX is required for the suppressive effect on mitochondrial mediated-cellular dysfunction (related to Figure 4).

Table S6 provides statistical analyses for the additional verification of the role of the WWOX SDR catalytic active site (related to Figure S3).

Table S7 provides statistical analyses supporting that alterations in the level of candidate genes result in modification of the cellular dysfunction (related to Figure 5-7).

Table S1. Summary of the RNAi screen testing for cellular dysfunction that is modulated by *WWOX* in the developing eye of *Drosophila*.

	Gene targeted		**RNAi line**	**Molecular function/pathway**	**Eye phenotype caused by eye-specific knockdown of gene expression using RNAi**	
					Reduced expression of candidate gene alone	**Modification by altered Wwox levels**
	mCherry (control)		BL35785	-	No phenotype	No phenotype
TCA cycle (includes processes converging in and out of the TCA cycle)						
1	*CG15400*		v7261	Glucose-6-phosphatase activity	No phenotype	No phenotype
2	*CG1516*		v105936	Pyruvate carboxylase activity	No phenotype	No phenotype
3	*CG7070*	*Pyruvate kinase (Pyk)*	v35165	Pyruvate kinase activity	No phenotype	No phenotype
4	*CG17725*	*Phosphoenolpyruvate carboxykinase (Pepck)*	v20529	Phosphoenolpyruvate carboxykinase (GTP) activity	No phenotype	No phenotype
5	*CG3127*	*Phosphoglycerate kinase (Pgk)*	v110081	Phosphoglycerate kinase activity	No phenotype	No phenotype
			v33797		No phenotype	No phenotype
6	*CG7010/ l(1)G0334*		v107209	Pyruvate dehydrogenase (acetyl-transferring) activity	No phenotype	No phenotype
7	*CG7430*		v106126	Dihydrolipoyl dehydrogenase activity	No phenotype	No phenotype
8	*CG8808*	*Pyruvate dehydrogenase kinase (Pdk)*	BL28635	Pyruvate dehydrogenase (acetyl-transferring) kinase activity	No phenotype	No phenotype
			v106641		No phenotype	No phenotype
9	*CG3626*		v107415	Pyruvate dehydrogenase (lipoamide) phosphatase regulator activity	No phenotype	No phenotype
10	*CG12151*	*Pyruvate dehydrogenase phosphatase*	v107271	Phosphoprotein phosphatase activity	No phenotype	No phenotype
11	*CG9709*	*Acyl-coenzyme A oxidase at 57D distal (Acox57D-d)*	v106733	Acyl-CoA oxidase activity	No phenotype	No phenotype

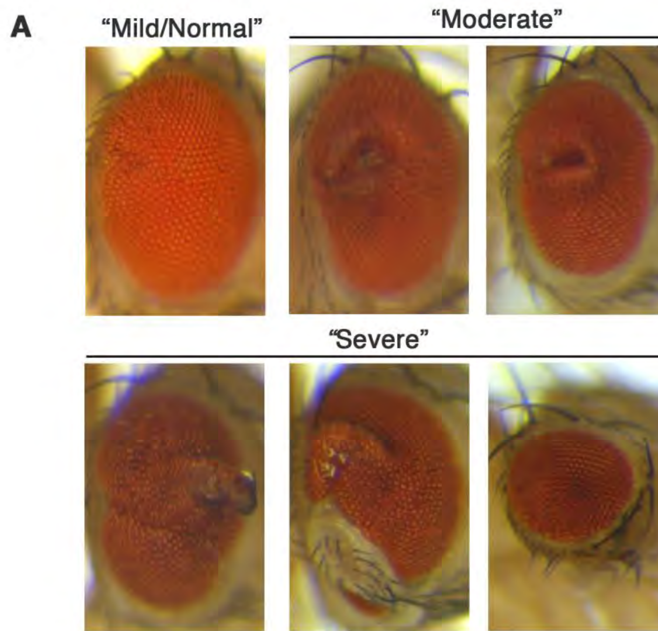
12	*CG11198*	*Acetyl-CoA carboxylase (ACC)*	BL34885	Acetyl-CoA carboxylase activity	No phenotype	No phenotype
13	*CG9390*	*Acetyl Coenzyme A synthase (AcCoAS)*	v100281	Acetate-CoA ligase activity	No phenotype	No phenotype
14	*CG3861*	*Knockdown(kdn)*	v26301	Citrate (Si)-synthase activity	No phenotype	No phenotype
			v107642		No phenotype	No phenotype
15	*CG9244*	*Aconitase(Acon)*	BL34028	Aconitate hydratase activity	No phenotype	No phenotype
			v103809		No phenotype	No phenotype
16	*CG6439*		v14443	Isocitrate dehydrogenase (NAD+) activity	No phenotype	No phenotype
17	*CG5028*		v103834	Isocitrate dehydrogenase (NAD+) activity	No phenotype	No phenotype
18	*CG11661*	*Neural conserved at 733F*	BL33686	Oxoglutarate dehydrogenase (succinyl-transferring) activity	No phenotype	No phenotype
19	*CG1065*	*Succinyl coenzyme A synthetase a subunit (Scsa)*	v107164	Succinate-CoA ligase (GDP-forming) activity	Loss of cells at the posterior end of the eye	No modification
20	*CG11963*	*skpA associated protein (skap)*	v105350	Succinate-CoA ligase (ADP-forming) activity	No phenotype	No phenotype
21	*CG10622*	*Succinate-CoA ligase (Sucb)*	v101554	Succinate-CoA ligase (GDP-forming) activity	No phenotype	No phenotype
22	*CG17246*	*Succinate dehydrogenase A (SdhA)*	v110440	Succinate dehydrogenase (ubiquinone) activity; Mitochondrial electron transport (Complex II)	No phenotype	No phenotype
23	*CG6666*	*Succinate dehydrogenase C (SdhC)*	v6031	Succinate dehydrogenase activity; Mitochondrial electron transport (Complex II)	No phenotype	No phenotype
24	*CG10219*		v101739	Succinate dehydrogenase (ubiquinone) activity; Mitochondrial electron transport (Complex II)	No phenotype	No phenotype

