Defining the role(s) of non-classical tumour suppressor Wwox in cellular function using Drosophila melanogaster genetic modelling

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by

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Abbreviations

°C – degrees Celsius

% – percentage

μg – micrograms

μL – microlitre

A – adenosine (in context of DNA)

A – alanine (in context of amino acid)

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

ATP – adenosine triphosphate

bp – base pairs

C – cytosine

CFS – common fragile site

CDD – conserved domain database

cDNA – complementary DNA

CIN – chromosomal instability

CoVa – cytochrome c oxidase subunit Va

CoVb – cytochrome c oxidase subunit Vb

da – daughterless

DEPC – diethyl pyrocarbonate

DNA – deoxyribonucleic acid

dNTP – deoxyribonuecleoside triphosphate

DMSO – dimethyl sulfoxide

EDTA – ethylene diamine tetra-acetic acid

en - engrailed

ETC – electron transport chain

EV – empty vector

ey – eyeless

F – phenylalanine

FLP – flippase

Foxo – forkhead box, sub-group O

FRT – flippase recognition target

G – guanosine

GFP – green fluorescent protein

GSH – glutathione

GWAS – genome wide association studies

HDL-C – high density lipoprotein-cholesterol

hh – hedgehog

HIF1α - hypoxia inducible factor 1α

IDH – isocitrate dehydrogenase

IMS – intermembrane space

kb – kilobase

kDa – kilodalton

L – lysine

LB – Luria Broth

LiCl – lithium chloride

LOH – loss of heterozygosity

M – Molar

MARCM – mosaic analysis with a repressible cell marker

mg – milligram

ml – millilter

mM – millimolar

mRNA – messenger RNA

N – asparagine

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

NLS – nuclear localisation sequence

ng – nanograms

ORF – open reading frame

P – proline

PBS – phosphate buffered saline

PBST – PBS + Tween

PCR – polymerase chain reaction

pmol – picomole

QTL – quantitative trait loci

R – arginine

RNA – ribonucleic acid

RNAi – RNA interference

ROS – reactive oxygen species

Rp49 – Ribosomal protein 49

rcf – relative centrifugal force

Scrib – scribbled planar cell polarity protein

SDR – short-chain dehydrogenase reductase

SDS – sodium dodecyl sulfate

Sima - similar

SOC – super-optimal broth with catabolite repression

SOD – superoxide dismutase

T – thymine (in context of DNA)

T – threonine (in context of amino acid)

Tgo - tango

TCA – tricarboxylic acid

TMRE – tetramethylrhodamine, ethyl ester

TNFα - tumor necrosis factor α

Tub – tubulin

U – uracil

UAS – upstream activator sequence

UTR – untranslated region

V – volts

VDRC - Vienna Drosophila Resource Centre

W – tryptophan

WW1 – 1st WW domain of WWOX

WW2 – 2nd WW domain of WWOX

WNP –WWOX mutant line carrying a triple mutation in the tryptophan 58 (W58F), asparagine 81 (N81A) and proline 84 (P84A) residues

WWOX – WW domain-containing oxidoreductase

Y – tyrosine

***Drosophila* nomenclature**

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

Abstract

The *WWOX* gene has been identified as the gene that spans the *FRA16D* common chromosomal fragile site (CFS), which is a frequent site of DNA instability in cancer. Perturbation of the *WWOX* gene has been reported in various cancers, with low *WWOX* levels correlating with poorer prognosis. Individuals who inherit a non-functional copy of *WWOX* have also been found to be at greater risk of developing cancer. WWOX has been implicated in various cellular pathways, however the role of WWOX in tumourigenesis is not yet fully defined. There is therefore a need to determine the normal function(s) of WWOX and how perturbation of these roles is likely to contribute to cancer. A model was previously established to examine the cellular function of the *Drosophila* orthologue, Wwox and to identify novel functional interactors. Loss of Wwox in *Drosophila* was not found to result in any obvious cellular dysfunction that manifested as a phenotype. The aim of this study was to identify the types of cellular dysfunction brought about by other genes that could be modulated by Wwox. As Wwox has previously been implicated in metabolic processes, particularly aerobic metabolism and redox homeostasis, an RNA interference (RNAi) screen was performed to identify the types of metabolic stress that can be modulated by altered Wwox levels. Wwox was found to regulate cellular homeostasis in cells with mitochondrial dysfunction, with a requirement for the active site of its short-chain dehydrogenase/reductase (SDR) enzyme. Other genetic effectors of the mitochondrial dysfunction were also identified as candidates for further investigation into the pathway(s) in which Wwox participates. The contributions of Wwox to two other models of cellular dysfunction were also examined. Wwox was found to have a role in a *Drosophila* model of intrinsic tumour suppression. In addition, Wwox was also shown to affect cells with chromosomal instability (CIN), with loss of Wwox resulting in oxidative stress, DNA damage and subsequently apoptosis of CIN cells. This study has identified roles for Wwox in three different novel models of cellular dysfunction. These findings provide further insight into the tumourigenic potential of WWOX and could contribute to the ultimate aim of designing therapeutics for treatment of cancers with low WWOX levels.

Chapter 1 - Introduction

Chromosomal fragile sites are regions of DNA that are susceptible to breakage upon exposure to replicative stress. Common fragile sites (CFS), which make up the larger class of these chromosomal fragile sites, are found in all individuals and have been associated with regions of DNA instability observed in various cancers. Breakage at numerous different fragile sites has been shown to be induced *in vitro* by the presence of aphidicolin, an inhibitor of DNA polymerase α (Glover *et al.*, 1984) and these fragile sites are also highly sensitive to environmental agents, such as caffeine, ethanol and tobacco (Yunis *et al.*, 1984; Kuwano *et al.*, 1987; Stein *et al.*, 2002). Whilst there are approximately 90 different CFS observed to date (Durkin and Glover, 2007), the sensitivity of CFS appears to be specific for different tissues and cells (Letessier *et al*., 2011; Huebner, 2011). Fragile sites *FRA3B* (on human chromosome 3p14.2) and *FRA16D* (located on chromosome 16q23.2) have been found as two of the most frequently observed fragile sites in lymphoblasts. These sites have also been identified as being among the most common sites of small homozygous deletions in cancers (Bignell *et al*., 2010), supportive of the notion that there is a correlation between chromosomal fragility observed *in vitro* and *in vivo* DNA instability in cancer.

In addition to homozygous deletions, the *FRA16D* region has also been associated with frequent loss of heterozygosity (LOH) and chromosomal translocations in multiple types of cancer, including prostate, breast, gastric carcinomas and multiple myelomas (Latil *et al.*, 1997; Driouch *et al.*, 1997; Mangelsdorf *et al.*, 2000; Paige *et al.*, 2000). Genomic analysis of this *FRA16D* region by two different research groups in 2000 had led to the identification of a novel gene spanning this fragile site (Bednarek *et al.*, 2000; Ried *et al.*, 2000). This gene, which is known mostly as the *WW domain containing oxidoreductase (WWOX)* and also, but less frequently, as the *fragile site FRA16D oxidoreductase (FOR)* gene, was found to span the region of homozygous deletions, LOH and translocations in the various cancers. Aberrant expression of WWOX has been reported in multiple types of tumours (Kuroki *et al.*, 2002; Aqeilan *et al.*, 2004a; O'Keefe *et al.*, 2006; Lewandowska *et al.*, 2009; Gardenswartz and Aqeilan, 2014), leading to the hypothesis that perturbation of WWOX function at the *FRA16D* fragile site plays a significant role in cancer development and progression.

1.1 WW domain-containing oxidoreductase (WWOX)

1.1.1 The human *WWOX* gene

The human *WWOX* gene is a large gene that spans 1.1Mb of genomic DNA and comprises nine exons (Figure 1.1). The full-length transcript is translated into a 46kDa protein of 414 amino acids consisting of several characterised functional elements, which includes two N-terminal WW domains as well as a short chain dehydrogenase/reductase (SDR) enzyme (Bednarek *et al.*, 2000). The tandem WW domains are encoded by exons 1-4 of the *WWOX* gene whereas exons 4-8 codes for the SDR enzyme. Alternative splicing of these exons has been proposed to give rise to various transcripts (Chang *et al.*, 2007), which in some cases are translated into truncated protein products lacking the complete SDR enzyme (Ishii *et al.*, 2003; Mahajan *et al.*, 2005). Several of these alternate transcripts, mostly those lacking the sequences between exons 4-8, have been detected in various cancer cells together with the absence of the full-length *WWOX* transcript (Figure 1.1).

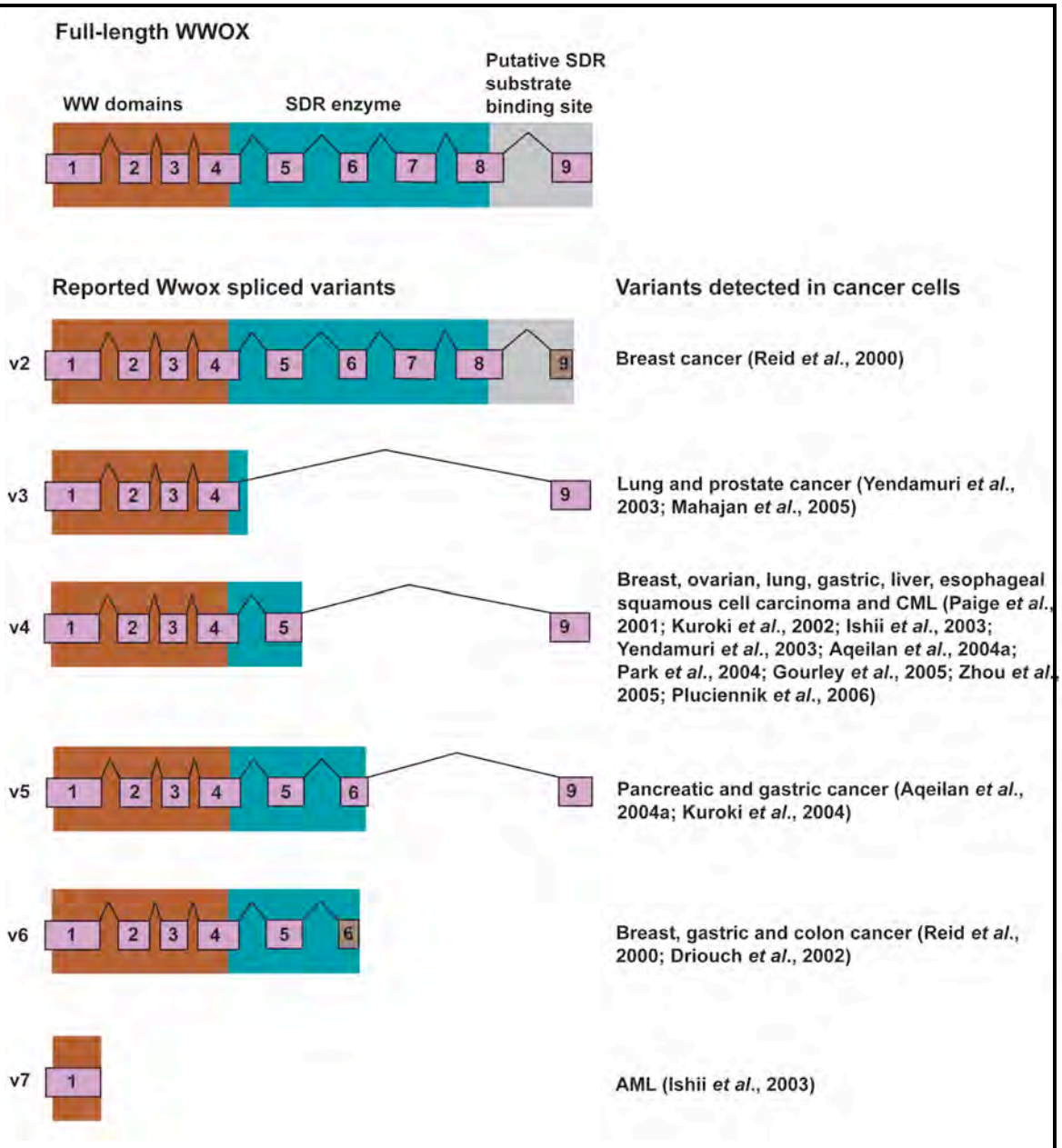

Figure 1.1 The human *WWOX* gene and its alternative spliced variants. The full-length *WWOX* transcript encodes for WW domains (indicated by the brown box) as well as a SDR enzyme (indicated by blue box). It has also recently been proposed that the SDR substrate-binding region could be C-terminal from the SDR enzyme (indicated by grey box) (Richards *et al*., 2015). Many alternatively spliced transcripts of *WWOX* (v2-v7) have been detected in different cancer cells, each with a different C-terminus and most of which lacking exons encoding a functional SDR enzyme. The v2 transcript contains an alternatively spliced exon 9, v3 - v5 transcripts contain deletions of exons 5-8, exons 6-8 and 7-8 respectively whilst v6 has an alternative exon 6 and absence of the remaining exons and v7 contains only exon 1.

1.1.2 WWOX protein structure

The WWOX protein is highly conserved across species, suggestive of WWOX having a biologically significant function (Figure 1.2) (Richards *et al.*, 2015). The unique feature of the WWOX protein is the presence of the WW domains together with an SDR enzyme, however it is not currently clear whether these two functional elements have distinct or cooperative roles in cell biology.

WW domains are typically characterised by the presence of two signature tryptophan (W) residues that are 20 amino acids apart and fold into a compact structure consisting of a triple strand anti-parallel β sheet with hydrophobic ligand-binding grooves (Ingham *et al.*, 2005). The WW domains of WWOX have been identified to be Group 1 WW domains that bind specifically to ligands with a proline-rich PPxY or LPxY motif and are involved in protein-protein interactions (Ludes-Meyers *et al.*, 2004; Abu-Odeh *et al.*, 2014). The first WW domain of WWOX contains the two signature tryptophan residues as well a tyrosine (Y33) and proline (P47) residue, which are both also essential for protein binding (Ludes-Meyers *et al.*, 2004; Chang *et al.*, 2003a; Chang *et al.*, 2003b). Phosphorylation of the Y33 residue in particular by tyrosine kinase Src has been shown to be crucial for protein-protein interactions and for the activation of WWOX in response to sex steroid hormones and stress stimuli (Chang *et al.*, 2003a; Aqeilan *et al.*, 2004b). The second WW domain, on the other hand, contains only one tryptophan residue and has been shown to be important in stabilizing the interactions with WWOX ligands (Ludes-Meyers *et al.*, 2004; Schuchardt *et al.*, 2013). In addition to the WW domains, a putative PEST domain and a nuclear localisation sequence (NLS) is also present in the N-terminus of the WWOX protein. PEST sequences are rich in proline (P), glutamate (E), serine (S) and threonine (T) residues and have been proposed to be a signal for rapid intracellular proteolysis (Rogers *et al.*, 1986). PEST-mediated proteolysis is thought to occur as an important regulatory mechanism for many metabolic enzymes as well as cell cycle proteins (Rechsteiner and Rogers, 1996).

SDR enzymes belong to a superfamily of NAD(P)(H)-dependent oxidoreductases (Kavanagh *et al.*, 2008). Members of this superfamily share conserved motifs and have common mechanisms. These enzymes have been found to be involved in various metabolic processes, including metabolism of steroids, amino acids, carbohydrates as well as xenobiotics and have an important role in signalling processes and NAD(P)(H) redox sensor mechanisms (Kavanagh *et al.*, 2008). One of the more well-characterised group of SDR enzymes are the 3β/17β-hydroxysteroid dehydrogenases which have been described

to have a wide range of substrates, mostly steroid hormones, and roles in processes such as estrogen and androgen metabolism and cholesterol biosynthesis (Moeller and Adams, 2006; Marchais-Oberwinfler *et al.*, 2011). These enzymes have also been associated with various metabolic diseases and cancer due to their role in hormone-signalling pathways and are now being considered as potential drug targets in treatment of such diseases (Oppermann *et al.*, 2003).

The SDR enzyme of WWOX contains both the TGxxxGxG co-factor binding motif and YxxxK active site motif that are characteristic of the classical SDR family of enzymes. WWOX also contains the less highly conserved upstream serine (S) and asparagine (N) residues that can contribute to catalytic function. Classical SDR enzymes have been reported to be involved in the oxidoreduction of hydroxy/keto groups in substrates including steroids, alcohols and growth factors in the presence of NAD(P)(H) cofactors (Kavanagh *et al*., 2008). However, the endogenous substrates and the exact biological reaction(s) that are catalysed by the WWOX SDR enzyme are yet to be identified. WWOX has recently been shown in an *in vitro* study to have dehydrogenase activity and is able to utilise both NAD^+ and $NADP^+$ as cofactors in oxidation reactions of selected steroid substrates; with higher levels of activity observed when NAD^+ was used (Saluda-Gorgul *et al.*, 2011). The steroid substrates were hormone steroids, which included progesterone, 17β-estradiol, estrone, testosterone, 5α-androstane-3,16-dione, 4-androstene-3,17-dione and 5α-dihydroprogesterone-*allo*. As WWOX is highly expressed in hormone-dependent tissues, it is possible that the steroids examined in the *in vitro* study could be endogenous substrates of the WWOX SDR enzyme; however further studies are required to confirm this. It has recently been postulated that the SDR substrate-binding site could be at a highly conserved region of the C-terminus of the WWOX protein (Figure 1.2) (Richards *et al.*, 2015), as substrate-determining regions of SDR enzymes are typically found C-terminal from the catalytic region (Kavanagh *et al.*, 2008). Identification of the SDR substrate-binding site will enable detection of the endogenous substrates and elucidate the pathways in which the WWOX SDR enzyme is involved.

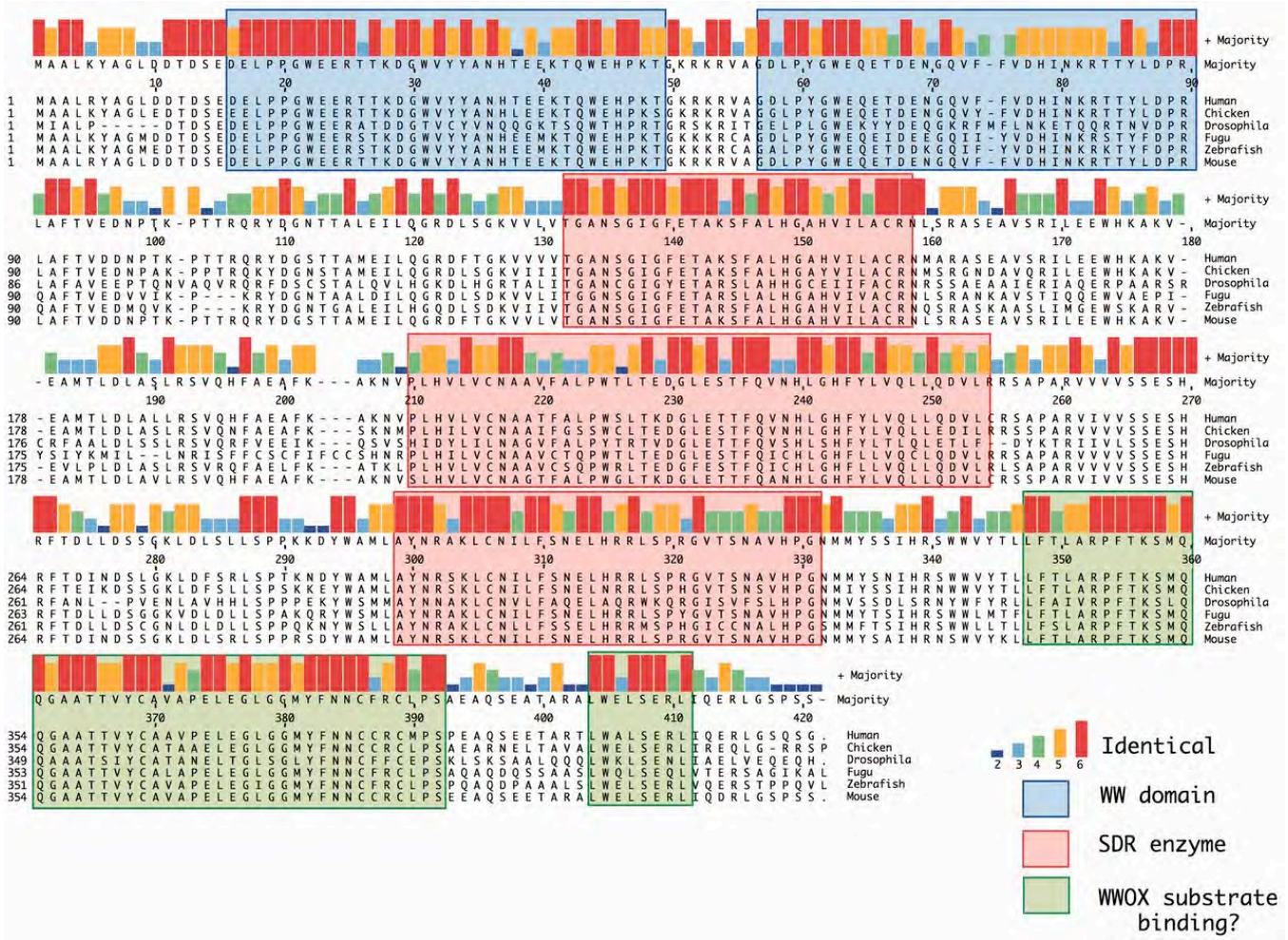

Figure 1.2 Sequence homology of the WWOX protein in human, chicken, *Drosophila*, fugu, zebrafish and mouse. Conserved sequences are highlighted, with different shading for the different domains – blue for the WW domains, red for the components of the SDR enzyme region with the highest homology and green for the putative SDR substrate binding site. The majority sequence is the consensus sequence, marked by the different colours for the various levels of identity (Figure obtained from Richards *et al*., 2015).

1.1.3 Expression and subcellular localisation of WWOX

Analysis of WWOX expression has revealed that WWOX is widely expressed in various mammalian tissues and cell types, suggesting that WWOX is likely to be involved in one or more primary biological processes (Chen *et al.*, 2004; Nunez *et al.*, 2006; Aqeilan *et al.*, 2008). Higher expression of WWOX has been detected in the reproductive, endocrine and exocrine organs as well as in cells of neural origin. The high expression of WWOX in hormone-regulated organs, including the testes, prostates and ovaries, coupled with the observation of impaired steroidogenesis in WWOX mutant mice, has led to the hypothesis that WWOX could have a role in steroid metabolism. Interestingly, the first WWOX germline mutation was reported in an individual with gonadal abnormalities, indicating that WWOX may have an important function in these endocrine organs (White *et al.*, 2012). The only other familial WWOX mutations identified thus far have been found to result in neuronal defects, such as mental retardation, epilepsy and ataxia (Mallaret *et al.*, 2014; Abdel-Salam *et al.*, 2014; Ben-Salem *et al*., 2014), pointing towards a crucial role for WWOX in neuronal function as well.

The cellular localisation of WWOX has always been and still remains a topic of great debate. Cytoplasmic localisation of WWOX was detected by various immunohistochemical analyses of normal and cancer tissues (Aqeilan *et al.*, 2004a; Nunez *et al.*, 2005a; Nunez *et al.*, 2005b) as well as in murine tissues (Aqeilan *et al.*, 2007). Analysis of *Drosophila* embryos and larval tissues also revealed cytoplasmic staining with ectopic expression of either the *Drosophil*a Wwox or human WWOX (O'Keefe *et al.*, 2005; O'Keefe *et al*., manuscript in preparation). Other research groups have reported WWOX to be mainly localised in the mitochondria in both human cancer and murine tissues (Chang *et al.*, 2001; Watanabe *et al.*, 2003). Translocation of WWOX from the mitochondria into the nucleus *in vitro* was observed in the presence of apoptotic stimuli, such as tumour necrosis factor (TNF) and staurosporine, as well as under confluent culture conditions. The presence of the WW domains of WWOX that contain a nuclear localisation sequence (NLS) between them was shown to be required for the translocation of WWOX into the nucleus whilst the SDR enzyme region had no effect on nuclear localisation but is necessary for mitochondrial localisation (Chang *et al.*, 2001; Bednarek *et al.*, 2001). The reported mitochondrial localisation of WWOX, however, has been contested by other studies (Bednarek *et al*., 2001; Ludes-Meyers *et al.*, 2003; Ludes-Meyers *et al.*, 2004). The latter research showed evidence for WWOX being located within the Golgi apparatus, attributed to the presence of the SDR region, but failed to detect

WWOX localisation in the mitochondria. It is unclear as to why there are discrepancies in the findings of these different studies. One explanation that could reconcile the seemingly disparate findings is that WWOX may actually be localised at the mitochondria-associated endoplasmic reticulum membrane (MAM), which links the mitochondria to the endoplasmic reticulum and contains many pre-Golgi secretory proteins. As specific experimental conditions are required in order to visualise protein in the MAM, many studies often fail to detect proteins in this subcellular localisation (Area-Gomez *et al*., 2009). On the other hand, it is also highly possible that WWOX could be present in various compartments of the cell and could have specific functions in different organelles or under different conditions.

1.2 Contribution of WWOX to cancer

1.2.1 WWOX expression in cancer cells

Following the identification of *WWOX* as the gene that spans the *FRA16D* region associated with DNA instability in cancer, analysis of *WWOX* expression in numerous different tumours and cancer cell lines has been carried out. Loss of heterozygosity (LOH), chromosomal translocations and homozygous deletions spanning the *WWOX* gene have been frequently observed in various types of cancers, including breast, ovary, pancreas, colon, stomach, lung and skin cancers (Paige *et al.*, 2001; Kuroki *et al.*, 2002; Lewandowska *et al.*, 2009; Gardenswartz and Aqeilan, 2014). These alterations often resulted in the complete loss of *WWOX* transcripts or production of aberrant transcripts lacking some, if not all, of the exons coding for the SDR enzyme. Low levels or absence of the normal WWOX protein have been reported in the tumours and cancer cell lines. Whilst the aberrant mRNAs are most likely to be subjected to nonsense-mediated decay, some of the transcripts are translated, resulting in truncated WWOX proteins without a functional SDR enzyme (Ishii *et al.*, 2003; Mahajan *et al.*, 2005).

In addition to the observed LOH and homozygous deletions, hypermethylation of the *WWOX* promoter and exon 1 has also been reported in lung, breast, ovarian, gastric and pancreatic cancers, correlating with an observed reduction or loss of WWOX protein in those cancers (Illiopoulos *et al.*, 2005; Nakayama *et al.*, 2009; Guo *et al.*, 2013; Yan and Sun, 2013). Use of a methylation inhibitor, 5-aza-deoxycytidine (DAC), resulted in an increase in *WWOX* expression in leukemic cells but not normal cells, supporting that methylation is one of the mechanisms by which WWOX is inactivated in cancer cells (Ishii *et al.*, 2003). Transcription of *WWOX* is also suppressed by binding of the epigenetic regulator/polycomb group protein Bmi1 to its promoter. Bmi1 has been shown to be overexpressed in small-cell lung cancer, correlating to lower *WWOX* levels in those tumours (Kimura *et al.*, 2011). Interestingly, previous studies did not detect many inactivating point mutations for WWOX in cancer and it is not known if any of the few reported mutations are actually rare polymorphisms (Paige *et al.*, 2001; Kuroki *et al.*, 2002; Ishii *et al.*, 2003; Yendamuri *et al.*, 2003). A recent study examining genetic alterations of WWOX in breast cancer however did report several changes that appear to be novel and may lead to loss of function and interestingly, the alterations were mostly in the SDR-coding region of the gene (Ekizoglu *et al.*, 2012). Furthermore, analysis of data from The Cancer Genome Atlas (TCGA) also revealed a number of point mutations, with

majority of them in the SDR region and only a few in the WW domains (Aldaz *et al.*, 2014). Several inherited mutations in WWOX have also recently been described, however whilst these mutations have been associated with gonadal abnormalities and neuronal defects, there is no reported evidence of tumourigenesis (White *et al.*, 2012; Mallaret *et al.*, 2014; Abdel-Salam *et al.*, 2014; Ben-Salam *et al*., 2014). Nevertheless, it is possible that any tumourigenic potential of those WWOX mutations are undetectable due to the short lifespan of those individuals with neuronal defects.

A detailed analysis was carried out to determine if the DNA instability observed at the *FRA16D* locus spanning *WWOX* is present in early stages of tumourigenesis, as early mutational events are more likely to contribute to cancer development, rather than being a consequence of tumourigenesis (Finnis *et al.*, 2005). Studies showing loss of WWOX protein in a large proportion of primary tumours suggest that alterations in WWOX do occur early on in cancer development (Aqeilan *et al.*, 2004a). Finnis *et al.* (2005) analysed the homozygous deletions at *FRA16D* in two cancer cell lines (KM12C and KM12SM), which arose from the same primary tumour but were at different stages of cancer progression. The KM12C cell line was obtained from the original tumour whereas the KM12SM cell line was from the tumour that had progressed on to metastasis. Although the two cancer cell lines had several different chromosomal aberrations, both lines had the exact same deletion breakpoints at *FRA16D*, indicating that the deletion spanning *WWOX* had occurred in the early stages of tumourigenesis, preceding the metastatic stage. There is also substantiating evidence of WWOX having a contribution to cancer development in recent studies that showed evidence for individuals with low levels of WWOX having a genetic predisposition to developing gliomas and lung cancer (Yang *et al.*, 2013; Yu *et al.*, 2014).

Whilst alterations in *WWOX* have been shown to occur during the early stages of tumourigenesis in some cancers, WWOX expression is also thought to have an effect on cancer progression as significant correlations between WWOX levels and tumour features have been detected in several studies. Aqeilan *et al.* (2004a) reported a positive correlation between WWOX levels and the grade of gastric tumours, in that a larger proportion of high-grade tumours were found to be negative for WWOX compared to tumours of a lower grade. Nunez *et al.* (2005a) on the other hand, did not detect correlations with tumour grade in ovarian cancers, but found a significant correlation between WWOX levels and the stage of cancer. Tumours at a more advanced stage were more frequently found to have complete loss of WWOX protein compared to tumours of an early stage. They also noted that in the cases of progressive cancer, patients with absent or low WWOX levels had

worse survival outcomes compared to patients with higher WWOX levels. Similarly, other studies in breast and colorectal cancer patients have also found that reduced WWOX expression is associated with poorer prognosis (Pluciennik *et al.*, 2006; Zelazowski *et al.*, 2011).

Positive correlations were also detected between WWOX and the estrogen receptor (ER) status in a substantial proportion of human breast carcinomas, with higher levels of WWOX detected in ER positive (ER+) breast carcinomas compared to ER negative (ER-) carcinomas (Ludes-Meyers *et al.*, 2003; Guler *et al.*, 2004; Nunez *et al.*, 2005a; Pluciennik *et al.*, 2006). A similar correlation was also reported between WWOX and progesterone receptor status (PR) (Nunez *et al.*, 2005b; Pluciennik *et al.*, 2006). Absence of these receptors has been observed in Wwox-negative mouse mammary tumours as well (Abdeen *et al.*, 2011), suggesting that there may be an association between WWOX expression levels and steroid hormone signalling. The presence of sex steroid hormones, such as 17β-estradiol and androgen, has previously been shown to result in activation of WWOX (Chang *et al.*, 2005a). Although the activation of WWOX by those hormones was shown to be independent of ER and androgen receptor (AR) in that study, nonetheless it implicates WWOX in steroid hormone pathways in the cell. These correlations, together with the high level of *WWOX* expression in hormone-regulated organs and the *in vitro* evidence of WWOX being able to metabolise steroid substrates (Saluda-Gorgul *et al.*, 2011), suggest that WWOX may have an important role in steroid signalling and/or metabolic pathways.

The findings from all these studies support the view that reduction in normal WWOX levels, and hence perturbation of its function, has negative implications for the cell and is associated with tumourigenesis. The reduction in WWOX levels in certain cancers has been observed together with alterations in another common fragile site gene, *FHIT*, which spans the most common fragile site, *FRA3B* and is also thought to act as a tumour suppressor (Ishii *et al.*, 2003; Aqeilan *et al.*, 2004a; Guler *et al.*, 2004). This has led to the postulation that fragile site genes are involved in similar molecular pathways and that perturbation of the pathways associated with fragile site genes could lead to cancer.

1.2.2 WWOX as a non-classical tumour suppressor

Frequent loss of *WWOX* mRNA and protein expression in various cancers has led to the proposal that WWOX may have a role as a tumour suppressor. The first evidence of tumour suppressor activity was demonstrated by Bednarek *et al.* (2001) when ectopic

expression of WWOX resulted in significant suppression of tumour growth both *in vitro* (in breast cancer cell lines grown in soft agar) and *in vivo* (in nude mice injected with breast cancer cells). Consistent with this are other studies that found that restoration of WWOX into WWOX-negative lung, pancreatic and prostate cancer cell lines induced apoptosis (Kuroki *et al.*, 2004; Fabbri *et al.*, 2005; Qin *et al.*, 2006). Furthermore, treatment of lung and breast cancer cells with methylation inhibitor DAC or with expression of microRNA-29 (which targets DNA methyltransferases) resulted not only in the activation of *WWOX* expression, but also led to suppression of tumour growth (Fabbri *et al.*, 2007; Iliopoulos *et al.*, 2007).

Whilst WWOX has been shown to have tumour suppressor activity, it does not appear to act as a classical tumour suppressor. According to Knudson's two hit hypothesis, inactivation of both alleles is typically required for the loss of function of tumour suppressor genes. In the case of WWOX however, inactivation of just one allele has been reported in cancer, with WWOX protein expression from the remaining functional allele detectable in tumours (albeit at lower levels compared to normal tissues). This suggests that reduction of WWOX expression is sufficient to disrupt the cellular processes in which WWOX is involved. Similarly, in a Wwox mutant mouse model generated by Aqeilan *et al.* (2007), both homozygous and heterozygous null mutants were found to develop spontaneous and induced tumours, from which they concurred that loss of one copy of Wwox is able to contribute to tumourigenesis. However, no tumour formation has been detected in other Wwox models, which include an independent null mutant mouse model generated by *Cre*-recombinase mediated deletion (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; O'Keefe *et al.*, 2011), as well as in humans with inherited mutations of WWOX (Abdel-Salam *et al.*, 2014; Mallarat *et al.*, 2014; Ben-Salam *et al.*, 2014). This has led to the suggestion that whilst loss of WWOX appears to have a contribution to tumourigenesis, WWOX may not behave as a classical highly penetrant tumour suppressor (Ferguson *et al.*, 2012) and further research is required to define its role in cancer development and/or progression. Identification of the molecular pathways in which WWOX is involved in could hence be crucial in elucidating the biological function of WWOX and subsequently, its role in cancer.

1.3 *In vitro* studies of WWOX function

Since the identification of WWOX as a non-classical tumour suppressor, many studies have been conducted to determine the biological function(s) of WWOX. Most of these are *in vitro* studies performed in normal and cancer cell lines. These *in vitro* studies have found numerous proteins that can physically bind to WWOX and have been useful in identifying the participation of WWOX in various pathways.

1.3.1 The role of WWOX in apoptosis

As WWOX has been found to suppress tumour growth and has the ability to induce apoptosis when reintroduced into cancer cells, numerous studies have been carried out to further characterise the role of WWOX in apoptosis. Phosphorylation of the Tyr33 residue in WWOX leading to its activation and translocation into the nucleus has been shown to be required for WWOX-mediated apoptosis in murine cells (Chang *et al.*, 2001). WWOX has since been demonstrated to interact with various components of the apoptotic pathway.

Initial studies in murine fibroblasts revealed upregulation of p53 expression and downregulation of apoptosis inhibitors Bcl-2 and Bcl-x_L expression by WWOX (Chang *et al.*, 2001; Chang *et al.*, 2003a). In addition to regulating p53 expression, WWOX was also shown to interact physically with p53 and to be required for p53-mediated apoptosis (Chang *et al.*, 2005b). Two other studies however failed to detect any physical interactions between WWOX and p53 and instead showed binding of WWOX through its WW domain to p73, which is a p53 homolog (Aqeilan *et al.*, 2004b; Lin *et al*., 2013). WWOX was also found to bind via its first WW domain to c-Jun N-terminal kinase 1 (JNK1), which acts to inhibit WWOX-mediated apoptotic activity (Chang *et al*., 2003b). This is supported by a later study that demonstrated that inhibition of JNK enhances WWOX-induced apoptosis in human hepatocellular carcinoma cells (Aderca *et al*., 2008).

WWOX has been shown to be able to induce caspase-mediated apoptosis through both the intrinsic and extrinsic pathways. Ectopic expression of WWOX resulted in activation of caspase-3 in various cancer cell lines. WWOX was found to promote mitochondrial-mediated apoptosis in prostate, breast, lung and leukemic cells, characterised by decreased expression of Bcl-2, release of cytochrome C from the mitochondria and activation of both caspase-3 and caspase-9 (Qin *et al.*, 2006; Iliopoulos *et al*., 2007; Zhang *et al.*, 2012; Cui *et al*., 2013). On the other hand, WWOX has also been

found to increase TNF∝-mediated cytotoxicity. WWOX is able to physically interact with the TNF∝ receptor-associated death domain protein (TRADD) of the TNF-signalling pathway, resulting in activation of caspase-8 (Chang *et al.*, 2003a). This is supported by the decreased expression of procaspase-8 and increased expression of caspase-3 in pancreatic cancer-derived cells when WWOX expression is restored (Nakayama *et al.*, 2008). In addition, WWOX can also bind to and inhibit the activity of the TNF∝-receptor-associated factor 2 (TRAF2), which is an inhibitor of TNF∝-mediated cell death (Chang *et al.*, 2003a). Furthermore, WWOX has been shown to physically bind to downstream targets of the pathway, such as MEK1 and IκBα to promote apoptosis (Li *et al.*, 2009; Lin *et al.*, 2011). WWOX also binds to the zinc finger-like peptide that regulates apoptosis (Zfra), which regulates the TNF∝ and JNK apoptotic pathways and appears to block the apoptotic activity of WWOX (Hong *et al.*, 2007a). From these studies, it is apparent that WWOX is able to mediate cell death through interactions with various components of the apoptotic pathways.

In addition to interacting with p53 and components of the TNF-signalling pathway, WWOX has also been shown to induce apoptosis through its interaction with complement protein C1q in serum. Whilst C1q is normally involved in the classical complement pathway that mediates antibody responses to foreign antigens, C1q was shown by Hong *et al.* (2007b) to be able to induce apoptosis of cancer cells independent of the classical complement pathway in the presence of WWOX. C1q is thought to activate WWOX and increase the formation of microvilli between cells, thus destabilizing the adherence properties of those cells and resulting in cell shrinkage and subsequently death. This interaction between WWOX and C1q has identified a novel role for both WWOX and C1q in mediating apoptosis. Another mechanism in which WWOX has been proposed to regulate cell death is through the TGF-β/Hyal2/WWOX/Smad4 signalling pathway (Nakayama *et al.*, 2008; Hsu *et al.*, 2009). Binding of transforming growth factor beta (TGF-β) to cell surface hyaluronidase Hyal-2 is thought to recruit activated WWOX to the surface, which in turns recruits SMAD family member 4 (Smad4). The Hyal2/WWOX/Smad4 complex then translocates into the nucleus where it is able to enhance the activation of Smad promoter, which subsequently leads to cell death.

The process of apoptosis in the cell is highly complex and is tightly regulated at each step of the different pathways. From all these studies, it is apparent that there are many facets to the role of WWOX in apoptosis. The participation of WWOX in different stages of various apoptotic mechanisms suggests that WWOX is central in regulating

apoptosis in the cell and that WWOX may mediate multiple apoptotic mechanisms in response to particular stimuli or cellular conditions.

1.3.2 Other reported WWOX binding partners

Upon classification of the first WW domain of WWOX as a Group 1 WW domain, numerous proteins containing PPxY motifs have been identified to be able to bind and interact with WWOX. WWOX has been shown to bind via its first WW domain to various transcription factors, such as activator protein-2γ (AP-2γ), C-Jun, runt-related transcription factor 2 (RUNX2), receptor tyrosine-protein kinase ErbB4 and most recently, the hypoxia-inducible factor 1 alpha (HIF1∝) and Kruppel-like transcription factor (KLF5) (Aqeilan *et al.*, 2004b; Aqeilan *et al.*, 2004c; Aqeilan *et al.*, 2005; Gaudio *et al.*, 2006; Aqeilan *et al.*, 2008; Abu-Remaileh and Aqeilan; 2014; Ge *et al*., 2014). Elevated activities of these transcription factors have been observed during tumourigenesis, suggesting that these transcription factors could be oncogenic if their transcriptional activities are not tightly regulated. Binding of WWOX has been shown in most cases to prevent these transcription factors from translocating into the nucleus to activate transcription, thus inhibiting their oncogenic activity under normal conditions.

In addition to these transcription factors, WWOX has also been found to bind to other PPxY motif proteins, with a few examples being Ezrin (a signal transducer that causes localisation of WWOX in apical membranes of parietal cells) and small integral membrane protein of the lysosome/late endosome (SIMPLE) (Ludes-Meyers *et al.*, 2004; Jin *et al.*, 2006). The interaction with Ezrin appears to be essential for apical membrane remodelling associated with H,K-ATPase recruitment. The biology behind the WWOX-SIMPLE interaction on the other hand is unclear – it was postulated that it might be important for mediating apoptosis as SIMPLE has been associated with both TNF∝ and NF-κB pro-apoptotic activity. Interestingly, mutations in SIMPLE have recently been associated with defects in the peripheral nervous system due to mislocalisation of the protein to the mitochondria (Lacerda *et al*., 2014). Apart from PPxY motifs, a recent study showed that WWOX is also able to bind to proteins with a LPxY motif, such as the ubiquitin E3 ligase ITCH. This physical association with ITCH results in polyubiquitination and consequently, stabilisation of WWOX as well as translocation of WWOX into the nucleus (Abu-Odeh *et al*., 2014).

Whilst most of the WWOX physical interactors have been found to bind to its WW domains, there are also a few studies that have reported binding of proteins to an unspecified region of WWOX downstream of the WW domains. The exact binding motif in that region has not been identified. Among those studies was a yeast two-hybrid analysis, which demonstrated that the human microtubule-associated protein Tau is able to bind to a region of the murine WWOX downstream from its WW domains (Sze *et al.*, 2004). This study showed that down-regulation of WWOX results in spontaneous phosphorylation of Tau and formation of neurofibrillary tangles, which has been associated with neurodegenerative diseases such as Alzheimer's. It was suggested that WWOX is involved in the regulation of Tau phosphorylation and could be protective against neurodegeneration. Interestingly, another study failed to detect formation of a WWOX-Tau complex and instead demonstrated that WWOX affects Tau phosphorylation and neuronal differentiation through binding to glycogen synthase kinase 3β (GSK3β) (Wang *et al.*, 2012). Two other proteins, Dishevelled protein 2 (Dvl2) of the Wnt/β-catenin pathway and Zfra (to regulate apoptosis), have been found to bind to both the WW domains and its downstream region, although the WW domain binding appeared to be of a stronger nature (Hong *et al*., 2007a; Bouteille *et al*., 2009).

Since the initial identification and characterisation of WWOX, numerous *in vitro* WWOX interactors have been identified, implicating WWOX in various molecular pathways. Whilst it is clear that WWOX has tumour suppressor activity and a central role in apoptosis, it appears that WWOX is also involved in various other processes contributing to the maintenance of cellular homeostasis. This view is supported by recent implications of WWOX having a role in neuronal and metabolic processes *in vivo*.

1.4 Participation of WWOX in neuronal function

WWOX was first suggested to have a role in neuronal function due to its high expression in neural tissues (Chen *et al*., 2004) and it was also proposed that WWOX has a protective role against neurodegeneration as loss of WWOX resulted in Tau hyperphosphorylation and decreased neurite outgrowth in cell lines (Sze *et al*., 2004; Wang *et al*., 2012). Rats carrying a spontaneous mutation in WWOX were reported to have epileptic seizures (Suzuki *et al.,* 2009) and it had also just been revealed that WWOX null mutant mice display a seizure phenotype as well (Mallaret *et al.,* 2014). However, it is only most recently that loss of WWOX has been associated with neuronal phenotypes in humans. Four different recessively inherited WWOX aberrations were identified in three independent studies of individuals that suffer from epilepsy, ataxia and mental retardation, with early death detected in some cases (Abdel-Salam *et al.,* 2014; Mallaret *et al*., 2014; Ben-Salem *et al*., 2014). These individuals were all found to be from consanguineous families of Arabic origin and are therefore likely to have multiple genes and/or non-coding sequences that are homozygous by descent. The detected homozygous aberrations in WWOX have been proposed to be the causative factor for these neuronal defects.

Three of the identified WWOX aberrations are mutations that result in amino acid substitutions. One of the mutations is in Exon 2 and results in a premature stop codon (Abdel-Salam *et al.,* 2014) whilst the other two are missense mutations, one of which is in proline 47, causing a loss of function of the first WW domain (Mallaret *et al.,* 2014). The second missense mutation is in a glycine residue (that is conserved across species) in exon 9 (Mallaret *et al.,* 2014), which is in a region that has recently been proposed to contain the SDR substrate-binding site (*Richard et al., 2015*). The fourth aberration is a deletion in exon 5, which encodes part of the SDR enzyme (Ben-Salem *et al*., 2014). This deletion results in exclusion of exon 5 from the transcript and introduction of a premature stop codon. The truncated transcript is presumably degraded by nonsense-mediated decay as there are lower *WWOX* transcript levels in those individuals. These aberrations all potentially result in loss of WWOX function, indicating that WWOX has a crucial role in neuronal function and that loss of WWOX can result in severe neuronal disorders.

1.5 WWOX and metabolism

1.5.1 Metabolic defects in murine Wwox mutant models

WWOX was first reported in the literature to have a role in metabolic pathways when Wwox null mutant mice generated by Aqeilan *et al.* (2007) were found to suffer from severe growth retardation and metabolic disorders resulting in early death. These mice exhibited severe bone metabolic disorder, with significant reduction in bone formation and increased bone resorption as well as elevated levels of RUNX2, a transcription factor important for osteoblast differentiation (Aqeilan *et al.*, 2008). It is thought that Wwox is normally involved in regulation of bone tissue formation by both reducing expression of RUNX2 as well as by physically suppressing its transcriptional activity. Recent studies have also detected elevated RUNX2 levels in WWOX-negative human osteosarcomas, suggesting that loss of WWOX may contribute to bone cancer through dysregulation of RUNX2 (Kurek *et al.*, 2010; Del Mare *et al.*, 2011).

In addition to bone metabolic disorders, the Wwox null mutant mice also displayed other signs of metabolic defects, such as reduced serum levels of proteins, carbohydrates and lipids (Aqeilan *et al.*, 2008). Further analysis revealed that the mice also have impaired steroidogenesis resulting in gonadal abnormalities (Aqeilan *et al.*, 2009). Microarray profiling in the gonads of affected mice revealed alterations in expression of 15 key genes involved in steroidogenic pathways. Whilst WWOX has previously been postulated to be involved in steroid metabolism (Bednarek *et al.*, 2000; Chang *et al.*, 2003a; Saluda-Gorgul *et al.*, 2011), this was the first evidence of WWOX having a functional role in steroidogenesis. The exact mechanism(s) by which WWOX regulates these pathways are still unclear. Intriguingly, it was indicated that the first WW domain of WWOX is crucial for this regulation of steroid enzymes (Aqeilan *et al.*, 2009), however it was not determined if the other regions of WWOX, such as the SDR enzyme region, could also have a contribution. A different study in bacterial systems later showed that WWOX is able to oxidise seven different steroid substrates *in vitro*, with this function being attributed to the SDR enzyme on the basis of it being an oxidoreductase/dehydrogenase enzyme (Saluda-Gorgul *et al.*, 2011).

In 2009, a second Wwox null mutant mouse model was described by Ludes-Meyer *et al.* (2009). These mice carried a Cre-mediated deletion of exon 1 of the *Wwox* gene whilst the first Wwox null mutant mice generated by Aqeilan *et al.* (2007) had deletions of exon 2, 3 and 4 of the *Wwox* gene. Nevertheless, complete loss of Wwox protein was

reported in both mouse models. Characterisation of this second mouse model revealed many similarities with the phenotypes observed from the first mouse model, supporting that the loss of Wwox is responsible for these phenotypes. Mice homozygous for the Cre-mediated deletion of Wwox exon 1 also exhibited severe metabolic defects, such as bone disorders, hypoglycemia and higher blood urea nitrogen levels, resulting in growth retardation and reduced lifespan. In addition, these mice had reduced bicarbonate levels in blood, suggestive of perturbation in the balance of acid/base levels which could result in metabolic acidosis.

Apart from the null mutant mice, a spontaneous Wwox mutant rat was also reported in 2009 (Suzuki *et al.,* 2009). These mutant rats carried a 13 base pair deletion in exon 9 of the *Wwox* gene and whilst there were similar levels of *Wwox* transcript, there was no detectable full length Wwox protein. Similar to the mouse models, Wwox mutant rats were found to have reduced lifespan and showed severe dwarfism. Although these rats did not appear to have severe metabolic bone disorders as seen with the mutant mice, they shared other phenotypes, such as gonadal abnormalities and high blood urea nitrogen levels, indicative of a metabolic disruption (Suzuki *et al.,* 2007). The mutant rats were also found to suffer from spontaneous and induced epileptic seizures due to abnormal neuronal excitability, suggestive of a role for Wwox in the central nervous system. Similar seizure phenotypes have now been reported in both null mutant mouse models (Mallaret *et al.,* 2014; Aqeilan, personal communication), however it is not known if the neuronal phenotype is linked to the metabolic defects in those mice.

1.5.2 WWOX as a susceptibility gene for metabolic diseases

Genome-wide association studies (GWAS) are widely used as a method to identify susceptibility genes for complex diseases in humans. Several studies have linked *WWOX* to metabolic traits or diseases arising from altered metabolism, supporting a role for WWOX in metabolic pathways. WWOX was first linked to low plasma high density lipoprotein-cholesterol (HDL-C) levels, a risk factor for coronary artery disease, through the association between a variant of the *WWOX* gene and low HDL-C levels from analyses of 9798 subjects across several different populations (Lee *et al.,* 2008) as well as in later studies in French-Canadian families (Iatan *et al.,* 2014). WWOX has also been identified as a quantitative trait loci (QTL) for triglyceride and HDL-C levels in a Spanish population

(Saez *et al*., 2010), with the latter also linked to WWOX through a study using a mouse QTL map (Leduc *et al*., 2011). Further analyses found that WWOX null mutant mice do have reduced levels of serum HDL-C, which is indicative of a role for WWOX in cellular lipid homeostasis as identified by GWAS (Iatan *et al.*, 2014). In addition to HDL-C and triglyceride levels, a GWAS for type 2 diabetes in a Chinese population had identified a genetic variant between *WWOX* and its neighbouring gene, *MAF* to be associated with reduced insulin secretion and increased fasting glucose, potentially increasing the risk of diabetes (Chang *et al*., 2012). Other subsequent studies also identified WWOX as a susceptibility locus for type 2 diabetes in East Asian populations (Cho *et al*., 2011; Sakai *et al*., 2013) as well as being a hypertension susceptibility gene (Yang *et al.*, 2012). The identification of WWOX as a possible susceptibility gene for these metabolic traits provides support for WWOX having an important role in metabolic pathways.

1.5.3 WWOX and aerobic metabolism

WWOX was first linked to aerobic metabolism in the *Drosophila* model established in our laboratory (O'Keefe *et al.,* 2011), in which alterations in the level of TCA cycle enzymes were observed in response to Wwox (described in greater detail in Section 1.8). A subsequent study using human cell lines also found that the steady-state levels of WWOX transcripts are dependent on the metabolic status of cells (Dayan *et al.*, 2013). WWOX levels are upregulated under conditions that induce oxidative phosphorylation whilst there is a decrease in transcript levels under glycolytic and hypoxic conditions. Subsequent to this, a role for WWOX in glucose metabolism has also been reported in a study using Wwox null mutant mouse embryonic fibroblasts (Abu-Remaileh and Aqeilan; 2014). The observations included increased glucose uptake and lactate levels as well as reduced oxygen consumption, low ATP and NADH levels and high NADPH levels associated with loss of Wwox. It was proposed that Wwox is normally involved in mitochondrial respiration by suppressing HIF1∝ activity (through binding to HIF1∝ via its first WW domain) and that loss of Wwox results in upregulation of HIF1∝ and subsequently, inhibition of the TCA cycle and enhanced glycolysis.

Altered metabolism is a particular area of interest in cancer research as it is recognised as one of the hallmarks of cancer cells (Hanahan and Weinberg, 2011). In the presence of oxygen, cells normally metabolise glucose through the tricarboxylic acid (TCA) cycle to generate energy and only undergo glycolysis to generate lactate from

glucose under anaerobic conditions. A link between altered metabolism and cancer was first proposed by Otto Heinrich Warburg, who showed that tumour cells tend to undergo glycolysis even in the presence of oxygen. This event of aerobic glycolysis has since been known as the Warburg effect and has been demonstrated in different types of cancers (Altenberg and Greulich, 2004; Hsu and Sabatini, 2008). The implication of various cancer-associated genes (such as the *MYC* oncogene and *p53* tumour suppressor gene) in the Warburg effect has provided further support for an association between altered metabolism and cancer (Kim and Dang, 2006; Koppenol *et al.*, 2011). Furthermore, identification of mutations in TCA cycle enzymes in various cancers has also contributed to the suggestion that metabolic reprogramming of cells has a significant role in tumourigenesis (Kim and Dang, 2006; Yan *et al.*, 2009). There is now increasing evidence that WWOX has an essential role in regulating cellular metabolism, leading to the hypothesis that one of the ways that decreased WWOX can contribute to tumourigenesis is through an involvement in the metabolic reprogramming of cells. Thus, identification and investigation of the different metabolic pathways in which WWOX has an effect is essential to elucidate its role in cancer.

1.6 Using *Drosophila* as an *in vivo* model system to study Wwox

WWOX has been shown to be involved in a wide range of processes, ranging from apoptosis to metabolism as well as most recently in neuronal function. Many of these findings are based on *in vitro* studies of mammalian cell lines, which have been a rich resource of information in terms of the identification of WWOX binding partners as well as its apoptotic function. *In vivo* studies however are crucial for determining which functions of WWOX are biologically significant. The WWOX mutant rodents have indeed been crucial in providing insight into the consequences brought about by loss of WWOX, in particular the metabolic implications. A role for WWOX in both metabolism and apoptosis has also been observed in our *Drosophila* model of WWOX, indicating that these roles of WWOX are of biological significance. An advantage of using *Drosophila* as an *in vivo* model to study WWOX function is that genetic analyses can easily be performed to further investigate these pathways.

