Functional characterisation of *coq8* in *Drosophila*

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Abstract

With the increasing number of novel mutations being discovered by whole genome and whole exome sequencing, functional studies are increasingly required to determine whether specific mutations are responsible for the disease phenotypes. *Drosophila*, with its vast set of genetic and molecular tools as well as robust behavioural assays, is an ideal model for functional characterisation.

Coenzyme Q biosynthesis is highly conserved from yeast to humans and involves a number of genes in the enzymatic pathway including COQ8A. The role of COQ8A in CoQ biosynthesis is not clear. However, mutations in COQ8A have been associated with autosomal recessive cerebellar ataxia, which is characterised by gait ataxia, cerebellar degeneration and CoQ_{10} deficiency. This project aimed to characterise the phenotypes resulting from the reduction of *coq8* expression (the *Drosophila* homologue of COQ8A) to develop a model of *coq8* deficiency that could be used to characterise COQ8A mutations functionally.

RNAi knockdown of *coq8* resulted in severe developmental delay, larval lethality, locomotor impairment, a decrease in ATP production, as well as developmental deficits and neurodegeneration in the *Drosophila* eye. Reintroduction of wild-type *Drosophila coq8* partially rescued the larval lethality, restored locomotor function and also primarily rescued the necrotic phenotype in the eye. This model could, therefore, be used to determine whether a specific mutation impaired function, such that it would not rescue the deficiency. As a proof-of-principle, two mutant variants of *coq8*, I295P and L520*, which were modelled on the human COQ8A mutations L277P and c.1506+1G>A (which results in a truncated protein) did not rescue the *coq8* deficiency, indicating that they disrupted normal *coq8* function. However, the reintroduction of human COQ8A did not restore function but instead exacerbated the necrotic and neurodegenerative phenotype in the eye suggesting that it may be impairing the mitochondrial function of wild-type *coq8*.

Drosophila provides the means to characterise disease-causing genetic mutations functionally. Here we have developed a model that can be used to study the role of *coq8* in *Drosophila* and have found that *Drosophila coq8* and human COQ8A differ in function.

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Dedication

Thank you for always pushing me to be a better version of myself, for always wanting me to be better and achieve so much more than I ever thought I could. Thank you for instilling the values and morals that you lived by so honourably. I just wish you were still here to see me achieve all the things you knew I could. There are a lot of things I am thankful for in my life, but none of them come close to you. I hope you knew how truly blessed I was to have had you in my life. You were the comfort I never knew I needed.

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Abbreviations

Abbreviation	Definition
%	Per cent
°C	Degrees Celsius
®	Registered
µg	Microgram
µg	Micrograms
µL	Microliters
µm	Micrometre
ADCK3	Aarf-domain containing kinase 3 (COQ8A)
ADP	Adenosine diphosphate
ARCA	Autosomal recessive cerebellar ataxia
arm	*armadillo*
ATP	Adenosine triphosphate
BSA	Bovine Serum Ablumum
C	Calyx
CABC1	Chaperone Activity of Bc1 Complex-Like
CNS	Central nervous system
CO_2	Carbon dioxide
coq8	Coenzyme Q_8 - *Drosophila*
COQ8A	Coenzyme Q_8A - human
CoQ_x	Coenzyme Q, x denotes the number of isoprenoid units which is species dependent
C-terminus	Carboxy-terminus, COOH-terminus
D42	Motor neuronal
da	*daughterless*
Dcp1	Decapping protein 1
dH_2O	Distilled H_2O
DH5α	DH5-alpha competent cells E.coli
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
dsRNA	Double-stranded RNA
ECL	Enhanced chemiluminescence

eGFP	Enhanced green fluorescent protein
elav	*Embryonic lethal abnormal visual system*
ey	*eyeless*
For	Forward
g	Grams
GAL4	Yeast-derived transcription factor
GFP	Green Fluorescent Protein
GMR	*glass multiple reporter*
HDS	Honest Significant Difference
HRP	Horseradish peroxidase
i.e.	Id est (that is)
IgG	Immunoglobulin G
IHC	Immunohistochemistry
KC	Kenyon cells
KD	Knockdown
KxGQ	Invariant motif in COQ8A
L	Litre
LB	Lysogeny broth Luria-Bertani
M	Molar
MB	Mushroom body
Mito-GFP	Mitochondrial Green Fluorescent Protein
mL	Millilitres
mM	millimolar
MMIC	Manawatu Microscopy & Imaging Centre
MRC	Mitochondrial respiratory chain
mRFP	Monomeric red fluorescent protein
MRI	Magnetic resonance imaging
mRNA	Messenger Ribonucleic acid
MTS	Mitochondrial targeting sequence
NGS	Normal goat serum
nm	Nanometre
N-terminus	Amino terminus, NH_2-terminus, amine-terminus
ORF	Open reading frame
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Triton X

PKL	Protein-kinase-like
PNS	Peripheral nervous system
PSNS	Parasympathetic nervous system
Rev	Reverse
RIPA	Radioimmunoprecipitation assay
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
rpm	Revolutions per minute
SB	Sample buffer
Sb	*Stubble*
SEM	Scanning Electron Microscope
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
ssRNA	Single-stranded RNA
TBST	Tris-buffered saline with Triton X
TE	Tris-EDTA
TF	Transcription factor
™	Trademark
UAS	Upstream activating sequence
VDRC	Vienna Drosophila RNAi Center

1 Introduction

1.1 Autosomal recessive cerebellar ataxia

Autosomal recessive cerebellar ataxias (ARCAs) are a heterogeneous group of rare inherited neurological disorders that affect the cerebellum and/or the spinal cord (Lagier-Tourenne et al., 2008; Palau & Espinós, 2006). Early-onset often occurs before 20 years of age and can include cerebellar ataxia, neurological defects, alteration to sensorimotor function, cognitive abnormalities, intellectual disability, uncoordinated gait and coenzyme Q (CoQ) deficiency (Anheim , Tranchant , & Koenig 2012; Fogel & Perlman, 2007). Affected individuals present in childhood with uncoordinated gait and cerebellar ataxia that progressively gets worse with age. The effect of CoQ deficiency can be highly heterogeneous including impairments in muscle, kidney and central nervous system (CNS) function (Luna-Sánchez et al., 2017). In some instances, CoQ deficiency is one of the more treatable effects of ARCA but has had varied responses to external supplementation of CoQ (Horvath et al., 2012; Jacobsen et al., 2017; Liu et al., 2014).

1.2 Coenzyme Q

Coenzyme Q (CoQ) is an essential component of metabolic function in all eukaryotes. It is a lipophilic molecule composed of a quinone head group and a polyisoprenoid tail, which embeds itself in the mitochondrial membrane (Doimo et al., 2014; Tauche, Krause-Buchholz, & Rödel, 2008; Tran & Clarke, 2007). The isoprenoid head group is responsible for the redox reactions involving quinone, semiquinone and hydroquinone (Dutton et al., 2000). The length of the isoprenoid tail is species dependent and most commonly contains ten repeating isoprenoid units in humans (CoQ_{10}), nine isoprenoid units in *C. elegans* (CoQ_9), eight isoprenoid units in *E. coli* (CoQ_8), and six in yeast (CoQ_6) (Fernández-Ayala, Jiménez-Gancedo, Guerra, & Navas, 2014). CoQ_{10} functions in adenosine triphosphate (ATP) production (Fernández-Ayala et al., 2014), biosynthesis of pyrimidines, modulation of apoptosis (Doimo et al., 2014), acts as an antioxidant (López-Lluch, Rodríguez-Aguilera, Santos-Ocana, & Navas, 2010) and as an electron transporter from complex I and complex II to complex III in the mitochondrial respiratory chain (MRC) (Crane, Hatefi, Lester, & Widmer, 1957;

Lagier-Tourenne et al., 2008; Turunen, Olsson, & Dallner, 2004). CoQ_{10} levels throughout the body are consistent with the dependence of the tissue for mitochondrial respiration (Lester & Crane, 1959).

Table 1.1 Table of the genes required for CoQ biosynthesis. *They are highly conserved across a number of higher organisms and are integral in roles such as cellular signalling, and CoQ biosynthesis. Gene names were obtained from https://www.yeastgenome.org/, http://flybase.org/ and https://www.ncbi.nlm.nih.gov/*

S. cerevisiae	***D. melanogaster***	**Human**
COQ1	*CG31005 (qless)* *CG10585 (pdss2)*	*COQ1A/PDSS1* *COQ1B/PDSS2*
COQ2	*CG9613*	*COQ2*
COQ3	*CG9249*	*COQ3*
COQ4	*CG32172*	*COQ4*
COQ5	*CG2453*	*COQ5*
COQ6	*CG7277*	*COQ6A* *COQ6B*
COQ7	*CG14437*	*COQ7*
COQ8	*CG32649 (coq8)*	*COQ8A* *COQ8B*
COQ9	*CG30493*	*COQ9*
COQ10	*CG9410*	*COQ10A* *COQ10B*

The biosynthesis of CoQ is orchestrated by a number of enzymes and occurs in different subcellular mitochondrial compartments (Doimo et al., 2014). The synthesis of the isoprenoid tail takes place in the cytosol in higher eukaryotes (Doimo et al., 2014) and is essential for the stability of the Q complex (He, Xie, Allan, Tran, & Clarke, 2014) which is a multi-enzyme complex containing biosynthetic enzymes and is integral for the biosynthesis of CoQ (Bentinger, Tekle, & Dallner, 2010; Casarin et al., 2008; He et al., 2014). In humans, 13 genes that are involved in the biosynthesis of CoQ (Table 1.1) each corresponding to a different step in the enzymatic pathway (Doimo et al., 2014). In yeast, *COQ4*, *COQ6, COQ7* and *COQ9* are thought to play an essential role in the formation of the Q complex (He et al., 2014). Mutations to any of the CoQ genes can result in catastrophic disease states and is broadly characterised as primary CoQ deficiency.

1.3 CoQ deficiency

CoQ deficiency is characterised as a decrease in CoQ_{10} levels in tissues and/or cells in the body and is observed in a clinically and genetically heterogeneous group of ataxias that can affect vital organs, the central and peripheral nervous systems (Casarin et al., 2008; Luna-Sánchez et al., 2017). Primary CoQ_{10} deficiency occurs when there are mutations in the genes that make up the Q complex responsible for CoQ biosynthesis (Casarin et al., 2008) whereas secondary CoQ deficiency occurs when mutations are in genes not directly associated with CoQ_{10} biosynthesis (Doimo et al., 2014). Confirmation of CoQ deficiency is most reliably determined via CoQ levels in a muscle biopsy (Rahman, Clarke, & Hirano, 2012). CoQ deficiency has a strong association with mitochondrial dysfunction (Grant, Saldanha, & Gould, 2010), an increase in reactive oxygen species (ROS) (Barca et al., 2016), stroke-like lesions (Horvath et al., 2012), caspase activation, neuronal apoptosis (Grant et al., 2010) and has been linked to malfunctions in the biosynthesis of CoQ.

1.4 COQ8A

Coenzyme Q8A (COQ8A previously known as ADCK3 and CABC1) encodes a highly conserved putative kinase that is involved in the regulation of CoQ biosynthesis (Barca et al., 2016; He et al., 2014; Mollet et al., 2008). Phylogenetic analysis identified COQ8A as an atypical kinase that is part of the UbiB protein kinase-like (PKL) family (He et al., 2014; Stefely et al., 2015). The UbiB family are found primarily in the mitochondria of eukaryotes (Pagliarini et al., 2008).

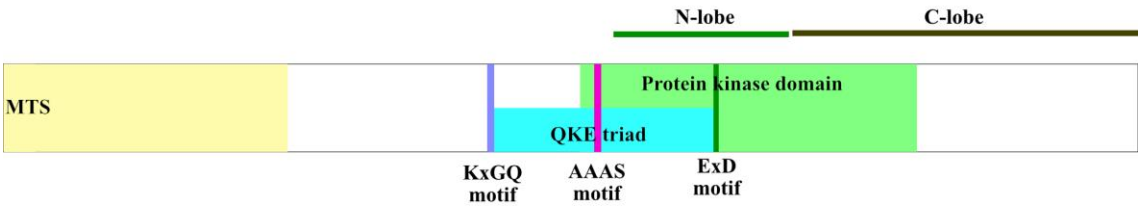


Figure 1.1 Schematic representation of COQ8A, indicating structural domains.
MTS = mitochondrial targeting sequence.

UbiB kinases are highly conserved from archaea to eukaryotes, but in *E.coli ubiB* (homolog of human COQ8A) is essential for the biosynthesis of CoQ_{10} (Poon et al., 2000) and carry several trademark features that make them unique (Stefely et al., 2015). These characteristics include an N lobe insert, an N-terminal extension containing a unique and invariant KxGQ motif, a non-canonical A-rich loop, and an ExD motif in the N lobe insert, an altered catalytic loop, a mitochondrial targeting sequence (MTS), an atypical active site and a selectivity for binding adenosine diphosphate (ADP) (Figure 1.1) (Stefely et al., 2015).

The KxGQ motif is integral for the proper function of COQ8A in the biosynthesis of CoQ and is highly conserved from yeast to humans (Figure 1.2). Mutations to the KxGQ motif results in a mutant phenotype in yeast and the inability for yeast to produce mitochondrial CoQ (Stefely et al., 2015).

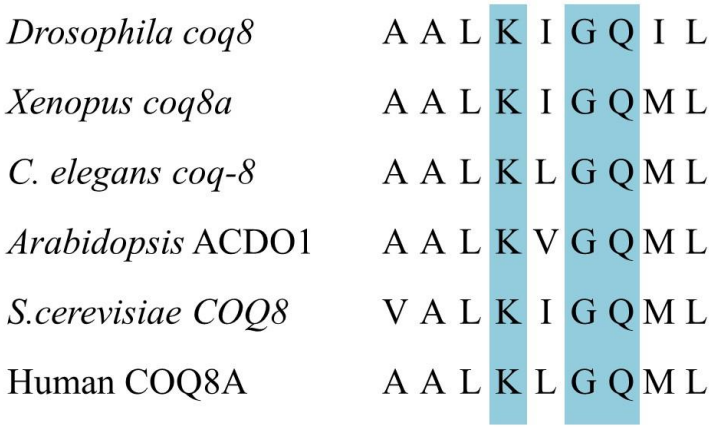


Figure 1.2 Conservation of the KxGQ motif.

The secondary structure of the N-terminal extension and the N lobe fold into a series of β sheets and α helices while the C lobe folds into α helices and β strands (Stefely et al., 2015). This secondary structure of the N-terminal extension occludes the substrate binding pocket by placing the KxGQ motif into the active site and ultimately preventing substrate binding (Stefely et al., 2015). This is an unusual feature of COQ8A as most PKL families will fold away from the substrate binding pocket as opposed to into it, where the KxGQ motif acts as an autoinhibitory domain (Stefely et al., 2015). The

inhibition of protein kinase activity in COQ8A is essential for the biosynthesis of CoQ (Stefely et al., 2015).

A QKE triad is also present and contains a salt bridge from the highly conserved K276 and Q397 residues of the KxGQ motif to the E405 residue of the ExD motif (Stefely et al., 2015). The QKE triad folds and prevents substrate binding near the A-rich loop which ultimately prevents kinase activity in the protein kinase domain (Stefely et al., 2015). The KxGQ domain and the QKE triad work as inhibitory mechanisms to prevent protein kinase activity and are what make COQ8A unique from other members of the UbiB family.

COQ8A localises to the mitochondrial cristae via an N-terminal MTS which is cleaved upon entry into the mitochondria (Cullen et al., 2016; Stefely et al., 2015). N-terminal FLAG-tagged COQ8A failed to colocalise to mitochondria when using MitoTracker® suggesting that the N-terminal of COQ8A is cleaved before entry (Cullen et al., 2016). COQ8A was FLAG-tagged at either the N-terminal or the C-terminal. Colocalisation was absent in the N-terminal FLAG-tagged COQ8A but present in the C-terminal FLAG-tagged COQ8A (Cullen et al., 2016). This suggested that the N-terminal of COQ8A was cleaved before entry into the mitochondria with this idea being supported by the cleavage of 162 amino acids in mature COQ8A (Stefely et al., 2015).

The MTS is located on proteins that are destined to function in mitochondria. These MTS are generally located on the N-terminal of the protein and often form amphiphilic helices (von Heijne, 1986). While MTS vary in sequence, dependent on function, the sequence itself seems to be 20 amino acids in length. Websites currently exist which aid in determining the presence of MTS in proteins. These websites are listed in Table 4.3.

While the role of COQ8A is mostly unknown, studies have tried to unravel the mystery surrounding COQ8A function (Cullen et al., 2016). It has been suggested that COQ8A may function in a regulatory capacity in the CoQ biosynthetic pathway by interacting with COQ3, COQ5, COQ7 and COQ9 (Cullen et al., 2016). While the exact function is unknown, in the absence of COQ8A it was noted that some of the CoQ proteins were unphosphorylated (Fernández-Ayala et al., 2014) suggesting that COQ8A may function in a regulatory capacity to stabilise or form the Q complex (Cullen et al., 2016; Stefely et al., 2016; Stefely et al., 2015).

1.5 COQ8A mutations

CoQ deficiency and cerebellar ataxia are often a result of COQ8A mutations in humans (Barca et al., 2016). Mutations in COQ8A can result in numerous disease phenotypes ranging from mild to extremely catastrophic (Horvath et al., 2012; Lagier-Tourenne et al., 2008). Clinical presentation can include infantile-onset cerebellar atrophy as determined by magnetic resonance imaging (MRI), seizures, complex ataxia-myoclonus, tremors, CoQ deficiency, slurred speech and uncoordinated gait (Barca et al., 2016; Horvath et al., 2012; Jacobsen et al., 2016 ; Liu et al., 2014; Stefely et al., 2015). A list of mutations and their associated phenotypes are listed in Table 1.2 with a schematic illustrating the mutations and their locations along the protein (Figure 1.3). The chemical analysis identified a decrease in the activity of complexes I+III and complexes II+III in the MRC (Liu et al., 2014) but is not always a consistent indicator of mutations to COQ8A.

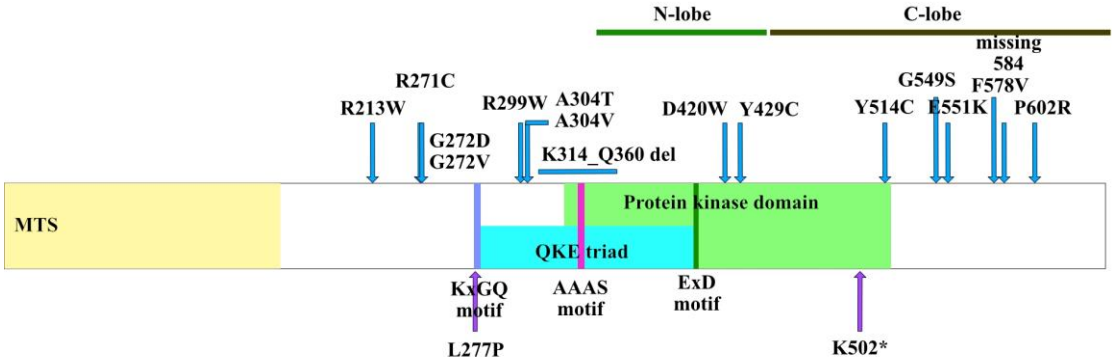


Figure 1.3 Schematic representation of the mutations found in COQ8A*. Mutations are listed in Table 1.2. The mutations indicated by blue arrows were found on the https://www.uniprot.org/uniprot/Q8NI60#family_and_domains website. The mutations indicated by purple arrows were the mutations used in this project and were found by the Minds for Minds research network (Jacobsen et al., 2017).*

With the increase in whole exome and genome sequencing, many COQ8A mutations are being identified (Bao et al., 2014; Horvath et al., 2012; Jacobsen et al., 2017; Mollet et al., 2008) and functional analysis of these mutations are necessary to determine whether the mutations are responsible for the disease state. Supplementation with

exogenous CoQ has varied results in patients with some responding well (Barca et al., 2016; Liu et al., 2014) and others showing no improvement at all (Horvath et al., 2012).

Table 1.2 Mutations found in COQ8A. *Mutations were found on the https://www.uniprot.org/uniprot/Q8NI60#family_and_domains website highlighting the genetic mutations currently identified in COQ8A. The purple rows highlight the mutations identified by Jacobsen et al. (2017) which have not been functionally characterised.*

Mutation	Change	Phenotypes	Publication
R213W	Missense Arg → Trp Highly conserved residue	Mild hypotonia, *talus valgus*, developmental delay, seizures, cerebellar ataxia, cerebellar atrophy, ptosis, vermis hypoplasia, epilepsy, muscle weakness, *epilepsia partialis continua*, CoQ_{10} deficiency stroke-like anomalies and hyperintensities. CoQ supplementation was not effective.	(Mollet et al., 2008) (Horvath et al., 2012)
R271C	Missense Arg → Cys Loss of interaction with V300 and Y361	Mild cerebellar ataxia, cerebellar atrophy, ptosis, CoQ_{10} deficiency and tremors.	(Horvath et al., 2012)
G272D	Missense Gly → Asp Steric clash with I342 and S340	Exercise intolerance, CoQ deficiency, hyperlactatemia, cerebellar ataxia, cerebellar atrophy, epilepsy, myoclonus, a decrease in respiratory chain activity in muscle	(Mollet et al., 2008) (Horvath et al., 2012) (Auré et al., 2004)
G272V	Missense Gly → Val Steric clash with I342 and S340 Highly conserved residue	Mild hypotonia, *talus valgus*, developmental delay, seizures, cerebellar ataxia, ptosis, vermis hypoplasia, *epilepsia partialis continua*, stroke-like anomalies and hyperintensities mild cerebellar atrophy, tremor, epilepsy and muscle weakness. CoQ_{10} supplementation was not effective.	(Mollet et al., 2008) (Horvath et al., 2012)
L277P	Missense Leu → Pro	CoQ_{10} deficiency, uncoordinated gait and cerebellar atrophy.	(Jacobsen et al., 2017)
R299W	Missense Arg → Trp Decreased stability Loss of interactions with S303 and E261	Cerebellar ataxia, cerebellar atrophy, epilepsy, muscle weakness, and cognitive defects,	(Stefely et al., 2015) (Horvath et al., 2012) (Hikmat et al., 2016)
A304T	Missense Ala → Thr Steric clash with P335 and A338	Mild cerebellar ataxia, cerebellar atrophy, epilepsy, muscle weakness, ptosis, CoQ_{10} deficiency, tremor	(Horvath et al., 2012)
A304V	Missense Ala → Val Steric clash with P335 and A338	Cerebellar ataxia, cerebellar atrophy, spasticity, tremor, epilepsy, myoclonus, muscle weakness, ptosis, CoQ_{10} deficiency.	(Horvath et al., 2012)
K314_Q360	Deletion Lys314_Gln360	Cerebellar ataxia, moderate disability, brisk reflexes and mild hearing loss.	(Lagier-Tourenne et al., 2008)
D420W	Frameshift Asp →Trp	Cerebellar ataxia, cerebellar atrophy, exercise intolerance, mental retardation and lactic acidosis.	(Lagier-Tourenne et al., 2008)

Y429C	Missense Try → Cys Decreased stability Loss of interaction with #446 and R419	Cerebellar ataxia, cerebellar atrophy, spasticity, tremor, epilepsy, muscle weakness, mild cognitive deficient, depression,	(Stefely et al., 2015) (Horvath et al., 2012)
K502*	Disrupts a canonical splice donor site in exon 12 predicted to inhibit transcriptional processing	CoQ deficiency, uncoordinated gait and cerebellar atrophy.	(Jacobsen et al., 2017)
Y514C	Missense Try → Cys Loss of H-bond with E605 backbone	Unsteady gait, generalised tonic-clonic seizures, absence seizures, gait increasingly atactic, lost ability to walk by 12 years, lost speech at 14 years old, feeding difficulties PEG inserted, sleeps a lot, drowsy, severe retardation, progressive cerebellar atrophy, enlarged ventricles, thinning corpus callosum, epileptiform activity and cerebellar ataxia	(Lagier-Tourenne et al., 2008) (Horvath et al., 2012)
G549S	Missense Gly → Ser Decreased stability Steric clash with I542	Slow motor development, progressive ataxia, muscle weakness, cognitive impairment, wheelchair-bound, horizontal nystagmus, mildly slurred speech, spastic tetraparesis with increased reflexes, bilateral dysmetria, tremor, depression and prominent cerebellar atrophy.	(Lagier-Tourenne et al., 2008) (Stefely et al., 2015) (Horvath et al., 2012)
E551K	Missense Glu → Lys Decreased stability. Highly conserved change from glutamic acid to lysine. Homozygous change Loss of H-bond with T548 backbone	Proximal muscle weakness, cerebellar ataxia, strabismus, tonic seizures, high CSF lactate, trunk hypotonia intellectual regression, unable to walk and speak after 12 years of age, stroke-like anomalies, *epilepsia partialis continua* cerebellar atrophy, epilepsy, ptosis, cognitive deficiency, decrease in respiratory chain activity in muscle fibroblasts and CoQ_{10} deficiency. CoQ_{10} supplementation was not beneficial	(Stefely et al., 2015) (Mollet et al., 2008) (Horvath et al., 2012)
F578V	Unknown pathological significance	Childhood-onset epilepsy, progressive cerebellar ataxia, stroke-like episodes and focal epileptic activity.	(Hikmat et al., 2016)
Missing 584		Unsteady gait, generalised tonic-clonic, absence seizures, gait increasingly atactic, lost ability to walk by 12 years, lost speech at 14 years old, feeding difficulties PEG inserted, sleeps a lot, drowsy, severe retardation, progressive cerebellar atrophy, enlarged ventricles, thinning corpus callosum, and epileptiform activity.	(Lagier-Tourenne et al., 2008) (Horvath et al., 2012)
P602R	Missense Pro → Arg Intragenic frameshift deletion Insertion c.[1812_1813insG]	Exercise intolerance, progressive cerebellar syndrome, cerebellar atrophy, CoQ deficiency and hyperlactatemia.	(Blumkin et al., 2014)

1.6 Case study

The Minds for Minds research network are a group of New Zealand researchers and clinicians who work collectively to better understand autism spectrum disorders and other neurodevelopmental disorders. The Minds for Minds research group assessed a female sibling pair who presented with cerebellar atrophy and cerebellar hypoplasia (Jacobsen et al., 2017). Clinically the children presented with CoQ deficiency, uncoordinated gait and cerebellar atrophy. Whole exome sequencing identified compound heterozygous inheritance of two single nucleotide polymorphisms (SNP). The first SNP was paternally inherited, this resulted in a non-synonymous missense change from Leucine to Proline (c.830T>C, p.Leu277Pro) in exon 6 (Jacobsen et al., 2017). This amino acid is highly conserved and located in the highly conserved and invariant KxGQ motif at the x position (Stefely et al., 2015). The second SNP, maternally derived, resulted in a novel mutation that disrupts a canonical splice donor site in exon 12 (c.1506+1G>A) (Jacobsen et al., 2017). It has been predicted that this SNP will destroy the splice donor site and inhibit transcriptional processing. Neither of these mutations have been previously characterised, and for this reason, functional studies are needed to confirm that the amino acid substitutions result in impaired functionality.

1.7 *Drosophila* as a model for functional analysis of human COQ8A mutations

Drosophila, the fruit fly, has been used to study genetics for many years with the iconic experiment carried out by Thomas Hunt Morgan which determined the sex-linked inheritance of eye colour in *Drosophila* (Morgan & Bridges, 1916). *Drosophila* are small, inexpensive, easy to care for; they have fast generation times, produce many offspring, and many genetic tools that have been developed for molecular and genetic analysis. Advances in both molecular genetics and *Drosophila* genetics have led to the increased use of *Drosophila* as a model for the investigation of the molecular mechanism behind human genetic diseases.

Drosophila can be used to study learning and memory (Fitzsimons & Scott, 2011), motor function (Rhodenizer, Martin, Bhandari, Pletcher, & Grotewiel, 2008), they can also be used to test the efficacy of drugs (Pandey & Nichols, 2011) and have been

previously used as a model to assess CoQ deficiency (Grant et al., 2010). With 75% of human disease-causing genes highly conserved in *Drosophila* (Bier, 2005) *Drosophila* provides the genetic stability to functionally characterise and probe the inner workings of uncharacterised human mutations.

1.7.1 *Drosophila* life cycle

The *Drosophila* life cycle (Figure 1.4) has four distinct stages which include the embryo, three larval stages, a pupal stage and finally the adult stage. When flies are raised at 25℃ first instar larvae emerge 24 hours after fertilisation. Each larval stage lasts roughly 24 hours each with the prepupa stage lasting 2-3 days before pupation occurs. Pupation is when morphogenesis occurs and typically lasts approximately five days before adults begin to emerge. During morphogenesis, the eyes, legs, antennae and body segments are produced. The day before the emergence of the adult fly, the wings and eyes become more visible, and the pupal casing darkens. During emergence (eclosion) adults emerge from the anterior end of the pupae. For up to eight hours after eclosion, female flies are sexually immature.

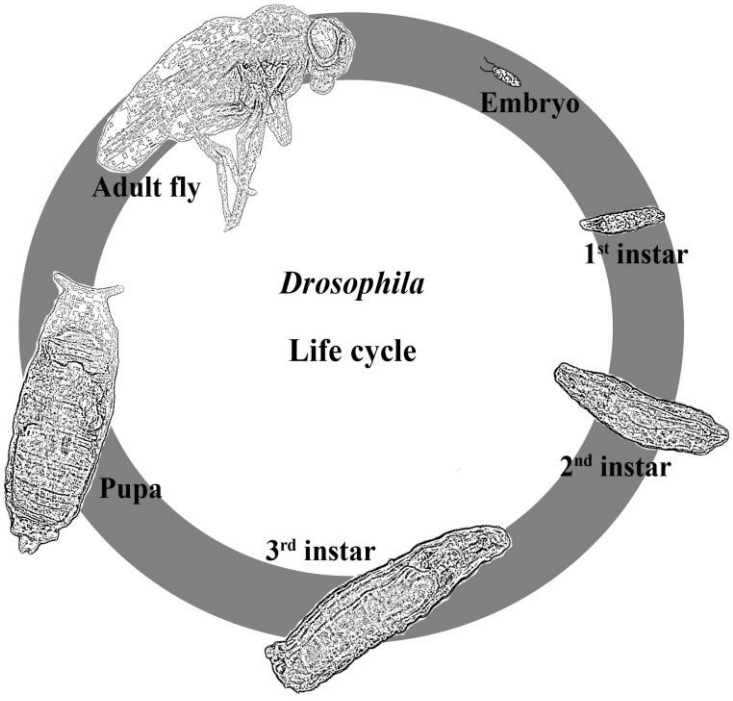


Figure 1.4 The life cycle of Drosophila melanogaster. *There are four distinctive stages in the Drosophila life cycle which include an embryo stage, three distinctive larval stages, a pupal stage and finally an adult stage. Development of Drosophila is temperature dependent and can be completed in 10 days at 25℃.*

When flies are raised at 25℃ the life cycle is completed in 7-10 days, at 21℃ it takes 10-14 days, and at 18℃ it takes 21-25 days (Suzuki, 1970). The generations times can be altered depending on the fly lines and driver lines used.

1.7.2 The *Drosophila* brain structure

The central brain includes the antennal lobes and the mushroom body which receive sensory input from the olfactory system. The optic lobes flank the central brain and work together to process visual input from the eyes. *Drosophila* contain a brain that is simple, yet sophisticated enough to assess memory and learning (Figure 1.5) (Bellen, Tong, & Tsuda, 2010). The *Drosophila* brain is composed of approximately 200,000 neurons which are highly organised. The mushroom body (MB) is the bilateral structure which is crucial for memory formation in the fly brain (McGuire, Le, & Davis, 2001). The MB is composed of 2500 Kenyon cells. These are the intrinsic neurons of the brain which project dendrites into the calyx (Heisenberg, 1998; Yang, Armstrong, Vilinsky, Strausfeld, & Kaiser, 1995). The Kenyon cell axons are bundled into α/β, α'/β' and γ MB lobes (Heisenberg, 1998) which project to the anterior of the brain forming a characteristic L-shape. The calyx functions as the primary sensory input into the mushroom body (Heisenberg, 1998; Zhu, Chiang, & Lee, 2003) from the antennal lobe (Yang et al., 1995).