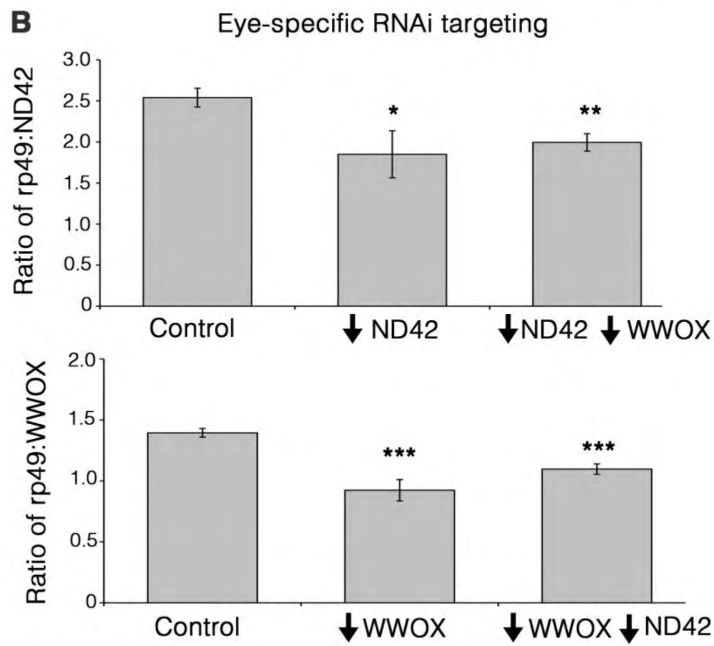
25	*CG4094/ l(1)G0255*		v105680	Fumarate hydratase activity	Mild disruption to the patterning of	No modification
					ommatidia	
26	*CG7998*	*Malate dehydrogenase 2 (Mdh2)*	v101551	Malate dehydrogenase activity	No phenotype	No phenotype
27	*CG5889*	*Malic enzyme b (Men-b)*	BL35486	Malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) activity	Extremely mild disruption in the patterning of ommatidia	No modification
			v100812		No phenotype	No phenotype
28	*CG10120*	*Malic enzyme (men/ME3)*	v104016	Malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) activity	No phenotype	No phenotype
29	*CG3523*		BL28930	Fatty acid synthase	No phenotype	No phenotype
			BL35775		No phenotype	No phenotype
30	*CG5009*		v103761	Palmitoyl-CoA oxidase activity	No phenotype	No phenotype
31	*CG8036*		BL32884	Transketolase activity	No phenotype	No phenotype
32	*CG4389*	Mitochondrial trifunctional protein α subunit (Mtp α)	v100021	Fatty acid beta-oxidation	No phenotype	No phenotype
Oxidative phosphorylation/Cell redox homeostasis						
33	*CG11793*	*Cu-Zn Superoxide dismutase (Sod1/Cu-ZnSOD)*	v31551	Removal of superoxide radicals; located in the cytoplasm	No phenotype	No phenotype
			v31552		No phenotype	No phenotype
			v108307		No phenotype	No phenotype
			BL29389		No phenotype	No phenotype
			BL24493		No phenotype	No phenotype
34	*CG8905*	*Manganese superoxide dismutase (Sod2/MnSOD)*	v110547	Removal of superoxide radicals; located in the mitochondria	No phenotype	No phenotype
35	*CG6871*	*Catalase*	v103591	Response to hydrogen peroxide	No phenotype	No phenotype
			v6283		No phenotype	No phenotype
36	*CG8913*	*Immune-regulated*	v101098	Response to oxidative stress	No phenotype	No phenotype

		catalase				
37	*CG31884*	*Thioredoxin-2 (Trx-2)*	BL34019	Disulfide oxidoreductase activity	No phenotype	No phenotype
			BL33721		No phenotype	No phenotype
38	*CG4181*	*Glutathione S transferase D2 (GstD2)*	v109123	glutathione transferase activity	No phenotype	No phenotype
39	*CG5164*	*Glutathione S transferase E1 (GstE1)*	v110529	glutathione transferase activity	No phenotype	No phenotype
40	*CG6673*	*Glutathione S transferase O2 (GstO2)*	v109255	glutathione transferase activity	No phenotype	No phenotype
41	*CG12529*	*Zwischenferment (Zw)*	v101507	glucose-6-phosphate dehydrogenase activity	No phenotype	No phenotype
42	*CG3896/ CG34399*	*NADPH oxidase (Nox)*	v102559	Oxidoreductase activity; calcium ion binding	No phenotype	No phenotype
43	*CG3944*	*NADH:ubiquinone reductase 23kD subunit precursor (ND23)*	v21748	NADH dehydrogenase (ubiquinone) activity; Mitochondrial electron transport (Complex 1 of ETC)	Disruption of ommatidia, with a range in severity	Enhanced by decreased *WWOX* levels and suppressed by increased *WWOX*
44	*CG6343*	*NADH:ubiquinone reductase 42kD subunit precursor (ND42)*	v14444	NADH dehydrogenase (ubiquinone) activity; Mitochondrial electron transport (Complex 1 of ETC)	Disruption of ommatidia, with a range in severity	Enhanced by decreased *WWOX* levels and suppressed by increased *WWOX*
45	*CG2286*	*NADH:ubiquinone reductase 75kD subunit precursor (ND75)*	v100733	NADH dehydrogenase (ubiquinone) activity; Mitochondrial electron transport (Complex 1 of ETC)	Disruption of ommatidia, with a range in severity	Enhanced by decreased Wwox levels and suppressed by increased Wwox

46	*CG2014*		v108457	NADH dehydrogenase (ubiquinone) activity; Mitochondrial electron transport (Complex 1)	No phenotype	No phenotype
47	*CG34085*	*Mitochondrial NADH-ubiquinone oxidoreductase chain 4 (ND4)*	v109553	NADH dehydrogenase (ubiquinone) activity; Mitochondrial electron transport (Complex 1)	No phenotype	No phenotype
48	*CG12140*	*Electron-transfer flavoprotein-ubiquinone dehydrogenase (ETFDH)*	v15508	Electron-transferring-flavoprotein dehydrogenase activity	No phenotype	No phenotype
49	*CG7580*	*Ubiquinol-cytochrome c reductase subunit 8*	v101371	Ubiquinol-cytochrome-c reductase activity; Mitochondrial electron transport (Complex III)	Disruption of ommatidia, with a range in severity	Enhanced by decreasing Wwox levels
50	*CG34067*	*Mitochondrial cytochrome c oxidase subunit I (Cox1)*	v109391	Cytochrome-c oxidase activity (Complex IV)	No phenotype	No phenotype
51	*CG34069*	*Mitochondrial cytochrome c oxidase subunit II (Cox2)*	v109278	Cytochrome-c oxidase activity; Mitochondrial electron transport (Complex IV)	No phenotype	No phenotype
52	*CG14724*	*Cytochrome c oxidase subunit V (CoVa)*	v44490	Cytochrome-c oxidase activity	Disruption of ommatidia, with a range in severity	Enhanced by decreased Wwox levels and suppressed by increased Wwox
53	*CG11015*	*Cytochrome c oxidase subunit V (CoVb)*	v30892	Cytochrome-c oxidase activity	Disruption of ommatidia, with a range in severity	Enhanced by decreased Wwox levels and suppressed by increased Wwox
54	*CG5818*	*Mitochondrial*	v40608	Translation	No phenotype	No phenotype

		ribosomal protein L4				
55	*CG16944*	*Stress-sensitive B (sesB)*	v104576	ATP:ADP antiporter activity	Loss of cells at the posterior end of the eye	No modification
56	*CG10523*	*Parkin (park)*	v104363	ubiquitin-protein ligase activity, response to oxidative stress, positive regulator of mitochondrial fission	No phenotype	No phenotype


A
"Mild/Normal"
"Moderate"
"Severe"


B
Eye-specific RNAi targeting
Ratio of rp49:ND42
3.0
2.5
2.0
1.5
1.0
0.5
0
*
**
Control
ND42
ND42 WWOX
Ratio of rp49:WWOX
2.0
1.5
1.0
0.5
0
***
***
Control
WWOX
WWOX ND42

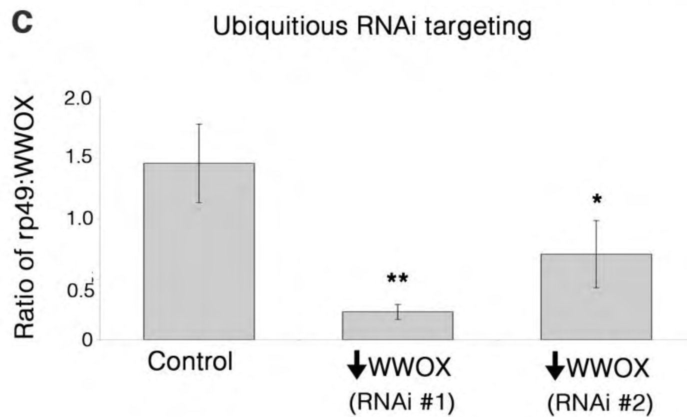

C
Ubiquitious RNAi targeting
Ratio of rp49:WWOX
2.0
1.5
1.0
0.5
0
**
*
Control
WWOX (RNAi #1)
WWOX (RNAi #2)