1.6.1 *Drosophila* as a model system to study gene function

Drosophila melanogaster is widely utilised as a model organism to study the function of human genes and to identify the mechanisms underlying human genetic diseases. The *Drosophila* genome has been fully sequenced and there is a *Drosophila* orthologue for 77% of human disease genes (Reiter *et al.*, 2001). This has paved the way for the study of "reverse genetics", in which mutations are generated in the fly and used in studies to gain better understanding of the normal function of the gene in question (O'Kane, 2003). *Drosophila* has not only been used extensively to study numerous cellular processes and diseases such as cancer (Bier, 2005), but is also used in therapeutic drug discovery studies (Pandey and Nichols, 2011).

Drosophila is a highly genetically manipulable organism and there are various genetic tools available for use, which include the GAL4-UAS expression system that allows for the ectopic expression of specific genes or constructs (Brand and Perrimon, 1993). RNA-mediated gene interference (RNAi) has also been introduced into the fly to study the consequences of knocking down the level of expression of specific genes and the establishment of the Vienna *Drosophila* Resource Center (VDRC) has made available RNAi fly lines for more than 88% of *Drosophila* genes (Dietzl *et al*., 2007). These RNAi lines have been widely used in the studies of reverse genetics and have been found to be

efficient and specific in the targeting of genes. These available techniques and resources have made *Drosophila* an ideal model organism to study the function of genes and to elucidate the molecular processes in which the genes are involved. In addition, the small size of the fly, short lifespan, fast generation time, ability to generate large numbers of progeny as well as the cost effectiveness of generating and maintaining fly lines are other advantages of using *Drosophila* as a model system.

1.7 *Drosophila* orthologue of WWOX

The *Drosophila* orthologue of WWOX was previously identified in our laboratory through database searching and phylogenetic analysis (O'Keefe *et al.*, 2005). The gene, *CG7221*, was identified from the *Drosophila* genome as having the highest similarity to the human *WWOX*, with 49% sequence identity at the protein level. This *Drosophila* gene contains 6 exons and codes for a 409 amino acid protein. Similar to the full-length human WWOX protein, the *Drosophila* Wwox consists of the two WW domains as well as the SDR enzyme (Figure 1.3), with the conserved TGxxxGxG cofactor binding site motif and the YxxxK catalytic active site motif. There is also high sequence conservation at the C-terminus of the proteins, which has been postulated to contain the SDR substrate-binding region (Richards *et al.*, 2015). High evolutionary conservation of the *WWOX* gene across distant species is indicative of WWOX having a biologically significant function (O'Keefe *et al.*, 2005; Richards *et al.*, 2015). A *Drosophila* model of WWOX was thus established to study the function of WWOX and to identify the biological pathway(s) in which WWOX is involved.

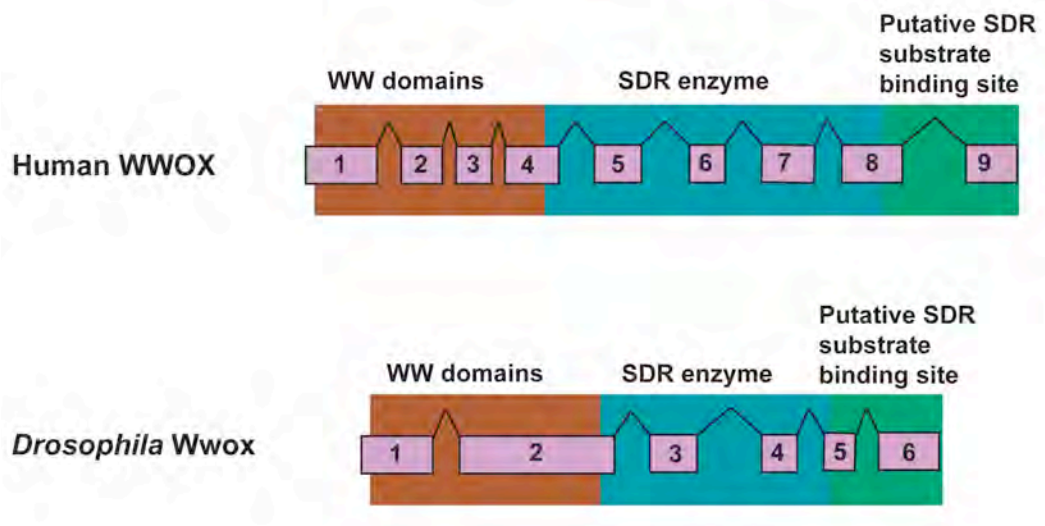

Figure 1.3 Domains of the *Drosophila* Wwox protein that are conserved with the human WWOX. Both the human WWOX and *Drosophila* Wwox proteins contain two WW domains and a SDR enzyme, which is characterised by the presence of conserved cofactor binding site and catalytic active site motifs. There is also conservation at the C-terminus region that has been hypothesised to contain the SDR substrate-binding site.

1.8 *Drosophila* model of WWOX

Following the identification of the *Wwox* orthologue in *Drosophila*, a null mutant, $Wwox^{1}$, was generated by homologous recombination to study the function of Wwox through reverse genetics (O'Keefe *et al*., 2005). Two premature stop codons and a +1 frameshift were introduced 24bp downstream of the start codon of the *Wwox* gene, resulting in the absence of any detectable Wwox protein in $Wwox^{1}$. An independent Wwox mutant, $Wwox^{f04545}$, which contains a piggyBac transposon inserted into the 2nd intron of the gene, was obtained from the Bloomington *Drosophila* Stock Center and there was no detectable WWOX protein in these flies as well (Thibault *et al*., 2004; O'Keefe *et al*., 2011). The Wwox null mutant flies were viable and displayed no obvious phenotype (O'Keefe *et al*., 2005; O'Keefe *et al*., 2007). From initial analyses, it was found that both $Wwox^{1}$ and $Wwox^{f04545}$ homozygous mutant flies were sensitive to ionizing radiation (O'Keefe *et al*., 2005), however further analyses indicated that the observed sensitivity was caused by background mutations instead of the Wwox mutations, as the transheterozygous mutant flies ($Wwox^{1}/Wwox^{f04545}$) did not exhibit such sensitivity (O'Keefe *et al*., 2007). These homozygous mutant flies were then subjected to 4 rounds of backcrossing to a wild-type strain, w^{1118}, to eliminate the background mutations that had likely arisen during the mutagenesis process (O'Keefe *et al*., 2007). All following analyses have been performed on the backcrossed mutant fly strains. In addition to the two Wwox null mutant fly lines, *Drosophila* lines with overexpression of Wwox were also established to determine the effects of ectopically increasing Wwox expression (O'Keefe *et al*., 2005; O'Keefe *et al*., 2011). The Wwox ectopic expression fly lines were also found to have no discernible phenotype.

1.8.1 Alterations in metabolic enzyme levels in response to Wwox

As the homozygous Wwox null mutant flies and the Wwox ectopic expression fly lines displayed no obvious phenotype, biochemical approaches were undertaken to identify changes that were occurring at a molecular level as a result of altering Wwox levels. Proteomic and microarray analyses were carried out to identify proteins and mRNAs that were altered in levels when Wwox was absent or ectopically expressed in *Drosophila* embryos and adults. The levels of proteins and mRNAs were measured in the two different Wwox mutants ($Wwox^{1}$ and $Wwox^{f04545}$) compared to a wild-type control fly line (w^{1118}).

Similar analyses were carried out using a *Drosophila* line with ectopic expression of Wwox (*da>Wwox*) compared to its appropriate wild-type control (*da>+*). 27 different proteins and/or mRNAs were found to be altered from these analyses, with a significant proportion of them being known or predicted to be involved in metabolic pathways (Figure 1.4, Table 1.1) (O'Keefe *et al*., 2011). In particular were various pathways that converge on the tricarboxylic acid (TCA) cycle, which suggest that the alteration of Wwox levels could have an effect on aerobic metabolism.

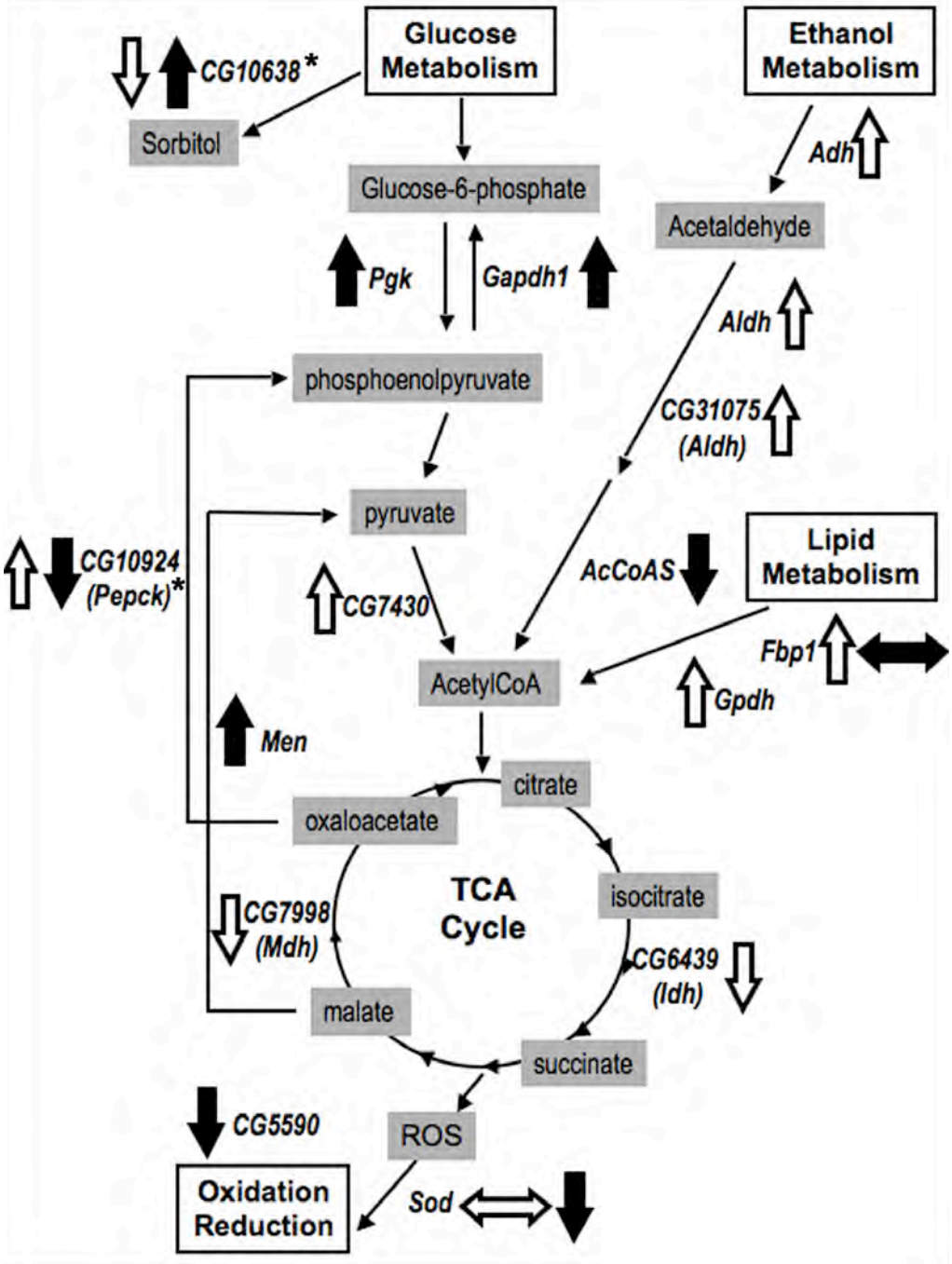

Figure 1.4 Metabolic enzymes that were altered in response to Wwox. The levels of various enzymes involved in metabolic pathways such as the TCA cycle, glucose, ethanol and lipid metabolism as well as redox homeostasis were altered in either Wwox null mutant flies or flies with ectopic expression of Wwox. The arrows indicate the direction of the change, with the side arrows indicative of a change in isoforms of the protein. The solid black arrows are indicative of changes in the Wwox null mutants whilst the white open arrows are changes in the Wwox ectopic expressing flies. (Figure obtained from O'Keefe *et al*., 2011).

Table 1.1 Fold change observed in expression of genes altered in response to Wwox (Obtained from O'Keefe et al., 2011).

Gene name/Symbol	Spot #	Protein Abundance			Transcript Abundance (measured by qPCR)			Molecular Function
		w^{1118} vs *Wwox mutants*		*da>+* vs *da>Wwox*	w^{1118} vs *Wwox* mutants		*da>+* vs *da>Wwox*	
		$Wwox^{1}$	$Wwox^{f04545}$	*da>Wwox*	$Wwox^{1}$	$Wwox^{f04545}$	*da>Wwox*	
Wwox/CG7221	1	n/d	n/d	1.49	-2.72	-17.38	172	Oxidoreductase activity
AEROBIC METABOLISM								
TCA CYCLE								
CG6439 (Idh)	2	-1.12	-1.19	n/c	-	-	-	isocitrate dehydrogenase (NAD+) activity
CG7998 (Mdh)	3	-1.30	-1.13	n/c	-	-	-	L-malate dehydrogenase activity
GLUCOSE METABOLISM								
Phosphoglycerate kinase (Pgk)/CG3127	4	n/c	n/c	1.23	-	-	-	phosphoglycerate kinase activity
CG7430	5	1.14	1.18	n/c	-	-	-	dihydrolipoyl dehydrogenase activity
Malic enzyme (Men) / CG10120	6	n/c	n/c	1.48	-	-	-	malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) activity
	7	n/c	n/c	1.43	-	-	-	
Glyceraldehyde-3-phosphate dehydrogenase1 (Gapdh1) /CG12055	8	n/c	n/c	1.22	-	-	-	glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity
CG10638	-	-	-	-	-2.15	-1.70	2.17	aldehyde reductase activity
CG10924 (PEPCK)	-	-	-	-	5.00	3.77	-1.82	phosphoenolpyruvate carboxykinase (GTP) activity
ETHANOL METABOLISM								
Alcohol dehydrogenase (Adh)/ CG3481	9	1.53	1.27	n/c	-	-	-	alcohol dehydrogenase (NAD) activity
Aldehyde dehydrogenase (Aldh)/ CG3752	10	1.25	1.36	n/c	-	-	-	aldehyde dehydrogenase (NAD) activity
Acetyl Coenzyme A synthase (AcCoAS) /CG9390	11	n/c	n/c	-1.57	-	-	-	acetate-CoA ligase activity
CG31075	12	1.14	1.23	n/c	-	-	-	aldehyde dehydrogenase (NAD) activity
	13	1.10	1.08	n/c	-	-	-	
LIPID METABOLISM								
Glycerol-3-phosphate Dehydrogenase (Gpdh)/ CG9042	14	1.16	1.21	n/c	-	-	-	glycerol-3-phosphate dehydrogenase (NAD+) activity
Fat body protein 1 (Fbp1)/CG17285	15	2.70	2.00	n/c	-	-	-	protein transporter activity
	16	n/c	n/c	3.04	-	-	-	
	17	n/c	n/c	1.60	-	-	-	
	18	n/c	n/c	-2.20	-	-	-	
OXIDATION REDUCTION								
Superoxide dismutase (Sod) /CG11793	19	2.15	1.64	-1.23	1.33	1.64	-1.10	antioxidant activity; superoxide dismutase activity
	20	-2.23	-1.53	n/c				
CG5590	21	n/c	n/c	-1.37	-	-	-	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor
OTHER CATEGORIES								
CG7470	22	n/c	n/c	-1.43	-	-	-	delta1-pyrroline-5-carboxylate synthetase activity; glutamate 5-kinase activity; glutamate-5-semialdehyde dehydrogenase activity
Suppressor of Profilin 2 (Sop2) /CG8978	-	-	-	-	-1.08	1.13	-1.23	actin binding
Hsp60C/CG7235	23	-1.08	-1.24	n/c	-	-	-	ATPase activity, coupled
CG2852	24	n/c	n/c	1.31	-	-	-	peptidyl-prolyl cis-trans isomerase activity
CG11089	25	1.13	1.21	n/c	-	-	-	phosphoribosylaminoimidazolecarboxamide formyltransferase activity; IMP cyclohydrolase activity
CG14526	26	n/c	n/c	-1.26	-	-	-	metalloendopeptidase activity
Bancal (bl)/CG13425	-	-	-	-	1.10	1.01	-1.29	mRNA binding; transcription factor binding
Prp19/CG5519	-	-	-	-	1.47	1.78	-1.52	ubiquitin-protein ligase activity
Tudor-SN/CG7008	27	n/c	n/c	-1.23	-	-	-	transcription coactivator activity
CG30152	-	-	-	-	1.13	1.23	-1.29	unknown
CG8193	28	n/c	n/c	1.22	-	-	-	monophenol monooxygenase activity

Average fold change ratios of protein abundance for 2D-DIGE spots and transcript abundance for candidates that exhibit significant changes in response to altered levels of Wwox in adult flies. n/d, protein was not detected; n/c, no change in protein abundance was detected.

1.8.2 Functional interactions between Wwox and metabolic enzymes

Functional studies were carried out to determine if Wwox has overlapping function(s) with any of the genes identified from the proteomic and microarray analyses. A genetic or functional interaction is defined as the phenomenon whereby alterations in the levels of two genes together produce a phenotype that is different from the additive phenotypes of the individual gene alterations (Mani *et al*., 2008). As flies with altered Wwox levels have been found to have no obvious phenotype, the approach taken to detect functional interactions was to firstly, alter expression of the individual candidate genes in *Drosophila* and examine those flies for a phenotype (Figure 1.5). Wwox levels were then altered together with that of the candidate gene to determine if altering Wwo*x* levels could modify the candidate gene phenotype. The basis of this approach is that by altering the expression of a candidate gene, this could affect the function of the gene product to a point where it becomes the rate-limiting step of the biological process that it is involved in. This will then lead to disruption of that biological process, resulting in an observable phenotype. If Wwox has a functional interaction with that candidate gene, then altering levels of Wwox could potentially rescue or further disrupt the function of the gene product in that biological process, which would then result in modification of the previously observed phenotype. Hence, any observed modification would be indicative of a functional interaction between Wwox and the particular candidate gene, suggesting that Wwox has a contribution to the same molecular process(es) as that of the candidate gene.

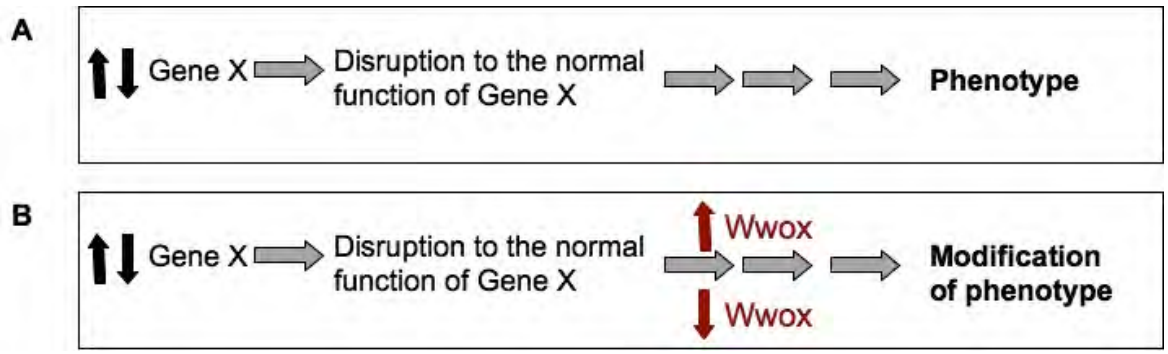

Figure 1.5 Model of the genetic studies carried out to identify functional relationships between Wwox and candidate genes. (A) Expression of Gene X is altered to the point that the function of its gene product is disrupted, resulting in a phenotype. **(B)** Wwox levels are then altered in that background to determine if Wwox can modify the phenotype, which would indicate if Wwox has a contribution to the same molecular process(es) as that of Gene X.

From these genetic studies, Wwox was found to have functional interactions with two genes, *CG6439* and superoxide dismutase, Sod1 (O'Keefe *et al.,* 2011). *CG6439* is the orthologue of the human isocitrate dehydrogenase 3β (IDH3β), which is the β subunit of the NAD(+)-dependent isocitrate dehydrogenase involved in catalysing the decarboxylation of isocitrate to 2-oxoglutarate in the mitochondria and is one of the key regulating enzymes of the TCA cycle (Kim *et al.,* 1999). Decreased *CG6439/ IDH3β* levels resulted in reduced survival of flies, which was further reduced by decreasing Wwox levels whilst ectopic Wwox expression led to an increase in the survival. A parallel study performed in human cancer cell lines found a positive correlation between endogenous WWOX and IDH1 levels (O'Keefe *et al.,* 2011). These studies are supportive of a functional relationship between Wwox and IDH genes. IDH mutations have been identified in various human cancers; whilst most mutations are found to be gain-of-function mutations resulting in production of an oncometabolite, loss-of-function mutations have also been reported (Ward *et al.,* 2012). The discovery of these IDH mutations has resulted in renewed interest in studies regarding altered metabolism having a contribution to cancer (Prenser and Chinnaiyan, 2011).

In the case of Sod1, which is also known as Cu-Zn superoxide dismutase (Cu-Zn Sod), two different peptides (with different isoelectric points) were identified from the proteomic analysis as being altered in level, suggesting that there could be two different Sod isoforms or modified forms altered in response to Wwox. Alterations in Sod1 transcripts were also found to result in inverse changes to Wwox levels in *Drosophila* and human cell lines (O'Keefe *et al.,* 2011). Sod1 catalyses the dismutation of superoxide anions to hydrogen peroxide and oxygen and loss-of-function Sod1 mutations have previously been shown to result in reduced lifespan in *Drosophila* (Parkes *et al.,* 1998). Loss of Wwox in these Sod1 mutants led to further reduction in lifespan, indicating that Wwox and Sod1 have a synergistic effect on the lifespan in *Drosophila* (O'Keefe *et al*., 2011).

As Sod1 is an antioxidant enzyme involved in maintaining the crucial balance of reactive oxygen species (ROS) in cells and ROS are also well known to be by-products of the TCA cycle, ROS levels were examined in the Wwox null mutants as well as in flies ectopically expressing Wwox to determine if there were changes in endogenous ROS levels in response to Wwox (O'Keefe *et al*., 2011). Endogenous ROS levels were measured in larvae using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), which is a cell-permeable probe that becomes fluorescent upon

oxidation by ROS. A decrease in overall endogenous ROS levels was observed in the two homozygous Wwox mutants compared to the wild-type control. Reciprocally, increased ROS levels were seen in two different *Drosophila* lines with ectopic overexpression of Wwox, suggesting that Wwox is involved in a process that results in the generation and/or regulation of ROS. It should be noted however that the ROS assay that was used is a measurement of overall ROS in the whole larvae and does not distinguish between the different types of ROS or its compartmentalization in the cell; nevertheless the data indicates that alteration of Wwox expression has an impact on ROS levels. Whilst oxidative stress resulting in increased ROS levels in the organism is normally associated with deleterious effects, ROS have been shown under normal physiological conditions to have an essential role in numerous cellular processes such as cell signalling and regulation (Thannickal and Fanburg, 2000), hence any disruption to the normal balance of cellular ROS levels would have detrimental effects to the cell.

Observations of functional interactions between Wwox and IDH and Sod1 as well as changes in the levels of ROS in response to Wwox indicate that Wwox has a contribution to metabolic processes in *Drosophila* (Figure 1.6), which is consistent with WWOX mammalian studies. In addition, there are also similarities between the *Drosophila* Wwox and mammalian WWOX in regards to its apoptotic function. There is evidence for *Drosophila* Wwox having a contribution to TNFα-mediated cell death through the modulation of caspase-3 activity (O'Keefe *et al.,* manuscript in preparation; included in Chapter 5). Ectopic expression of Wwox results in enhancement of TNFα-mediated cytotoxicity whilst there is decreased levels of cell death when Wwox is absent. Whilst such a role has previously been shown for mammalian WWOX through *in vitro* studies (Chang *et al.*, 2001), this is the first *in vivo* study that demonstrates a requirement for WWOX in this extrinsic apoptotic pathway. The conserved roles of WWOX in apoptosis and metabolism indicate that these function(s) of WWOX are of primary biological significance. The capability of performing genetic analyses in *Drosophila* makes it a powerful tool for the further investigation of these biological role(s) of WWOX in an *in vivo* context.

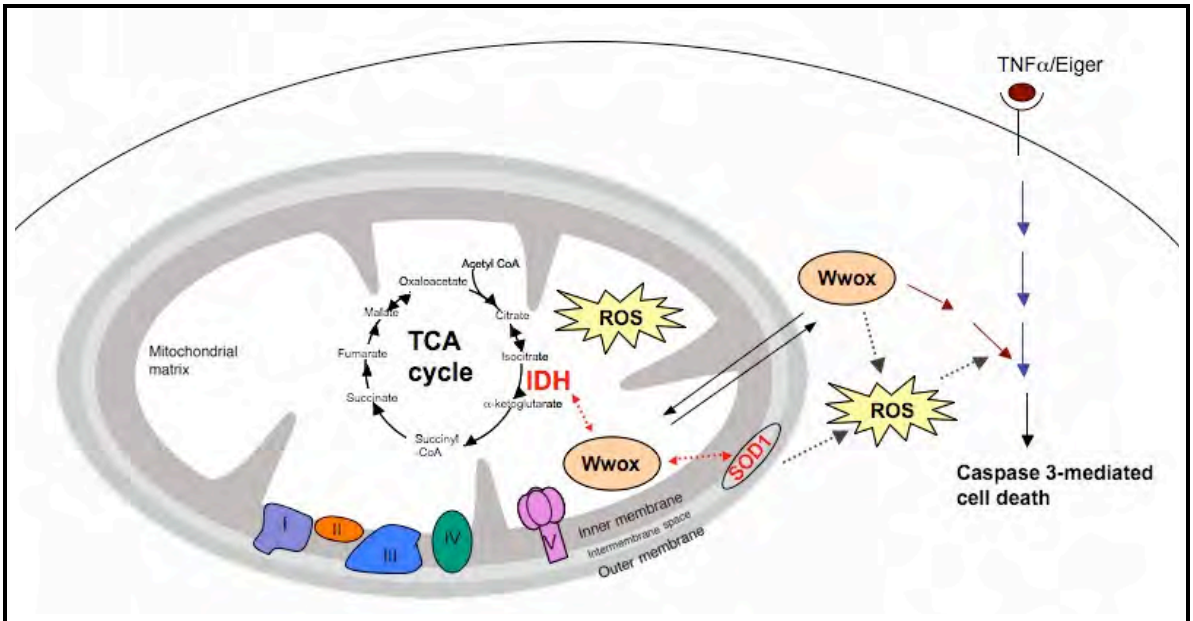

Figure 1.6 Model of the cellular contributions of Wwox identified in *Drosophila*. Wwox has been shown to functionally interact with isocitrate dehydrogenase (Idh) and superoxide dismutase Sod1 in *Drosophila* and mammalian studies have shown that Wwox can translocate from the cytoplasm to the mitochondria. These functional interactions indicate that Wwox has an impact on metabolic processes. Wwox has also been shown to affect ROS levels and to have a role in TNF∝/Eiger cytotoxicity through caspase 3-mediated cell death.

1.9 Aims of this study

WWOX has been identified as a non-classical tumour suppressor gene that has a significant contribution to cancer initiation and progression. Through various studies, predominantly *in vitro* work, WWOX has been implicated in numerous cellular processes including apoptosis and metabolism. Whilst these *in vitro* studies have identified roles for WWOX in many important cellular pathways, an *in vivo* model is required to determine the specific function(s) of WWOX that are of biological significance. In addition, an *in vivo* model that allows comprehensive research into those pathways is essential for the identification of potential therapeutic targets. *Drosophila melanogaster* is an ideal model organism for such studies as it is an *in vivo* model that allows genetic dissection of the apoptotic and metabolic pathways to which WWOX has been shown to have a conserved contribution. The *Drosophila* Wwox model that has previously been established can be used for extensive research into these pathways, which has been limited in the WWOX mutant mouse models by their reduced lifespan.

This study was aimed at using the *Drosophila* model of Wwox to gain novel information regarding the biological processes that Wwox has an effect on, namely metabolism. As the lack of a visible phenotype in *Drosophila* Wwox mutant and overexpression flies has precluded the direct study of reverse genetics, this study was focussed on identifying the types of cellular dysfunction that can be modulated by Wwox. The approach that had been taken was to target other genes to generate a sensitised background and to determine if Wwox had any contribution to the resultant cellular dysfunction. Three different models of cellular dysfunction were examined in this study, with a focus on the types of dysfunction that have been associated with metabolic genes.

The first aim of this study was to identify the types of metabolic dysfunction that Wwox is able to modulate (Chapter 2 & 3) as Wwox was previously shown to have a contribution to metabolic processes. An RNA interference (RNAi) screen was performed to identify a contribution by Wwox to metabolic dysfunction (Chapter 3). Different Wwox overexpression lines carrying mutations in critical amino acid residues of Wwox were established to identify the functional element(s) of Wwox that are involved in its metabolic modulation (Chapter 2 and 3). Further characterisation of the identified metabolic dysfunction was also performed (Chapter 4).

The second model examined was an intrinsic model of tumour suppression that had previously been shown to be regulated by metabolic activity. As Wwox was identified to have a role in exogenous TNFα-mediated cell death, it was hypothesised that Wwox could also contribute to an endogenous function of TNFα in clones of cells that are mutant for a

tumour suppressor protein, Scribbled (Scrib). Scrib mutant clones are eliminated from the *Drosophila* eye tissue through a mechanism involving its surrounding wild-type cells as a form of intrinsic tumour suppression (Brumby *et al.*, 2003). This mechanism is mediated by TNFα signalling and requires the involvement of metabolic activity (Igaki *et al*., 2009; Kanda *et al.*, 2011). This study investigated the involvement of Wwox in the elimination of these tumourigenic Scrib mutant cells (Chapter 5).

The other model of cellular dysfunction that was used in this study was a *Drosophila* model of chromosomal instability (CIN). CIN, which can result in aneuploidy, is often observed in cancer cells and is associated with poor prognosis, drug resistance and cancer relapses (Carter *et al*., 2006). However, as CIN is only common to cancer cells and is not a property of normal cells, it was suggested that the specific targeting of CIN cells could be used as a form of cancer therapy. A *Drosophila* CIN model was established to identify mechanisms in which apoptosis can be induced specifically in CIN cells without affecting normal cells (Shaukat *et al*., 2011). Initial results had indicated that CIN cells are sensitive to the targeting of metabolic genes and induction of metabolic stress, thus the effects of decreasing Wwox expression in CIN cells was investigated in parallel to the study of other metabolic genes in this model (Chapter 6).

The ultimate aim of this study was to identify novel models of cellular dysfunction in which Wwox is involved, in order to define the biological role(s) of Wwox and to enable further dissection of the contribution of Wwox to tumourigenesis. The information gained from this study will be used to inform the direction of future WWOX research, with the underlying objective of elucidating the role of WWOX in cancer development and/or progression.

Chapter 2 - Establishment of *Drosophila* lines ectopically expressing mutations in the different functional elements of Wwox

2.1 Introduction

The *WWOX* gene contains sequences that are highly conserved across species, from humans to the evolutionarily distinct sea sponge (O'Keefe *et al.*, 2005; Richards *et al.*, 2015), indicative of WWOX having a significant role in biology. Sequence homology has revealed distinct functional domains, which include well-known motifs characteristic of WW domains and a short-chain dehydrogenase/reductase (SDR) enzyme. Both the WW domains and SDR enzymatic region are subjects of great interest with respect to the biological role of WWOX, although the WW domains have been the main focus in the literature to date. The identification of WW domain binding partners has been used as a common approach in attempts to elucidate WWOX function (Ludes Meyer *et al.*, 2004; Aqeilan *et al.*, 2004a; Aqeilan *et al.*, 2004b; Aqeilan *et al.*, 2005; Abu-Odeh *et al.*, 2014; Abu-Remaileh & Aqeilan, 2014). On the contrary, not much is currently known regarding the SDR enzymatic region of WWOX – neither its enzymatic activity nor its endogenous substrates have been identified. However, alternatively spliced *WWOX* transcripts that have been detected in various cancers often include the WW domain region but not all the necessary SDR-coding exons (Ried *et al.*, 2000; Paige *et al.*, 2001; Driouch *et al.*, 2002; Kuroki *et al.*, 2002; Lewandowska *et al.*, 2009; Gardenswartz *et al.*, 2014). Some of these transcripts have been shown to be translated into truncated forms of WWOX protein that lack a functional SDR enzyme (Ishii *et al.*, 2003; Mahajan *et al.*, 2005), leading to the hypothesis that loss of SDR function has a significant contribution to tumourigenesis. Hence, there appears a need for the identification of WWOX SDR function.

WWOX has also been reported to contain both nuclear and mitochondrial localisation signals (Chang *et al.*, 2001; Chang *et al.*, 2007). The nuclear localisation signal is located between the WW domains whilst the mitochondrial localisation signal is said to be within the SDR enzyme region, although the exact sequence has not been identified. Furthermore, high conservation of some of the remaining sequences suggests that there could be other functional elements that are yet to be defined. As SDR enzymes typically contain their substrate-binding sequence C-terminal to their catalytic domain (Kavanagh *et al.*, 2008), it has been postulated that the highly conserved sequences at the C-terminal region of WWOX (downstream of its catalytic region) could contain the specific binding site for the WWOX SDR substrates (Richards *et al.*, 2015).

Mutational studies are widely used to investigate the role of different functional elements within a protein. An advantage of using *Drosophila* as a model system is the feasibility of ectopically expressing constructs containing targeted mutations (using the binary GAL4-UAS system, Figure 2.1) in order to determine the effect of the different mutations. These constructs can be expressed in a cell- or tissue-specific manner (Brand & Perrimon, 1993). Although alterations in Wwox expression alone have not been found to result in any detectable phenotype *in Drosophila* thus far, the use of the GAL4-UAS system allows for expression of the mutant Wwox constructs in *Drosophila* with sensitised backgrounds (where there is loss of other genes that would result in detectable phenotypes that can be modulated by Wwox, described in Chapter 3). This enables the identification of the amino acid residues that are crucial for Wwox function (or at least a particular aspect of its function).

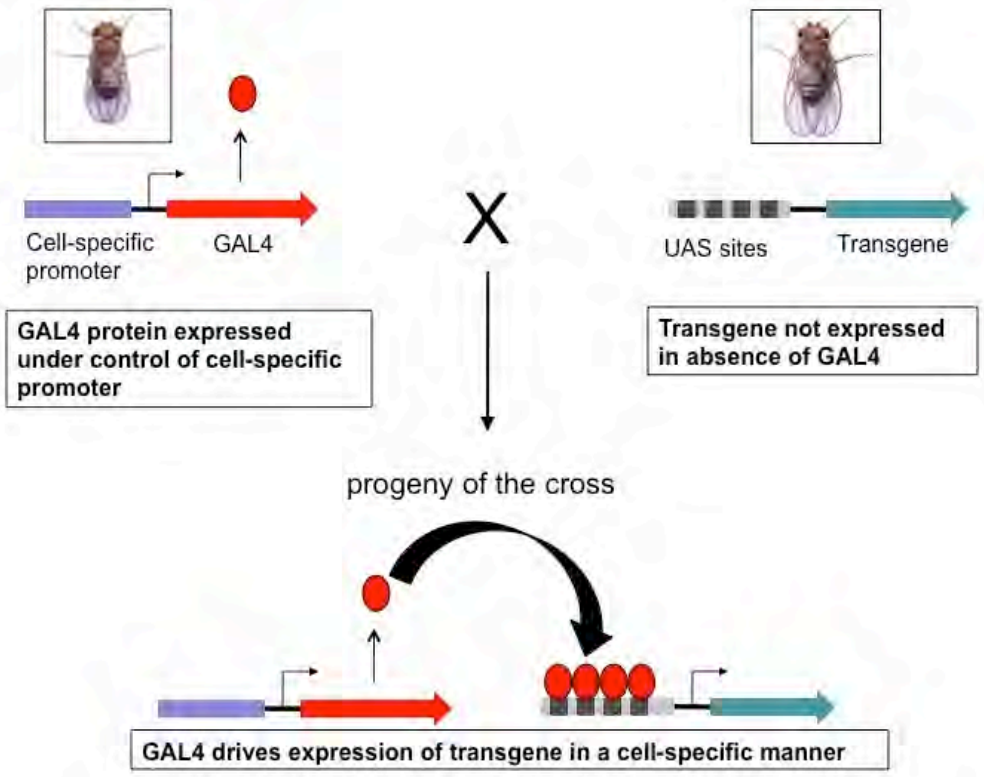

Figure 2.1 Expression of transgenes in a cell-specific or tissue-specific manner in *Drosophila* using the binary GAL4-UAS system. Exogenous yeast GAL4 protein is produced in specific cells at a particular developmental time under the control of a cell-specific promoter. A fly line carrying this GAL4 construct is crossed to a fly line carrying the desired transgene that is not expressed in the absence of GAL4. The transgene is only expressed when GAL4 protein is present and binds to the Upstream Activator Sequence (UAS) of the transgene. Progeny from the cross that carry both constructs will thus have GAL4-driven expression of the desired transgene in a cell-specific manner.

The aim of the experiments described in this chapter was to establish *Drosophila* transformant lines carrying mutations in the different functional elements of an ectopically expressed Wwox protein. As the WW domains and the SDR enzymatic region have been identified as the main functional elements of WWOX thus far, mutagenesis was targeted specifically at the critical amino acid residues within these elements. Specific amino acid residues within the WW domains of WWOX have been identified to be involved in mediating the binding of WWOX to numerous ligands, some of which are transcription factors whose activities are modulated by WWOX (Chang *et al.*, 2003; Aqeilan *et al.*, 2004a; Aqeilan *et al.*, 2004b; Aqeilan *et al.*, 2005; Gaudio *et al.*, 2006). As for the SDR enzymatic region, SDR enzymes are typically characterised by the presence of well-

established motifs that are highly conserved and there have been various studies demonstrating that the loss of particular amino acids within those motifs is able to affect the function of various SDR enzymes (Oppermann *et al.*, 1997; Nakajin *et al.*, 1998; Filling *et al.*, 2002). Based on these studies, mutagenesis was carried out on these amino acids that are known or predicted to be crucial for Wwox function. Mutations in the targeted amino acid residues were generated by site-specific mutagenesis and verification studies were carried out firstly, to confirm the presence of the mutations and secondly, to determine if these mutations have any effect on the overall stability of the ectopic Wwox protein.

2.2 Material and Methods

Drosophila stocks and husbandry

Drosophila 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 25ºC. The ey-GAL4 and ZH-attP-68E line stocks were obtained from Bloomington *Drosophila* Stock Center (Indiana University).

Identification of conserved motifs

Identification of conserved motifs in the Wwox protein was carried out using the Conserved Domain Database (CDD, NCBI). Protein sequences of both the human WWOX and *Drosophila* Wwox protein were obtained from NCBI, Ensembl and Flybase. Conserved domains that were identified were also compared with the identified motifs that had been reported in the literature.

Generation of constructs carrying mutations in the functional domains of Wwox

Site-specific mutagenesis was carried out to generate constructs with mutations in targeted amino acid residues in either the WW domains or the SDR enzyme region of Wwox. A construct carrying the Y29R mutation was previously generated by Kristie Lee and Donna Denton whilst constructs harbouring the T127A and Y288F mutation respectively have been generated by Alexander Colella. A glycerol stock containing the open reading frame (ORF) of *Drosophila* Wwox in a TOPO vector was obtained from Sonia Dayan in order to generate constructs with mutations in the second WW domain and in amino acid 277 of the SDR enzyme region. Plasmid DNA was isolated from colonies grown from the glycerol stock using the GenElute^{TM} Plasmid Miniprep Kit (Sigma-Aldrich) and mutagenesis was performed on plasmid DNA to introduce the W58F, N81A, P84A and P277A mutations into the Wwox transgene. Plasmid DNA was amplified using Pfu polymerase (Roche) and mutation-specific primers (W58F and P277A primers, Table 2.1) to obtain product of full-length Wwox ORF with the desired mutation(s). PCR conditions were optimised for each

primer pair and carried out in a MJ Research PTC-200 Peltier Thermal cycler. PCR conditions for W58F primers were 95ºC for 45 seconds, followed by 18 cycles of 95ºC for 45 seconds, 45ºC for 1 minute and 68ºC for 6 and half minutes whilst PCR conditions for P277A primers had an annealing temperature of 50ºC for 1 minute. PCR products were then treated with Dpn1 (New England Biolabs (NEB)) to digest the methylated, non-mutated parental DNA template and transformed into DH5α chemically competent cells. Plasmid DNA was isolated and a restriction digest was carried out using restriction enzyme BsrG1 (NEB) to verify presence of the insert. Sequencing was then performed using M13F primer specific to the TOPO vector to verify presence of the mutation. Plasmid DNA carrying the desired W58F mutation was then used for further mutagenesis to introduce the N81A and P84A mutations using the N81AP84A primers (Table 2.1). Amplification of DNA, transformation of Dpn1-treated product in bacteria, isolation of plasmid DNA and verification of insert were carried out as previously described with two exceptions – 3% dimethyl sulfoxide (DMSO) was used in the DNA amplification step and the PCR conditions were 95ºC for 1 minute, followed by 18 cycles of 95ºC for 50 seconds, 45ºC for 50 seconds and 68ºC for 4 and half minutes, followed by a final extension step at 68ºC for 7 minutes. The constructs carrying the desired mutation(s) were then subcloned into the Gateway® pTW-attB vector by LR clonase recombination (Invitrogen/Life Technologies). Presence of the constructs in the Gateway® pTW-attB vector was confirmed by digestion with BsrG1 and DNA sequencing was performed using pUAST-F primer (to confirm the presence of the mutation and integrity of the remaining sequence as well as the orientation of the insert) and UAS-Rev primer (to verify presence of upstream UAS sites that will enable expression of transgene in *Drosophila*). The six different constructs with the desired mutations as well as a construct carrying the wild-type copy of the Wwox ORF were then microinjected to generate different transgenic *Drosophila* lines.

Oligonucleotides used for DNA amplification and sequencing

The oligonucleotides used are standard PCR grade oligonucleotides obtained from Geneworks (Adelaide, Australia) and are presented as 5' to 3' in Table 2.1

Table 2.1 List of oligonucleotides used for DNA amplification and sequencing

Mutation-specific primers		
W58F	Fwd	CGAATTACCCTTGGGCTTCGAGAAGTACTACG
	Rev	GCTAGTACTTCTCGAAGCCCAAGGGTAATTCG
N81AP84A	Fwd	GCAACGAACGGCTGTGGATGCTCGTTTGGCAT
	Rev	ATGCCAAAGCAGCATCCACAGCCGTTCGTTGC
P277A	Fwd	GCATCACCTTTCGGCGCCGCCGGAGAAATAC
	Rev	GTATTTCTCCGGCGGCGCCGAAAGGTGATGC
Primers for amplification of genomic DNA		
Primers for amplifying DNA region for detection of the WW domain mutations and the T127A mutation in the SDR domain		
Fwd (specific to vector upstream of Wwox ORF)		GAAGAGAACTCTGAATAGGG
Rev (specific to vector downstream of Wwox ORF)		GTCACACCACAGAAGTAAGG
Primers for amplifying DNA region for detection of the Y288F and P277A mutations		
Fwd (specific to Exon 2 of Wwox)		GCTCTCGACTTGAGCTCTTTGC
Rev (specific to Exon 6 of Wwox)		GACTATCTAGAATCATTGGTGGTAGCATAATGCA
Primers for DNA sequencing		
Primers for vector sequencing		
M13F (specific to TOPO vector)		GTAAAACGACGGCCAG
pUAST-F (specific to pTW-attB vector)		GAAGAGAACTCTGAATAGGG
UAS-Rev (UAS sites verification primer)		CCCTATTCAGAGTTCTCTTC
Primers for genomic sequencing		
Detection of the Y29R, W58F, N81A, P84A and T127A mutations (pUAST-F, vector-specific primer upstream of Wwox ORF)		GAAGAGAACTCTGAATAGGG
Detection of Y288F and P277A mutations (Wwox Exon 2 specific primer)		GCTCTCGACTTGAGCTCTTTGC

Transformation of plasmids into bacteria

DH5α chemically competent cells were used for all transformations. Cells were thawed on ice from storage at -80ºC and 50µL of cells were added to 1-3µL of the Dpn1-treated PCR product (of the desired DNA). Mixture was incubated on ice for 30 minutes, heat shocked at 42ºC for 50 seconds before chilling on ice again for another 2 minutes. 450µL of SOC medium (2% bactotryptone, 0.5% yeast extract, 100mM sodium chloride, 2.5mM potassium chloride, 10mM magnesium chloride, 10mM magnesium sulphate, 20mM glucose) was added to the mixture and incubated at 37ºC for 1 hour. Cells were then pelleted at 3000rcf for 5 minutes. 400µL of supernatant was removed and the pellet was resuspended in the remaining 100µL of SOC and plated on solid L-Broth (LB) media (1% (w/v) amine A, 0.5% yeast extract, 1% sodium chloride pH7.0) supplemented with either kanamycin (100mg/mL, for TOPO vector selection) or ampicillin (100mg/mL, for pTW-attB vector selection). Plates were left to dry at room temperature and incubated at 37ºC overnight.

Generation of *Drosophila* transgenic lines

Microinjection of constructs containing the mutations into *Drosophila* embryos were performed by BestGene Inc. (Chino Hills, California, USA) and Dr. Clare van Eyk (The University of Adelaide) to generate *Drosophila* transgenic lines carrying those mutations in the desired docking site (68E1 on the third chromosome). These microinjections were all carried out according to the standard protocol using the FlyC31 system (Bischof *et al.*, 2007). Flies that eclosed from injected embryos were crossed individually to w^{1118} flies and the progeny from those crosses were screened for transformants (identified based on the presence of red ocelli as well as light yellow-orange eye colour from the *white* mini-gene present in the inserted construct). Stocks were generated by balancing the insertions over the dominantly marked TM6B balancer (using the *w; TM2/TM6B* stock) for insertions on the third chromosome.

Generation of *Drosophila* recombinant lines

Drosophila recombinant lines expressing the Wwox mutations in the developing eye were generated (Figure 2.2) for use in functional studies (Chapter 3). These lines all carry a recombinant third chromosome with the eyeless-GAL4 (ey-GAL4) promoter as well as either the wild-type or a mutant copy of Wwox. Virgin female flies of the balanced transgenic lines were crossed to male w^{1118} flies and recombinant progeny were identified based on eye colour and presence of red ocelli. The recombinant chromosomes were then balanced over the TM6B balancer to generate stocks and the genotype of the generated recombinant stocks were confirmed by DNA sequencing.

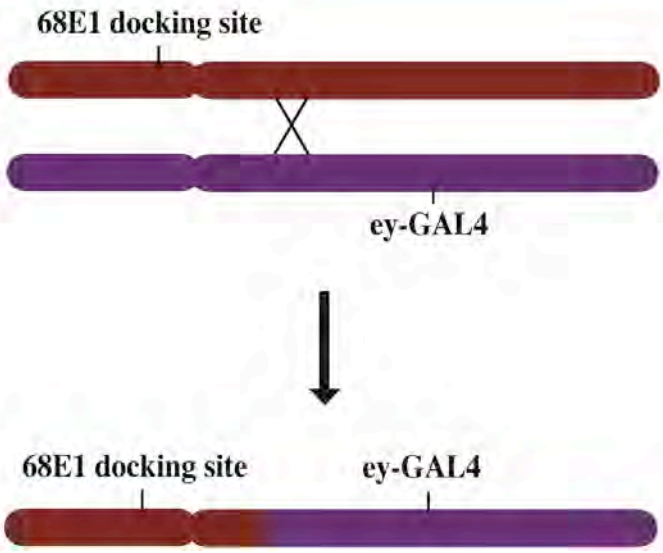

Figure 2.2 Generation of *Drosophila* recombinant lines carrying ey-GAL4 promoter and the different Wwox constructs inserted into the docking site at the 68E1 chromosomal location. Both sites are on the third chromosome although the exact insertion site of ey-GAL4 is not known.

DNA extraction from *Drosophila* adult flies

15-30 adult flies (per genotype) were collected in an eppendorf tube and incubated at -20ºC for 1 hour. The flies were then homogenised with a pestle in 400µL Buffer A (100mM Tris-HCl, pH 7.5, 100mM EDTA, 100mM NaCl and 0.5% SDS) and incubated at 65ºC for 30 minutes. 800µL of 1:2.5 [5M]KAc:[6M]LiCl solution was added and samples were incubated on ice for 10 minutes. The samples were then spun at 10000rcf for 15 minutes and the supernatant was transferred into a new eppendorf tube. DNA was precipitated with isopropanol and spun down at 10000rcf for 15 minutes before a wash with cold ethanol, followed by another spin down at 10000rcf for 5 minutes. Supernatant was removed and the DNA pellet was resuspended in 1x Tris EDTA (TE).

PCR amplification of genomic DNA

PCR amplification was performed using the Expand Long Template PCR Kit (Roche) with buffer 2 (according to manufacturer's protocols) in a MJ Research PTC-200 Peltier Thermal cycler to detect the presence of Wwox mutant transgenes in the recombinant transgenic flies. Two different PCR amplification reactions were set up. One of the PCR amplification reactions was to amplify the region for detection of the mutations in the WW domains as well as for detection of the T127A mutation in the SDR domain. The cycling conditions were 94ºC for 2 minutes, followed by 30 cycles of 94ºC for 45 seconds, 50ºC for 5 seconds and 68ºC for 2 minutes 30 seconds (with an additional 20 seconds per cycle for the last 20 cycles), followed by a final additional extension step of 68ºC for 7 minutes. The other PCR amplification reaction was to amplify the region for detection of the other two mutations in the SDR domain (Y288F and P277A) with the following cycling conditions: 94ºC for 2 minutes, followed by 20 cycles of 94ºC for 45 seconds, 55ºC for 45 seconds and 68ºC for 2 minutes 30 seconds, followed by an additional 20 cycles of 94ºC for 45 seconds, 50ºC for 45 seconds and 68ºC for 2 minutes and 30 seconds (with an additional 20 seconds per cycle), and a final additional extension step of 68ºC for 7 minutes. The PCR primers that were used are as stated in Table 2.1 (see primers for amplification of genomic DNA). The amplified DNA was then sequenced to verify the mutations (see Method for DNA sequencing and Table 2.1 for the primers used for genomic sequencing).

DNA sequencing

Sequencing analyses were performed by the Australian Genome Research Facility Ltd (AGRF), Adelaide. 150-300ng of template was sent along with 20ng of primer per reaction to AGRF. The primers used for vector and genomic sequencing are described in Table 2.1. Data was analysed and images prepared using Geneious (version 5.5.6, Biomatters).

SDS PAGE and Western blot analyses

Five adult flies (per genotype) were frozen at -80ºC for protein extraction. Protein extraction was carried out by homogenisation in 100µL 2x Laemmli Buffer (0.5M Tris HCl pH6.8, 80% glycerol, 10% SDS, 2-β-mercaptoethanol). Samples were boiled at 95ºC

for 5 minutes and spun down at 14000rcf. 7µL of the BenchmarkTM Pre-stained Protein Ladder (Invitrogen) was used as a molecular weight determinant. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) of proteins was carried out using the Bio-Rad Mini PROTEAN® 3 Cell Kit with the 10% resolving gel and 4% stacking gel prepared according to manufacturer's guidelines. Proteins were transferred to nitrocellulose membrane using a Mini Trans-Blot® Electrophoresis Transfer Cell Kit (Bio-Rad) at 100V for 1 hour at room temperature and membranes were briefly incubated with 0.1% Ponceau S in 5% acetic acid to determine if the transfer had been successful. The nitrocellulose membranes were then blocked with 10% skim milk in blocking buffer (100mM Tris pH7.5, 100mM NaCl, 0.1% Tween-20) for 1 hour at room temperature and washed with blocking buffer for 30 minutes (with 3 x 10 minute washes) before incubating with primary antibody (diluted in 10% skim milk in blocking buffer) overnight at 4ºC and secondary antibody (diluted in 5% skim milk in blocking buffer) for 1 hour at room temperature. The membranes were washed with blocking buffer for 30 minutes (with 3 x 10 minute washes) after each incubation with the antibodies. Detection of proteins was performed by fluorescence visualisation using a Typhoon Trio+ variable mode imager (Amersham Biosciences). Images were taken using the Typhoon Scanner Control program and processed using ImageJ software. For detection of Wwox, the anti-N-terminal WWOX antibody (O'Keefe *et al.*, 2005) was used at 1:1000 with a DyLight 649 Anti-Rabbit secondary antibody (Vector Laboratories) was used at 1: 2500. For the loading control, a mouse monoclonal anti-α-tubulin (Sigma) was used at 1: 2000 with an anti-mouse Cy3 (Jackson Laboratories) used at 1: 2000.

2.3 Generation of *Drosophila* lines ectopically expressing mutations in the known functional domains of Wwox

The *Drosophila* Wwox protein shares 49% sequence identity with that of the human WWOX (O'Keefe *et al.*, 2005), which includes highly conserved motifs of the two WW domains and the SDR enzymatic region (Figure 2.3). Mutations were generated in these conserved motifs of Wwox and ectopically expressed in *Drosophila* (Table 2.2). Amino acid residues that are critical for WWOX function were identified either by motif analyses (using the Conserved Domain Database, NCBI) as well as based on reported studies conducted in mammalian systems on WWOX. These identified residues are generally conserved in the *Drosophila* Wwox protein, which highlights the potential functional importance of these residues.

Figure 2.3 Sequence alignment of human WWOX and *Drosophila* Wwox proteins showing high conservation of functional motifs. The functional elements include two WW domains (blue boxes), the SDR region (shaded red box; with the conserved co-factor binding and catalytic active site motifs in green boxes) and conserved regions at the C-terminus that have been postulated to be the putative substrate binding domain (Richards *et al.*, 2015) (shaded purple boxes). The amino acid variant associated with thyroid cancer risk factor was highlighted with an orange box. The amino acids that were targeted for mutagenesis were indicated with red font. The symbols used to signify conservation are as follows: asterisk (*) to indicate identical residue, colon (:) to indicate conserved residues with strongly similar properties and period (.) to indicate conserved residues with weakly similar properties. Sequence alignment was performed using Clustal Omega (http://www.ebi.ac.uk).