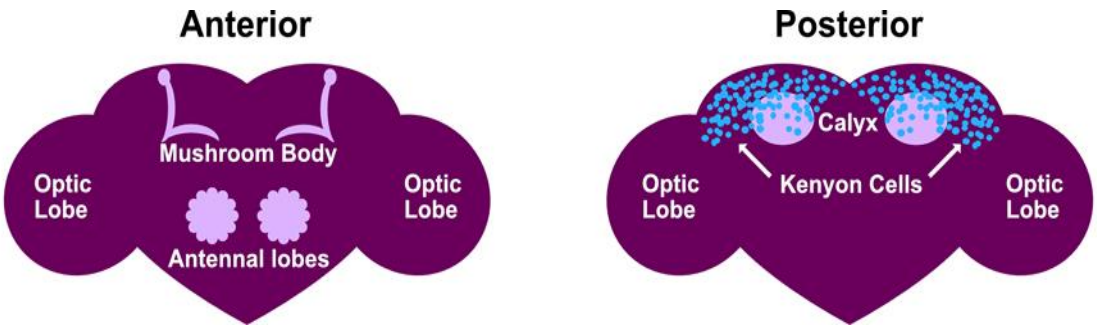


Figure 1.5 Schematic representation of the Drosophila brain. *Anterior view contains the mushroom body lobes and the antennal lobes. The optic lobes are visible from both sides of the brain. The posterior view shows the cell bodies of the Kenyon cells and the calyx.*

With over 600 causative genes identified in intellectual disability disorders (Morales et al., 2002; van der Voet, Nijhof, Oortveld, & Schenck, 2014) a robust model system is necessary to enable functional characterisation of these genes. Due to the heterogeneity of the disease states present in intellectual disability disorders, *Drosophila* has been used to aid in our understanding of the molecular basis of human disease (Morales et al., 2002). *Drosophila* has been used to functionally characterise neurological and neurodegenerative disorders including Fragile X syndrome (Morales et al., 2002), Parkinson's disease (Pesah et al., 2004), Alzheimer's disease (Sofola et al., 2010), neurodegeneration (Barone & Bohmann, 2013) and other congenital diseases (Moulton & Letsou, 2016).

1.7.3 The *Drosophila* compound eye

The *Drosophila* compound eye is an elaborate and extraordinary structure composed of roughly 800 ommatidia (Kumar, 2012). Each ommatidium is composed of specialised neurons, the photoreceptors, as well as non-neuronal cells including cone cells, primary pigment cells, bristle cells, tertiary pigment cells, and interommatidial cells (Freeman, 1996; Johnson & Cagan, 2009; Kumar, 2012). To be able to construct a structure so grand an elaborate network of cellular interactions are necessary (Johnson & Cagan, 2009). Any impairments in the development of the compound eye are visually evident as a disruption in ommatidial patterning, impaired adhesion of ommatidia and disruption to bristle integrity is seen. Therefore it is an excellent model to investigate the impact of genetic mutations on cellular interactions (Johnson & Cagan, 2009). During embryonic development, the optic nerve and retina are derived from the CNS and are therefore considered to be a part of the CNS (Johnson & Cagan, 2009). In this case, the retina and optic nerve can be used to assess the involvement a genetic mutation may have in neurodegeneration (London, Benhar, & Schwartz, 2013).

The *Drosophila* eye can be used as a surrogate indicator of the effect of protein in the brain which is why the eye has been used to model Parkinsons (Faust et al., 2009; Pesah et al., 2004), Alzheimer's disease (Sofola et al., 2010) and cellular differentiation (Freeman, 1996). Protocols have been established to prepare *Drosophila* brains and retina for live images to aid in the study of neuronal development, cellular function and neurodegeneration (Warrick et al., 1998; Williamson & Hiesinger, 2010).

1.8 *Drosophila* as a genetic toolbox

Over the last ~30 years, many genetic tools have been developed to aid in the genetic and molecular analysis using *Drosophila*, which make *Drosophila* an appealing model for studying human disease.

These tools include the UAS/GAL4 system which utilises the yeast-derived transcription factor GAL4 and the GAL4-dependent Up-stream Activating Sequence (UAS) which is tethered to a transgene or gene of interest (Kakidani & Ptashne, 1988). This system allows genes to be expressed in a cell and/or tissue-specific manner in *Drosophila* (Duffy, 2002). In conjunction with the UAS/GAL4 system, there are a vast library of RNA interference (RNAi) lines available from the Vienna *Drosophila* RNAi Center (VDRC, Vienna, Austria) for the knockdown of many *Drosophila* genes (Dietzl et al., 2007). Any gene can be expressed in a highly cell and/or tissue-specific manner. Also, genes can be switched on or off at a specific developmental stage to assess what impact the gene may have in *Drosophila* development.

Advances in both molecular genetics and *Drosophila* genetics have led to the increased use of *Drosophila* as a model for neurodegeneration (Barone & Bohmann, 2013). With the increase in susceptibility and prevalence of neurodegenerative disorders such as Parkinson's disease (Pesah et al., 2004) and Alzheimer's disease (Sofola et al., 2010) a model system such as *Drosophila* can aid in further exploration of the pathways and mechanisms underpinning these disorders.

A study by Grant et al. (2010) used *Drosophila* a model to functionally characterise mutations associated with *qless*, the *Drosophila* homologue of the human PDSS gene, which is the first gene in the biosynthetic pathway of CoQ_{10}. This gene is responsible for the lengthening of the isoprenoid side chain that embeds in the mitochondrial membrane. It was identified that *qless* is essential for neural growth and protects *Drosophila* against mitochondrial dysfunction (Grant et al., 2010) and demonstrates that *Drosophila* can be used as a model to examine the function of genes involved in CoQ biosynthesis. It was also determined that dietary supplementation with CoQ_{10} could rescue the neural undergrowth.

1.8.1 The UAS-GAL4 System is used for cell and/or tissue-specific expression of a transgene in *Drosophila*

Targeted gene expression enables transgene expression in a cell and/or tissue-specific manner during any or all stages of development (Duffy, 2002). Previously used targeted gene expression had been limited to heat shock, which required the transgene to be under the control of a promoter which was activated once heated to a specific temperature (Ashburner & Bonner, 1979). The second method was promoter specific and enabled expression in a specific subset of cells in a defined region (Zuker, Mismer, Hardy, & Rubin, 1988) however; both options lack the finesse and high specificity required for an in-depth understanding of the role and function of a gene of interest. In response to this, the UAS/GAL4 system was development by Brand and Perrimon (1993).

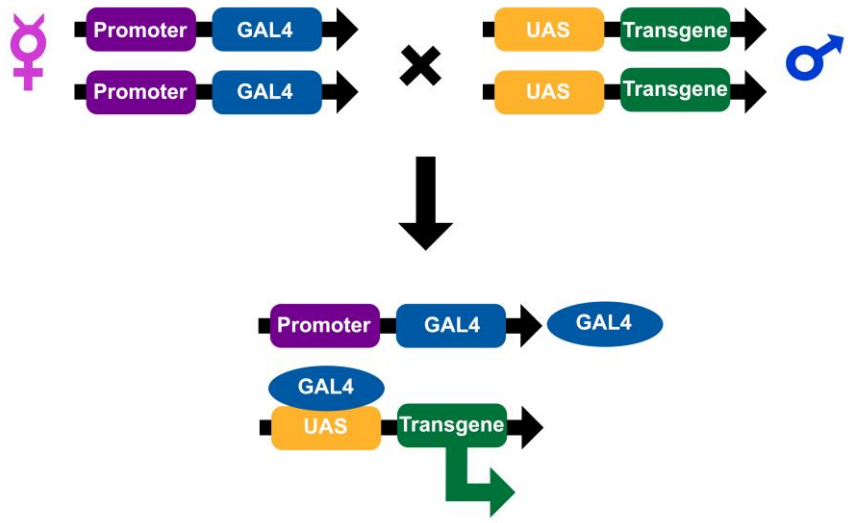


Figure 1.6 Temporal and spatial expression of transgenes using the UAS/GAL4 system in Drosophila. *Virgin female flies (left) harbour a construct containing the cell and/or tissue-specific promoter, known as the driver, which is fused upstream of the yeast transcription factor GAL4. Virgin female flies are then crossed to males (left) which contain the GAL4-dependent upstream activating sequence (UAS) which is upstream of the transgene. Progeny from these crosses (bottom) contain the GAL4 transcription factor and the GAL4-depedent upstream activating sequence. GAL4 is able to bind to the UAS which results in cell and/or tissue specific expression of the transgene.*

The UAS-GAL4 binary system utilises the yeast transcription factor (TF) GAL4 and the GAL4-dependent upstream-activating system (UAS), which enables cell and/or tissue-specific expression of transgenes in *Drosophila* (Brand, 1993). In one fly line resides a tissue-specific promoter fused upstream of the GAL4 transcription factor and is termed a "GAL4 driver" (Brand, 1993). In a second fly line, the transgene is found downstream of the GAL4-dependent UAS which is inactive in the absence of GAL4. When these fly lines are crossed their progeny contain both the promoter-GAL4 and the UAS-transgene enabling the binding of GAL4 to UAS and ultimately the expression of the transgene. This system allows for targeted gene expression or silencing within *Drosophila*.

The advantage of this system is the specificity of transgene expression which, in instances when a transgene results in a lethal phenotype this can be avoided by restricting expression to a specific cell and/or tissue type to further characterise the function and ability of the transgene. With a large number of GAL4 driver's available, transgene expression can occur anywhere in *Drosophila*.

1.8.2 GAL4 driver lines

Five main GAL4 driver lines used in this study were used to drive strong ubiquitous expression of the transgene using *da*-GAL4 (Cronmiller & Cummings, 1993), weaker ubiquitous expression using *arm*-GAL4 (Akiyoshi, Kaneuch, Aigaki, & Suzuki, 2014; Peifer, Rauskolb, Williams, Riggleman, & Wieschaus, 1991; Riggleman, Wieschaus, & Schedl, 1989), pan-neuronal expression using *elav*-GAL4 (Campos, Rosen, Robinow, & White, 1987), *D42*-GAL4 was used to drive expression in motor neurons and *glass multiple reporter* (*GMR*)-GAL4 drives expression in the post-mitotic eye.

The *daughterless* (*da*) gene is ubiquitously expressed in somatic cells during embryogenesis (Vaessin, Brand, Jan, & Jan, 1994) and functions as a regulatory mechanism in sex determination, neurogenesis and oogenesis (Cronmiller & Cummings, 1993). Following germ band retraction, the expression profile of *da* becomes more specific with expression evident in the ventral side of the developing ventral cord of the CNS (Vaessin et al., 1994). An increase in *da* expression can also be seen in the salivary glands, gut and muscle until cuticle formation. Expression can also be seen in the parasympathetic nervous system (PSNS) and is evident in the imaginal discs in a similar intensity to that of the CNS (Vaessin et al., 1994). The *da*-GAL4 driver is a driver in which GAL4 has been inserted within the *da* enhancer and is thus

under the control of the regulatory elements that drive expression of *da*. *da*-GAL4 will be useful in determining the global effect of a transgene in *Drosophila.*

Due to the strong expression profile of *da,* it was decided that *armadillo (arm)* would be used as it is a weaker ubiquitous driver. *arm* is expressed throughout *Drosophila* embryogenesis (Akiyoshi et al., 2014; Peifer et al., 1991; Riggleman et al., 1989). Expression of *arm*-GAL4 is evident throughout development with highest levels occurring during the early to mid-embryogenesis and also during early pupal stages (Akiyoshi et al., 2014; Riggleman et al., 1989). a*rm* is uniformly expressed and is also one of the more abundant transcripts present in the cell (Riggleman et al., 1989). Due to the broad and constant expression of *arm*-GAL4, it was decided to use it as a weaker ubiquitous driver (Rewitz, Yamanaka, & O'Connor, 2010).

The *embryonic lethal abnormal visual system* (*elav*) is located on the X-chromosome in *Drosophila* (Campos et al., 1987) and is expressed during development in the embryonic nervous system (Robinow & White, 1988), the salivary glands (Campos et al., 1987) and in all post-mitotic neurons (Rewitz et al., 2010).

The fluorescent reporter Apoliner was used in conjunction with *elav*-GAL4 to aid in the detection of early apoptotic events. Apoliner is a fluorescent reporter that is capable of detecting caspase activity which is evident during apoptosis (Bardet et al., 2008). Apoliner is a sensor that is composed of two fluorescent proteins, monomeric red fluorescent protein (mRFP) and enhanced green fluorescent protein (eGFP), which are fused together by a caspase cleavage site (Bardet et al., 2008). The mRFP fluorophore contains a transmembrane domain while the eGFP fluorophore contains a nuclear targeting signal. Caspases cleave the fluorophores at the caspase-sensitive linker site resulting in the fluorophores separating from each other. This results in eGFP translocating to the nucleus while mRFP is left at the membranes (Bardet et al., 2008). Therefore, upon caspase activation eGFP will be present in the nucleus while mRFP is present at the membranes.

As the human COQ8A mutant phenotype involves motor dysfunction (such as gait ataxia), *D42*-GAL4 was used to drive expression in motor neurons. *D42* is expressed in the peripheral nervous system (PNS), the ventral nerve cord, the brain, sensory neurons and the motor neurons in *Drosophila* (Pilling, Horiuchi, Lively, & Saxton, 2006;

Sanyal, 2009; Schroll et al., 2006; Yeh, Gustafson, & Boulianne, 1995). *D42* expression is also present in the central brain and ventral ganglia (Parkes et al., 1998).

The *glass multiple reporter* (*GMR*)-GAL4 driver is commonly used to drive expression of a transgene in the developing eye (Li, Li, Zheng, Zhang, & Xue, 2012) specifically in the post-mitotic cells posterior to the morphogenetic furrow (Freeman, 1996). However, it was later identified that the expression of *GMR*-GAL4 was also present in the developing wing, the larval brain, the leg and the trachea (Li et al., 2012). For this study, we will be focusing on the expression of transgenes in the developing eye to examine any neurological involvement of the transgenes.

The *eyeless (ey)* gene plays a vital role in eye development not only in *Drosophila* but throughout the animal kingdom (Hauck, Gehring, & Walldorf, 1999). *ey* is expressed in the developing nervous system and the eye during morphogenesis (Halder, Callaerts, & Gehring, 1995). *ey* is known as a master control gene as it is responsible for the initial activation of the eye development pathway (Halder et al., 1995). Initially, *ey* is expressed in the embryonic ventral nerve cord before being expressed in the larval developing imaginal discs and eye discs which is anterior to the morphogenetic furrow (Halder et al., 1995). Expression of *ey* mutants can result in a decrease or complete absence of the *Drosophila* compound eye. However even with the loss of the compound eye due to *ey* mutants the simple *Drosophila* eye is still present (Halder et al., 1995). The use of the *ey*-GAL4 driver line will enable a quantitative measure based on the size of the resulting compound eye. *ey*-GAL4 is expressed earlier in eye development to that of *GMR*-GAL4 and will highlight the importance of the gene function in these earlier stages.

1.8.3 RNAi-mediated knockdown of target gene expression

RNA interference (RNAi) (Figure 1.7) is a natural defence mechanism that is present in all living organisms. This mechanism enables protection from viral infection via the introduction of double-stranded RNA (dsRNA). Introduction of long dsRNA into an organism results in activation of the endonuclease Dicer which cuts the dsRNA into smaller 19-21 nucleotide segments known as small interfering RNA (siRNA). These siRNAs are then bound by the RNA-induced silencing complex (RISC) which separates the siRNA into two separate single-stranded RNA (ssRNA) molecules with one known as the guide strand acting as a template for gene silencing and the other the passenger

strand which is degraded. The RISC bound guide strand then forms a complex with Argonaute proteins. The Argonaute complex containing the guide strand binds to the target messenger RNA (mRNA) and cleaves the mRNA. The target mRNA is then degraded preventing expression of the target gene. This process ultimately results in a decrease in expression of the target gene (Fire et al., 1998) and aids in characterising the function of a gene. The VDRC has developed a library of fly lines harbouring RNAi constructs that target most genes in *Drosophila.* RNAi can be used in conjunction with the UAS/GAL4 system which enables the silencing of target genes in a cell and/or tissue-specific manner.

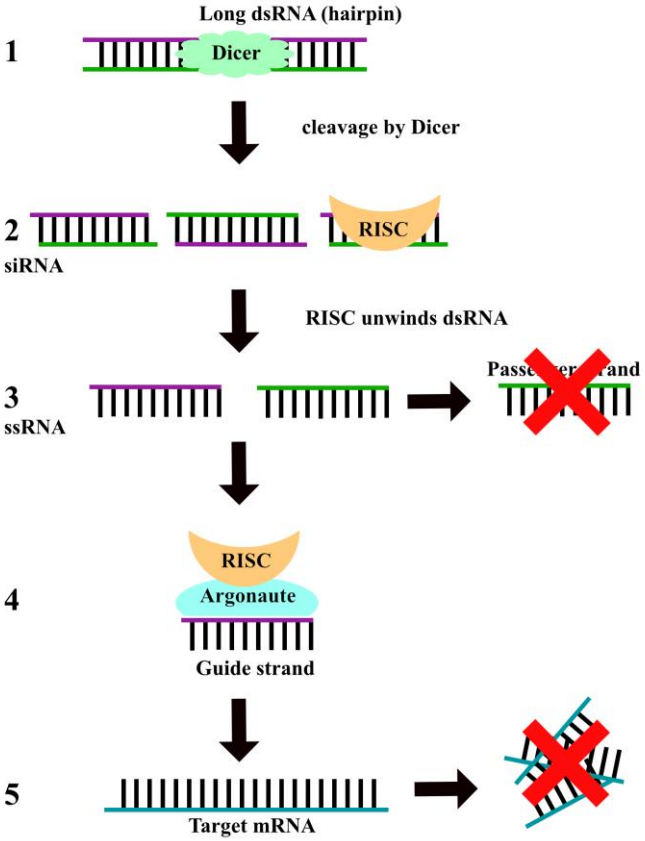


Figure 1.7 RNA interference mechanism. *RNAi is a natural mechanism used to silence foreign dsRNA in an organism. 1. Long double-stranded RNA (dsRNA), also known as a hairpin, is introduced into the target organism/cell. Dicer, an endonuclease protein, binds to the dsRNA and cleaves it into 19-25 nucleotide segments of small-interfering RNA (siRNA). 2. The multi-protein complex RISC binds to siRNA and unwinds the small dsRNA fragments into single-stranded RNA (ssRNA). 3. ssRNA separates into two strands known as a guide strand, which binds to RISC and Argonaut, and the passenger stand which is degraded. 4. RISC and Argonaut bind to the guide stand. 5. The target mRNA is then cleaved by the RISC and Argonaut complex. Degradation of the target mRNA occurs after cleavage resulting in the silencing of gene expression.*

While each RNAi construct was bioinformatically designed to target only one gene, a potential limitation is off-target effects which could result in a mutant phenotype that is not specific to RNAi silencing of the target gene. When ordering RNAi lines from VDRC, it is often beneficial to order two or more RNAi lines targeted to a gene as some RNAi lines are less effective or reliable than others (Dietzl et al., 2007). Some fly lines may also require RNAi enhancers to increase the expression of an RNAi line. In *Drosophila*, Dicer-2 binds to siRNA to enhance RNAi expression (Dietzl et al., 2007).

2 Aims of this Thesis

With the increased number of genetic mutations being identified by whole genome and/or exome sequencing, a model system is needed to characterise these mutations functionally. *Drosophila* is a highly functional model organism that can be used to study learning and memory, neuronal development and neurodegeneration. The Minds for Minds research network in New Zealand (Jacobsen et al., 2017) identified two COQ8A SNPs in two human siblings. Compound heterozygous inheritance of COQ8A mutations resulted in a heterogeneous disease state that included cerebellar ataxia, cerebellar atrophy, and CoQ deficiency.

This project aimed to characterise the phenotypes resulting from the reduction of coq8 in *Drosophil*a to develop a model of coq8 deficiency. This model could be used to functionally characterise the impact of the mutations on *coq8* function.

I hypothesise knockdown of *Drosophila coq8* results in a degenerative phenotype and that this degenerative phenotype can be rescued by reintroduction of wild-type *coq8*, but not the two mutants.

The specific objectives are as follows:

1. Characterisation of the phenotypes resulting from RNAi knockdown of *coq8* in *Drosophila*
2. Generation and characterisation of flies that overexpress wild-type and mutant forms of *coq8*
3. Determination of whether wild-type or mutant *coq8* can rescue RNAi knockdown
4. Generation and characterisation of flies that overexpress wild-type and mutant forms of human COQ8A
5. Determination of whether wild-type or mutant COQ8A can rescue RNAi knockdown

3 Methods

3.1 Maintenance of *Drosophila* melanogaster strains

Flies were housed in 30 mL v-bottomed vials (Labserv) or 100 mL glass bottles containing eight mL or 40 mL of standard fly food, respectively (per L: cornmeal, yeast, sugar, agar, molasses, methyl 4-hydroxybenzoate and ethanol. Flies were raised at 18°C, 22°C or 25°C, depending on experimental parameters as described in the results section. Flies were kept on a 12-hour light-dark cycle. Flies were anesthetised using CO2 when setting crosses or collecting virgin females or FlyNap® (Carolina Biological Supply Company) when anaesthesia was required for longer than 2-3 minutes, such as when imaging whole flies or fly heads. *Drosophila* strains used during this study are listed in appendix 9.1, 9.2 and 9.3.

3.2 Genetic Crosses

Ten males and ten females of each appropriate genotype were placed in bottles and allowed to mate for one week before being removed from the bottles. Approximately 12 days later adult progeny began to emerge. Bottles were cleared in the morning and any females that emerged within eight hours were considered virgin as females do not reach sexual maturity until at least ten hours post-eclosion. Virgin females were housed in vials for five days to verify virginity, as evidenced by the lack of fertilised eggs in the vial. Virgin females were then crossed to the appropriate males with the desired genotype. For initial characterisation experiments, flies were raised in vials. Rescue experiments, where vast quantities of progeny were needed, were carried out in bottles. Seven days after crosses were set the adult flies were removed, and the bottles were incubated at the appropriate temperatures until most of the adult flies had emerged. Progeny were then assessed for fundamental experimental purposes which could have included progeny counts, imaging or further analysis.

3.3 Assessment of larval survival and phenotypes

Flies of the appropriate genotypes were housed in vials and allowed to mate for seven days before they were removed. The fly food containing the larvae was emptied into a petri dish and washed in dH_2O and larvae were isolated. The number of larvae at each

developmental stage (1st 2nd and 3rd instar) were counted. Comparisons were made between the *coq8* RNAi lines and the control. Representative larvae from each genotype were placed at -20℃ until they were incapacitated and then imaged as described in section 3.5.

3.4 Assessment of pupal and adult survival

Flies of the appropriate genotypes were housed in vials and allowed to mate for seven days before they were removed. Progeny counts were carried out once most adults had emerged from the control cross. This was approximately 20 days after setting the cross but varied depending on incubation temperature and genotype. Flies were anesthetised with FlyNap® then the number of adults of each sex were counted. Pupal counts were carried out once all adults had been removed from the fly bottles by counting the number of empty pupae on the sides of the bottles.

For crosses that contained a balancer chromosome, pupae were not counted as their genotype could not be determined until the adult stage (i.e. whether they contained the balancer chromosome or the chromosome carrying the transgene).

3.5 Imaging of larvae and adult flies

Flies and larvae were anesthetised using FlyNap® and placed on a piece of white card for imaging. The Olympus SZX12 microscope and an Olympus LG-PS2 bifurcated light source combined with DP controller imaging software were used to capture images. Composite images were then generated in Adobe Photoshop CC 19.0 release and/or Affinity Photo version 1.5.1.54. For the detection of fluorophores, the Olympus U-RFL-T mercury burner was used in conjunction with the microscope described above.

3.6 Brain dissection

Brain dissections were carried out on adult flies. Adult flies were anesthetised with CO2 then placed in PFAT fixative (4% paraformaldehyde, 1X PBS, 0.1% Triton-X and 5% DMSO). Adult flies were fixed for 2 hours at room temperature before being washed in PBST (1X PBS, 0.5% Triton-X). Flies were then transferred to a petri dish containing ice-cold PBST. Using the Olympus VMZ 1X-4X Japan stereoscope and two sharp tweezers, heads were detached from bodies and the hard-outer head capsule was

removed to isolate the brain. The trachea and air-sacs were then removed from the outer surface of the brain. Freshly dissected brains were placed in fresh PBST on ice for up to 20-30 minutes until dissections were complete. Fly debris was then removed from brains by incubating in PBST with 3.7% formaldehyde and heptane by inverting the tube ten times. After the brains settled at the bottom of the tube, the upper heptane phase was discarded. The brains were then further fixed and cryoprotected in PBST with 3.7% formaldehyde and 5% DMSO for 20 minutes. After two 5-minute washes with methanol, brains were stored in methanol at -20℃ until processing for immunofluorescence.

3.7 Immunofluorescence

Brains were rehydrated in 50% methanol and 50% PBST for 5 minutes. They were then washed four times with PBST before blocking for 2 hours with immunobuffer (5% Normal Goat Serum (NGS) in PBST). The immunobuffer was then removed before the addition of primary antibodies (Table 3.1). Antibodies were diluted in immunobuffer, added to fly brains and placed on an orbital rocker overnight at room temperature. Primary antibody was removed, and brains were washed twice with PBST before three longer washes (>20 minutes) in PBST. PBST was removed then brains were incubated in the appropriate secondary antibodies (Table 3.2) diluted in immunobuffer, and left overnight shaking on an orbital shaker at 4℃. Secondary antibodies were then removed and brains washed in PBST before being mounted in Antifade (PBS+ 4% N-propyl gallate). For mounting the brains, two coverslips were affixed approximately 5 mm apart with nail polish to create a channel for the brains to be placed in which prevented the brains from being flattened by the top coverslip. The coverslip edges were sealed with nail polish and slides were stored at 4℃ in the dark before imaging by confocal microscopy.

Table 3.1 List of primary antibodies used for immunohistochemistry on whole mount brains.

Antigen	Clone	Class	Host	Source	Dilution
Nc82	AB_2314866	Monoclonal	Mouse	DSHB	1:50
Dcp1	Asp216	Polyclonal	Rabbit	Cell Signalling Technology	1:100
c-Myc	9E10	Monoclonal	Mouse	DSHB	1:50
Elav	9F8A9	Monoclonal	Mouse	DSHB	1:100
Cytochrome c	7H8.2C12	Monoclonal	Mouse	ThermoFisher	1:100
GFP	Ab290	Polyclonal	Rabbit	Abcam	1:5000
GFP	12A6	Monoclonal	Mouse	DSHB	1:50
COQ8A	ab221193	Polyclonal	Rabbit	Abcam	1:500
Rpd3	Ab1767	Polyclonal	Rabbit	Abcam	1:10,000

Table 3.2 Secondary antibodies used for immunohistochemistry on whole mount brains. *All secondary antibodies were obtained from ThermoFisher Scientific. These secondary antibodies were used to detect the primary antibodies from Table 3.1 via fluorescent detection.*

Name	Fluorophore	Dilution
Goat anti-mouse IgG	AlexaFluor® 488	1:500
Goat anti-rabbit IgG	AlexaFluor® 488	1:500
Goat anti-mouse IgG	AlexaFluor® 555	1:500
Goat anti-rabbit IgG	AlexaFluor® 555	1:500
Goat anti-mouse IgG	AlexaFluor® 647	1:500
Goat anti-rabbit IgG	AlexaFluor® 647	1:500

3.8 Confocal Microscopy

Confocal microscopy was carried out at the Manawatu Microscopy & Imaging Centre (MMIC) at Massey University with a Leica SP5 DM6000B Scanning Confocal Microscope. Processing of confocal images was carried out using ImageJ and Adobe Photoshop CC 19.0 release editing software.

3.9 Scanning Electron Microscopy

Two days before processing, flies were removed from food containing vials and placed in vials containing a Kimwipe soaked with a 1% sucrose solution and left for two days to allow them to clean their eyes. After two days flies were anesthetised using FlyNap® and placed in fixative (1˚ EMFix, 3% glutaraldehyde, 2% formaldehyde in 0.1M phosphate buffer, pH 7.2). Flies were then taken down to MMIC for processing using the FEI Quanta 200 Environmental Scanning Electron Microscope. Karnovsky fixative (3% glutaraldehyde, 2% formaldehyde in 0.1 M phosphate buffer, pH 7.2 plus Triton X-100). Flies were fully emerged before being taken down to MMIC for processing. Samples were fixed for an additional eight hours at room temperature before being washed with phosphate butter (0.1M, pH7.2). Samples were then dehydrated using a graded ethanol series (25%, 50%, 75%, 95% and 100%). The Polaron E3000 series II critical point drying apparatus was used to dry the samples using liquid CO2. Samples were then mounted on to aluminium stubs and coated with gold (Baltec SCD 050 putter coater). Samples were then viewed and imaged using the FEI Quanta 200 Environmental Scanning Electron Microscope at an accelerating voltage of 20 kV. This process was carried out by Ms Niki Minards at the Massey Microscopy and Imaging Center (MMIC), Institute of Fundamental Sciences, Palmerston North.

3.10 Preparation of whole cell lysates

Lysates using adult flies: Flies were anesthetised in FlyNap® and heads removed with a razor blade. Heads were placed in an Eppendorf tube that was kept on ice. Twenty heads were isolated per genotype and 100 µL Radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-Cl - pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140mM NaCl. Before use, add: 1 mM PMSF) + 1X Complete Mini protease inhibitors (Roche) was added to the tube. Heads were homogenised for 30-45 seconds using a motorised pestle. The tubes were incubated on ice for ten minutes then placed in a 4℃ centrifuge (Heraeus™ Fresco™ 21 Microcentrifuge) and centrifuged at 13,000g for 5 minutes. The upper aqueous phase was transferred to a new pre-chilled Eppendorf tube and centrifuged again at 13,000g for 5 minutes at 4℃. The supernatant was then transferred to a new pre-chilled tube and stored at -80℃.

Lysates using larvae: Instead of using adult flies 3rd-instar larvae were used instead. Fifteen to twenty wandering third instar larvae were collected from the walls of the vials and placed in an Eppendorf tube that was kept on ice. The remaining steps were the same as previously described for adult flies.

3.11 BCA Assay

Cell lysates were removed from -80°C and allowed to thaw in an ice bucket. The colourimetric protein quantitation assay (Pierce™ BCA Protein Assay Kit, Thermo Scientific™) was used as per manufacturer's instructions. Samples were diluted 1:100 in RIPA buffer and pipetted into a transparent 96 well plate (Greiner Bio-One CELLSTAR® 96 well transparent polystyrene plates) alongside the standards. Following a 30 minute incubation, the plate was read on a plate reader (BioTek PowerWave HT Microplate Spectrophotometer) at 562 nm with Gen5™ analysis software (BioTek).

3.12 Negative Geotaxis assay

The Negative Geotaxis assay described by Barone and Bohmann (2013) was used to assess locomotor abnormalities in *Drosophila*. Thirty female flies from each genotype were anesthetised and placed in flat bottom vials without food so they were able to climb without the distraction of food or other stimuli that may affect their ability to climb. The vial was gently banged against a hard surface which resulted in the flies moving to the bottom of the vial, and then the number of flies that climbed above 5cm in 10 seconds were counted. These parameters were used as preliminary experiments carried out to evaluate the assay indicated that most control flies climbed above 5cm in 10 seconds. The assay was repeated ten times on each vial of flies. As flies of some genotypes showed poor climbing ability and appeared to display a startle response at the beginning of the assay when they were banged to the bottom of the vial, and flies were also assessed on their ability to climb 5cm in 30 seconds to allow time for them to recover.