Figure S1. RNAi targeting of mitochondrial complex genes with WWOX and its effects. **(A)** Categories of eye phenotype severity used to quantify the effects of altered WWOX levels together with knockdown of genes encoding components of the mitochondrial respiratory chain. Eyes were grouped into three different categories: “mild/normal” – normal organisation or mild disruption in the patterning of the ommatidia; “moderate” –the loss of photoreceptor cells in the eye field forming a cavity in the eye; “severe” – significant disruption in the structure and development of the eye (tissue outgrowth, presence of ectopic structures in the eye or substantial loss of cells resulting in overall decreased eye size). **(B)** Successful RNAi targeting for WWOX and ND42 in *Drosophila* heads. ND42 and WWOX mRNA levels were measured in *Drosophila* heads with eye-specific RNAi knockdown of those genes. Significant reduction in WWOX and ND42 levels were observed in flies expressing either the WWOX RNAi, ND42 RNAi or both RNAi lines together. ***$p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$ compared to the respective control. **(C)** Comparing the knockdown efficiency of WWOX by ubiquitious targeting between two independent WWOX RNAi constructs. Higher level of knockdown was observed in WWOX RNAi #1 compared to WWOX RNAi #2 and this result corresponds to the level of enhancement by the two different lines (WWOX RNAi #1 has a stronger effect than WWOX RNAi #2). ** $p \leq 0.01$, * $p \leq 0.05$

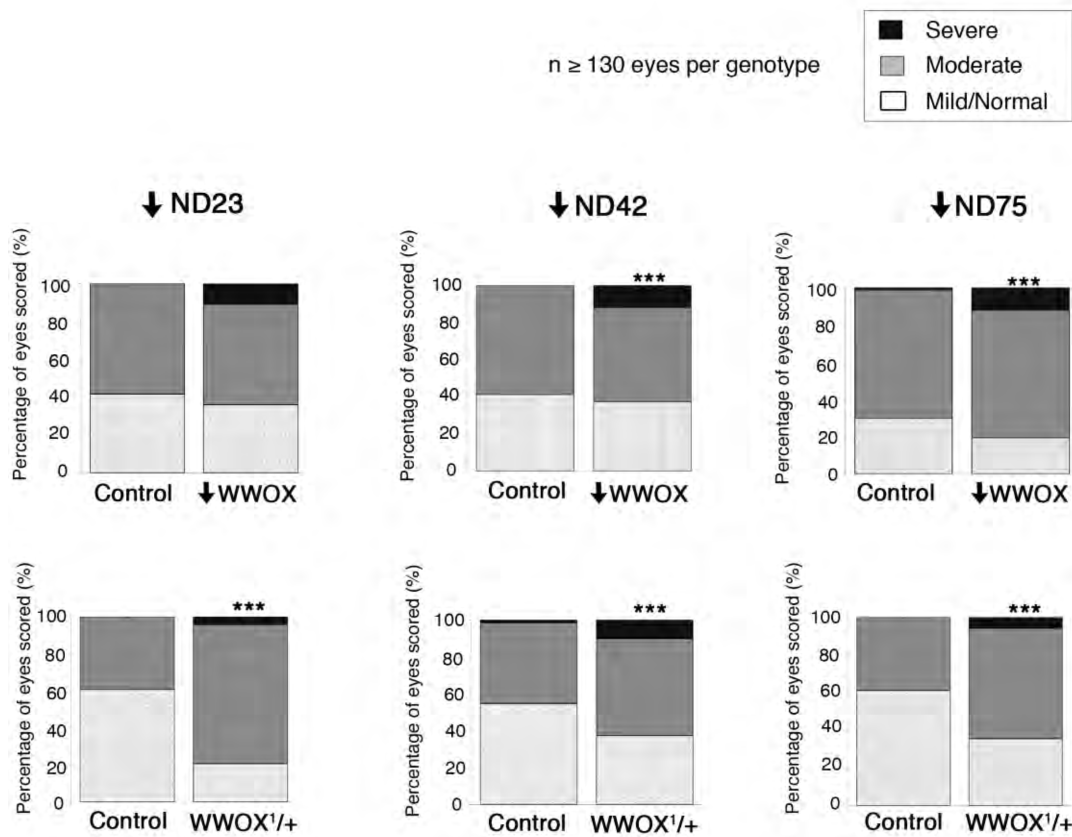


Figure S2. Independent verification that reduced WWOX expression exacerbates the cellular dysfunction caused by decreased expression of three different mitochondrial Complex I genes (ND23, ND42 and ND75). Decreased WWOX expression using two other independent lines (different RNAi line v22536, *WWOX*^{1}/+ is heterozygous for a WWOX null mutation) increases the proportion of eye phenotypes indicative of cellular dysfunction. Eyes were scored based on severity of the phenotype (severe, moderate or mild/normal) for each genotype. Chi-square test analyses were performed using GraphPad Prism. ***$p \leq 0.001$ when comparing to the respective controls, exact p values as well as further statistical analyses are provided in Table S2. $n \geq 130$ eyes per genotype.

Table S2. Statistical analyses of the severity of eye phenotypes exhibited by flies with reduced WWOX expression together with reduced expression of six different mitochondrial complex genes.

Adult eye phenotypes		
n>150 eyes per genotype	Control vs WWOX$^{RNAi\ 1}$	
	Chi-square test comparing all three categories (severe, moderate and mild/normal)	Fisher's exact test comparing severe phenotypes with the other phenotypes
ND23^{RNAi}	**Enhancement** (p<0.0001 ***)	**Enhancement** (p<0.0001 ***)
ND42^{RNAi}	**Enhancement** (p<0.0001 ***)	**Enhancement** (p<0.0001 ***)
ND75^{RNAi}	**Enhancement** (p<0.0001 ***)	**Enhancement** (p<0.0001 ***)
CG7580^{RNAi}	**Enhancement** (p<0.0001 ***)	**Enhancement** (p<0.0001 ***)
CoVa^{RNAi}	**Enhancement** (p<0.0001 ***)	**Enhancement** (p=0.0003 ***)
CoVb^{RNAi}	**Enhancement** (p=0.0107 *)	**Enhancement** (p=0.0073 **)

n>130 eyes per genotype	Control vs WWOX$^{RNAi\ 2}$		Control vs WWOX^{1}/+	
	Chi-square test	Fisher's exact test	Chi-square test	Fisher's exact test
ND23^{RNAi}	**Enhancement** (p<0.0001 ***)	**Enhancement** (p<0.0001 ***)	**Enhancement** (p<0.0001 ***)	**Enhancement** (p=0.0001 ***)
ND42^{RNAi}	**Enhancement** (p=0.0002 ***)	**Enhancement** (p<0.0001 ***)	**Enhancement** (p<0.0001 ***)	**Enhancement** (p<0.0001 ***)
ND75^{RNAi}	**Enhancement** (p<0.0001 ***)	**Enhancement** (p<0.0001 ***)	**Enhancement** (p<0.0001 ***)	**Enhancement** (p<0.0001 ***)

Larval eye disc outgrowth phenotype	
n≥50 eye discs per genotype	Control vs WWOX$^{RNAi\ 1}$ (Fisher's exact test)
ND23^{RNAi}	**Enhancement** (p<0.0001 ***)
ND42^{RNAi}	**Enhancement** (p=0.0034 **)
ND75^{RNAi}	**Enhancement** (p<0.0001 ***)

For the adult eye phenotypes, chi-square test analyses were firstly performed to compare all three categories of phenotype between the different genotypes. Further analysis using Fisher's exact test were also carried out as comparisons of the severe phenotypes with the other phenotypes (combined moderate and mild/normal categories). For the larval eye outgrowth phenotype, Fisher's exact test was using to determine significance when comparing presence and absence of the outgrowth phenotype. All analyses were performed with GraphPad Prism. The different WWOX lines used were v108350 (WWOX$^{RNAi\ 1}$), v22536 (WWOX$^{RNAi\ 2}$) and a heterozygous WWOX null mutation (WWOX^{1}/+).