Table 2.2 Mutations generated to target critical amino acid residues in Wwox

Identified functional amino acid residues		Mutations generated in the identified amino acid residues		Known information regarding the functional residues/elements (from mammalian studies/current literature)
		Nucleotide substitution	Corresponding amino acid substitution	
WW domains				
1st WW domain	Y29	TAT → CGT	Y29R	Y33R substitution in mammalian systems (Y29 in *Drosophila*) abolished binding of proteins to the WW domain of WWOX (Chang *et al.*, 2003b; Aqeilan *et al.*, 2004a, 2004b, 2005, Gaudio *et al.*, 2006).
2nd WW domain	W58	TGG → TTC	W58F	Substitution of tryptophan residues (to phenylalanine or alanine) in WW domain proteins could either affect their function or result in structural changes in the protein (Chen *et al.*, 1997; Koepf *et al.*, 1999; Komuro *et al.*, 1999b).
	W58 N81 P84	TGG → TTC AAT → GCT CCT → GCT	W58F N81A P84A (WNP)	Simultaneous substitutions of such critical residues can result in a greater negative effect (on protein structure and/or function) than the single mutations (Komuro *et al.*, 1999a; Ludes Meyer *et al.*, 2004).
SDR enzymatic 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.*, 1997; Filling *et al.*, 2002).
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).
Identified risk polymorphism	P277	CCG → GCG	P277A	Corresponding variant A282 in humans associated with increased risk of thyroid cancer (Cancemi *et al.*, 2011).

2.3.1 Mutations in the WW domains of Wwox

The WW domains of WWOX are Group 1 WW domains that are able to bind to proline-rich proteins, specifically proteins with a PPxY motif (Ludes Meyer *et al.*, 2004; Hu *et al.*, 2004) as well as proteins with LPxY motif (Abu-Odeh *et al.*, 2014). WW domains typically consist of approximately 40 amino acids that form a three-stranded, anti-parallel ß-sheet containing two ligand-binding grooves (Sudol *et al.*, 2005). There are notably two signature tryptophan (W) residues spaced approximately 20-22 amino acids apart in WW domains, with the first tryptophan located in the first ß-strand and the second tryptophan in the third ß-strand (Hu *et al.*, 2004, Sudol *et al.*, 2005). These tryptophan residues have an important role in the structure and function of WW domain proteins (Sudol *et al.*, 2001). Both signature tryptophan residues are present in the first WW domain (WW1) of the human WWOX and *Drosophila* Wwox proteins. In the second WW domain (WW2) however, the second characteristic tryptophan residue is absent and is replaced by a tyrosine (Y) residue in the human WWOX and an asparagine (N) residue in *Drosophila.*

Various studies have identified numerous ligands that are able to bind, through their PPxY motifs, to the WW domains of WWOX (Ludes Meyer *et al.*, 2004; Hu *et al.*, 2004, Aqeilan *et al.*, 2004a; Aqeilan *et al.*, 2004b, Aqeilan *et al.*, 2005; Ingham *et al.*, 2005; Gaudio *et al.*, 2006; Abu-Odeh *et al.*, 2014), most of which are to the WW1 domain. WWOX has been found to bind, through its WW1 domain, to proteins such as p73, ErbB-4, c-Jun and AP2γ and suppress their transcriptional activity. Binding to such proteins is mediated by phosphorylation of the first tyrosine residue (Y33) of the WW1 domain. Substitution of that tyrosine residue with an arginine residue (Y33R) results either in reduction or, in many cases, abolishment of binding to those proteins (Chang *et al.*, 2003b; Aqeilan *et al.*, 2004a; Aqeilan *et al.*, 2004b, Aqeilan *et al.*, 2005, Gaudio *et al.*, 2006). It is thought that the tyrosine residue, which is located in the second ß-strand, makes up part of an “aromatic cradle” structure required for ligand binding, which is abrogated by the arginine substitution (Aqeilan *et al.*, 2005). Hence, it appears that this tyrosine residue in the WW1 domain of WWOX is essential for its binding activity. A mutation in the corresponding first tyrosine residue of *Drosophila* Wwox was thus generated (Table 2.2).

The function of the WW2 domain of WWOX appears to differ from that of the WW1 domain. It has been suggested by many studies that the WW2 domain is not directly involved in protein-protein binding; however a protein array mapping human WW domain protein-protein interactions did identify 16 ligands that were able to bind to the WW2 domain, although the identity of those 16 ligands were not specified (Hu *et al.*, 2004).

Nonetheless, it has been shown that the WW2 domain is unable to bind to the some of the binding partners of the WW1 domain, which is thought to be due to the absence of the second signature tryptophan residue in the hydrophobic ligand-binding groove of the WW2 domain (Schuchardt *et al.*, 2013). Instead, it is postulated that the function of the WW2 domain is to stabilise the binding between the WW1 domain and its ligands. Indeed, WW domain proteins often have multiple WW domains within them, which are thought to contribute to the binding avidity of their target ligands (Chong *et al.*, 2010). It has previously been shown that the WW1 domain of WWOX on its own is structurally disordered and mostly unfolded, however when the WW2 domain is also present in tandem, the WW1 domain assumes a folded conformation and is able to bind more strongly to its ligands, such as ErBb4 (McDonald *et al.*, 2012; Schuchardt *et al.*, 2013). Mutation of the tyrosine residue (Y85) that is in place of the absent characteristic second tryptophan residue as well as mutation of a neighbouring proline residue (P88) in the WW2 domain reduces binding of the WW1 domain to one of its other ligands, SIMPLE (Ludes Meyer *et al.*, 2004), indicating that these residues are important for WW2 domain function.

Two mutant lines were generated to test the function of the WW2 domain in *Drosophila* (Table 2.2). One of the mutant lines contains a substitution of the only signature tryptophan residue with a phenylalanine residue (W58F) whilst the other mutant line contains simultaneous mutations of W58, the arginine residue (N81) that is in place of the absent second signature tryptophan residue in *Drosophila* as well as the neighbouring proline residue (P84). Such substitutions have been shown to affect the function and/or folding of other WW domains, with simultaneous substitutions of such critical residues often found to confer a greater effect (Chen *et al.*, 1997; Koepf *et al.*, 1999; Komuro *et al.*, 1999a; Komuro *et al.*, 1999b; Ludes Meyer *et al.*, 2004).

2.3.2 Mutations in the SDR enzymatic region of Wwox

The SDR enzymatic region of WWOX is classified as a classical SDR domain based on the presence of the typical glycine-rich NAD(P)-binding motif (TGxxxGxG) as well as an active site motif (YxxxK). The standard reaction mechanism of such active site motifs is a 4-pro-S hydride transfer and proton relay involving the conserved tyrosine and lysine residue, a water molecule as well as nicotinamide (NAD). The substrate binding normally occurs in the C-terminal region of such enzymes, which determines specificity of the reaction (Kavanagh *et al.*, 2008). Whilst the endogenous substrate(s) of the SDR enzymatic region of WWOX are yet to be identified, it has been postulated that WWOX is involved in regulating steroid metabolism through this domain. High expression of WWOX has been found in endocrine tissues, such as ovary, prostate and testis (Bednarek *et al.*, 2000), and WWOX mutant mice have been shown to exhibit impaired steroidogenesis (Aqeilan *et al.*, 2009). WWOX was also shown, in an *in vitro* study, to be able to oxidise seven different steroid substrates using both NAD^+ and $NADP^+$ cofactors, with higher activity observed when NAD^+ is used (Saluda-Gorgul *et al.*, 2011). Hence, it appears that the dehydrogenase activity of WWOX *in vivo* would be dependent on $NAD(P)^+$ levels in the cell and would in turn, be involved in maintaining cellular $NAD(P)^+$ /NAD(P)H levels.

In *Drosophila*, both the NAD(P) co-factor binding motif and the catalytic active site motif in the SDR region of Wwox are highly conserved with that of the human protein. As there have been no reported mutagenesis studies of these motifs in WWOX thus far, mutations were generated in these motifs based on mutagenesis of other SDR enzymes in the literature (Table 2.2). For the active site motif (YxxxK), the tyrosine (Y) residue is recognised as a critical catalytic residue of SDR enzymes and has been proposed to act as the catalytic base (Rafferty *et al.*, 1995; Tanaka *et al.*, 1996; Hwang *et al.*, 2005). Substitution of the active site tyrosine residue with a phenylalanine (F) residue has been shown to completely abolish enzymatic activity of various SDR enzymes (Nakajin *et al.*, 1998; Filling *et al.*, 2002, Hwang *et al.*, 2005). As for the cofactor-binding motif, a substitution of the threonine (T) residue with an alanine (A) residue was previously shown to alter co-factor binding preference and resulted in loss of most of its enzymatic activity (Oppermann *et al.*, 1997; Filling *et al.*, 2002). Apart from those essential residues, the proline residue at codon 282 in the SDR region of WWOX (corresponding amino acid 277 in *Drosophila*) is also of particular interest as an alanine variant at that codon has been

associated with an increased risk of thyroid cancer in humans, potentially through changes to the protein structure (Cancemi *et al.*, 2011).

2.4 Verification of *Drosophila* lines expressing mutations in the known functional domains of Wwox

Constructs carrying the different Wwox mutations were microinjected into *Drosophila* embryos to be integrated into the *Drosophila* genome in a site-specific manner using the ϕC31 system (Bischof *et al.*, 2007). The ϕC31 68E1 chromosomal site was chosen as a docking site as it is known not to span any genes or functional regions of DNA thus far. Use of that docking site has been shown to produce reasonable rates of integration into the genome (Bischof *et al.*, 2007) and has been widely used without any reported negative effects. Integration of the different constructs into a common chromosomal site in the genome allows for comparison of Wwox protein levels in the different mutant lines as well as identification of mutation-specific effects on Wwox function.

Verification analyses were performed on stocks of transgenic *Drosophila* carrying the different Wwox mutations. Sequence analyses were carried out to verify that the transgenic lines contain the desired mutation(s) (Figure 2.4). Transgenic lines that carry the inserted constructs were then crossed to flies carrying a ubiquitous promoter expressing GAL4 protein. Progeny from that cross that carry both the inserted Wwox mutant construct and the GAL4 promoter have ubiquitous expression of the Wwox mutant protein. These lines all contain the endogenous wild-type Wwox gene and are expressing the Wwox mutant protein ectopically. Western blot analyses were performed to determine if the generated mutation(s) have any effect on the stability of the ectopic Wwox protein (Figure 2.5, Appendix A: Figure A1, A2).

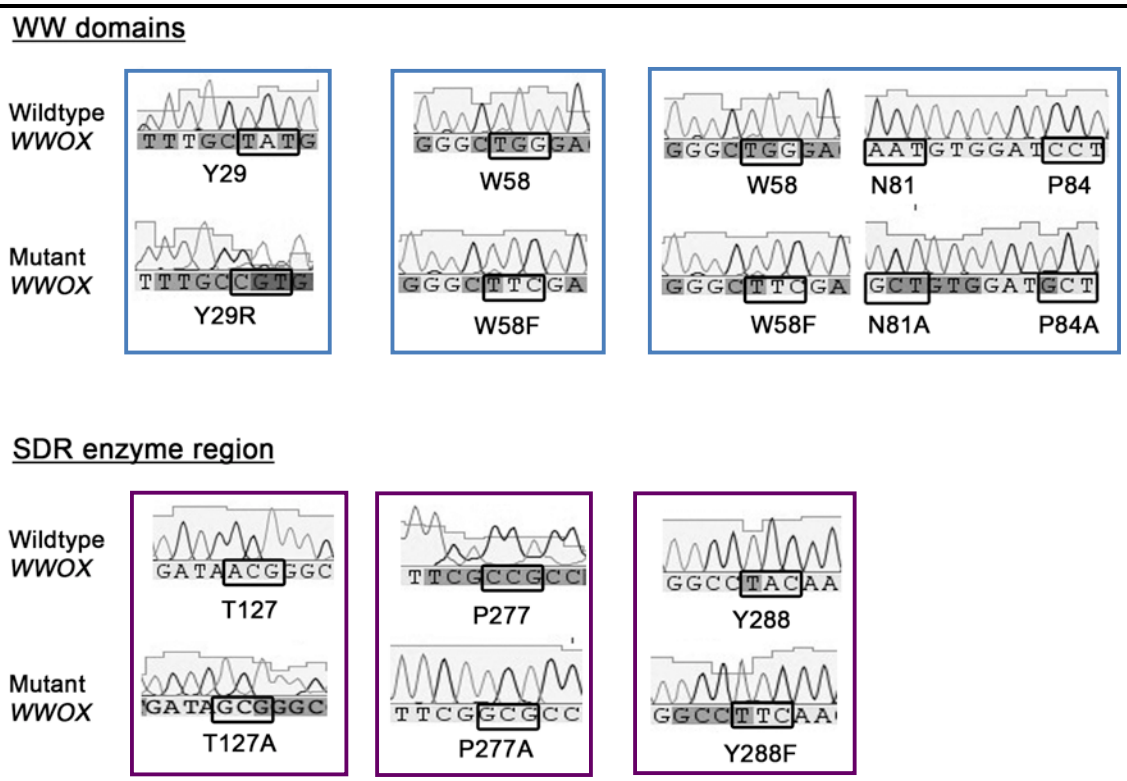

Figure 2.4 Sequence analyses of the ectopic *Wwox* mutations in the different *Drosophila* transgenic lines. The top panels (with the blue boxes) are of the three *Drosophila* lines with mutations in the WW domains (Y29R, W58F and W58N81AP84A) whilst the bottom panels are of the lines with mutations in the SDR enzyme region (T127A, P277A and Y288F). Both the wild-type and mutant sequences are shown, with the targeted codons highlighted with black boxes.

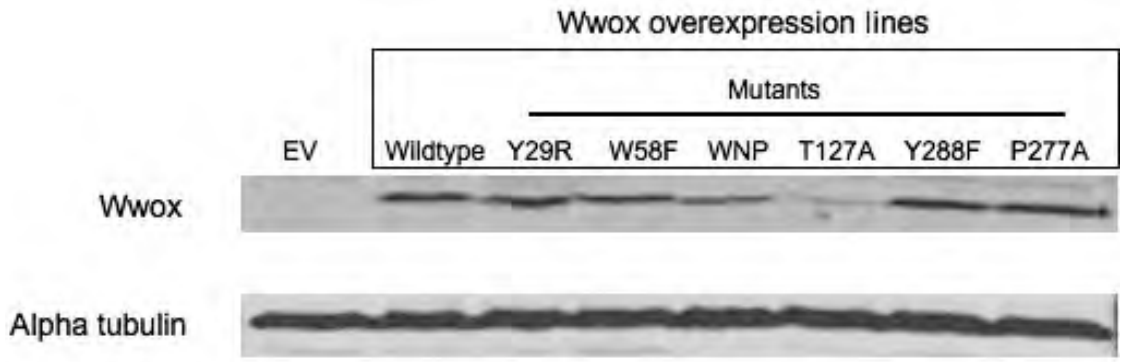

Figure 2.5 Western blot analysis of the different *Drosophila* ectopic Wwox mutant lines. Ectopic Wwox protein expression was determined for the six different mutant lines and compared to the control expressing ectopic wild-type Wwox protein. The mutant lines are labelled according to their specific mutations, with WNP designated to the WW2 domain triple mutant (W58FN81AP84A). Endogenous Wwox was undetectable using the standard Western blot analysis method, as seen in the empty vector (EV) control that is not expressing any ectopic Wwox protein. Alpha tubulin was used as a loading control.

The endogenous Wwox gene is expressed at very low levels in *Drosophila* and is often undetectable on a standard Western blot analysis without the use of enhancer kits to amplify the signal (O'Keefe *et al.*, 2005; O'Keefe *et al.*, 2011). This is evident in the empty vector (EV) control, which is not expressing any ectopic Wwox protein and is only expressing the endogenous Wwox (Figure 2.5). A high level of Wwox protein was detected for the *Drosophila* line ectopically expressing a wild-type copy of Wwox (in addition to its own endogenous Wwox). Four out of the six mutant lines (Y29R, W58F, P277A, Y288F) all had fairly similar levels of Wwox protein when compared to the ectopic wild-type control. It was observed that there were slightly lower levels of Wwox protein in the W58FN81AP84A triple mutant (WNP) compared to the control in that Western blot analysis, however that slight difference was not always as apparent (Appendix A: Figure A1). The T127A mutant line, however, clearly showed lower levels of Wwox than the control or the other mutant lines and this was consistently observed in two other independent Western blot analyses (Appendix A: Figure A1, A2), suggesting that this particular mutation results in decreased stability of the Wwox protein. The T127A mutant carries a substitution in the cofactor-binding motif in the SDR enzyme region of Wwox (Table 2.2) and it has been shown that the same mutation in another SDR enzyme

results in changes to cofactor binding preference and loss of some of its SDR enzymatic activity (Oppermann *et al.*, 1997; Filling *et al.*, 2002). It was thought that the threonine residue in the cofactor-binding motif is not directly involved in binding to the cofactor, but rather, is responsible for stabilising the framework for cofactor positioning. Hence, it is possible that this particular mutation destabilises the framework and affects the folding of the protein, thus resulting in overall decreased stability and subsequently lower levels of Wwox protein detected.

2.5 Summary

In summary, six different ectopic Wwox mutant lines were established, with three lines carrying mutations in the WW domains and three lines with mutations in the SDR enzymatic region. Establishment of these mutant lines has enabled studies to be carried out to differentiate the contribution of the different functional domains of Wwox, both within and outside the scope of this project. It should be noted that there are other WW domain residues that have recently been identified or predicted to also have some functional contribution. Additional mutagenesis can be carried out in the future in those newly identified residues to further investigate any findings from the use of these established mutant lines.

Chapter 3 – Identification of novel metabolic processes in which Wwox has a significant contribution

A portion of this chapter has been written up as a manuscript for publication:-
<u>Choo, A.</u> , O'Keefe, L.V., Lee, C.S., Gregory, S.L., Shaukat, Z., Colella, A., Lee, K., Denton, D. and Richards, R. I. (2014) The SDR enzymatic activity of tumour suppressor WWOX is required for its functional interaction with the mitochondrial respiratory genes. (To be submitted for publication)

Additional experiments that were not included in the manuscript have been described in the sections below (Summary and Additional Material and Methods) and the results from those additional experiments are included in Appendix B.

Summary

The lack of a visible, measurable phenotype associated with altered Wwox expression in *Drosophila* has made it difficult to directly study the role of Wwox in relation to cellular function. Nonetheless, Wwox has been shown to have a significant contribution to cellular processes when altered together with various other genes in *Drosophila* (O'Keefe *et al.*, 2011; Chapter 5- O'Keefe *et al.*, manuscript in preparation; Chapter 6- Shaukat *et al.*, 2014; Cheng Shoou Lee, personal communication). This highlights that Wwox has an important role in regulating cellular function, although the effects are more evident at a phenotypic level under cellular stress in *Drosophila*. Hence, in order to further examine the contributions of Wwox to cellular function, studies were conducted in a sensitised background where expression of other genes have been altered to result in cellular dysfunction, with the aim of identifying the types of cellular dysfunction that can be modulated by altered Wwox expression. *In vivo* RNA interference (RNAi) screens were conducted to identify phenotypes indicative of cellular dysfunction that could be modified by decreasing or increasing Wwox levels (Figure 3.1). As Wwox has been proposed to have a role in regulating metabolism and have been shown to affect the levels of metabolic genes (O'Keefe *et al.*, 2011), one of the aims of this project was to identify novel contributions of Wwox to metabolic pathways. Thus, the RNAi screens were aimed at identifying cellular dysfunction caused by defects in metabolic genes, with an emphasis

placed on genes involved in regulation of the tricarboxylic acid (TCA) cycle and related oxidative processes (Figure 3.2) as these are processes that Wwox has previously been associated with (O'Keefe *et al.*, 2011).

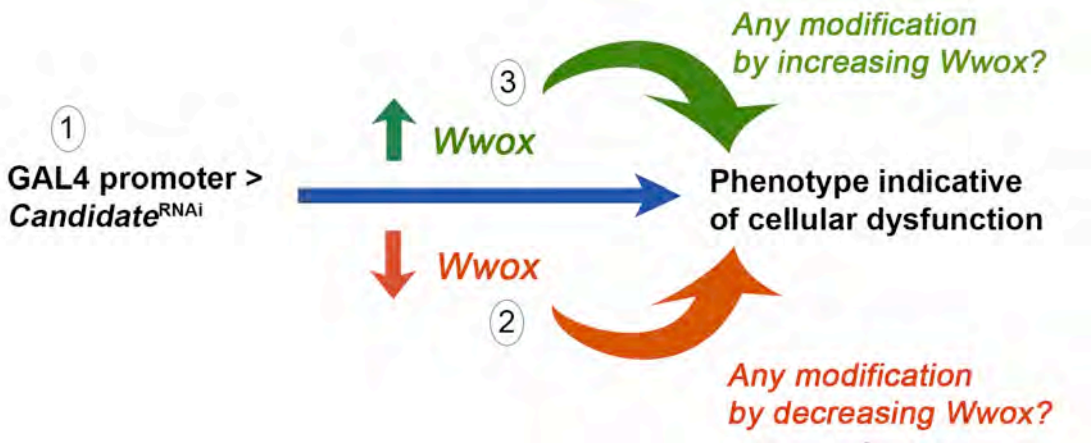

Figure 3.1 Model of the *in vivo* RNAi screens performed to identify metabolic genes that would give rise to cellular dysfunction that could be modulated by altered Wwox levels in *Drosophila*. Different GAL4 promoters were used to drive RNAi targeting of genes in various tissues. (1) RNAi was used to reduce expression of those metabolic genes to a point where the function of the gene is disrupted beyond a threshold required for normal cellular morphology or function, hence resulting in some form of a measurable phenotype. Wwox levels were then altered (decreased by RNAi targeting (2) or increased by expressing Wwox cDNA or ORF (3)) in that background to determine if Wwox was able to modify the phenotype previously observed. Modification of the phenotype by Wwox would indicate that Wwox is able to modulate the cellular dysfunction caused by reduced expression of those metabolic genes and that Wwox has a significant contribution to the same metabolic processes.

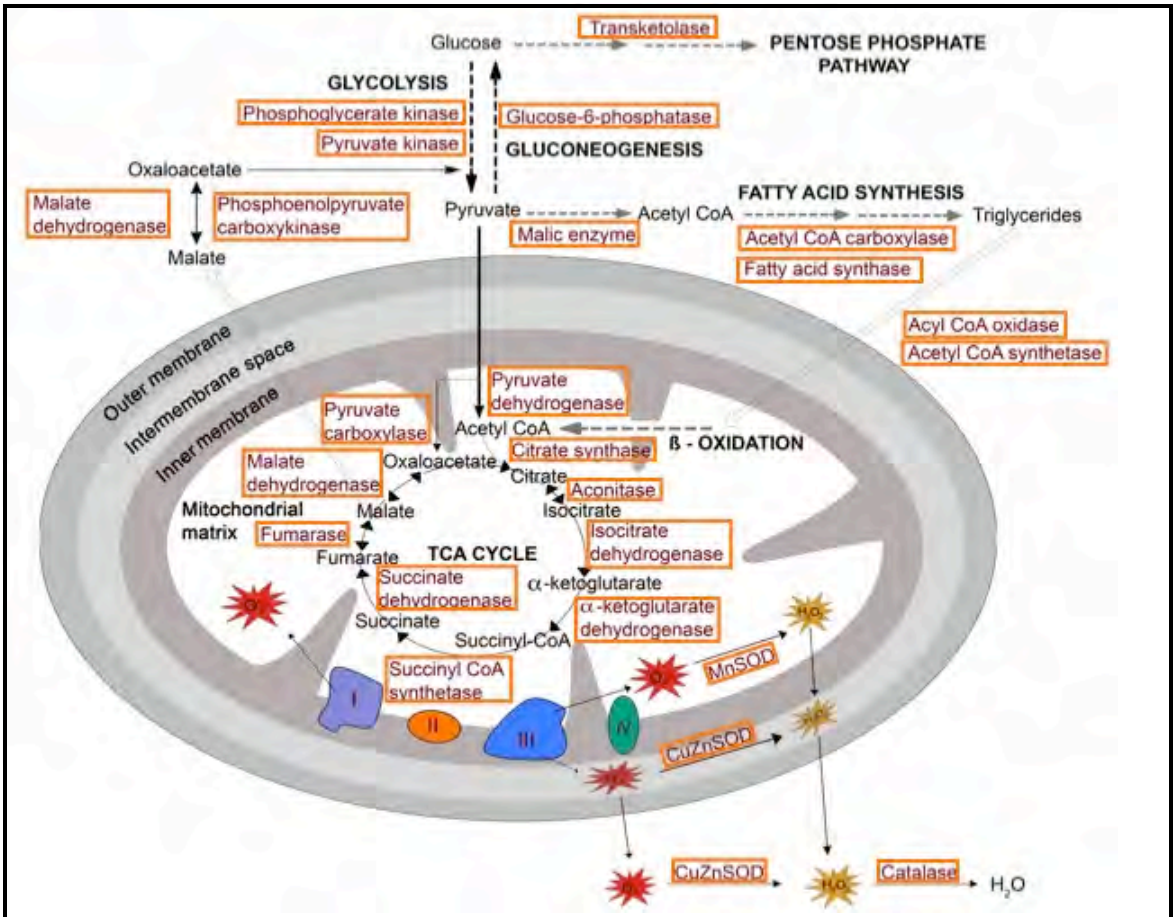

Figure 3.2 Metabolic enzymes targeted in the *in vivo* RNAi screens in *Drosophila*. Genes encoding the enzymes highlighted with the orange boxes were targeted using RNAi in the screens. The genes were chosen on the basis of their involvement in metabolic processes, particularly the TCA cycle and related oxidative processes, which Wwox has been shown to be involved in. The range of genes tested in the screens was determined by the availability of RNAi lines targeting those genes at the time of each screen.

In order to identify Wwox-associated metabolic pathways through RNAi targeting of candidate genes, an informative assay was required. Therefore, various types of *in vivo* RNAi screens were performed to establish the most appropriate and informative assay that could be used. In the first *in vivo* RNAi screen, the daughterless-GAL4 (da-GAL4) promoter was used to reduce expression of the targeted genes ubiquitiously in *Drosophila.* The phenotype (indicative of cellular dysfunction) that was examined was the survival of the flies to adulthood. The da-GAL4 promoter drives expression of the RNAi constructs starting from early embryogenesis and throughout the development of the organism (Cronmiller and Cummings, 1993). Thus, the reduction in expression of metabolic genes

beyond a particular threshold could lead to an effect on the ability of the flies to survive to adulthood. This phenotype was chosen as it would enable the identification of pathways that are essential for the survival of the organism and any modification of the phenotype by Wwox would therefore be of significant value. A similar screen was previously performed on candidate genes that had been identified from the Wwox proteomic analysis (O'Keefe *et al.*, 2011). In that screen, Wwox was found to functionally interact with *CG6439/ IDH3β* in maintaining viability of the flies (O'Keefe *et al.*, 2011). *CG6439/ IDH3β* is an integral part of the TCA cycle, indicating that this function of Wwox is linked to TCA cycle function. Following on from that screen, this subsequent da-GAL4 screen was performed as a more comprehensive screen of metabolic genes, with the focus on testing all the genes involved in the TCA cycle as well as related metabolic processes in order to gain better insight into the contribution of Wwox to metabolism.

Using the survival assay, 35 genes were tested for an effect when there is ubiquitious reduction in their expression levels. The targeting of 19 of those genes resulted in complete lethality, which was not rescued by altered Wwox levels (Appendix B: Table B1). Reduced viability (indicated by lower survival proportions) was observed for six of the 35 genes when tested at either 25ºC or 18ºC. However, it was noted that the survival rate of the flies was affected by environmental factors that were difficult to control, such as fluctuations in temperature that can affect the expression levels of GAL4 protein (Duffy, 2002) and presence of microorganisms. Consequently, there was substantial variability in the results obtained between experiments, which often made it difficult to ascertain the effect of Wwox on the survival of those flies (Appendix B: Table B1). Such variability was also observed in a previous screen using this particular assay, although at a lesser extent (O'Keefe *et al.*, 2011). The targeting of important metabolic genes is likely to cause the flies to be more sensitive to environmental factors compared to targeting of other genes that are less essential for cellular viability. The variability in the results and observations of lethal phenotypes suggested that whilst this assay has proven to be useful in providing some information regarding Wwox function in the past, the establishment of more suitable assays could enable investigation of Wwox function in greater detail.

Another small-scale RNAi screen was also conducted using the hedgehog-GAL4 (hh-GAL4) promoter to reduce expression of the targeted genes only in posterior cells in order to avoid causing lethality to the organism (Appendix B: Table B2). Indeed, only one out of the 18 genes tested resulted in complete lethality when targeted. For six of the genes, the adult flies that eclosed exhibited a "crinkled-wing" phenotype (Appendix B: Figure B1). Although the "crinkled-wing" phenotypes were of interest as they were

phenotypes indicative of oxidative stress (Xie *et al.*, 2010), there was no obvious modification of the phenotypes by altered Wwox levels and it was difficult to determine if there were any slight modifications due to the nature of the phenotype. Thus, a different RNAi screen with a more amenable phenotype was required. Of specific interest was a phenotype that exhibited a gradation in severity such that changes due to modifying genes, such as Wwox, could be demonstrated to be statistically significant.

A subsequent RNAi screen was performed in the *Drosophila* eye as such a screen allows for the manipulation of essential biological processes without usually affecting the overall viability of the organism and often produces phenotypes that can be easily modified. The *Drosophila* eye has long been used as a model system to dissect biological pathways – it is thought that two-thirds of all the vital genes in *Drosophila* are involved in normal eye development, with most of these genes involved in general cellular processes (Thaker *et al.*, 1992; Thomas & Wassaman, 1999), hence making the eye a good system in which to identify the role of Wwox in general metabolism and cell redox homeostasis. The ey-GAL4 promoter was used to alter expression of the genes of interest in the developing eye (Hauck *et al.*, 1999). 56 genes were tested in this screen. This assay appeared to be informative as none of those genes resulted in lethality to the organism when targeted and in the case of 10 genes, eye phenotypes were observed; amongst which were some that were modified by altered Wwox levels.

The complete results from this ey-GAL4/RNAi screen are described in detail in the following manuscript. In summary, Wwox was found to modulate cellular dysfunction caused by decreased expression of six different genes, each of which encoded a component of the mitochondrial respiratory complexes. RNAi targeting of either of the six genes resulted in a similar range of eye phenotypes indicative of cellular dysfunction, such as defects in cell growth, differentiation and survival. It has previously been shown that use of some of these RNAi lines to target these mitochondrial respiratory complex genes result in defects in mitochondrial activity (Shaukat *et al.*, 2014), consistent with the phenotypes being due to defects in mitochondrial function rather than any off-target RNAi effects. The alteration of Wwox levels was demonstrated to have an effect on the range of eye phenotypes produced, with decreased Wwox expression exacerbating the cellular dysfunction whilst ectopic overexpression of Wwox was able to rescue some of the tissue disruption caused by the mitochondrial defects. The results indicate that Wwox is involved in regulating cellular homeostasis in cells with mitochondrial dysfunction. As ectopic expression of wild-type Wwox was shown to suppress the effects of mitochondrial dysfunction, the six ectopic Wwox mutant lines (described in Chapter 2) were tested to

determine the functional domain(s) involved in the suppression (results described in the manuscript and in Appendix B: Figure B3, Table B3). This suppressive function of Wwox was demonstrated to be dependent upon its SDR enzyme activity as ectopic expression of the Wwox SDR catalytic active site mutation was unable to suppress the tissue disruption caused by mitochondrial defects. This result was further verified using an independent SDR catalytic active site mutant line in which the mutant construct was inserted into a different chromosomal location (51C1) in the genome (Appendix B: Figure B4, Table B4).

The outcomes of this study demonstrate that Wwox has a role in the maintenance of cellular homeostasis in response to mitochondrial dysfunction, which is dependent on its SDR enzymatic activity. The result of this study is of significance as mitochondrial dysfunction has been shown to be involved in promoting tumour progression (Santidrian *et al*., 2013). Previous work in the field of WWOX research has mainly been focussed on identifying binding partners of its WW domains whilst there is currently not much known about the SDR enzyme of Wwox. This study has demonstrated that the SDR enzyme of Wwox has a significant contribution to its cellular function. This highlights the importance of identifying the SDR enzymatic activity of Wwox as well as its endogenous SDR substrates as they may be good therapeutic targets to improve the prognosis of patients with low Wwox levels in tumours. Further characterisation of the mechanism(s) by which reduced Wwox function, in conjunction with mitochondrial defects, results in such cellular dysfunction will also provide better understanding of how perturbation of Wwox, as seen in many cancers, can contribute to tumourigenesis.

Additional Material and Methods

The material and methods included here are of the experiments that are additional to those in the manuscript.

Drosophila stocks and husbandry

All crosses were performed at 25ºC unless stated otherwise. The da-GAL4 and hh-GAL4 stocks were obtained from Bloomington *Drosophila* Stock Center. The RNAi lines were obtained from either the Vienna *Drosophila* Resource Centre (VDRC) or Bloomington. The UAS-*Wwox*^{RNAi} stocks that were used in the assays were v22536 (for the survival assays) and v108350 (for the hh-GAL4 and initial ey-GAL4 assays) from VDRC. Both lines have been shown to have a reduction in Wwox expression by quantitative real-time PCR (qRT-PCR) analysis (Appendix B: Figure B2). The UAS-Wwox lines that were used have been designated as "ORF2" (for the survival assays) and "cDNA1" (for the hh-GAL4 and initial ey-GAL4 assays). Both lines contain random insertion of the wild-type Wwox construct and have been verified by Western blot analyses to be ectopically expressing Wwox protein when crossed to a promoter (O'Keefe *et al.*, 2005; O'Keefe *et al.*, 2011; Tanya Henshall, personal communication). The verification studies of the ey-GAL4 assays were performed using UAS-Wwox lines that contain site-specific insertion (chromosomal locations 68E1 and 51C1) of the wild-type and mutant Wwox constructs.

Generation of *Drosophila* transgenic lines with constructs inserted in the 51C1 chromosomal location

Microinjections of constructs containing the wild-type Wwox and the Y288F mutation into *Drosophila* embryos were performed by Joanne Milverton and Dr. Clare van Eyk (The University of Adelaide) to generate *Drosophila* transgenic lines carrying the mutation in the desired docking site (51C1 on the second chromosome). These microinjections were all carried out according to the standard protocol using the FlyC31 system (Bischof *et al.*, 2007). Flies that eclosed from injected embryos were crossed individually to w^{1118} flies and the progeny from those crosses were screened for transformants (identified based on the

presence of red ocelli as well as light yellow-orange eye colour from the *white* mini-gene present in the inserted construct). The identified transformants would carry one copy of the insertion and stocks were generated by balancing the insertions over the dominantly marked CyO balancer (using the w; Gla/CyO stock) for insertions on the second chromosome.

Real-Time quantitative Reverse Transcription PCR (qRT-PCR)

RNA isolation and purification

Male flies (0-1 day old) were snap frozen in liquid nitrogen and kept at -80ºC until RNA extraction was carried out. 5 flies were used for each sample and three biological samples were prepared for each genotype. The flies were homogenised in 1mL Trizol™ (Life Technologies) – 100µL of Trizol™ was first added and the tissue was homogenised using a pestle before the remaining 900µL of Trizol™ was added. The homogenate was then passed through a 20-gauge needle ten times before being pelleted by centrifugation at 13, 200 rpm for 10 minutes at 4ºC. The supernatant was then transferred to a new RNAse free tube. 200µL of chloroform was added to each tube and the tubes were inverted vigorously by hand to allow mixing of the supernatant with the chloroform. The samples were then centrifuged at 13, 200 rpm for 15 minutes at 4ºC and 500µL of the upper aqueous phase was transferred to another fresh RNAse free tube. An equal amount (500µL) of 100% ethanol was added and the samples were vortexed before loading onto an RNeasy® column (Qiagen). The remaining purification steps were carried out according to the RNeasy® mini kit instructions, with the RNA eluted in 30µL of RNAse-free water. The samples were then stored at -80ºC.

Reverse transcription reaction

Reverse transcription to produce complementary DNA (cDNA) was carried out using 1µg of RNA (which have been DNase treated with DNAse I (Life Technologies), according to the manufacturer's guidelines) and Superscript III® (Life Technologies). 100ng of random

hexamers was used in a 20uL reverse transcription reaction, which was performed at 25ºC for 5 minutes, 50ºC for 1 hour followed by 72ºC for 15 minutes.

Quantitative real-time PCR (qPCR)

The cDNA produced from the reverse transcription reactions were diluted by adding 80μL of RNAse-free water (1/5 dilution) and 5μL was used as a template for a qPCR reaction with 1.26pmol of each primer and 1x Power SYBR® Green Master Mix (Life Technologies) in the final reaction volume of 25μL. A standard curve was produced by serial dilution of a cDNA sample (neat, 1/2, 1/5 and 1/10) and used for each primer set. Reactions for each cDNA sample were performed in triplicate in a 96 well plate. The reactions were performed using an ABI Prism® 7000 Sequence Detection System (Applied Biosystems) with the following cycling conditions: 50ºC for 2 minutes, 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. A dissociation curve was produced for each primer pair to verify the amplification of only a single product in each reaction. The relative amount of product in each reaction was then quantified using the Relative Standard Curve Method (Applied Biosystems) and the ABI Prism® 7000 SDS programme. The results were exported to Microsoft Excel for further data analysis and the quantity of product in each reaction for the *Wwox* primers were normalised to that of the housekeeping gene (*Ribosomal protein 49, Rp49*) primers to obtain the relative expression level of *Wwox* transcript for each sample. The average value and standard deviations of the triplicate reactions for each sample was calculated and statistical significance was determined using a two-tailed Student's t-test for each $Wwox^{RNAi}$ sample compared to the wild-type control, with a significance cut-off value of $p<0.05$.

The primers used were standard PCR grade and obtained from Geneworks (Adelaide, Australia), as presented below 5' to 3':

Rp49 Fwd: GACGCTTCAAGGGACAGTATCTG
Rp49 Rev: AAACGCGGTTCTGCATGAG

Wwox Fwd: ATTGTGCTGTCATCCGAGTCG
Wwox Rev: ATTCTCCACGGGCAGGTTG

Survival assay

Drosophila crosses were set up as shown in Figure 3.3 (and as previously described in O'Keefe *et al.*, 2011). Majority of the crosses were performed at the standard temperature of 25°C. For some of the crosses that were found to result in lethality when performed at 25°C, the crosses were repeated at a lower temperature of 18°C. As the GAL4-UAS system is temperature-dependent, the use of the lower temperature (18°C) could result in a weaker phenotype and prevent lethality of the flies. The daughterless-GAL4 (da-GAL4) promoter was used to drive ubiquitous expression of the RNAi constructs in the different Wwox backgrounds. All progeny that carry the TM6B balancer do not have expression of the RNAi construct and so only the non-TM6B progeny have the desired genotype. The number of TM6B progeny was used as an indication of the survival rate in each vial and only the crosses with a minimum of 30 TM6B progeny were included in the analyses. 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. The ratio of non TM6B:TM6B progeny for each experimental cross was then compared to the corresponding control cross. Statistical analyses were performed using the chi-square test with $p=0.05$ as cut off value for significance (GraphPad Prism). Further verification crosses were carried out for the genes that showed significant reduction in survival when targeted.

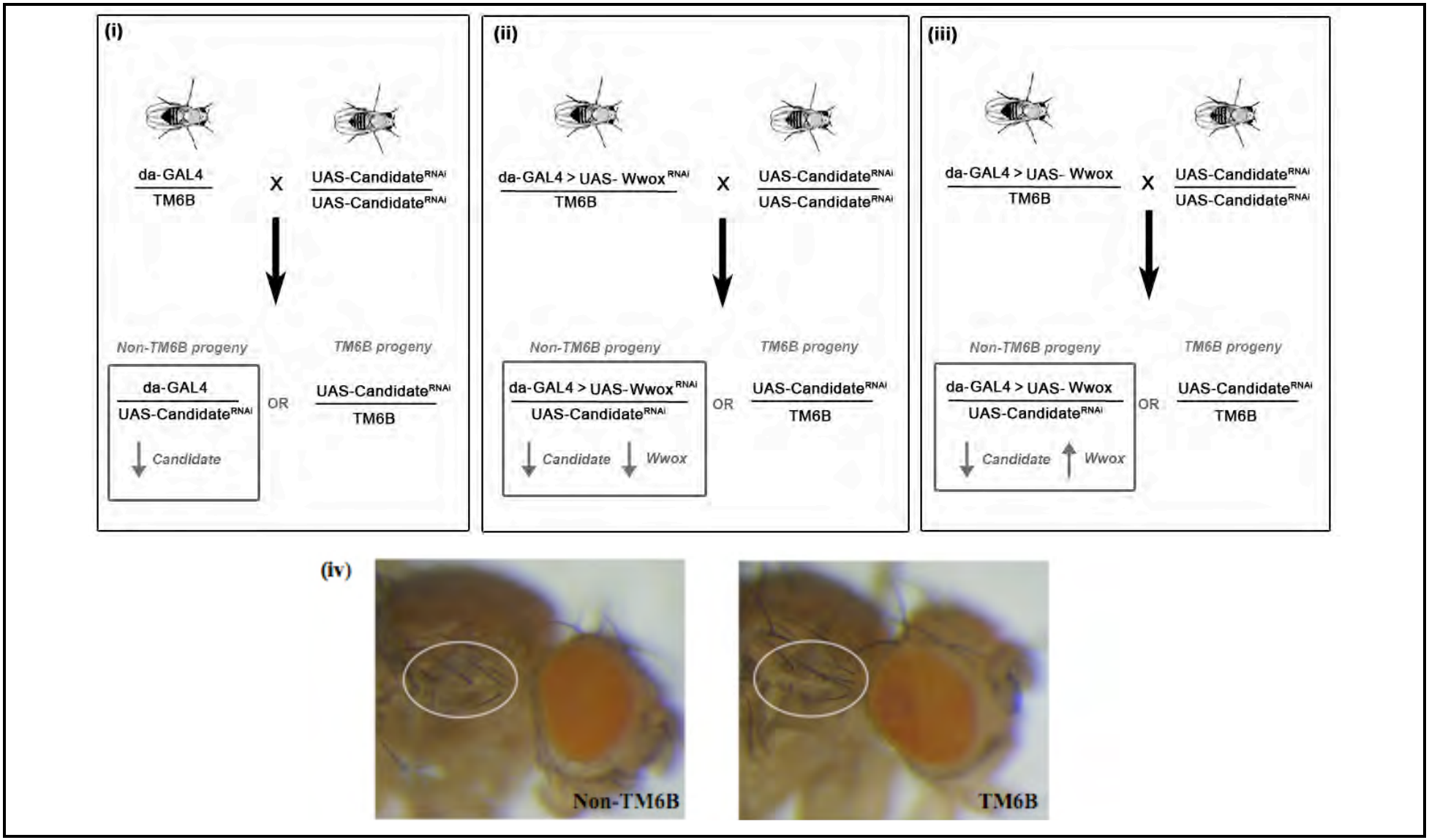
(i)
da-GAL4
TM6B
X
UAS-Candidate^RNAi
UAS-Candidate^RNAi
Non-TM6B progeny
TM6B progeny
da-GAL4
UAS-Candidate^RNAi
Candidate
OR
UAS-Candidate^RNAi
TM6B
(ii)
da-GAL4 > UAS- Wwox^RNAi
TM6B
X
UAS-Candidate^RNAi
UAS-Candidate^RNAi
Non-TM6B progeny
TM6B progeny
da-GAL4 > UAS-Wwox^RNAi
UAS-Candidate^RNAi
Candidate
Wwox
OR
UAS-Candidate^RNAi
TM6B
(iii)
da-GAL4 > UAS- Wwox
TM6B
X
UAS-Candidate^RNAi
UAS-Candidate^RNAi
Non-TM6B progeny
TM6B progeny
da-GAL4 > UAS- Wwox
UAS-Candidate^RNAi
Candidate
Wwox
OR
UAS-Candidate^RNAi
TM6B
(iv)
Non-TM6B
TM6B

Figure 3.3 Crosses used in the survival assay screen. Crosses were set up to obtain progeny with ubiquitious expression of RNAi construct targeting the candidate gene (da-GAL4> UAS-Candidate^{RNAi}) with **(i)** normal levels of Wwox, **(ii)** decreased levels of Wwox and **(iii)** ectopic expression of wild-type Wwox. A TM6B balancer (*ln(3LR)TM6, Hu e*) was used in the crosses to provide an indication of the survival rate in each cross. The TM6B balancer contains a dominant allele of *Antennapedia (Hu),* which manifests itself as additional bristles on the humerus and allows the distinction between flies that carry that balancer and flies that do not carry the balancer. The bristles that are used for this distinction are marked with the white circles. **(iv)** Flies without the TM6B balancer (non-TM6B) have the characteristic wild-type three macrochaetae (long bristles) on the humeri whilst TM6B flies have more than three bristles. Progeny without the TM6B balancer will have the desired genotype. According to Mendel's law of segregation, each cross should generate progeny with the two different genotypes (non TM6B:TM6B) at a 1:1 ratio if the genotype of the flies do not have any effect on survival ability. Any effect on survival ability when the candidate genes are targeted (non TM6B progeny) would result in changes to the ratio of non TM6B:TM6B progeny. The ratio of non TM6B:TM6B progeny for cross (ii) and (iii) were compared to that of cross (i) to determine if there was any effect on survival when Wwox levels were altered. In the diagrams, the UAS-Candidate^{RNAi} constructs are depicted as being on the third chromosome for simplicity, however RNAi constructs on the first and second chromosomes were also used, with the same resulting genotypes (Figure adapted from O'Keefe *et al.*, 2011).

Statement of Authorship

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Contribution to the paper	Conceived and designed the experiments, analysed the data, contributed reagents/materials/analysis tools, contributed to revision of manuscript		
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Contribution to the paper	Contributed reagents/materials/analysis tools; design and generation of the T127A and Y288F constructs		
Signature		Date	13/11/2014

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Contribution to the paper	Contributed reagents/materials/analysis tools; design and generation of the Y29R construct		
Signature		Date	5/12/14

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Contribution to the paper	Contributed reagents/materials/analysis tools; design and generation of the Y29R construct		
Signature		Date	8/12/14

Name of Co-Author	Robert I. Richards		
Contribution to the paper	Conceived and designed the experiments, analysed the data, contributed reagents/materials/analysis tools, writing and revision of manuscript and is the corresponding author		
Signature		Date	8/12/14

The SDR enzymatic activity of tumour suppressor WWOX is required for its functional interaction with the mitochondrial respiratory complexes.

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Abstract

Common chromosomal fragile site *FRA16D* is a frequent site of DNA instability in various types of cancer, with the *WWOX* gene that spans it often being perturbed. WWOX has been shown to be able to suppress tumour growth, however the mechanism of this suppression has not yet been fully delineated. WWOX participates in pathways involving aerobic metabolism and regulation of reactive oxygen species. The WWOX protein contains two WW domains as well as a short-chain dehydrogenase/reductase (SDR) enzyme. Herein is described a genetic analysis in the model organism *Drosophila melanogaster* conducted to identify novel functional interactions between WWOX and components of metabolic pathways. WWOX was found to functionally interact with mitochondrial respiratory complex genes, with the active site in the SDR enzyme of WWOX shown to be required for its participation. Significantly, increased levels of WWOX expression were able to suppress tissue disruption caused by defects in the mitochondrial respiratory chain, whereas decreased levels of WWOX exacerbate the phenotype. This demonstrates that WWOX, through its SDR enzyme activity, and mitochondrial function have a cooperative effect on the maintenance of cellular homeostasis.

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 cancer (Richards, 2001). More than 200 different fragile sites have been induced to appear in human cells *in vitro*, 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 (Smith *et al*., 2007).

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. Reduced levels of full-length WWOX have been reported in multiple types of cancers (Paige *et al*., 2001; Lewandowska *et al*., 2009; Gardenswartz *et al*., 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 tumourigenesis (Yang *et al*., 2013; Yu *et al*., 2014). Aberrant WWOX transcripts are often observed in tumours 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 be translated into truncated protein products that lack the complete SDR enzyme (Ishii *et al*., 2003; Mahajan *et al*., 2005). This brings about the possibility that loss of the SDR enzymatic activity of WWOX has a significant contribution to tumourigenesis.

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 tumour suppressor activity (Bednarek *et al*., 2001) although it does not appear to act as a classical tumour suppressor. Low levels of WWOX, rather than complete absence of WWOX, are often observed in tumours, indicating that the tumour suppressor function of WWOX does not fit Knudson's two hit hypothesis. An increase in incidence of tumour 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 Meyer *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; Mallarat *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; Aqeilan *et al*., 2009; Ludes Meyer *et al*., 2009; Suzuki *et al*., 2009). Evidence of WWOX having a contribution to metabolism has also been reported in *Drosophila* (O'Keefe *et al*., 2011). Whilst loss of WWOX has no obvious effect on the viability and lifespan of *Drosophila*, proteomic and microarray analyses of flies with altered levels of WWOX protein revealed quantitative and qualitative alterations in various metabolic proteins and/or mRNAs, 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 (IDH) 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 (MEFs) has also 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 1∝ (HIF-1∝) (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* transcription was observed under conditions that promote oxidative phosphorylation, whilst 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. This metabolic role for WWOX is further supported by genome-wide association studies

that showed associations between WWOX and triglyceride and HDL cholesterol levels in humans (Saez *et al*., 2010; Iatan *et al*., 2014).

Altered metabolism is now recognised 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 tumours include those affecting the tricarboxylic acid cycle (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 tumourigenesis. 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.

Results

Screening metabolic pathways for visible cellular dysfunction in *Drosophila*

A single gene orthologue of WWOX is found in a diverse range of organisms from the sea sponge to primates, indicating that a primary biological function has been conserved through evolution. *Drosophila* has a single WWOX orthologue, therefore the extensive resources in this model organism render it ideal for the genetic identification and dissection of the primary pathway(s) in which WWOX participates. 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). In order 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 if 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 due to its 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 expressed in the developing eye using the ey-GAL4 promoter and 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 (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 (Table S1). The six genes (*ND23, ND42, ND75, CG7580, CoVa* and *CoVb*) all encode components of the mitochondrial respiratory chain. Whilst some flies displayed mild or no disruption in eye tissue (Figure 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 (Figure 1B). More severe eye phenotypes were observed in a small percentage of flies (Figure 1C-C’’), where there was outgrowth of eye tissue (Figure 1C), presence of ectopic cuticle structures within the eye field (Figure 1C’) or substantial loss of cells resulting in an overall decrease in eye size (Figure 1C”). These phenotypes indicate that there is significant cellular dysfunction caused by decreased expression of these mitochondrial respiratory complex genes. Targeting of *ND42* and *CoVa* by different RNAi lines have previously been shown to result in defects in mitochondrial activity in the *Drosophila* eye discs (Ambrus *et al*., 2013) and we have also previously shown that the use of our RNAi line to target *ND42* result 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.

There was considerable variation in the severity of phenotype displayed by flies of the same genotype and even between the eyes of an individual fly. Hence, in order to determine if altered WWOX levels could significantly modulate the cellular dysfunction observed, a system was established to score the range of these phenotypes. 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”; See Supplementary Methods; Figure S1). This allowed for the quantification of the number of eyes displaying considerable tissue disruption caused by mitochondrial defects and enabled the determination of whether altered WWOX levels were able to enhance or suppress the tissue disruption in a statistically significant manner.

Loss of WWOX exacerbates cellular dysfunction caused by mitochondrial defects

Reduced WWOX expression was found to enhance the cellular dysfunction caused by mitochondrial defects, with significantly higher proportions of adult eyes exhibiting 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 (Figure 2A) ($p \leq 0.01$, 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 (Figure S2, Table S2). These results indicate that reduced WWOX expression contributes to the dysfunction caused by loss of these mitochondrial respiratory complex genes.

Similar enhancement of cellular dysfunction by decreased WWOX was observed at an earlier stage of development - in third instar larval eye imaginal discs, which are the developmental precursors to the adult eye (Figure 2B-C). An outgrowth phenotype, characterised as a protrusion from the eye disc, was seen in a small proportion of larvae when mitochondrial dysfunction is induced and is indicative of defects in cellular differentiation and proliferation (Figure 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). 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 morphogenetic furrow (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 compared to Complex I gene alone (Figure 2C). This data further support the conclusion that reduced WWOX expression contributes to mitochondrial-mediated cellular dysfunction.

Reactive oxygen species are effectors of the mitochondrial-mediated cellular dysfunction

Mitochondrial respiratory complex dysfunction has been shown to promote tumour 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 (Figure 3A), which demonstrates that the mitochondrial dysfunction can result in high levels of ROS. Further experiments were then performed to determine if the ROS that are produced are effectors of the outgrowth phenotype or whether they are by-products of mitochondrial dysfunction that are independent of the phenotype. ROS levels in cells are regulated by antioxidant enzymes such as superoxide dismutases (SOD) and Catalase to maintain redox homeostasis. SOD1 and SOD2 are responsible for the removal of superoxide anions in the mitochondria and cytoplasm by converting those molecules to the cell-permeable hydrogen peroxide that are then converted by Catalase to water molecules. Overexpression of Catalase, SOD1 or SOD2 was able to significantly reduce the proportion of larval eye discs with the outgrowth phenotype (Figure 3B, Table S3). Conversely, decreased expression of SOD1 or SOD2 significantly increased the proportion of the outgrowth phenotype (Figure 3C, Table S3). These results indicate that the ROS that are produced by mitochondrial dysfunction are contributing factors to the production of the outgrowth phenotype.

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 overexpressed together with reduced expression of the mitochondrial respiratory complex genes in order to determine if increased WWOX is able to suppress the phenotype. The phenotypes at the adult stage were examined to ascertain whether increasing levels of WWOX had any significant lasting effect throughout development of the eye. The proportion of adult eyes displaying any considerable tissue disruption was compared to the proportion 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 (Figure 4) ($p<0.001$, Table S4). 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 WWOX is unable to overcome.

The 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 a short-chain dehydrogenase/reductase (SDR) enzyme. The WW domains have been shown to physically interact with various proteins with a PPxY motif (Aqeilan *et al*., 2004; Hu *et al*., 2004; Abu-Odeh *et al*., 2014) 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 function of the SDR enzyme. It is classified as a SDR enzyme based on amino acid sequence homology including 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.

In order 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 (Figure 5A-B, Table S5). 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 SDR proteins (Oppermann *et al*., 1997; Nakajin *et al*., 1998; Koepf *et al*., 1999; Filling *et al*., 2002; Cancemi *et al*., 2002; Aqeilan *et al*., 2004). 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. The transgenes were inserted into the same site in the *Drosophila* genome to minimise insertional effects and to ensure that any observed differences are due to the particular mutation.

Comparable levels of WWOX protein were observed in *Drosophila* ubiquitiously expressing the wild-type and mutant forms of WWOX except for the mutant form that contains a mutation in the co-factor binding site in the SDR region (T127A) (Figure 5C), which suggests that the mutation results in a significant reduction in stability of the WWOX protein. The same mutation was previously shown to alter co-factor binding preference and abolish some but not all enzymatic activity of another SDR enzyme (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 stabilising the framework for cofactor positioning. Hence, it is possible that whilst this mutation may not necessarily abolish all enzymatic SDR activity, it is able to affect the folding and thus, overall stability of the WWOX protein resulting in lower levels of protein being produced.