3.13 Quantification of DNA and RNA

The DeNovix DS-11 Spectrophotometer was used to measure concentrations of either DNA or RNA. Samples were purified, and 1 µL of sample was used for measuring the concentration. The arm was wiped with a damp Kimwipe to clear it of previous contaminants. 1 µL of the appropriate blank was used before measuring samples. A damp Kimwipe was used between each measurement. 1 µL of sample was placed on the arm and absorbance was measured at 260 and 280 nm. The concentration of DNA or RNA was calculated from the absorbance at 260 nm. The purity of the DNA was assessed via the 260/280 nm ratio. A ratio of >1.8 and >2.0 indicated pure DNA and RNA, respectively.

3.14 Preparation of plasmid DNA

3.14.1 Transformation

DNA received from GenetiVision was initially centrifuged at 6,000g for 1 minute at 4°C to ensure all DNA was at the bottom of the tube. Distilled H_2O was then added to the DNA and vortexed for 1 minute to resuspend. The tube was then placed in a heat block (Thermolyne DB28125 Dri-Bath, Marshall Scientific) at 50°C for 15 minutes to dissolve the DNA.

Competent cells (DH5α) were removed from -80°C and placed in an ice box to thaw. Once thawed 40 µL of competent cells were placed in a new Eppendorf tube, and 2 µL of plasmid DNA was added to it. Tubes were then incubated for 30 minutes on ice then heat shocked in a 42°C water bath for 30-45 seconds. Tubes were then placed back on ice for 2 minutes before 500 µL of Luria-Bertani (LB) media (For 1 L of LB: 500 mL dH2O, 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl, adjust pH to 7.5 with NaOH, add dH2O to make it up to 1 L) was added and then they were incubated at 37°C for 45 minutes. 100 µL of the cell culture was added to the appropriately labelled LB-agar plate supplemented with ampicillin (amp concentration: 100 µg/mL). The remaining culture was spun down and the supernatant removed. The pellet was then resuspended in 100 µL of LB-media. Plates were left to dry for 10 minutes then incubated at 37°C overnight.

3.14.2 Large-scale preparation of plasmid DNA

A total of 5 mL of LB-media containing ampicillin (100 µg/mL) was placed in a 15 mL tube. A single colony was selected from the ampicillin plates and placed in the LB-amp media. Tubes were placed at 37℃ to allow growth. Once adequate growth had occurred 1 mL of the cell culture was added to a flask (200 µL ampicillin:100 mL LB-media). Flasks were then placed on an orbital shaker at 220- 225 rpm at 37℃ overnight. The PureLink HiPure Plasmid Filter Purification kit (Invitrogen) was used for midi preparation of plasmid DNA and was used as per the manufacturer's instructions.

3.14.3 Small-scale preparation of plasmid DNA

LB-media containing ampicillin (100 µg/mL) was placed in 15mL tubes. A single colony was selected and placed in the media. Tubes were placed on an orbital shaker at 220 - 225 rpm at 37℃ overnight. The DNA-spin Plasmid Purification Kit (iNtRON) was used for mini preparation of plasmid DNA and was used as per manufacturer's instructions.

3.15 DNA precipitation

DNA precipitation was carried out using 1 volume of DNA; 1/10 volume to 3M sodium acetate; the 2 volumes (DNA volume + 1/10 volume sodium acetate) of 100% absolute Ethanol. DNA was spun at 13,000g for 10 minutes at 4℃. The supernatant was removed, and the pellet was washed with 70% ethanol. The DNA was spun again at 13,000g for 5 minutes at 4℃. The pellet was then air dried and resuspended in Tris-EDTA (TE) buffer.

3.16 DNA sub-cloning of *coq8*-myc

3.16.1 pUASTattB vector

The pUASTattB vector is often used for expression of transgenes in *Drosophila*. The pUASTattB vector contains the GAL4-dependent UAS which is crucial for expression of *Drosophila* transgenes. The *coq8*-myc construct arrived in the pUC57 vector and needed to be removed and placed into the pUASTattB vector for use in characterisation experiments.

The pUASTattB vector was digested with XhoI and XbaI, so the plasmid was open and ready for ligation with the insert. The *coq8*-myc containing pUC57 was digested with XhoI and XbaI to release the *coq8*-myc insert from pCU57 for further ligation (methods 3.16.5).

3.16.2 Restriction Digest

Restriction digests were carried out using plasmid DNA (appendix 9.8) and the appropriate restriction enzymes (appendix 9.4). For preparative digests required for DNA subcloning, the following reaction mixes (table 3.3) were prepared.

Table 3.3 Restriction digest reaction recipe.

Component	Volume 40 µL total
DNA 5 µg	x µL
Restriction enzyme (each)	0.5 µL
10X Buffer	4 µL
Water	Make up to 40 µL

For analytical restriction digests, a total volume of 10 µL was used. Up to 1 µg of DNA was used for each reaction which also contained 1X buffer, 0.5 µL enzyme and dH_2O up to 10 µL.

Samples were placed in at either 21℃ or 37℃ for 2 hours depending on the activation temperatures of the restriction enzyme. A 1% agarose gel containing 0.5 µg/µL ethidium bromide (to visualise DNA bands) in 1X TAE was poured into the gel dock. The appropriate comb was selected, and the gel was poured to the desired thickness. The gel was left to set for 30 minutes. Samples were then removed from the water bath, and the appropriate amount of 1X loading dye was added to each tube. Samples were left on ice until needed.

3.16.3 Running an agarose gel.

A 1% agarose gel was made with TAE and ethidium bromide. Once set was placed into the Mini-Sub® Cell GT Horizontal Electrophoresis System (Bio-RAD) and 1X TAE

containing 3 µg of ethidium bromide (EtBr), was added to the tank until the gel was just submerged. Samples were loaded in the appropriate lane and were run alongside the 1kb plus DNA ladder (Invitrogen). The gel was run at 100V using a PowerPac™ Basic Power Supply (BIO-RAD) until the dye front was two-thirds of the way down the gel (>45 minutes). If the gel was to be imaged, it was imaged on the Gel Doc™ XR+ Gel Documentation system.

3.16.4 Gel purification

Once bands had separated enough on the gel, it was placed on a UV block and bands removed for each appropriate construct using a sterile steel surgical blade (Swann-Morton). The gel slices were placed in new Eppendorf tubes, and the PureLink™ Quick Gel extraction kit (Life Technologies) was used as per manufacturer's instructions to purify vector and insert DNA. The purified DNA was then run on a new 1% gel and the concentrations assessed based on the gel. Once concentrations were verified ligation mixtures were set up.

3.16.5 DNA Ligation

After purifying the plasmid DNA, ligation mixtures were set up as described in Table 3.4. Ligation mixtures contained 100ng of vector DNA was used with a 5:1 vector to insert ratio which included 10X T4 DNA ligase reaction buffer and T4 DNA ligase (New England BioLabs). Ligation mixtures were placed at 18℃ overnight and transformed the next day.

Table 3.4 Ligation mixtures.

Ligation mixture	**pUAST 1**	**Ligase**	**Buffer**	***coq8*-insert**
A	0.5 µL	1 µL	1 µL	7.5 µL
C	0.1 µL	1 µL	1 µL	7.9 µL

Ligation mixture	**pUAST 2**	**Ligase**	**Buffer**	***coq8*-insert**
B	0.5 µL	1 µL	1 µL	7.5 µL
D	0.1 µL	1 µL	1 µL	7.9 µL

3.16.6 Cloning restriction digest

Colonies that had grown on LB-ampicillin plates from the ligation mixtures were isolated and transformed (methods 3.14.1), and a mini preparation (section 3.14.3) was carried out to determine whether cloning was successful. Each colony was placed in a separate tube, and transformation protocols followed (methods 3.14.1). DNA was purified and measured using Nanodrop (methods 3.13). Restriction digests (methods 3.16.2) were set up for each colony and were cut with XhoI and XbaI to determine whether both the *coq8*-myc insert and the pUASTattB were present in the clones. The presence of bands at the appropriate size would result in successful cloning.

3.16.7 DNA Sequencing

The pUASTattB/*coq8*-myc plasmid was sequenced to confirm that there were no errors in the sequence. The primers are described in (Table 3.5). Sequencing was performed by the Massey Genome Service (Institute of Fundamental Sciences, Massey University).

Table 3.5 Primer sequences used for sequencing reactions.

Oligo Name	Primer Sequence (5'-3')	Tm°	Source
***coq8*-For**	TCTACCGCCACGAGTTCATC	65.2	Sigma
***coq8*--Rev**	ACTCCCTGGCGATTGTTCTC	65.4	Sigma

3.17 Generation of transgenic flies

3.17.1 Preparation of plasmid DNA

Plasmid DNA was precipitated as previously described (methods 3.15). Plasmid DNA was resuspended in 50 µL of injection buffer (50 mM KCl, 0.1 mM sodium phosphate buffer, pH 6.8,) and stored at -20℃ until needed.

3.17.2 Egg harvesting

Two days before microinjection, three bottles of flies containing the VK37 C31 landing site (Table 9.1) were transferred into three small plastic beakers. Each fly containing beaker was then placed onto polystyrene Petri dishes (Fisher Scientific) (referred to as

egg plates) containing standard fly food with yeast paste spread on top to increase laying efficiency and housed at 25℃. The day of microinjection females were transferred to new egg plates for an hour enabling females to release any old eggs they may be harbouring. During this time a large glass Petri containing silicon beads was placed in a 200℃ oven. The silicon plate was used to dehydrate the embryos before injection. Once the hour had elapsed, the silicon beads were removed from the oven and the lid placed on top. The egg plates were replaced and left for 20-30 minutes. After this time, eggs were collected with a fine paintbrush and brushed onto a slide containing a strip of double-sided tape to hold the eggs in place. Once eggs were secured, two blunt tweezers were used to dechorionate the embryos. Tweezers were used to gently pierce the chorion and then the embryo was gently removed from the casing and placed on a separate piece of double-sided tape affixed to a microscope slide with the posterior end oriented to the right edge of the tape. Dechorionation continued for 5-10 minutes before the slide was placed on the silicon beads for 5-7 minutes to dehydrate the embryos so they would not burst during microinjection. The embryos were then covered in halocarbon oil 700 (Sigma-Aldrich) to prevent further dehydration and reduce cytoplasmic leakage once injected.

3.17.3 Microinjection

A very sharp microfilament (A-M Systems), which was pulled by a laser-based micropipette puller (P-2000, Sutter Instrument®) was used to inject plasmid DNA into the posterior end of the embryo. 2 µL of *coq8*-myc DNA was loaded into the very tip of the microfilament using a 20 µL Eppendorf Microloader™ tip. The microfilament was then placed into a micro-manipulator (Leica) and lowered into the slide containing the dechorionated embryos and submerged into the halocarbon oil. Microinjections were carried out on the Olympus CKZ inverted compound microscope which was attached to the Femto jet express Eppendorf microinjector which utilised pressurised nitrogen gas. A tiny amount of DNA was injected into the embryos. Once a sufficient number of embryos had been injected the slides were placed in a humidity chamber (QNA international MIC-101) with approximately 2 atmospheres of oxygen added to the vessel and the vessel was placed at 18℃ overnight. The next day more oxygen was added to the vessel, and it was placed at 21℃ overnight.

3.17.4 Larval collections

On the third day after injections, any larvae that were alive and had developed to the first instar larval stage were collected and placed on a small piece of Kimwipe (Kimberly-Clarke Kimwipe). Once there were 10-20 larvae on a piece of Kimwipe, it was placed in a 30 mL vial containing standard fly food. The Kimwipe was moistened, so it was in contact with the fly food enabling larvae to move and eat. Vials were labelled and placed at 25°C until adults emerged. It was important that the Kimwipe was moistened each day to prevent larvae from drying out.

3.17.5 Establishing a transgenic fly line

Flies that emerged following microinjection (G_0), all had white eyes and were crossed to CyO virgin females or males which contained the curly balancer chromosome on the second chromosome. Progeny produced from these crosses were either white eyed or orange eyed. Those that were curly winged and orange eyed (G_1) contained the *coq8*-myc construct and the balancer chromosome which prevented recombination in females. Males and females were crossed together to produce homozygous progeny. Males and virgin females that had wild-type wings and orange eyes (G_2) were selfed to generate a stable homozygous line.

3.18 ATP Assay

The ATP assay was carried out using the ATP Bioluminescence Assay Kit CLS II (Sigma-Aldrich) as per manufacturer's instructions.

3.19 Statistical analysis

Experiments were carried out in triplicate to determine statistical significance for progeny counts. The data was then subjected to a one-way ANOVA test followed by post hoc analysis using the Tukey HSD test with a significance level of $p<0.05$. Means +/- standard errors were then graphed and significance levels indicated as either * = $p<0.05$ or ** = $p<0.01$.

Negative geotaxis assays were carried out on 30 females flies per genotype and trials were repeated ten times per genotype. The number of flies that passed the 5cm mark were divided by the total number of flies in the vial (n=30). A one-way ANOVA was carried out on the data followed by post hoc analysis using the Tukey HSD test. Means

+/- standard error were then graphed, and significance levels indicated as either * = $p<0.05$ or ** = $p<0.01$.

3.20 Mitochondrial Targeting Sequence software

The amino acid sequence was run through each program to determine the presence of a MTS on *Drosophila coq8.*

Table 3.6 Software used to determine the presence of a mitochondrial targeting sequence.

Website Name	Description	Website address
MitoFates	A prediction tool for identifying putative mitochondrial presequences and cleavage sites	http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi
TargetP 1.1 Server	Predicts the subcellular location of eukaryotic proteins.	http://www.cbs.dtu.dk/services/TargetP/
TPpred 2.0	Prediction of cleavage sites of mitochondrial targeting peptides	https://tppred2.biocomp.unibo.it/welcome/default/index
iPSORT	Subcellular localisation sire predictor for N-terminal sorting signals	http://ipsort.hgc.jp/#predict

4 Results

In humans, ARCA2 is homozygous recessive, and thus usually occurs via compound heterozygous inheritance in which both parents carry a single pathogenic mutation but present no symptoms. As an additional diagnostic approach whole genome and whole exome sequencing are often implemented clinically to determine the genetic mutations associated with the phenotype. What is not clear is whether the identified genetic mutations are responsible for the phenotype. Functional characterisation aids in our understanding of how the gene works and what mutant phenotypes these mutations induce. *Drosophila*, with its set of genetic tools for gene manipulation, provide the means to functionally characterise COQ8A mutations in a rapid and inexpensive manner. The initial objective of this project was to knock down the *Drosophila* homologue of human COQ8A via the UAS/GAL4 system and characterise the resulting phenotypes.

The UAS/GAL4 system is a powerful binary system used in *Drosophila* to turn genes on or off in a cell and/or tissue specific manner. GAL4 is not endogenously expressed in *Drosophila* providing a highly specific system that functions with high fidelity. Targeting gene expression in this way allows for in-depth investigations into cellular and molecular function in individual subsets of cells and/or tissues.

Mutations in *coq8*, the *Drosophila* homologue of COQ8A and COQ8B, are homozygous lethal. Therefore we expected that a robust RNAi-mediated knockdown of *coq8* should also be lethal. To that end, the *da*-GAL4 driver was selected. *da* is expressed ubiquitously throughout *Drosophila* during most stages of development (Vaessin et al., 1994), thus when placed under control of the *da* promoter, GAL4 drives a high level of ubiquitous expression. A second ubiquitous driver, *arm*-GAL4 was also selected. *arm* is ubiquitously expressed through most stages of development but at a weaker level than *da* (Rewitz et al., 2010). The rationale for using *arm*-GAL4 was to confirm the results obtained with *da*-GAL4. If a lower level of knockdown of *coq8* allowed survival to a later developmental stage, these larval and/or adults could be phenotypically characterised. As the clinical presentations seen in those individuals affected with COQ8A mutations included neuronal phenotypes together with cerebellar atrophy and motor dysfunction, it was also desirable to select a brain-specific driver to characterise the neuronal phenotypes resulting from knockdown of *coq8*. *elav* is

essential for development in the embryonic nervous system (Robinow & White, 1988), and is expressed in all post-mitotic neurons of the CNS and the PNS as well as in post-mitotic neurons (Jiménez & Campos-ortega, 1987; Rewitz et al., 2010). Following selection of these drivers, the first task was to confirm the expression patterns associated with each of the core driver lines used in this project by examining the expression of GFP. CD8::GFP is a plasma membrane-targeted GFP which is ideal for visualisation of neuronal processes.

4.1 Characterisation of the expression pattern associated with the three main GAL4 driver lines

The use of these three driver lines will provide a profile of the function of *coq8* in *Drosophila*. Five virgin females from each driver line were crossed to five UAS-CD8-GFP males (methods 3.2). The UAS-CD8-GFP flies enabled visualisation of the expression patterns associated with each driver line utilising GFP for visualisation. Representatives were selected for each larval stage and then placed at -20°C until they were incapacitated. The remaining larvae were left in the vial until pupation and finally eclosion which was when they emerged as adult flies. Larvae and adult flies were imaged (methods 3.5). Adult fly brains were then dissected (methods 3.6) and imaged via confocal microscopy (methods 3.8).

When driven by *da*-GAL4, ubiquitous expression of GFP was observed throughout all stages of larval development, pupation and adulthood (Figure 4.1). Please note that the visualisation of GFP is partially occluded in adult flies due to the head and body casing. Expression in the brain was concentrated in the mushroom body lobes (Anterior) and the calyx (Posterior). The mushroom body is essential for memory and learning (McGuire et al., 2001) while the calyx provides sensory input into the mushroom body (Heisenberg, 1998). Overall, the expression is strong. This is consistent with previous work that suggested expression is ubiquitously expressed in *da* (Vaessin et al., 1994).

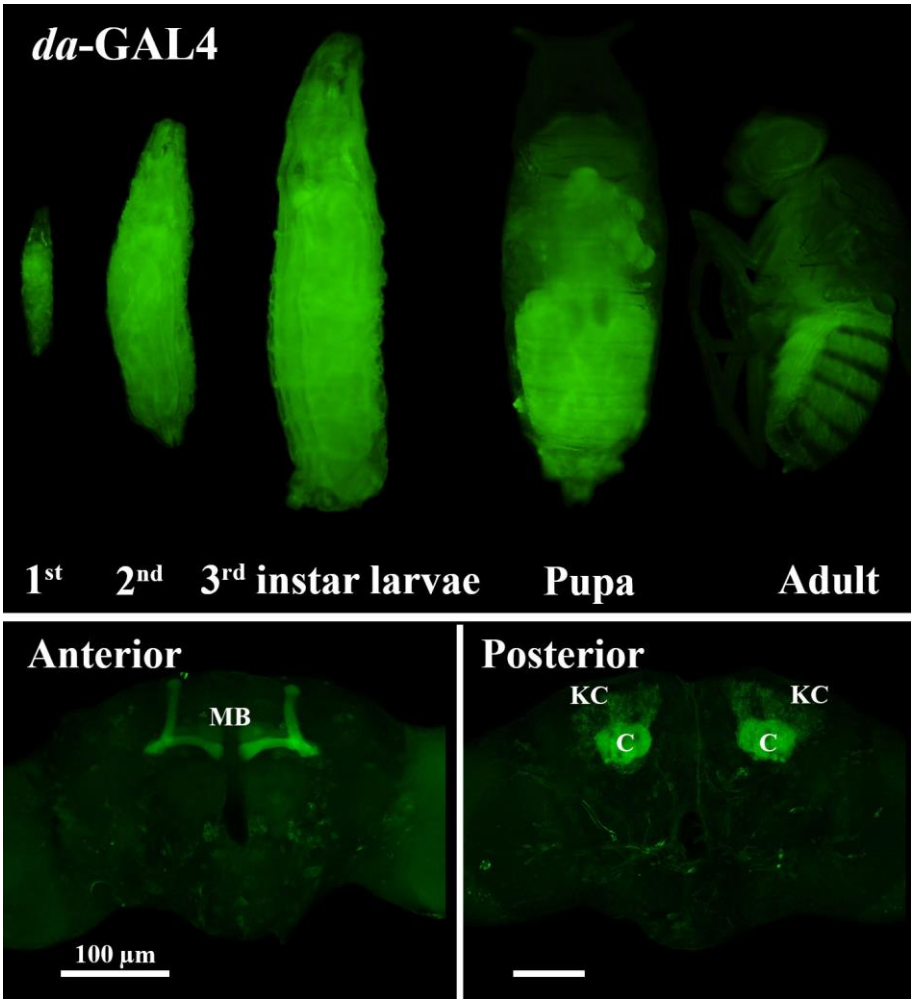


Figure 4.1. Expression patterns for the ubiquitous driver da-GAL4*. Expression is highlighted during all stages of development from larvae to adult and throughout the brain. MB = mushroom body, C = Calyx, KC = Kenyon cells.*
Genotype shown was UAS-CD8::GFP/da-GAL4.

arm-GAL4 is lethal when homozygous, so it is maintained as a heterozygous stock over a balancer chromosome. The balancer chromosome contains multiple inversions which prevent crossing-over and recombination. Balancer chromosomes have many different phenotypes that are chromosome dependent which makes it effortlessly distinguishable from other phenotypes enabling easy detection of flies that contain the transgene and flies that do not. Balancer chromosomes also prevents a homozygous recessive lethal stock from losing the recessive phenotype. Balancer chromosomes are a unique and significant part of *Drosophila* functional studies.

arm-GAL4 was used in conjunction with the TM3 balancer which results in stubbled (*Sb*) bristles and serrate wings which have a nick taken out of them. Any progeny that contain stubble does not contain the transgene and acts as a negative control for the experiment. A crossing scheme (Figure 4.3) was constructed to indicate the inheritability of the transgene when using the *arm*-GAL4 driver line. *arm*-GAL4 drove a similar expression pattern (Figure 4.2) to that seen in *da*-GAL4 (Figure 4.1) with GFP expressed ubiquitously throughout the body and during all stages of development. Expression was also seen in the MB lobes and also in the calyx.

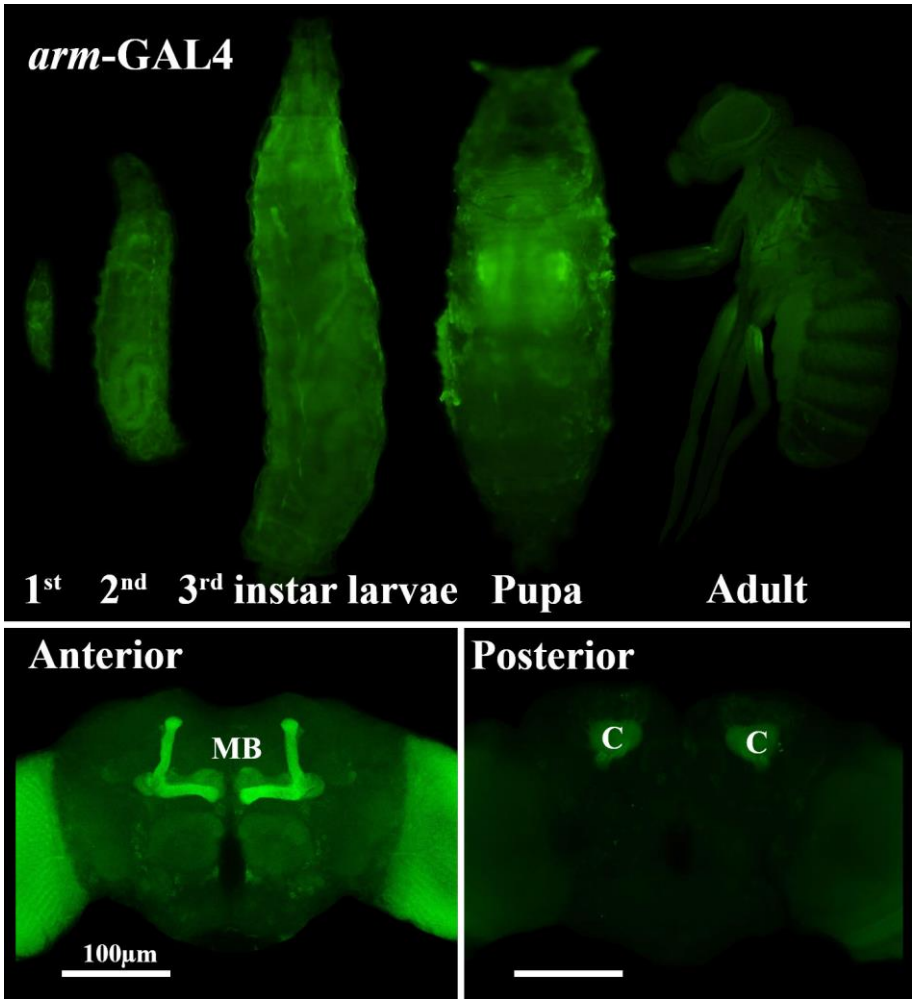


Figure 4.2 Expression patterns for the weak ubiquitous driver arm-GAL4. *Indicating ubiquitous expression during all stages of development but weaker than da-GAL4. MB = mushroom body, C = calyx. Genotype shown was UAS-CD8::GFP/arm-GAL4.*

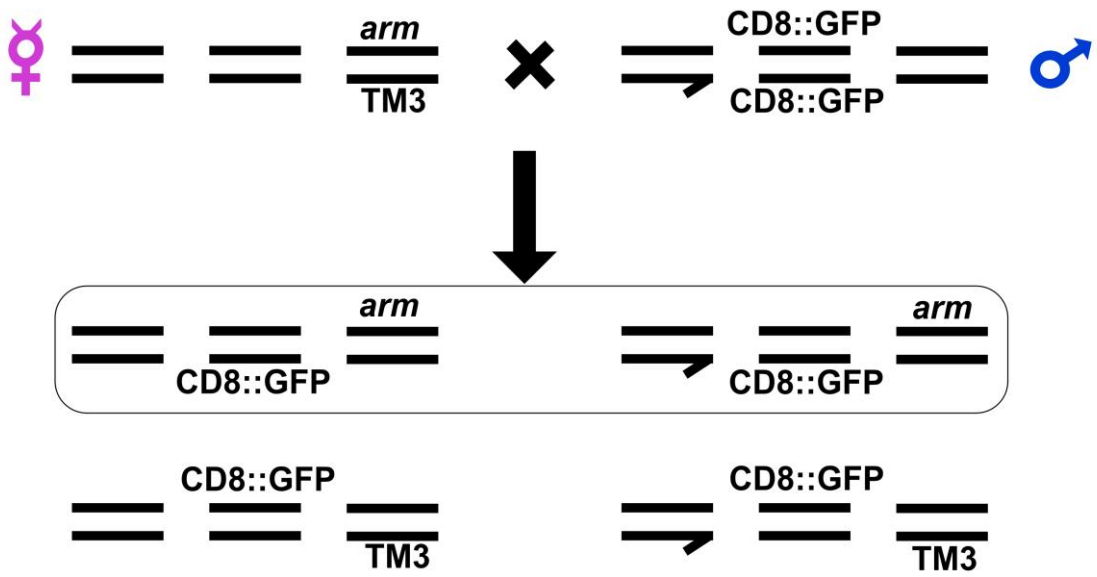


Figure 4.3 Crossing scheme using the weak ubiquitous driver arm-GAL4*. arm-GAL4 is maintained as a heterozygous stock over the TM3 balancer chromosome which results in a distinctive mutant phenotype resulting it stubbled adult progeny. Virgin females containing the arm-GAL4 driver line maintained over the TM3 balancer chromosome were crossed to adult males containing the UAS-CD8::GFP. Progeny that resulted from the cross resulted in males and females that contained UAS-CD8::GFP and arm-GAL4 which results in expression of the transgene. Males and females were also produced that contained UAS-CD8::GFP and the TM3 balancer chromosome. In the absence of arm-GAL4 there is no expression of the transgene and progeny lacking the transgene contain stubbled bristles.*

Mutations in human COQ8A result in various neurological phenotypes including cerebellar degeneration and ataxia. The *elav*-GAL4 driver was used to knockdown *coq8* in all neurons to characterise the impact reduced *coq8* expression has on neuronal development and function in *Drosophila*. Expression of CD8::GFP driven by *elav*-GAL4 (Figure 4.4) indicates a more specific expression pattern. When compared to the ubiquitous *da*-GAL4 (Figure 4.1) and *arm*-GAL4 (Figure 4.2), *elav*-GAL4-driven GFP is expressed at a higher level in the larval brain (Figure 4.4, circles). In the adult brain (Figure 4.4, anterior-posterior), expression is also higher in the mushroom body (anterior) and the calyx (posterior). It is also worth noting that expression is seen in the salivary glands in larvae (outside the circles) which was previously described (Campos et al., 1987).

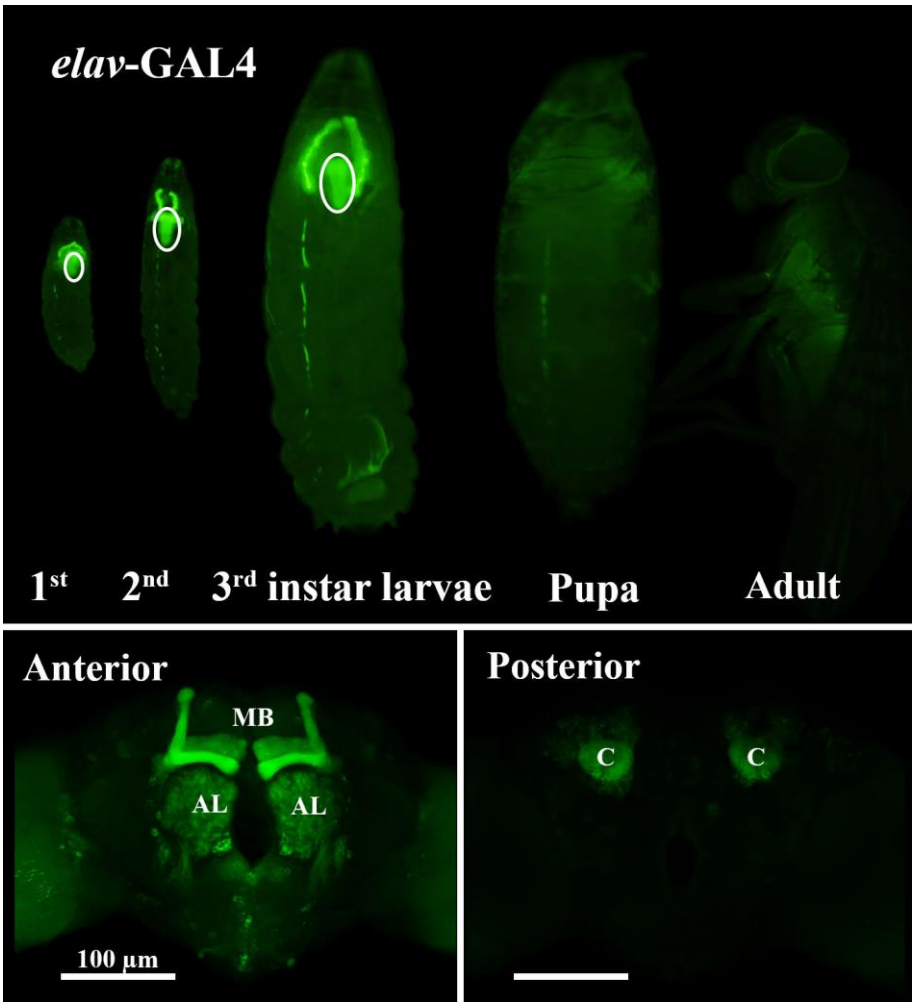


Figure 4.4. Expression patterns using the pan-neuronal driver elav-GAL4. *Expression patterns of the highly specific pan-neuronal elav-GAL4 driver using CD8-GFP. Genotype was UAS-CD8::GFP/elav-GAL4*

Expression profiles highlighted the specificity of each driver line used in this project. *da*-GAL4 (Figure 4.1) and *arm*-GAL4 (Figure 4.2) can be used to characterise the overall function of *coq8* in *Drosophila*. Due to the clinical presentation in humans with COQ8A mutations the *elav*-GAL4 driver (Figure 4.4) will enable characterisation of the impact *coq8* has on the neurons and more specifically the brain. By expressing *coq8* in the brain alone, we can further characterise the function and expression patterns of *coq8*.