Table S3. Statistical analyses of the presence or absence of eye phenotypes exhibited by flies with increased WWOX expression together with reduced expression of six different mitochondrial complex genes.

n>150 eyes per genotype	Control vs $WWOX^{cDNA}$
$ND23^{RNAi}$	**Suppression** ($p<0.0001$ ***)
$ND42^{RNAi}$	**Suppression** ($p<0.0001$ ***)
$ND75^{RNAi}$	**Suppression** ($p<0.0001$ ***)
$CG7580^{RNAi}$	**No change** ($p=0.2767$ n.s.)
$CoVa^{RNAi}$	**Suppression** ($p<0.0001$ ***)
$CoVb^{RNAi}$	**Suppression** ($p<0.0001$ ***)

Fisher's exact test analyses were performed using GraphPad Prism to compare the proportion of flies exhibiting considerable tissue disruption ("phenotype") to the proportion of flies with no considerable tissue disruption ("no phenotype").

Table S4. Mutations generated to target amino acids that are known or predicted to be crucial for WWOX function.

Predicted functional amino acid residues		Mutations generated to replace predicted functional amino acid residues in *Drosophila*		Known information regarding such residues
		Nucleotide substitution	Amino acid substitution	
WW domains				
1st WW domain	Y29	TAT → CGT	Y29R	Corresponding Y33R mutation in mammalian systems abolished binding of partner proteins to the WW domain and resulted in reduced apoptotic function (Chang et al., 2003).
2nd WW domain	W58	TGG → TTC	W58F	Substitution of tryptophan residues (to phenylalanine) in WW domain proteins could either affect their function or result in structural changes in the protein (Koepf et al., 1999).
SDR region				
Cofactor binding site	T127	ACG → GCG	T127A	T→A substitution in the cofactor binding motif resulted in changes in cofactor binding preference and abolished some enzymatic activities of another SDR protein (Oppermann et al., 2997; Filling et al., 2002).
Identified risk polymorphism	P277	CCG → GCG	P277A	Corresponding variant A282 in humans associated with increased risk of thyroid cancer (Cancemi et al., 2011).
Catalytic active site	Y288	TAC → TTC	Y288F	Y→ F substitution in the active site completely abolished enzymatic activity of other SDR proteins (Nakajin et al., 1998; Filling et al., 2002).

Table S5. Statistical analyses of the modification of eye phenotypes by increased WWOX expression for all WWOX over-expression mutant lines together with reduced expression of ND23.

n>250 eyes per genotype	Compared to EV control	Compared to wildtype WWOX
Wildtype WWOX	**Suppression** (p=0.001 **)	-
Y29R	**Suppression** (p<0.0001 ***)	No difference (p=0.4285 n.s)
W58F	**Suppression** (p=0.0002 ***)	No difference (p=0.5888 n.s.)
T127A	**Suppression** (p=0.0154 *)	No difference (p=0.1669 n.s.)
P277A	**Suppression** (p<0.0001 ***)	No difference (p=0.1217 n.s.)
Y288F	**Enhancement# (p=0.0223 *)**	Difference (p<0.0001 ***)

Fisher's exact test analyses were performed using GraphPad Prism to compare the proportion of flies exhibiting considerable tissue disruption ("phenotype") to the proportion of flies with no considerable tissue disruption ("no phenotype").

The significant difference detected for the Y288F mutant line compared to EV control is due to a enhancement with the Y288F mutant line, not a suppression; the significance was not included in Figure 4 as the significance shown in that figure was to demonstrate suppressive effects.

Comparisons were also made between the wildtype and mutant WWOX lines to demonstrate that the Y288F mutant is the only line that shows any significant difference in suppression ability compared to the wildtype WWOX.