As previously seen, overexpression of the wild-type WWOX protein was able to suppress the tissue disruption caused by decreased expression of ND23, demonstrated by the higher proportion of eyes displaying no considerable tissue disruption. Similar suppression was evident with overexpression of four mutant forms of WWOX (Y29R, W58F, T127A and P277A), however the Y288F mutant was unable to rescue the tissue disruption (Figure 5D, Table S6), rather the tissue disruption appears to be slightly stronger in this mutant. This lack of suppression by the Y288F mutant was also observed when tested with two other Complex I genes, ND42 and ND75 (Figure S3, Table S7). 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 wild-type and Y288F mutant form of WWOX (Figure 5C), 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.

Discussion

In this study, genetic analyses were utilised to identify phenotypes indicative of cellular dysfunction that could be modulated by altered levels of WWOX. WWOX has previously been shown to have 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*, reduction in the individual expression of 10 genes was found to result in cellular dysfunction and WWOX was able to modulate the cellular dysfunction seen with 6 of these genes, all of which are components of the mitochondrial respiratory chain. Loss of WWOX exacerbated the cellular dysfunction, resulting in a higher proportion of eyes displaying severe phenotypes in adults and outgrowth of tissue in the larval eye imaginal discs. These severe adult eye 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 tumourigenesis. 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 tumourigenic potential of cancer cells (Santidrian *et al*., 2013). One of the major effects of mitochondrial dysfunction is the production of large amounts of ROS, which leads to tumour 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 be driven by high levels of ROS. WWOX has been found to have an effect on ROS levels, although the resultant effect appears to be dependent on the context (O'Keefe *et al*., 2011; Shaukat *et al*., 2014). We have previously shown that decreasing WWOX levels in cells that have 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.

Significantly, increased WWOX expression was able to suppress the tissue disruption caused by Complex I and IV genes, as demonstrated by lower proportions of eyes exhibiting any considerable disruption. This indicates that increasing WWOX levels is able to provide a more favourable outcome for cells that have mitochondrial dysfunction. This finding appears to be consistent with the observed up-regulation of WWOX expression in damaged mitochondria of mice with retinal defects (Chen *et al*., 2005) and that increasing WWOX expression is able to 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 supportive of WWOX having a functional relationship with the mitochondria. A recent study has also provided evidence pointing towards reduced mitochondrial respiration and enhanced glycolysis in WWOX mutant mouse embryonic fibroblasts (Abu-Remaileh and Aqeilan, 2014). Thus it is possible that WWOX may have a role in regulating the balance between glycolysis and mitochondrial respiration and that this could be a mechanism by which Wwox is regulating cellular homeostasis in cells with mitochondrial defects.

Structure-function analyses were carried out to identify the amino acid residues required for the interaction between WWOX and the mitochondrial complex genes. Four out of the five lines carrying mutations in specific amino acid residues retained the ability to suppress the tissue disruption, indicating that those amino acid residues are not essential for the modulation of mitochondrial dysfunction. One of the mutant lines (Y29R) contains a mutation equivalent to the mammalian Y33R mutation that has been shown to abrogate the apoptotic function of WWOX at the nuclear level (Chang *et al*., 2003). This indicates that this suppressive function of WWOX is independent of its ability to induce apoptosis through Tyr33 phosphorylation. The W58F mutant contains a mutation in the only tryptophan residue in the second WW domain. The second WW domain of WWOX is slightly different to other WW domains as it contains only one tryptophan residue, instead of the two signature tryptophan residues that are characteristic of WW domains. This WW domain of WWOX is thought to augment the binding of the first WW domain to its binding partners, as the first WW domain assumes a folded conformation only when the second WW domain is present in tandem (Schuchardt *et al*., 2013). These specific residues in the WW domains of WWOX are therefore not critical for this mitochondrial-related function of WWOX. As for the mutations in the SDR region, both the alanine polymorphism associated with increased risk of thyroid cancer (P277A) as well as the specific mutation in the co-factor binding site (T127A) failed to abolish the suppression. Whilst the T127A mutant did not appear to suppress as well as the other lines, this could

be due to either its lower expression level or a decrease in SDR enzymatic activity. The T127A mutation is unlikely to completely abolish all SDR enzymatic activity as such a mutation in a different SDR enzyme was previously shown to retain some SDR enzymatic activity (Oppermann *et al*., 1997; Filling *et al*., 2002).

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 localisation signal has been identified to be within its SDR region (Chang *et al*., 2001). Whilst the exact enzymatic reaction(s) catalysed 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. Mitochondrial dysfunction has been proposed to have a role in cancer development and in tumour progression and metastasis. An increasing number of cancer mutations have been found in components of the mitochondrial respiratory chain as well as in TCA cycle enzymes, indicating that alterations in the metabolic and cellular redox state of cells have significant contributions to tumourigenesis (Kroemer and Pouyssegur, 2008; Carew *et al*., 2002; Wallace *et al*., 2012). This study shows that in circumstances where cells have defective mitochondria, loss of Wwox leads to further disruption in cellular homeostasis whilst increased Wwox expression has a protective effect. Further investigations would be required to gain better understanding of the mechanism(s) in which WWOX is able to modulate the mitochondrial-mediated cellular dysfunction, as this could help elucidate its role in promoting cancer progression. Detection of aberrant transcripts that lack coding sequences for this SDR enzyme activity in numerous cancers is consistent with the loss of SDR enzymatic function of WWOX contributing to tumourigenesis.

Material 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, 1.7% tegosept). Crosses were carried out at 25ºC. The w^{1118} , *ey-GAL4*, UAS-SOD1, UAS-SOD2 and UAS-Catalase stocks were obtained from Bloomington *Drosophila* Stock Center (Indiana University, USA). $WWOX^{1}$ has previously been described (O'Keefe *et al*., 2005; O'Keefe *et al*., 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). Constructs carrying the desired mutation were subcloned into the *Drosophila* transformation vector (Gateway® pTW-attB, obtained from the *Drosophila* Genomics Resource Centre, Indiana University, USA) by LR clonase recombination according to manufactor's guidelines (Life Technologies, CA, USA). Constructs were then microinjected into the ZH-68E line (FBst0328401) by standard methods to obtain germline transformants. Mutations in the transformed *Drosophila* lines were verified using sequence analyses by Australian Genome Research Facility Ltd (AGRF), Adelaide, Australia.

Primers used for the mutagenesis reactions were as follows (5'→ 3')
Y29R : GACGGCACCGTTTGCCGTGTGAACCAGCAGGGA
W58F : CGAATTACCCTTGGGCTTCGAGAAGTACTACG
T127A: CACCGCGCTGATAGCGGGCGCAAATTG
Y288F: GGAGCATGATGGCCTTCAACAATGCCAAGC
P277A: GCATCACCTTTCGGCGCCGCCGGAGAAATAC

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 (1:2500, Vector Laboratories, Burlingame, CA), mouse monoclonal anti-α-tubulin (1:2000, 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 1x phosphate buffered saline (PBS) and fixed in 4% formaldehyde for 20 minutes. Three 10-minute washes were performed in PBST (1xPBS + 0.3% Triton-X-100). Discs were incubated with PBST containing 5% fetal calf serum for 1 hour before incubation with primary antibody overnight at 4ºC, followed by three 20-minute washes with PBST and 2 hour incubation with second antibody at room temperature in the dark before another round of three 20-minute washes with PBST. Primary antibodies used were mouse anti-Elav-9F8A9 (1:10, Developmental Studies Hybridoma Bank, University of Iowa, Iowa) and rabbit anti-phospho-histone H3 (S10) (1:1500, Cell Signaling, Massachusetts, USA). Second antibodies were anti-mouse Cy3 (1:200, Jackson Laboratories, West Grove, PA) and anti-rabbit-Dylight 649 (1:200, Vector Laboratories, Burlingame, CA). Discs were mounted in 80% glycerol and fluorescence visualised using a Zeiss Axioplan 2 microscope and 10x PlanApo objective. Images compiled using Axiovision (Carl Zeiss) and Adobe Photoshop software.

Detection of ROS

CellROX® Deep Red Reagent (Life Technologies, CA, USA) 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 5μm CellROX® in D22 media for 15 minutes (in the dark at room temperature). The discs were then subjected to two quick washes with 1X PBS, fixed with 3.7% formaldehyde for 5 minutes and then mounted in 80% glycerol for fluorescence visualisation. The absorption/emission maxima of the dye is ~644/665nm. Images were taken using the 20x PlanApo objective.

Light microscopy

Photographs of exterior adult *Drosophila* eye 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. Anterior of 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 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 disorganisation in the patterning of the ommatidia; “moderate” –loss of photoreceptor cells forming a cavity in the surface of the eve; “severe” – significant disruption in the gross structure and development of the eye (eg. 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. Further analyses were also performed using Fisher’s exact test to compare two categories – “severe” vs combined “moderate + mild/normal” in order to determine if reduced WWOX levels specifically affects 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 to 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 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 Fisher’s exact test with $p=0.05$ as cut off value for significance. All statistical analyses were performed using GraphPad Prism. Percentage of eyes or eye discs in each category was plotted for visual representation using Microsoft Excel.

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Conflict of Interest statement.

The authors declare that there are no conflicts of interest.

Author contributions

A. Choo designed research, conducted experiments, analysed data and wrote the manuscript; L.O'Keefe designed and supervised research, interpreted data and contributed to revision of manuscript; C.S. Lee, S.L. Gregory and Z. Shaukat designed methods, analysed data and contributed to revision of manuscript; A. Colella, K. Lee, D. Denton designed methods and analysis tools; R.I. Richards conceived the project, supervised research, interpreted data and wrote the manuscript.

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Figures

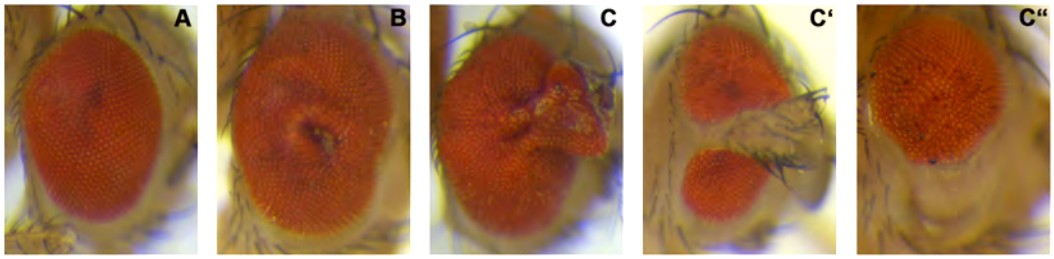


Figure 1. Adult eye phenotypes indicative of cellular dysfunction caused by reduced expression of mitochondrial respiratory complex genes. Decreased expression of *ND23*, *ND42*, *ND75*, *CG7580*, *CoVa* and *CoVb* all result in a range of phenotypes, ranging from **(A)** mild or no disruption to the patterning of ommatidia, **(B)** loss of photoreceptor cells forming a cavity in the surface of the eye **(C-C’’)** severe phenotypes indicative of defects in development, which include **(C)** outgrowth of the eye tissue, **(C’)** presence of ectopic structures within the eye field, **(C”)** substantial loss of cells resulting in an overall decrease in eye size.

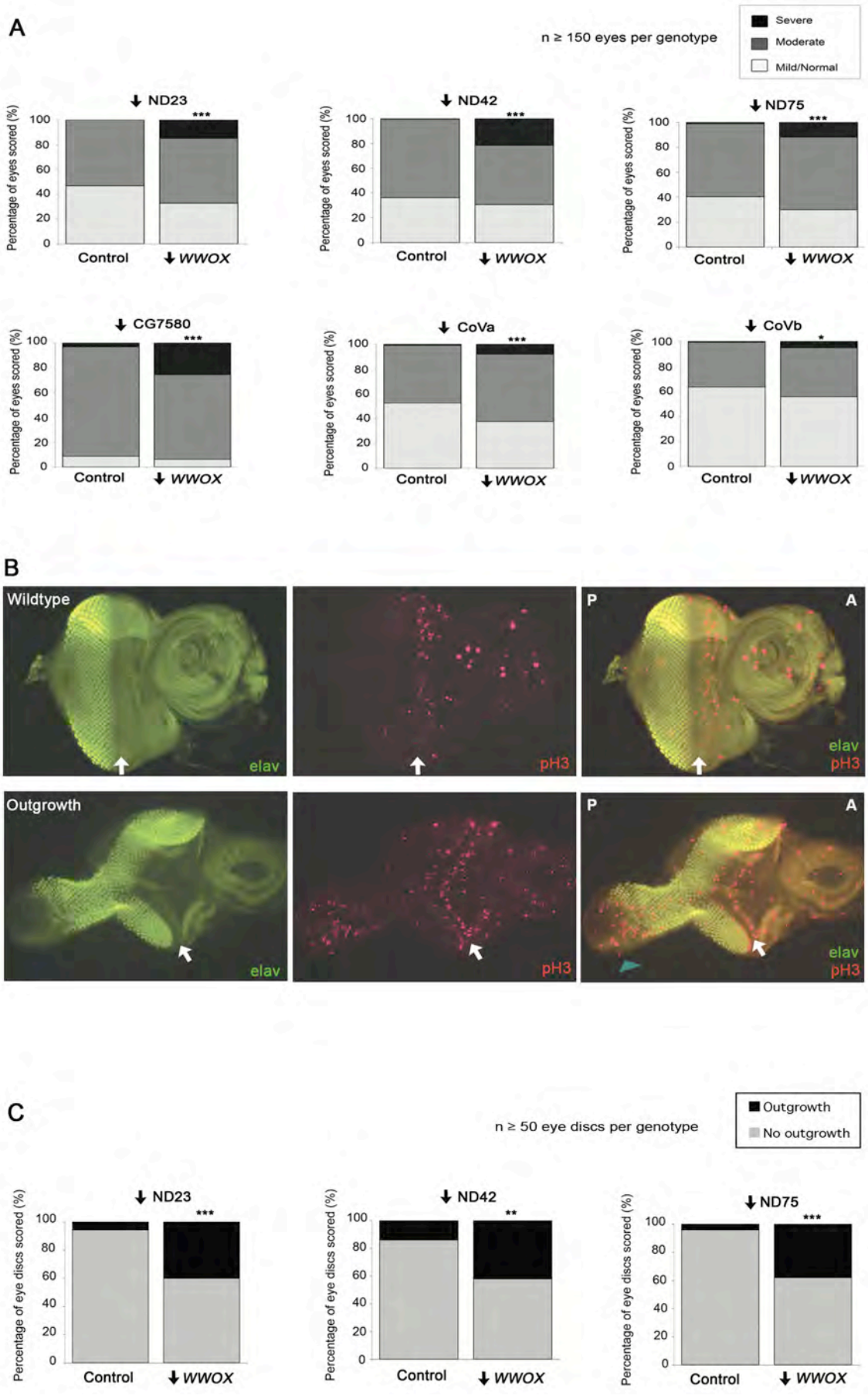

A
n ≥ 150 eyes per genotype
Severe
Moderate
Mild/Normal
↓ ND23
↓ ND42
↓ ND75
↓ CG7580
↓ CoVa
↓ CoVb
Percentage of eyes scored (%)
Control
↓ WWOX
B
Wildtype
Outgrowth
elav
pH3
P
A
C
n ≥ 50 eye discs per genotype
Outgrowth
No outgrowth
Percentage of eye discs scored (%)

Figure 2. Decreased WWOX expression exacerbates the cellular dysfunction caused by reduced expression of mitochondrial respiratory complex genes. Reduced WWOX levels result in higher proportions of adult and larval eye phenotypes indicative of cellular dysfunction. **(A)** Percentage of adult eyes in the different phenotypic categories (severe, moderate and mild/normal). *$p<0.01$, ***$p\leq0.001$ compared to respective controls determined by chi-square test. p values and further statistical analyses in Table S2. $n\geq150$ eyes per genotype. **(B)** Outgrowth phenotype in larval eye discs. The protrusion is characterised as an increased region of differentiated cells (neuronal cells stained with anti-elav, green) posterior from the morphogenetic furrow (MF, indicated by the white arrows). Differentiation of cells in the eye disc normally 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) typically 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 (indicated by the blue arrowhead). **(C)** Percentage of larval eye discs with the outgrowth phenotype. ** $p\leq0.01$, ***$p\leq0.001$ compared to control determined by Fisher's exact test (Table S2). $n\geq50$ dics per genotype.

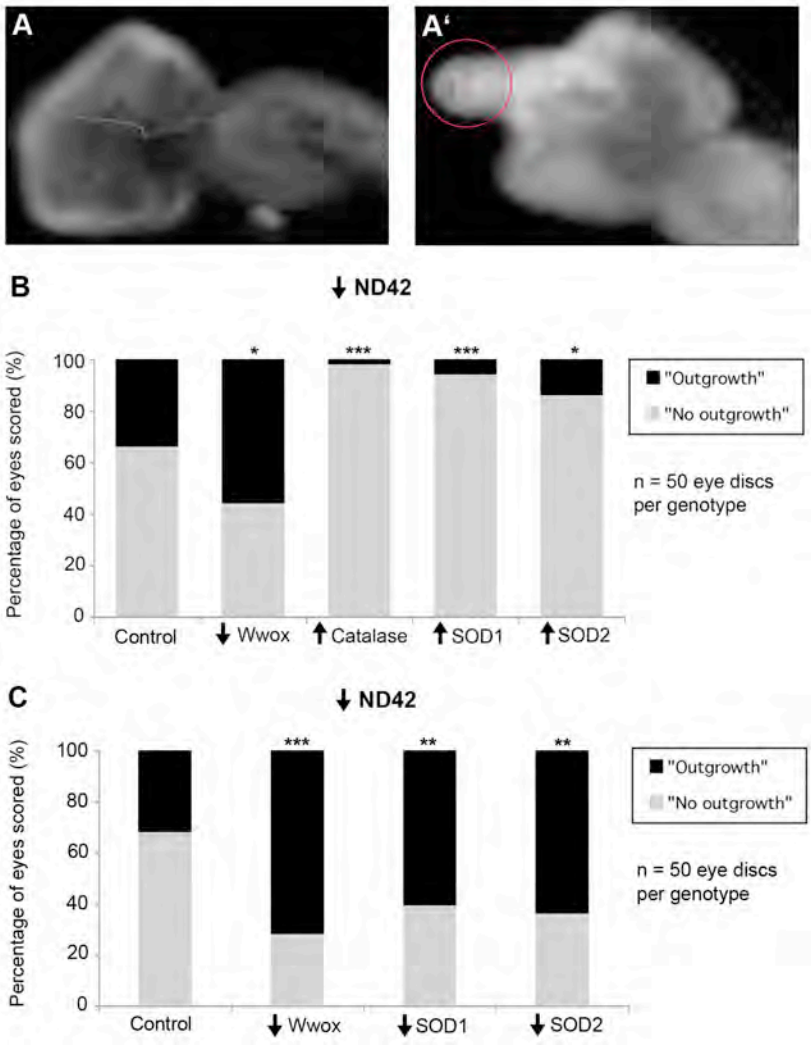


Figure 3. Reactive oxygen species (ROS) are effectors of the larval eye disc outgrowth phenotype. (A) Presence of ROS detected by CellRox® staining in the outgrowth region of the affected larval eye discs. Punctuate staining is observed in the outgrowth region of affected discs (A') but not in wild-type 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, ***p≤0.001 compared to respective controls determined by Fisher's exact test. Exact values presented in Table S3. n=50 dics 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 the antioxidant enzymes Catalase, SOD1 or SOD2 result in significantly lower proportion of the outgrowth phenotype compared to the control (overexpression of an empty vector). **(C)** Decreased expression of SOD1 or SOD2 result in significantly higher proportion of the outgrowth compared to the control (expressing a non-specific RNAi).

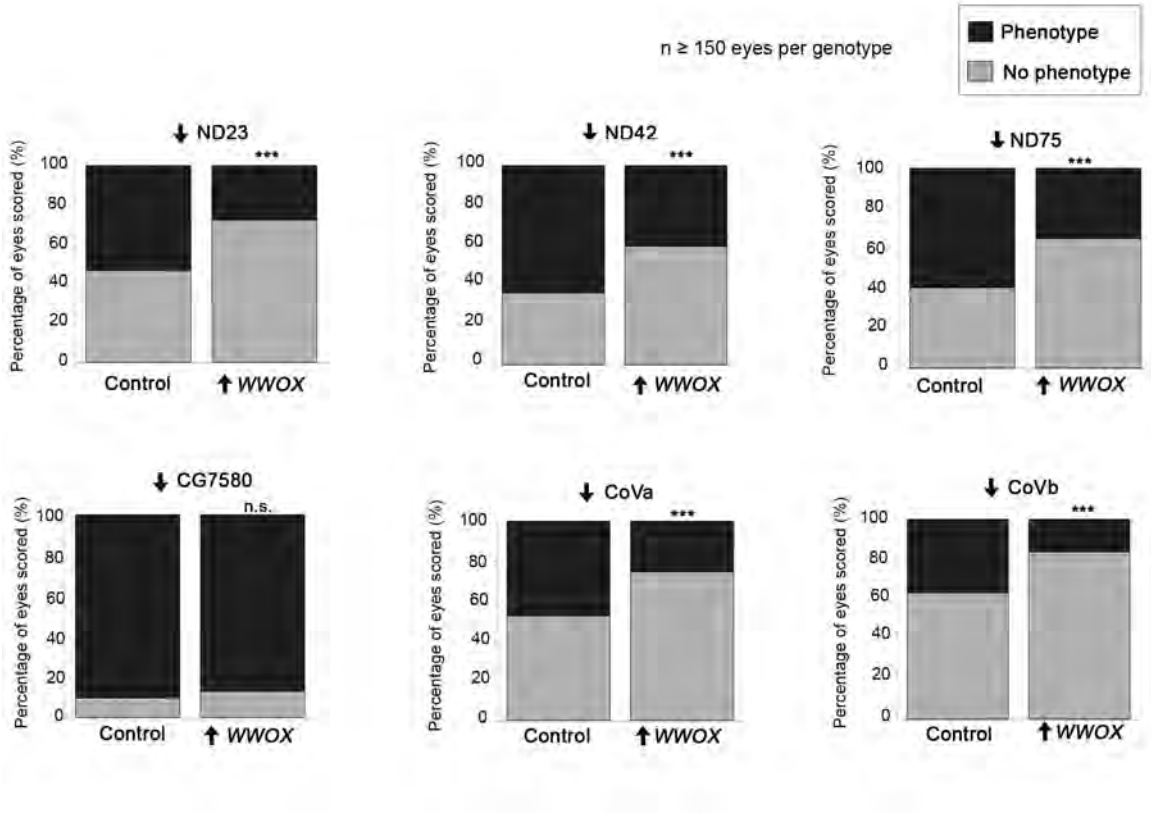


Figure 4. Increased WWOX expression rescues some of the cellular dysfunction caused by reduced expression of mitochondrial respiratory complex genes. Overexpression of *WWOX* cDNA results in higher proportion of normal eyes when expressed together with decreased levels of *ND23*, *ND42*, *ND75*, *CoVa* and *CoVb* but not with *CG7580*. Eyes scored based on the presence or absence of a phenotype. ***$p \leq 0.001$ compared to respective controls determined by Fisher's exact test (Table S4), n.s. indicates non-significance. $n \geq 150$ eyes per genotype.

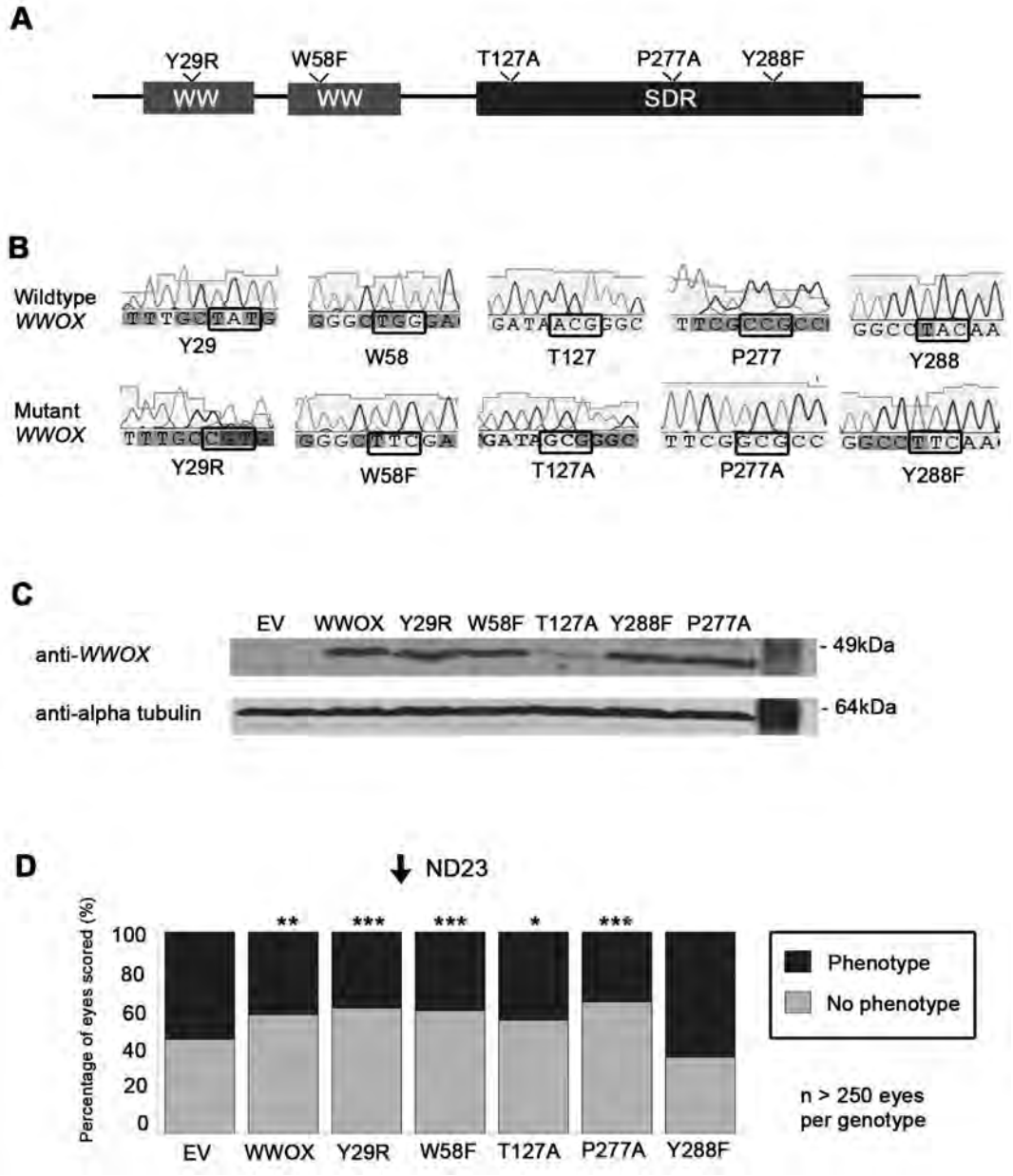


Figure 5. Catalytic active site in the SDR region of WWOX required in its suppression of tissue disruption caused by mitochondrial defects. (A) Amino acid residues mutated in the different functional elements of WWOX. **(B)** Sequencing results of nucleotide substitutions that give rise to the different amino acid mutations. **(C)** Comparable levels of WWOX protein detected by Western blot analysis in WWOX mutant lines compared to the wild-type 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. **(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 to EV control. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ indicative of suppression compared to EV control as determined by Fisher's

exact test, p values and further statistical analyses in Table S6. n≥250 eyes per genotype. In the image of the Western blot analysis presented, a lane was removed from the gel image between the W58F and T127A samples during processing of the image, however this does not affect the data that is presented.

Supplementary Material

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 (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 the alteration in the levels of antioxidant enzymes results in modification of the cellular dysfunction (related to Figure 3).

Table S4 provides statistical analyses supporting that increased WWOX expression suppresses the cellular dysfunction (related to Figure 4).

Table S5 provides details regarding the mutations generated in the different domains of WWOX (related to Figure 5).

Table S6 provides statistical analyses supporting that the SDR active site of WWOX is required for its suppression of cellular dysfunction (related to Figure 5).

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 5).

Table S7 provides statistical analyses for the additional verification of the role of the WWOX SDR catalytic active site (related to Figure S3).

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 ommatidia	No modification
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 catalase*	v101098	Response to oxidative stress	No phenotype	No phenotype
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)	Eye defects, 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)	Eye defects, 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)	Eye defects, 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)	Eye defects, 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	Eye defects, 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	Eye defects, with a range in severity	Enhanced by decreased Wwox levels and suppressed by increased Wwox
54	*CG5818*	*Mitochondrial ribosomal protein L4*	v40608	Translation	No phenotype	No phenotype
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-ligase activity,positive regulator of mitochondrial fission	No phenotype	No phenotype

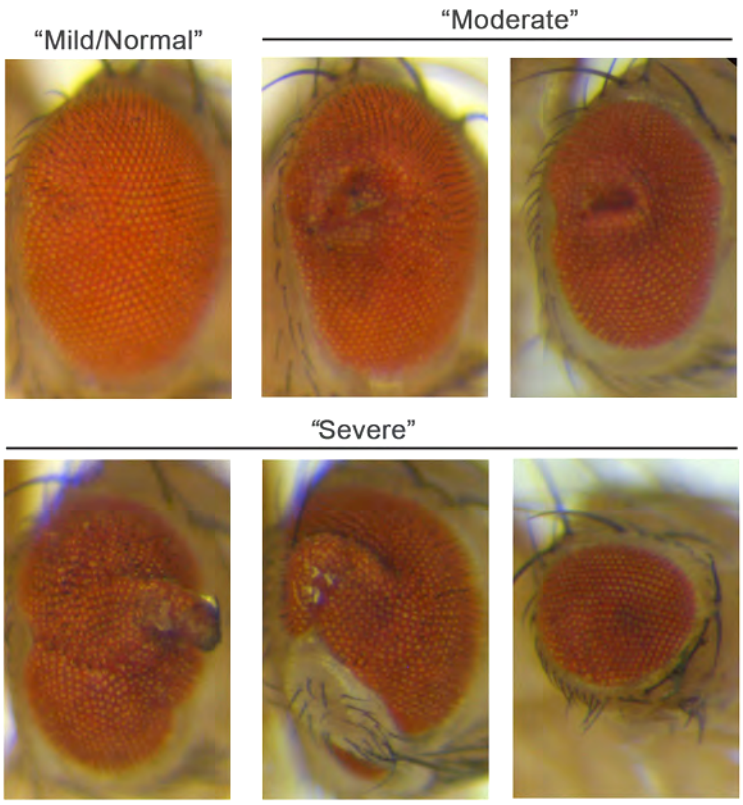


Figure S1. Categories of eye phenotype severity used to evaluate the effects of reduced WWOX levels on the mitochondrial-mediated cellular dysfunction. Eyes were grouped into three different categories: “mild/normal” – normal or mild disruption in the patterning of the ommatidia; “moderate” –loss of photoreceptor cells in the eye field forming a cavity in the surface of 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 decreased eye size).

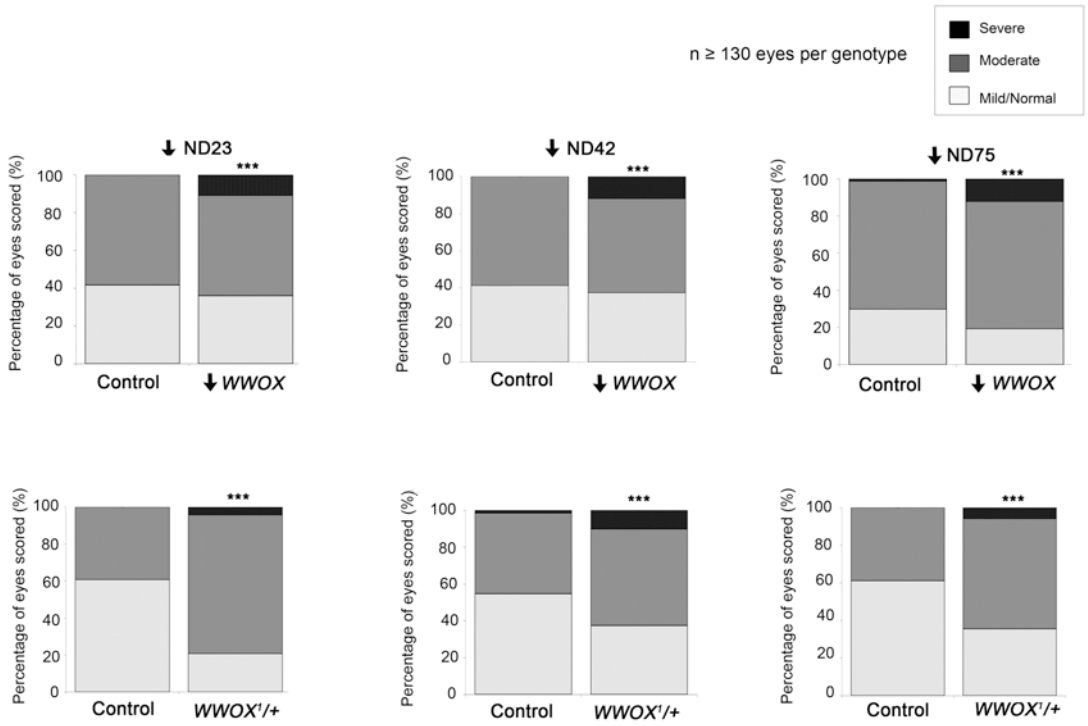


Figure S2. Independent verification that reduced *WWOX* expression exacerbates the cellular dysfunction caused by decreased expression of three different Complex I genes (*ND23, ND42* and *ND75*). Decreased *WWOX* expression using two other independent lines (different RNAi line v22536 and heterozygous WWOX null mutation, $WWOX^{1}$/+) increases the proportion of eye phenotypes indicative of cellular dysfunction. Eyes were scored based on severity of the phenotype (severe, moderate and 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 S3. $n \geq 130$ eyes per genotype.

Table S2. Statistical analyses of the effects of reduced WWOX expression on the mitochondrial-mediated phenotypes.

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 performed to compare all three phenotypic categories between the different genotypes whilst Fisher's exact test analyses were carried out as comparisons of the severe phenotypes with the other phenotypes (combined moderate and mild/normal phenotypes). 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 modification of the larval eye disc outgrowth phenotype by altered levels of antioxidant enzymes.

Overexpression of antioxidant enzymes	
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 *)
Decreased expression of antioxidant enzymes	
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 **)

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.

Table S4. Statistical analyses of the effects of increased WWOX expression on the mitochondrial-mediated phenotype.

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 S5. Mutations generated to target the amino acid residues that have been identified or are 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.*, 1997; 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 S6. Statistical analyses of the effects of the different WWOX mutations on the mitochondrial-mediated phenotype.

n>250 eyes per genotype	Compared to EV control	Compared to wild-type *WWOX*
Wild-type 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 mitochondrial-mediated phenotype in this experiment was generated by targeting expression of the Complex I gene, *ND23*.

[#] 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 5 as the significance shown in that figure was only in reference to the suppression phenotypes.

Comparisons were also made between the wild-type 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 wild-type WWOX.

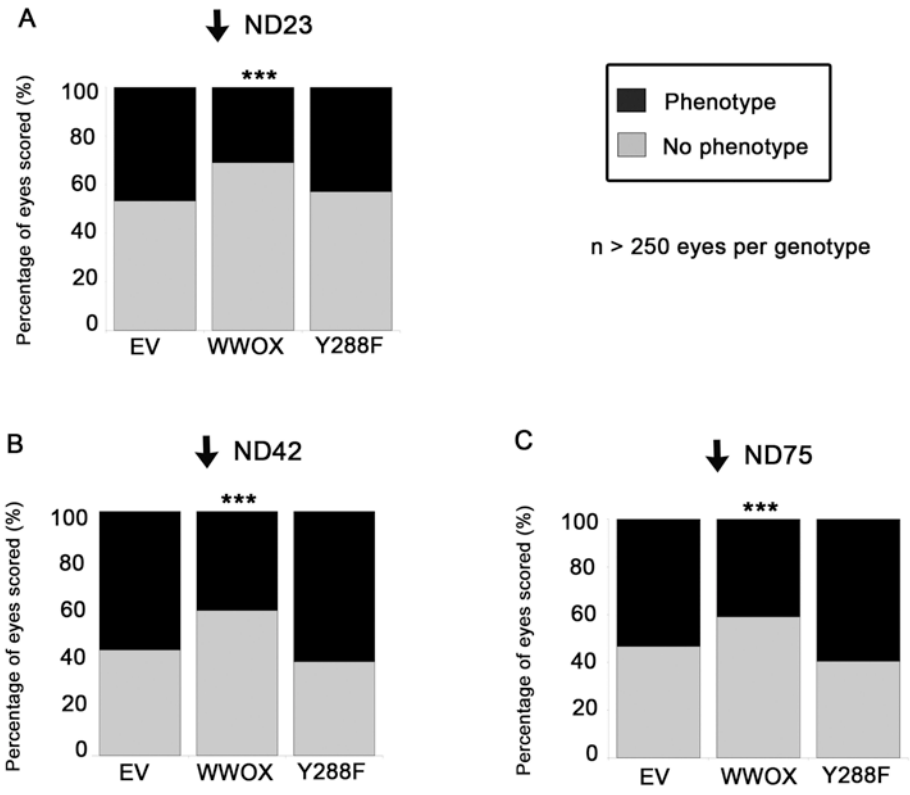


Figure S3. Additional analyses supporting that the SDR catalytic active site is required for the suppressive function of WWOX on mitochondrial defect-mediated tissue disruption. Both ectopic wild-type WWOX and Y288F mutant form of WWOX (mutation in the SDR active site) were tested for their ability to suppress tissue disruption caused by decreased expression of three mitochondrial Complex I genes – (**A**) *ND23*, (**B**) *ND42* and (**C**) *ND75* in the *Drosophila* eye. Whilst expression of the ectopic wild-type WWOX 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 5). 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 S7.

Table S7. Statistical analyses of the additional experiments testing the suppressive function of the Y288F mutant compared to wild-type WWOX on the mitochondrial -mediated tissue disruption.

n>250 eyes per genotype		**Compared to EV Control**	**Compared to wild-type Wwox**
ND23	Wild-type Wwox	**Suppression** (p<0.0001 ***)	-
	Y288F mutant	No change (p=0.3077 n.s.)	Difference (p=0.0004 ***)
ND42	Wild-type Wwox	**Suppression** (p<0.0001 ***)	-
	Y288F mutant	No change (p=0.2045 n.s.)	Difference (p<0.0001 ***)
ND75	Wild-type 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 lines tested in these experiments were all inserted into the 68E1 chromosomal location.

Chapter 4 - Identification of other genetic contributors to the mitochondrial-mediated cellular dysfunction

A portion of this chapter (Figures 4.1, 4.5 and 4.6) has been included in the manuscript prepared for publication (Chapter 3). Those results are described in greater detail in this chapter and are included here to provide context to the overall findings of this work.

4.1 Introduction

Defects in cell growth, differentiation and cell survival are common properties of cancer cells; therefore the identification of enabling factors that give rise to or contribute to such defects can provide insight into the affected processes that lead to tumourigenesis and can also be considered as therapeutic targets for cancer treatment (Vogelstein & Kinzler, 1993). Whilst loss of Wwox in *Drosophila* by itself has no obvious effect on cellular function, reducing Wwox levels in a sensitised background, where cells have a defect in mitochondrial function, is detrimental to the cell (Chapter 3). Inducing mitochondrial dysfunction in the developing *Drosophila* eye was found to result in phenotypes characteristic of defects in cell growth, differentiation and survival, in particular an outgrowth phenotype in larval eye imaginal discs. Reducing Wwox levels, in conjunction with such mitochondrial dysfunction, resulted in higher levels of such phenotypes being observed whilst increasing Wwox levels was able to moderate the observed phenotypes, indicating that Wwox is involved in the maintenance of cellular homeostasis in cells with mitochondrial dysfunction (Chapter 3). However, it is unclear how Wwox contributes to these phenotypes as loss of Wwox alone in *Drosophila* does not appear to induce mitochondrial dysfunction (Chapter 6; Cheng Shoou Lee, personal communication), hence further investigation into the mitochondrial-mediated cellular dysfunction is required. The aim of this work is to identify other genetic contributors that are rate-limiting factors in the pathway responsible for the observed mitochondrial-mediated phenotype.

In order to identify other genetic contributors to the mitochondrial-mediated cellular dysfunction, modification assays were performed using the phenotypes caused by decreased expression of the mitochondrial complex genes. As described in Chapter 3, the first phenotypes observed were at the adult stage in the fully developed eye (Figure 4.1A-D). These phenotypes include loss of photoreceptor cells resulting in a cavity within the eye (Figure 4.1A), outgrowth of eye tissue (Figure 4.1B), presence of ectopic structures

within the eye (Figure 4.1C) and severe loss of tissue resulting in a decrease in eye size (Figure 4.1D). These eyes all have the same genetic makeup, however the results represent a wide range of phenotypes. As the mitochondrial dysfunction was induced throughout eye development, starting from early embryogenesis in the eye primordia through to adulthood, the phenotypes produced in the adult eye are the result of defects in eye morphogenesis. The adult eye is derived from the eye imaginal disc where specification of the eye is controlled by a complex network of signalling events involving various morphogens. The range of phenotypes observed in the adult eye hence suggests that there was aberrant cellular signalling within the eye imaginal disc such that cells were able to adopt different fates. This also indicates that it could be more informative to examine the changes occurring at an earlier stage of eye development. Thus, third instar larval eye imaginal discs were examined for any significant effects when mitochondrial dysfunction is induced (Figure 4.1E-F). Abnormal protrusions from the eye imaginal discs were observed (Figure 4.1E). There are no known reports of such a phenotype in the literature to date. These protrusions appeared to be an extension of the eye tissue, as a portion of the additional tissue was positive for elav staining which is a marker for differentiated neurons and is indicative of the presence of photoreceptor cells (Figure 4.1F). Abnormal distribution of mitotic cells, as marked by anti-phospho-histone H3 staining, were also observed in these protrusions, indicating that there is proliferation of cells driving the outgrowth of tissue. This larval eye disc outgrowth phenotype was used as the main assay to identify modifier genes and effectors that are involved in promoting or restricting the cellular dysfunction caused by mitochondrial defects.

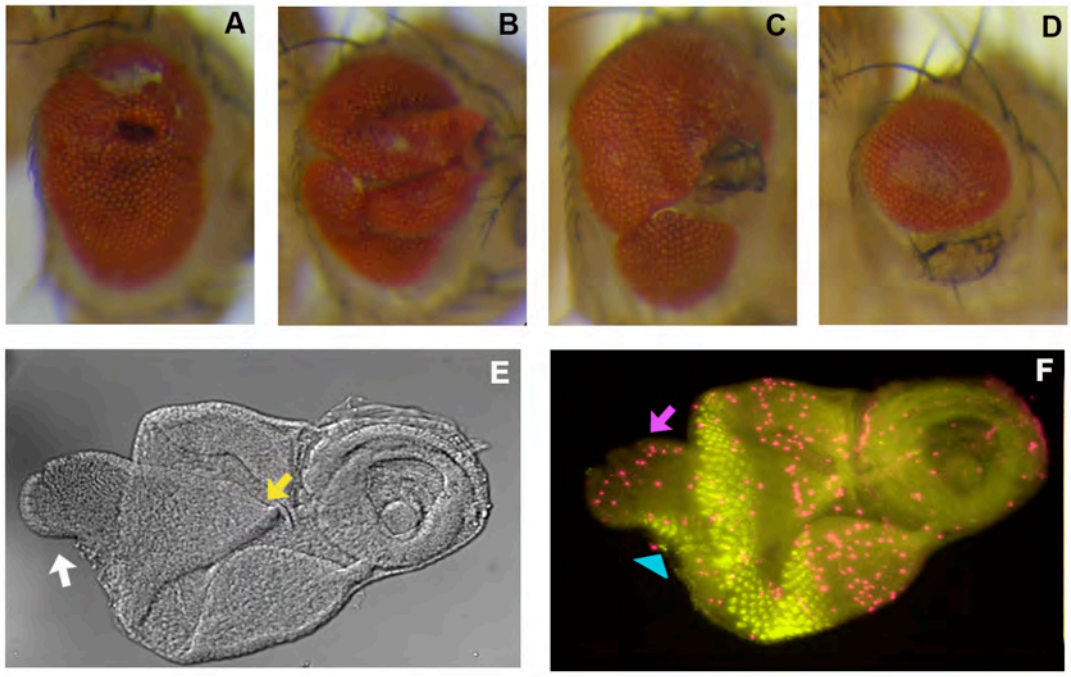


Figure 4.1 Phenotypes that are used as assays to characterise the mitochondrial defect-mediated cellular dysfunction. Selected genes are tested for their ability to modify these phenotypes in order to determine the effectors of the observed cellular dysfunction. The identification of these phenotypes and the contribution of Wwox to the frequency of the observed phenotypes had been previously reported in Chapter 3. **(A-D)** Phenotypes observed in the adult eye - **(A)** Loss of photoreceptor cells forming a cavity within the eye field, **(B)** Outgrowth of eye tissue, **(C)** Presence of ectopic structures within the eye field and **(D)** Severe loss of eye tissue resulting in a decrease in eye size. **(E-F)** Outgrowth phenotype observed in third instar larval eye imaginal disc, which are developmental precursors to the adult eye. **(E)** Structure of the larval eye-antennal disc visualised by Normaski optics. Abnormal protrusion indicated by arrows - the yellow arrow is indicative of where the protrusion was observed to begin and the white arrow indicates the tip of the protrusion. **(F)** Immunohistochemistry performed on the affected eye discs indicates that there are photoreceptor cells within the protrusion (indicated by the blue arrowhead, photoreceptor cells marked in green by anti-elav staining) as well as increased number of mitotic cells – mitotic cells are stained red by anti-phospho-histone H3 and the purple arrow indicates the abnormal presence of the mitotic cells in the protrusion. Additional immunohistochemical staining images in Appendix C: Figure C1.

4.2 Material 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, 1.7% tegosept). Crosses were carried out at 25ºC unless stated otherwise. The RNAi lines obtained from VDRC are as follows: *CG6343/ND42*^{RNAi} – v14444, *CG7221/Wwox*^{RNAi} – v108350, *CG11793/SOD1*^{RNAi} – v108307, *CG8905/SOD2*^{RNAi} – v110547, *CG4006/Akt*^{RNAi}- v103703, *CG45051/Sima*^{RNAi} – v4550, *CG11987/Tango*^{RNAi} – v10735, *CG3143/Foxo*^{RNAi} – v30557, *CG7986/Atg18a*^{RNAi} – v22643, *CG2286/ND75*^{RNAi} – v100733. The lacZ^{RNAi} line was provided by Richard Carthew whilst the mCherry^{RNAi} line (BL35785) was obtained from TRiP, Bloomington. The ey-GAL4 stock, ectopic expression lines and FRTCova^{tend} stock were obtained from Bloomington and are as follows: ey-GAL4- BL8227, UAS-Catalase - BL24621, UAS-SOD1 – BL24750, UAS-SOD2 –BL24494, UAS-Foxo – BL9575, FRTCova^{tend} – BL33839. The UAS-Dp110 stock was kindly provided by Donna Denton. The empty vector (EV) line was previously generated by Joanne Milverton. The stocks for MARCM III analyses (*eyFLP, UAS-mCD8-GFP; +/CyO; Tub-GAL4FRT82BTub-GAL80/TM6B* and *+/CyO; FRT82B/TM6B*) were kindly provided by Helena Richardson

Generation of recombinant *ey > ND42*^{RNAi} screening line

As both the *ND42*^{RNAi} construct and the ey-GAL4 promoter are located on the third chromosome, a stock carrying a recombinant chromosome (*ey, ND42*^{RNAi}*/TM6B*) was generated to obtain flies carrying both constructs on the same chromosome in order to be used as a screening line. Virgin female flies carrying the *ND42*^{RNAi} construct were crossed to male flies carrying the ey-GAL4 promoter. Virgin female progeny from that cross were collected and crossed to wild-type w^{1118} male flies to allow for recombination to occur in the germline of the females. Male progeny (from that second cross) that displayed tissue disruption in the adult eye were selected as those flies carried the recombinant *ey, ND42*^{RNAi} chromosome. Individual male flies were crossed to virgin female flies of the *w; TM2/TM6B* genotype to generate stocks where the recombinant chromosome is balanced over the dominantly marked TM6B balancer. The screening line will be referred to as "*ey*

> *ND42*^{RNAi}" in the following text; with the symbol " > " to indicate that the *ND42*^{RNAi} is constantly being expressed under the ey-GAL4 promoter.

Light microscopy

Photographs of exterior adult *Drosophila* eye 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. Anterior of eye is positioned on the right of all images.

Quantification of the adult eye phenotypes

Adult eyes were scored for a phenotype according to the categories described in Chapter 3 (representative images of each phenotypic category in the manuscript in Chapter 3). Eyes were scored from multiple independent crosses under identical conditions and counts were pooled to obtain the final tally per genotype. Percentage of eyes in each category was plotted for visual representation using Microsoft Excel. For the analyses of reduced expression of antioxidant enzymes, the proportion of phenotypes was compared between flies with normal and reduced Wwox expression. Statistical significance was determined using chi-square test with p=0.05 as cut off value for significance (GraphPad Prism) with a minimum of 100 eyes scored per genotype. For the analyses of reduced Foxo expression, the proportion of phenotypes was compared between flies with normal and altered (either reduced or increased) Wwox expression with a minimum of 300 eyes scored per genotype. Chi-square test analysis was used to determine statistical significance between the three different categories. Additional analyses were carried out using Fisher's exact test. For reduced Foxo and Wwox expression, the proportion of severe phenotypes were compared to determine if reduced Wwox levels increased the proportion of severe phenotypes whilst for reduced Foxo and increased Wwox expression, the phenotypes were regrouped to two categories – "phenotype" (combined severe and moderate classes) and "no phenotype" (mild/normal class) to determine if increased Wwox was able to suppress the tissue disruption. GraphPad Prism was used for all the analyses with p=0.05 as cut off value for significance.

Quantification of larval eye disc outgrowth phenotype

Eye-antennal imaginal discs were dissected from wandering third instar larvae and separated from the larval brain. The intact eye discs were then examined for the presence or absence of an outgrowth, with a minimum of 50 eye discs examined per genotype and approximately the same number of eye discs examined for each genotype per dissection. Any non-intact or damaged eye discs were discarded and excluded from the analysis. The proportions of eye discs with and without the outgrowth were compared to the control genotype. Statistical significance was determined using Fisher's exact test with p=0.05 as a cut off value for significance (GraphPad Prism). The percentage of eye discs in each category (outgrowth or no outgrowth) was plotted for visual representation using Microsoft Excel.

Immunohistochemistry

Fluorescence visualisation was performed using a Zeiss Axioplan 2 microscope and 20x PlanApo objective unless stated otherwise. Images are compiled using Axiovision (Carl Zeiss) and Adobe Photoshop software.

Detection of reactive oxygen species (ROS):

CellROX® Deep Red Reagent was used to detect presence of reactive oxygen species (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 5μm CellROX® in D22 media for 15 minutes (in the dark at room temperature). The discs were then subjected to two quick washes with 1X PBS, fixed with 3.7% formaldehyde for 5 minutes and then mounted in 80% glycerol for fluorescence visualisation. The absorption/emission maxima of the dye is ~644/665nm. Images were taken using the 20x PlanApo objective.

Antibody staining:

Staining of the eye imaginal discs with anti-elav and anti-phospho-histone H3 was carried out as described in the 'Materials and Methods' section of the manuscript in Chapter 3. Staining of phosphorylated Akt was performed using the same protocol, with 1/100 dilution of anti-phospho-Akt (Ser473) antibody (Cell Signaling). Images were taken at a

set exposure time using the 20x PlanApo objective. Images were also taken using the 63x PlanApo objective and processed for presentation. A region of 900 x 675 pixels was selected as representation of the image.

Clonal analyses of reduced mitochondrial function in the developing *Drosophila* eye

Clones with reduced mitochondrial function were generated in the developing *Drosophila* eye using the Mosaic analysis with a repressible cell marker (MARCM) III system. Reduced mitochondrial function was induced by either knocking out a Complex I gene, *ND75* in clones of eye tissue in a wild-type background or by generating clones that are homozygous mutant for a Complex IV gene, *CoVa* in an overall heterozygous animal. Wwox levels were reduced specifically in those clones by RNAi targeting. Adult eyes with normal and with reduced Wwox levels were examined for a phenotype using light microscopy.

4.3 Results

4.3.1 Establishment of the *ey > ND42^{RNAi}* recombinant screening line

In order to identify modifier genes and effectors that can affect the larval outgrowth phenotype, a screening line was established. The screening line carries a recombinant chromosome that consists of both the ey-GAL4 promoter and an RNAi construct targeting one of the Complex I genes, *ND42* (Figure 4.2). The presence of both constructs on the same chromosome results in the flies having continuous reduction of *ND42* by RNAi in the *Drosophila* eye from early development onwards.

Flies from this screening line were outcrossed to wild-type w^{1118} flies for validation of the larval eye disc outgrowth phenotype (Figure 4.3A Cross 1). The outgrowth phenotype was detected in a proportion of the progeny from that cross as expected (Figure 4.3B), albeit at slightly higher proportions than previously observed when the cross was performed in a different manner with the ey-GAL4 promoter and *ND42*^{RNAi} construct on different chromosomes (Chapter 3). This increase in the severity of the phenotype observed with the use of the screening line recombinant stock flies is likely to be due to the mitochondrial defects being continuously induced in the stock of flies over generations, thus having an overall greater effect, whereas in the previous cross, the mitochondrial defects were only induced in the progeny but not in the parental stocks. Nevertheless, when flies from the screening line were crossed to flies carrying an RNAi construct targeting *Wwox*, significantly higher proportions of the outgrowth phenotype were observed in the progeny that have reduced expression of both *ND42* and *Wwox* compared to the progeny that only have reduced expression of *ND42* (Figure 4.3B Cross 2, Appendix C: Table C1, p=0.0006), consistent with the previous results (Chapter 3). Furthermore, no enhancement of the outgrowth phenotype was seen when the screening line flies were crossed to flies carrying a RNAi construct targeting either lacZ or mCherry, which are both sequences that are not present in *Drosophila* (Figure 4.3B Cross 3 & 4, Appendix C: Table C1). These flies are a control for the activation of the RNAi machinery and the lack of an enhancement with these two lines demonstrates that any enhancement observed with the use of RNAi lines (e.g. *Wwox*^{RNAi}) is not due to non-specific activation of the RNAi machinery.