4.2 Characterisation of the phenotypes resulting from RNAi knockdown of *coq8* in *Drosophila*

After characterising expression of the driver lines (section 4.1) the impact of ubiquitous knockdown of *coq8* was examined. In *Drosophila*, gene-specific knockdown is achieved via the expression of an inverted repeat RNAi that is fused downstream of a UAS. A library of UAS-RNAi lines targeting most genes in the *Drosophila* genome has been generated by the VDRC. The RNAi library was bioinformatically designed by using *Drosophila* genome sequences that utilised explicitly targeted inverted repeats to known protein-coding genes (Dietzl et al., 2007). In doing so, the off-target effects were minimised. However, it has been previously mentioned that not all RNAi lines are equally efficacious in knocking down gene expression (Dietzl et al., 2007) and, for that reason, more than one RNAi line has been developed for a single gene, including *coq8.* Two UAS-RNAi fly lines (termed *coq8* KD and *coq8* KD2), each targeting a different sequence within *coq8*, were ordered from the VDRC.

4.2.1 Assessment of survival following *da*-GAL4-mediated knockdown of *coq8*

Ten *da*-GAL4 virgin females were crossed to either ten control, *coq8* KD or *coq8* KD2 and placed at 21℃ (methods 3.2). Progeny counts were carried out 20 days post cross and were carried out as previously described (methods 3.2). Pupae, males and females (Figure 4.5) indicated a significant decrease in *coq8* KD when compared to control (ANOVA, post hoc Tukey HSD Test, $p<0.01$ pupae, males and females) and a significant decrease in *coq8* KD when compared to *coq8* KD2 (ANOVA, post hoc Tukey HSD Test, $p<0.01$ for pupae, males and females). These results suggest that larval lethality was observed in *coq8* KD progeny. A significant decrease was also seen between control and *coq8* KD2 adult males (ANOVA, post hoc Tukey HSD Test, $p<0.01$).

These data suggest that the decrease in the number of adult males is due to the absence of *coq8* in both *coq8* KD and *coq8* KD2. It also suggests that *coq8* KD is more effective at knocking down *coq8* than *coq8* KD2. The knockdown of *coq8* had a more significant effect on survival in *coq8* KD flies when compared to both control and *coq8* KD2. The

difference in the number of adult females and the number of pupae between control and *coq8* KD2 was not significant suggesting that *coq8* KD2 may not be as capable of knocking down *coq8* as *coq8* KD. However, further investigations were needed to support this idea.

Progeny counts (Figure 4.5) indicated that *coq8* KD had an adverse effect on survival in *Drosophila*. To further characterise those observations larval counts were carried out seven days post cross to assess development and survivability. Five *da*-GAL4 virgin females were crossed to either five control, *coq8* KD or *coq8* KD2 males and left for seven days. Adults were removed, and the contents of each vial examined (methods 3.3). Initial observations indicated a severe developmental delay in *coq8* KD flies but not in control or *coq8* KD2 (Figure 4.6) suggesting that the loss of *coq8* was responsible for the observed phenotype.

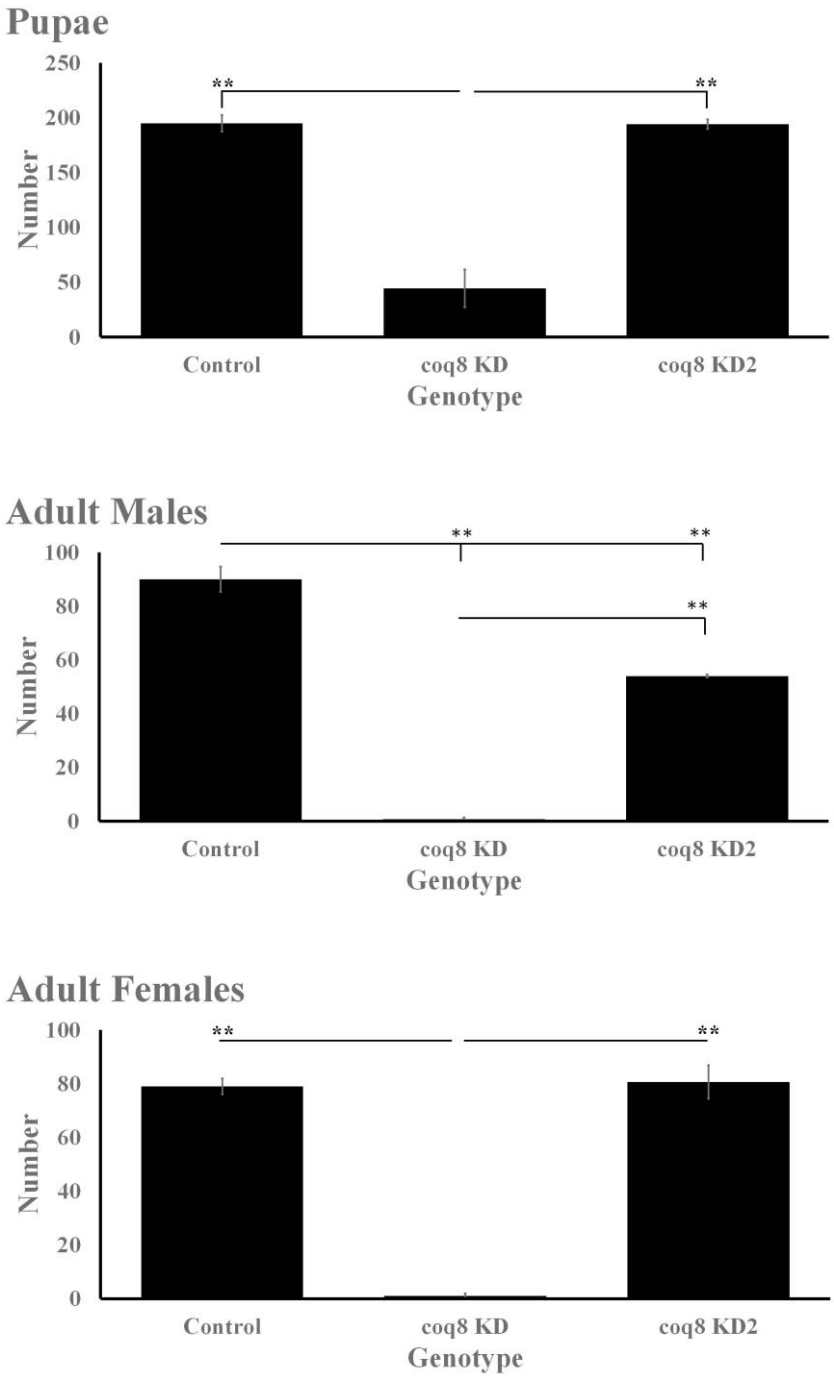


Figure 4.5 Progeny counts for the characterisation of coq8 knockdown using the ubiquitous driver da-GAL4. *Flies were raised at 21℃ and supplemented with normal fly food. Males, females and pupa were counted 20 days post cross. Genotypes shown are w1118/+;daGAL4/+ (control); w1118/+;da-GAL4/coq8KD and w1118/+;da-GAL4/coq8KD2. Means +/- standard error. ANOVA, post hoc Tukey HSD Test. Statistical significance * = p<0.05 and ** = p<0.01. Crosses carried out in triplicate.*

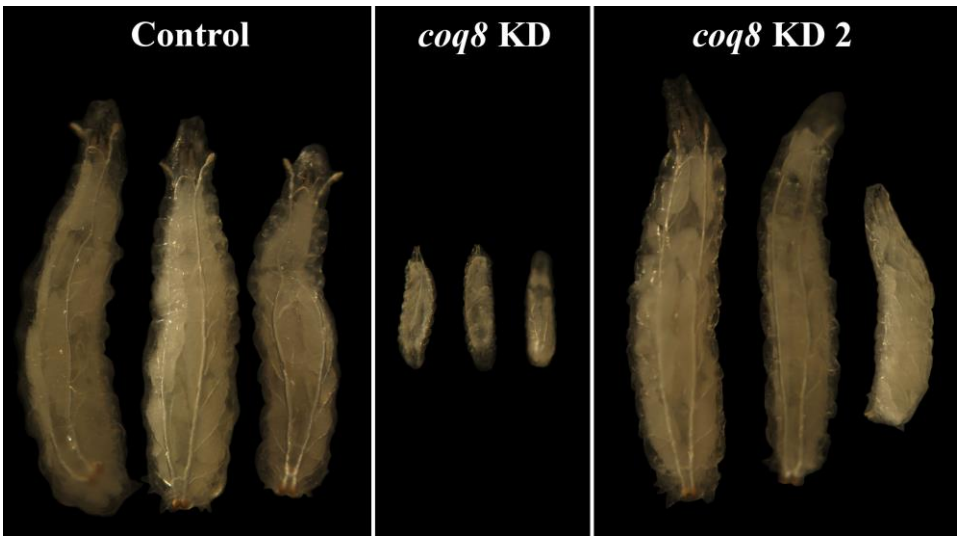


Figure 4.6 Larval phenotype from the ubiquitous knockdown of coq8. *Images were taken 7 days post egg laying. Severe developmental delay was seen in coq8 KD larvae but not in coq8 KD2 larvae. Genotypes shown are w^{1118}/+;daGAL4/+ (control); w^{1118}/+;da-GAL4/coq8KD (coq8 KD) and w^{1118}/+;da-GAL4/coq8KD2 (coq8 KD2).*

To quantify these observations larvae were counted (methods 3.3) and compared (methods 3.5). Counts for first instar larvae were similar across all three crosses (Table 4.1). Differences were seen for the 2nd and 3rd instar larvae with *coq8* KD larvae showing severe developmental delay when compared to control and *coq8* KD2. Again, this suggested that a more severe phenotype was seen in *coq8* KD progeny versus *coq8* KD2 progeny.

Table 4.1 Larval counts from the ubiquitous knockdown of coq8 in Drosophila.

Larval Stages	Control	*coq8* KD	*coq8* KD2
1st	31	20	14
2nd	24	3	27
3rd	19	0	14

Control and *coq8* KD2 larvae showed similar larval numbers across all larval stages with slightly less 1st instar larvae present in *coq8* KD2. The results obtained with *coq8* KD are consistent with the lethal phenotype expected from the loss of *coq8* expression. The lack of phenotype from *coq8* KD2 indicated that it is not effective at knocking down *coq8*. Therefore it was decided only to use *coq8* KD. Reintroduction of wild-type

partially rescued the knockdown phenotype confirming the specificity of the RNAi for the target mRNA (section 4.4.3).

4.2.2 Assessment of survival following *arm*-GAL4-mediated knockdown of *coq8*

Due to the larval lethality and severe developmental delay seen with the use of the strong ubiquitous driver, *da*-GAL4 (section 4.2.1), we decided to use the weaker ubiquitous driver *arm*-GAL4.

A similar crossing scheme to that used for the *da*-GAL4 experiments was implemented. Adult progeny of each genotype were counted 27 days post cross (methods 3.2), at which time all viable adults had emerged. Larval phenotypes could not be assessed because the stubble phenotype is only evident in adults. Therefore larvae cannot be correctly identified as to whether they contain the chromosome harbouring *coq8* KD or the balancer chromosome (in which case no knockdown can occur). In the control cross, approximately equal numbers of males and females with and without stubble phenotype emerged, as expected (Figure 4.7). This indicates that the presence of the *arm*-GAL4 driver (in the absence of *coq8* KD) does not affect viability. However, no non-stubble adults emerged from the *coq8* KD cross. Therefore, an expression of *coq8* KD was 100% lethal. There were significantly less *coq8* KD progeny when compared to the control (ANOVA, post hoc Tukey HSD Test, $p<0.05$) and *arm*-GAL4 progeny (in the absence of *coq8* KD) (ANOVA, post hoc Tukey HSD Test, $p<0.05$).

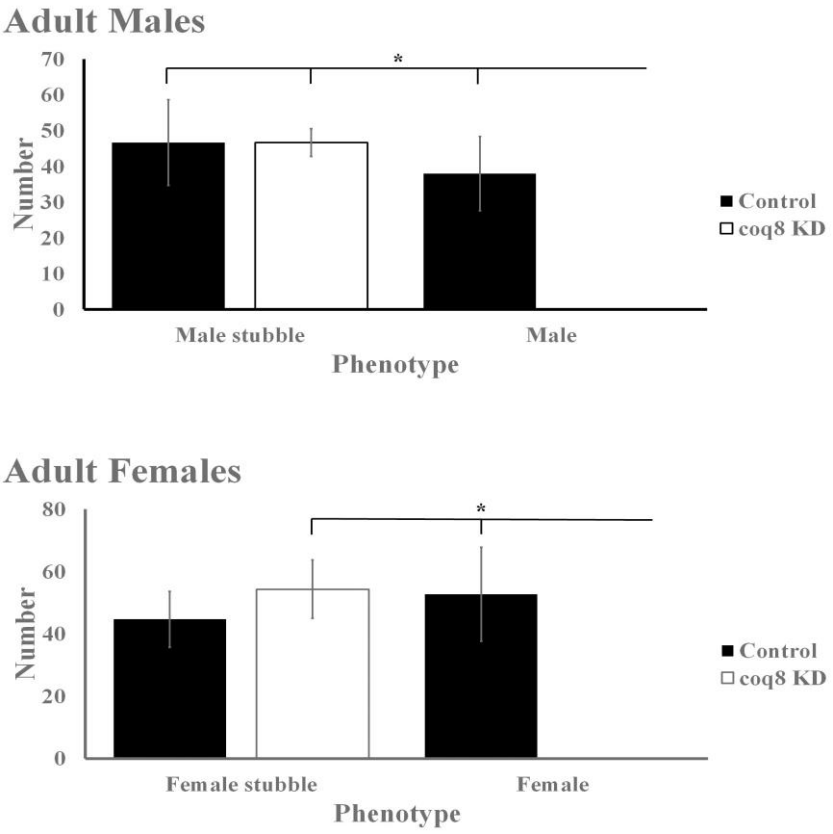


Figure 4.7 Progeny counts for the characterisation of coq8 knockdown using the ubiquitous driver arm-GAL4. *Flies were raised at 21°C and supplemented with normal fly food. Males and females were counted 27 days post cross. Genotypes shown are w^{1118}/+;arm-GAL4/+ (control); and w^{1118}/+;coq8KD/+; arm-GAL4/+ (coq8 KD). Means +/- standard error, ANOVA, post hoc Tukey HSD Test, * = p<0.05. Crosses were carried out in triplicate.*

4.2.3 Assessment of phenotypes following *elav*-GAL4-mediated knockdown of *coq8*

Due to the lethality associated with the ubiquitous knockdown on *coq8* in *Drosophila* and therefore the inability to produce viable progeny for behavioural analysis it was necessary to isolate the knockdown of *coq8* to the neurons. The pan-neuronal driver, *elav*-GAL4, was used to assess the neurological impact of *coq8* knockdown in *Drosophila*.

4.2.3.1 Analysis of the impact pan-neuronal knockdown of *coq8* had on survival

Ten *elav*-GAL4 virgin females were crossed to ten males from either control or *coq8* KD genotypes (methods 3.2). Progeny was counted 24 days post cross (methods 3.4). There was no statistical significance for pupal or female progeny counts (Figure 4.8) between control and *coq8* KD flies. However, male progeny counts showed a significant decrease in the number of *coq8* KD males when compared to control males (ANOVA, post hoc Tukey HSD Test, $p<0.01$).

These results suggest that the absence of *coq8* in the neurons of *Drosophila* resulted in male lethality but had no effect on female survival or overall pupal production. This semi-lethal phenotype enabled generation of female progeny for further analysis.

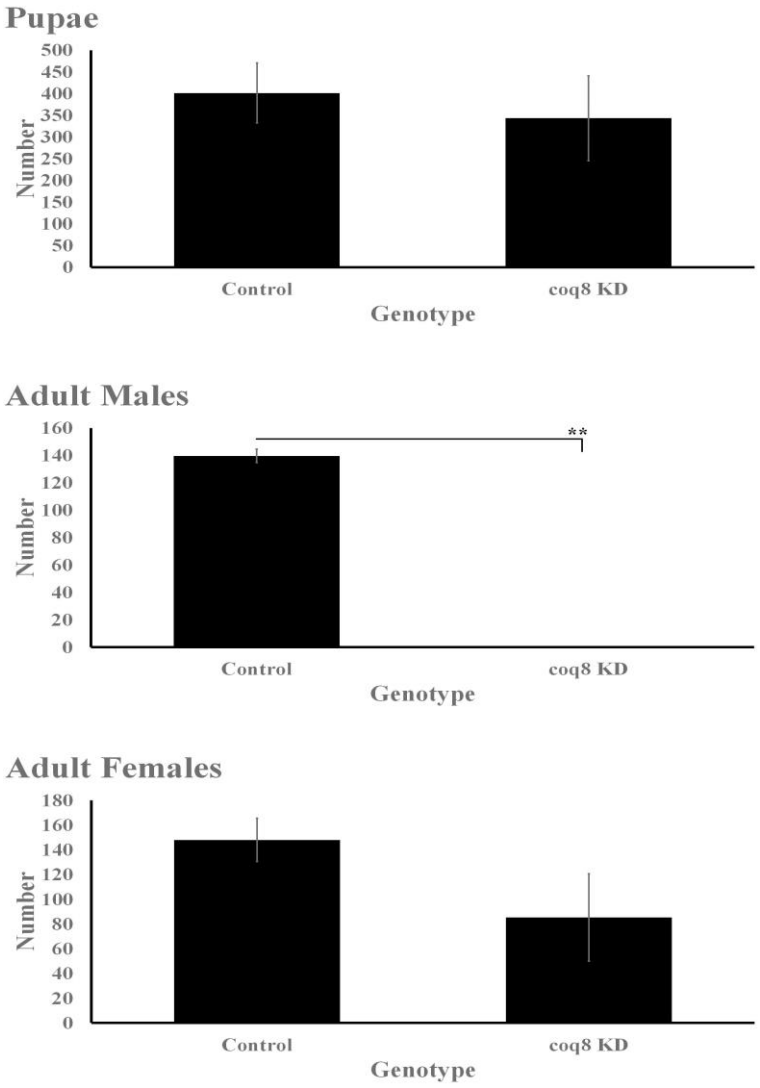


Figure 4.8 Progeny counts for the knockdown of coq8 in Drosophila using the pan neuronal driver elav-GAL4. *Flies were raised at 21℃ and supplemented with normal fly food. Progeny were counted 24 days post cross. Genotypes shown are w^{1118}/+;elav-GAL4/+ (control) and w^{1118}/+;elav-GAL4/coq8KD. Means +/- standard error, ANOVA, post hoc Tukey HSD Test. Statistical significance * = p<0.05 and ** = p<0.01. Crosses were carried out in triplicate.*

4.2.3.2 Characterisation of the expression patterns associated with the pan-neuronal knockdown of *coq8*

Mutations in human COQ8A result in various neurological phenotypes including cerebellar degeneration and ataxia. To characterise the impact of reduced *coq8* expression has on neuronal development and function in *Drosophila*, the *elav*-GAL4 driver was used to knockdown *coq8* in all neurons. *elav*-GAL4 females were crossed to either control or *coq8* KD male flies (methods 3.2). Flies were raised at 21℃ in vials. Once adults emerged, brains were dissected (methods 3.6) and subjected to immunohistological analyses (methods 3.7 and 3.8).

Initial immunofluorescence was carried out using the anti-nc82 antibody to mark and highlight the gross structure of the brain and an anti-caspase, Dcp-1, antibody that detects the early markers of caspase activity in *Drosophila* (Foo, Song, & Cohen, 2017*)*. Nc82 is a marker of presynaptic active zones in the brain (Wagh et al., 2006). Anti-nc82 binds Bruchpilot, a marker of active zones and thus highlights the neuropil, the synaptically dense region of the brain to allow an assessment of overall brain architecture.

There were no anatomical differences seen between *coq8* KD and the control as observed by anti-nc82 immunofluorescence (Figure 4.9). 1 µm sections were taken via confocal microscopy and showed no apparent vacuoles or anomalies in the structure of the brain (cross-section). The anti-caspase antibody showed no specific staining so gave no indication of apoptosis or caspase activity in the brain. The use of Apoliner employed an alternative approach to examining apoptosis.

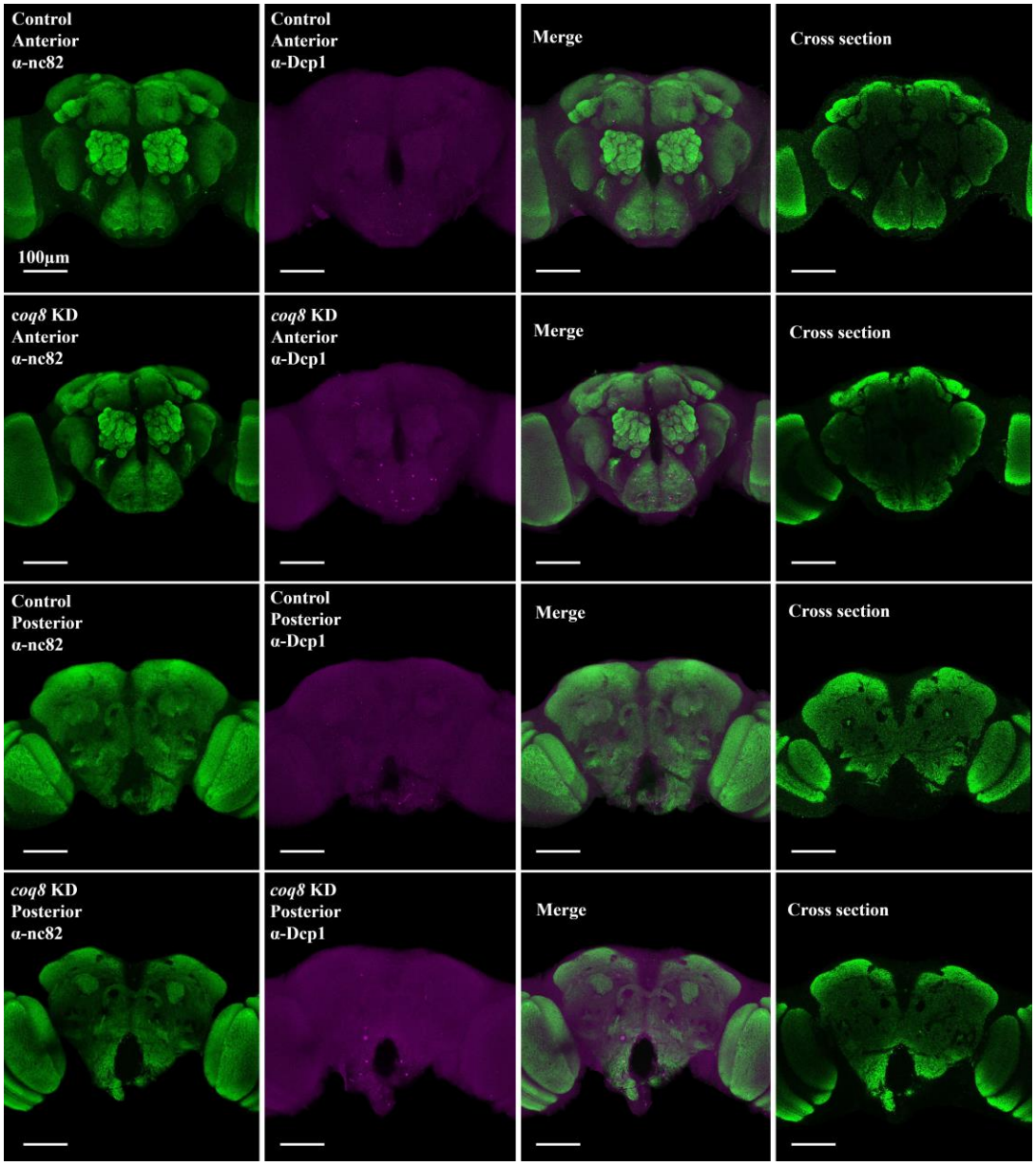


Figure 4.9 Analysis of the gross structure of the Drosophila brain and the impact coq8 KD has on apoptosis. *The gross structure of the Drosophila brain was highlighted using the α-nc82 antibody to stain the neuropil. In order to assess caspase activity, the Dcp1 antibody was used. Genotypes shown are w^{1118}/elav-GAL4; (control) and w^{1118}/elav-GAL4;coq8KD/+; (coq8 KD).*

4.2.3.3 Analysis of the impact the loss of *coq8* had on apoptosis in the *Drosophila* brain

Apoliner is a fluorescent reporter that can detect early caspase activity. Apoliner is composed of two fluorescent reporters, enhanced green fluorescent protein (eGFP) and monomeric red fluorescent protein (mRPF) which are tethered to each other by a caspase sensitive cleavage site (Bardet et al., 2008). mRPF contains a transmembrane domain while eGFP contains a nuclear targeting signal. Upon caspase activation, the caspase-sensitive cleavage site is cleaved, and eGFP translocates into the nucleus while mRPF maintains at the membranes. (Bardet et al., 2008). A new fly line was generated that contained both *elav*-GAL4 and Apoliner. *Elav*-GAL4 female virgin flies were crossed to Apoliner males to create a homozygous stock for this assay (methods 9.5). The resulting e*lav*-GAL4;Apoliner virgin females were crossed to the control and *coq8* KD males (methods 3.2), and brains of the resulting progeny were analysed via confocal microscopy (methods 3.6 and 3.8). No differences in nuclear GFP localisation was observed between control and *coq8* knockdown brains (Figure 4.10). Zooming in on the glomeruli, which are involved in olfactory learning (Semmelhack & Wang, 2009), showed that there was no detectable apoptosis present in the brain (Figure 4.10). This was the second mechanism used to assess the presence of apoptosis in the brain and the second instance where detection was abated. Crossing schemes can be found in appendix 9.5.

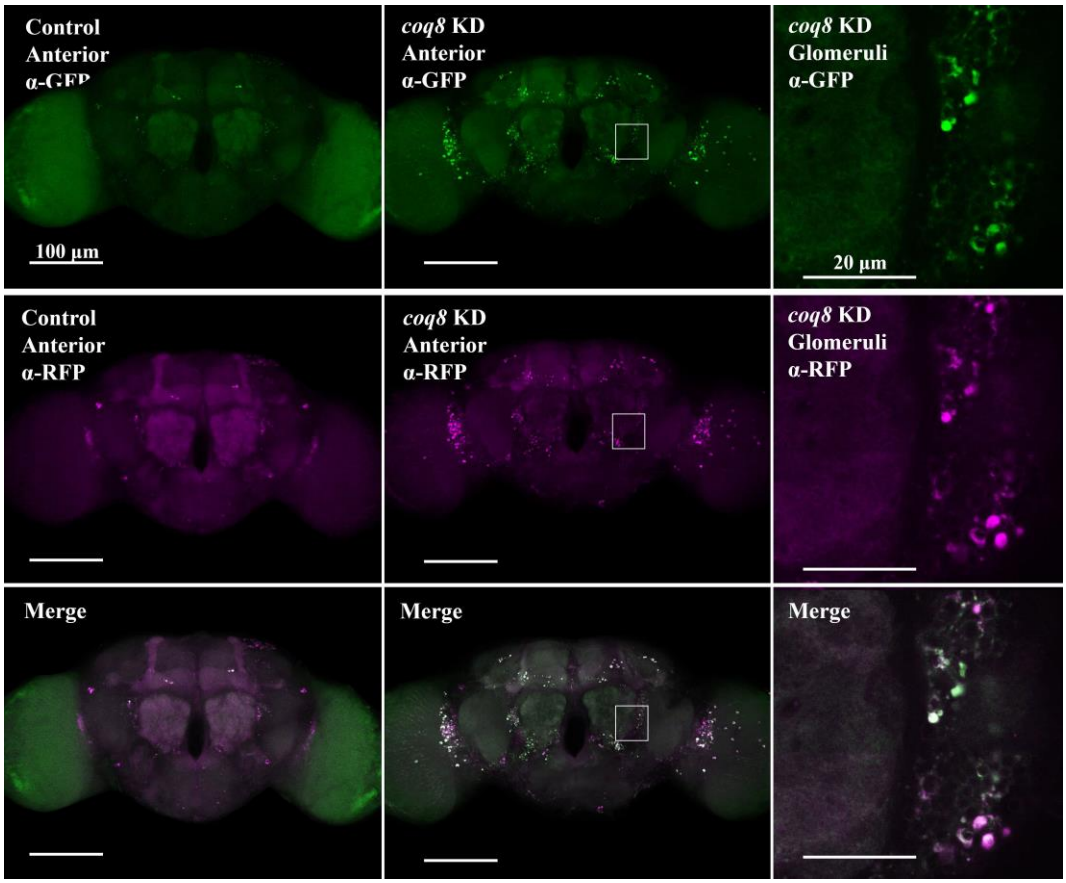


Figure 4.10 Analysis of apoptosis using Apoliner and elav-GAL4*. In the absence of coq8, brains show no increase in apoptosis in the brain. Indicating that in the absence of coq8 there is no change in apoptotic activity. Genotypes shown are* w^{1118}*/elav-GAL4; (control) and* w^{1118}*/elav-GAL4;coq8 KD/Apoliner; (coq8 KD).*

4.2.3.4 Negative Geotaxis assay to assess locomotor ability in flies lacking *coq8*

Viable *coq8* KD progeny were produced when expressed using the pan-neuronal driver, *elav*-GAL4. Viable progeny enabled further analysis to be carried out on *coq8* KD adult flies. Locomotor ability is often reduced in people afflicted with COQ8A mutations, so the progeny produced from *elav*-GAL4 crosses were used to characterise this mutant phenotype in *Drosophila*.

Pan-neuronal knockdown of *coq8* was semi-lethal in *Drosophila* (section 4.2.3) which enabled the survival of female progeny, but lethality was present in *coq8* KD males. Female survivors displayed locomotor impairment which was characterised by a delayed ability or inability to climb. To further characterise this, negative geotaxis assays were carried out. Negative geotaxis assays utilised the flies natural ability to climb once startled. A standard distance was determined by the capacity of most control

flies to climb up the sides of the test vial. This distance was 5cm in 10 seconds. Due to the locomotor impairment seen in the *coq8* KD flies, flies were assessed again at 30 seconds to see if an increase in recovery time would allow for an increase in climbing ability for *coq8* KD flies. Flies were collected 27 days post cross and assayed.