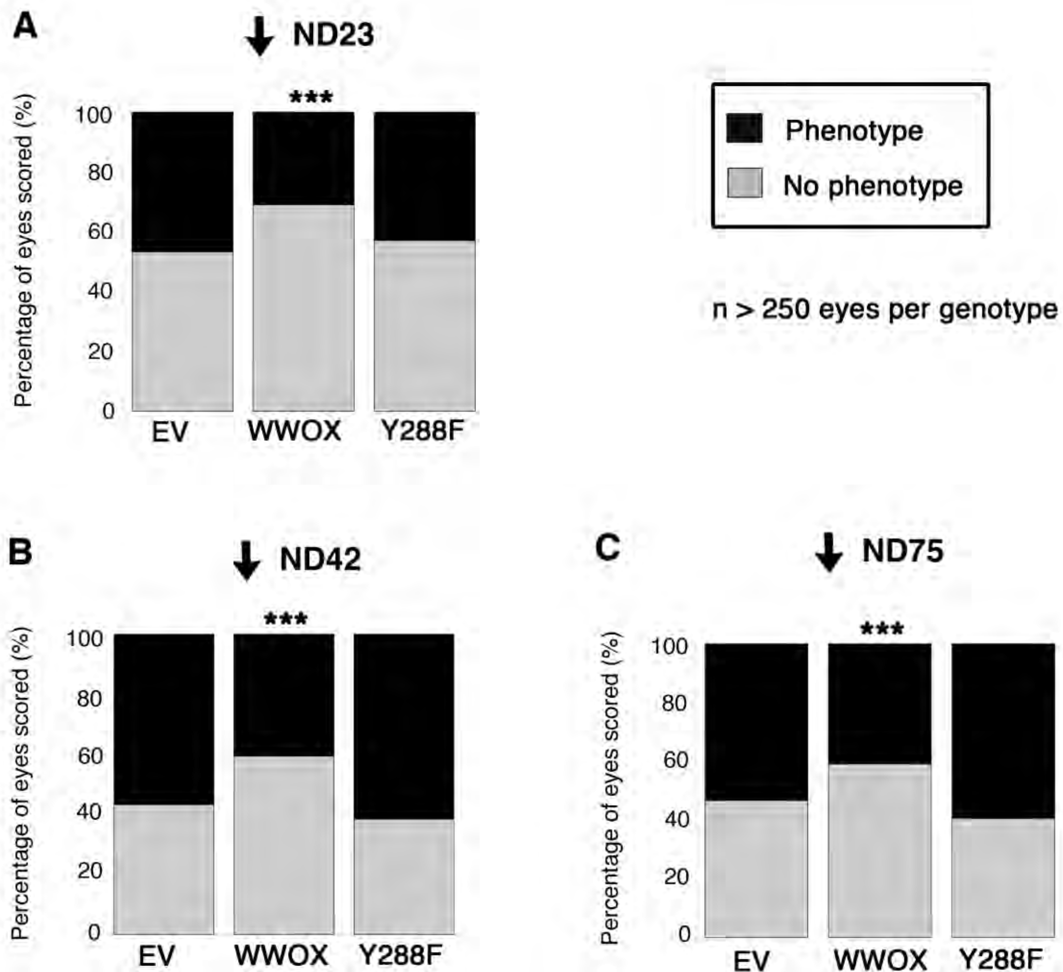


Figure S3. Additional analyses supporting that the SDR catalytic active site is required for the suppressive function of Wwox on mitochondrial defect-mediated tissue disruption. Both ectopic wildtype Wwox and Y288F form of Wwox (mutation in the SDR active site) were tested for their ability to suppress tissue disruption caused by decreased expression of different mitochondrial complex genes – (**A**) ND23, (**B**) ND42 and (**C**) ND75 in the *Drosophila* eye. Whilst expression of the ectopic wildtype Wwox consistently resulted in suppression of the tissue disruption, expression of the Y288F mutant form was unable to suppress the tissue disruption caused by defects in all three genes. The lines tested in these experiments were all inserted into the 68E1 chromosomal location. The lines have previously been tested with ND23 (Figure 4). In this experiment, the lines were again tested with ND23 as well as with the two other genes (ND42 and ND75). *** $p \leq 0.0001$ indicative of suppression compared to the empty vector (EV) control as determined by Fisher's exact test, p values and further statistical analyses in Table S6.

Table S6. Statistical analyses of the additional experiments testing the suppressive function of the Y288F mutant compared to wildtype WWOX on mitochondrial defect-mediated tissue disruption in the *Drosophila* eye.

n>250 eyes per genotype		**Compared to EV Control**	**Compared to wildtype WWOX**
ND23	Wildtype WWOX	**Suppression** ($p<0.0001$ ***)	**-**
	Y288F mutant	No change ($p=0.3077$ n.s.)	Difference ($p=0.0004$ ***)
ND42	Wildtype WWOX	**Suppression** ($p<0.0001$ ***)	**-**
	Y288F mutant	No change ($p=0.2045$ n.s.)	Difference ($p<0.0001$ ***)
ND75	Wildtype WWOX	**Suppression** ($p<0.0001$ ***)	**-**
	Y288F mutant	No change ($p=0.0643$ n.s.)	Difference ($p<0.0001$ ***)

Fisher's exact test analyses were performed using GraphPad Prism to compare the proportion of flies exhibiting considerable tissue disruption ("phenotype") to the proportion of flies with no considerable tissue disruption ("no phenotype"). The WWOX lines tested in these experiments were all inserted into the same chromosomal location.

Table S7. Statistical analyses of the modification of the larval eye disc outgrowth phenotype by altered levels of candidate genes.

Overexpression of candidate genes	
n= 50 eye discs per genotype	Compared to control (ey, ND42^{RNAi}, EV)
WWOX^{RNAi}	**Enhancement** (p=0.0439 *)
UAS-Catalase	**Suppression** (p<0.0001 ***)
UAS-SOD1	**Suppression** (p=0.0008 ***)
UAS-SOD2	**Suppression** (p=0.0338 *)
UAS-Foxo	**Suppression** (p=0.0002 ***)
Decreased expression of candidate genes	
n= 50 eye discs per genotype	Compared to control (ey, ND42^{RNAi}, lacZ^{RNAi})
WWOX^{RNAi}	**Enhancement** (p=0.0001 ***)
SOD1^{RNAi}	**Enhancement** (p=0.0052 **)
SOD2^{RNAi}	**Enhancement** (p= 0.0025 **)
Akt^{RNAi}	**Suppression** (p=0.0004 ***)
Atg^{RNAi}	**Enhancement** (p=0.0262*)
WWOX^{RNAi}	**Enhancement** (p=0.0085 **)
Sima^{RNAi}	**Enhancement** (p=0.0268 *)

Fisher's exact test analyses were performed using GraphPad Prism to compare the proportion of larval eye discs with and without the outgrowth phenotype between genotypes.

Appendix B

Responses and amendments in response to examiners' comments

Comments of examiner 1:

Introduction:

Comment 1: *Page 4, FRA16D is not necessarily the second most active CFS as recent evidence shows that this depends on cell/tissue type.*

Amendment: Page 4, paragraph 1, sentence 1- "the second most" has been changed to "one of the most".

Comment 2: *Page 5, Original reference for ErbB4-WWOX interaction is by Aqeilan, et al. Cancer Research 2005.*

Amendment: Page 5, paragraph 1, sentence 5- "(Schuchardt, *et al*. 2013)" has been changed to "(Aqeilan*, et al*. 2005b)".

Comment 3: *In Table 1.1, in over-expression of WWOX in cancer cells is able to suppress their tumorigenicity both in vitro and in vivo, you can add also paper by Aqeilan and colleagues in 2012 in Cancer Research on suppressing osteosarcoma.*

Amendment: Page 11, table 1.1, a new reference (Del Mare and Aqeilan 2015) has been added to the statement of "over-expression of WWOX in cancer cells is able to suppress their tumorigenicity both *in vitro* and *in vivo*".

Comment 4: *In Table 1.2, the Aqeilan group has also generated a conditional KO that was published in 2013 in J. Cellular Physiology. Also the Aqeilan lab has published development of mammary tumors in Wwox-heterozygous mice on the C3H genetic background, a paper that was published in Oncogene in 2011.*

Amendment: Page 12, table 1.2- a new column with the information of Wwox KO that was published in Abdeen *et al*. 2013 has been added.

Page 10 paragraph 1, sentence 1- "Additionally, Aqeilan *et al.* (2007) demonstrated that a complete knock-out of *Wwox* expression in mice (*Wwox-/-*) leads to the development of osteosarcomas (Table 1.2), whilst heterozygous *Wwox* mice (*Wwox+/-*) have a 5-fold increase in the development of spontaneous tumors compared to wild-type mice (Aqeilan*, et al.* 2007c)." **has been changed to**

"Additionally, it has been demonstrated that a complete knock-out of *Wwox* expression in mice (*Wwox*$^{-/-}$) leads to the development of osteosarcomas (Table 1.2), whilst heterozygous *Wwox* mice (*Wwox*$^{+/-}$) are more susceptible to the development of spontaneous tumors compared to wild-type mice (Aqeilan*, et al.* 2007c; Abdeen, *et al*. 2011)."

Comment 5: *Page 17/18 metabolic diseases, paper by M. Abu-Ramaileh on hypoglycaemia and functional interaction between WWOX and HIF1a could be included.*

Amendment: Page 18, paragraph 1- A new reference (Abu-Remaileh and Aqeilan 2014) has been added to sentence 1

Chapter 3:

Comment 6: *How can it be sure that the effect of the truncated Wwox version used in here, Wwox*^{trun169}*, is solely responsible for the phenotypes observed given that the endogenous Wwox is intact? Do levels/localization of endogenous Wwox change upon Wwox*^{trun169} *overexpression? Can Wwox*^{trun169} *rescue phenotypes in Wwox-null background?*

Response: Ectopic expression of Wwox^{trun169} alone does not cause any phenotype, however it shows activity similar to ectopic expression of full length Wwox in some assays but not others. As all of these experiments were carried out in a wild-type background, any contribution from the endogenous *Wwox* gene cannot be excluded. We did not determine whether there was any change to the abundance or the localization of endogenous Wwox in the background of Wwox^{trun169} overexpression. It is possible that over-expression of Wwox^{trun169} could change the levels of endogenous *Wwox* or other genes, resulting in the observed phenotypes in different functional assays. Future experiments can use microarray and/or proteomic analyses to determine genes, whose expression is altered in flies with ectopic expression of Wwox^{trun169}.