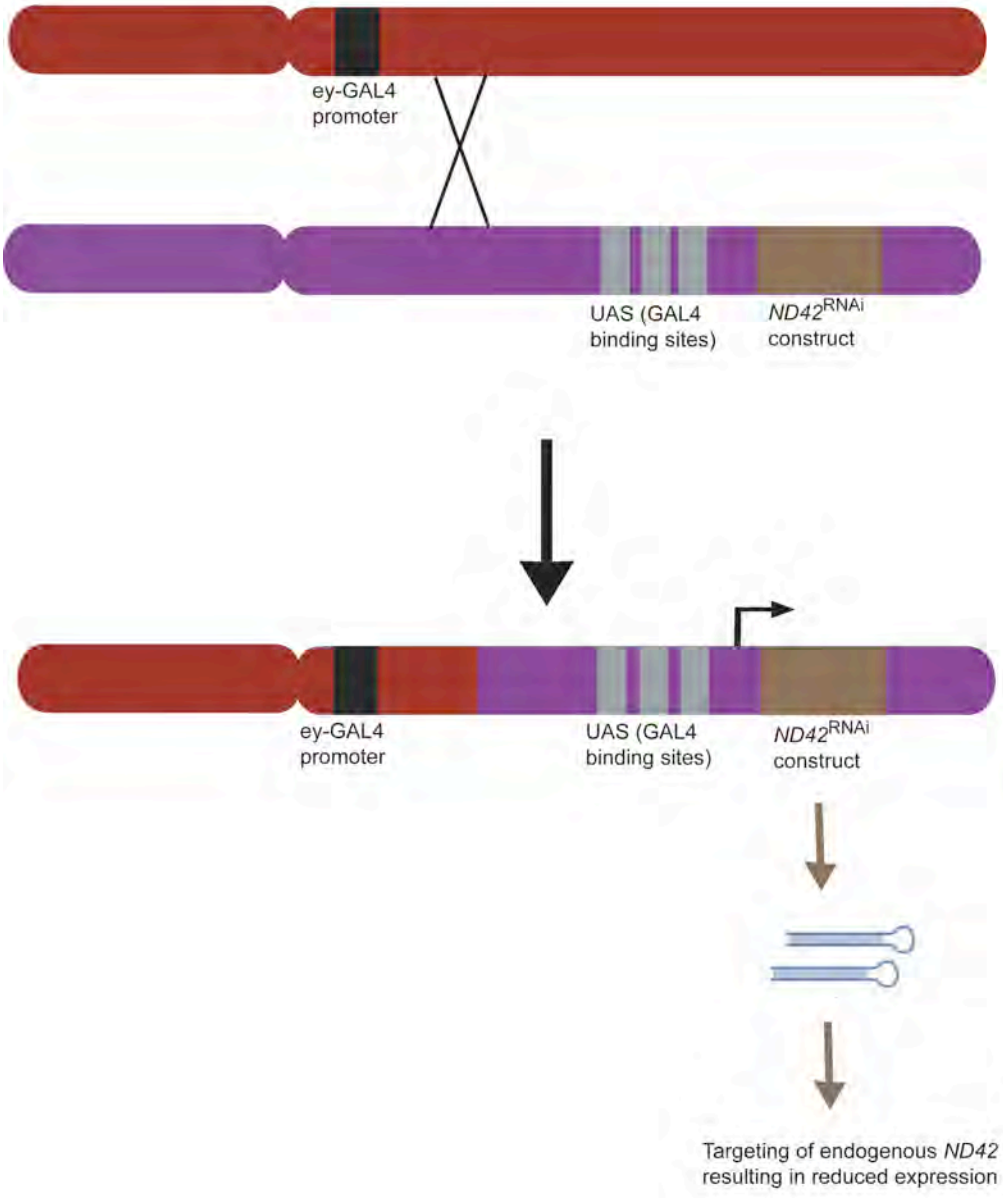


Figure 4.2 Generation of the *ey > ND42^{RNAi}* recombinant screening line that carries both the eyeless-GAL4 (ey-GAL4) promoter and an RNAi construct targeting *ND42*. Recombination between the two homologous chromosomes results in production of a recombinant chromosome that carries both the ey-GAL4 promoter and the RNAi construct targeting *ND42* downstream of Upstream Activator Sequences (UAS, which are GAL4-binding sites). GAL4 protein, which is produced under the control of eyeless promoter in cells of the developing *Drosophila* eye, binds to the UAS sites and drives expression of the *ND42*^{RNAi} construct. Flies that carry this recombinant chromosome will therefore have reduced expression of ND42 and this stock of flies was used to screen for candidate genes that can modify its larval outgrowth phenotype (see Figure 4.4). The loci presented in this figure are arbitrary as the exact locations of both constructs are unknown.

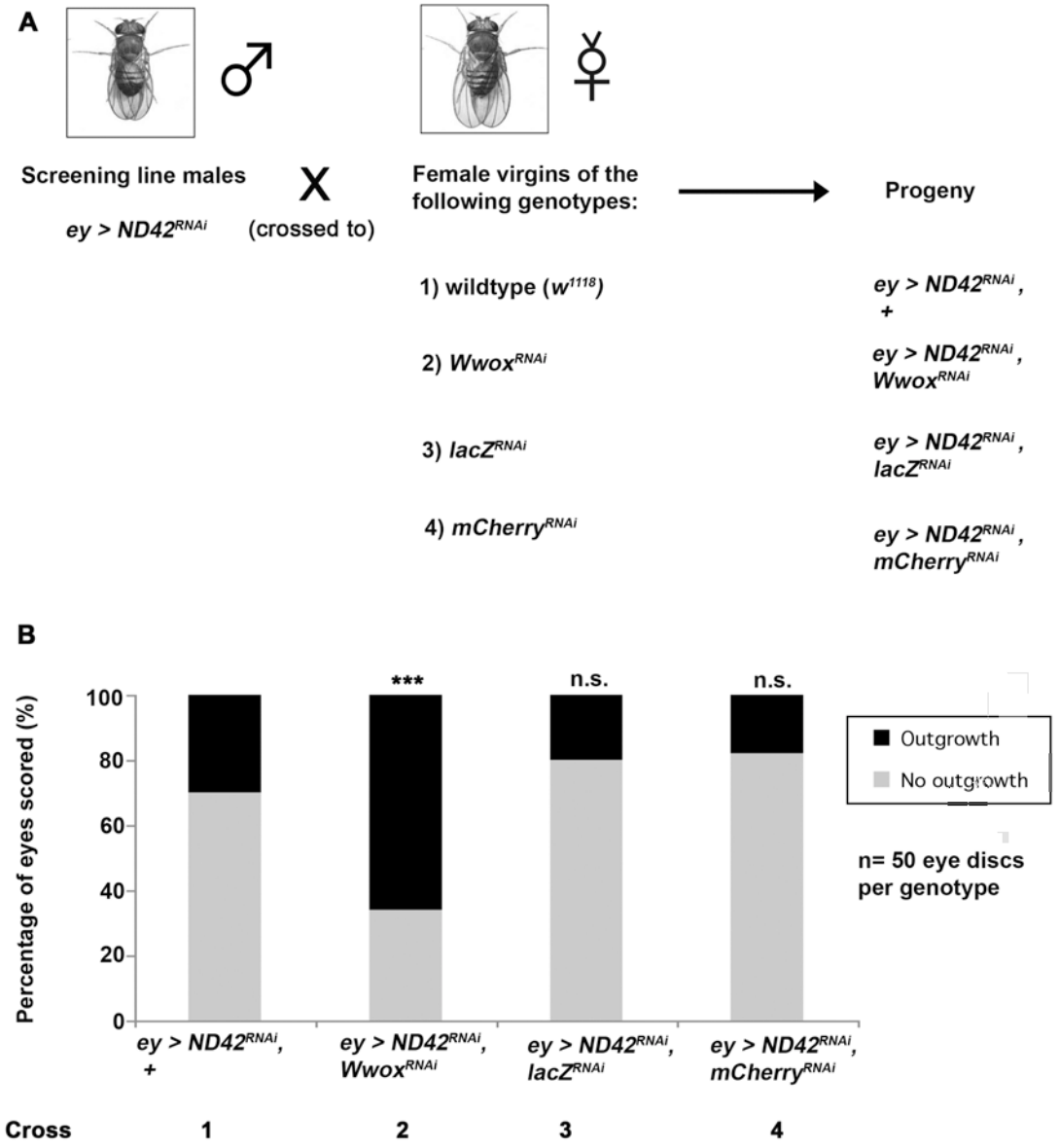


Figure 4.3 Verification of the larval eye disc outgrowth phenotype with the *ey > ND42^{RNAi}* screening line. (A) Crosses were set up to compare the larval eye disc outgrowth phenotype among the following genotypes: reduced *ND42* expression with (1) a wild-type background, (2) reduced Wwox levels, (3-4) non-specific activation of the RNAi machinery through targeting of non-*Drosophila* genes such as (3) lacZ and (4) mCherry. Male flies of the *ey > ND42^{RNAi}* screening line were crossed to female virgins of those genotypes and the progeny were assayed for the outgrowth phenotype. **(B)** Presence of the outgrowth phenotype was detected in progeny from the crosses using the *ey > ND42^{RNAi}* screening line. There was no significant difference in the proportion of the outgrowth phenotype when comparing the wild-type background (1) to the backgrounds with non-specific activation of the RNAi machinery (3 & 4) but higher proportion of the outgrowth phenotype was observed when Wwox levels were reduced (2), *** p=0.0006 (Appendix C: Table C1). n= 50 eye discs per genotype.

Once the screening line had been established and validated, it was then used to test for modifiers that can affect the larval eye disc outgrowth phenotype. Flies from the screening line that carry the recombinant chromosome were crossed to flies carrying either an RNAi construct targeting a candidate gene or a transgene encoding the cDNA of a candidate gene (Figure 4.4). The progeny from that cross contained (and thus expressed) both the recombinant chromosome and the candidate gene construct. The proportion of the outgrowth phenotype observed in those progeny was compared to that of the progeny from the control cross, which carried only the recombinant chromosome. Any significant difference in the proportion of the outgrowth phenotype is indicative of a modification by that candidate gene, thus identifying the candidate gene as a modifier or effector of the phenotype.

It should be noted that the GAL4-UAS system is temperature-sensitive; hence variation in the severity of the phenotypes observed between different experiments is expected due to slight uncontrollable fluctuations in temperature (as well as other environmental factors) between experiments. Flies with decreased Wwox expression were used as a positive control for the assay in all experiments as reduced Wwox expression has been shown to result in significantly higher proportion of the outgrowth phenotype (Chapter 3).

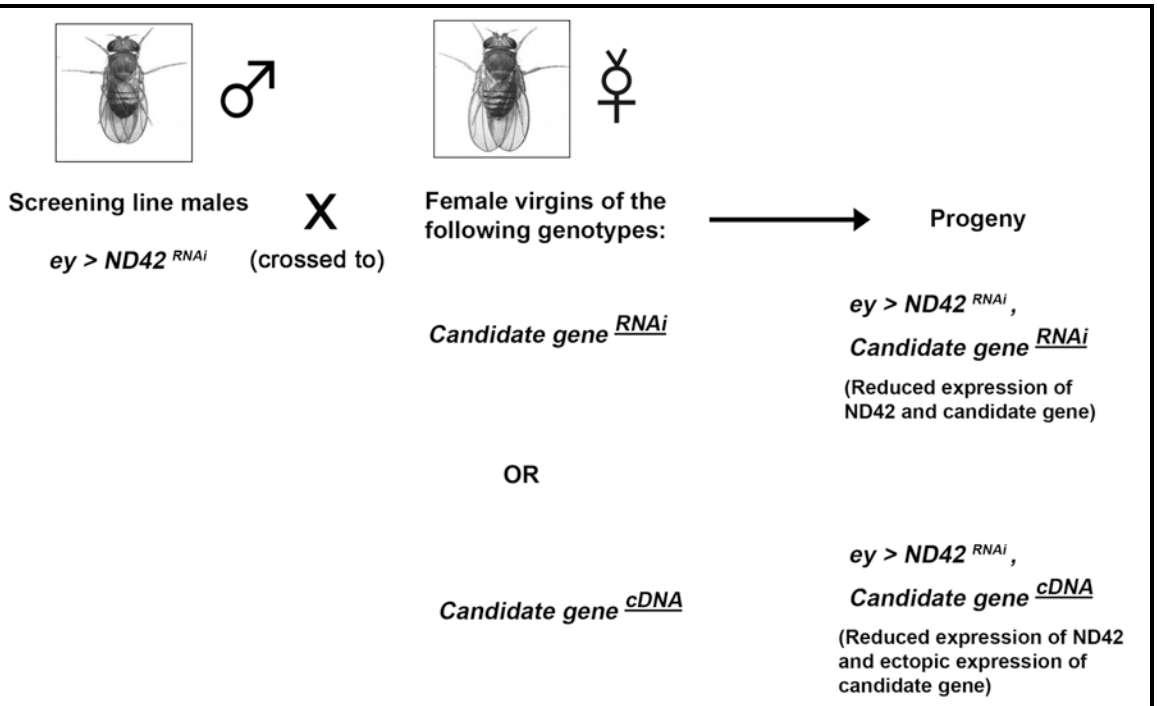


Figure 4.4 Model of the screen performed to identify modifiers of the larval eye disc outgrowth phenotype. Males of the *ey > ND42*^{RNAi} screening line were crossed to female virgins carrying either an RNAi construct targeting the candidate gene or a construct expressing the cDNA of the candidate gene. The progeny would have reduced expression of *ND42* and the candidate gene or reduced expression of *ND42* and ectopic expression of the candidate gene respectively. The progeny were assayed for the larval eye disc outgrowth phenotype and the proportions of the phenotype were compared to an appropriate control (lacZ^{RNAi} for the RNAi lines and empty vector (EV) for the ectopic expression lines). Statistical significance was determined by Fisher's test analysis to identify the genes that are able to modify the outgrowth phenotype.

4.3.2 Reactive oxygen species are effectors of the cellular dysfunction caused by mitochondrial defects

Mitochondria are known sources of reactive oxygen species (ROS) in cells and inhibition of the mitochondrial respiratory chain has been shown to result in increased levels of ROS (Kowaltowski *et al.*, 2009; Sharma *et al.*, 2011). Targeting of the Complex I gene, *ND42* by RNAi in the posterior region of the *Drosophila* wing disc was found to produce ROS that was detectable by CellRox® staining (Chapter 6 - Shaukat *et al.*, 2014). High levels of ROS produced by mitochondrial dysfunction have been shown to induce damage to cellular components and affect signalling pathways (Marchi *et al.*, 2012). In addition, increased production of ROS by mitochondrial dysfunction has been demonstrated to promote tumour progression and metastasis of cancer cells (Ishikawa *et al.*, 2008; Pelicano *et al.*, 2009; Sharma *et al.*, 2011; Taddei *et al.*, 2012). Evidently the high levels of ROS induced by mitochondrial dysfunction have very negative effects on the cell. Thus it was proposed that decreased expression of these mitochondrial respiratory chain genes would result in high levels of ROS, resulting in cellular dysfunction and subsequently the outgrowth phenotype in the *Drosophila* larval eye discs. Furthermore, Wwox has been shown to be involved in the regulation of ROS (O'Keefe *et al.*, 2011; Chapter 6- Shaukat *et al.*, 2014). Loss of Wwox expression in whole *Drosophila* larvae was found to result in overall decreased ROS levels (O'Keefe *et al.*, 2011), however the ROS that were measured in that assay were in the context of the whole organism and the effect of Wwox on ROS was undetermined at the cellular level in specific tissues. Further work was performed to examine the effect of Wwox on ROS in the *Drosophila* wing disc and found that decreased expression of Wwox in a sensitised background (in flies with chromosomal instability) resulted in high levels of ROS (Chapter 6 - Shaukat *et al.*, 2014). Although it is still unclear how decreased expression of Wwox result in an effect on ROS or the context in which this occurs, it appears that altering Wwox expression can affect the level of ROS in cells. As both mitochondrial dysfunction and altered Wwox levels can affect ROS levels, ROS were examined as candidate effectors of the cellular dysfunction and resultant outgrowth phenotype in the *Drosophila* larval eye imaginal disc.

In order to determine if ROS could be driving the outgrowth phenotype in the *Drosophila* larval eye imaginal disc, CellRox® staining was first performed to determine if there are detectable levels of ROS in discs that contain the outgrowth. The CellRox® Deep Red Reagent is a fluorescence probe designed for detection of oxidative stress in live cells. The cell-permeable dye is introduced into cells in a reduced state that is non-fluorescent

and is oxidised into a fluorescence state by ROS present in the cells, which can then be detected by fluorescence microscopy. Punctuate fluorescent staining was detected in the outgrowth region of the eye discs, indicating that ROS are present in the outgrowth (Figure 4.5, Appendix C: Figure C2). Whilst the staining appeared to only be detected in the outgrowth region of the outgrowth-containing eye discs, it is likely that there are ROS present in the entire disc but may only be detectable using a more sensitive stain or method. Nevertheless, the result suggest that ROS were present in high levels that were sufficient to be detected by CellRox® staining in the outgrowth region and so further experiments were then carried out to determine if the detected ROS are causes of the outgrowth phenotype or are merely byproducts of mitochondrial dysfunction.

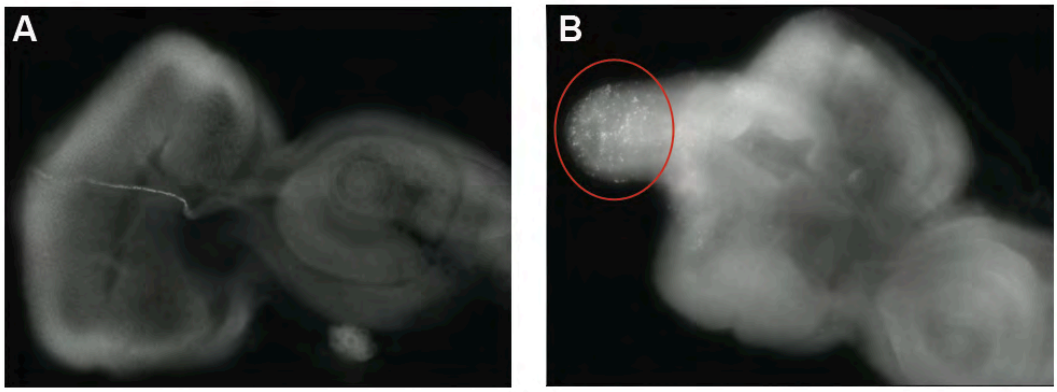


Figure 4.5. Presence of ROS in the outgrowth region of the eye imaginal disc in third instar larvae as detected by CellRox® staining. (A) No punctuate staining was detected in wild-type eye disc. Staining of the wild-type eye disc provides an indication of the level of background staining. **(B)** Punctuate staining corresponding to presence of ROS was observed in the outgrowth region of the eye disc (circled in red). Additional images are provided in Appendix C: Figure C2.

Mitochondrial ROS are important molecules that act as signalling messengers at physiological levels (Kowaltowski *et al.*, 2009; Marchi *et al.*, 2012). The majority of the mitochondrial ROS are produced by the mitochondrial respiratory chain, with the primary ROS being superoxide anions ($O_2^{\cdot-}$). Superoxide anions are highly reactive and are often converted to hydrogen peroxide (H_2O_2), a more stable form of ROS, by superoxide dismutases (SOD). There are two SODs located within the cell – copper/zinc dismutase (Cu-ZnSOD/SOD1), which is found in the cytoplasm, nucleus and mitochondrial intermembrane space (IMS) and manganese dismutase (MnSOD/SOD2) located in the

mitochondrial matrix. As superoxide anions are unable to diffuse across cell membranes, SOD1 and SOD2 have been proposed to be essential for controlling mitochondrial ROS levels in the IMS and mitochondrial matrix respectively by conversion of the superoxide anions to H_2O_2 (Papa *et al.*, 2014). H_2O_2, which is membrane permeable, can diffuse within the cell and act as signalling molecules in a number of essential cellular processes, including cell cycle and stress responses (Starkov, 2010). The physiological level of H_2O_2 is normally kept in check by antioxidant enzymes such as catalase, gluthathione peroxidase and thioreductase, which metabolises H_2O_2 to water and oxygen molecules. Ectopic expression of Sod1, Sod2 or Catalase individually in *Drosophila* have been shown to reduce ROS levels and confer resistance to oxidative stress (Orr *et al.*, 1994; Sohal *et al.*, 1995; Wang *et al.*, 2012b), hence these antioxidant enzymes were used to test if ROS are effectors of the outgrowth phenotype.

Transgenes of Sod1, Sod2 or Catalase were expressed individually in larvae that carry the recombinant *ey > ND42*^{RNAi} screening chromosome (as described in Figure 4.4) to determine if increased expression of these antioxidant enzymes are able to modify the proportions of the larval eye disc outgrowth phenotype. Increased expression of Catalase, Sod1 or Sod2 was found to result in a significant suppression of the phenotype, with lower proportion of eye discs detected with the outgrowth phenotype (Figure 4.6A, Appendix C: Figure C3, Table C2). On the other hand, decreased expression of Sod1 or Sod2 by RNAi targeting was found to enhance the outgrowth phenotype, with higher proportion of larvae displaying the outgrowth phenotype (Figure 4.6B, Appendix C: Figure C4, Table C3). Decreased expression of Catalase by RNAi targeting was not examined as the RNAi construct targeting Catalase was balanced over a CyO balancer that was not distinguishable at the larval stage, hence those flies could not be used for any larval analyses. Increased or decreased expression of the antioxidant enzymes themselves did not result in any phenotype (Appendix C: Figure C3, C4), thus the effect of the antioxidants on the *ey > ND42*^{RNAi} outgrowth phenotype is not additive. Taken together, these results demonstrate that ROS are effectors of the outgrowth phenotype as reduction of ROS levels by ectopic expression of these antioxidant enzymes resulted in a decrease in the proportions of the outgrowth phenotype whilst increasing ROS levels led to an increase in the proportions.

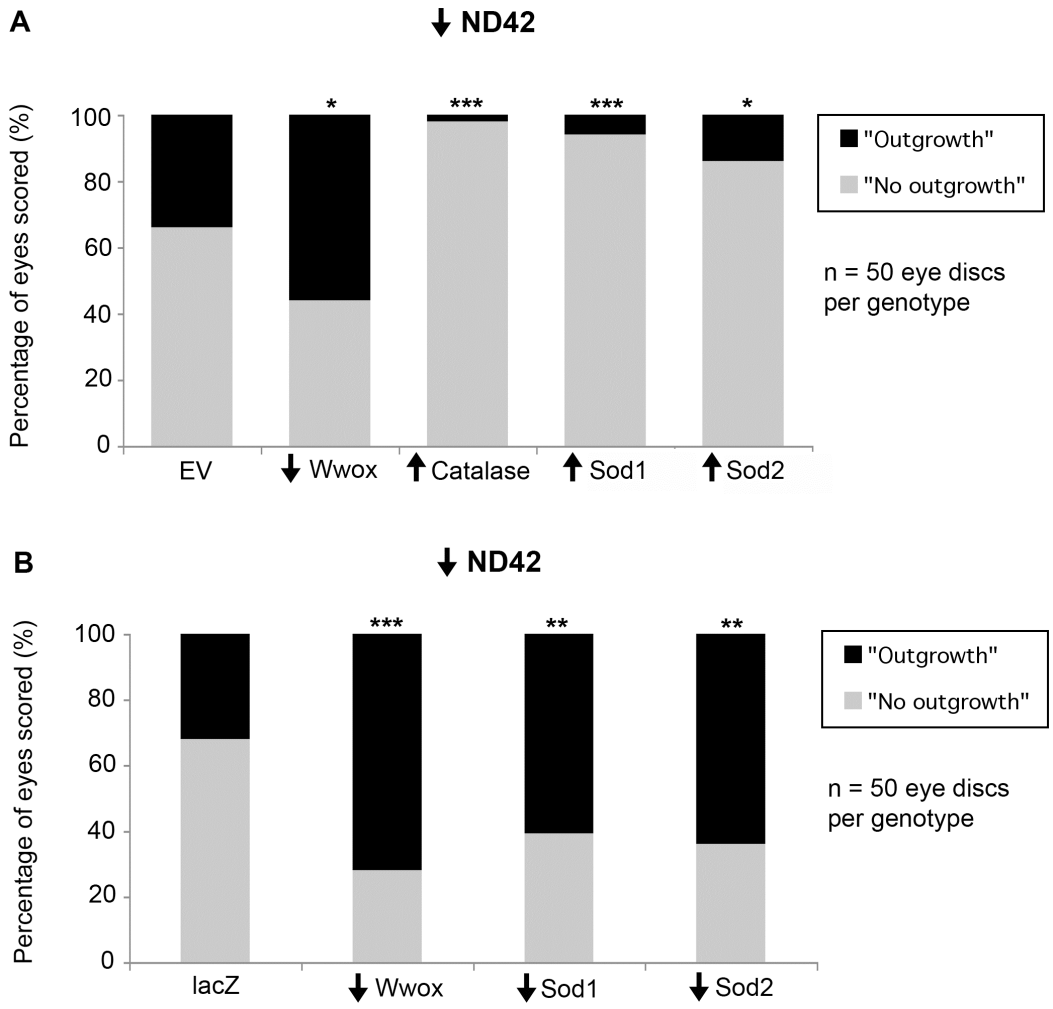


Figure 4.6 ROS are effectors of the larval eye disc outgrowth phenotype. (A) Ectopic expression of the antioxidant enzymes Catalase, Sod1 or Sod2 resulted in suppression of the larval eye disc outgrowth phenotype, with lower proportions of the phenotype present (2%, 6% and 14% respectively) compared to the *ey > ND42^{RNAi} > EV* flies (34%). The *ey > ND42^{RNAi} > EV* flies carried an empty vector (EV) as a control for ectopic expression lines. *p<0.05, ***p<0.001 when compared to the EV control, exact p values in Appendix C: Table C2. **(B)** Reduced expression of *Sod1* or *Sod2* by RNAi targeting resulted in enhancement of the phenotype, with higher proportions observed (61% and 64% respectively) compared to the *ey > ND42^{RNAi} > lacZ^{RNAi}* flies (32%). The *ey > ND42^{RNAi} > lacZ^{RNAi}* flies were used as a control for RNAi lines as there is non-specific activation of the RNAi machinery in those flies. **p<0.01, ***p<0.001 when compared to the lacZ^{RNAi} control, exact p values in Appendix C: Table C3. The *ey > ND42^{RNAi} > Wwox^{RNAi}* flies were used as a positive control for the assay in both experiments as reduced expression of *Wwox* by RNAi targeting has been shown to result in enhancement of the phenotype. n =50 eye discs per genotype for both experiments. Replicate results in Appendix Figure C: C3 and C4.

As ROS were found to be able to drive the outgrowth phenotype, further experiments were carried out to determine if inducing high levels of ROS by itself (without first inducing mitochondrial dysfunction through targeting of the mitochondrial respiratory complexes) was able to result in cellular dysfunction and produce similar phenotypes. Decreased expression of Sod1, Sod2 or Catalase individually (by RNAi targeting using the ey-GAL4 driver) did not have any effect in the adult eye (Chapter 3) or in larval eye discs (Appendix C: Figure C4) when performed at 25ºC, which was the standard temperature used for all crosses. The GAL4-UAS system is known to be temperature sensitive, with higher temperatures resulting in increased GAL4 activity (Duffy, 2002); thus the crosses were repeated at 29ºC to achieve greater levels of RNAi targeting of those genes. Phenotypes that were similar to those induced by mitochondrial defects were observed in the adult eye, albeit at low proportions and not as severe, when expression of Sod1 or Catalase was reduced (Figure 4.7A-B). Similarly, when larval eye discs with reduced Sod1 or Sod2 expression were examined, protrusions (alike those caused by mitochondrial dysfunction) were detected in 2-4% of discs (Figure 4.7C).

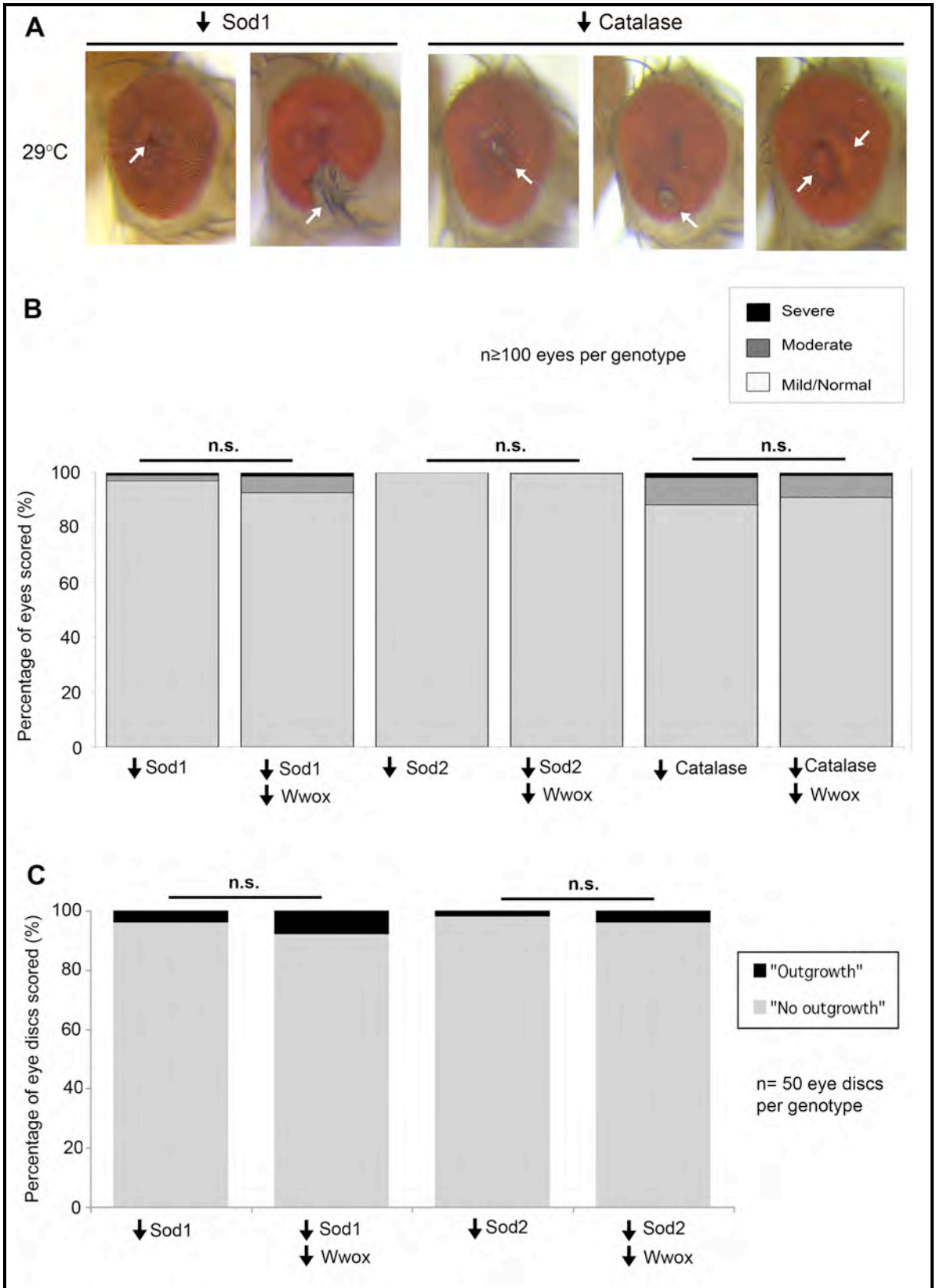


Figure 4.7 Increased ROS levels resulted in similar phenotypes to those caused by mitochondrial dysfunction in the *Drosophila* adult eye and larval eye discs. (A) Disruption to the eye tissue was observed in the adult eye when Sod1 or Catalase expression was decreased at 29°C. The phenotypes include loss of photoreceptor cells resulting in a cavity in the eye (panel 1 and 3), presence of ectopic structures (panel 2 and

4), as well as slight outgrowth of eye tissue (panel 5). **(B)** Quantification of the eyes that display such tissue disruption. The proportions of eyes with reduced Sod1 or Catalase expression that exhibited any phenotypes were low (3% and 12% respectively) and the proportions did not increase significantly with reduced Wwox expression (p=0.0988 and p=0.7811 respectively). No obvious tissue disruption was observed with reduced SOD2 expression (with normal or low Wwox levels) out of the 295 and 267 eyes examined respectively (p=0.4751**)**. **(C)** Quantification of the outgrowth phenotype observed in larval eye discs. The eye disc outgrowth phenotype was observed at low proportions in larvae with reduced Sod1 or Sod2 expression (4% and 2% respectively) and there was no significance difference in the proportion of the phenotype when there were normal or low Wwox levels (p=0.6777 and p=1.000 respectively).

The observation of similar phenotypes by just decreasing expression of these antioxidant enzymes alone further support that increased levels of ROS are able to contribute to the generation of such phenotypes. As ROS are able to act as signalling molecules, it is likely that such increases in ROS levels would result in aberrant activation of various pathways, leading to the production of such phenotypes. The lower proportions of phenotype observed with decreased expression of the antioxidant enzymes at the high temperature compared to that seen with induction of mitochondrial defects at a low temperature suggest that there are higher ROS levels being produced by inhibition of the mitochondrial respiratory chain. Interestingly, decreased expression of Wwox did not appear to result in higher proportion of these phenotypes when caused by decreased expression of these antioxidant enzymes (Figure 4.7B-C). This suggests that the contribution of Wwox to cellular dysfunction may only be apparent in the context of mitochondrial defects or when ROS levels are increased beyond a particular threshold whereby there is a certain level of cellular dysfunction already present. The latter seems to be supported by clonal analyses studies where loss of mitochondrial function in clones of the developing *Drosophila* eye did not result in any phenotype, even in the absence of Wwox in the clones or in the entire eye (Appendix C: Figure C5), suggesting that loss of Wwox only has a negative effect in the developing *Drosophila* eye when there is a sufficient baseline level of mitochondrial dysfunction resulting in high levels of ROS.

4.3.3 Involvement of Akt signalling in the mitochondrial defect-mediated cellular dysfunction

Mitochondrial dysfunction and high levels of ROS production have been shown to affect various signalling pathways leading to pathological conditions such as tumourigenesis, neurodegeneration and inflammatory diseases (Hamanaka and Chandel, 2010). Several recent studies in particular have shown that inhibition of the mitochondrial respiratory complex result in increased phosphorylation of the *v-AKT* murine thymoma viral oncogene (AKT) and subsequently, activation of AKT-mediated signalling pathways, leading to malignancy (Pelicano *et al.*, 2006; Sharma *et al.*, 2011; Santidrian *et al.*, 2013). AKT proteins, which are also known as protein kinase B (PKB), are serine-threonine kinases that are activated upon phosphorylation in response to various cellular stimuli and can modulate the function of a number of other proteins involved in glucose metabolism, protein synthesis as well as cell growth, proliferation and survival (Brazil and Hemmings, 2001; Manning and Cantley, 2007). Aberrant AKT activation has been reported in numerous cancers and has been described as one of the most common molecular changes in tumour cells (Bellacosa *et al.*, 2005).

As it has been shown that mitochondrial respiratory Complex I dsyfunction results in high levels of mitochondrial ROS and promotes tumourigenesis through AKT activation (Sharma *et al.*, 2011), experiments were performed to determine if the larval eye disc outgrowth phenotype (resulting from Complex I dysfunction and in which ROS has been shown to be an effector) could be caused by aberrant AKT signalling. Whilst there are three known AKT proteins in humans, there is only one identified AKT orthologue (Akt) in *Drosophila.* Activation of the *Drosophila* Akt, through phosphorylation of its serine residue (S) at amino acid 505 (which corresponds to the essential amino acid S473 in mammals), has been shown to be required for cell growth and survival (Scanga *et al.*, 2000). A monoclonal antibody specific for phosphorylated Akt (pAkt) at that serine residue (human pAKT-S473/*Drosophila* pAkt-S505) was used to detect for aberrant activation of Akt signalling in the larval eye disc outgrowth phenotype (Figure 4.8A). Eye discs that are ectopically expressing Dp110 (the *Drosophila* orthologue of phosphatidylinositol-4,5-bisphosphate 3-kinase, Pi3K92E) using the ey-GAL4 driver, were used as a positive control for pAkt staining as high levels of Dp110 have been shown to result in increased Akt phosphorylation and activity (Scanga *et al.*, 2000). Detectable pAkt staining was observed in the Dp110-overexpressing discs localised around individual ommatidia (Figure 4.8A, second row panels) compared to the weaker staining in wild-type

eye discs corresponding to low levels of endogenous pAkt (Figure 4.8A, first row panels). pAkt staining was also detectable in the affected discs (Figure 4.8A, third row panels), suggesting that there is elevated Akt activation in the eye discs that have mitochondrial dysfunction.

The observation of elevated pAkt staining in the eye discs with the outgrowth phenotype suggested that aberrant Akt signalling could be responsible for causing the phenotype. In order to test this, Akt levels were decreased by RNAi targeting to examine if reduction in Akt is able to rescue the outgrowth phenotype. Use of this particular RNAi line to target Akt has previously been shown to strongly reduce Akt protein levels as well as pAkt function (Ye *et al.*, 2012). Reduction of Akt alone did not result in any phenotype in the larval eye disc (Appendix C: Figure C6) but it was found to significantly suppress the outgrowth phenotype caused by decreased expression of ND42, with a lower proportion of eye discs exhibiting the phenotype compared to the corresponding control (Figure 4.8B, Appendix C: Figure C6). This result supports the involvement of aberrant Akt signalling in driving the outgrowth phenotype.

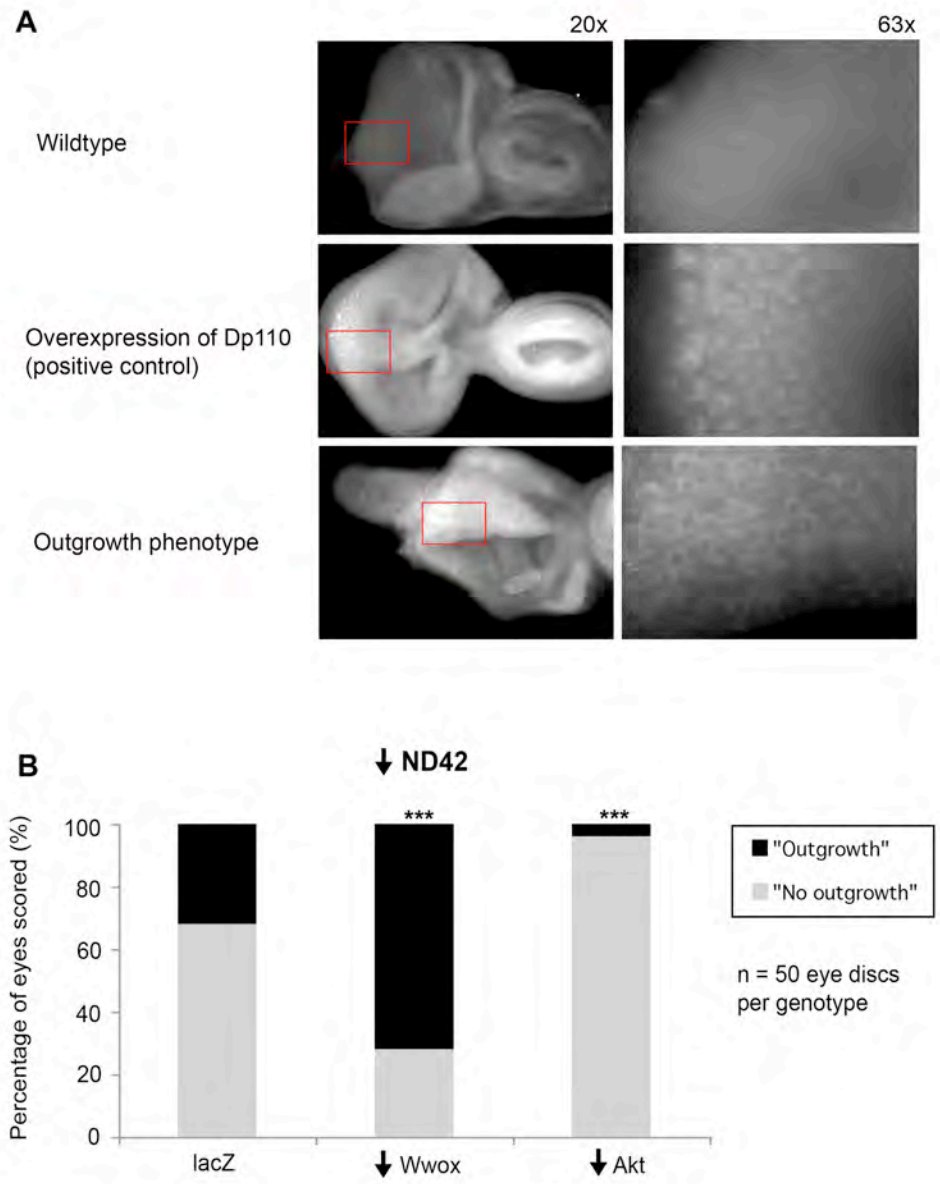


Figure 4.8. Contribution of Akt signalling to the larval eye disc outgrowth phenotype induced by mitochondrial dysfunction. (A) Detection of increased pAkt staining in third instar larval eye imaginal discs. Low levels of pAkt staining were present in the wild-type eye disc whilst increased pAkt staining 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 20x PlanApo objective whilst panels on the left were taken using the 63x PlanApo objective. The 63x images were taken of the area highlighted with the red boxes in the 20x 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 to the lacZ control (34%) (***p=0.0004). n=50 eyes per genotype. Replicate results in Appendix Figure C: C6, Table C3.

4.3.4 Decreased expression of *Drosophila HIF1-∝* and autophagy gene *Atg18* have parallel effects on mitochondrial-mediated cellular dysfunction

One of the frequently studied downstream targets of AKT signalling is the hypoxia-inducible factor 1 alpha (HIF-1∝). Activated AKT results in an increase in HIF-1∝ protein levels, potentially through indirect stabilization of the HIF-1∝ protein (Zundel *et al.*, 2000; Jiang *et al.*, 2001). HIF-1∝ is a transcription factor that is normally unstable under normoxic conditions but is involved in the cellular response to hypoxic stress through the regulation of numerous genes implicated in metabolic adaptation, cell growth, differentiation and survival (Rankin and Giaccia, 2008; Weidemann and Johnson, 2008). Overexpression of HIF-1∝ has been reported in various cancers (Mabjeesh and Amir, 2007), with the role of HIF-1∝ in tumourigenesis being of considerable interest as tumours are often thought to be in a hypoxic state. Most studies are indicative of activated HIF-1∝ having a pro-tumorigenic function, with the overexpression of HIF-1∝ promoting angiogenesis and metastasis of cancer cells, resulting in poor prognosis for patients (Semenza, 2002). Intriguingly, there are a few studies suggestive of HIF-1∝ having the potential to negatively regulate tumour growth through induction of apoptosis and/or autophagy (Sowter *et al.*, 2001; Rankin and Giaccia, 2008; Chiavarina *et al.*, 2010). These studies highlight the possibility of contrasting roles for HIF-1∝ in different cancer cell types and in the presence of other genetic alterations as well as at different stages of tumour progression. It has been proposed that some of the differences could be attributed to induction of autophagy by HIF-1∝. HIF-1∝ is thought to induce autophagy as an adaptive response to mediate tumour cell survival in the initial stages of tumourigenesis, however prolonged autophagy can result in cell death (Rankin and Giaccia, 2008; Yang *et al.*, 2011). In addition, activation of HIF-1∝ in cancer-associated fibroblasts has been shown to promote tumour growth through induction of autophagy but results in suppression of tumour growth when induced directly in epithelial cancer cells (Chiavarina *et al.*, 2010).

WWOX has previously been linked to HIF-1∝ (Abu-Remaileh and Aqeilan, 2014; Dayan *et al.*, 2013; Dayan *et al.*, unpublished). WWOX was shown to physically bind to HIF-1∝ in HEK293 cells and decrease HIF-1∝ activity in mouse embryonic fibroblasts (MEFs) (Abu-Remaileh and Aqeilan, 2014). It was proposed that under normoxic conditions, WWOX inhibits HIF-1∝ activity to suppress glycolysis and promote oxidative phosphorylation, a pathway that involves the mitochondria (Abu-Remaileh and Aqeilan,

2014) whilst in hypoxic conditions, WWOX levels are downregulated (Dayan *et al.*, 2013, Abu-Remaileh, 2014). In addition to the negative regulation of HIF-1∝ activity by WWOX, HIF-1∝ is in turn able to inversely regulate WWOX expression in human cells, indicative of a reciprocal relationship between WWOX and HIF-1∝ (Dayan *et al.*, unpublished). Whilst there has been no genetic interaction detected between Wwox and HIF-1∝ in *Drosophila* thus far (Cheng Shoou Lee, personal communication), the identified relationship between the two proteins in mammalian cells suggest that HIF-1∝ could have a contribution to Wwox-mediated processes, such as the cellular response to mitochondrial defects. Indeed, increased levels of HIF-1∝ have been observed in response to mitochondrial dysfunction (often with increased AKT signalling) (Freije *et al.*, 2012; Ma *et al.*, 2013). On this basis, decreased expression of Similar (Sima), the *Drosophila* orthologue of HIF-1∝, as well as its dimerisation partner, HIF-1ß/Arnt (Tango in *Drosophila*) were induced to determine if the HIFs have any contribution to the mitochondrial-mediated larval eye disc outgrowth phenotype (Figure 4.9, Appendix C: Figure C7).

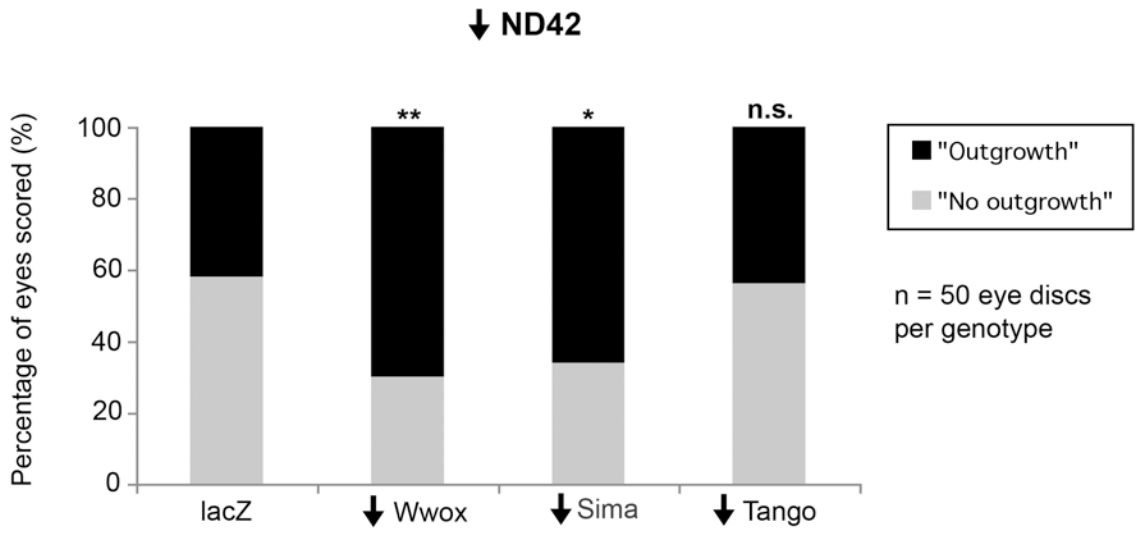


Figure 4.9 Decreased expression of Sima, but not Tango, results in enhancement of the larval eye disc outgrowth phenotype. Reduced Sima expression by RNAi targeting results in higher proportion of the outgrowth phenotype (66%) compared to the lacZ control (42%) (*p=0.0268) whilst reduced expression of Tango did not lead to any significant change in the proportion of the phenotype (44%, p=0.2299 n.s.). The result obtained with decreased Sima is parallel with that obtained with decreased Wwox. n=50 eyes per genotype. Replicate results in Appendix C: Figure C7, Table C3.

Unexpectedly, decreased expression of Sima resulted in a significant increase in the proportion of the outgrowth phenotype, parallel to what was observed with decreased expression of Wwox (Figure 4.9, Appendix C: Figure C7). On the other hand, decreased expression of HIF-1β /Arnt orthologue, Tango (Tgo) did not result in any significant change. It should be noted that decreased expression of either Sima or Tgo alone did not result in any phenotype (Appendix C: Figure C7); hence the enhancement by reduced Sima is not due to an additive effect. This would suggest that loss of Sima, but not Tgo, has a negative impact on cellular homeostasis when there is mitochondrial dysfunction, which may be a possibility as Sima has been shown to be involved in estrogen-related receptor (dERR)-mediated pathways independent of Tgo (Li *et al.*, 2013). Interestingly, WWOX has also been proposed to have a role in dERR-associated pathways as loss of WWOX expression has been found to correlate with lack of estrogen receptor status in breast cancer cells (Nunez *et al.*, 2005a). However it is also possible that the lack of modification by Tgo is due to insufficient reduction of Tgo expression using RNAi targeting such that its function is not disrupted, hence the involvement of Tgo in the mitochondrial dysfunction-induced phenotype cannot be ruled out as of yet.

Of particular interest are recent studies that have demonstrated that Sima is a negative regulator of cell growth (Romero *et al.*, 2007) and that overexpression of Sima leads to induction of autophagy (Löw *et al.*, 2013). Autophagy has been shown to be a protective mechanism in restricting metastatic growth caused by mitochondrial complex I defects (Santidrian *et al.*, 2013), hence experiments were performed to determine if autophagy could be a mechanism to restrict the larval eye disc outgrowth. Expression of an essential autophagy gene, *Atg18* was decreased by RNAi targeting to ascertain if reduced autophagic activity can result in enhancement of the outgrowth phenotype (Figure 4.10, Appendix C: Figure C8, Table C3). Decreased autophagy resulted in higher proportions of larval eye disc outgrowth phenotype compared to the corresponding control, indicating that autophagy is able to restrict this cellular dysfunction caused by mitochondrial defects.

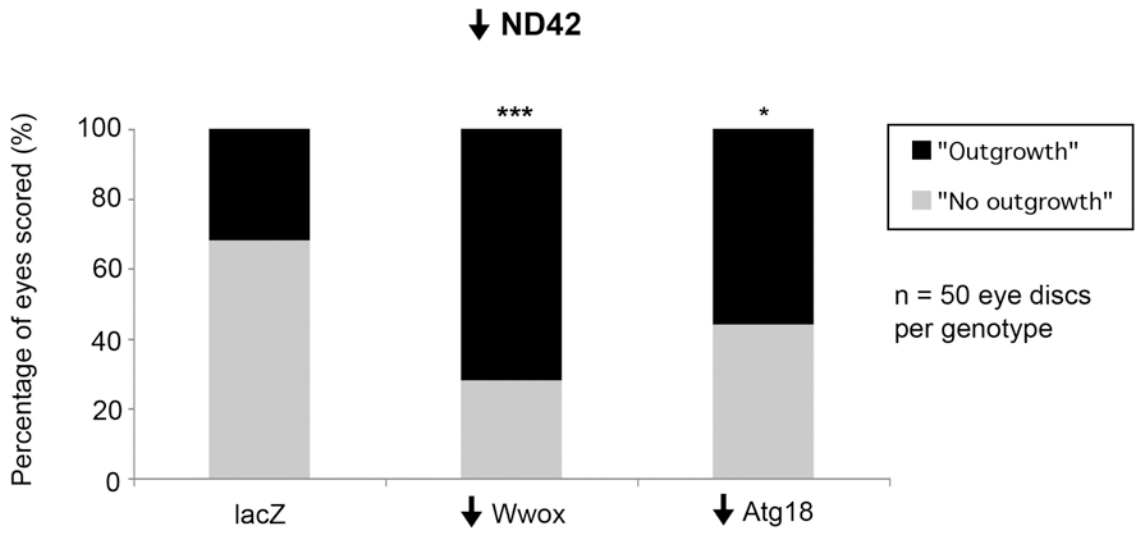


Figure 4.10 Reduced autophagic activity results in enhancement of the cellular dysfunction caused by mitochondrial defects. Decreasing expression of an autophagy gene, *Atg18*, leads to a significantly higher proportion of larval eye disc outgrowth phenotype (56%) compared to the lacZ control (32%), *p=0.0262. n=50 eye discs per genotype. Further analyses in Appendix C: Figure C8, Table C3.

4.3.5 Increased Forkhead box O (Foxo) expression suppresses the cellular dysfunction caused by mitochondrial defects

Another well-known target of AKT signalling in both mammals and *Drosophila* is the forkhead box O (FOXO) transcription factor (Jünger *et al.*, 2003; Kramer *et al.*, 2003; Greer and Brunet, 2005; Calnan and Brunet, 2008). FOXO transcription factors are able to function as tumour suppressors 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 signalling results in phosphorylation of FOXO in response to cellular stimuli, such as insulin as well as oxidative stress (Vurusaner *et al.*, 2012). Phosphorylation of FOXO causes it to be retained in the cytoplasm instead of translocating into the nucleus to activate transcription of its target genes, thus inhibiting its tumour suppressive function. AKT-mediated inhibition of FOXO activity has been reported in various cancers and has been thought to be a contributing factor to tumour progression (Greer and Brunet, 2005).

Whilst there are at least four different FOXO proteins in mammals, there is only one FOXO orthologue in invertebrates, including *Drosophila*. The *Drosophila* Foxo 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). As activated Akt signalling is thought to contribute to the larval eye disc outgrowth phenotype, inhibition of Foxo activity could be a mechanism for causing such dysfunction. Thus, overexpression of Foxo was tested to ascertain if it could rescue the cellular dysfunction caused by decreased ND42 levels (Figure 4.11). The fly line used to overexpress Foxo has previously been shown to result in higher levels of Foxo that is phosphorylated by Akt in the presence of insulin (Puig *et al.*, 2003). Overexpression of Foxo by itself did not result in any obvious morphological phenotype in the larval eye discs, although the adult flies appeared to have smaller eyes. However, when Foxo was overexpressed in the larval eye discs with decreased levels of ND42, it was able to suppress the larval eye disc outgrowth phenotype, with a lower proportion of eye discs displaying that phenotype (Figure 4.11, Appendix C: Figure C9). Thus, it appears that increasing Foxo activity is able to restrict the mitochondrial defect-mediated cellular dysfunction, suggesting that Foxo activity has a protective or compensatory effect.