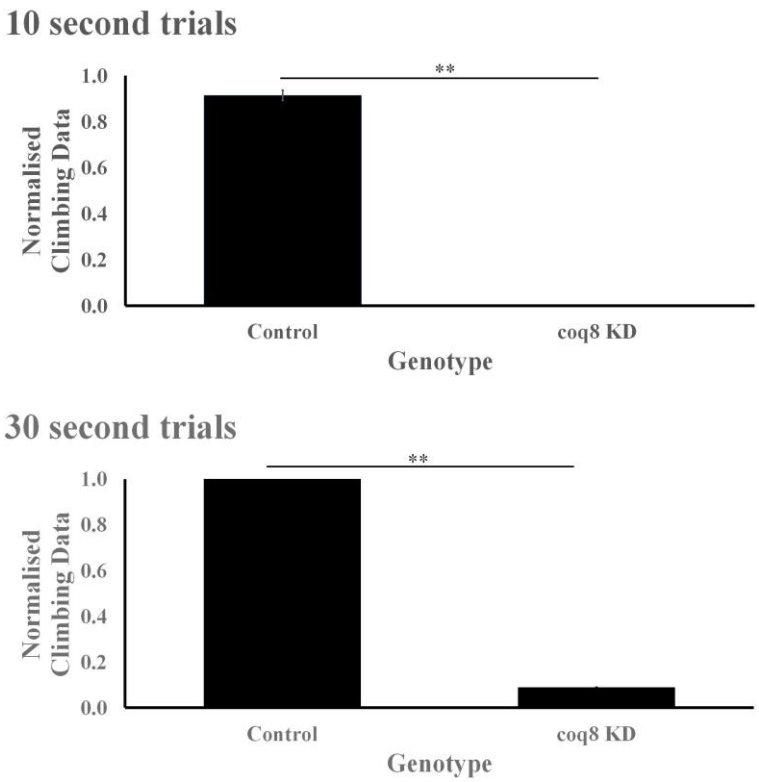


***Figure 4.11 Negative geotaxis assays used to determine locomotor ability**. The assay was carried out using progeny from the elav-GAL4 crosses in (section 4.2.3.1). Control and coq8 KD flies were used. Means have been normalised by dividing the number of flies that passed the 5cm line by the total number of flies for each trial (n=30). Flies were initially assayed at 10 seconds to assess locomotor ability before being assessed at 30 seconds to assess recovery in coq8 knockdown flies. Genotypes shown are w^{1118}/elav-GAL4; (control) and w^{1118}/elav-GAL4;coq8KD/+; (coq8KD). Means +/- standard error. ANOVA, post hoc Tukey HSD test. Statistical significance * = p<0.05 and ** = p<0.01.*

Negative geotaxis assays (methods 3.12) indicated that at both the 10 second and 30 second trials (Figure 4.11) control flies performed significantly better than *coq8* KD flies (ANOVA, post hoc Tukey HSD Test, p<0.01). At 10 seconds no *coq8* KD flies had crossed the line whereas almost all control flies had. At 30 seconds only 10% of *coq8* KD flies had crossed the line whereas most control flies had crossed the line. This indicates that increasing the recovery time enabled *coq8* KD progeny to recover enough to cross the 5cm mark. However, at 30 seconds, control flies were beginning to wander

back down the vial. These results indicated that pan-neuronal knockdown of *coq8* in *Drosophila* pan-neurons resulted in a significant decrease in locomotor ability.

4.2.4 Characterisation resulting from the knockdown of *coq8* in the *Drosophila* compound eye

In humans, COQ8A mutations often result in cerebellar atrophy and neurodegeneration in affected individuals (Barca et al., 2014; Blumkin et al., 2014; Gerards et al., 2010). This phenotype is associated with the loss of COQ8A function in the brain. The compound eye was used to characterise this phenotype in *Drosophila.*

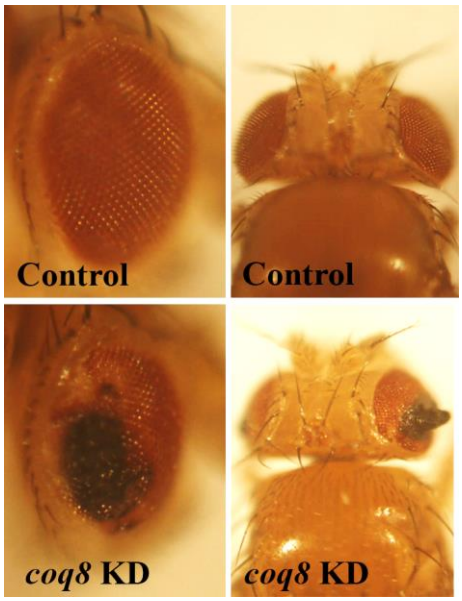


Figure 4.12 Assessment of apoptosis and neurodegeneration in the compound eye. *GMR-GAL4 expression of control and coq8 KD flies. Control flies show normal development of the Drosophila compound eye. In the absence of coq8 necrosis is present. The top down view of the eye showed a necrotic tumour protruding from the compound eye. Genotypes shown are w^{1118}/+;GMR-GAL4/+ (control); and GMR-GAL4/coq8KD (coq8 KD).*

The highly elaborate structure of the *Drosophila* compound eye is composed of a remarkably orchestrated and logical arrangement of over 800 ommatidia (Kumar, 2012). The cell-to-cell interactions and organisation of this structure are critical for proper development and function. The compound eye can be used to assess neurological disorders due to the retina and optic lobe being a part of the CNS. This means the retina and the optic lobe can be used to assess the impact a gene may have in neuronal or

neurodegenerative disorders (Johnson & Cagan, 2009) The *GMR*-GAL4 driver can be used to drive expression of a transgene during the later stages of eye development (Li et al., 2012).

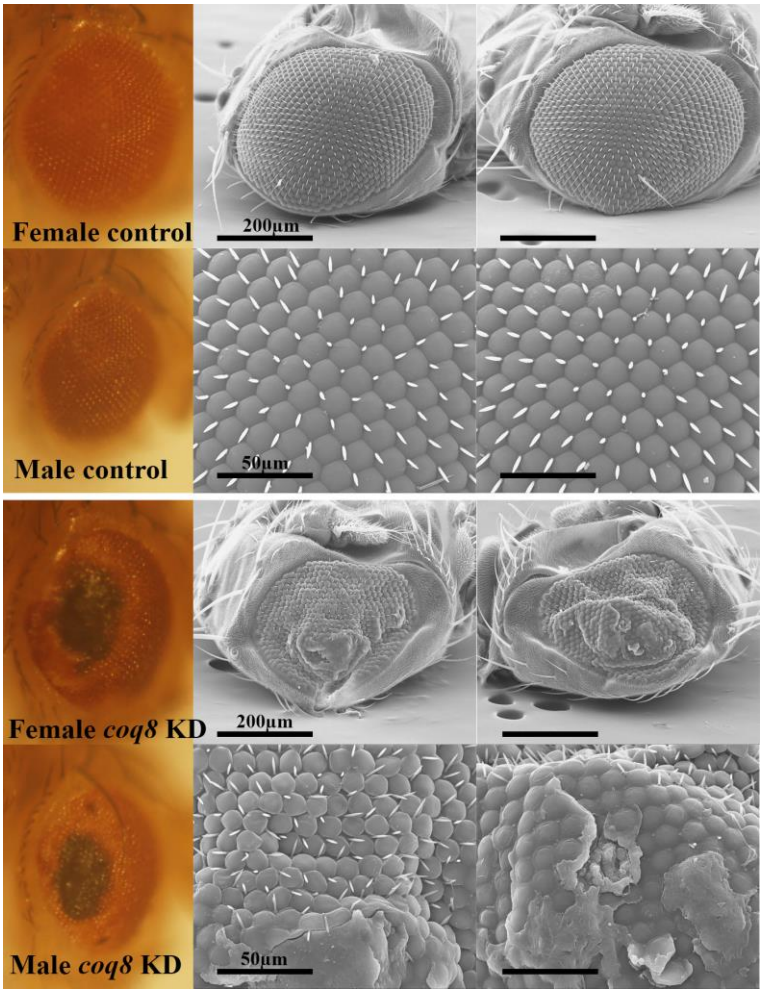


Figure 4.13 Neurodegeneration in the Drosophila eye. *The use of the eye specific driver, GMR-GAL4, enabled the detection of neurodegeneration in the photoreceptors of the compound eye. Control flies contain a regular array of ommatidia with bristles spaced accordingly throughout the eye. coq8 KD eyes contain necrotic patches and a loss of overall integrity of the eye. Genotypes shown are* w^{1118}*/+;GMR-GAL4/+ (control); and GMR-GAL4/coq8 KD (coq8 KD).*

Five virgin *GMR*-GAL4 females were crossed to five *coq8* KD or control males (methods 3.2). Flies were raised at 25°C in vials and housed for 30 days before imaging (methods 3.5). Control flies showed normal eye development with a regular array of ommatidia (Figure 4.12) and (Figure 4.13) whereas *coq8* KD presented with black necrotic patches (*coq8* KD). The necrotic patches varied in size and intensity but were never observed in control eyes. Over time the necrotic patches worsened which was

indicative of neurodegeneration due to the loss of *coq8*. In the severest cases, tumours protruded from the eye (Figure 4.12 *coq8* KD). Scanning electron microscopy (SEM) (methods 3.9) revealed a breakdown in the gross structure of the *coq8* KD eye (Figure 4.13) with a complete loss of adhesion between ommatidia. Bristles were in disarray and often missing. This suggests that *coq8* is required for maintenance of ommatidia structure and cell survival in the eye.

4.2.4.1 Characterisation resulting from the knockdown of *coq8* in the photoreceptors of the eye during all stages of development

The presence of necrosis in the compound eye using the *GMR*-GAL4 driver indicated that the knockdown of *coq8* results in neurodegeneration in this structure. Next, we wanted to determine the effect the knockdown of *coq8* has on the eye during all stages of eye development. The *GMR*-GAL4 driver is only active from the later larval stages so to characterise the effect c*oq8* has during all stages of eye development the *ey*-GAL4 driver was chosen. The *ey* driver, like the *arm*-GAL4 driver, is lethal as a homozygous stock, so it is maintained over a CyO balancer chromosome which displays a curly wing phenotype.

Five virgin *ey*-GAL4 females were crossed to five male flies from either control or *coq8* KD flies (methods 3.2). Flies were raised at 21℃ and 18℃. The knockdown of *coq8* had a very severe impact on survival as no *coq8* KD containing progeny survived to eclosion. Pupae could not be counted due to the presence of the balancer chromosome. As there were no survivors, the impact on eye development could not be assessed with this driver. However, *ey* is also expressed in the developing nervous system (Halder et al., 1995) suggesting that coq8 may be critical for development in other *ey*-GAL4 expressing regions of the body.

4.2.5 Characterisation of the influence *coq8* had on ATP production in *Drosophila*

Human CoQ functions in the mitochondria as an electron transporter from complex I and complex II to complex III in the MRC (Crane et al., 1957; Lagier-Tourenne et al., 2008; Turunen et al., 2004). The MRC is responsible for the production to ATP in the mitochondria of eukaryotes. In patients with mutations to COQ8A, which can include a

decrease in CoQ biosynthesis, there was also a correlation with the decrease in ATP levels in cells and tissues in the body. Some cells and/or tissues are more responsive to the decrease in ATP due to their reliability on CoQ to function (Lester & Crane, 1959). To assess ATP levels for this project, control and *coq8* KD flies were used in conjunction with the ATP Bioluminescence Assay Kit CLS II (Roche Applied Science). Examination of the impact of ubiquitous knockdown of *coq8* had on ATP levels in *Drosophila* was vital in determining whether the knockdown of *coq8* would result in a similar phenotype to that seen in humans. As the ubiquitous knockdown of *coq8* in *Drosophila* resulted in the larval lethality, first instar larvae were collected and used in this assay. Bottles were used to house progeny to obtain more significant numbers of *coq8* KD larvae. Ten *da*-GAL4 virgin flies were crossed to either control or *coq8* KD males. They were placed in 100 mL bottles with standard fly food.

One week post cross, wandering 3rd instar larvae were collected from control bottles and were placed on ice in Eppendorf tubes. From the same experimental crosses, 1st instar larvae were collected from *coq8* KD bottles. Larvae had not progressed any further for *coq8* KD progeny. Light emission data was normalised to the blank and compared (Figure 4.14). Control lysates were used as the standard and were compared to *coq8* KD lysates. The *coq8* KD ATP levels were less than the control. This was consistent with the decrease in ATP production often found in people with COQ8A mutations (Gerards et al., 2010).

The 25℃ samples, which had an increase in *coq8* KD expression, resulted in a decrease in ATP production. These results indicated that ATP production in *coq8* KD flies is less than that of control flies. This is consistent with previous case studies and the dependence on *coq8* for the production of ATP. These data are preliminary and must be repeated for accuracy.

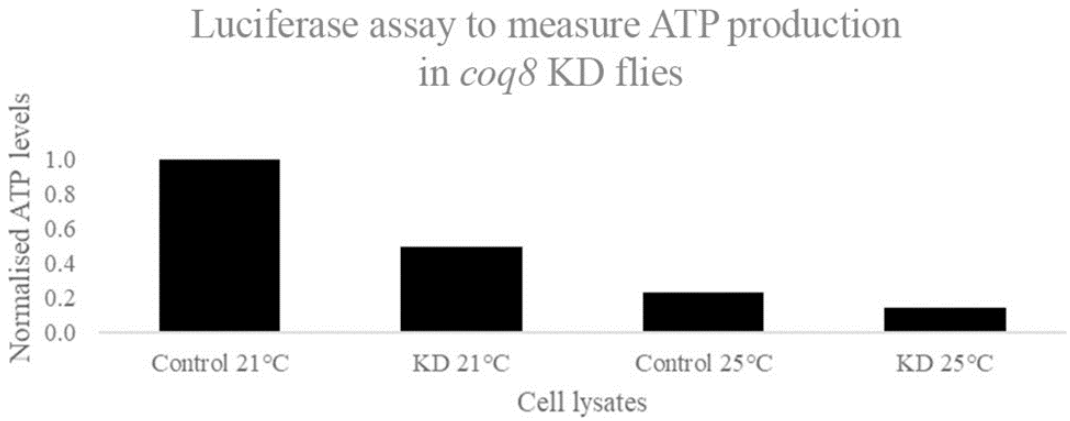


Figure 4.14 Analysis of the ATP levels associated with the loss of coq8. *Flies were raised at 21℃ and 25℃. Heads were collected for 21℃ samples and larvae for 25℃. Samples were prepared as per manufacturer's instructions. Values were normalised and compared. Samples were carried out in duplicated and normalised to the control. Genotypes shown are w^{1118}/+;elav-GAL4/+ (control) and w^{1118}/+;elav-GAL4/coq8 KD (coq8 KD).*

4.3 Generation and characterisation of flies that overexpress wild-type and mutant forms of *Drosophila coq8*

Ubiquitous knockdown of *coq8* in *Drosophila* resulted in larval lethality and severe developmental delay (section 4.2.1 and 4.2.2), whereas pan-neuronal knockdown had no visible impact on brain development but did impair climbing ability (section 4.2.3). Also, knockdown of *coq8* in the compound eye resulted in the loss of ommatidial integrity, necrosis and neurodegeneration (section 4.2.4) and finally, the loss of *coq8* resulted in a decrease in ATP production (section 4.2.5). Together, these phenotypes indicated that *coq8* is an essential gene for survival and locomotor ability in *Drosophila*. These measurable phenotypes allowed RNAi-mediated knockdown of *coq8* to be used as a model for the knockdown of *coq8* in *Drosophila*. Based on this model, it is expected that reintroduction of wild-type *coq8* will alleviate the loss of *coq8*. However, the introduction of a mutant form of *coq8* with an impaired function would not. To functionally characterise the mutations found by Jacobsen et al. (2017), the appropriate DNA constructs corresponding to the *Drosophila* variants of these COQ8A mutations were generated.

4.3.1 Generation of fly stocks that overexpress wild-type and mutant forms of *Drosophila coq8*

The yeast homologue *COQ8* has been widely characterised and is essential for CoQ biosynthesis (He et al., 2014). Sequence alignments between yeast, human and *Drosophila* (appendix 9.6, Figure 4.15) were used to determine the variants needed for this project.

The alignment of human *COQ8A,* yeast *COQ8* and *Drosophila coq8* (annotation symbol CG32649) showed 53% amino acid identity and 70% similarity between human COQ8A and *Drosophila coq8*, 42% amino acid identity and 61% similarity between human COQ8A and yeast *COQ8,* 43% amino acid identity and 64% similarity between yeast *COQ8* and *Drosophila coq8*. The paternally derived human SNP was characterised by as a non-synonymous missense change from Leucine to Proline (c.830T>C, p.Leu277Pro) (Jacobsen et al., 2017). This amino acid change is predicted to disrupt the highly conserved KxGQ domain that is found in the N-terminal of

COQ8A. In humans, the amino acid sequence at the motif is KLGQ (L277P) while the corresponding mutation in *Drosophila* is I295P which is an isoleucine change to proline at position 295. The KxGQ domain contains an invariant KxGQ motif which folds and occludes the active site of the enzyme Stefely et al. (2015).

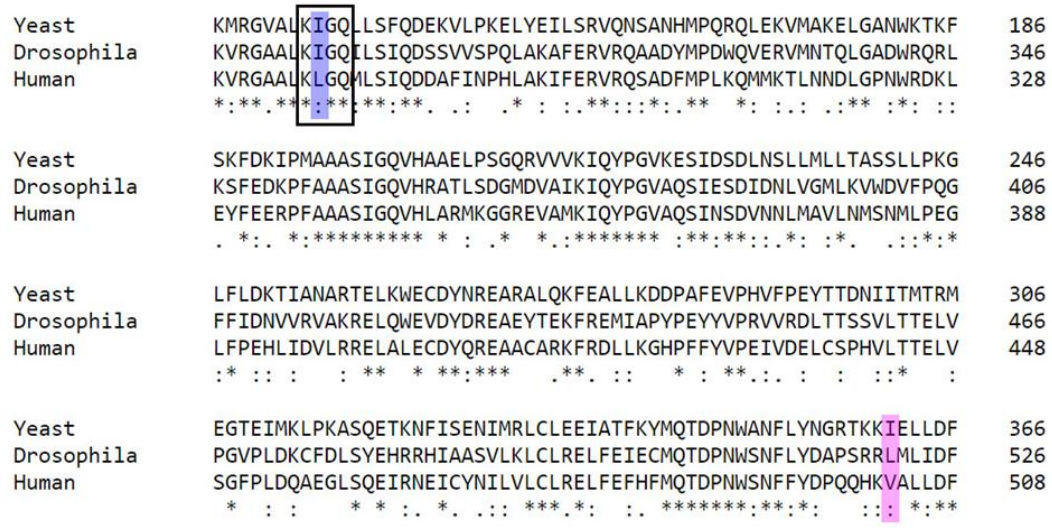


Figure 4.15 Sequence alignment to identify the COQ8A mutants found by Jacobsen et al. (2017). *The blue box highlights the human L277P mutation/Drosophila I295P with the black box indicating the presence of the highly conserved KxGQ motif. The pink box highlights the human K502*/Drosophila L520* mutants that result in a truncated protein product. The sequence alignment was carried out using Clustal Omega https://www.ebi.ac.uk/Tools/msa/clustalo/*

The maternally-inherited mutation disrupts a canonical splice site donor in exon 12 (c.1506+1G>A) and is predicted to result in translation through an intron for 19 amino acids until it reaches a premature STOP codon resulting in a truncated protein product. The last amino acid of the wild-type sequence before the intron is K502. The splice site mutation cannot be modelled as the introns are not conserved in *Drosophila*. Therefore the *Drosophila* sequence was truncated at the corresponding amino acid, L520, to model the truncation of the C-terminal domain. As there was no available antibody for *Drosophila coq8*, a myc-tag was placed on the N-terminal of each *Drosophila coq8* construct to enable detection via immunohistochemistry. The myc-tagged wild-type *coq8* (from here on referred to as myc-*coq8*) I295P and L520* sequences were produced by GenScript® and cloned into the pUASTattB transformation vector for

generation of transgenic *Drosophila* (Groth, Fish, Nusse, & Calos, 2004). This was also performed by Genscript®, pUASTattB contains multiple cloning sites upstream of a UAS which, when used in conjunction with GAL4 driver lines, drives expression of the transgene. It also contains a mini-white gene for selection of transgenic *Drosophila* and an attB site for PhiC31-mediated transgenesis. High-quality plasmid DNA was prepared (section 3.14.2) and sent to GenetiVision for production of transgenic flies.

4.3.2 Characterisation of survival resulting from the strong ubiquitous expression of wild-type and mutant forms of *coq8* in a wild-type background

Before investigating whether any of the transgenes could rescue the knockdown phenotype, it was imperative to determine whether overexpression of myc-*coq8*, I295P or L520* affected survival when expressed in a wild-type background. As *coq8* functions as a dimer, it was possible that introduction of the mutant forms could interfere with normal function even when expressed in a wild-type background. Flies containing myc-*coq8*, I295P or L520* variants of *coq8* were crossed to the strong ubiquitous driver *da*-GAL4 (methods 3.2). Control flies and *coq8* KD flies were included in these experiments as reference points for wild-type and lethal phenotypes. Progeny counts were carried out 23 days post cross (methods 3.4).

While the results are preliminary, progeny counts (Figure 4.16) indicated that, while similar numbers of pupae were produced for control, myc-*coq8*, I295P and L520* genotypes, there was a decrease in the number of adult males and females produced by L520* flies. There was also a slight decrease in the number of adult progeny for myc-*coq8*. In these experiments, progeny were not produced for the *coq8* KD cross which was consistent with previous findings (section 4.2.1). These experiments must be repeated to determine whether the results are reproducible.

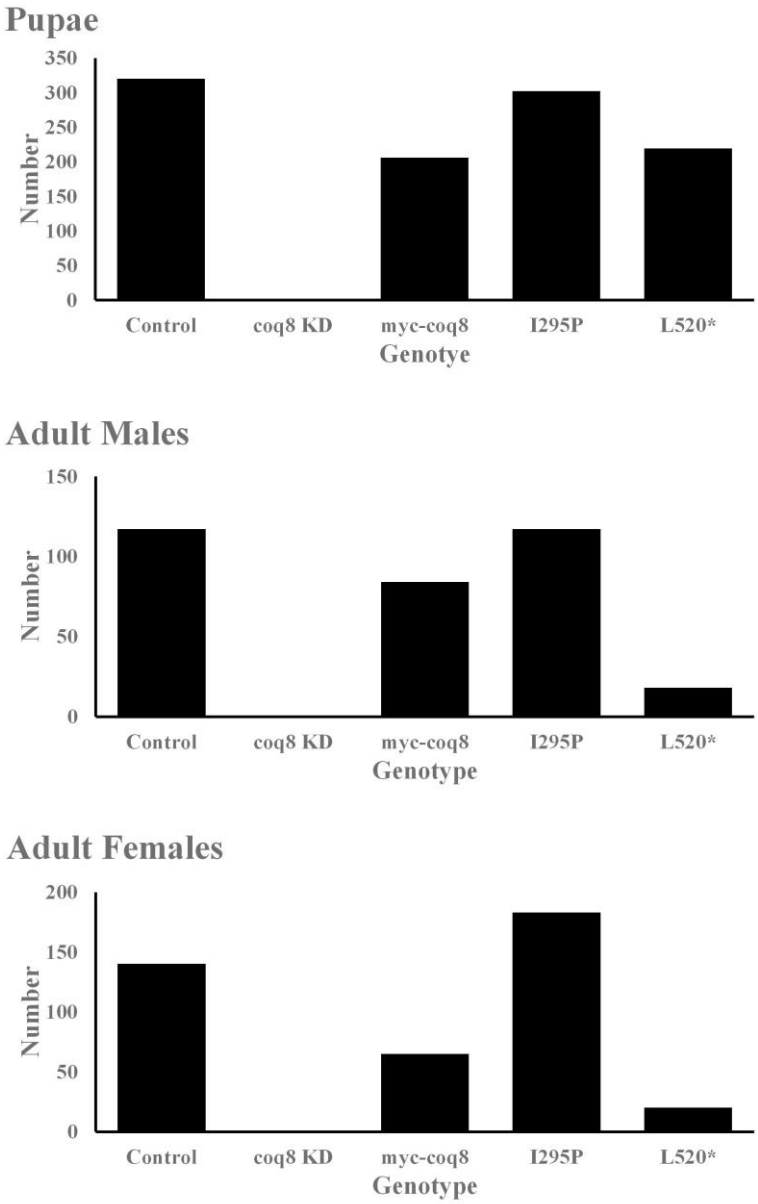


Figure 4.16 Progeny counts to assess the impact of the strong ubiquitous expression of coq8 variants on survival. *Only one cross was carried out for these experiments so in order to reach statistical significance more crosses are necessary. Genotypes shown are* w^{1118}*/+;daGAL4/+ (control);* w^{1118}*/+;da-GAL4/coq8 KD (coq8 KD) and* w^{1118}*/+;da-GAL4/myc-coq8 (myc-coq8),* w^{1118}*/+;daGAL4/I295P(I295P),* w^{1118}*/+;daGAL4/+ ;daGAL4/L520* (L520*).*

4.3.3 Characterisation of survival resulting from the highly specific pan-neuronal expression of wild-type and mutant forms of *coq8* in a wild-type background

Knockdown of *coq8* using the highly specific pan-neuronal driver *elav*-GAL4 was semi-lethal in *Drosophila* (section 4.2.3.1). Some female *coq8* KD survivors presented with locomotor impairment (section 4.2.3.3) which was of interest due to the human mutant phenotype which results in gait ataxia and uncontrolled movements (Jacobsen et al., 2017).

Five *elav*-GAL4 virgin females were crossed to five males of either control, *coq8* KD, myc-*coq8*, I295P or L520* genotypes (methods 3.2). Flies were raised in 30 mL vials. Progeny was counted 24 days post cross (methods 3.4).

Progeny counts (Figure 4.17) indicated that knockdown of *coq8* in the neurons was lethal in both males and females in these experiments. However, previous experiments in which there were female survivors, had been carried out in glass bottles, whereas this experiment was carried out in smaller plastic vials. The difference in containers, plus fluctuations in air conditioning temperature, which was not holding a consistent temperature at this time, were thought to be contributing factors during these experiments. If the temperature within the vials were slightly higher, this would result in increased GAL4 activity and therefore increased RNAi expression which is likely to increase female lethality. Pan-neuronal expression of myc-*coq8* or the *coq8* mutants did not appear to have an impact on survival in *Drosophila*.

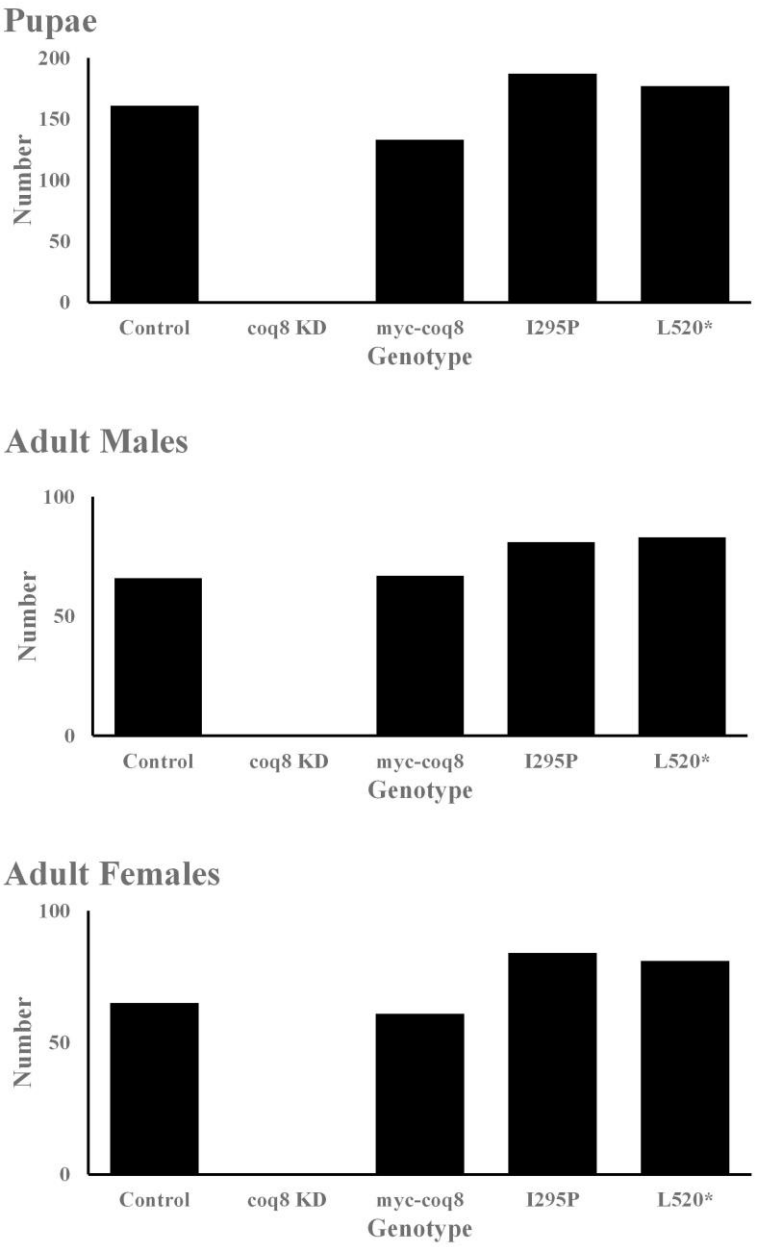


Figure 4.17 Analysis of the impact of pan-neuronal expression of coq8 variants on survival. *Only one cross was carried out for these experiments so in order to reach statistical significance more crosses are necessary. Genotypes are w^{1118}/+; elav-GAL4/+ (control); w^{1118}/+; elav-GAL4/coq8 KD (coq8 KD) and w^{1118}/+; elav-GAL4/myc-coq8 (myc-coq8). w^{1118}/+; elav-GAL4/I295P (I295P), w^{1118}/+; elav-GAL4/L520* (L520*).*

4.3.4 Negative Geotaxis assay to assess locomotor ability in flies expressing wild-type or mutant forms of *coq8* in a wild-type background

In humans, clinical presentation can include cerebellar atrophy and a decrease in motor skills (Barca et al., 2014; Jacobsen et al., 2017; Stefely et al., 2016), therefore in order to determine whether pan-neuronal overexpression of myc-*coq8* or mutant *coq8* altered locomotor activity in a wild-type fly, a negative geotaxis assay was performed (methods 3.12).

Five *elav*-GAL4 virgin females were crossed to each *Drosophila* variant and raised at 21°C in 30 mL vials (methods 3.2). After 25 days adult female survivors were collected, and locomotor ability assessed using the negative geotaxis assay (section 3.12). Control flies were used as the positive reference sample, and all data was normalised to the total number of flies per trial (n=30).

It was evident that there were some defects present in the *coq8* KD progeny. They were a lot slower in their movements and not as quick to respond after being startled but did attempt to climb. Upon closer inspection, they were holding their wings in an unusual position and would often flick them. When the assay was carried out it was clear that *coq8* KD progeny were not able to climb after being startled. Initial runs suggested that the banging on the bench was preventing the flies from recovering quick enough to climb. Therefore flies were given an extra 20 seconds (for a total of 30 seconds) to climb up the vial to allow them time to recover.

Negative geotaxis assays (Figure 4.18) determined that across both time trials control, myc-*coq8* and I295P flies exhibited normal climbing ability. However, *coq8* KD and L520* flies had a significant decrease in climbing ability when compared to all other genotypes at both time points (ANOVA, post hoc Tukey HSD Test, $p<0.01$) indicating that the loss of *coq8* results in a decrease in climbing ability. This also suggests that expression of L520* may be reduced and also affects climbing ability.