We are not sure whether ectopic expression of Wwox^{trun169} can also function in these assays in a *Wwox* null background. Although we have tried to test this, it was found that *Wwox* homozygous mutations showed inconsistent results in different functional assays leading us to propose a compensatory mechanism as a possble explanation for this.

Amendments: Page 29, paragraph 1- "We hypothesized that homozygous Wwox mutant flies are able to develop a compensatory mechanism. The compensatory mechanism has also been suggested in both homozygous *Relish*^{E20} mutant *Drosophila* and homozygous *egfl7* mutant zebrafish. While these mutants do not show phenotype, heterozygous *Relish*^{E20} mutation in flies and reduced *egfl7* levels in zebrafish induce sleep defects and vasular defects respectively (Williams *et al.* 2007, Rossi *et al.* 2015), suggesting that there is a compensatory mechanism developed in both of these models. In contrast, heterozygous *Wwox* mutation also does not induce phenotype in flies, and this may because the perturbation has not reached the threshold to induce phenotype in flies." has been added after the last sentence of this paragraph.

Page 29, paragraph 2, sentence 1- "However" has been changed to "Although *Wwox* mutant flies do not show any phenotype".

Comment 7: *In mammalian cells, WW1 domain of WWOX has been shown to be crucial for its DDR function. This seems to be consistent with intact function of Wwox*^{trun169} *and hence worth discussion.*

Amendment: Page 83, paragraph 1- "First WW domain of WWOX has also been shown to be required for ATM-mediated DNA damage response [54]. Although it has been shown that reduced *Mad2* levels can induce DNA damage [34], it is not known whether the DNA damage is the cause of lethality or the consequence of spindle assembly checkpoint defects. Future studies are required to confirm the conserved Wwox function in DNA damage repair in *Drosophila*, and determine whether the suppression of DNA damage can rescue the lethality of flies with reduced *Mad2* levels." has been added after the last sentence of this paragraph.

Comment 8: *Would it be possible to include any marker to verify the outcome of Mad2 reduction on spindle checkpoint assembly? Same for ND proteins regulating mitochondrial function?*

Response: Yes, it would, and we have verified the outcomes of both *Mad2* reduction and ND42 reduction using some markers. Shaukat *et al.* (2012) have used Hoechst 33342 to stain for chromosomes in *Drosophila* third instar larval brains (Shaukat, *et al.* 2012). Cells undergoing clear anaphase in each brain were scored as normal or defective. Defective anaphase included those with either chromosomal bridges or lagging chromosomes. It was found that brains with reduced *Mad2* levels have more than 20% defective anaphases compared to normal brains (Shaikat, *et al.* 2012), thus verifying the outcome of Mad2 reduction on spindle checkpoint assembly. Similarly, the effect of reduced *ND42* levels on mitochondrial function has been verified by tetramethylrhodamine ethyl ester (TMRE) staining (Shaukat, *et al.* 2014). Dysfunctional mitochondria have a decreased mitochondrial membrane potential, and thus fail to sequester the TMRE. It has been shown that reduced *ND42* levels results in loss of membrane potential (Shaukat, *et al.* 2014), suggesting that ND42 protein is important for the regulation of mitochondrial function.

Comment 9: *The results of this chapter is consistent with previous findings demonstrating that WW1 domain of WWOX is required for its pro-apoptotic function, probably through interaction with p73 or other alike proteins, while SDR domain is required for WWOX metabolic function. In future studies, it might be required to prove this point using genetic studies in flies (modeling WW1 mutation or overexpressing SDR alone).*

Response: Currently, our laboratory is mutating the putative substrate binding site of SDR domain to identify the substrate of SDR. This would enable us to determine the enzymatic and biological functions of SDR. *Drosophila* can then be used as a genetic model to determine small molecules that are able to compensate for the SDR function of WWOX (as discussed in chapter 5.3). "*The SDR enzyme region of Wwox is required for some of the metabolic functions of Wwox, but neither the substrate(s) nor the product(s) are known. It has been hypothesized that the loop region within SDR domain (residues 265-285), as well as its extended C-terminal sequence, are substrate binding sites (Kavanagha, et al. 2008; Abu-Remaileh, et al. 2015; Richards, et al. 2015). Future studies could mutate these regions, in order to undertake high throughput screens for molecules that bind wild-type, but not mutant*

WWOX protein. This would allow for the identification of candidate substrates and products for WWOX enzymatic function."

Amendment: Page 83, paragraph 1- "The pro-apoptotic function of Wwox^{trun169} is consistent with a previous study showing that the first WW domain of WWOX is required for inducing p73 pro-apoptotic function. Thus, it is possible that Wwox^{trun169} contributes to Eiger/TNFα-mediated cell death through interaction with p73." has been added after sentence 3 of this paragraph.

Chapter 4

Comment 10: *Can author show that WWOX localizes in mitochondria in fruit flies?*

Response: Endogenous Wwox is present at a very low level and is not detectable by immunostaining. Immunostaining of ectopically expressed Wwox in *Drosophila* embryos showed diffuse cytoplasmic localisation which is not consistent with specific mitochondrial localization (O'Keefe *et al.* 2005).

Comment 11: *In page 129, "As reduced WWOX expression allows the survival of DNA damaged cells"…can you provide a reference?*

Response: Our data showed that reduced *Prp19* levels induced DNA damage in cells, and decreased *Wwox* levels prevented these cells from dying. Together, this suggests that reduced *WWOX* expression increases the survival of DNA damaged cells. There is also precedence in the literature, as Lin *et al*. 2011 have also shown that inactivation of another CFS gene, *FHIT*, protects cell from DNA damage-induced death (Lin *et al.* 2011).

Amendment: Page 130, line 1- "It has also been shown that down-regulation of *FHIT* CFS gene prevents cells from DNA damaged-induced killing [94], suggesting that both *WWOX* and *FHIT* CFS genes share a similar function." has been added to the last sentence.

Comment 12: *In mamalian cells, WWOX and YAP have been shown to physically interact with ErbB4 specifically and not EGFR (ErbB1). ErbB1 does not get cleaved as ErbB4. This has to be corrected and emphasized.*

Amendment: Page 130, paragraph 1, sentence 3-8 - "Functional interactions between WWOX, YAP (human homolog for Yki) and EGFR have also been shown previously [46]. YAP can bind with the EGFR cytoplasmic fragment and promote its translocation to the nucleus, as well as activate transcriptional activity of this EGFR fragment. Translocation of the EGFR fragment to the nucleus has been correlated with poor prognosis in cancer cells [84]. On the other hand, WWOX can compete with YAP for the EGFR fragment binding and inhibit EGFR activity by sequestering it in cytoplasm [46]. The identification of Yki and EGFR as WWOX functional interactors in *Drosophila* suggests that the roles for WWOX in the regulation of both EGFR and Hippo pathways are conserved throughout evolution."

has been changed to

"In contrast to *Drosophila*, humans have four EGFR family members, including EGFR (also known as ErbB1), ErbB2, ErbB3 and ErbB4. Functional interactions between WWOX, YAP (human homolog for Yki) and ErbB4 have previously been shown in mammalian cells [46]. YAP can bind with the ErbB4 cytoplasmic fragment and promote its translocation to the nucleus, as well as activate its transcriptional activity. Translocation of the ErbB4 fragment to the nucleus has been correlated with poor prognosis in cancer cells [95]. WWOX can compete with YAP for binding the ErbB4 fragment and inhibit ErbB4 activity by sequestering it in cytoplasm [46]. Although the role of WWOX in the regulation of ErbB1 activity in mammalian cell remains to be determined, the identification of Yki and EGFR as WWOX functional interactors in *Drosophila* suggests that the contribution of WWOX in the regulation of both EGFR and Hippo pathways, at least, are conserved throughout evolution."