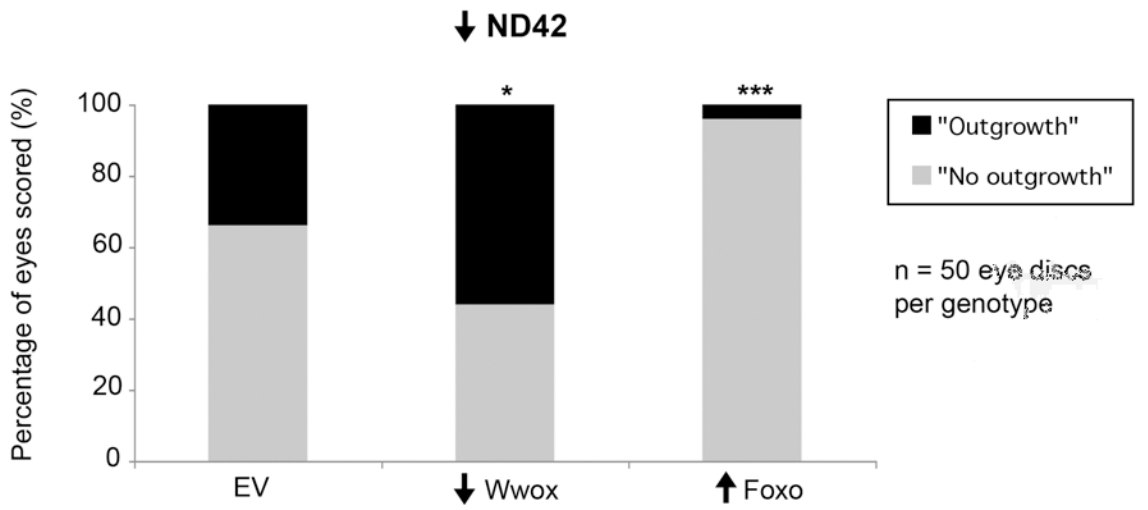


Figure 4.11 Larval eye disc outgrowth phenotype suppressed by increased levels of Foxo. Ectopic expression of Foxo results in lower proportion of the outgrowth phenotype caused by decreased levels of ND42 (4%) compared to the empty vector (EV) control (34%), ***p=0.0002 n=50 eye discs per genotype. Further analyses in Appendix C: Figure C9, Table C2.

4.4 Summary of results and discussion

A targeted analysis was undertaken to identify other genetic interactors that can modulate the cellular dysfunction caused by mitochondrial defects in the developing *Drosophila* eye. As Wwox has previously been shown to be able to modify those phenotypes, the aim of this work is to identify other genetic contributors that can provide better understanding of the pathways affected.

The larval eye disc outgrowth phenotype was chosen as the main assay in which the targeted analysis was conducted as changes at the cellular level could be observed. The phenotype was used as a readout of the cellular dysfunction caused by mitochondrial defects and the central focus of the analyses was the modification of the phenotype by different candidate genes or molecules. The expression of different candidate genes was altered by either RNAi targeting or expression of the cDNA or ORF of the gene using the GAL4-UAS system.

High levels of ROS were detected by CellRox® staining in the outgrowth region of the larval eye discs when mitochondrial dysfunction was induced and the proportion of the observed outgrowth was modified by altering levels of the antioxidant enzymes Catalase, Sod1 or Sod2. Furthermore, similar phenotypes at the larval and adult stage were observed when the levels of these antioxidant enzymes were decreased in a wild-type background in the developing *Drosophila* eye. These results indicate that high levels of ROS have a causal role in the observed cellular dysfunction. Wwox has previously been shown to play a significant role in processes where ROS act as signalling molecules and has an effect on ROS levels in various backgrounds (O'Keefe *et al.*, 2011; Chapter 5- O'Keefe *et al.*, manuscript in preparation; Chapter 6 -Shaukat *et al.*, 2014). However, Wwox appears to have differing effects on ROS levels in different contexts, indicating that the relationship between Wwox and ROS is rather complex. In this case, Wwox was unable to modify or produce any phenotype when tested together with decreased levels of antioxidant enzymes directly or in clones of cells with mitochondrial dysfunction, suggesting that the modifying effects of Wwox is only detectable using this assay when there are sufficient levels of ROS present to begin with.

High levels of phosphorylated Akt were also observed in the larval eye discs with mitochondrial defects and decreased Akt expression was able to suppress the cellular dysfunction, indicative of the phenotypes being caused by increased Akt signalling, which can be a result of high ROS levels. Interestingly, Wwox expression has previously been shown to promote dephosphorylation of AKT (Hu *et al.*, 2012), hence it is possible that the

contribution of Wwox to the cellular dysfunction caused by mitochondrial defects is through the regulation of Akt activity. This could be tested by directly examining the effects of altering Wwox levels on the phosphorylation status of Akt. It would also be of interest to determine if the SDR enzymatic activity of Wwox is directly responsible for the dephosphorylation of Akt.

Downstream targets of Akt signalling such as Sima (*Drosophila* orthologue of HIF-1∝) and Foxo were tested for an effect on the mitochondrial defect-induced phenotypes. As Akt signalling have been shown to result in an increase in Sima activity, the level of Sima was decreased to determine if it could result in suppression of the phenotype. Unexpectedly, decreased levels of Sima were found to enhance the phenotype, suggesting that Sima has a protective, rather than a pernicious, effect on the detected cellular dysfunction. Similarly, inhibition of autophagy by targeting one of its essential components resulted in enhancement of the phenotype, demonstrating that autophagy is used as a mechanism to restrict the cellular damage caused by mitochondrial defects. As HIF-1∝ has been shown to be involved in induction of autophagy, which in some instances can inhibit cellular growth, it is possible that the protective role of HIF-1∝ in this context is related to autophagy and may be of interest for further examination. Work from a recent study demonstrated that WWOX is able to suppress autophagy in order to induce apoptosis in human squamous cell carcinoma cells upon treatment with an anticancer drug, methotrexate (Tsai *et al.*, 2013). That is the first report of WWOX having a role in regulation of autophagy. Although that study describes autophagy as a mechanism to promote cancer cell survival, there is also other evidence to indicate that autophagy can act as a protective response by cells to inhibit cancer growth (Santidrian *et al.*, 2013), hence it would be of interest to examine the effect that low Wwox levels may have on autophagic activity in different cancer cells. Of interest also is the potential lack of involvement of Tgo, the *Drosophila* orthologue of HIF-1β. Additional RNAi and mutant lines for Tgo are required to rule out the involvement of Tgo in this model. Nonetheless, the contribution of Sima could be through Tgo-independent pathways, such as ones mediated by dERR. As loss of WWOX expression in breast cancer cells has been reported to correlate with lack of the estrogen receptor status and it has been hypothesised that WWOX could have a role in estrogen-associated pathways (Nunez *et al.*, 2005a), a role for Wwox in dERR-associated pathways should be tested.

Foxo, which is the other downstream target of Akt signalling, has been shown to have tumour suppressive abilities by transcriptionally regulating a number of essential genes involved in various critical processes such as cell cycle arrest and DNA damage

repair. The transcriptional activity of Foxo has been shown to be inhibited through phosphorylation by activated Akt, as seen in various cancers. Increased expression of Foxo was found to suppress the cellular dysfunction caused by mitochondrial defects, indicating that Foxo activity could have a protective effect in cells with mitochondrial dysfunction. Indeed, Foxo has been shown to be a cellular sensor of oxidative stress and its target genes are involved in detoxification of ROS (Storz, 2011). There is currently no evidence of any interaction between Wwox and Foxo in the literature, although the AKT/Foxo pathway has been associated with other CFS genes, such as *FHIT* and *Parkin* (Kelley and Berberich, 2011; Todd and Staveley, 2013). Preliminary data, arising from the work described herein, appear to be suggest that loss of Foxo could result in similar phenotypes as those caused by mitochondrial defects and that Wwox may be able to genetically interact with Foxo as well (Appendix C: Figure C10); however additional RNAi and mutant lines for Foxo have to be tested in order to validate these preliminary results. Nevertheless, the work described herein has identified Foxo as a genetic interactor of this pathway and potentially a novel candidate interactor for Wwox.

In conclusion, the work described in this study has led to the identification of various genetic modifiers of the mitochondrial-induced cellular dysfunction in addition to Wwox (Figure 4.12). This work highlights the power of *Drosophila* genetic modelling, which enables the genetic dissection of the pathway(s) of interest. Many, if not all, of these identified modifiers have been found to be implicated or have been suggested to have some contribution to other Wwox-mediated pathways; therefore it is possible that Wwox is able to modulate the mitochondrial dysfunction through an effect on these genetic modifiers.

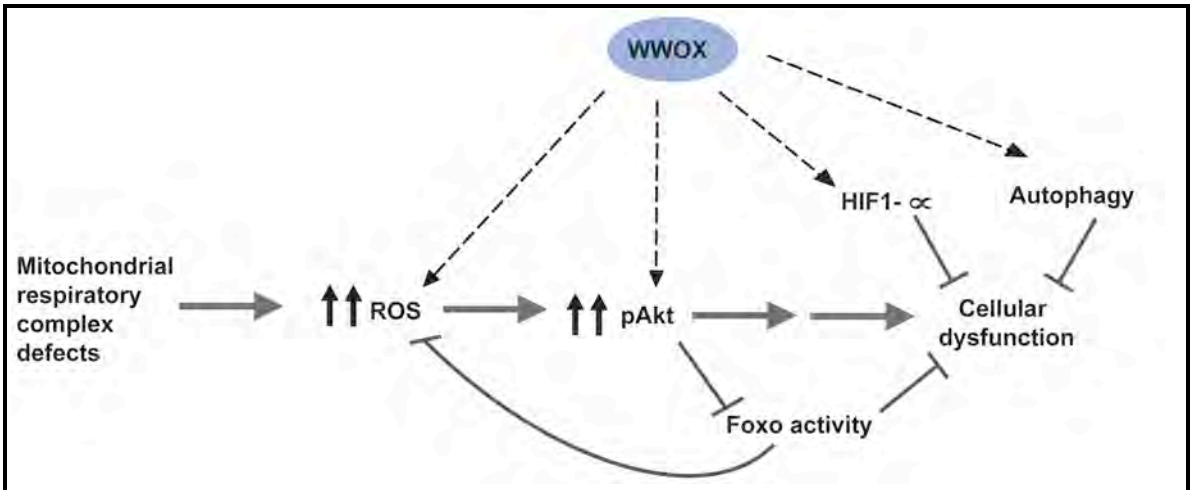


Figure 4.12 Model for the mitochondrial-mediated cellular dysfunction that is modulated by Wwox. Defects in mitochondrial respiratory complexes result in high levels of ROS that leads to activation of Akt signalling pathways and subsequently cellular dysfunction presenting as a larval eye disc outgrowth phenotype as well as severe adult eye phenotypes. Akt signalling can result in inhibition of Foxo activity and Foxo, which has been proposed to act as a tumour suppressor and is involved in the detoxification of ROS, has a protective effect against the mitochondrial dysfunction. The cellular dysfunction has also been demonstrated to be restricted by HIF-1∝ and autophagy. Wwox has been shown to play a role in the mitochondrial-mediated cellular dysfunction observed and is known to interact with various identified components of this mitochondrial-mediated pathway – Wwox has been implicated in the regulation of ROS, Akt signalling, HIF-1∝ activity as well as autophagy (with preliminary data suggesting that Wwox could also genetically interact with Foxo); therefore it is possible that Wwox modulates the mitochondrial-mediated cellular dysfunction through its association with one or more of these effectors.

Chapter 5 – Identification of a contribution by Wwox to a *Drosophila* model of intrinsic tumour suppression

This chapter has been written up as part of a manuscript for publication: -
O'Keefe, L.V., Lee, C.S., Choo, A., and Richards, R. I. Conserved role of tumour suppressor WWOX in TNF∝-mediated cell death. (To be submitted for publication)

The results from this work are included in the manuscript as Figure 3 (Panels A-L) and Supplementary Figure S2 (Panels A-F). The extent of the work is summarised below.

Summary

The progression of cells to a neoplastic state not only involves the acquirement of distinct biological capabilities by the tumour cells but also depends on the interactions between the cancerous cells and their surrounding cells or tissue (Hanahan and Weinberg, 2011). Competitive cell interactions result in the survival of cells of a higher fitness level, which in most cases involves the proliferation of cancerous cells at the expense of its neighbouring cells (Wagstaff *et al*., 2013). However, in some cases, the competitive cell interactions can act as a form of intrinsic tumour suppression whereby cells of tumorigenic potential are eliminated by normal neighbouring cells in an effort to restrict tumour progression. One such model in *Drosophila* is that of the *Scribbled* mutant clones (Brumby *et al.*, 2003).

Scribbled (Scrib) is a cell polarity protein that acts as a tumour suppressor in both *Drosophila* and mammals (Bilder and Perrimon, 2000; Dow *et al.*, 2003; Zhan *et al.*, 2011). Complete loss of Scrib in *Drosophila* results in neoplastic overgrowth (Bilder and Perrimon, 2000; Bilder *et al*., 2000), however somatic clones of Scrib homozygous mutant cells in a developing wild-type eye disc do not lead to overgrowth despite the ability of the Scrib mutant cells to undergo ectopic proliferation (Brumby *et al.*, 2003). The neoplastic overgrowth is instead restricted by the elimination of a large proportion of the Scrib mutant cells through TNFα/Eiger-induced JNK-dependent cell death mediated by the surrounding wild-type cells (Brumby *et al.*, 2003; Igaki *et al.*, 2009). The TNFα/Eiger signalling appears to originate from within the clones themselves but the presence of the surrounding wild-type cells is required for the elimination of the clones to occur (Igaki *et al.*, 2009). This TNFα/Eiger-induced cell death of Scrib mutant clones has been found to be controlled by genes involved in mitochondrial function and metabolic energy production,

which indicate that metabolic activity has a role in this model of intrinsic tumour suppression (Kanda *et al.*, 2011). Wwox has been found to contribute to exogenous TNFα/Eiger-mediated cell death in the *Drosophila* eye and wing disc (as described in the following manuscript), hence the aim of the work described in this chapter was to establish if Wwox also has a role in the endogenous TNFα/Eiger-induced cell death of Scrib mutant clones.

Mitotic mutant clones of cells can be generated in the developing *Drosophila* eye using the *M*osaic *A*nalysis with a *R*epressible *C*ell *M*arker (MARCM) method (Lee and Luo, 1999). The eyeless promoter is used to drive expression of a flippase (FLP) that induces FRT-mediated recombination between a wild-type allele and an allele carrying the mutation of interest, resulting in random clones of cells homozygous for that mutation within the eye disc (Figure 5.1). Cells that undergo the FRT-mediated recombination and are homozygous for the mutation will express a Green Fluorescent Protein (GFP); hence the mutant clones can be identified by fluorescence visualisation of GFP.

A *Scrib* null mutant allele was used to generate the homozygous Scrib mutant clones (Figure 5.1A) and the eye imaginal discs of late third instar larvae were examined for GFP visualisation of the clonal area. In 25% of the larval eye discs, the discs resembled masses of overproliferating cells containing high levels of GFP, which presumably correlates to a neoplastic overgrowth of the Scrib mutant clones. However, in 75% of the eye discs, only a small proportion of cells was found to be GFP-expressing due to the majority of the Scrib mutant clones being eliminated, which is similar to what has been reported in the literature (Brumby *et al.*, 2003; Igaki *et al.*, 2009; Kanda *et al.*, 2011). Whilst the elimination of those Scrib mutant clones mediated by the surrounding wild-type cells was able to restrict any neoplastic overgrowth in those discs, the remaining Scrib mutant clones that were not eliminated were able to cause disruption to the tissue. The remaining Scrib mutant clones were impaired in their ability to differentiate, which is evident from the absence of photoreceptor cells in some regions where the GFP-expressing Scrib mutant clones are present. As described in the literature, the Scrib mutant clones were also able to cause disruption to its surrounding tissue (Brumby *et al.*, 2003). The tissue disruption was more evident in the adult eye, where the clones result in disruption to the ommatidial patterning and in some cases, necrosis in the eye.

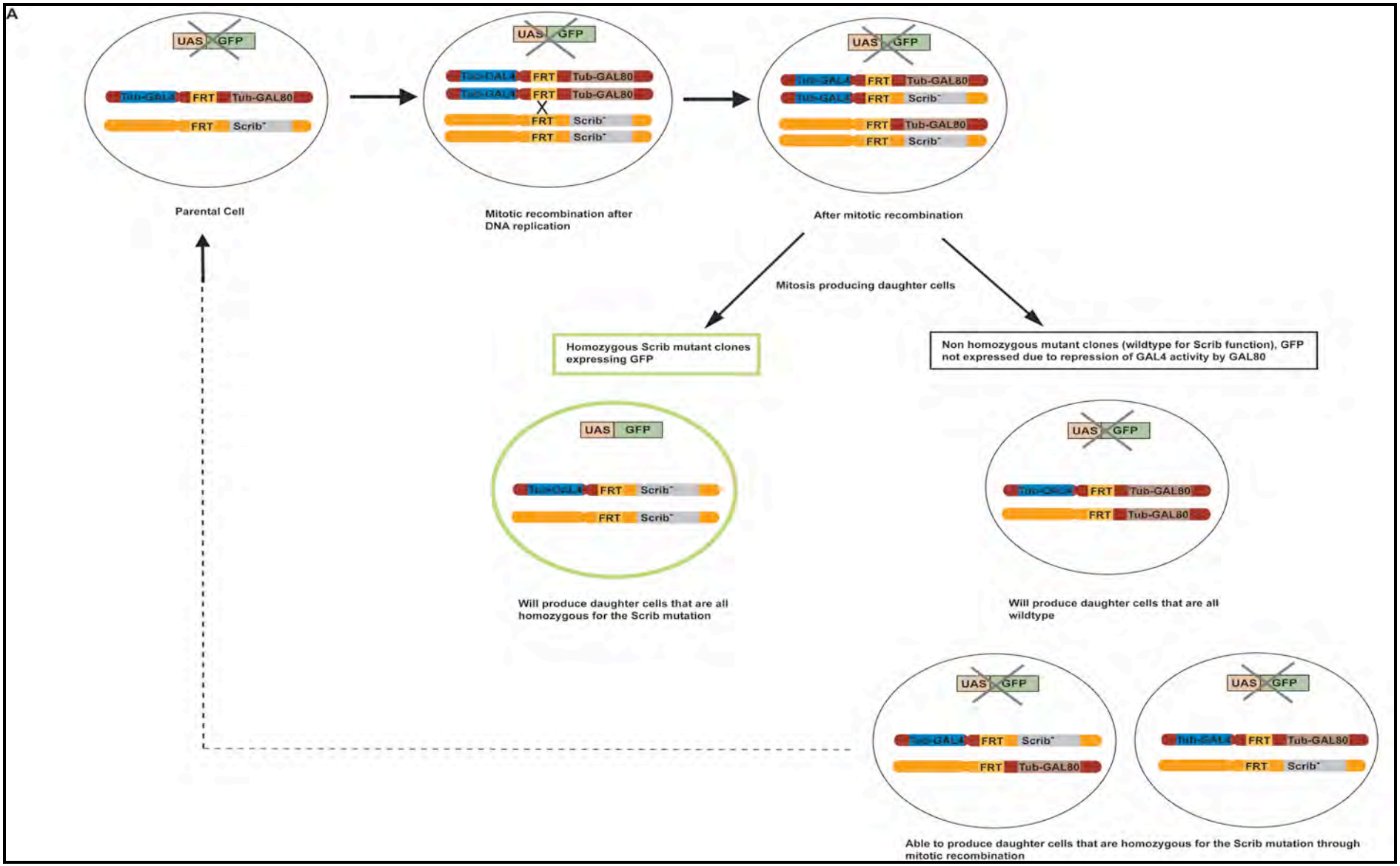
In order to determine if Wwox has a role in the TNFα/Eiger-mediated elimination of the Scrib mutant clones, Wwox expression was reduced (by RNAi targeting) specifically in the Scrib mutant clones (Figure 5.1B). Reduction of Wwox specifically in the Scrib mutant clones resulted in a higher proportion of third instar larvae with eye

imaginal discs that resembled masses of overproliferating cells containing high levels of GFP (62% compared to the 25% in the Scrib mutant clones alone). As for the remaining eye imaginal discs that exhibited normal morphology, they were found to contain a larger area of GFP-expressing clones when compared to the Scrib mutant clones alone. These results together indicate that reduction of Wwox expression in the Scrib mutant clones leads to reduced elimination of the clones. Reduction of Wwox in control wild-type clones did not result in any proliferating mass of cells or any increase in the clonal area, which supported that the observed increase in the proportion of remaining Scrib mutant clones as a result of reduced Wwox levels was not due to any additive effect. In fact, reduction of Wwox in the control wild-type clones was found to result in a decrease in clonal area. This observation is similar to what has been reported for other metabolic genes, where reduced expression of those genes also resulted in increased clonal area of Scrib mutant clones but decreased clonal area when in a wild-type context (Kanda *et al*., 2011).

Consistent with what was observed before, the non-eliminated Scrib mutant clones (in this case, with reduced Wwox) resulted in disruption to the tissue, causing loss of photoreceptor cells in the larval eye imaginal discs and subsequently disruption in the ommatidial patterning in the adult eye. Comparison of adult eyes revealed that reduction of Wwox in the Scrib mutant clones resulted in a significant decrease in eye size as well as an increase in the number of necrotic spots compared to the Scrib mutant clones alone. This increase in tissue disruption is consistent with the eye discs having a higher number of non-eliminated Scrib mutant clones when Wwox level is reduced. Furthermore, there was a decrease in the viability of adults, with a lower number of flies surviving to adulthood compared to the flies containing Scrib mutant clones alone. This decrease in viability is potentially due to the flies dying before eclosion as a consequence of overproliferation of the non-eliminated Scrib mutant clones, which appears to be consistent with the higher proportion of larvae displaying eye discs with masses of GFP-expressing cells. The observed increase in the severity of the adult eye phenotype and decrease in viability further supports that loss of Wwox leads to reduced elimination of Scrib mutant clones and that Wwox is involved in the TNFα/Eiger-induced elimination of the tumourigenic Scrib mutant cells.

Concluding remarks

Tumour progression is influenced by the interactions between cancerous cells and the tumour microenvironment provided by the surrounding normal tissue (Brumby *et al.*, 2003). Scrib mutant cells have the potential to result in neoplastic overgrowth, however in the clonal context, physiological TNFα/Eiger signalling leads to elimination of some of the Scrib mutant clones, thus preventing overproliferation of the Scrib mutant cells. The elimination of these clones is mediated by the surrounding wild-type cells as a form of intrinsic tumour suppression (Igaki *et al.*, 2009) and is regulated by metabolic activity (Kanda *et al.*, 2011). Wwox has been demonstrated to have an involvement in the elimination of the Scrib mutant clones and the results obtained appear to be parallel with that of reduced expression of other metabolic genes (Kanda *et al.*, 2011). The observed contribution of Wwox to the elimination of tumourigenic cells in this model provides *in vivo* evidence that Wwox plays an essential role in tumour suppression.


A
UAS
GFP
Tub-GAL4
FRT
Tub-GAL80
Scrib-
Parental Cell
Mitotic recombination after DNA replication
After mitotic recombination
Mitosis producing daughter cells
Homozygous Scrib mutant clones expressing GFP
Non homozygous mutant clones (wildtype for Scrib function), GFP not expressed due to repression of GAL4 activity by GAL80
Will produce daughter cells that are all homozygous for the Scrib mutation
Will produce daughter cells that are all wildtype
Able to produce daughter cells that are homozygous for the Scrib mutation through mitotic recombination

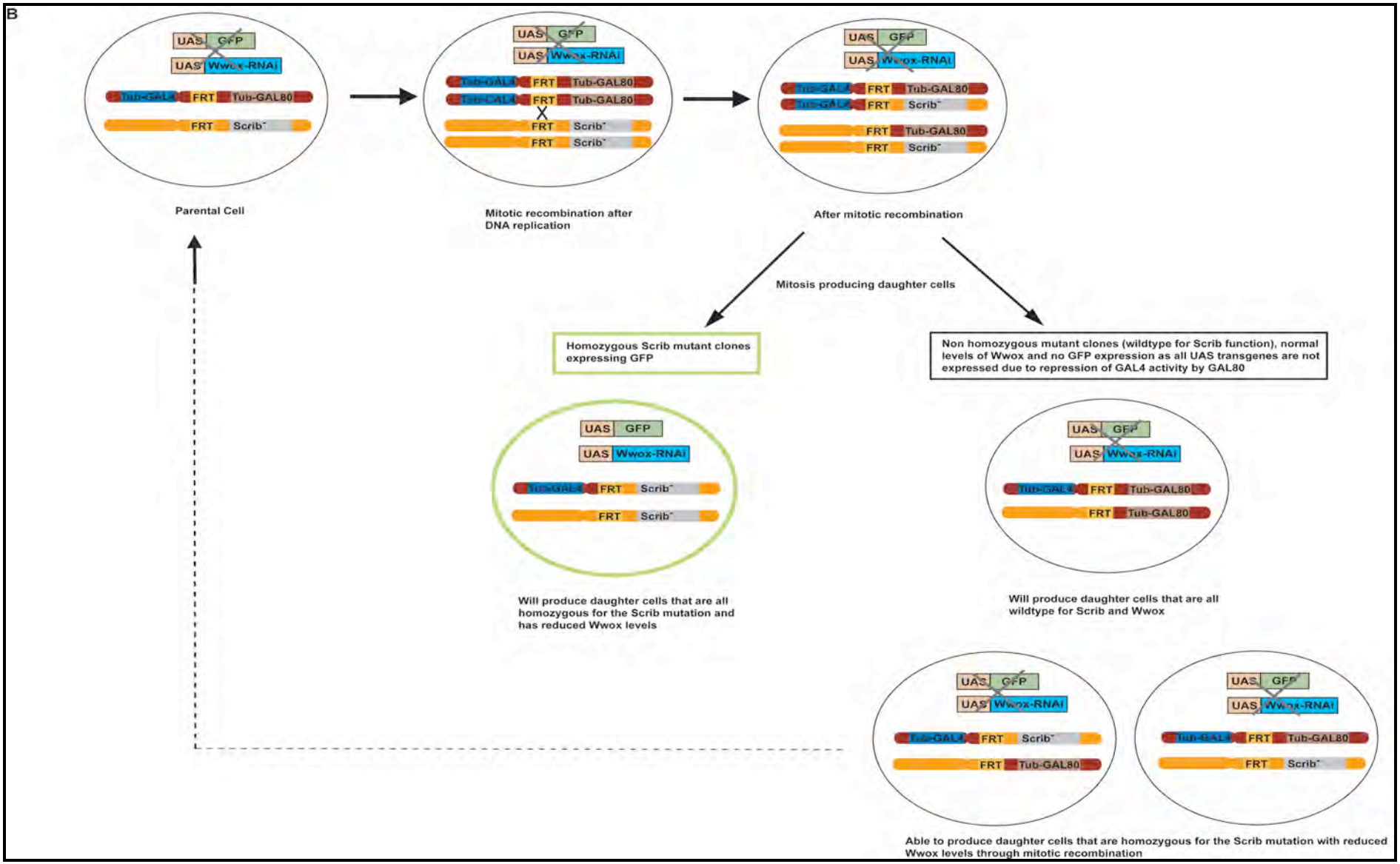

B
UAS GFP
UAS Wwox-RNAi
Tub-GAL4 FRT Tub-GAL80
FRT Scrib-
Parental Cell
Mitotic recombination after DNA replication
After mitotic recombination
Mitosis producing daughter cells
Homozygous Scrib mutant clones expressing GFP
Non homozygous mutant clones (wildtype for Scrib function), normal levels of Wwox and no GFP expression as all UAS transgenes are not expressed due to repression of GAL4 activity by GAL80
Will produce daughter cells that are all homozygous for the Scrib mutation and has reduced Wwox levels
Will produce daughter cells that are all wildtype for Scrib and Wwox
Able to produce daughter cells that are homozygous for the Scrib mutation with reduced Wwox levels through mitotic recombination

Figure 5.1 Generation of mitotic homozygous mutant clones in a heterozygous *Drosophila* using the *M*osaic *A*nalysis with a *R*epressible *C*ell *M*arker (MARCM) system. The eyeless (ey) promoter is used to express a flippase (FLP) in the developing *Drosophila* eye (not shown in the diagram), which induces FRT-mediated recombination in mitotic cells. Mitotic recombination occurs between the FRT sites present on the homologous chromosomes in the cell, resulting in the exchange of chromosome arms distal to the recombination site. A tubulin-GAL80 (Tub-GAL80) construct is present on one of the chromosome arms distal to the FRT site and results in expression of GAL80 protein under the control of the tubulin promoter. The GAL80 protein is able to repress activity of the GAL4 protein expressed under the control of a separate tubulin promoter (Tub-GAL4) proximal to the FRT site. The GAL4 protein binds to Upstream Activator Sequences (UAS) and in the absence of GAL80, drives expression of transgenes downstream from the UAS, such as the UAS-GFP transgene; however in the presence of GAL80, the UAS-transgenes are not expressed. When mitotic recombination occurs, this results in exchange of chromosome arms, one carrying the Tub-GAL80 construct and the other with the mutant allele of interest. Segregation of the sister chromatids during mitosis can then lead to daughter cells that are homozygous for the mutation – these cells do not carry the Tub-GAL80 construct, therefore they are positively marked with GFP (circled in green). The other daughter cells that can be produced are not homozygous for the mutation and will all carry the Tub-GAL80 construct hence do not express GFP. The daughter cells that are either homozygous mutant or wild-type will go on to produce daughter cells of the same genotype whilst the daughter cells that carry one copy of the mutation and one copy of the Tub-GAL80 construct are able to produce daughter cells that are homozygous through mitotic recombination. **(A)** Generation of homozygous clones carrying a mutant null allele for Scrib ($Scrib^{-}$). As the effects of the Scrib mutation are recessive, presence of one wild-type copy of Scrib is sufficient to carry out normal Scrib function. Only the GFP-expressing cells have complete loss of Scrib function and are the clones of interest. **(B)** Generation of homozygous Scrib mutant clones with reduced expression of Wwox. The flies carry an additional transgene, which is an RNAi construct targeting Wwox under the control of UAS (UAS-Wwox RNAi). The UAS-Wwox RNAi transgene is only expressed in the presence of GAL4 activity, hence only the daughter cells that are homozygous for the Scrib mutation will express the UAS-Wwox RNAi transgene and will have reduced levels of Wwox. In this case, the GFP positive clones are homozygous Scrib mutants with reduced Wwox expression. The other daughter cells will carry the UAS-Wwox RNAi transgene but the transgene will not be expressed due to the repressive activity of the GAL80 protein.

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By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Contribution to the paper	Performed experiments (Wwox and Scrib mutant clonal analyses), analysed the data, contributed to writing of manuscript		
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Abstract

WWOX is an extremely large gene spanning the *FRA16D* Common Chromosomal Fragile Site. Altered *WWOX* levels have been observed in a wide variety of cancers. While WWOX can act to suppress the growth of tumors, its normal role and functional contributions to cancer have not been fully defined. We have previously reported evidence for the participation of WWOX in cellular metabolism and herein we investigate the contribution of WWOX to apoptotic cell death. Using *Drosophila* as an *in vivo* model system, we find that altered expression of WWOX modulates ectopic TNFα/Egr-mediated phenotypes. This supports an *in vivo* pro-apoptotic role for WWOX. We also show that WWOX activity is required for an endogenous TNFα/Egr-mediated process whereby clones of cells mutant for the cell polarity regulator *Scribbled* are eliminated from a developing epithelial tissue. We report a positive correlation between WWOX levels and TNFαÆgr-mediated Caspase 3 activity. We 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.

Introduction

Evasion of cell death and altered metabolism are recognized as hallmarks of cancer cells, while DNA instability is also recognised as one of the enabling characteristics (Hanahan and Weinberg, 2011). The *FRA16D* Common Chromosomal Fragile Site (CCFS) spanning the WWOX gene participates in each of these phenomena and therefore its perturbation in cancer cells presents 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 (Yunis and Soreng, 1984; Glover *et al*., 2005). There are ~200 such sites in the human genome displaying a hierarchy of sensitivity *in vitro* that is matched by the frequency with which these sites show *in vivo* DNA instability in various cancers (O'Keefe and Richards, 2006; Mrasek *et al*., 2010). Of these sites, *FRA3B* and *FRA16D* have been shown to be the most frequent regions of recurrent homozygous deletion in cancer (Bignell *et al*., 2010). Damage at *FRA16D* is an early event in tumorigenesis since precisely the same deletion has been detected in cell lines derived from a primary tumor (KM12S) and secondary metastasis (KM12SM) from the same patient, despite these cells having quite distinct karyotypes (Finnis *et al*., 2005). 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 (O'Keefe and Richards, 2006). DNA instability at these sites, resulting in deletion(s) and / or localised rearrangements, is associated with alterations to CCFS-associated gene expression (Ried *et al*., 2000).

Altered expression of WWOX has been reported for many different cancer cell types (Salah *et al*., 2010). In addition, a low expression allele of WWOX was found at a higher frequency in lung cancer patients, consistent with decreased WWOX as a predisposing factor for tumorigenesis (Yang *et al.*, 2013). Also WWOX hypomorphic mice showed an increased incidence of B-cell lymphoma (Ludes-Meyers *et al.*, 2007). Mice heterozygous for WWOX exhibit higher rates of tumour growth, however the tumour cells still express WWOX protein indicating a lack of the typical 'second-hit' somatic mutation characteristic of classical tumour suppressors (Aqeilan *et al.*, 2007). Correlation of higher WWOX expression with better prognosis has been reported for various types of cancer including colon, breast and bladder (Pluciennik *et al*., 2006; Zelazowski *et al.*, 2011, Lin *et al.*, 2013). Thus it appears that reduced levels of WWOX activity are sufficient for a 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* (Bednarek, *et al*. 2001, Fabbri *et al*., 2005; Nakayama *et al*., 2008). 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 belongs to a super-family of short-chain dehydrogenases/reductase (SDR) enzymes that share a conserved 'Rossmann' fold which acts as a dinucleotide binding scaffold for NAD(P)(H), in addition to two WW domains that facilitate binding to other proteins with PPxY motifs (Kavanagh *et al.*, 2008). 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 (Chang *et al*., 2010, Salah *et al*., 2010). A role for WWOX in metabolism has been established through the analysis of knockout models in mouse, rat and *Drosophila* (Aqeilan *et al.*, 2007a; Aqeilan *et al.*, 2007b; Ludes-Meyers *et al.*, 2007; Ludes-Meyers *et al.*, 2009; Suzuki *et al*., 2009; O'Keefe *et al.*, 2011). In addition, patients with WWOX mutations also show significant growth retardation suggestive of a similar effect (Abdel-Salam *et al*., 2014). 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 (Dayan *et al.*, 2013). WWOX has also been widely reported to play a role in cell death pathways. A pro-apoptotic role for WWOX *in vitro* has previously been reported for many different cancer cell types; multiple myeloma (Zhang *et al.*, 2014), colon (Nowakowska *et al*., 2014), gall bladder (Wei *et al*., 2013), cervical (Qu *et al*., 2013), leukaemia (Cui *et al*., 2013), glioblastoma (Chiang *et al*., 2012), hepatoma (Hu *et al*., 2013), lung (Fabbri *et al*., 2005; Zhang *et al*., 2012; Zhou *et al*., 2010), ovarian (Xiong *et al*., 2010), breast (Illopolous *et al*., 2007), pancreatic (Nakayama *et al*., 2008), glioblastoma (Kosla *et al*., 2011), prostate (Qin *et al*., 2006) and squamous epithelia (Lai *et al*., 2005). However the contribution of WWOX to apoptotic pathways *in vivo* has not been determined for any of the knockout models. *Drosophila* is an effective system to uncover those aspects of the contribution of WWOX to cellular pathways that are conserved in evolution and therefore likely to be of biological significance in human cancer. Herein we describe an in *vivo* role for WWOX in TNFα-mediated cell death within the developmental context of this whole animal model.

Results

Altered WWOX modulates ectopic Egr/TNFα eye phenotypes

Drosophila has a single ortholog with homology to TNFα encoded by the *EDA-like cell death trigger* or *Eiger (Egr)* gene (Igaki *et al*., 2002; Moreno *et al*., 2002; Kauppila *et al*., 2003). Ectopic over-expression of Egr/TNFα in the eye during its development has previously been shown to result 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 (Figure 1A; Kanda *et al*., 2011). This Egr/TNFα phenotype was completely suppressed by RNAi-mediated knockdown of *wengen,* a gene that encodes the *Drosophila* TNF receptor (*TNFR*) thus confirming the specificity of the ectopic Egr/TNFα-mediated phenotype (Figure 1B; Kanda *et al*., 2002; Kauppila *et al*., 2003).

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 *Drosophila* eye (Kanda *et al*., 2011). The WWOX gene has previously been identified as participating in aerobic metabolism in *Drosophila* and thus also represents a candidate for contributing to Egr/TNFα-mediated cell death (O'Keefe *et al*., 2011). 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 on the surface of the eye as well as an increase in eye size (Figure 1C-D). 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 (Figure S1B-D).

Ectopically expressed WWOX has previously been shown to enhance the *in vitro* cytotoxicity of TNFα in various tissue culture cell lines (Chang *et al.,* 2001). Ectopic expression of WWOX alone does not result in any obvious cell death-induced phenotype in the biological context of the *Drosophila* eye (Figure S1H). Consistent with its effect on TNFα-cytotoxicity *in vitro,* 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 significant decrease in eye size (Figure 1E-F). A decrease in adult eye size was also observed with ectopic over-expression of an open reading frame (ORF) encoding WWOX (Figure S1E). The level of ectopic WWOX expression for each of these lines was confirmed by western blot analysis (Figure S1I). Thus, increased or decreased WWOX levels were able to modify the Egr/TNFα-mediated

cell death phenotype in reciprocal directions consistent with a role for WWOX in contributing to cell death via this signalling pathway.

Altered WWOX has no obvious effect on ectopic p53 or hid eye phenotypes

WWOX has been shown to act synergistically with p53 to promote cell death in various tissue culture cell lines (Chang *et al.*, 2001). Ectopic expression of the *Drosophila* orthologue of p53 tumor suppressor results in a phenotype with a decrease in eye size and significant disruption to ommatidial patterning accompanied by loss of pigment and the presence of small necrotic lesions (Figure 2A; Ollman *et al.*, 2000). However, decreased or increased WWOX expression had no significant effect on this rough eye phenotype (Figure 2B-C). Ectopic expression of the pro-apoptotic gene head involution defective (hid) also resulted in a distinct phenotype characterised by a greater reduction in eye size and almost complete loss of ommatidial structures (Figure 2D; Grether *et al*., 1995). However, no obvious modification of this phenotype was observed with either decreased or increased levels of WWOX activity (Figure 2E-F). Hence there was no evidence for WWOX being able to contribute to, or further promote, cell death phenotypes mediated either by p53 or hid. Thus there is specificity in the role of WWOX in modulating ectopically over-expressed Egr/TNFα-mediated cell death in the *Drosophila* eye.

WWOX contributes to endogenous *Egr*/TNFα dependent elimination of *Scribbled* mutant clones

Egr/TNFα has been shown to be required as an endogenous pro-apoptotic signal for the elimination of clones of the cell polarity regulator and tumor suppressor gene *Scribbled (Scrib)* in *Drosophila* imaginal eye discs (Igaki *et al*., 2009; Ohsawa *et al*., 2011). Clones of *Scrib* mutant cells are restricted in their ability to overgrow despite their ability to undergo ectopic proliferation (Brumby *et al*., 2003). *Scrib* mutant cells within the clones are eliminated and replaced by proliferation of the surrounding wild-type cells (Brumby *et al.*, 2003, Igaki *et al.*, 2009). The elimination of the *Scrib* mutant cells is mediated by a Egr/TNFα signal that appears to originate from within the clones themselves (Igaki *et al.*, 2009). Clones of *Scrib* mutant cells generated using the *M*osaic *A*nalysis with a *R*epressible *C*ell *M*arker (MARCM) system are positively labelled with GFP expression (Lee and Luo, 1999). Many cells of the randomly generated mutant clones are eliminated, however this is not complete and some remain as visualized by random patches of GFP positive cells in developing eye discs (Figure 3A-A', Brumby *et al.*, 2003; Igaki *et al.*, 2009; Kanda *et al.*, 2011). These *Scrib* mutant clones that are not eliminated result in a

disruption to the normal patterning of differentiation as visualised by Elav staining (Figure 3B-B' and 3C-C'). These remaining clones of *Scrib* mutant cells continue to develop and give rise to adult eye phenotypes characterised by patches of roughness where ommatidial organisation and development has been disrupted (Figure 3I).

Since WWOX has been shown to modify phenotypes resulting from ectopic over-expression of Egr/TNFα, we also tested whether WWOX can contribute to the endogenous requirement for Egr/TNFα in the elimination of *Scrib* mutant clones. When WWOX levels were reduced within the *Scrib* mutant clonal tissue of developing discs, a significant increase in patches of GFP positive cells was observed despite no overall change to disc size (Figure 3D-D', G-H). In contrast, control clones of wild-type cells with reduced WWOX expression did not show any such increase in total area of GFP expression (Figure S2). They did however show a decrease in total area of GFP expression despite no overall change to disc size (Figure S2A-D). This is consistent with results previously reported for clones of various metabolic genes within the developing eye imaginal disc where a decrease in GFP expression was observed (Kanda *et al.*, 2011). The GFP positive cells of *Scrib* mutant clones with decreased WWOX expression are also disrupted in the patterning of differentiation (Figure 3E-E', F-F'). A more obvious disruption to Elav staining in surrounding wild-type cells was also observed. Together these findings demonstrate a contribution from WWOX specifically within clones of *Scrib* mutant cells in the developing eye disc for them to be eliminated in response to an Egr/TNFα-mediated pro-apoptotic signal. This is evidence for an *in vivo* functional role for WWOX in TNFα-mediated cell death.

Decreased WWOX expression in *Scrib* mutant clones also resulted in further disruption to the adult eye phenotype with these eyes showing a significant decrease in size, as well as an increase in the frequency and size of black necrotic lesions on the surface of the adult eye (Figure 3J-L*).* This enhanced phenotype also corresponds to an observed decrease in overall viability for the flies with decreased WWOX expression in *Scrib* mutant clones (31.9% survival) compared to the flies with the *Scrib* mutant clones alone (74.1% survival) (**p=0.0016). No decrease in viability was observed for flies with decreased WWOX expression alone. 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 different genetic backgrounds (Brumby *et al.*, 2003; Igaki *et al.*, 2009; Kanda *et al.*, 2011). 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) (Figure S3). These results confirm that there is a decrease in the effectiveness of elimination of *Scrib* mutant clones when WWOX activity is reduced specifically within the clones or when WWOX activity is reduced or completely removed from all cells of the animal. Rescue of the *Scrib* mutant clonal phenotype with ectopic expression of Egr/TNFα has previously been reported (Igaki *et al*., 2009). However, adult eyes of flies ectopically expressing WWOX cDNA were comparable to those of *Scrib* control alone suggesting that increased levels of WWOX were not sufficient to promote apoptosis (and subsequent elimination) of cells within the *Scrib* mutant clones (Figure S3).

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

Nuclear localisation of WWOX has been reported to be necessary for the pro-apoptotic functions of WWOX in mammalian cells (Chang *et al*., 2001). Although endogenous levels of WWOX are too low to be detected in *Drosophila*, we have previously shown cytoplasmic localization of ectopically expressed WWOX during embryonic development (O'Keefe *et al*., 2005). Thus, we also determined the localisation of ectopically expressed WWOX in differentiated cells of the developing eye disc. Ectopic expression with *GMR-GAL4* results in expression of 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 (Figure S4). A similar pattern of cytoplasmic WWOX expression was observed in the presence of ectopic Egr/TNFα expression.

Given the small size and complex organisation of cells in the developing eye disc, Egr/TNFα expression was also tested in cells in the posterior compartment of the developing 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 surrounding the DAPI stained nuclei in the posterior half of the disc (Figure 4A-D). In the presence of ectopic Egr/TNFα expression, the wing discs are much smaller and there is disruption to the region of the disc marked by GFP expression (Figure 4E). In addition to GFP expression at the posterior margin of the disc, there also appears to be a region of weaker GFP expression extending into the central wing pouch region of the disc. Closer examination of the GFP expression in the posterior region showed that ectopic WWOX remains clearly cytoplasmic as staining was once

again observed complementary to the DAPI stained nuclei (Figure 4E-H). Thus there was no evidence *in vivo* for nuclear localisation of WWOX in response to ectopic Egr/TNFα expression in eye or wing imaginal discs.

Ectopic Egr/TNFα promotes apoptosis in the larval imaginal wing disc

Ectopic Egr/TNFα expression alone resulted in wing discs that were significantly decreased in size with disruption to the region of the disc marked by GFP expression (Figure 4I, M). In addition to cytoplasmic GFP expression at the posterior margin of the disc, there also appears to be a region of weaker GFP expression extending into the central wing pouch region of the disc where GFP appears punctate (Figure 4M-M'). This is suggestive of cells undergoing cell death in this region and immunostaining of Caspase 3 was carried out to determine resultant levels of apoptosis. Negligible levels of Caspase 3 staining were observed in control discs (Figure 4J) whilst high levels were observed in the central pouch region of discs ectopically expressing Egr/TNFα (Figure 4N-N'). In addition, Caspase 3 staining was found to extend beyond the GFP region of the wing pouch in two distinct regions (Figure 4N', asterisks). The extremities of these regions correspond to regions previously reported in a process termed apoptosis induced apoptosis (AiA) and shown to require JNK activation (Perez-Garjio *et al.*, 2013; Fernández *et al.*, 2014). Caspase 3 staining specifically at the tip of these two regions in the anterior portion of the disc 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. Loss of Egr/TNFα has been shown to be required for this death signal that is required for inducing apoptosis at a distance from the cell death signal. Here, we show that overexpression of Egr/TNFα in the posterior compartment is sufficient to induce AiA in addition to a more broad region of Caspase 3 staining that includes the central wing pouch region, further confirming Egr/TNFα as a key activating signal for this process.

Cubitis interruptus (Ci) staining was used to determine the identity of cells originating from the anterior portion of the wing disc. In the control disc the region corresponding to Ci staining is complementary to the GFP expression pattern in the posterior region under control of *hh-gal4* (Figure 4I,K-L). However, in response to ectopic Egr/TNFα co-expression in the posterior region there is now a region of Ci positive cells overlapping with the GFP positive cells in the central pouch region (Figure 4O-O', P-P'). Thus there no longer is a clear distinction between cells from the Ci staining portion of the disc (i.e. the

wild-type cells from the anterior region) with GFP positive cells from the posterior part of the disc (i.e. cells expressing ectopic Egr/TNFα).

WWOX regulates Caspase 3 activity in TNFα-mediated cell death

Levels of cleaved Capsase-3 staining were determined in wing discs ectopically expressing Egr/TNFα together with altered WWOX activity. Ectopic expression of Egr/TNFα alone in the posterior region resulted in Caspase 3 staining extending throughout the wing pouch and beyond the region of GFP expression including the two distinct regions previously reported for apoptosis induced apoptosis (Figure 5A-B; Perez-Garjio *et al.,* 2013). Decreased WWOX activity together with ectopic expression of Egr/TNFα in the posterior portion of the disc resulted in a significant decrease in the total Caspase 3 staining while retaining the distinct regions of Caspase 3 staining (Figure 5C-D). Conversely, increased WWOX expression resulted in a significant increase in the total region of Caspase 3 staining with loss of the obvious regions resulting from apoptosis induced apotosis (Figure 5E-F). Instead there seems to be a larger area of Caspase3 staining extending throughout the central wing pouch region. Quantification of the relative area of Caspase 3 staining for a number of discs revealed each of these observed changes was statistically significant (Figure 5G). Thus there is evidence supporting a role for WWOX in regulating the area of Caspase 3 spreading to anterior region in response to the ectopically expressed Egr/TNFα in the posterior region of this developing epithelial tissue.

Egr/TNFα-mediated cell death phenotypes are modified by altered SOD activity

Phenotypes resulting from ectopic expression of Egr/TNFα have previously been shown to be mediated by increased levels of ROS (Igaki *et al.,* 2009; Kanda *et al.,* 2011). We were able to confirm this in larval wing discs by increased staining for CellRox® in the posterior region of the developing disc as well as in the specific regions corresponding to apoptosis induced apoptosis (Figure 6A-C). We then 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 hydrogen peroxide as an intermediary in the detoxification process and 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 was able to obviously suppress the Egr/TNFα eye (Figure 6D-F) supporting the role for ROS in generating the Egr/TNFα-mediated rough eye phenotypes.

Discussion

Altered expression of WWOX has been reported in many different cancers and correlates with frequent DNA instability at the *FRA16D* locus (O'Keefe and Richards, 2006). Low levels of WWOX have been associated with poor prognosis in patients with various forms of cancer (Pluciennik *et al.*, 2006; Zelazowski *et al.*, 2011; Lin *et al.*, 2013). Thus, this highlights the need to understand the normal functions of WWOX for the development of novel therapeutic interventions aimed at improving clinical outcomes. WWOX has an established role as a metabolic regulator as determined by *in vivo* studies from rodent and *Drosophila* endogenous mutant models as well as by *in vitro* mammalian cell culture systems (Aqeilan *et al.*, 2007a; Aqeilan *et al.*, 2007b; Ludes-Meyers *et al.*, 2007; Ludes-Meyers *et al.*, 2009, Suzuki *et al*., 2009, O'Keefe *et al.*, 2011; Dayan *et al.*, 2013). However, most studies to date on the pro-apoptotic functions of WWOX have determined the effect of restoration of WWOX function via ectopic overexpression, and in some cases the requirement for WWOX function by RNA interference-mediated knockdown, in various cultured cancer cells *in vitro*. In this study we have investigated the *in vivo* pro-apoptotic functions of the conserved WWOX protein from *Drosophila* in the context of this whole animal model system.

We have shown here that altered WWOX levels can modify cell death-induced rough eye phenotypes resulting from ectopic expression of Egr/TNFα in *Drosophila*. Reactive oxygen species (ROS) are known to be principle effector molecules of TNFα-mediated cell death (Kanda *et al*., 2011). We have previously shown ectopic expression of WWOX giving high levels of ROS whilst reduced levels of WWOX show decreased ROS in developing *Drosophila* larvae (O'Keefe *et al.*, 2011). Therefore, one of the likely mechanisms by which WWOX contributes to the Egr/TNFα-mediated cell death pathway is via its regulation of ROS. At least to some extent, this occurs through regulation of the subset of ROS that are also responsive to enzymes of the 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 (O'Keefe *et al.*, 2011). Thus the cell death promoting effects of WWOX may only become effective in an *in vivo* situation once cells have received significant levels of damage. This damage could be reflected by cellular levels of ROS as we have shown with ectopic Egr/TNFα and are consistent with a non-classical role in tumor suppression. 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 (Palacino *et al*., 2004; Trapasso *et al*., 2008; Karras *et al*., 2014). 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. In this way the common fragile site associated genes could act as suppressors of tumor growth via their ability to promote apoptosis of cells with increased levels of ROS and consequently a heightened potential for accumulating deleterious mutations.

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. In mouse L929 cells, an ectopic increase in WWOX was found to enhance TNFα-mediated cell death (Chang *et al*., 2001). We find here that while WWOX has a clear role to play in the Egr/TNFα-mediated cell death pathway in *Drosophila*, no role for altered WWOX in other well-characterised cell death pathways was evident. WWOX was previously shown to be an essential component of p53-mediated apoptosis in NIH3T3 cells (Chang *et al*., 2001). However, in human glioblastoma cells the pro-apoptotic activity of WWOX was shown to be specific to cells with a mutant form of p53 suggesting other factors may also be involved (Chiang *et al*., 2012). In addition, *in vitro* nuclear localisation of pro-apoptotic WWOX was also reported in L929 cells (Chang *et al., 2001*). However, we found no *in vivo* evidence for nuclear localization of WWOX in the presence of ectopic TNFα expression. Although endogenous WWOX levels are too low to be detected, we show clear localization of over-expressed WWOX in the cytoplasm of two different developing larval tissues as well as during embryonic development, as previously reported (O'Keefe *et al*., 2005). Conflicting reports appear in the literature for the location of WWOX protein to various cytoplasmically localised organelles including Golgi and mitochondria (Bednarek *et al*., 2001; Chang *et al*., 2001). However, an absence of nuclear translocation of WWOX has also been reported for the pro-apoptotic functioning of p73 in leukaemic cells also suggesting the cellular response may vary depending on other factors (Lin *et al*., 2013).

Significantly we determined an *in vivo* contribution by WWOX in the endogenous Egr/TNFα-mediated process of Intrinsic Tumor Suppression in *Drosophila*. This is an established system where clones of cells carrying deleterious mutations are generated within a developing epithelium and must be eliminated in a process mediated by the surrounding wild-type cells such that they do not proceed to become tumorigenic. The outcomes of competitive cell interactions in this way are essential contributing factors to

the development of tumors *in vivo* (Wagstaff *et al*., 2013). We report here that reduction, or absence, of WWOX activity decreased the effectiveness of this elimination process. Although relatively mild effects of decreased WWOX expression were observed on GFP expressing mutant cells in the eye imaginal discs, more striking effects were observed at later stages. Significant disruption to adult eye development was observed with obvious necrotic lesions present on the surface of adult eyes. It has been previously reported that inhibition of apoptosis (by ectopic expression of P35) in *Scrib* mutant clones gave rise to similarly enhanced adult eye phenotypes supporting a pro-apoptotic role for WWOX in this process (Brumby and Richardson, 2003). 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 and therefore able to be replaced by the surrounding wild-type cells. This finding is consistent with a better prognosis as reported for cancer patients with increased levels of WWOX activity (Park *et al*., 2004; Pluciennik *et al.,* 2006; Zelazowski *et al.,* 2011). Understanding the functions of WWOX in the genetically tractable *Drosophila* system have therefore identified cellular pathways and processes that can be targeted therapeutically to improve the outcomes for patients with WWOX deficient cancers.

Materials and Methods

Fly lines and crosses: *w^{1118}*, *UAS-Dmp53* (Ollman *et al.*, 2000), *GMR-GAL4*, *GMR-hid* (Grether *et al*., 1995) and *hh-GAL4* (Evans *et al*., 2009 and Ibrahim *et al*., 2013) 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 Drosophila RNAi Center. Ectopic Eiger/TNFα expression construct (*UAS-egr^{+w}*) was kindly provided by Professor Miura (Igaki *et al*., 2009). *WWOX^{1}*, *WWOX^{2}*, *UAS-WWOX ORF$^{\#1}$* and *UAS-WWOX cDNA$^{\#1}$* have previously been described (O'Keefe *et al*., 2005). MARCMIII, FRT82B,Scrib^{1} and FRT82B stocks were kindly provided by Helena Richardson. *Drosophila* 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 25ºC.

Analyses of Adult Eyes: Photographs of exterior adult female *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 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, *WWOX$^{\text{RNAi}}$* transgene or *UAS-WWOX* transgene with either *FRT82B* as a control, or *FRT82B scrib^{1}* to express the transgene in *scrib^{1}* clones. Timed lays were carried out for all analyses. For analyses of eye discs, 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 were analysed per genotype). 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 (550-3000 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 (O'Keefe *et al*., 2011). 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 and the percentage of progeny were graphed using Microsoft Excel.