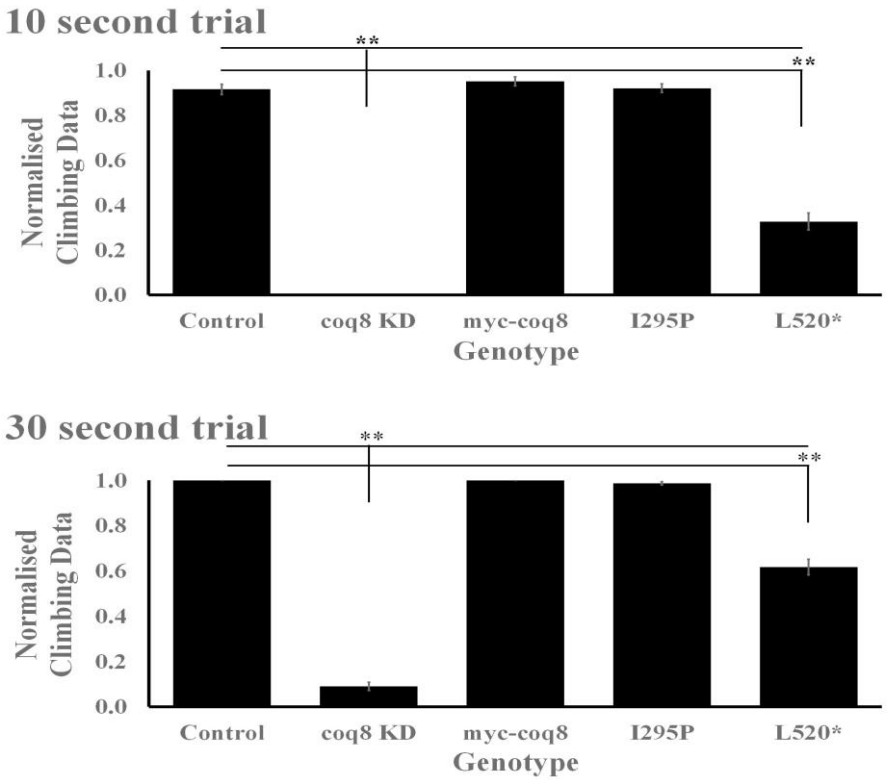


Figure 4.18 Negative geotaxis assay using the pan-neuronal elav-GAL4 driver. *Flies raised at 21℃. Trials were run for 30 seconds each and successful climbers were counted once they crossed the 5cm line. Genotypes are w^{1118}/+; elav-GAL4/+ (control); w^{1118}/+;elav-GAL4/coq8KD (coq8 KD) and w^{1118}/+;elav-GAL4/myc-coq8 (myc-coq8), . w^{1118}/+; elav-GAL4/I295P (I295P), w^{1118}/+; elav-GAL4/L520* (L520*). Means +/- standard error. ANOVA, post hoc Tukey HSD Test. Statistical significance * = p<0.05 and ** = p<0.01.*

4.3.5 Characterisation resulting from the expression of wild-type or mutant forms of *coq8* in the *Drosophila* compound eye

The compound *Drosophila* eye is a highly elaborate structure that requires a number of cellular processes for proper development. The retina of the *Drosophila* eye is often used to assess neuronal degeneration (London et al., 2013) due to the retina and optic nerve being derived from the CNS (Johnson & Cagan, 2009). The knockdown of *coq8* in the *Drosophila* compound eye resulted in a loss of integrity of the lattice structure of the eye, necrosis and neurodegeneration (section 4.2.4). In order to characterise the impact the *Drosophila* variants, myc-*coq8*, I295P and L520*, had on eye development when expressed in a wild-type background eye morphology was analysed here.

Five virgin *GMR*-GAL4 females were crossed to five male flies from either control, *coq8* KD, myc-*coq8*, I295P or L520* (methods 3.2). Flies were raised at 25℃ in vials and housed for 30 days before imaging (methods 3.5). By raising flies at 25℃ a more severe knockdown of the transgene occurred. Control flies showed normal eye development (Figure 4.19) with a healthy array of ommatidia, bristles and a consistent shape to the eye. The eyes of *coq8* KD flies showed the irregular ommatidia arrangement and disrupted bristle formation as well as black necrotic patches and a break down in the integrity of the eye. The black necrotic patches observed were of varying sizes and intensities but were only present in *coq8* KD fly eyes. The remaining *coq8* variants, myc-*coq8*, I295P and L520*, had normal ommatidia and eye development which was similar to the control.

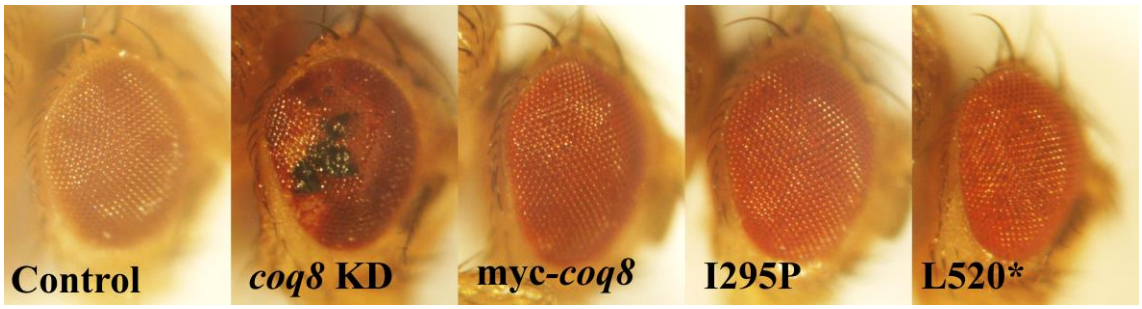


Figure 4.19 GMR-GAL4 characterisation experiments to assess the involvement Drosophila coq8 variants have on the compound eye. *Genotypes shown are w^{1118}/+;GMR-GAL4/+ (control); GMR-GAL4/coq8KD (coq8 KD), GMR-GAL4/myc-coq8(myc-coq8), GMR-GAL4/I295P (I295P), GMR-GAL4/L520*(L520*).*

Overexpression of myc- *coq8* or the I295P mutant did not appear to alter survival, climbing ability or eye development, however pan-neuronal expression of L520* in a wild-type background resulted in a semi-lethal phenotype in *Drosophila* at the pupal stage and also resulted in severe defects in locomotor activity as measured by the negative geotaxis assay, indicating that it may be acting as a dominant negative effect.

4.4 Determination of whether wild-type or mutant *Drosophila coq8* could rescue the *coq8* knockdown phenotype

A model of *coq8* deficiency was characterised in section 4.2. Ubiquitous knockdown of *coq8* using both the strong *da*-GAL4 and weak a*rm*-GAL4 drivers resulted in larval lethality and severe developmental delay, pan-neuronal knockdown of *coq*8 under the control of the highly specific *elav*-GAL4 driver resulted in a semi-lethal phenotype and reduced locomotor function, and knockdown of *coq8* in the post-mitotic eye with *GMR*-GAL4 resulted in disruption to ommatidial development, necrosis, and neurodegeneration.

4.4.1 Generation of *Drosophila* fly lines that express wild-type or mutant forms of *coq8* in a knockdown background

To assess whether the myc-*coq8*, I295P or L520* transgenes could rescue the knockdown phenotypes described above, they were first recombined into the same fly line containing the UAS-*coq8* inverted repeat RNAi construct, such that when crossed to the appropriate GAL4 driver, the transgene would be expressed concurrently with *coq8* RNAi. The sequence alignment (Figure 4.15) depicts the three variants that were generated for these rescue experiments. A crossing scheme can be found in appendix 9.5.

4.4.2 Characterisation of the phenotypes resulting from ubiquitous expression of wild-type or mutant *coq8* in *coq8* KD flies when raised at 21°C

Ten adult male flies of each genotype, described above, were crossed to ten *da*-GAL4 virgin flies and raised at 21°C (methods 3.2). Once the adult progeny had emerged adult flies, pupae and dead larvae were counted (methods 3.4). Images of the fly bottles were taken at the end of each week to provide a visual representation of pupal development between the genotypes (Figure 4.20).

Control flies developed normally and were used as a positive control for developmental progress. It was evident in week one that lethality and/or severe developmental delay

was present in all genotypes lacking *coq8*. By week three most control flies had emerged. While a small number of pupae were present in KD;myc-*coq8* bottles, there were no pupae present in *coq8* KD, KD;I295P or KD;L520*. This suggested that reintroduction of wild-type *coq8* resulted in a partial rescue.

Progeny from these crosses were counted and compared (Figure 4.21). Dead larvae were counted for these experiments to quantify the lethality associated with *coq8* expression in *Drosophila*. Dead larvae containing the *coq8* KD RNAi were small and immature which indicated that lethality occurred early in larval development. There was a significant increase in the number of dead larvae in KD;L520* (ANOVA, post hoc Tukey HSD Test, <0.05) when compared to the all other genotypes. Larval lethality was evident in *coq8* KD progeny as larvae did not reach pupation. However, a small number of pupae were present in KD;myc-*coq8* and KD;I295P bottles suggesting that reintroduction of wild-type *coq8* or I295P partially rescued larval lethality. Adult progeny counts showed normal development for control flies but a complete absence of adults in all other genotypes (ANOVA, post hoc Tukey HSD Test, $p<0.01$ for males, $p<0.05$ for females). These results indicated that reintroduction of wild-type *coq8* and I295P were able to partially rescue larval lethality resulting in pupation in a small minority of individuals but was unable to result in viable adults.

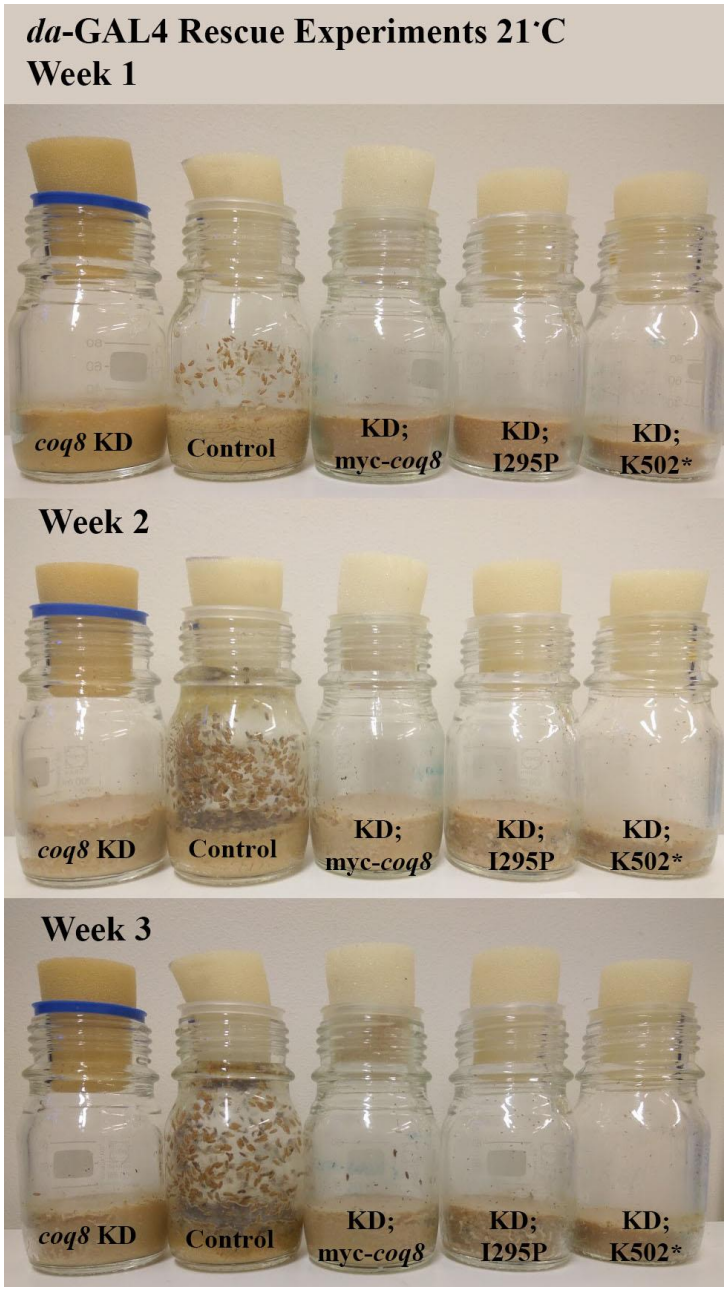
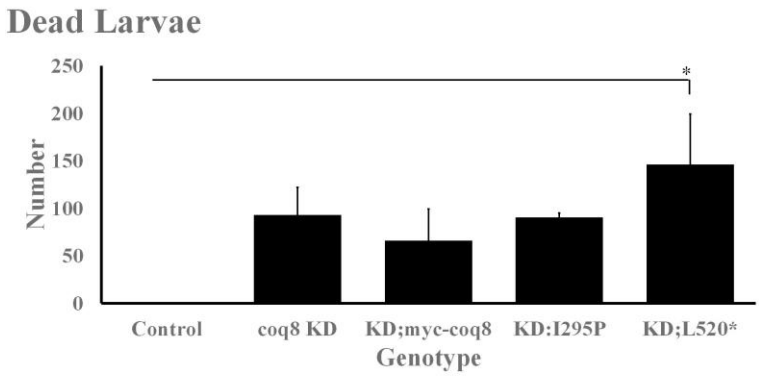
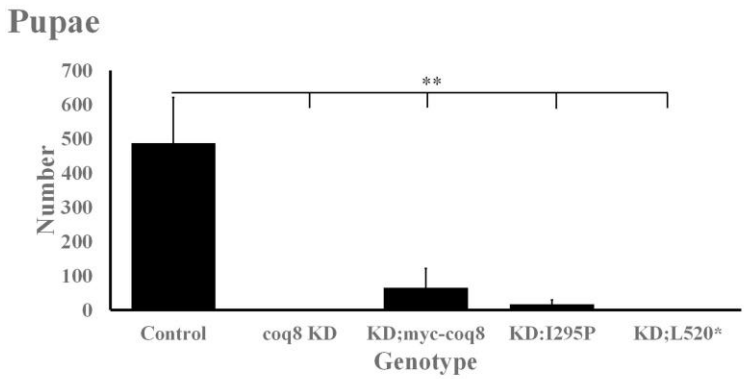
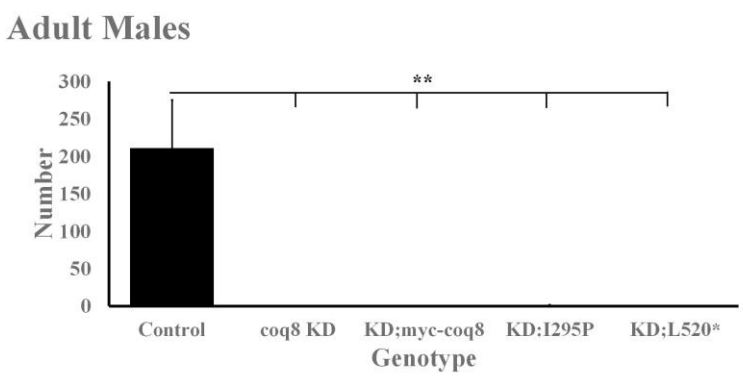


Figure 4.20 Photos of the fly bottles from da-GAL4 crosses taken at 1, 2 and 3 weeks post cross. *Images indicate the difference seen in the development of Drosophila under the strong ubiquitous driver. Genotypes shown are w^{1118}/+;daGAL4/+ (control); w^{1118}/+;da-GAL4/coq8KD (coq8 KD) and w^{1118}/+;da-GAL4/ coq8KD;myc-coq8 (KD;myc-coq8), w^{1118}/+;daGAL4/ coq8KD ;I295P (KD;I295P), w^{1118}/+, daGAL4/ coq8KD/L520*(KD;L520*).*







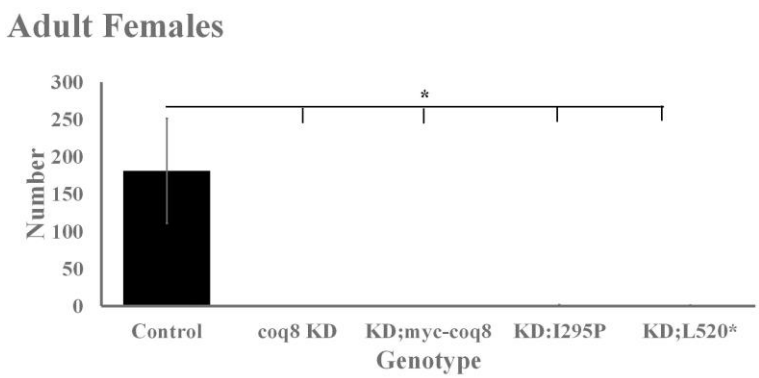


Figure 4.21 Progeny counts from rescue experiments using the strong ubiquitous driver da-GAL4. *Flies were raised at 21℃ and housed in 100 ml glass bottles. Progeny were counted 26 days post cross. Genotypes shown are w^{1118}/+;daGAL4/+ (control); w^{1118}/+;da-GAL4/coq8KD (coq8 KD) and w^{1118}/+;da-GAL4/ coq8KD;myc-coq8 (KD;myc-coq8), . w^{1118}/+;daGAL4/ coq8KD ;I295P (KD;I295P), w^{1118}/+, daGAL4/ coq8KD/L520* (KD;L520*). Means +/- standard error. ANOVA, post hoc Tukey HSD Test. Statistical significance * = p<0.05 and ** = p<0.01. Crosses carried out in triplicate.*

4.4.3 Characterisation of the phenotypes resulting from ubiquitous expression of wild-type or mutant *coq8* in *coq8* KD flies when raised at 18°C

GAL4 activity is temperature sensitive; at lower temperatures, GAL4 activity is lower, which results in reduced transgene expression, whereas an increase in temperature results in increased GAL4 activity and subsequently an increase in transgene expression (Duffy, 2002). For this reason, it was decided that a decrease in temperature may lead to an increase in survival. Due to the inability to rescue the knockdown phenotype at 21°C, it was decided to repeat the experiments at 18°C to determine whether rescue might be improved when *coq8* RNAi expression was reduced.

Crosses were set up as previously described (section 3.2) but were raised at 18°C. Progeny were counted 26 days post cross (section 3.4). No significant differences were seen in the dead larval counts for any of the genotypes (Figure 4.22). A partial rescue was seen in KD;myc-*coq8* pupae which had significantly more pupae than all other *coq8* KD containing flies (ANOVA, post hoc Tukey HSD Test, $p<0.01$). A minimal amount of KD;myc-*coq8* adult males emerged and did not appear to have any noticeable defects. As expected, there was significantly more control adult flies when compared to all *coq8* KD containing flies (ANOVA, post hoc Tukey HSD Test, $p<0.01$). To partially rescue the knockdown phenotype wild-type *coq8* was reintroduced and resulted in the emergence of a small number of viable male offspring.

Raising flies at a lower temperature increased the number of pupae produced by KD;myc-*coq8* flies and also the emergence of a small number of viable males. This suggests that decreasing *coq8* RNAi expression in *Drosophila* resulted in partial rescue of the knockdown phenotype.

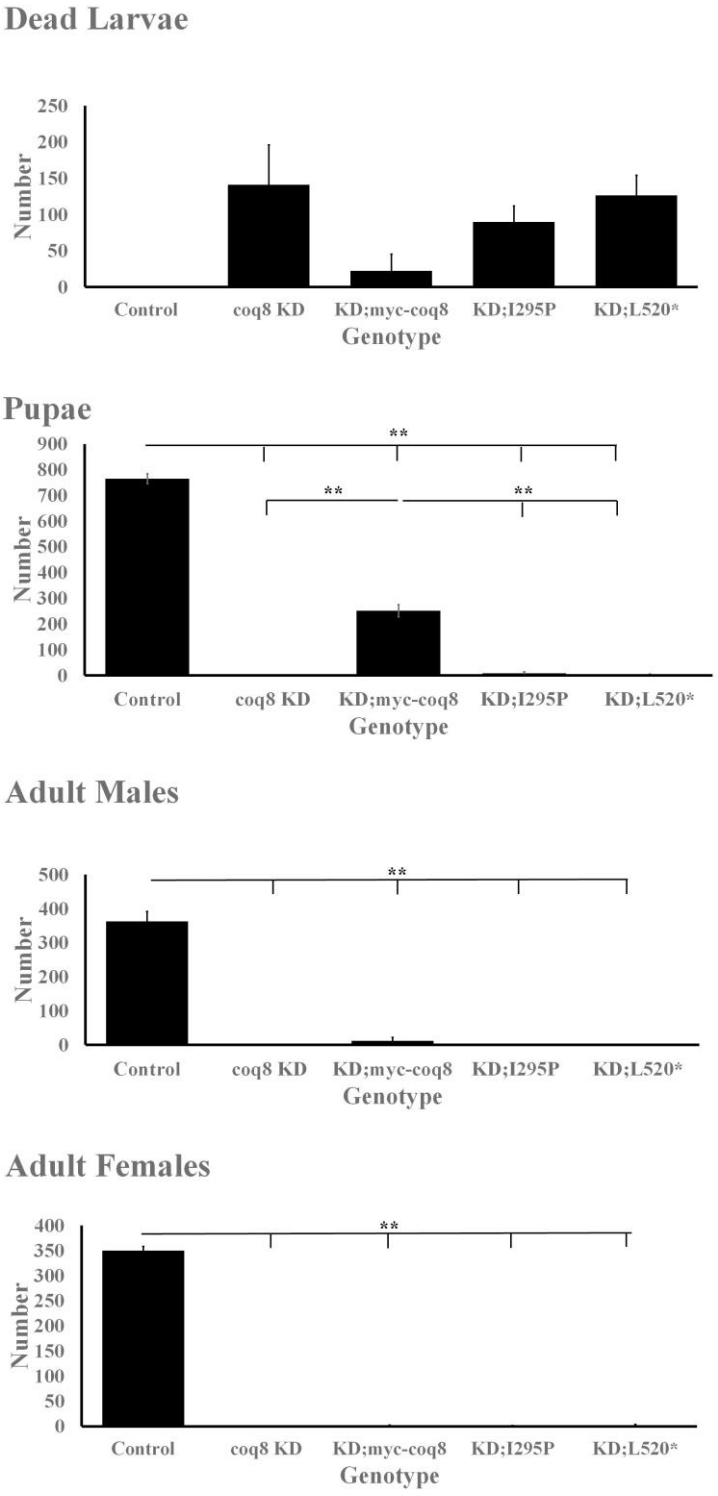


Figure 4.22 Progeny counts for the rescue experiments at 18°C using the strong ubiquitous driver da-GAL4*. Flies were raised at 18°C and dead larvae counted to determine the effect Drosophila variants have on survival. Genotypes shown are w^{1118}/+;daGAL4/+ (control); w^{1118}/+;da-GAL4/coq8KD (coq8 KD) and w^{1118}/+;da-GAL4/ coq8KD;myc-coq8 (KD;myc-coq8), . w^{1118}/+;daGAL4/ coq8KD ;I295P (KD;I295P), w^{1118}/+, daGAL4/ coq8KD/L520*(KD;L520*). Means +/- standard error. ANOVA, post hoc Tukey HSD Test. Statistical significance * = p<0.05 and ** = p<0.01. Crosses carried out in triplicate.*

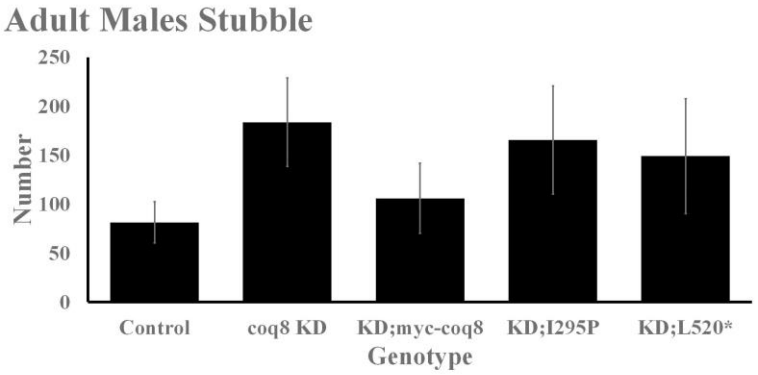
4.4.4 Characterisation of the phenotypes resulting from the weak ubiquitous expression of wild-type or mutant *coq8* in *coq8* KD flies raised at 21°C

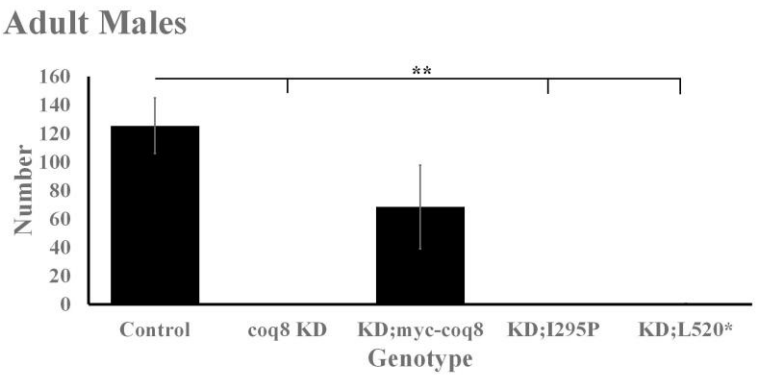
Due to the inability to rescue lethality using the strong ubiquitous driver *da*-GAL4 at 21°C, the *arm*-GAL4 driver was used with the rationale that weaker RNAi expression could result in an increase in rescue with the reintroduction of wild-type *coq8*.

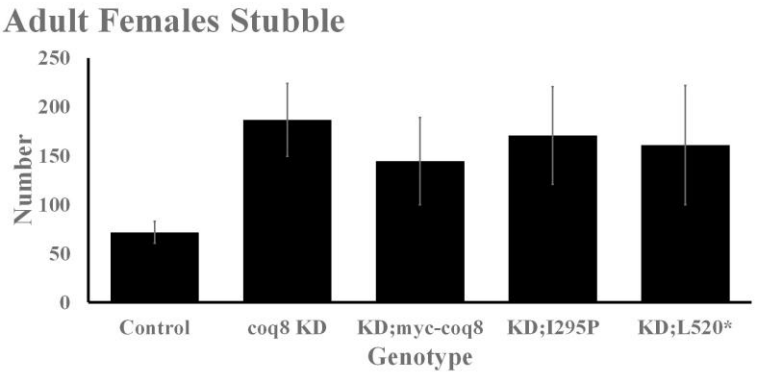
Ten *arm*-GAL4 virgin females were crossed to ten males from either control, *coq8* KD, KD;myc-*coq8*, KD;I295P or KD:L520*. Flies were crossed and raised at 21°C (methods 3.2). Adult flies were counted 26 days post cross (methods 3.4). For these experiments, pupae were not counted as the pupal genotype could not be determined due to the presence of a balancer chromosome. For these crosses, flies that contained the Sb balancer chromosome did not contain the driver line needed for the successful expression of the transgene (Figure 4.3). Any flies that emerged with standard wild-type bristles contained the driver and the transgene. The presence of the balancer chromosome in these crosses provide an internal control for the experiment as those flies did not express the transgene.

The *Sb* in vial controls showed no significant differences in males or females (Figure 4.23). A partial rescue in adult males expressing both the driver and the transgenes, KD;myc-*coq8*, was seen with the absence of males in all other *coq8* KD containing flies. While there was no significant difference seen between control males and KD;myc-*coq8* there was with all other genotypes (ANOVA, post hoc Tukey HSD Test, $p<0.01$). Lethality was evident in females expressing the driver and the transgene for all *coq8* KD containing flies with a significant decrease in progeny when compared to the control (ANOVA, post hoc Tukey HSD Test, $p<0.01$). The knockdown phenotype in female flies was partially rescued by the reintroduction of wild-type coq8. However, rescue was not seen in any other genotypes that contained *coq8* KD (ANOVA, post hoc Tukey HSD Test, $p<0.01$).

A partial rescue was seen with the reintroduction of wild-type *coq8* in male progeny for KD;myc-*coq8* crosses. This indicates that the lethality associated with *coq8* KD flies is due to the loss of *coq8*.







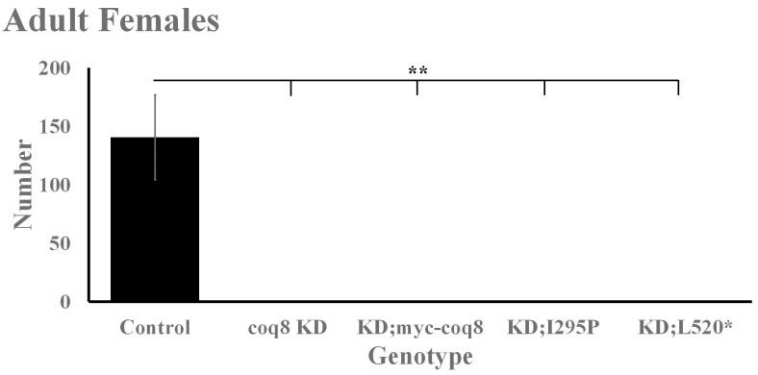


Figure 4.23 Progeny counts for rescue experiments using the weak ubiquitous driver arm-GAL4. *Sb=no transgene. Genotypes shown are w^{1118}/+;arm-GAL4/+ (control); w^{1118}/+;coq8KD/+; arm-GAL4/+ (coq8 KD) and w^{1118}/+; coq8KD;myc-coq8/+; arm-GAL4/+(KD;myc-coq8), w^{1118}/+; coq8KD;I295P/+; arm-GAL4/+(KD;I295P), w^{1118}/+; coq8KD;L520*/+; arm-GAL4/+(KD;L520*). Means +/- standard error. ANOVA, post hoc Tukey HSD Test. Statistical significance * = p<0.05 and ** = p<0.01. Crosses were carried out in triplicate.*

4.4.5 Characterisation of the phenotypes resulting from the pan-neuronal reintroduction of wild-type or mutant *coq8* into a knockdown background raised at 21℃

Pan-neuronal knockdown of *coq8* resulted in a semi-lethal phenotype (section 4.2.3.1) and impaired locomotor ability (section 4.2.3.4). The following rescue experiments assessed the ability for *coq8* variants to rescue the previously characterised *coq8* knockdown phenotype. As previously described (section 4.4.1) the *coq8* inverted repeat was recombined into the same fly line as either myc-*coq8,* I295P or L520* in order co-express the *coq8* variants in a knockdown background. Ten males from each fly construct were then crossed to ten virgin *elav*-GAL4 females (methods 3.2).

Initial observations revealed a developmental delay present in *coq8* KD flies (Figure 4.24) week one saw a similar phenotype across all crosses with development occurring sooner in control flies. By the end of week two, control flies had begun to emerge while all *coq8* KD containing flies had not. This developmental delay was worse in KD;L520* flies with a large number of pupae still occupied by the time progeny counts were carried out.