Comment 13: *Does manipulation of WWOX levels change that of Drp1 or other mitochondrial genes/products related to fission?*

Response: We have performed microarray and proteomics analyses to identify genes, whose transcripts and proteins levels were altered in response to aletered levels of *Wwox* (O'Keefe *et al.* 2011). None of the genes involved in the regulation of mitochondrial dynamics were identified in these analyses, suggesting that alteration of *Wwox* levels does not change the

expression of mitochondrial fission genes. Future experiments can verify these results by using quantitative PCR.

Comment 14: *Do authors speculate that WWOX's SDR domain plays a crucial role in its mitochondrial function?*

Response: We think both WW and SDR domains are important for mitochondrial function. Wwox may compete with Yki for its binding partners that recognize WW domains, and suppress the function of Yki for up-regulating mitochondrial fusion proteins (Nagaraj *et al.* 2012). On the other hand, we have shown that SDR catalytic site is important for the suppression of mitochondrial defects (Choo *et al.* 2015).

Comment 15: *Fig 4B requires panel explanation or addition of subtitle of the different phenotypes.*

Amendment: The names of phenotypes have been added to the Fig 4B.

Comment 16: *How can authors explain the discrepancy that Yki overexpression is known to be associated with increased organ size while authors show loss of head or eyes (Fig 4).*

Response: Ectopic expression of Yki using promoters, such as *GMR-gal4* (Zhang *et al.* 2008, Ren *et al.* 2010) and *engrailed-gal4* (Goulev *et al.* 2008), has previously been shown to induce tissue overgrowth. In this study, a different promoter, *eyeless-gal4 (ey-gal4)*, was used to ectopically over-express Yki. *ey-gal4* drives the expression of target gene in the region of the eye disc that is anterior to the morphogenetic furrow, and consists of undifferentiated cells (Halder *et al.* 1995). Ectopic over-expression of Yki in these cells caused defects in differentiation, and led to phenotypes characterised by loss of head structures. As described on page 125, paragraph 2, sentence 3- "*The "headless" phenotype has also been observed previously by Jiao et al (2001) following ectopic expression of different transcription factors using the ey-gal4 promoter (Jiao et al. 2001). Thus, the "headless" phenotype observed this study, followed by ectopic expression of Yki, is due to the use of eyeless-gal4 promoter.*"

Chapter 5

Comment 17: *Complete deletion of Wwox in the mouse or human is associated with severe phenotype including premature lethality. This is in sharp contrast with the phenotype of Wwox null mutation in flies. It would be important to give some insight on how to explain this discrepancy.*

Response: This has been discussed in page 29, paragraph 1- "*We hypothesized that homozygous Wwox mutant flies are able to develop a compensatory mechanism. The compensatory mechanism has also been suggested in both homozygous Relish^{E20} mutant Drosophila and homozygous egfl7 mutant zebrafish. While these mutants do not show phenotype, heterozygous Relish^{E20} mutation in flies and reduced egfl7 levels in zebrafish induce sleep defects and vasular defects respectively (Williams et al. 2007, Rossi et al. 2015), suggesting that there is a compensatory mechanism deeloped in both of these models. In contrast, heterozygous Wwox mutation also does not induce phenotype in flies, and this may be due to the perturbation has not reached the threshold to induce phenotype in flies.*"

Comment 18: *The results by Lee indicate that increased WWOX levels is associated with mitochondrial fission. How can the overexpression model of WWOX levels in flies be translated to physiological terms (ratio between endogenous vs. O/E)? Or in other words what physiological conditions are associated with increased WWOX levels and hence can lead to impaired mitochondrial function?*

Response: In this study, we have shown that over-expression of Wwox can contribute to Drp1-mediated mitochondrial fission, and cause lethality in flies with defects in mitochondrial fusion induced by reduced *Marf* levels. Consistently, reduced *Wwox* levels rescued the survival of flies with reduced *Opa1* levels (induced mitochondrial fission). Together these suggest that Wwox can contribute to mitochondrial fission under these experimental conditions. Altered *Wwox* levels have formed the basis of investigating *Wwox* function in *Drosophila*, and has revealed the consequence of reduced *Wwox* levels, leading to the understand of the pathways to which *Wwox* contributes.

As discussed in page 34, paragraph 1- "*WWOX expression has also been correlated with different metabolic states of cells; it is increased when cells rely on OXPHOS and decreased when glucose is provided solely as a carbon source (glycolysis) (Figure 1.11) (Dayan, et al.*

2013). In the glycolytic state, more mitochondria are fragmented (Guido, et al. 2012) and the cell is sensitized to apoptosis (Frank, et al. 2001; Lee, et al. 2004; Estaquier and Arnoult 2007). It is possible that the cell down-regulates WWOX levels during this state in order to keep mitochondrial fission in check and prevent excess cell death (Figure 1.11)."

Comment 19: *Have authors examined whether WWOX loss or haploinsufficiency are associated with altered mitophagy and fission genes/products in cancer data bases; i.e TCGA?*

Response: Although we have not check the association of loss of WWOX with altered miochondrial fission/fusion genes using TCGA database, in response to this question, we have now searched the association between *Wwox* and *Drp1* levels in cancer cells. An amendment has been made.

Amendment: Page 181, line 2- "It has been shown that *Drp1* levels are upregulated in several cancer cell lines, including MDA-MB 231 breast and A549 lung cancer cell lines (Rehman *et al.* 2012, Zhao *et al.* 2013). *Drp1* levels are also higher in invasive breast cancer cell lines (MDA-MB 231 and MDA-MB 436) compared to non-metastatic cancer cell line (MCF-7) (Zhao *et al.* 2013), suggesting that mitochondrial fission has been hijacked in these cancer cells. Interestingly, *WWOX* expression is abolished in A549 lung cancer cell line (Fabbri *et al.* 2005), and it is 2.5 fold increased in MDA-MB 231 breast cancer cell line compared to MCF7 breast cancer cell line (Bednarek *et al.* 2001), demonstrating that *WWOX* levels are indeed inversely correlated with the expression of *Drp1* in these cancer cells. This supports the hypothesis that cancer cells down-regulate *Wwox* levels, in order to inhibit the mitochondrial fission." has been addeed to this paragraph.

Examiner 2

1. Queries and suggstions for clarifications:

Comment 20: *Cell fate = cell death? There are a few places where the term "cell fate" is being used to refer to apoptosis, and not in the usual sense of different paths of differentiation (e.g. epidermal vs neural). E.g. pg130 para2, line 1: "The role of WWOX in cell fate decisions, or more specifically in caspase activation, is conserved…" i.e. or in other words,*

Wwox is affecting cell fate decisions via caspase activation, Again on pg 131, having stated that "mitochondrial fission can contribute to cell death pathways, it reads "This suggests that Wwox can regulate cell fate decisions by modulating mitochondrial dynamics."..... and persumbably thereby regulating cell death. Can the author reconsider whether this is a correct use of the term "cell fate".