Western blot analyses: 30 female adult flies (0-1 day old) per sample were collected and Western blot analyses were performed as previously described (O'Keefe et al., 2005). Primary antibodies used were anti-C-DmWWOX antibody (1:1000) (O'Keefe *et al*., 2005) and mouse monoclonal anti-α-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 with PBST (1xPBS + 0.3% Triton-X-100) for 20 minutes three times 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 (O'Keefe *et al*., 2005)), anti-cleaved caspase 3 antibody (1:100, Cell Signaling), anti-Elav-9F8A9 (1:10, Developmental Studies Hybridoma Bank) and anti-Ci antibody (1:100) were used as primary antibodies. After that, discs were washed with PBST for 20 minutes three times and blocked with PBSTF for 30 minutes, followed by incubation of secondary antibody in the dark environment 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 with PBST for 20 minutes three times again 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 was detected

using the fluorogenic probe CellRox® (Life Technologies) as described previously (Shaukat *et al.*, 2014).

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Author Contributions

L.V.O conceived and designed the approach, performed experiments, analysed data and prepared the manuscript. C.S.L and A.C. performed experiments, analysed data and contributed to preparation of the manuscript. R.I.R conceived and designed the approach, analysed data and prepared the manuscript.

Conflict of Interest

The authors declare no competing commercial interests in relation to the submitted work.

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Figures

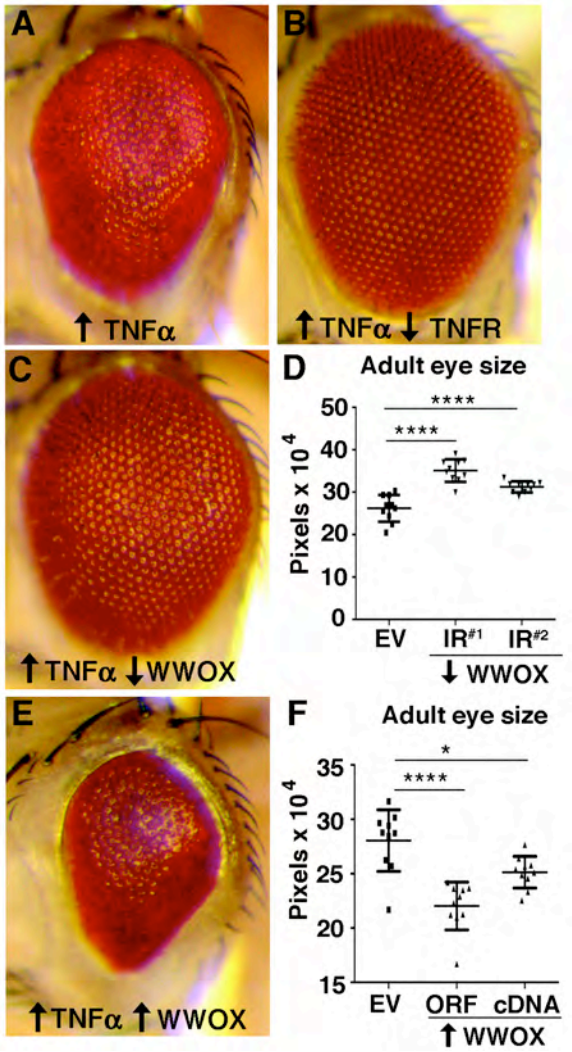

Figure 1. Altered WWOX modifies ectopic Egr/TNFα-mediated eye phenotype. (A) Ectopic expression of Egr/TNFα (GMR>*egr*$^{+w}$>*EV*) results in a decrease in eye size and disruption to ommatidial patterning. **(B)** The ectopic Egr/TNFα phenotype is completely suppressed by decreased levels of TNFR (GMR>*egr*$^{+w}$>*wengen/TNFR-IR*). **(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<0.05 and ****=p <0.0001.

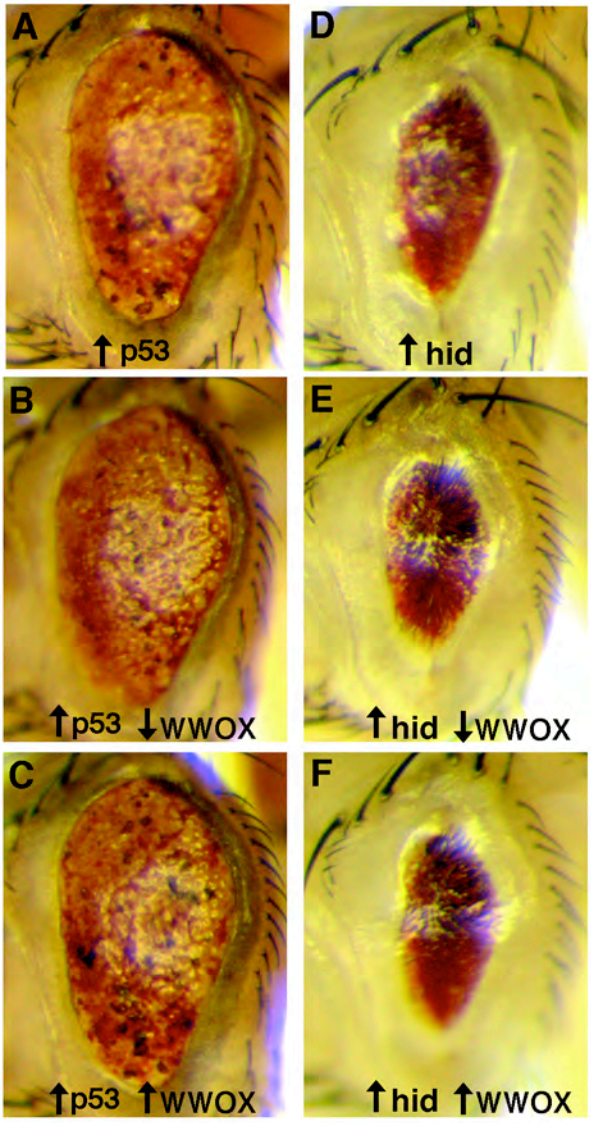

Figure 2. Altered WWOX has no effect on ectopic p53 or hid eye phenotypes. (A) Ectopic expression of Dmp53 in the developing eye *(GMR>DmP53>EV)* at 18^{o}C in the adult eye results in a phenotype characterized by decrease in eye size and significant disruption to ommatidial patterning accompanied by 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.

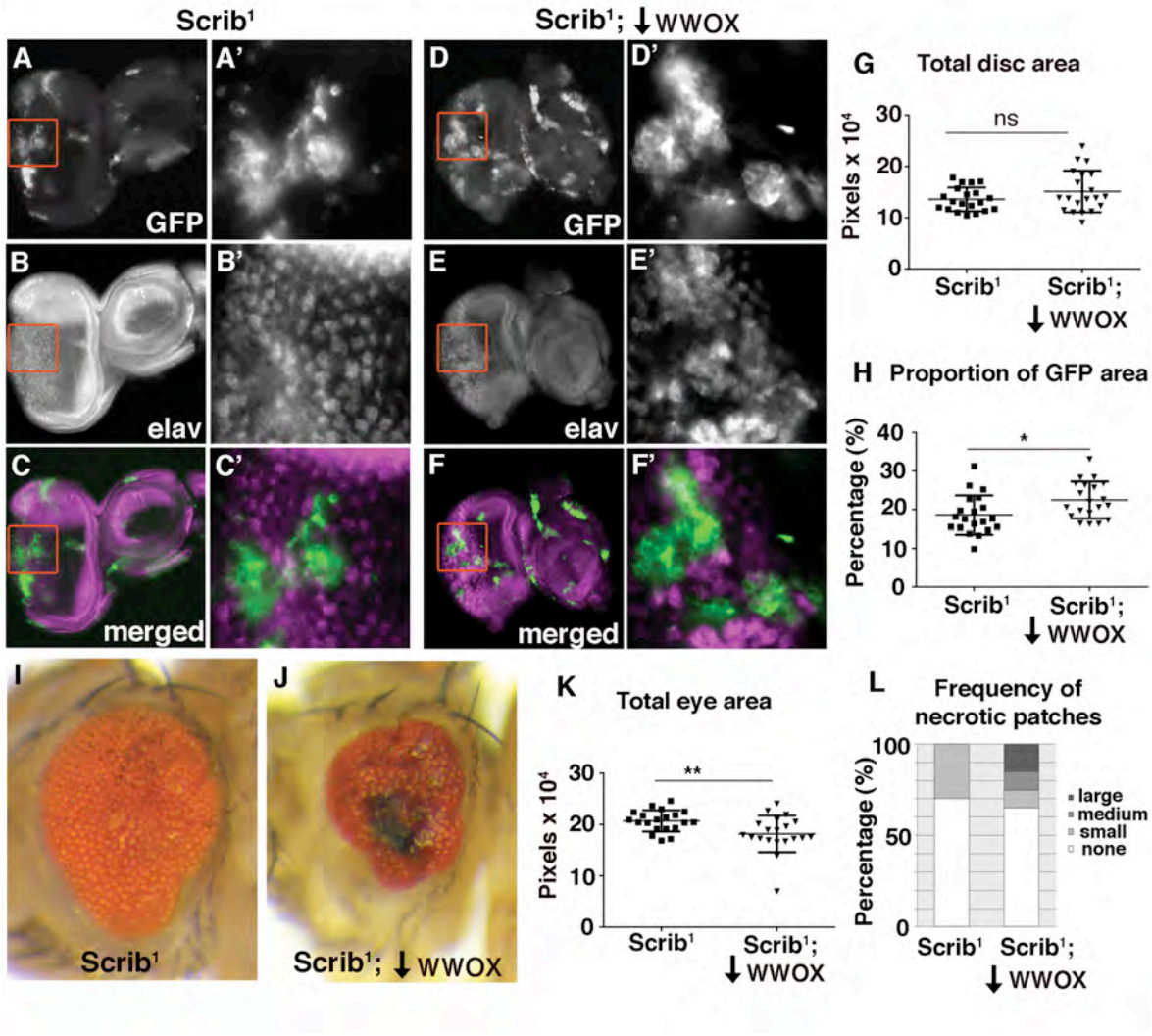

Figure 3. WWOX is required for elimination of *Scribbled (Scrib)* mutant clones (Scrib^{1}). (A-A') Clones of cells mutant for *Scrib* generated in the eye using the MARCM system are positively labelled with GFP. **(B-B')** Elav staining reveals absence of differentiated photoreceptors within a portion 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 total area of the eye disc containing *Scrib* clones with and without WWOX knockdown revealed no significant difference. **(H)** 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. (Significant disruption to eye disc morphology was observed in 13/52 of the *Scrib^{1}* clones and 31/50 *Scrib^{1};WWOX-IR* clones and these were not included in these analyses.) **(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 (550-3000 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.

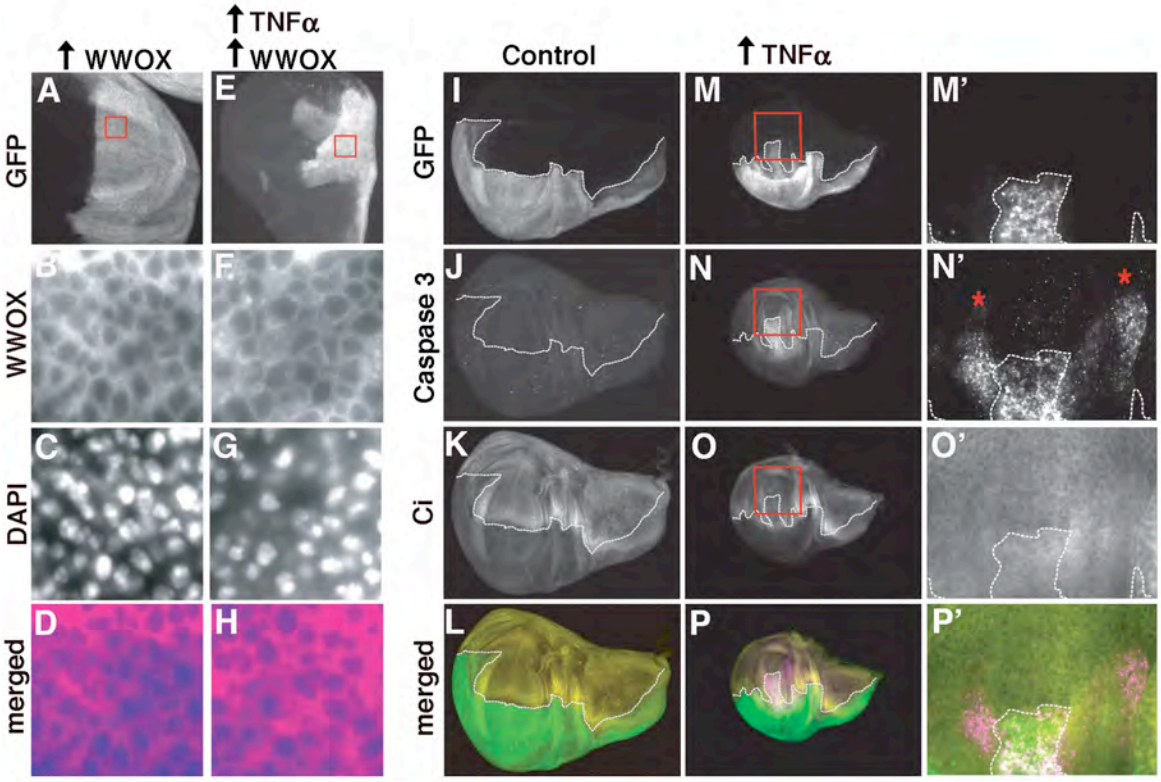

Figure 4. Ectopic Egr/TNFα has no obvious effect on cytoplasmically localized WWOX and promotes apoptosis in the larval imaginal wing disc. (A-D) Ectopic expression of WWOX alone with *hh-gal4* results in WWOX localisation to areas complementary to the DAPI stained nuclei of eye-imaginal discs of wandering third instar larvae. **(E-H)** Ectopic expression of WWOX in the presence of ectopic Egr/TNFα expression also results in cytoplasmic localisation of WWOX. **(I)** Control disc showing ectopic expression in the posterior region of wing discs using *hh-GAL4* visualized by co-expression of GFP. **(J)** Caspase 3 staining revealed low levels of apoptosis in control imaginal wing discs. **(K)** Ci staining of control discs show staining of the anterior compartment and is complementary to the region of GFP expression. **(L)** Merged image where GFP is green, Caspase 3 staining is magenta and Ci is yellow. **(M-M')** 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. **(N-N')** 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). **(O-O')** Staining of the anterior compartment with Ci reveals expression beyond the boundary and overlapping with the region of GFP expression. **(P-P')** 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 M'-P'.

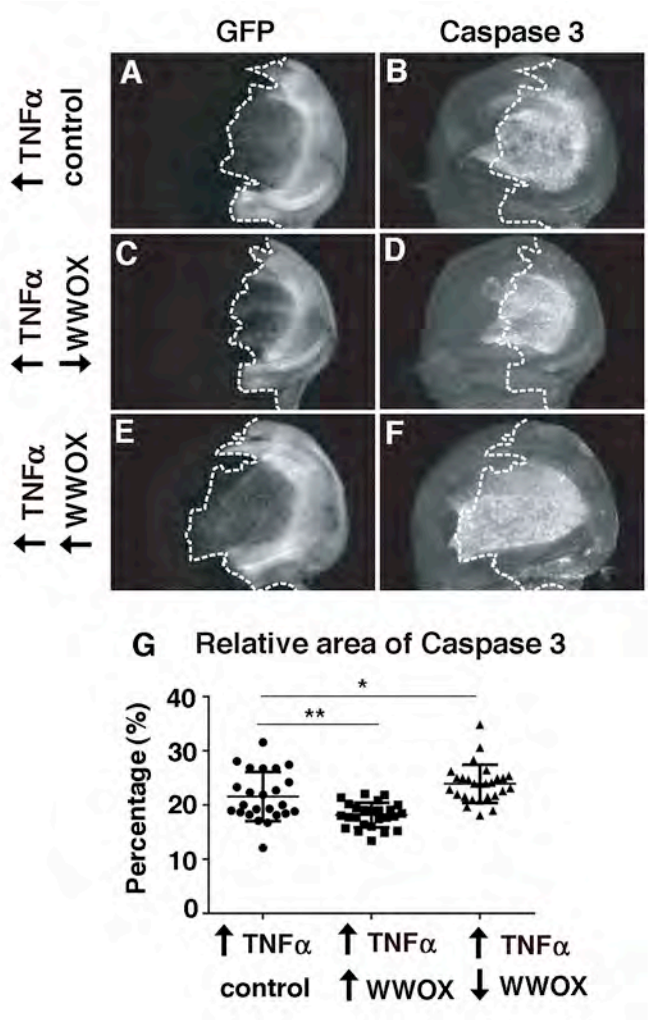

Figure 5. WWOX modifies Caspase3 staining in wing pouch in response to ectopic Egr/TNFα. **(A)** Ectopic expression of GFP and Eiger/TNFα with *hh-GAL4* in the posterior portion of wing discs of wandering third instar larvae, GFP showing the regions of ectopic expression. **(B)** Caspase 3 staining reveals 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 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 *=$p<0.05$, **=$p<0.005$.

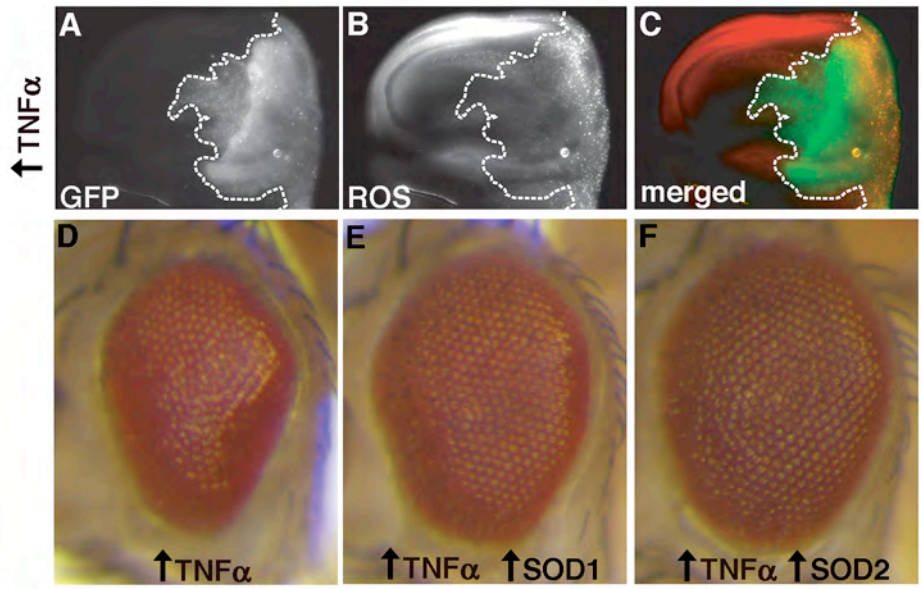

Figure 6. Ectopic expression of Egr/TNFα gives increased ROS and is suppressed by increased SOD activity. (A) Ectopic expression of GFP and Egr/TNFα with *hh-GAL4* in the posterior portion of wing discs of wandering third instar larvae, GFP showing the regions of ectopic expression (outlined by dotted line). **(B)** Puncate CellRox® staining revealed increased ROS levels at posterior edge of disc.

(C) Merged image with GFP in green and CellRox® in red. **(D)** Ectopic expression of Egr/TNFα in the eye (GMR>*egr*$^{+w}$>*EV*) results in a decrease in eye size and disruption to ommatidial patterning. **(E-F)** The ectopic Egr/TNFα phenotype is suppressed by increased levels of SOD1 or SOD2.

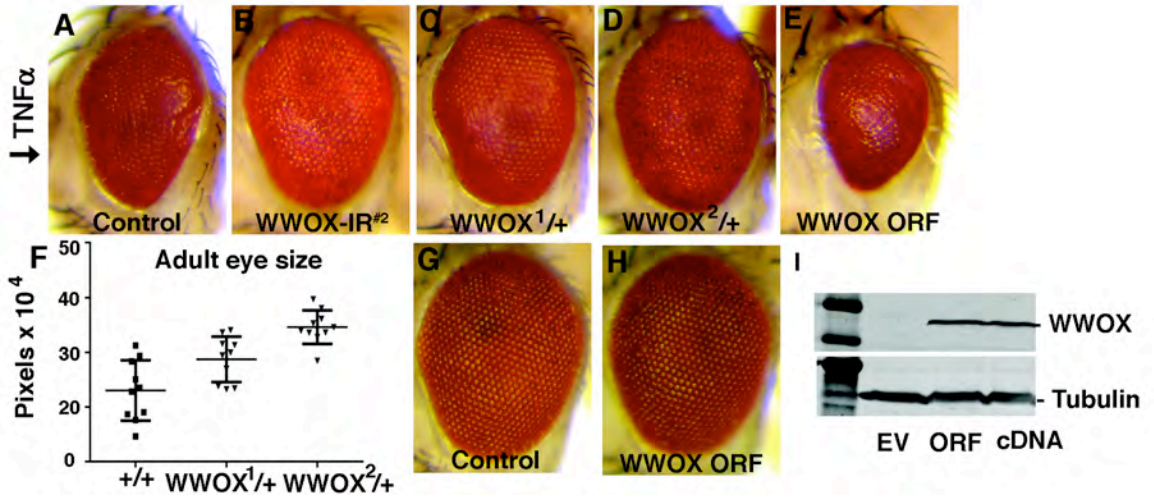

Figure S1. Altered WWOX modifies ectopic Egr/TNFα-mediated eye phenotype. (A) Ectopic expression of Egr/TNFα (GMR>*egr*$^{+w}$>+) results in a decrease in eye size and disruption to ommatidial patterning. **(B)** Decreased expression of WWOX by RNAi knockdown (*GMR>egr*$^{+w}$*>WWOX-IR*$^{\#2}$) resulted 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)** Wild-type control eye *(GMR>EV)* showing regular ordered arrays of ommatidia. **(H)** 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. **(I)** Western blot analysis of the relative levels of WWOX protein expressed in each of the ectopic expression lines compared to an α-tubulin control.

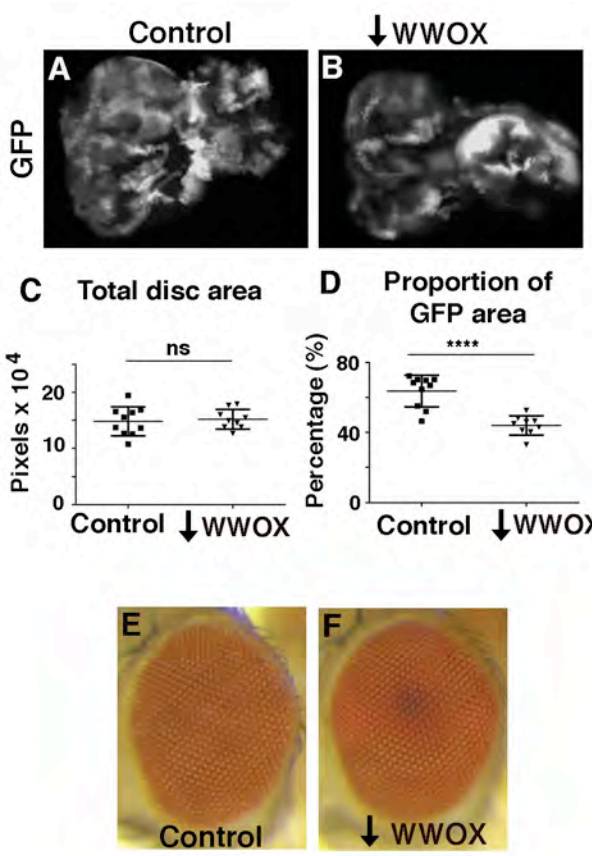

Figure S2. Decreased WWOX in wild-type clones does not cause increased GFP in imaginal discs or disrupt patterning in adult eyes. (A) Control clones of wild-type cells generated in the eye using the MARCM system are positively labeled with GFP. **(B)** Clones of wild-type cells with decreased WWOX expression are positively labeled with GFP. **(C)** Quantification of total disc area of wild-type clones with and without WWOX knockdown revealed no significant difference. **(D)** Quantification of the proportion of GFP expressing cells demonstrated a significant decrease when WWOX expression was decreased in clones of cells compared to the control wild-type clones. **(E)** Clones of wild-type cells have no effect on adult eye development. **(F)** Clones of wild-type cells with decreased WWOX expression have no effect on adult eye development. Genotypes used in these experiments: Control wild-type clones (*FRT;+) = (ey-FLP1,UAS-mCD8-GFP;;tub-GAL4 FRT82B tub-GAL80/ FRT82B);* Wild-type clones with decreased WWOX expression (*FRT;WWOX-IR) = (ey-FLP1,UAS-mCD8-GFP; UAS-WWOX-IR$^{\#2}$/+;tub-GAL4 FRT82B tub-GAL80/ FRT82B).* Significance indicated by ****=p<0.0001, ns=not significant.

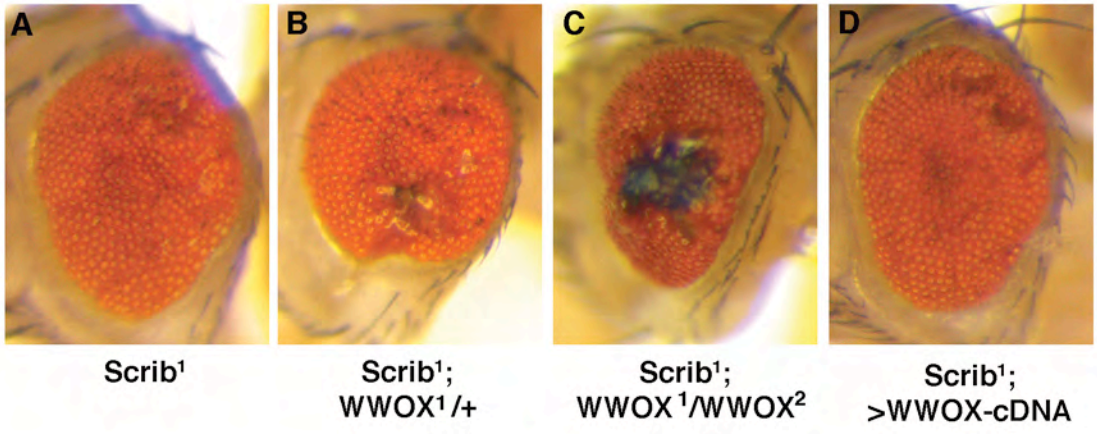

Figure S3. WWOX is required for elimination of *Scribbled (Scrib)* mutant clones in adult eyes. (A) Clones of cells mutant for *Scrib* generated in the eye using the MARCM system result in a mild adult rough eye phenotype. **(B)** Decreased WWOX expression throughout the whole animal (*Scrib*^{1}; *WWOX*^{1}*/+)* 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. **(D)** Increased expression of WWOX cDNA in the *Scrib* mutant clones was not able to obviously modify the phenotype of *Scrib* mutant clones. 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}*), Scrib*^{1}*;↑WWOX = (ey-FLP1,UAS-mCD8-GFP; UAS-WWOX cDNA/+;tub-GAL4 FRT82B tub-GAL80/ FRT82B scrib*^{1}*).*

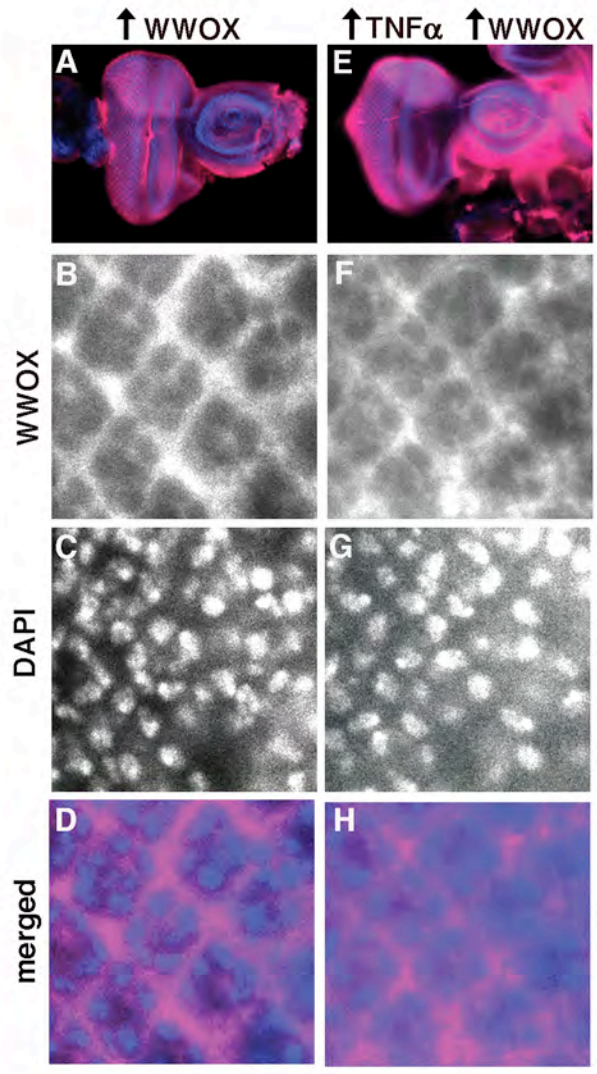

Figure S4. Ectopic Egr/TNFα has no effect on the cytoplasmical localization of WWOX in the eye imaginal disc. (A-D) Ectopic expression of WWOX alone with *GMR-gal4* results in WWOX localisation to areas complementary to the DAPI stained nuclei of eye-imaginal discs of wandering third instar larvae. **(E-H)** 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.

Chapter 6 – The effects of reducing Wwox levels in cells with chromosomal instability

This work is part of a manuscript that has been published: -
Shaukat, Z., Liu, D., Choo, A., Hussain, R., O'Keefe, L., Richards, R., Saint, R. and Gregory, S. L. (2014) Chromosomal instability causes sensitivity to metabolic stress. Oncogene, doi: 10.1038/onc.2014.344.

This work has contributed to the following results in the manuscript (Figure 1B, Figure 2A, B, C, E, E', H, H', I, I', Figure 4A, G, G', Figure 6A, Supplementary Table 1, Supplementary Figures 1B and 1C). The extent of the work is summarised below.

Summary

Chromosomal instability (CIN) is the state in which cells fail to maintain stable chromosome number and integrity due to an increased rate of gaining or losing chromosomes during cell division, resulting in aneuploidy (Geigl *et al.*, 2007). High levels of CIN are frequently observed in advanced tumours and have been associated with poor prognosis, drug resistance and cancer relapses (Carter *et al.*, 2006). However, as high levels of CIN are a common property specific to cancer cells and not normal cells, it has been proposed that selective killing of CIN cells could be used as a target for cancer therapy.

A *Drosophila* model of CIN has previously been established, in which decreased expression of a spindle assembly checkpoint protein, Mitotic arrest deficient 2 (Mad2), resulted in high levels of lagging chromosomes and chromosomal bridges that are characteristic of CIN cells (Shaukat *et al.*, 2011). Previous work using this *Drosophila* Mad2 knockdown ($Mad2^{RNAi}$) model has shown that it is possible to selectively kill CIN cells by targeting specific kinases and phosphatases (Shaukat *et al.*, 2011). These genes were found to be required for the viability of cells with a CIN phenotype but did not affect the viability of normal cells when reduced in expression. Among the kinases and phosphatases tested, a few of them were found to have a role in metabolism. This was of particular interest as altered metabolism is a hallmark of cancer cells, in which cancer cells often undergo metabolic reprogramming which provides a proliferative advantage over normal cells (Hanahan and Weinberg, 2011). The observation that the reduction in some metabolic genes can result in cell death of CIN cells specifically suggest that whilst the

metabolic reprogramming that accompanies tumourigenesis could provide a growth advantage for cancer cells, it could also occur at a cost to the overall resistance and viability of those cancer cells. Hence, it was hypothesised that a potential mechanism of selectively killing CIN cells is by exploiting the differential metabolic status of cancer cells to induce cell death specifically in those cells without affecting normal cells. The work described in the following manuscript was focussed on investigating whether CIN cells are more susceptible to metabolic stress compared to normal cells. As Wwox has been shown to have a metabolic contribution to cells, decreased expression of Wwox was tested to determine its effect in CIN cells compared to normal cells.

Using the *Drosophila Mad2*^{RNAi} CIN model that was previously established, initial work had shown that ubiquitious reduction of Wwox expression in flies with CIN resulted in lethality to the organism. As ubiquitious induction of CIN or loss of Wwox individually did not compromise the viability of the organism, the observation of the lethal phenotype caused by loss of Wwox in CIN cells indicate that CIN cells are sensitive to alterations in Wwox levels. Further investigations were then carried to identify the mechanism by which reduced Wwox levels can lead to lethality specifically in CIN cells. These investigations were performed in the *Drosophila* third instar larval wing disc using the engrailed-GAL4 (en-GAL4) promoter, which drives expression of desired transgenes in the posterior half of the disc.

In order to identify how reduced Wwox levels leads to the lethal phenotype in CIN cells, an assay was conducted to determine if reduced Wwox levels are able to result in apoptosis specifically in CIN cells. Whilst decreased expression of Mad2 and Wwox individually did not result in apoptosis as detected by acridine orange staining, high levels of staining were observed in the double knockdown (*Mad2*^{RNAi}, *Wwox*^{RNAi}) cells, indicating that loss of Wwox is able to induce apoptosis in CIN cells but not normal cells. Similar results were obtained by targeting other metabolic genes, such as IDH and antioxidant enzymes including Catalase and SOD1.

Previous work has shown that there is a functional interaction between Wwox and the mitochondrial respiratory complexes and demonstrated that altering Wwox levels has an effect in cells with mitochondrial dysfunction (Chapter 3). Cells with reduced Wwox expression were examined to determine if there is any effect on mitochondrial activity. A tetramethylrhodamine ethyl ester (TMRE) dye was used to label active mitochondria – the positively charged dye permeates cell membranes and accumulates in the negatively charged mitochondria. Decreased expression of a Complex I gene, *ND42*, results in defective mitochondria with decreased membrane potential and failure to sequester the

TMRE dye, resulting in absence of TMRE staining. Loss of Wwox by itself, on the other hand, had no visible effect on TMRE staining, indicating that loss of Wwox by itself does not have any significant impact on mitochondrial activity that was detectable using this assay. However, when tested in CIN cells, it was found that loss of Wwox resulted in high levels of TMRE staining, indicative of hyperpolarization of mitochondria. Hyperpolarization is a characteristic of overactive mitochondria and has been shown to result in oxidative stress (Terhzaz *et al.*, 2010). This result indicates that loss of Wwox is able to result in mitochondrial stress although the effects are only evident in a sensitised background and may have different consequences depending on the context.

Based on the observation that decreased Wwox levels in CIN cells result in mitochondrial stress, further experiments were carried out to determine if there is activation of an oxidative stress response and subsequently, generation of reactive oxygen species (ROS). ThiolTracker™ staining was used to detect an oxidative stress response as it stains for the presence of reduced glutathione (GSH) that is produced in response to oxidative stress whilst CellRox® staining detects the presence of ROS. Reduced expression of Wwox by itself did not result in any change in ThiolTracker™ or CellRox® staining, however reduction of Wwox levels in CIN cells led to high levels of GSH and ROS, indicative of redox stress. Similar results were again obtained with reduced expression of other metabolic genes. These results indicate that whilst such alterations in these metabolic genes have no drastic impact on normal cells, they are able to result in significant redox stress in CIN cells.

Following on from that, it was also observed that reduced Wwox levels (as well as reduced levels of other metabolic genes) resulted in DNA damage in CIN cells. Increased levels of γ-H2AX, a marker of double strand breaks, were detected in the double knockdown cells but not in the individual single knockdown cells. The DNA damage is likely to be caused by the increased levels of ROS as a consequence of the redox stress.

As targeting of metabolic genes, including Wwox, in CIN cells were found to result in oxidative stress and DNA damage, further experiments were performed to determine if the observed oxidative stress is responsible for the apoptosis of the CIN cells. Overexpression of antioxidant enzymes was able to rescue the lethality of those cells. Hence, it appears that reduction in the levels of Wwox or other metabolic genes in CIN cells results in apoptosis through generation of oxidative stress and DNA damage. Although in these experiments no oxidative stress was detectable in CIN cells alone, further investigation by increasing the level of CIN showed that these cells are already undergoing cellular stress. Targeting of these metabolic genes increased the level of

oxidative stress to a point where the CIN cells are no longer able to cope, resulting in cell death.

Concluding remarks

CIN is a common characteristic of cancer cells, which provides diversity to the tumours and can promote tumour progression as well as resistance to cancer treatment. Metabolic reprogramming is one of the ways that cancer cells are able to promote their growth and many studies have proposed using the differential metabolic status of cancer cells as a target for cancer therapy. The work described herein demonstrates that the induction of CIN in cells causes sensitivity to metabolic stress. As CIN cells have to adapt to the cellular environment in order to drive tumourigenesis, these cells are already dealing with cellular stress, rendering them more sensitive to any increase in the levels of stress compared to normal cells. Reduction in the levels of metabolic genes (including Wwox) result in high levels of mitochondrial and oxidative stress and subsequently, cell death specifically in CIN cells but not normal cells. These results support that metabolic interventions could be effective in selectively targeting CIN cells in cancer therapy.

Statement of Authorship

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Signature		Date	7/10/14

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Chapter 7 – Discussion

7.1 Summary of results

The main aim of this study was to identify novel role(s) of the non-classical tumour suppressor Wwox in cellular function. A *Drosophila* model has previously been established to study the biological function of Wwox (O'Keefe *et al.*, 2005); however, the *Drosophila* null mutants show no obvious phenotype or significant cellular dysfunction, which has made it difficult to study the role of Wwox by reverse genetics. Hence, in order to define the role(s) of Wwox in cellular function using this *Drosophila* model, a different approach was taken, which was to identify the types of cellular dysfunction that Wwox is able to modulate. Three different models of cellular dysfunction were examined in this study. As Wwox has been implicated in metabolic pathways (Aqeilan *et al.*, 2008; Aqeilan *et al.*, 2009; O'Keefe *et al.*, 2011; Dayan *et al.* 2013; Abu-Remaileh and Aqeilan, 2014), the contribution of Wwox to cellular function was examined in three different contexts that have been associated with metabolism.

The initial work of this study was focussed on identifying the types of metabolic dysfunction that can be modulated by altered Wwox levels. As altered metabolism is one of the cancer hallmarks (Hanahan and Weinberg, 2011), identification of the types of metabolic dysfunction that Wwox is able to modulate could provide better understanding of how decreased levels of Wwox can affect metabolic homeostasis and contribute to the metabolic reprogramming that often accompanies tumourigenesis. Metabolic stress was induced in *Drosophila* by targeting various metabolic genes and the level of Wwox was altered to determine if Wwox is able to modulate the resultant phenotypes. Use of the ey-GAL4 promoter to drive RNAi targeting of the metabolic genes in the developing *Drosophila* eye was found to be effective in producing distinct phenotypes in the adult eye and a scoring system was established to assess for modifications of these phenotypes.

Wwox was found to modify the phenotypes produced by the targeting of six different genes, all of which encode specific components of the mitochondrial respiratory complexes. The phenotypes produced were indicative of defects in growth, differentiation and cell survival, which are cellular properties often associated with cancerous cells. Decreased Wwox expression enhanced the phenotype in both the adult eye and at the larval stages whilst increasing Wwox expression suppressed the phenotype, indicating that Wwox is able to regulate cellular homeostasis in cells with mitochondrial dysfunction. It

was also shown, through a structure-function analysis, that the SDR enzymatic activity of Wwox is required for its regulation of mitochondrial-mediated cellular dysfunction.

Further characterisation of the mitochondrial-mediated cellular dysfunction revealed that deficiencies in the mitochondrial respiratory complexes result in such defects through high levels of ROS production and activation of the Akt signalling pathway. The mitochondrial-induced defects were regulated by altered levels of the antioxidant enzymes Catalase, Sod1 and Sod2. Decreased Akt expression and increased levels of its downstream target, Foxo was found to suppress the defects. The dysfunction was also shown to be restricted by autophagy and HIF-1∝. Wwox has previously been shown from *in vitro* studies to be associated with regulation of ROS levels, Akt signalling, HIF-1∝ binding and autophagy, hence it is possible that Wwox could contribute to the mitochondrial-mediated phenotype by modulating those processes *in vivo*.

The contribution of Wwox was also examined in a *Drosophila* model of intrinsic tumour suppression that has been shown to be regulated by metabolic activity. Scribbled (Scrib) is a cell polarity protein and loss of Scrib results in neoplastic overgrowth. However, when Scrib mutant cells are generated in clones in the developing *Drosophila* eye disc, most of the clones are eliminated by TNFα/Eiger signalling. Elimination of the clones has been demonstrated to involve metabolic activity and is mediated by the surrounding wild-type cells. Wwox was shown to be involved in the elimination of these Scrib mutant clones, with loss of Wwox resulting in higher proportions of remaining mutant clones and subsequently, a more negative effect on the organism at the end of development. This study demonstrates that Wwox plays a significant role in intrinsic tumour suppression.

The last model that was examined in this study was a *Drosophila* model of chromosomal instability (CIN). CIN, which is characteristic of many solid tumours, contributes to the diversity of cancer cells and enables them to acquire properties such as rapid proliferation, avoidance of cellular defence mechanisms and altered metabolism. CIN cells are able to cope with high levels of cellular stress, which allows them to confer tumorigenic properties; however there is data to indicate that there is a threshold to the level of oxidative stress that is tolerated by CIN cells. CIN cells were found to be sensitive to metabolic stress and reduction of Wwox levels was found to cause mitochondrial and oxidative stress leading to DNA damage and cell death specifically in CIN cells but not normal cells. This result demonstrates that whilst loss of Wwox in *Drosophila* has no obvious negative effect in cells, it has a detrimental effect in cells with some level of cellular dysfunction (resulting in high levels of oxidative stress and DNA damage) and in

this context, the reduction in Wwox levels can be manipulated to selectively kill off CIN cells without affecting normal cells.

7.2 Implications for WWOX-related research

The work from the RNAi screen has shown that altering Wwox levels has an effect in cells with mitochondrial dysfunction. It was demonstrated that in cells that are defective in mitochondrial function, loss of Wwox leads to further disruption in cellular homeostasis whereas increased Wwox expression has a protective effect. This finding could be relevant to cancer research as there is emerging evidence that deficiencies in mitochondrial respiratory complexes are able to promote tumour progression and metastasis (Sharma *et al.*, 2011; Ma *et al.*, 2013; Santidrian *et al.*, 2013). Indeed, genetic modifiers of the observed mitochondrial dysfunction (high ROS production, Akt signalling, autophagy as well as Foxo and HIF-1∝ activity) have all been shown to have a role in tumourigenesis. Wwox has also been shown from other studies to be associated with these genetic modifiers. This study appears to be consistent with the idea that in cells with tumourigenic potential, loss of Wwox could act as an enabling factor to further promote tumourigenesis whereas increasing Wwox expression could have a protective role. A recent study in mouse embryonic fibroblasts suggests that loss of WWOX can result in a switch towards enhanced glycolysis and concomitantly, reduced mitochondrial respiration (Abu-Remaileh and Aqeilan, 2014). Hence, WWOX could be involved in the regulation of the balance between glycolysis and mitochondrial respiration in cells and this could be a mechanism by which Wwox is regulating cellular homeostasis in cells with mitochondrial defects.

In addition to its association with tumourigenesis, mitochondrial defects have also been demonstrated to contribute to neuronal dysfunction in some cerebellar ataxias (Koutnikova *et al.*, 1997; Zeviani *et al.*, 2007). As inherited mutations in WWOX have recently been shown to result in autosomal recessive cerebellar ataxia and neuronal defects (Mallaret *et al.*, 2014; Abdel-Salam *et al.*, 2014; Ben-Salem *et al.*, 2014), the results demonstrating that loss of Wwox could render cells to be more sensitive to the effects of mitochondrial dysfunction may also be of some interest to this area of WWOX research.

This study also highlights that the SDR enzymatic activity of Wwox is required for its contribution to cells with mitochondrial dysfunction. Not much is currently known regarding the exact SDR enzymatic activity of Wwox, however the presence of alternatively spliced transcripts lacking exons coding for a functional SDR enzyme in

various tumours suggest that loss of the SDR enzymatic activity has a significant contribution to tumourigenesis (Paige *et al.,* 2001; Driouch *et al.,* 2002; Ishii *et al.,* 2003; Aqeilan *et al.,* 2004a; Kuroki *et al.,* 2004; Mahajan *et al*., 2005). Previous studies on WWOX have been mainly focussed on identifying binding partners of the WW domain of WWOX and little has been done to investigate the SDR enzyme activity. This work indicates that the SDR enzyme has an important contribution to WWOX function, thus the identification of the reactions and substrates catalysed by the WWOX SDR enzyme should be the focus of future investigations in Wwox-related research.

Scrib mutant clones are used as a model of intrinsic tumour suppression in *Drosophila*, as the clones are eliminated in a process mediated by their surrounding wildtype cells to prevent neoplastic growth (Brumby *et al.,* 2003; Igaki *et al.,* 2009; Kanda *et al.*, 2011). Wwox has been shown to be involved in the elimination of these tumourigenic clones, which demonstrates that Wwox plays an important role in tumour suppression. The establishment of a contribution by Wwox to this intrinsic tumour suppression model supports that *Drosophila*, in particular this model, is a good system in which to study the role of Wwox in tumourigenesis and can complement other studies that are being performed in mammalian systems.

The work that was done in the *Drosophila* CIN model illustrates that whilst the effects of having low Wwox levels is not evident in normal cells in *Drosophila,* it has been detected to have negative effects (such as increased oxidative stress and DNA damage) in CIN cells. As CIN cells are already coping with high levels of cellular stress, these negative effects are able to cause apoptosis of those CIN cells. Similar results were observed with other metabolic genes, which provides support that metabolic interventions could be effective in selectively targeting CIN cells in cancer therapy.

7.3 Limitations of this study

The main limitation of this study has been the lack of phenotype or cellular dysfunction caused by loss of Wwox alone in *Drosophila*. Whilst loss of Wwox has a drastic effect in mice, the effect of low Wwox expression in *Drosophila* appears to be subtle in a wild-type background and is only evident in a sensitised background. Alterations to Wwox levels in a wild-type background do not show any detectable cellular dysfunction, hence it is difficult to study its contribution to cellular function. This study was thus aimed at identifying the types of cellular dysfunction that Wwox can modulate, with the basis of the study being phenotypes produced by other genes. However, these phenotypes are not fully penetrant and the modification of the phenotypes by Wwox is by altering the proportion of flies with the phenotype and not by producing a different or additional phenotype. Whilst these analyses have allowed us to determine whether Wwox has an effect on those phenotypes and have provided novel contributions regarding the cellular function of Wwox, studying the phenotypes does not provide information of the exact mechanism(s) in which Wwox affects those phenotypes. Hence, part of the work was focussed on identifying other modifiers of the phenotype – this informs the direction of future studies which will be aimed at examining the effects of Wwox on the function of the identified modifiers. Although this *Drosophila* model has provided novel insight into Wwox function, it should be complemented by work done in mammalian systems, particularly cancer cell lines to demonstrate the involvement of the identified contributions to tumourigenesis.

RNAi targeting has been used widely in this study to decrease expression and subsequently the function of various genes. The use of RNAi is quite standard for screening large number of genes in *Drosophila*, however there is no guarantee that the level of knockdown by RNAi targeting is sufficient to affect the function of the gene. A negative result is not indicative of a lack of contribution by that gene and cannot be used to rule out the participation of that gene to the pathway in question. Thus, whilst positive results from an RNAi screen are of significance, the negative results do not rule out the possibility of any interaction. In those cases where interactions were observed, the efficiency of the RNAi lines to specifically target the particular gene of interest should be demonstrated and the likelihood of off-target effects need to be taken into consideration. In this study, independent RNAi lines were used when possible, either to target the same gene or to target different genes that have the same function or are involved in the same pathway or protein complex. Furthermore, many of the lines have previously been used

and shown to be efficient in targeting the particular gene. There are also fairly effective algorithms established to predict the likelihood of off-target effects (Dietzl *et al.*, 2007). Nonetheless, loss-of-function mutations should also be used to validate the results. For many genes, however, there are no mutations available and the generation of loss-of-function mutations require a considerable effect (although this should be significantly easier in the very near future with the establishment and current optimisation of the CRISPR/CAS technology in *Drosophila*). Another issue with using mutant lines is that homozygous or transheterozygous mutations of essential genes are often lethal to the organism, thus preventing such analyses and heterozygous mutants can only be used in cases of haploinsufficiency.

7.4 Future directions

This study has identified three different models of cellular dysfunction that can be modulated by Wwox. These models can form the basis of future studies to define the exact mechanism(s) by which Wwox is able to regulate cellular function in *Drosophila.* In the first model, Wwox was shown to be involved in regulating cellular homeostasis in cells with mitochondrial dysfunction through its SDR enzymatic activity. Further understanding of the biological reaction(s) that is catalysed by the Wwox SDR enzyme as well as its substrates could provide insight into how loss of Wwox could lead to tumourigenesis and could contribute to the design of therapeutic targets to improve the prognosis of cancer patients with low Wwox levels. This study has also identified several other genetic modifiers of the mitochondrial dysfunction phenotype. Further investigations can be carried out to determine if Wwox is able to modulate the mitochondrial dysfunction through interactions with these other genetic modifiers. Preliminary work stemming from this study is suggestive of Wwox having a functional interaction with the transcription factor Foxo, however future studies will need to be done to verify a functional relationship. Wwox has not previously been associated with Foxo, hence identification of a functional interaction between Wwox and Foxo would be novel. Foxo is thought to act as a tumour suppressor through its regulation of various genes involved in cell cycle arrest, DNA damage repair and detoxification of ROS, thus identified interactions between Wwox and Foxo could be relevant to tumourigenesis.

Wwox was also shown to have a role in a *Drosophila* model of intrinsic tumour suppression by reducing the elimination of Scrib mutant clones from the developing

Drosophila eye. Other work has shown that the elimination of Scrib mutant clones also requires other metabolic genes and proposed an association between altered metabolism and sensitivity to cell death in cancer cells (Kanda *et al.*, 2011). It is thought that these metabolic genes could contribute to the elimination of Scrib mutant clones through regulation of ROS or ATP levels. Hence, further work could be performed to determine if reducing Wwox in the Scrib mutant clonal system has any effect on ROS or ATP levels in that context and whether such metabolic alterations are responsible for the ability of tumourigenic cells to evade cell death in the absence of Wwox.

7.5 Concluding remarks

Since the identification of *WWOX* as the gene that spans the *FRA16D* fragile site in 2001, numerous studies have reported reduced levels of WWOX in various types of cancer as well as the association of low WWOX levels with a higher risk of developing cancer and poorer prognosis for patients. However the contribution of WWOX to tumourigenesis has not been fully delineated, thus many studies have been focussed on investigating the cellular contribution of WWOX in order to elucidate its role in cancer cell biology. *Drosophila* has been used as a model system to identify the conserved function(s) of WWOX that are of primary biological significance. Although the lack of a phenotype has been limiting in the study of Wwox function in *Drosophila*, nonetheless this study has identified three models of cellular dysfunction that Wwox has a contribution to and has defined important roles for Wwox in cellular function. Significantly, this study has shown that the SDR enzymatic activity of Wwox has an essential role in the maintenance of cellular homeostasis. Whilst there have been many WWOX structure function analyses performed *in vitro*, none of them have identified such a crucial contribution of the WWOX SDR enzyme. This study thus highlights key contributions resulting from an *in vivo* model for WWOX. This *Drosophila* Wwox model can be used for further analyses of its SDR enzyme, through a combination of biochemical analyses, such as metabolomics, and genetic manipulation to identify its substrates and enzymatic activity. In the past 15 years since the identification of the WWOX gene, there has been slow but steady progress in the research towards understanding the contribution of WWOX to tumourigenesis. However, combined with the available WWOX cancer cell lines and mouse mutant models, this *Drosophila* model holds promise to be a powerful tool in elevating WWOX research to the next level – the identification of targets (such as its endogenous substrates) that will aid in

the design of therapeutics to improve the prognosis of cancer patients with low WWOX levels.

Appendix A

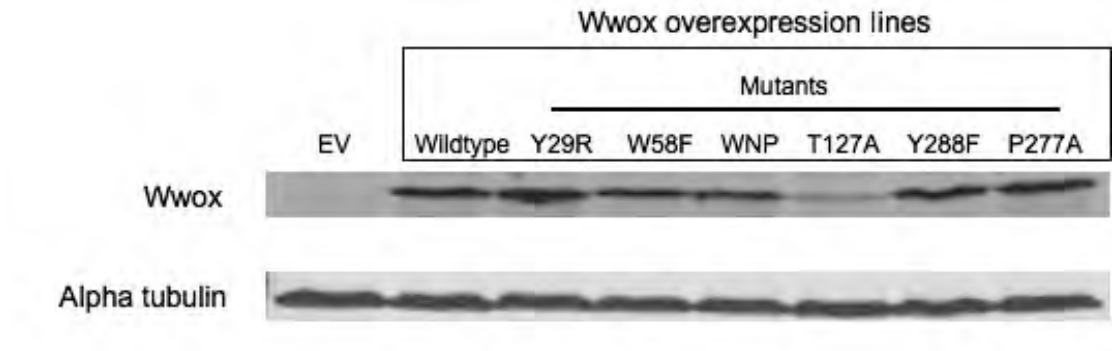

Figure A1. Additional Western blot analysis of the six different *Drosophila* ectopic Wwox mutant lines for verification of Wwox protein levels (Supporting figure for Figure 2.5). A second Western blot analysis was performed using a different set of protein preps. A lower level of ectopic Wwox protein was again observed for the T127A mutant compared to the wild-type control as well as compared to all the other mutant lines. A decrease in protein level for the WNP mutant is not as significant in this Western blot analysis compared to that previously observed in Figure 2.5.

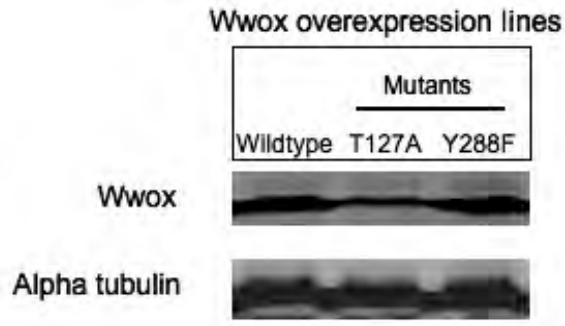

Figure A2. Additional Western blot analysis to verify the levels of ectopic Wwox protein in the two SDR enzymatic region mutant lines (Supporting figure for Figure 2.5 and Figure A1). A third Western blot analysis was performed using a different set of protein preps. A lower level of ectopic Wwox protein was again observed for the T127A mutant line compared to the wild-type control as well as compared to the other line carrying a different mutation in the SDR enzymatic region (Y288F).