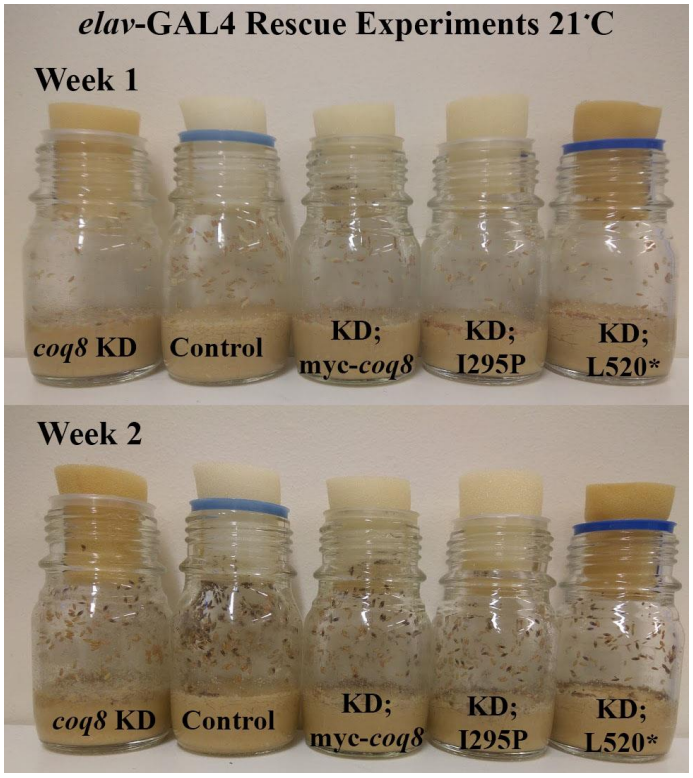


Figure 4.24 Photos of fly bottles for elav-GAL4 rescue experiments. *Images were taken 1 and 2 weeks post cross. Genotypes shown are* w^{1118}*/+; elav-GAL4/+ (control);* w^{1118}*/+;elav-GAL4/coq8KD (coq8 KD) and* w^{1118}*/+;elav-GAL4/ coq8KD ;myc-coq8, .(KD;myc-coq8)* w^{1118}*/+; elav-GAL4/ coq8KD;I295P (KD;I295P),* w^{1118}*/+; elav-GAL4/ coq8KD;L520* (KD;L520*).*

Progeny counts (Figure 4.25) indicated a significant decrease in the number of adult males between control and *coq8* KD (ANOVA, post hoc Tukey HSD Test, $p<0.01$), KD;myc-*coq8* (ANOVA, post hoc Tukey HSD Test, $p<0.05$), KD;I295P (ANOVA, post hoc Tukey HSD Test, $p<0.01$) and KD;L520* (ANOVA, post hoc Tukey HSD Test, $p<0.01$). A significant increase in adult males was seen in KD;myc-*coq8* when compared to *coq8* KD (ANOVA, post hoc Tukey HSD Test, $p<0.01$), KD;I295P (ANOVA, post hoc Tukey HSD Test, $p<0.05$) and KD;K502* (ANOVA, post hoc Tukey HSD Test, $p<0.01$) suggesting that the reintroduction of wild-type *coq8* was able to partially rescue the knockdown phenotype. The introduction of the mutants into the knockdown background, KD;I295P and KD;L520*, saw a worsening of the knockdown phenotype in males. There was a significant decrease between KD;myc-*coq8* and both KD;I295P (ANOVA, post hoc Tukey HSD Test, $p<0.05$) and KD;L520* (ANOVA, post hoc Tukey HSD Test, $p<0.01$) suggesting that in the presence of the *coq8* mutants survival is reduced.

Adult female progeny counts showed a significant derease between control females and both KD;I295P (ANOVA, post hoc Tukey HSD Test, $p<0.05$) and KD;L520* (ANOVA, post hoc Tukey HSD Test, $p<0.01$). This suggests that expression of the mutant variants in the knockdown background does not rescue the *coq8* knockdown phenotype. There were no significant differences seen between control, *coq8* KD and KD;myc-*coq8*. However, there was a significant decrease in the number of adult females between KD;myc-*coq8* and both KD;I295P (ANOVA, post hoc Tukey HSD Test, $p<0.01$) and KD;L520* (ANOVA, post hoc Tukey HSD Test, $p<0.01$). These results suggest that the introduction of either mutant variant to the knockdown background resulted in a significant decrease in the number of adult females

Pan-neuronal knockdown of *coq8* was lethal in males and semi-lethal in females. There was no effect on pupal development in these crosses indicating that lethality occurs during the pupal stage. Reintroduction of wild-type *coq8* into the knockdown background rescued male lethality but the reintroduction of the *coq8* mutants did not. L520* reduced survival in *coq8* KD females similarly to what was seen when *coq8* variants were expressed in wild-type flies (section 4.3.3).

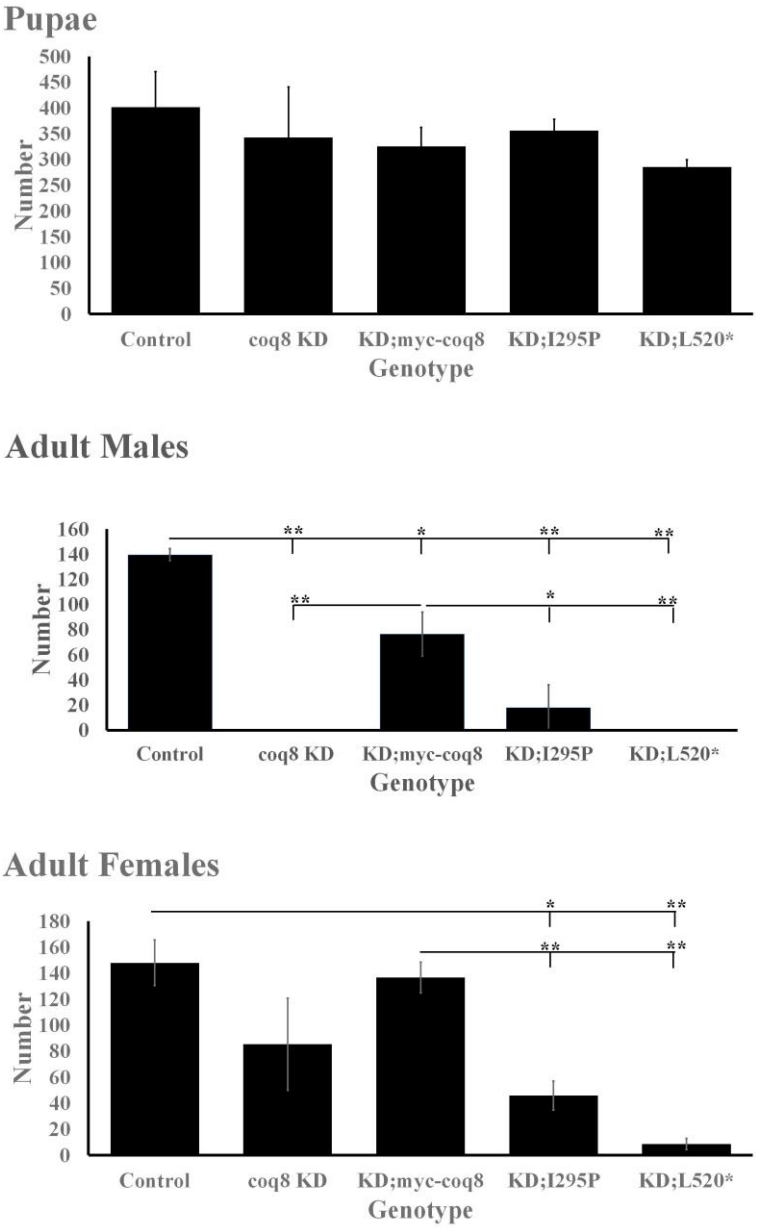


Figure 4.25 Progeny counts for rescue experiments using the pan-neuronal driver elav-GAL4. *Flies were raised at. 21℃. Genotypes shown are w^{1118}/+; elav-GAL4/+ (control); w^{1118}/+;elav-GAL4/coq8KD (coq8 KD) and w^{1118}/+;elav-GAL4/ coq8KD ;myc-coq8, .(KD;myc-coq8) w^{1118}/+; elav-GAL4/ coq8KD;I295P (KD;I295P), w^{1118}/+; elav-GAL4/ coq8KD;L520* (KD;L520*). Means +/- standard error. ANOVA, post hoc Tukey HSD Test. Statistical significance * = p<0.05 and ** = p<0.01. Crosses carried out in triplicate.*

4.4.6 Negative Geotaxis assay to assess locomotor ability resulting from the reintroduction of wild-type or mutant *coq8* into a knockdown background raised at 21℃

In section 4.2.3.4, it was demonstrated that knockdown of *coq8* resulted in impaired locomotor function in the negative geotaxis assay, indicating that expression of *coq8* in neurons could be essential for normal locomotor function in *Drosophila*. This result was consistent with the clinical presentation exhibited in patients with COQ8A mutations where deficiencies can include the loss of fine motor skills or gait ataxia (Barca et al., 2016; Jacobsen et al., 2017).

Flies of the appropriate genotypes were assessed via the negative geotaxis assay (methods 3.12) to examine whether wild-type or mutant variants of *coq8* could rescue the impaired locomotor function resulting from knockdown of *coq8*.

During both 10 second and 30 second trials (Figure 4.26), most control flies and KD;myc-*coq8* flies had crossed the 5cm line and were not significantly different from each other. Control flies and KD;myc-*coq8* climbed significantly better than *coq8* KD, KD;I295P and KD;L520* flies (ANOVA, post hoc Tukey HSD Test, $p<0.01$) suggesting a full rescue of locomotor ability with the reintroduction of wild-type *coq8*. Additionally KD;L520* flies performed worse than *coq8* KD flies indicating that introduction of the L520* mutant resulted in a worsening in locomotor ability.

The negative geotaxis assay was also repeated with flies that were raised at 18℃. The 10 second trials indicated that control flies had significantly better locomotor ability than all other genotypes (ANOVA, post hoc Tukey HSD Test, $p<0.01$). However, the locomotor ability of KD;myc-*coq8* flies was still significantly better than *coq8* KD and KD;L520* flies (ANOVA, post hoc Tukey HSD Test, $p<0.01$). This indicates that while KD;myc-*coq8* locomotor ability was not restored to that of the control, it was still significantly better than *coq8* KD and KD;L520* flies.

The 30 second trials showed no statistical significance between the control flies and KD;myc-*coq8* suggesting that reintroduction of the wild-type, myc-*coq8*, into the knockdown background resulted in the full rescue of locomotor ability. There was a significant decrease between the control and both KD;I295P and KD;L520* suggesting that while the rescue was seen in KD;myc-*coq8* introduction of the mutants into the

knockdown background had an adverse effect on locomotor ability. In contrast of the climbing data at 21℃ (Figure 4.26) KD;L520* flies performed similarly to that of *coq8* KD flies suggesting that expression of KD;L520* may have decreased when flies are raised at 18℃. Reintroduction of wild-type *coq8* was able to rescue the loss of locomotor ability due to the loss of *coq8*. Partial rescue was seen with KD;I295P, whereas KD;L520* flies performed worse than *coq8* KD at 21℃ suggesting a dominant negative effect.

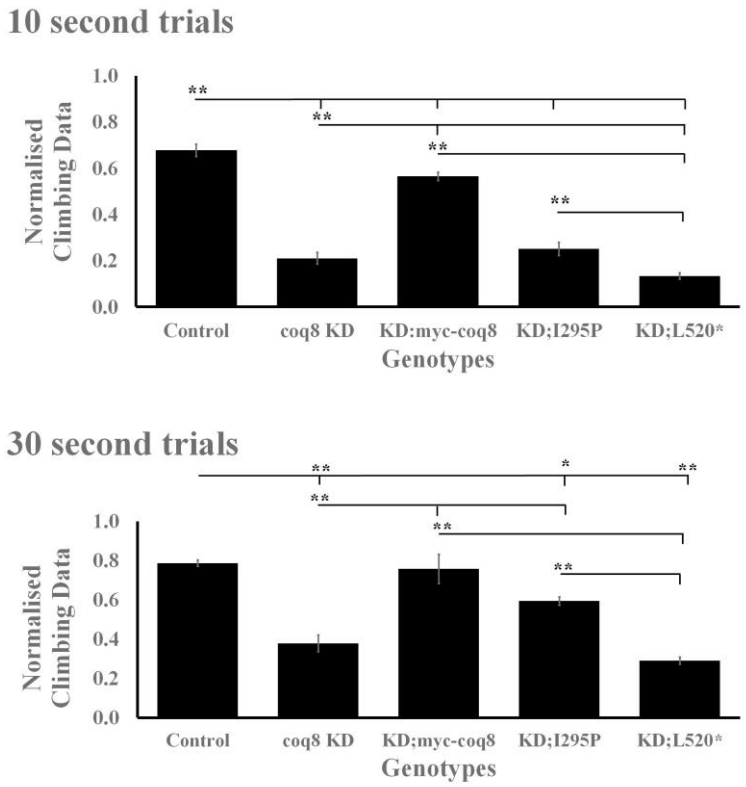


Figure 4.26 Negative geotaxis assay to assess locomotor ability in rescue experiments. *Flies were raised at 18℃. Genotypes shown are w^{1118}/+; elav-GAL4/+ (control); w^{1118}/+;elav-GAL4/coq8KD (coq8 KD) and w^{1118}/+;elav-GAL4/ coq8KD ;myc-coq8, .(KD;myc-coq8) w^{1118}/+; elav-GAL4/ coq8KD;I295P (KD;I295P), w^{1118}/+; elav-GAL4/ coq8KD;L520* (KD;L520*). Means +/- standard error. ANOVA, post hoc Tukey HSD Test. Statistical significance * = p<0.05 and ** = p<0.01.*

4.4.7 Characterisation of the phenotypes resulting from the expression of wild-type or mutant *coq8* in the compound eye of *coq8* KD flies

4.4.7.1 Characterisation of the compound eye using light microscopy

It was previously established that knockdown of *coq8* under the control of the eye-specific driver *GMR*-GAL4 resulted in black necrotic patches and neurodegeneration in the *Drosophila* eye (section 4.2.4). Moreover, SEM images indicated a loss of cohesion and overall structure of the ommatidia suggesting that *coq8* plays a vital role in the proper development of the *Drosophila* compound eye.

Flies containing the appropriate genotype were crossed to *GMR*-GAL4 (methods 3.2) to determine whether the introduction of wild-type or mutant variants of *coq8* could rescue the deficits in ommatidia development and reduce neurodegeneration (as observed by necrotic patches) resulting from knockdown of *coq8*. Flies were raised in vials and placed at either 25℃, 21℃ or 18℃ until imaged 30-60 days post cross (methods 3.5).

Representative images from an analysis of 50 pairs of eyes were shown for each genotype at each temperature. Control males and females across all three temperatures showed normal development and ommatidia arrangement (Figure 4.27). *coq8* KD flies presented with black necrotic patches at all three temperatures. Females presented with a more severe phenotype than males. Reintroduction of wild-type *coq8*, KD;myc-*coq8*, resulted in smaller necrotic patches (21℃) if not a complete lack of necrosis in the eye (18℃). KD;I295P fly eyes displayed a phenotype that was worse than KD;myc-*coq8* but not as severe as *coq8* KD alone which was evident across all temperatures. Of all the genotypes KD;L520* had the most severe mutant phenotype suggesting that introduction of L520* into the knockdown background caused a worsening of the previously described knockdown phenotype (section 4.2.4). KD;L520* flies raised at 25℃ presented with the worst mutant phenotype of all as the casing surrounding the eye was also affected by the impending necrosis.

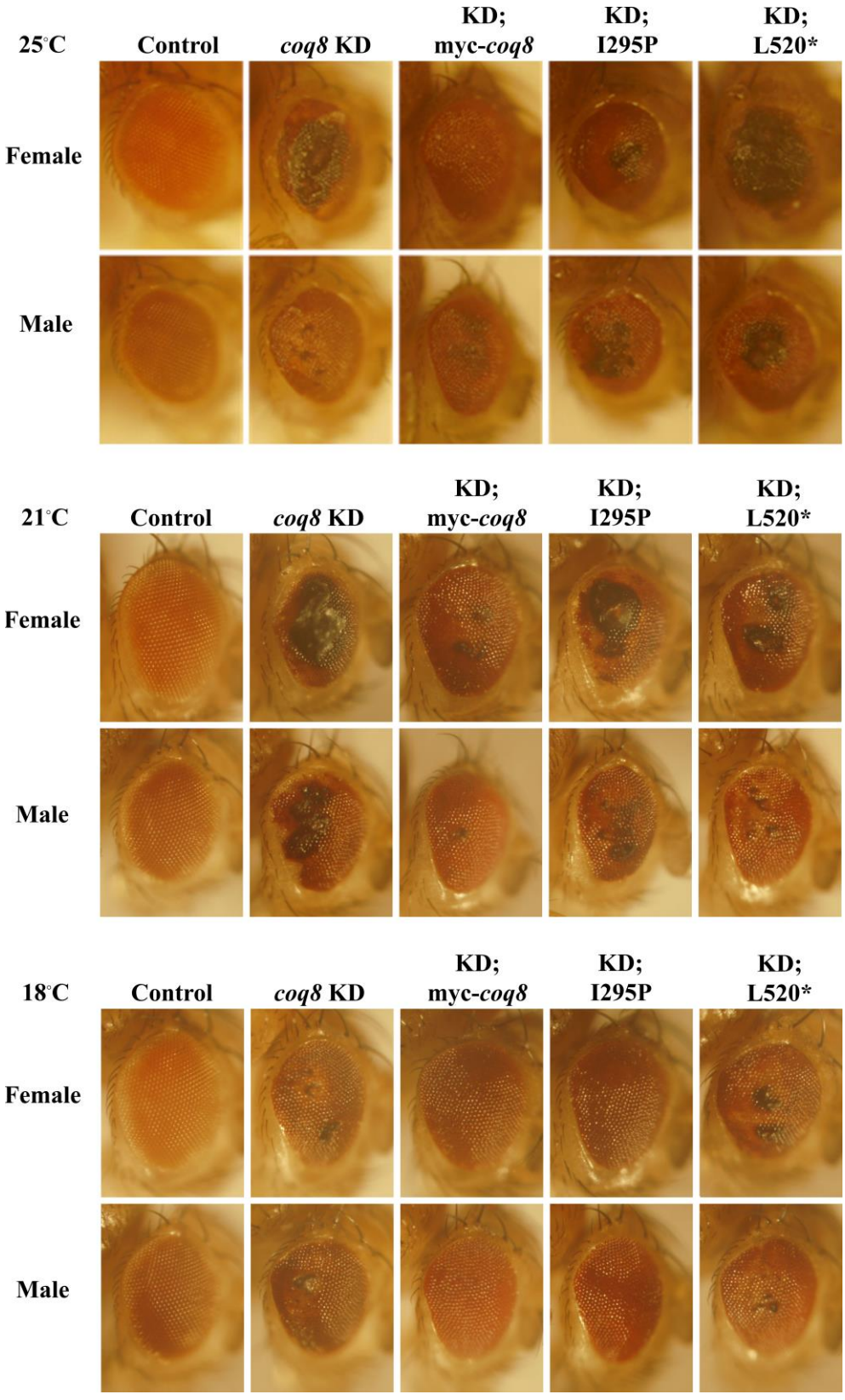


Figure 4.27 Analysis of the impact rescue experiments have on the compound eye. *GMR-GAL4 rescue experiments carried out at 25°C, 21°C, and 18°C. The eye specific driver is a good indicator of neurodegeneration in the compound eye. Genotypes shown are w^{1118}/+;GMR-GAL4/+ (control), GMR-GAL4/coq8KD, GMR-GAL4/ coq8KD ;myc-coq8, GMR-GAL4/ coq8KD ;I295P, and GMR-GAL4/ coq8KD ;L520*.*

4.4.7.2 Characterisation of the compound eye using scanning electron microscopy at 21°C

Scanning electron microscopy images provided higher resolution of the *Drosophila* eye which enabled further analysis of the lattice structure of the compound eye. Flies were raised at 21°C and prepared as previously described before being sent to MMIC for SEM processing (methods 3.9).

Control flies (Figure 4.28) displayed normal eyes with a regular array of plump ommatidia and bristles. By knocking down, *coq8* in the eye necrosis was evident in *coq8* KD flies with ommatidia fused together and a loss of most bristles. Reintroduction of wild-type *coq8* (KD;myc-*coq8*) presented with necrosis, mostly absent bristles and an irregular array of ommatidia. KD;I295P flies had necrosis in the eye, fused and misshapen ommatidia, and a loss of bristles cells. KD;L520* eyes had a more significant loss of ommatidia arrangement with a loss of bristles and necrosis present in the eye. While reintroduction of wild-type *coq8* did not rescue the knockdown phenotype, it did result in a retention of more bristles than the other *coq8* KD is containing flies.

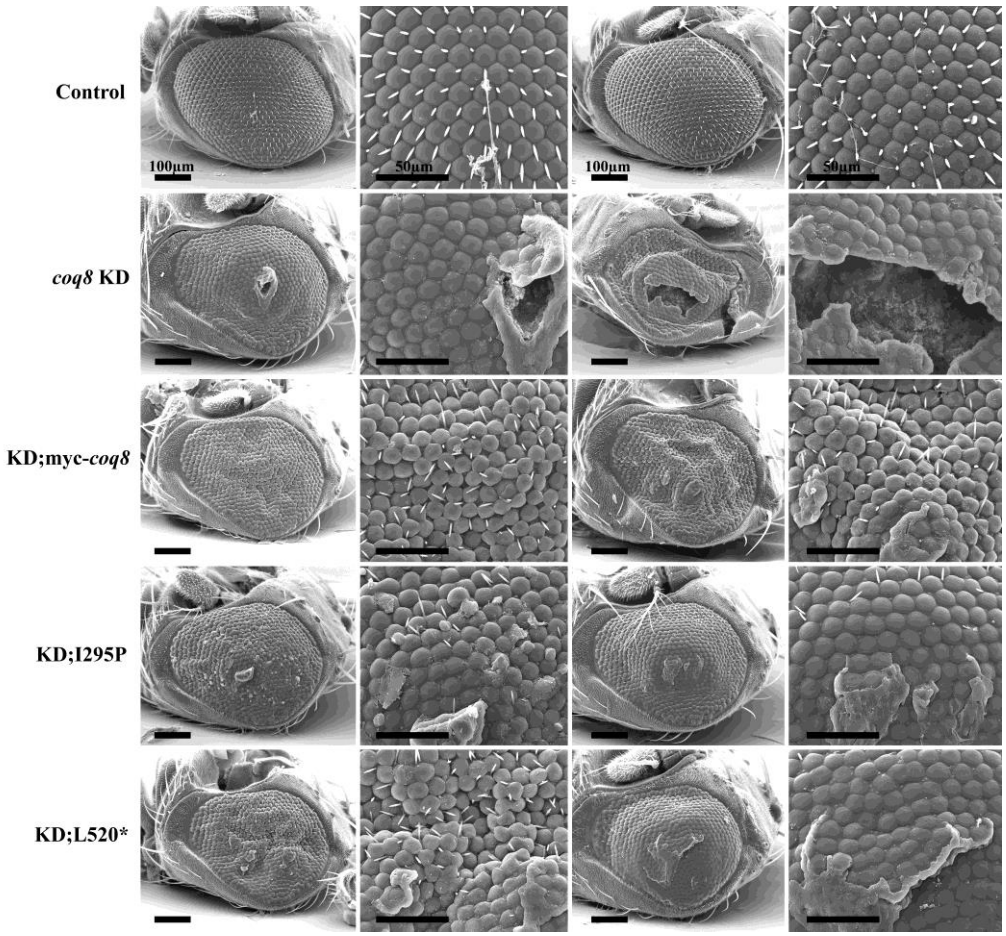


Figure 4.28 Determination of the impact reintroduction of wild-type coq8 has in coq8 KD eyes*. Flies were raised at 21℃ and images taken via SEM. Genotypes shown are* w^{1118}*/+;GMR-GAL4/+ (control); GMR-GAL4/coq8KD, GMR-GAL4/ coq8KD ;myc-coq8, GMR-GAL4/ coq8KD ;I295P, and GMR-GAL4/ coq8KD ;L520*. The two sets of images were used to show phenotypic range.*

4.4.8 Characterisation of the phenotypes resulting from the expression of wild-type or mutant *coq8* in the compound eye of *coq8* KD flies

Knocking down *coq8* in the developing *Drosophila* eye using the *ey*-GAL4 driver expectedly resulted in lethality which was likely due to the decrease in *ey*-GAL4 expression elsewhere in the body. *ey*-GAL4 is also expressed in the developing embryonic nervous system (section 4.2.4.1) (Halder et al., 1995). To determine whether expression of *coq8* could rescue lethality, *ey*-GAL4. Females were crossed to males from either control, *coq8* KD, KD;myc-*coq8*, KD;I295P or KD;L520* genotypes and raised at 21°C (methods 3.2). From these crosses survivors were only present in KD;myc-*coq8* crosses (Figure 4.29). This suggested that reintroduction of wild-type *coq8* enabled rescue of lethality. However, a severe mutant phenotype remained which varied considerably between individuals. Due to the absence of *coq8* in these rescue experiments, it indicated that, for standard development of the compound eye, *coq8* must function in a wild-type capacity. KD;myc-*coq8* flies presented with either the complete absence of a compound eye or at best a fraction of the full sized compound eye.

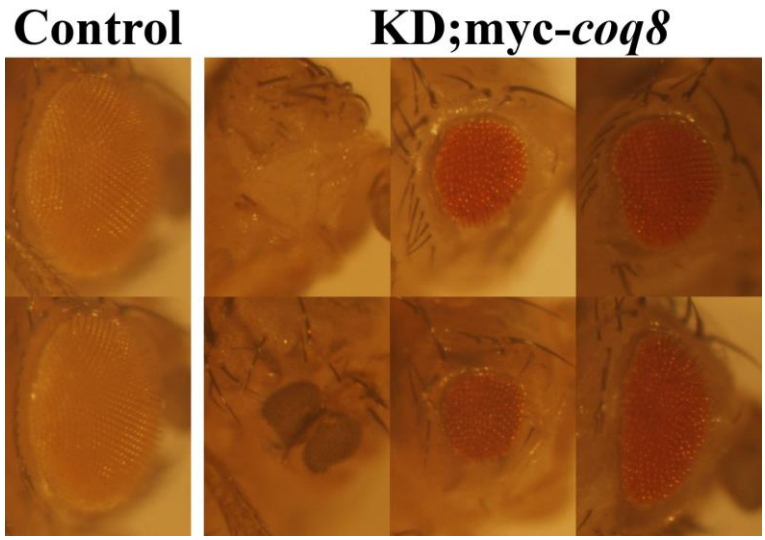


Figure 4.29 ey rescue experiments with flies raised at 21°C. *Only KD-myc-coq8 flies survived*

4.5 Characterisation of the expression patterns associated with the wild-type and mutant forms of *Drosophila coq8*

After completing the characterisation experiments using the *Drosophila coq8* variants, it was vital to characterise the expression patterns of wild-type and mutant *Drosophila coq8* transgenes. To do so *elav*-GAL4 virgin females were crossed to each *Drosophila* variant (methods 3.2). Brains were used to visualise the expression of *coq8* via immunohistochemistry.

4.5.1 Characterisation of *coq8* expression patterns in the *Drosophila* brain

An N-terminal myc tag was added to each construct to visualise the expression patterns associated with wild-type and mutant *coq8*. Five *elav*-GAL4 virgin females were crossed to five males of either EGFP-KASH (negative control), myc-MEF2-VP16 (positive control) or myc-*coq8* genotypes (methods 3.2) to characterise expression in the fly brain. Fly brains were dissected (methods 3.6) from adult flies then immunohistochemistry (methods 3.7) was carried out before detection via confocal microscopy (methods 3.8).

Myc expression (Figure 4.30) was absent in EGFP-KASH (negative control) highlighting the specificity of α-myc staining in the brain. MEF2-VP16 is a transcription factor, and myc-MEF2-VP16 was detected in neuronal nuclei (MEF2-VP16). The myc-*coq8* expression was cytosolic and evident as halos present in the Kenyon cells surrounding the calyx (myc-*coq8* zoom).

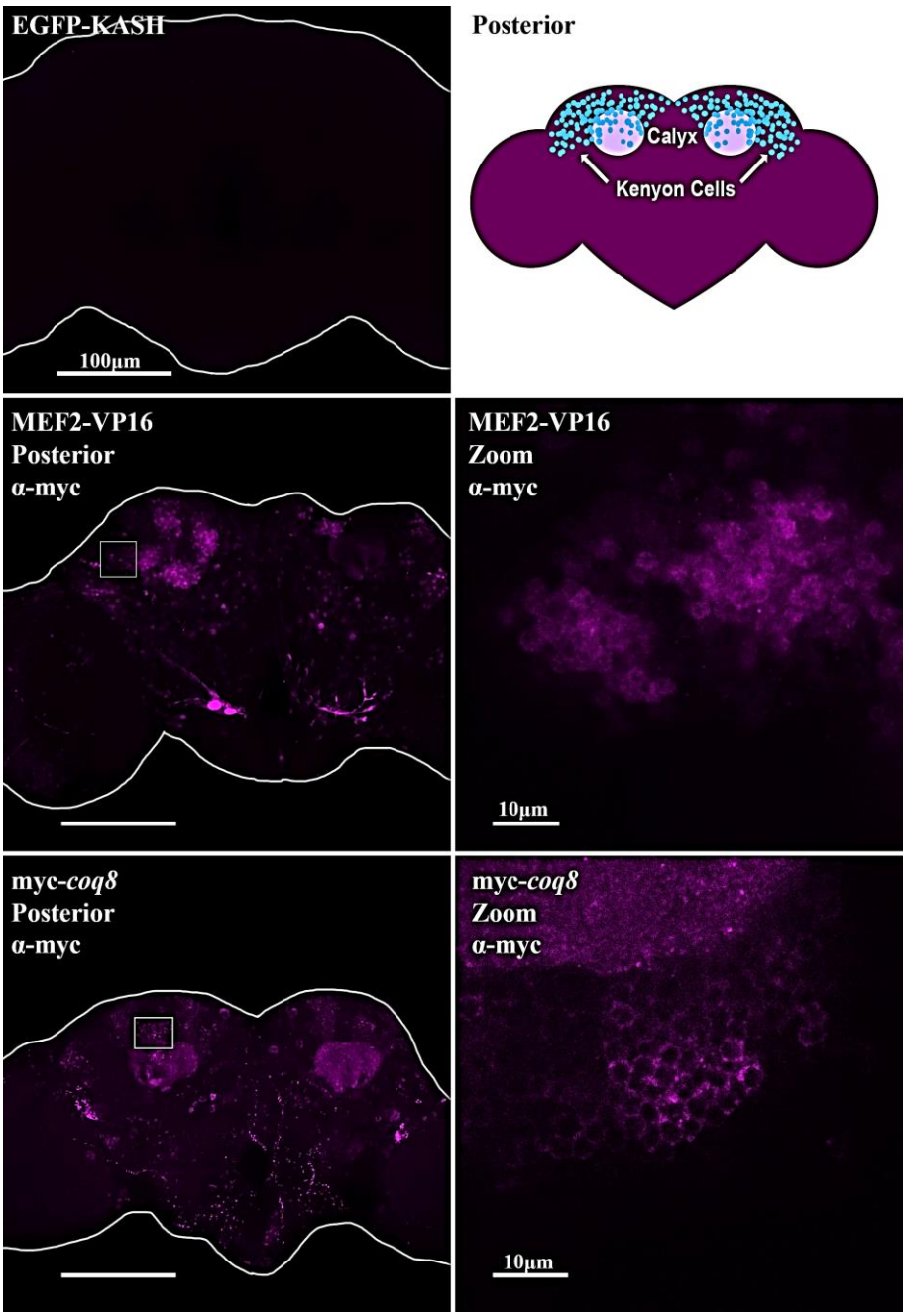


Figure 4.30 Analysis of myc expression in wild-type coq8 fly brains. *Immunohistochemistry was carried out on positive control (myc-MEF2-VP16) and myc coq8. The negative control EGFP-KASH showed no myc expression, MEF2-VP16 showed expression in nuclei. myc expression for myc-coq8 showing cytosolic localisation Genotypes shown are w^{1118}/+; elav-GAL4/+ (control); w^{1118}/+;elav-GAL4/myc-coq8 (myc-coq8) and w^{1118}/+;elav-GAL4/myc-MEF2-VP19, .(MEF2-VP16) w^{1118}/+; elav-GAL4/EGFP-KASH (negative control).*

Further analysis of subcellular distribution was examined by co-labelling with α-Rpd3. Rpd3 is a histone deacetylase and thus could be used as a nuclear marker to confirm that the expression of myc-*coq8* was in the cytoplasm surrounding the nuclei. The expression profile for *Drosophila coq8* variants varied in intensity (Figure 4.31) with myc-*coq8* having the highest α-myc expression which also appeared to be more widespread. I295P was expressed at a lower level and had a more variable expression pattern. It would be advisable to repeat the experiments to see whether there is a difference between I295P expression and myc-*coq8*. L520* expression was present in a minimal amount around the optic lobes but not in the antennal lobes like myc-*coq8*. Wild-type *coq8* expression was predominantly seen in the antennal lobes located in the anterior portion of the fly brain (Figure 4.31, white circle).