Response: I agree with the suggestion of the examiner, and have made the following amendments:

Page 121, paragraph 1, sentence 3- "cell fate decisions" is changed to "apoptosis"

Page 125, paragraph 1, sentence 1- " cell fate determination" is changed to "controlling cell proliferation"

page 127, paragraph 1, sentence 1- " cell fate decisions" is changed to "regulating cell death"

page 176, paragraph 2, sentence 3- "cell fate decisions" is changed to "cell survival"

Comment 21: *Information of Prp19- On pg. 127, it says "....genes involving cell fate decisions, including Prp19, Yki and EGFR." I know that Yki and EGFR are components of signaling pathways that can affect gene expression, and thereby lead to changed in cell fate- but how does Prp19 affect cell fate? Actually the thesis says very little about the molecular & biological function of Prp19 so a bit more information on what is known about this gene is warranted.*

Amendments: The phrase of "cell fate decisions" has been changed to "regulating cell death" as detailed above. In addition, the following has been added to page 122, paragraph 3, sentence 1: "Prp19 has been shown to be involved in a wide range of biological pathways, such as pre-mRNA splicing (Cheng *et al.* 1993, Ajuh *et al.* 2000, Chan *et al.* 2005), DNA damage repair (Paulsen *et al.* 2009, Maréchal *et al.* 2014), the regulation of cell death (Lu *et al.* 2007, Sato *et al.* 2010, Benjamin *et al.* 2014), cell cycle control (Hofmann *et al.* 2013, Benjamin *et al.* 2014) and proteolysis (Sihn *et al.* 2007)."

Page 129, paragraph 2 sentences 1 and 2- "Reduction of *Prp19* levels, a novel interactor of Wwox, induced DNA damage and led to cell death in *Drosophila*. This cell death involved Eiger/TNFα secretion and caspase activation." has been changed to "Reduction of *Prp19*

levels, a novel interactor of Wwox, induced DNA damage and led to cell death in *Drosophila*, suggesting that the functions of Prp19 in the regulations of DNA damage repair and cell death are conserved in *Drosophila*. The cell death induced by reduced *Prp19* levels involved Eiger/TNFα secretion and caspase activation."

Comment 22: *Pg125 paragraph 1 "Wwox can suppress Yki activity". Yorkie is a TF whose subcellular localisation is regulated by phosphorylation. So activity has a very specific meaning in this case. Is the author is saying that Wwox is affecting Yki phosphorylation or localisation? If so they should make it more explicit. If not, then it would be better to just say "Wwox can suppress the lethality induced by Yki expression"*

Amendment: Page 125, paragraph 2, sentence 1- "Wwox can suppress Yki activity" is changed to "Wwox can suppress the lethality induced by Yki over-expression."

Mitochondrial fission phenotype

The effects on mitochondria are very interesting (a good area for future research) but need clarification.

Comment 23: *Page 127, paragraph 2, line 6. "When Drp1 was ectopically over-expressed in wing discs, all cells in the scored region contained mitochondria with the same appearance; they were either all tubular (Figure 7B i-i'), a mixture of tubular and round (Figure 7B ii-ii') or all fragmented (Figure 7B iiiiii')." Is this truly saying that for each disc, the cells display only one phenotype- normal (tubular), mixed or fragmented? If this is correct, it warrants a little more explanation. One would think, given the usual variablity of GAL4 drivers, that cells within a disc would exhibit a range of the three phenotypic strengths.*

Response: Indeed, there is a small amount of variation of mitochondrial morphology in some of the analyzed wing discs, and the following amendments have been made.

Amendments: Page 127/128, paragraph 2- "When Drp1 was ectopically over-expressed in wing discs, all cells in the scored region contained mitochondria with the same appearance; they were either all tubular (Figure 7B i-i'), a mixture of tubular and round (Figure 7B ii-ii') or all fragmented (Figure 7B iii-iii'). 49% of wing discs with ectopic expression of Drp1 showed fragmented mitochondria in the scored region (Figure 7C), confirming that increased *Drp1* levels can induce mitochondrial fission."

has been changed to

"When Drp1 was ectopically over-expressed in wing discs, all cells in the scored region contained mitochondria with the same appearance; they were either all tubular (Figure 7B i-i'), a mixture of tubular and round (Figure 7B ii-ii') or all fragmented (Figure 7B iii-iii'). This was consistent for 85% of analyzed wing discs, while the remaining 15% of analyzed wing discs have a mixture of cells with different mitochondrial morphology (not all cells have same mitochondrial morphology) (Table S2). This may because the variety of GAL4 expression between different cells in these wing discs. As mitochondrial fission is important for the induction of cell death [47, 48, 56], we compared the percentage of wing discs, in which all cells in the scored region showed fragmented mitochondria, between different genotypes. 49% of wing discs with ectopic expression of Drp1 showed fragmented mitochondria (all cells in the scored region) (Figure 7C and Table S2), confirming that increased *Drp1* levels can induce mitochondrial fission."

Page 135, "Analyses of mitochondrial morphology" section- "As the entire scored region of each wing discs contained mitochondria with the same appearance, wing discs were classified into three classes based on their mitochondrial morphology in the scored region as shown in Figure 7B."

has been changed to

"The percentage of wing discs, in which all cells in the scored region showed fragmented mitochondria (Figure 7B iii-iii'), was compared between each genotype (Figure 7C)."

Comment 24: *"However, 49% of wing discs with ectopic expression of Drp1 showed fragmented mitochondria in the scored region (Figure 7C), confirming that increased Drp1 levels can induce mitochondrial fission." It isn't clear why "However" is used, i.e. why is this counter to the first statement? Also we should be told what proportion of the remaining 51% discs had cells with tubular mitochondria, and what proportion showed a mixed mitochondrial phenotype.*

Amendment: Page 128, line 1: "However" is deleted.

Page 174: A new table (Table S2) has been added to summarize the proportion of wing discs for different mitochondrial morphology.

Ezymatic activity of SDR

Comment 25: *There are several places in the thesis [Pg. 72. Last line of abstract. Pg 177 paragraph 2, line 1. Pg180 last line] where it is stated that the enzymatic activity of SDR is required. To be able to say this one would have needed to test a version of Wwox in which SDR catalytic domain was present but had an inactivating mutation. Given the* $Wwox^{trun169}$ *transgene had a deletion of the SDR domain, it would seem that one can only say that the SDR domain was required.*

Amendments:

Page 72, last sentence- "SDR enzyme activity" has been changed to "SDR region".

Page 81, paragraph 2, last sentence- "SDR enzyme activity" has been changed to "SDR region".

Page 82, line 2- "SDR enzyme function" has been changed to "SDR region".

Page 178, paragraph 1, last sentence- "SDR enzyme activity" has been changed to "SDR region".

Page 182, paragraph 2, sentence 4- "SDR enzymatic function" has been changed to "SDR enzyme region".

Page 182, paragraph 2, last sentence- "SDR enzymatic function" has been changed to "SDR enzyme region".

Typographic errors

Comment 26:

Pg125, paragraph 2. "it was found that ***the*** *majority"*

Pg126, paragraph 3. ".....mode of cell death, ***the*** *caspase inhibitor p35…." and "Ectopic expression of* ***the*** *caspase inhibitor p35"*

Pg128, line 16. "Together, ***the*** *results herein…."*

Pg 153. "(as pointed ***to*** *by arrow)"*

*Pg 99, Fig 1 legend. line 3. "****short****-chain"*

Amendment: All the typographic errors have been corrected.

Comment 27: *Pg 156, Fig 4B. These images could be made larger and clearer. Also what is the reason for the two right hand panels- are they showing something different? If they are just two examples of the same category, can you make that clear?*

Response: Page 156, Fig 4B has been made larger and the names of phenotypes have been added.

References

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