Appendix B

Table B1. Summary of the *in vivo* RNAi screen testing for cellular dysfunction (reduced viability) that is modulated by *Wwox* using the da-GAL4 driver.

	Gene targeted		RNAi line	Molecular function/pathway	Decreased viability caused by ubiquitious knockdown of gene expression through RNAi targeting	
					Reduced expression of candidate gene alone	Modification by altered Wwox levels
TCA cycle (includes processes converging in and out of the TCA cycle)						
1	*CG1516*		v105936	Pyruvate carboxylase activity	No phenotype	No phenotype
2	*CG3127*	*Phosphoglycerate kinase (Pgk)*	v110081	Phosphoglycerate kinase activity	Inconsistent reduction in viability	Indeterminable (due to inconsistency)
3	*CG7010/ l(1)G0334*		v107209	Pyruvate dehydrogenase (acetyl-transferring) activity	Lethal	No modification
4	*CG8808*	*Pyruvate dehydrogenase kinase (Pdk)*	v106641	Pyruvate dehydrogenase (acetyl-transferring) kinase activity	No phenotype	No phenotype
5	*CG9709*	*Acyl-coenzyme A oxidase at 57D distal (Acox57D-d)*	v106733	Acyl-CoA oxidase activity	No phenotype	No phenotype
6	*CG12262*		v107820	Acyl-CoA dehydrogenase activity	No phenotype	No phenotype
7	*CG9390*	*Acetyl Coenzyme A synthase (AcCoAS)*	v100281	Acetate-CoA ligase activity	No phenotype	Indeterminable
8	*CG3861*	*Knockdown(kdn)*	v107642	Citrate (Si)-synthase activity	Lethal	No modification
9	*CG9244*	*Aconitase(Acon)*	v103809	Aconitate hydratase activity	Inconsistent reduction in viability	Indeterminable

10	*CG12233*	*lethal (1) G0156*	v106091	Isocitrate dehydrogenase (NAD+) activity	Lethal	No modification
11	*CG7755*		v25544	Isocitrate dehydrogenase (NAD+) activity	No phenotype	No phenotype
12	*CG32026*		v30432	Isocitrate dehydrogenase (NAD+) activity	No phenotype	No phenotype
13	*CG7176*	*Isocitrate dehydrogenase*	v42915	Isocitrate dehydrogenase (NADP+) activity	Lethal at 25°C, reduced viability/lethal at 18°C	No modification at both temperatures
			v42916		Reduced viability	No modification
14	*CG3483*		v101958	Isocitrate dehydrogenase (NAD+) activity	No phenotype	No phenotype
15	*CG1065*	*Succinyl coenzyme A synthetase a subunit (Scsa)*	v107164	Succinate-CoA ligase (GDP-forming) activity	Lethal	No modification
16	*CG17246*	*Succinate dehydrogenase A (SdhA)*	v110440	Succinate dehydrogenase (ubiquinone) activity; Mitochondrial electron transport (Complex II)	Lethal at 25°C, no phenotype at 18°C	No modification at both temperatures
17	*CG6666*	*Succinate dehydrogenase C (SdhC)*	v6031	Succinate dehydrogenase activity; Mitochondrial electron transport (Complex II)	Lethal (at 25°C and 18°C)	No modification at both temperatures
18	*CG4094/ l(1)G0255*		v105680	Fumarate hydratase activity	Lethal (at 25°C and 18°C)	No modification at both temperatures
19	*CG5889*	*Malic enzyme b (Men-b)*	v100812	Malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) activity	Reduced viability or lethal (inconsistent)	Indeterminable due to inconsistency
Oxidative phosphorylation/Cell redox homeostasis						
20	*CG3896/ CG34399*	*NADPH oxidase (Nox)*	v102559	Oxidoreductase activity; calcium ion binding	No phenotype	No phenotype

21	*CG3944*	*NADH:ubiquinone reductase 23kD subunit precursor (ND23)*	v21748	NADH dehydrogenase (ubiquinone) activity; Mitochondrial electron transport (Complex 1 of ETC)	Lethal	No modification
22	*CG6343*	*NADH:ubiquinone reductase 42kD subunit precursor (ND42)*	v14444	NADH dehydrogenase (ubiquinone) activity; Mitochondrial electron transport (Complex 1 of ETC)	Lethal	No modification
23	*CG2286*	*NADH:ubiquinone reductase 75kD subunit precursor (ND75)*	v100733	NADH dehydrogenase (ubiquinone) activity; Mitochondrial electron transport (Complex 1 of ETC)	Lethal	No modification
24	*CG2014*		v108457	NADH dehydrogenase (ubiquinone) activity; Mitochondrial electron transport (Complex 1)	Lethal	No modification
25	*CG12140*	*Electron-transfer flavoprotein-ubiquinone dehydrogenase (ETFDH)*	v15508	Electron-transferring-flavoprotein dehydrogenase activity	Lethal	No modification
26	*CG7580*	*Ubiquinol-cytochrome c reductase subunit 8*	v101371	Ubiquinol-cytochrome-c reductase activity; Mitochondrial electron transport (Complex III)	Lethal	No modification
27	*CG4181*	*Glutathione S transferase D2 (GstD2)*	v109125	Glutathione transferase activity	Lethal at 25°C, reduced viability at 18°C	No modification at both temperatures
28	*CG5826*	*Peroxiredoxin 3 (Prx3)*	v105619	Thioredoxin peroxidase activity	No phenotype	No phenotype

29	*CG7834*		v110434	Electron carrier activity/oxidative phosphorylation	Lethal at 25°C, no phenotype at 18°C	No modification at both temperatures
30	*CG1633*	*Thioredoxin peroxidase 1*	v109125	Glutathione transferase activity	Reduced viability	No modification
Other related metabolic processes						
31	*CG17654*	*Enolase*	v110090	Phosphopyruvate hydratase activity / glycolysis	Lethal	No modification
32	*CG12390*	*defective in the avoidance of repellents (dare)*	v38682	NADPH-adrenodoxin reductase activity; ferredoxin-NADP+ reductase activity; electron carrier activity	Lethal	No modification
33	*CG14816*	*Phosphoglycerate mutase 5 (Pgam5)*	v110219	Phosphoprotein phosphatase activity	No phenotype	No phenotype
34	*CG8193*	*Prophenoloxidase 2 (PPO2)*	v26843	Phosphoglycerate kinase activity	No phenotype	No phenotype
35	*CG2621*	*Shaggy(sgg)/ GSK-3β*	v7005	Protein serine/threonine kinase activity	No phenotype	No phenotype
			v101538		Lethal	No modification

Table B2. Summary of the *in vivo* RNAi screen testing for cellular dysfunction that is modulated by *Wwox* using the hh-GAL4 driver.

	Gene targeted		RNAi line	Molecular function/pathway	Decreased survival/wing phenotypes caused by knockdown of gene expression in all posterior cells through RNAi targeting	
					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*	*Glucose-6-phosphatase*	v7261	Glucose-6-phosphatase activity	No phenotype	No phenotype
2	*CG7070*	*Pyruvate kinase*	BL35165	Pyruvate kinase activity	Short crinkled wing	No clear modification
3	*CG17725*	*Phosphoenolpyruvate carboxykinase (Pepck)*	v20529	Phosphoenolpyruvate carboxykinase (GTP) activity	No phenotype	No phenotype
4	*CG7010/ l(1)G0334*		BL33721	Pyruvate dehydrogenase (acetyl-transferring) activity	No phenotype	No phenotype
5	*CG7430*		v106126	Dihydrolipoyl dehydrogenase activity	Small crinkled wings	No clear modification
6	*CG8808*	*Pyruvate dehydrogenase kinase (Pdk)*	BL28635	Pyruvate dehydrogenase (acetyl-transferring) kinase activity	No phenotype	No phenotype
7	*CG9390*	*Acetyl Coenzyme A synthase (AcCoAS)*	v100281	Acetate-CoA ligase activity	Small crinkled wings	No modification
8	*CG3861*	*Knockdown(kdn)*	v107642	Citrate (Si)-synthase activity	Small crinkled wings	No modification
9	*CG9244*	*Aconitase(Acon)*	BL34028	Aconitate hydratase activity	No phenotype	No phenotype
10	*CG5028*		v103834	Isocitrate dehydrogenase (NAD^+) activity	Short crinkled wings	No modification
11	*CG4094/ l(1)G0255*		v105680	Fumarate hydratase activity	Small crinkled wings	No clear modification

12	*CG5889*	*Malic enzyme b (Men-b)*	BL35486	Malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) activity	No phenotype	No phenotype
13	*CG10120*	*ME3*	v104016	Malate dehydrogenase (decarboxylating) (NADP+) activity	No phenotype	No phenotype
14	*CG11198*	*Acetyl-CoA carboxylase (ACC)*	BL34885	Acetyl-CoA carboxylase activity	Lethal	No modification
15	*CG11661*	*Neural conserved at 733F*	BL33686	Oxoglutarate dehydrogenase (succinyl-transferring) activity	Reduced viability	No modification
16	*CG3523*	*Fatty acid synthase (FASN)*	BL35775	Fatty acid synthase activity	No phenotype	No phenotype
			BL28930		No phenotype	No phenotype
Oxidative phosphorylation/Cell redox homeostasis						
17	*CG3896/ CG34399*	*NADPH oxidase (Nox)*	v102559	Oxidoreductase activity; calcium ion binding	No phenotype	No phenotype
18	*CG31884*	*Trx-2*	BL34019	Disulfide oxidoreductase activity	No phenotype	No phenotype

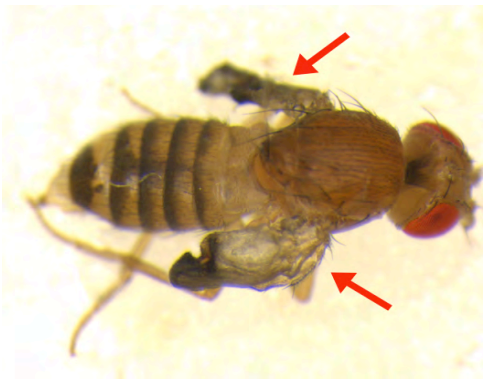

Figure B1. **Crinkled wing phenotype (indicated with red arrows) observed with the targeting of metabolic genes using the hh-GAL4 driver.** The metabolic genes that resulted in such phenotypes when targeted are listed in Table B2. It was found that any modification to such a phenotype was difficult to determine unless there was an obvious rescue of the phenotype or if it resulted in complete lethality. Modifications that had slight effects were difficult to determine.

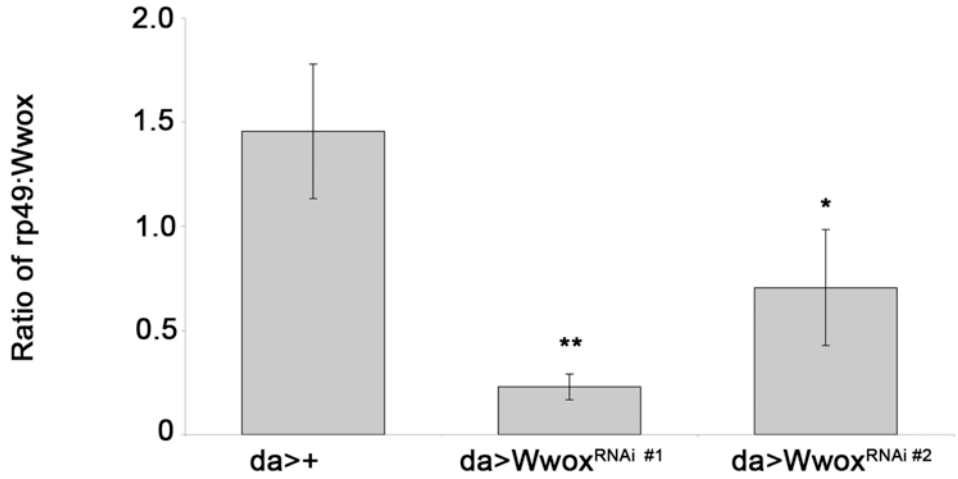

Figure B2. qRT-PCR levels of *Wwox* transcript in flies ubiquitiously expressing RNAi constructs targeting the *Wwox* gene. The da>*Wwox*$^{\text{RNAi #1}}$ flies (which were expressing construct v108350) had approximately 85% reduction in *Wwox* transcript levels compared to the da>+ control flies (which were not expressing any RNAi constructs targeting *Wwox*) (**p=0.003). The da>*Wwox*$^{\text{RNAi #2}}$ flies expressed construct v22536 and were detected to have approximately 50% reduction in *Wwox* transcript levels in this experiment (*p=0.038).

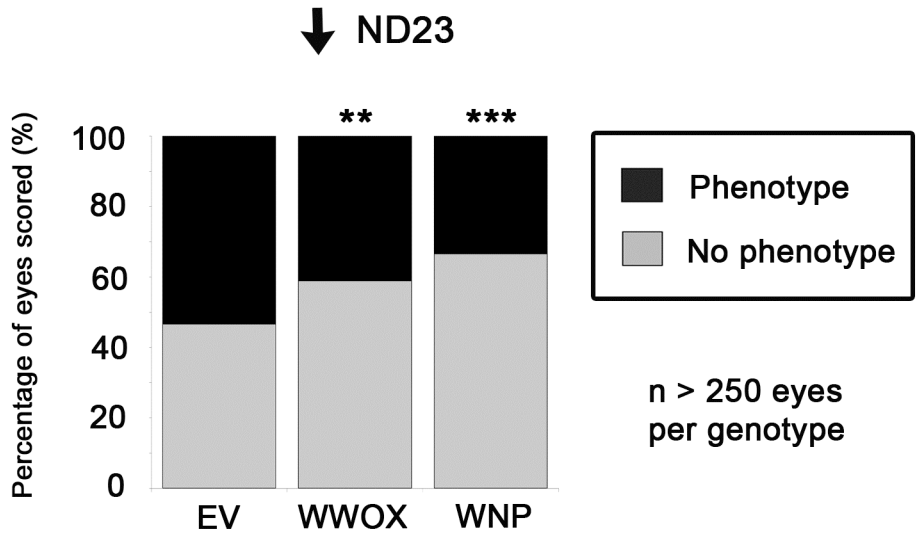

Figure B3. The WNP triple mutation has no effect on the ectopic Wwox suppression of mitochondrial dysfunction-mediated tissue disruption. The WNP mutations consist of the W58F, N81A and P84A mutations in the 2nd WW domain of Wwox (described in Chapter 2). As seen with ectopic expression of wild-type Wwox, ectopic expression of the WNP triple mutant form of Wwox was also able to result in suppression of the tissue disruption caused by decreased ND23 expression. This demonstrates that those amino acid residues are not required for this suppressive function of Wwox. Proportion of eyes with presence or absence of phenotype were compared to the EV control. **p=0.001, *** p≤0.0001 indicative of suppression compared to EV control as determined by Fisher's exact test, statistical analyses presented in Table B3.

Table B3. Statistical analyses of the suppressive function of the WNP mutant compared to wild-type Wwox on mitochondrial defect-mediated tissue disruption in the *Drosophila* eye.

n>250 eyes per genotype	**Compared to EV**	**Compared to wild-type Wwox**
Wild-type Wwox	**Suppression** (p=0.001 **)	-
WNP mutant	**Suppression** (p<0.0001 ***)	No difference (p=0.0706)

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").

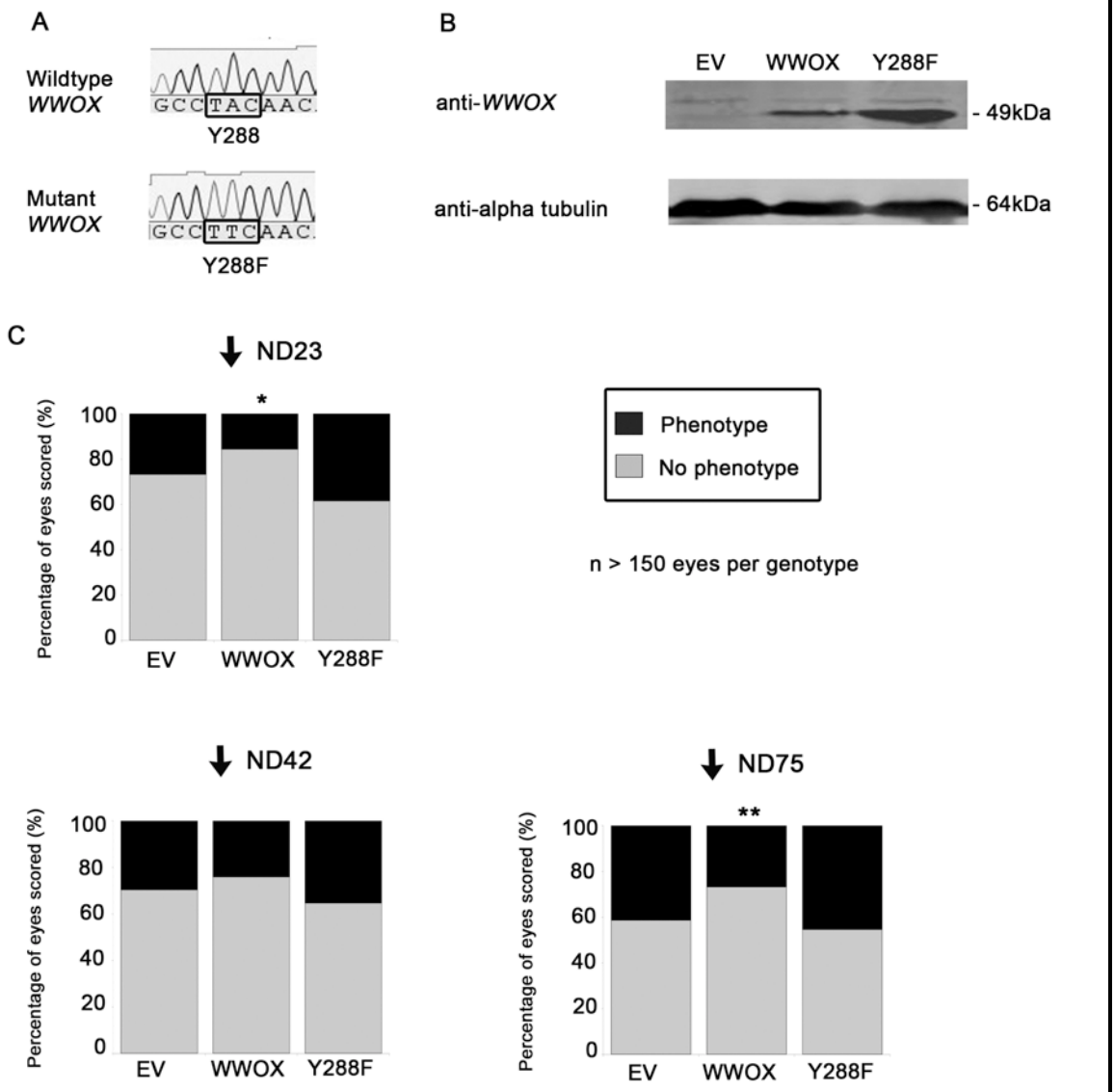

Figure B4. Differential site insertion of the ectopic SDR active site mutation (Y288F) has minimal effect on its inability to suppress the tissue disruption induced by mitochondrial defects in the *Drosophila* eye. Both the ectopic wild-type Wwox and Y288F mutation were tested for their ability to suppress the mitochondrial defect-mediated tissue disruption when inserted into a different chromosomal location (51C1) in the *Drosophila* genome. **(A)** Sequence analyses provided verification for the genotypes of the lines used in these experiments. **(B)** Western blot analysis of flies ubiquitiously expressing the Wwox constructs showed that the lines were both ectopically expressing Wwox protein. Alpha tubulin was 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 lower level of Wwox protein was observed in this wild-type Wwox line compared to the Y288F mutant (as well as compared to the wild-type Wwox expressed from the 68E1 chromosomal location shown in the manuscript). **(C)** Ectopic wild-type Wwox and the Y288F form (expressed from the 51C1 chromosomal location) were tested

for their ability to suppress tissue disruption caused by decreased expression of ND23, ND42 and ND75. *$p \leq 0.05$, **$p \leq 0.01$ indicative of suppression when compared to EV control, determined by Fisher's exact test (Table B4). Ectopic wild-type Wwox was able to significantly suppress tissue disruption caused by decreased expression of ND23 and ND75 but not ND42, although there was a trend towards a suppressive effect. This lack of suppression with ND42 differs from the result seen with the 68E1 insertion line, which could be due to the lower levels of Wwox protein expressed from the 51C1 chromosomal location that may not be sufficient to have a consistent suppressive effect. The ectopic Y288F form, on the other hand, was consistently unable to suppress the tissue disruption, similar to what was observed for the 68E1 insertion line. The Y288F line exhibited tissue disruption similar to that of the EV line and had a much stronger phenotype than that of the wild-type Wwox, even for the ND42-mediated disruption (Table B4). These results are supportive of the previous results that the SDR active site is required for the Wwox-mediated suppression of tissue disruption caused by mitochondrial defects.

Table B4. Statistical analyses of the suppressive function of the ectopic Y288F mutation compared to wild-type Wwox (when inserted into the 51C1 chromosomal location) on mitochondrial defect-mediated tissue disruption in the *Drosophila* eye.

n>250 eyes per genotype		**Compared to EV**	**Compared to wild-type Wwox**
ND23	Wild-type Wwox	**Suppression** (p=0.0115 *)	-
	Y288F mutant	**Enhancement** (p=0.0191 *)	**Difference** (p<0.0001 ***)
ND42	Wild-type Wwox	No change (p=0.2303 n.s.)	-
	Y288F mutant	No change (p=0.2120 n.s.)	**Difference** (p=0.0068 **)
ND75	Wild-type Wwox	**Suppression** (p=0.0015 **)	-
	Y288F mutant	No change (p=0.3722 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 lines tested in these experiments were all inserted into the 51C1 chromosomal location. Ectopic wild-type Wwox was able to suppress tissue disruption caused by decreased expression of ND23 and ND75 whilst the Y288F mutant form was unable to result in a suppressive effect. Rather, the phenotype observed with the Y288F form was stronger, resulting in an enhancement when tested with ND23, which has also been observed with the Y288F line in the 68E1 chromosomal location although not always consistently. As for the ND42-mediated tissue disruption, although ectopic wild-type Wwox did not result in a suppression, nonetheless, the tissue disruption observed for the ectopic Y288F mutant line was still stronger compared to the wild-type Wwox, which supports that the SDR active site is required in the regulation of the level of tissue disruption, consistent with all previous results.

Appendix C

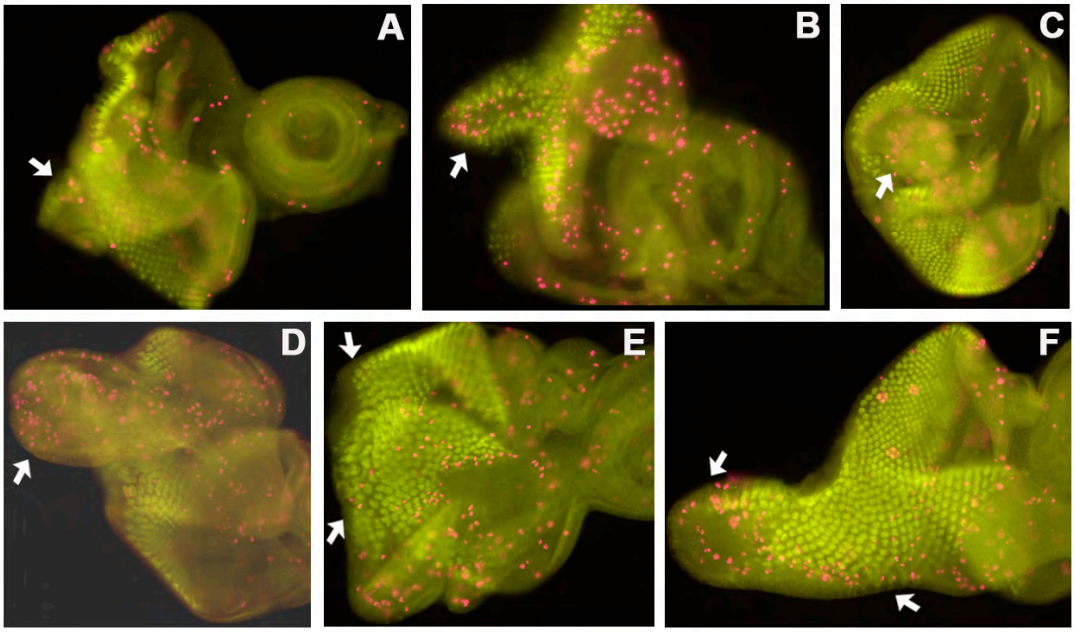

Figure C1. Additional images showing elav and pH3 staining in the affected third instar larval eye imaginal disc when mitochondrial defects were induced. Protrusions from the eye disc were observed (indicated with the white arrows). Elav staining (green) shows the presence of differentiated neurons whilst mitotic cells are marked by pH3 staining (red). Mitotic cells were observed in the protrusions in all images and presence of photoreceptor cells were clearly seen in some of the protrusions (A, B, E, F), supporting that the protrusion is an outgrowth of eye tissue. Images (**A-B**) are images of the whole eye-antennal disc whilst for images (**C-F**), only the eye portion of the eye-antennal disc is displayed as those discs were larger in size compared to wild-type discs or had longer outgrowths. (Related to Figure 4.1)

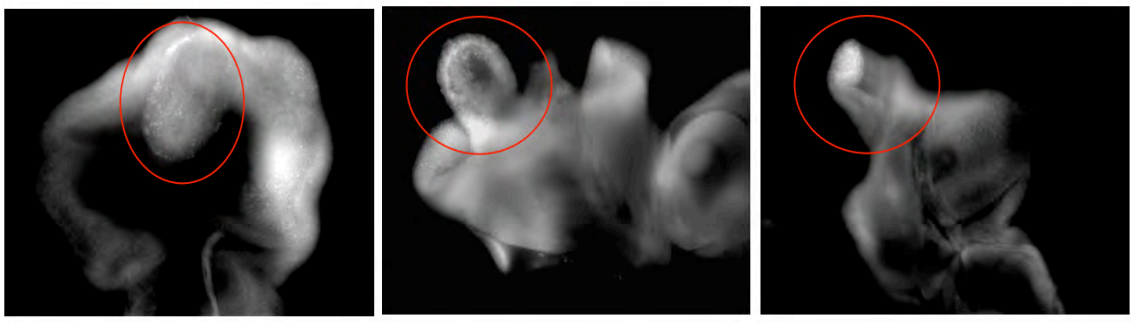

Figure C2. Additional images showing presence of ROS in the outgrowth region of the third instar larval eye imaginal discs. Punctuate CellRox® staining was detected in the outgrowth regions (circled in red) (Related to Figure 4.5)

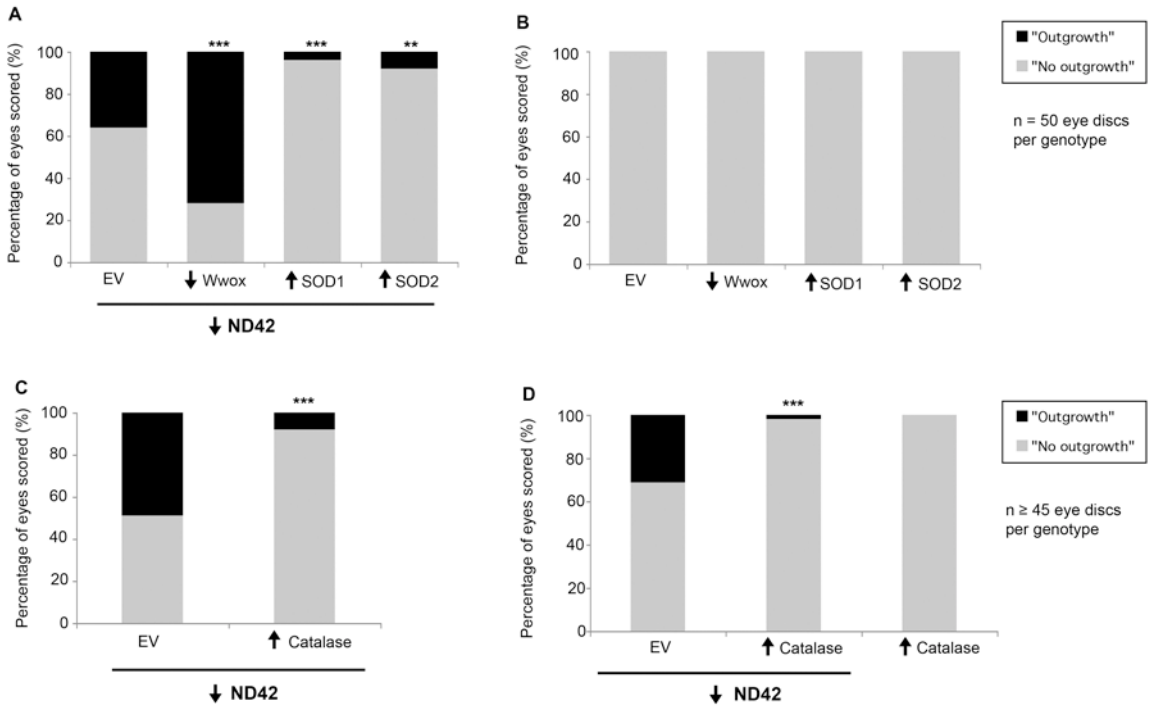

Figure C3. Additional evidence supporting that increased levels of antioxidant enzymes leads to suppression of the larval eye disc outgrowth phenotype. (A) Additional results showing that increased expression of Sod1 or Sod2 resulted in significant suppression of outgrowth phenotype caused by decreased expression of ND42 (4% and 8% respectively compared to the 36% in the EV control, **p=0.0013, ***p<0.0001). **(B)** Increased expression of Sod1 or Sod2 as well as decreased Wwox expression alone did not result in the outgrowth phenotype. **(C-D)** Increased expression of Catalase (with decreased *ND42* expression) resulted in significant suppression of the phenotype in two other independent experiments (8% and 2% compared to 49% and 31% of the respective EV controls, ***p<0.0001) while **(D)** increased expression of Catalase alone did not result in any outgrowth phenotype. Further details of the statistical analysis and number of eyes discs per genotype in Appendix C: Table C2.

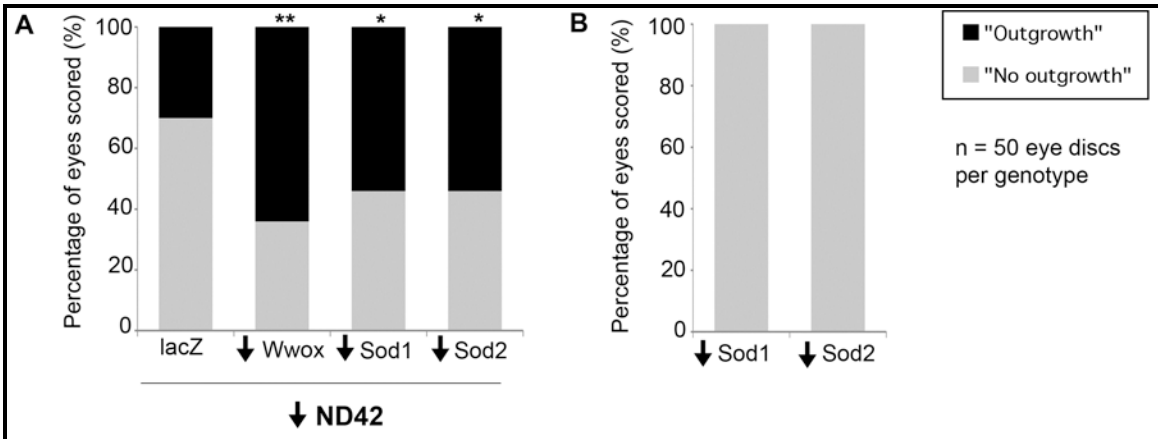

Figure C4. Further additional evidence supportive of ROS as effectors of the larval eye disc outgrowth phenotype. (A) Additional result demonstrating that reduced expression of *Sod1* or *Sod2* by RNAi targeting resulted in significant enhancement of the outgrowth phenotype caused by decreased expression of *ND42*. Reduced Sod1 or Sod2 both resulted in 54% of eye discs with outgrowth compared to the 30% in the lacZ control, *p=0.0253 (Appendix C: Table C4). **(B)** Reduced expression of Sod1 or Sod2 alone did not result in any outgrowth phenotype. n =50 eye discs per genotype for all experiments.

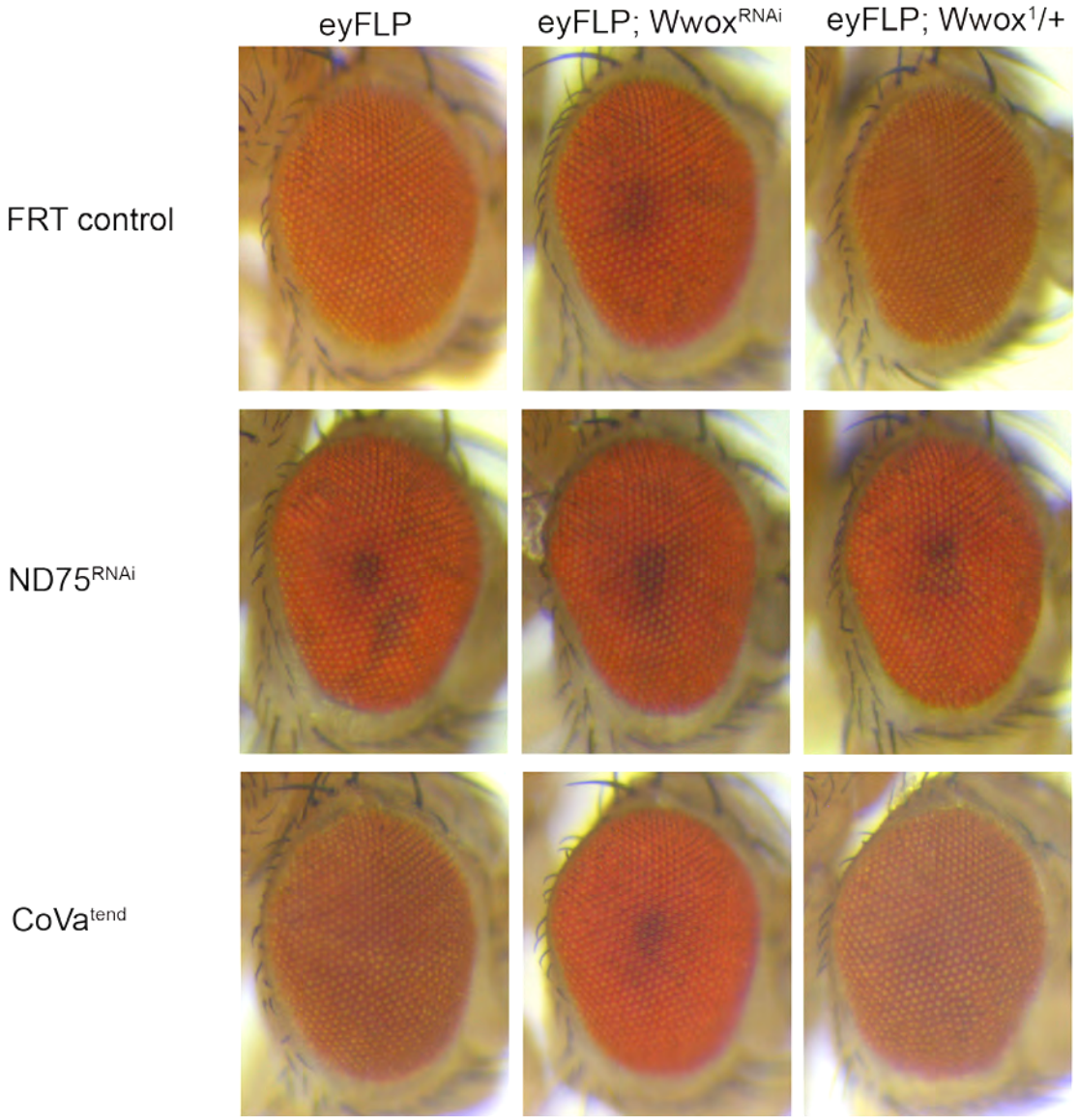

Figure C5. Inducing mitochondrial defects in clones of the developing *Drosophila* eye did not result in any observable tissue disruption. Reduced mitochondrial function was induced either by reducing expression of a Complex I gene, *ND75* in clones of the eye (by RNAi targeting) or by generating eye clones that homozygous mutant ($CoVa^{tend}$) for a Complex IV gene, *CoVa* in a CoVa heterozygous mutant fly. The absence of observable tissue disruption suggests that the previously observed phenotypes are only produced when there is mitochondrial dysfunction in the entire eye. Decreased expression of Wwox by RNAi targeting specifically in those clones ($Wwox^{RNAi}$) or loss of 1 copy of Wwox in the entire eye ($Wwox^{1}/+$) did not have any enhancing effect, indicating that the previously observed genetic interactions between Wwox and these mitochondrial respiratory chain genes are only evident when there is a level of mitochondrial dysfunction that is sufficient to cause a phenotype in the first instance.

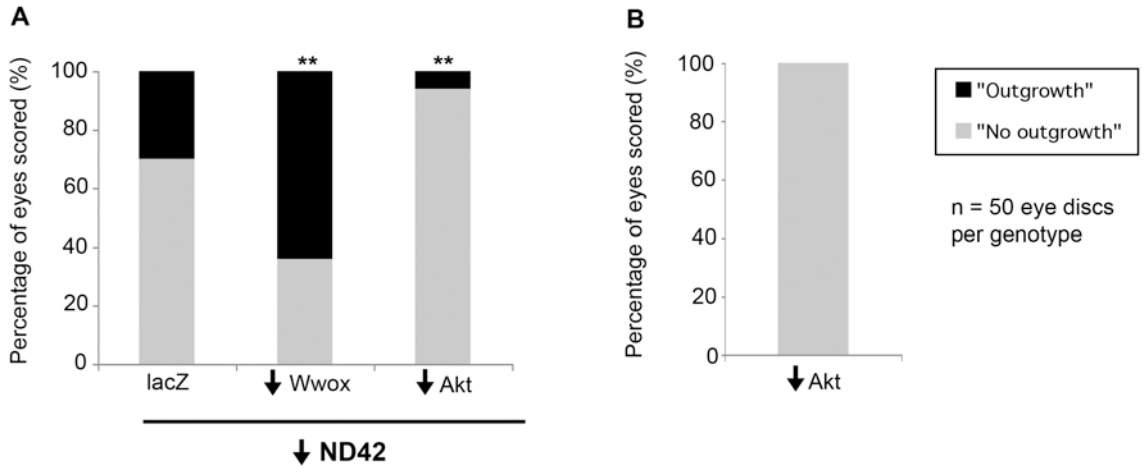

Figure C6. Additional evidence supporting that decreased Akt expression results in suppression of the larval eye disc outgrowth phenotype. (A) Additional result demonstrating that reduced expression of Akt resulted in significantly lower proportion (6%) of eye discs with the outgrowth phenotype compared to the lacZ control (30%), **p=0.0033 (Appendix C: Table C3). **(B)** Reduced expression of Akt alone did not result in the outgrowth phenotype. n=50 eye discs per genotype for all experiments.

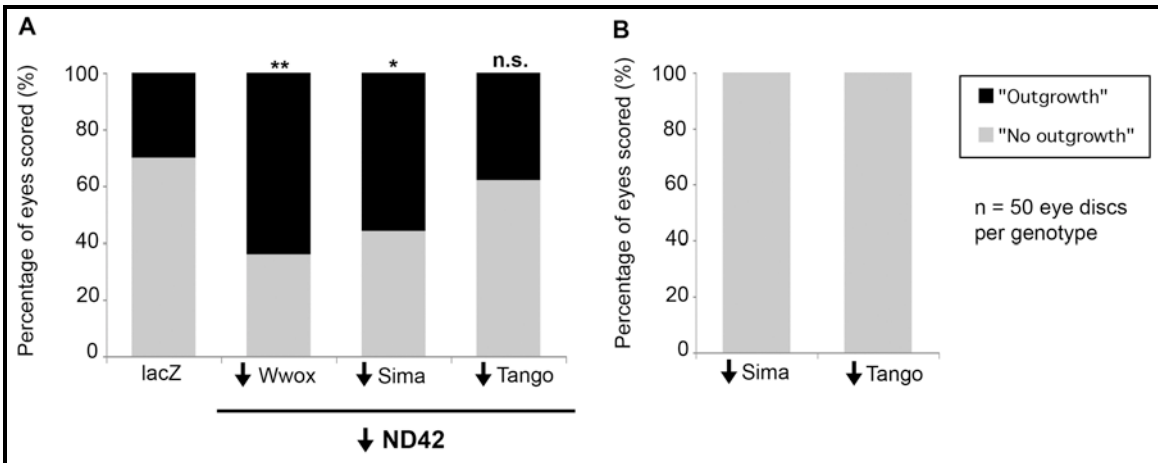

Figure C7. Additional evidence supporting that decreased Sima expression but not Tango results in enhancement of the larval eye disc outgrowth phenotype. (A) Additional result showing that reduced expression of Sima resulted in a significantly higher proportion (56%) of eye discs with the outgrowth phenotype compared to the lacZ control (30%), *p=0.0149, but reduced expression of Tango did not result in any significant change in phenotype proportions (38%) compared to the lacZ control, p=0.5269 n.s. (Appendix C: Table C3). **(B)** Reduced expression of either Sima or Tango alone did not result in any outgrowth phenotype. n= 50 eye discs per genotype for all experiments.

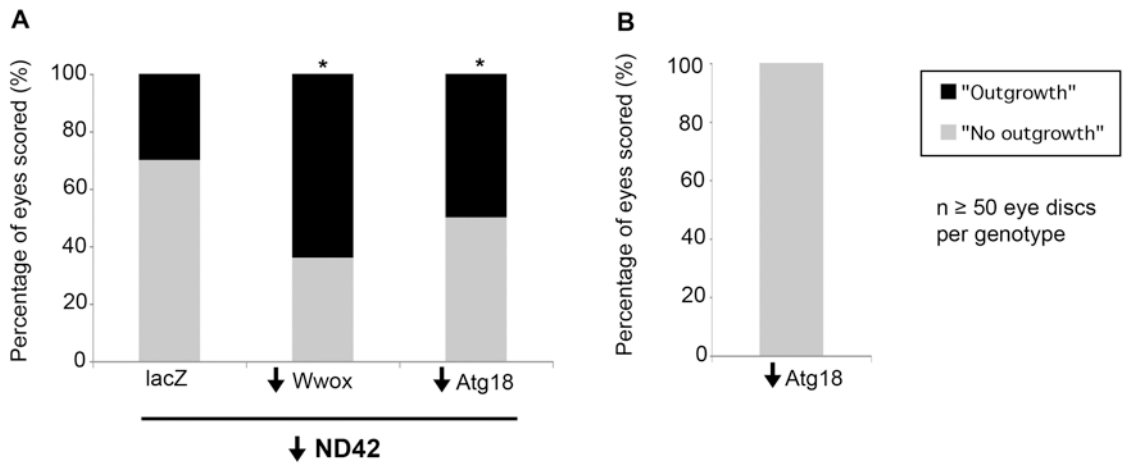

Figure C8. **Additional evidence supporting that autophagy restricts the larval eye disc outgrowth phenotype. (A)** Additional result demonstrating that reduced expression of Atg18 resulted in a significantly higher proportion (50%) of eye discs with the outgrowth phenotype compared to the lacZ control (30%), *p=0.0456 (Appendix C: Table C3). **(B)** Reduced expression of Atg18 alone did not result in the outgrowth phenotype. $n \geq 50$ eye discs per genotype for all experiments.

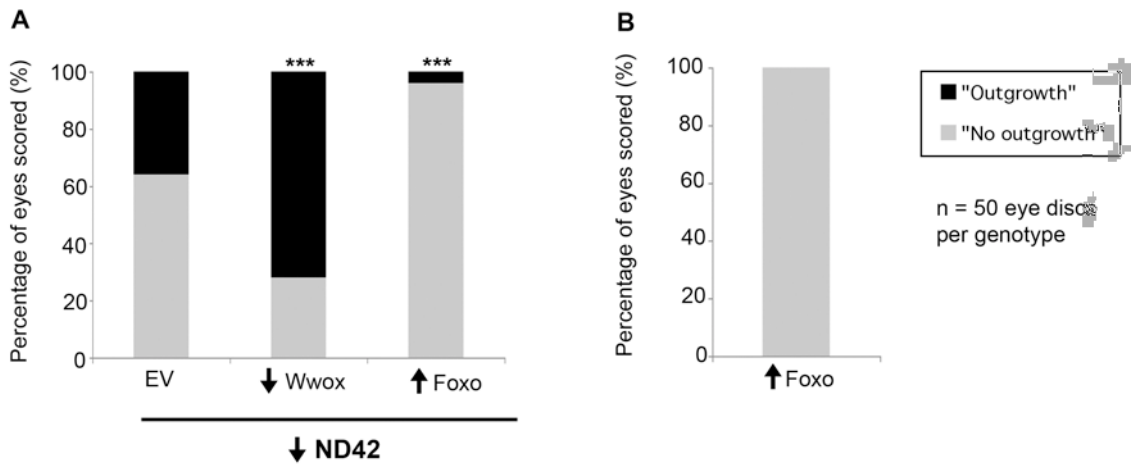

Figure C9. Additional evidence supportive of increased Foxo expression having a suppressive effect on the larval eye disc outgrowth phenotype. (A) Additional result showing that increased Foxo expression resulted in significant suppression of the outgrowth phenotype caused by decreased expression of ND42 (4% of eye discs with outgrowth compared to the 36% in the EV control, ***p<0.0001, Appendix C: Table C3). **(B)** Increased expression of Foxo alone did not result in any outgrowth phenotype phenotype. n =50 eye discs per genotype for all experiments.

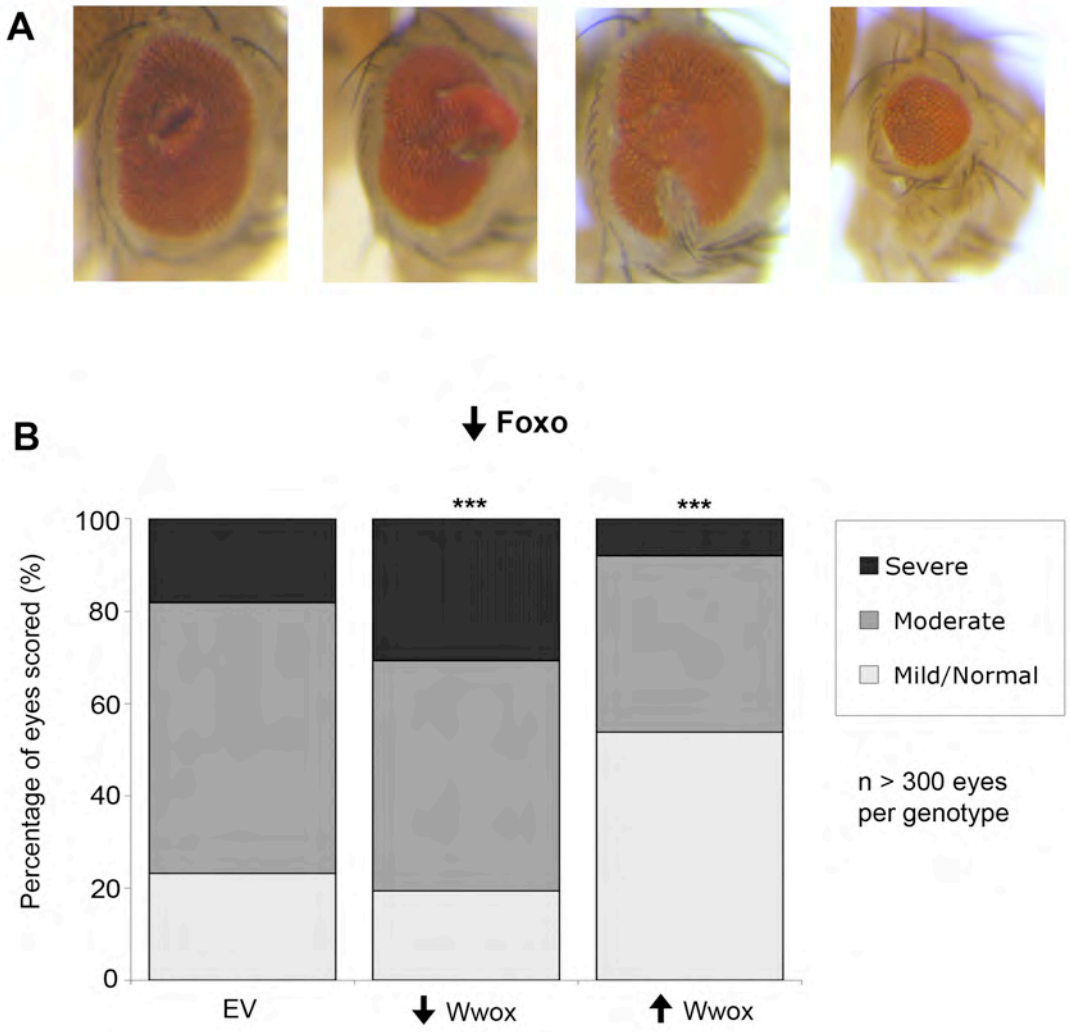

Figure C.10 Preliminary results suggesting that reduced Foxo expression by itself using RNAi targeting results in adult eye phenotypes similar to those induced by mitochondrial dysfunction and are also affected by altered Wwox expression. (A) Phenotypes observed in the adult eye, which includes the loss of photoreceptor cells forming a cavity within the eye field, outgrowth of eye tissue, presence of ectopic structures as well as severe loss of tissue resulting in decreased eye size. **(B)** Modification of the Foxo-induced phenotypes with altered Wwox expression. Decreased Wwox levels resulted in an enhancement of the phenotype, with a higher proportion of eyes displaying the severe phenotypes, ***$p \leq 0.0001$ compared to the EV control. Conversely, increased Wwox expression resulted in significantly less tissue disruption overall, ***$p < 0.0001$ compared to the EV control. n> 300 eyes per genotype. It should be noted that these results were obtained using a RNAi line that has not been verified to be efficient in specifically targeting Foxo levels, hence these results have to validated using an independent RNAi line that has been shown to be able to target Foxo specifically and efficiently.

Table C1. Statistical analyses verifying the validity of the *ey > ND42*^{RNAi} recombinant screening line and the genetic interaction between *Wwox* and *ND42* (Related to Figure 4.3).

Larval eye disc outgrowth phenotype	
n=50 eye discs per genotype	**Compared to *ey > ND42*^{RNAi} > +**
ey > ND42^{RNAi} *> Wwox*^{RNAi}	Enhancement (p=0.0006***)
ey > ND42^{RNAi} *> lacZ*^{RNAi}	No change (p=0.3558 n.s.)
ey > ND42^{RNAi} *> mCherry*^{RNAi}	No change (p=0.2414 n.s.)

Table C2. Statistical analyses of *ey > ND42*^{RNAi} modifier screen with different candidate^{cDNA} lines. (Related to Figure 4.6A, 4.11; Appendix C: Figure C3, C9).

(Larval eye disc outgrowth phenotype	
n≥50 eye discs per genotype	**Compared to *ey > ND42*^{RNAi} *> EV***
Set 1	
ey > ND42^{RNAi} *> Wwox*^{RNAi} (control)	Enhancement (p=0.0439*)
ey > ND42^{RNAi} *> UAS-Catalase*	Suppression (p<0.0001***)
ey > ND42^{RNAi} *> UAS-SOD1*	Suppression (p=0.0008***)
ey > ND42^{RNAi} *> UAS-SOD2*	Suppression (p=0.0338*)
ey > ND42^{RNAi} *> Foxo*	Suppression (p=0.0002***)
Set 2 (excluding *UAS-Catalase)* (Replicate results)	
ey > ND42^{RNAi} *> Wwox*^{RNAi} (control)	Enhancement (p=0.0006***)
ey > ND42^{RNAi} *> UAS-SOD1*	Suppression (p<0.0001***)
ey > ND42^{RNAi} *> UAS-SOD2*	Suppression (p=0.0013**)
ey > ND42^{RNAi} *> Foxo*	Suppression (p<0.0001***)
Set 2 for *UAS-Catalase* (Replicate results) (n≥45 eye discs per genotype)	
ey > ND42^{RNAi} *> UAS-Catalase*	Suppression (p<0.0001***)
Set 3 for *UAS-Catalase* (Replicate results) (n≥48 eye discs per genotype)	
ey > ND42^{RNAi} *> UAS-Catalase*	Suppression (p<0.0001***)

Table C3. Statistical analyses of *ey > ND42*^{RNAi} modifier screen with different candidate^{RNAi} lines. (Related to Figure 4.6B, 4.8, 4.9, 4.10; Appendix Figure C: C4, C6, C7, C8).

Larval eye disc outgrowth phenotype	
n≥50 eye discs per genotype	**Compared to *ey > ND42^{RNAi} > lacZ^{RNAi}***
Set 1 for SOD1, SOD2, Akt and Atg18	
ey > ND42^{RNAi} > Wwox^{RNAi} (control)	Enhancement (p=0.0001***)
ey > ND42^{RNAi} > SOD1^{RNAi}	Enhancement (p=0.0052**)
ey > ND42^{RNAi} > SOD2^{RNAi}	Enhancement (p=0.0025**)
ey > ND42^{RNAi} > Akt^{RNAi}	Suppression (p=0.0004***)
ey > ND42^{RNAi} > Atg18^{RNAi}	Enhancement (p=0.0262*)
Set 1 for Sima and Tango	
ey > ND42^{RNAi} > Wwox^{RNAi} (control)	Enhancement (p=0.0085**)
ey > ND42^{RNAi} > Sima^{RNAi}	Enhancement (p=0.0268*)
ey > ND42^{RNAi} > Tango^{RNAi}	No change (p=0.2299 n.s.)
Set 2 (Replicate results)	
ey > ND42^{RNAi} > Wwox^{RNAi} (control)	Enhancement (p<0.0001***)
ey > ND42^{RNAi} > SOD1^{RNAi}	Enhancement (p=0.0253*)
ey > ND42^{RNAi} > SOD2^{RNAi}	Enhancement (p=0.0253*)
ey > ND42^{RNAi} > Akt^{RNAi}	Suppression (p=0.0033**)
ey > ND42^{RNAi} > Atg18^{RNAi}	Enhancement (p=0.0456*)
ey > ND42^{RNAi} > Sima^{RNAi}	Enhancement (p=0.0149*)
ey > ND42^{RNAi} > Tango^{RNAi}	No change (p=0.5269 n.s.)

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