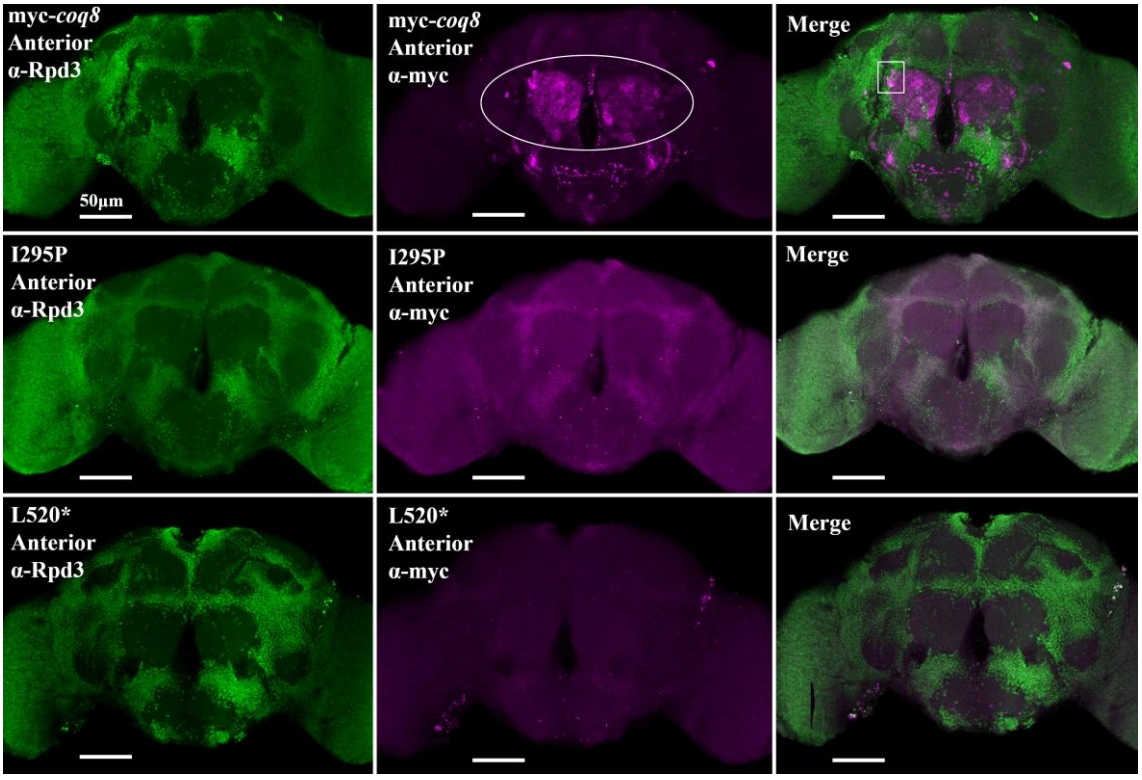


Figure 4.31 Neuronal expression of the Drosophila variants under the control of elav-GAL4. *anti-myc was used to visualise expression of the Drosophila coq8 variants. anti-Rpd3 is a marker of the neuropil Genotypes shown are w^{1118}/+; elav-GAL4/+ (control); /+;elav-GAL4/myc-coq8, .(myc-coq8) w^{1118}/+; elav-GAL4/I295P (I295P), w^{1118}/+; elav-GAL4/L520* (L520*). Images were captured via confocal microscopy.*

High magnification of the area surrounding the antennal lobe of myc-*coq8* brains (Figure 4.32) indicated Rpd3 expression (green) was localised in the nuclei whereas myc expression (magenta) was cytosolic. Colocalisation between Rpd3 and myc did not occur in the merged image highlighting the fact that myc-*coq8* expression was present in the cytosol and not in the nuclei. These results showed that myc-*coq8* was expressed in the cytosol and that the level of *coq8* expression decreased in the I295P and L520* variants. This could suggest that due to the *coq8* mutations there could be a decrease in protein production and possibly degradation of *coq8* mRNA.

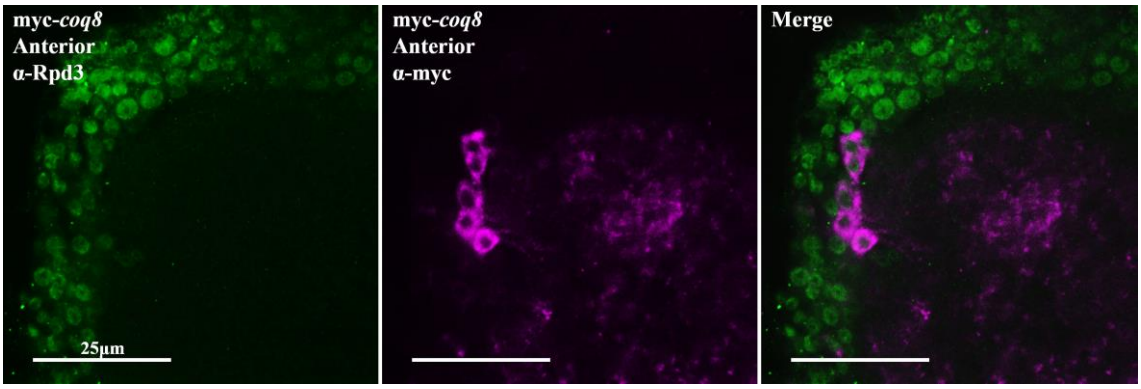


Figure 4.32 High magnification of an optical slice through the Kenyon cell layer of the brain to characterise myc-coq8 expression. *1 µm thick slices were imaged. Rpd3 (green) and myc (magenta) expressed in myc-coq8 fly brain. Genotypes shown are w^{1118}/+; elav-GAL4/+ (control); /+;elav-GAL4/myc-coq8, .(myc-coq8) w^{1118}/+; elav-GAL4/I295P (I295P), and w^{1118}/+; elav-GAL4/L520* (L520*).*

4.5.2 Characterisation of *coq8* expression patterns in the mitochondria of the *Drosophila* brain

After determining the cytosolic localisation of wild-type *coq8* (section 4.5.1), it was necessary to determine whether myc-*coq8* localised to mitochondria using mitoGFP, which consists of GFP fused to a mitochondrial matrix targeting domain (LaJeunesse et al., 2004). A fly line harbouring both the *D42*-GAL4 driver, which drives strong expression in motor neurons, and UAS-mitoGFP, a mitochondrial localised GFP, were crossed to wild-type *coq8*.

Immunofluorescent detection in the brain indicated that *coq8* and mito-GFP appeared adjacent to each other but lacked colocalisation in mitochondria as indicated by a lack of white in the merged channel (Figure 4.33). myc-*coq8* staining appeared diffuse in comparison to mito-GFP expression.

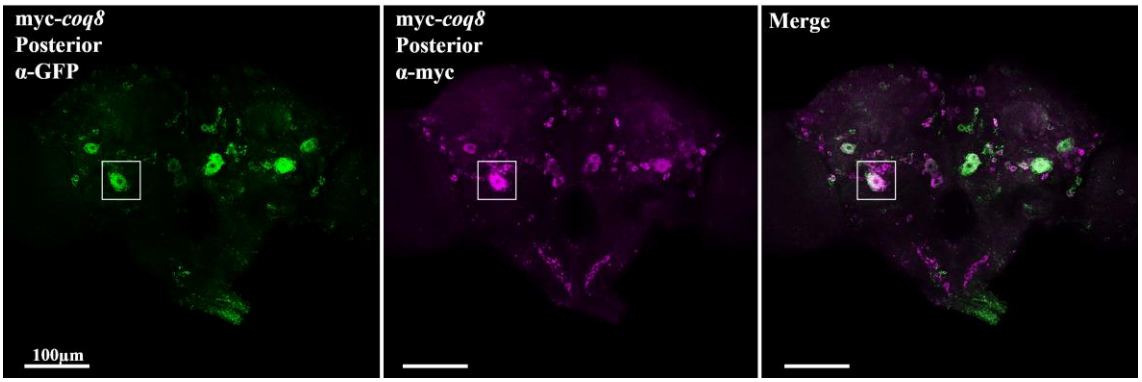


Figure 4.33 Mitochondrial localisation of Drosophila coq8 variants via immunofluorescent detection in the neurons*. Genotypes shown are w^{1118}/D42-GAL4;myc-coq8/+; UAS-mito-GFP, .(myc-coq8)*

High magnification captures were taken (Figure 4.34) and showed a lack of colocalisation between mito-GFP and myc-*coq8*. As myc-*coq8* colocalisation was not seen in these experiments, it was imperative to determine the reasoning for it. A study by Cullen et al. (2016) identified a mitochondrial targeting sequence present on the N-terminal of human COQ8A. In the study, COQ8A was FLAG-tagged on both the N- and C-terminus to determine colocalisation using Mito-tracker. Although both constructs were functional, colocalisation in mitochondria was absent in N-terminally

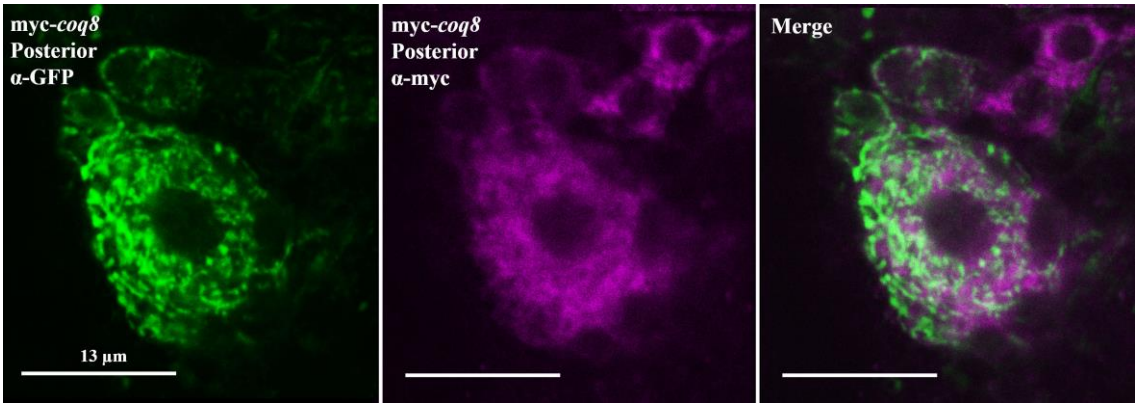


Figure 4.34 High magnification images of mitochondrial localisation in the brain of myc-coq8 flies*. Genotypes shown are w^{1118}/D42-GAL4;myc-coq8/+; UAS-mito-GFP, .(myc-coq8).*

tagged COQ8A, but present in C-terminally tagged COQ8A.

When the N-terminally myc-tagged results obtained here were compared to Cullen et al. (2016) the staining patterns were similar suggesting that functional *coq8* is being produced (as observed by rescue of the *coq8* KD phenotype) and that the N terminal is likely being cleaved and the functional portion of the protein that is localised to mitochondria is not being detected Due to this it was determined that a second *coq8* expressing fly, *coq8*-myc, with the myc tag on the C-terminal would be produced to assess mitochondrial expression.

4.6 Subcloning of *coq8*-myc and generation of transgenic flies

To further investigate the mitochondrial localisation of *coq8,* a second *Drosophila coq8* construct was created that contained a myc-tag placed on the C-terminus of the protein, which was referred to as *coq8*-myc. The DNA construct was synthesised by GenScript® and provided in the plasmid cloning vector pUC57 (appendix 9.8). Therefore it was necessary to subclone *coq8*-myc into pUASTattB (appendix 9.8) for germline transformation of *Drosophila*.

4.6.1 Cloning *coq8*-myc

The pUASTattB and pUC57/*coq8*-myc plasmids were both digested with XhoI and XbaI (methods 3.16.1). The pUASTattB vector was then treated with 1 µL of calf intestinal alkaline phosphatase (CIP) to prevent self-ligation. Both digests were run on an agarose gel (methods 3.16.3) (Figure 4.36 Gel A), and the 2,034 bp *coq8*-myc fragment and 8,489 bp linearised pUASTattB vector were gel purified (methods 3.16.4), (Figure 4.36 Gel B) then ligated (Table 3.4) (methods 3.16.5) and transformed into E.*coli* (methods 3.14.1).

Following transformation, mini preparation (methods 3.14.3) of plasmid DNA was performed on eleven individual clones. These were digested with XhoI and XbaI (Figure 4.35) to identify recombinant clones. Clones 1 and 4 released the expected fragments of 8,489bp (vector) and 2,034bp (*coq8*-myc).

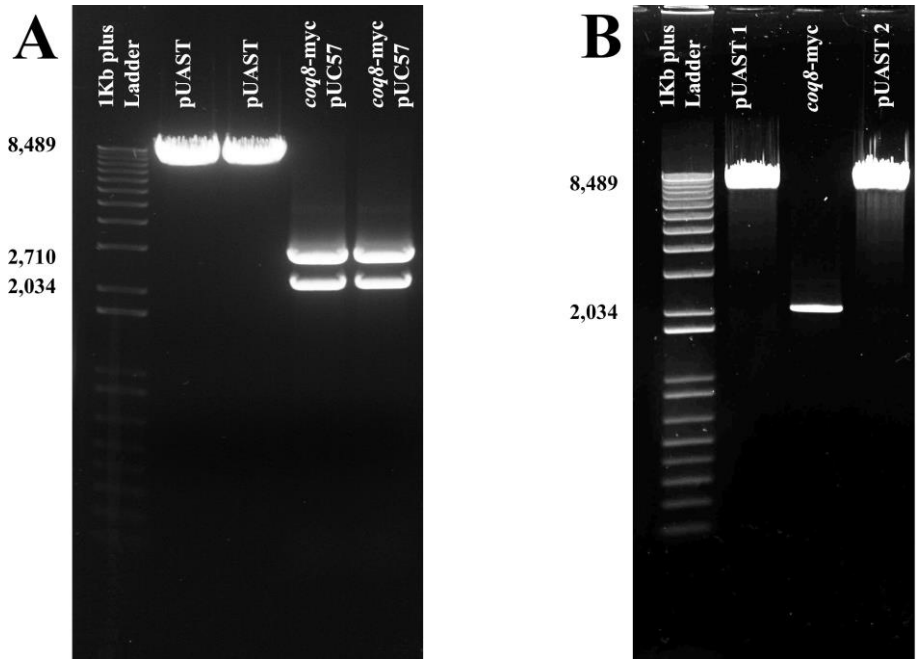


Figure 4.36 Cloning gels. *Gel A: Lanes:1. 1kb plus ladder; 2 and 3, pUAST vector digested with XhoI and Xba; 4. pUC57/coq8-myc digested with XhoI and XbaI. Size is in base pairs.*
Gel B: Gel purified fragments for DNA ligation. Lanes: 1. Kb plus ladder, 2. pUAST, 3. coq8-myc; 4. pUAST. Size in base pairs.

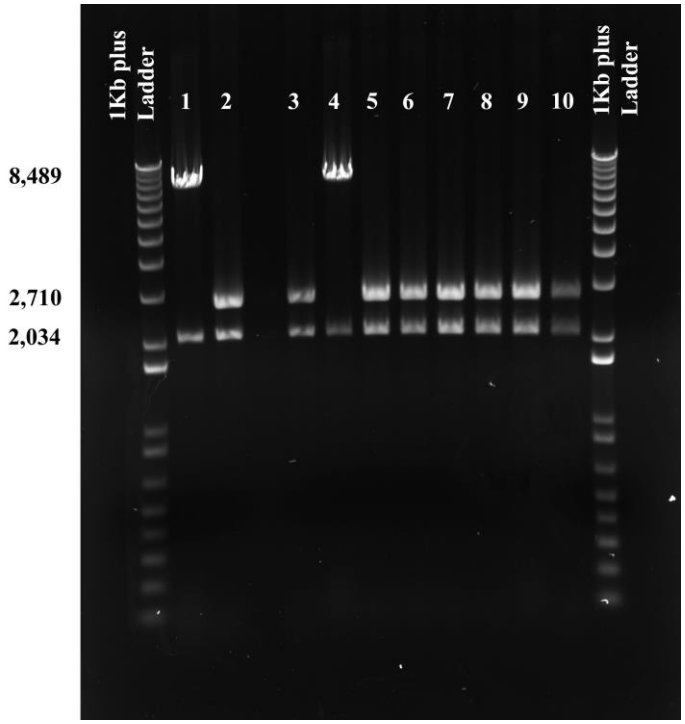


Figure 4.35 Restriction digests of plasmid clones. *Each clone was digested with XhoI and XbaI to determine if the vector and insert were present in the clones. Size in base pairs.*

Clone 1 was selected, and further restriction digests were carried out to confirm the presence of the *coq8*-myc insert (Figure 4.37). The correct fragment sizes were obtained indicating that *coq8*-myc had been successfully cloned into pUASTattB. The insert was sequenced with forward and reverse primers (methods 3.16.7) which confirmed the presence of *coq8*-myc and that the sequence was 100% correct.

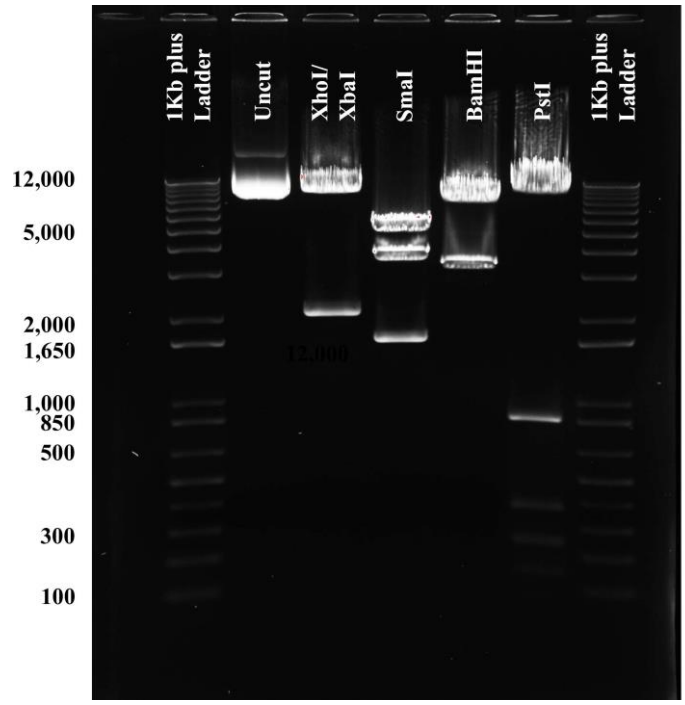


Figure 4.37 Analytical restriction digests of pUAST/coq8-myc clone #1. Size in base pairs.

Table 4.2 Analytical digest for identification of coq8-myc

Restriction enzyme	Expected Bands (bp)	Activation temperature (°C)
Uncut	10,523	37°C
XhoI/XbaI	8,489	37°C
	2,034	
SmaI	5,253	25°C
	3,615	
	1,655	
BamHI	7,380	
	3,165	
PstI	8,835	37°C
	849	
	372	
	243	
	151	
	56	

4.6.2 Generation of transgenic *coq8*-myc flies

Midi preparations of pUAST/*coq8*-myc were performed (methods 3.14.2) to obtain high-quality DNA for microinjection. The plasmid was precipitated and resuspended in injection buffer (methods 3.15) and then microinjected into *Drosophila* embryos which carried a PhiC31 attP landing site at position VK37 (methods 3.17.3). A transgenic line was successfully obtained, and genetic crosses were then performed to obtain a stable homozygous line (methods 3.17.15).

4.6.3 Characterisation of the mitochondrial localisation of N- and C-terminally tagged *coq8* in the *Drosophila* brain

Due to the possibility of an MTS present on the N-terminal of *Drosophila* myc-*coq8,* a second construct was made with the myc tag on the C-terminal (section 4.6). It was previously determined (section 4.5.2) that colocalisation was absent in N-terminally tagged wild-type *coq8* with similar results reported in human COQ8A (Cullen et al., 2016).

Five *D42* -GAL4;mito-GFP virgin females were crossed to five males that contained either myc-*coq8* or *coq8*-myc (methods 3.2) to determine mitochondrial localisation.

coq8-myc and myc-*coq8* had very similar expression profiles for mitoGFP (green) and myc (magenta) which suggested that both *coq8* constructs were present at similar levels in the brain. High magnification optical slices (Figure 4.40) that were 1 µm thick showed that *coq8*-myc colocalised with mitoGFP (white) whereas myc-*coq8* did not.

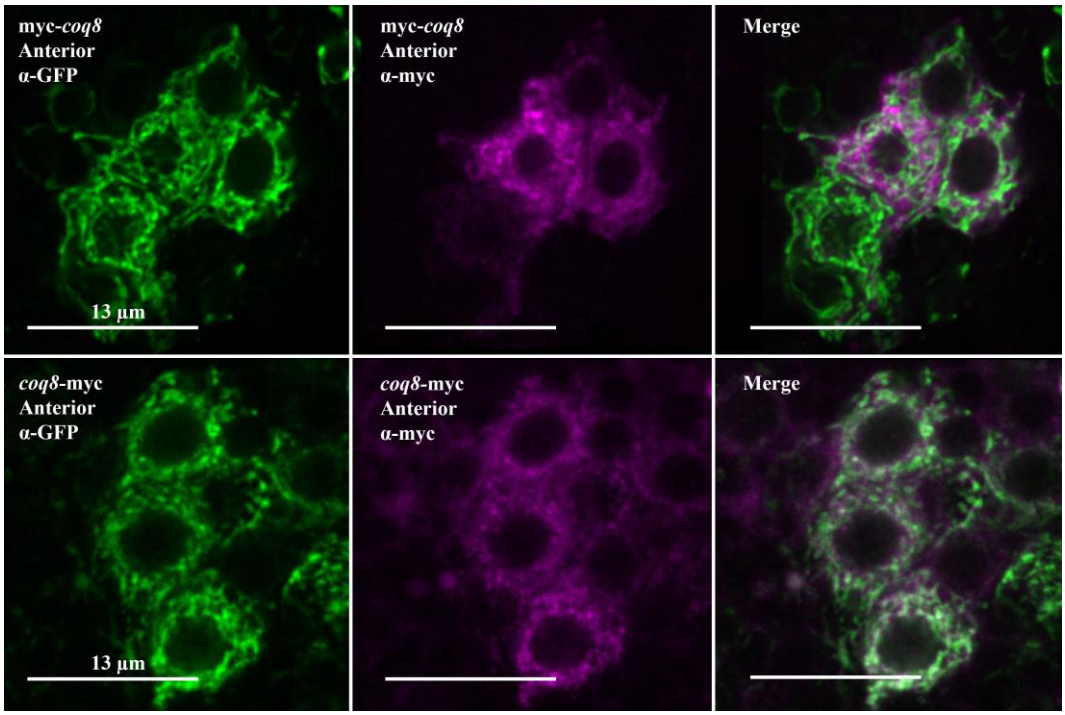


Figure 4.38 Mitochondrial localisation of N- and C-terminally tagged Drosophila coq8. *Immunofluorescent detection using mito-GFP and anti-myc in the neurons. Genotypes shown are w^{1118}/D42-GAL4;myc-coq8/+; UAS-mito-GFP, .(myc-coq8) and w^{1118}/D42-GAL4;coq8-myc/+; UAS-mito-GFP, .(coq8-myc).*

These results suggest, similar to that of Cullen et al. (2016), the possibility of an MTS present on the N-terminal of *coq8* which prevented myc-*coq8* from colocalising with mitoGFP as it was cleaved before entry into the mitochondria.

4.6.4 Using software to predict MTS present on *Drosophila coq8*

Due to the differences that were seen in the mitochondrial localisation experiments (section 4.6.3) the following software was used to predict the presence of an MTS. Two of the four websites (Table 4.3) identified a 56 aa targeting sequence which was consistent with the findings by Cullen et al. (2016) where a mitochondrial targeting

sequence was present on the N-terminal of COQ8A. To further characterise this in *Drosophila* further experiments will be needed.

Table 4.3 Websites used to determine the possibility of an MTS present on Drosophila coq8

Website	Protein ID	Protein length	MTS length
TPpred 2.0	*Drosophila* coq8	661	No
MitoFates	*Drosophila* coq8	661	56
TargetP 1.1	*Drosophila* coq8	661	56
iPSORT Prediction	*Drosophila* coq8	661	No

4.7 Generation and characterisation of flies that overexpress wild-type and mutant forms of human COQ8A

The initial rescue experiments were performed using *Drosophila* wild-type and mutant *coq8*. The next logical step was to determine whether wild-type human COQ8A was able to rescue the *coq8* KD phenotype. Human COQ8A and *Drosophila coq8* are functionally conserved thus *Drosophila* could be used as a model for testing the impact of mutations on human COQ8A function.

In humans, there are two forms of COQ8, COQ8A and COQ8B. Mutations to these genes result in very different mutant phenotypes. Patients with mutations in COQ8A present with cerebellar atrophy and gait ataxia with a decrease in CoQ levels (Horvath et al., 2012; Jacobsen et al., 2017; Lagier-Tourenne et al., 2008) while patients that present with COQ8B mutations have nephrotic conditions (Ashraf et al., 2013). It is unknown whether *Drosophila coq8* or human COQ8A have similar functions in *Drosophila* so these and subsequent experiments will provide a better understanding of this. Sequences can be found in appendix 9.7 with plasmid maps in appendix 9.8.

4.7.1 Generation of fly stocks that overexpress wild-type or mutant forms of human COQ8A

Wild-type human COQ8A and constructs corresponding to the maternal and paternal mutations were generated to characterise the expression of the human COQ8A variants (section 1.6 and Figure 4.15). L277P was generated via the incorporation of the point mutation into the wild-type open reading frame (ORF). The splice site mutation could not be modelled as if all the introns were included, the construct (appendix 9.7) was too large to synthesise, therefore the wild-type sequence was included up until the last amino acid prior to the splice site (L520*) and then the following sequence was included that was predicted to be expressed if the splice site was mutated. This corresponded to 29 amino acids until a stop codon was reached. The constructs were made as previously described (section 4.3.1) and were carried out by Dr Helen Fitzsimons. A crossing scheme can be found in appendix 9.5.

4.7.2 Characterisation of survival resulting from the strong ubiquitous expression of wild-type and mutant forms of human COQ8A in a wild-type background

Before determining whether overexpression of the wild-type human COQ8A, L277P or K502* human variants could rescue the *coq8* knockdown phenotype (section 4.2) expression of each variant was assessed to determine the impact on development and survival in a wild-type background.

Five virgin *da*-GAL4 virgins were crossed to five males from either control, *coq8* KD, COQ8A, L277P, or K502* genotypes. Ubiquitous knockdown of *Drosophila coq8* was previously characterised (section 4.2.1) and indicated larval lethality before pupation as well as severe developmental delay. Two independent crosses were counted for these experiments. Progeny was counted 24 days post cross (methods 3.4).

Progeny counts (Figure 4.39) showed similar pupal development across all genotypes with 100% lethality seen in the *coq8* KD pupae. While the number of COQ8A pupae was similar to the control, it appeared that in both males and females only around half the number eclosed when compared to the other genotypes, suggesting that human COQ8A might be causing lethality at the pupal stage. These observations are interesting but preliminary and must be repeated to determine if it reaches statistical significance.

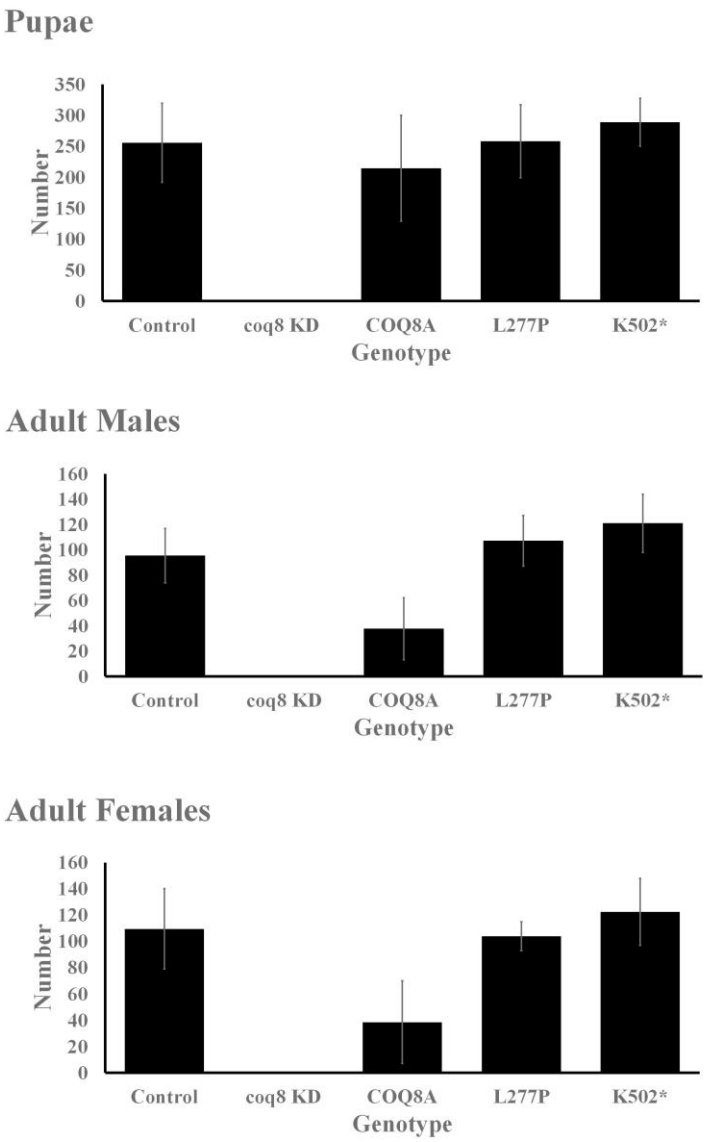


Figure 4.39 Progeny counts for human COQ8A using the strong ubiquitous driver da-GAL4 at 21°C. *Progeny were counted 22 days post cross. The human COQ8A variants were expressed in the wild-type background to see the effects these mutations had on fly survival. Genotypes shown are w^{1118}/+;daGAL4/+ (control); w^{1118}/+;da-GAL4/coq8KD (coq8 KD) and w^{1118}/+;da-GAL4/COQ8A (COQ8A), w^{1118}/+;daGAL4/L277P (L277P), and w^{1118}/+, daGAL4/K502*(K502*).*

4.7.3 Characterisation of human COQ8A expression patterns in the *Drosophila* brain

To examine whether wild-type and mutant variants of COQ8A localised to mitochondria, D42;UAS-mito-GFP flies were crossed to either COQ8A, L277P or K502* and brains dissected (methods 3.6) and processed for immunofluorescence as previously described (methods 3.7 and 3.8). An anti-COQ8A (anti-ADCK3) antibody was used to detect COQ8A (Table 3.1).

Expression in control fly brains (Figure 4.40) showed nonspecific staining for both mito-GFP (green) and COQ8A (magenta) indicating a lack of human COQ8A expression, which was expected, however, mitoGFP expression was unusually low in these brains, and this experiment will need to be repeated. COQ8A brains showed specific COQ8A staining with a similar cytosolic expression profile to that seen in *Drosophila coq8* (section 4.6.3). A similar pattern of expression was observed in α-COQ8A expression was highest in L277P brains. COQ8A expression was absent in K502* brains which suggested that there was no expression of COQ8A in these flies consistent with the introduction of a premature stop codon resulting in the absence of COQ8A expression in the brain via nonsense-mediated decay of mRNA or protein degradation. While the expression pattern for the human variants is similar to that seen in the *Drosophila* variants (section 4.5.2) the amount of expression is somewhat different with wild-type COQ8A having less expression than L277P which was unexpected. High magnification optical slices were captured (Figure 4.41) to visualise the colocalisation of both mito-GFP and COQ8A in the fly brain. The mitochondrial localisation was observed for both wild-type COQ8A and L277P suggesting that human COQ8A is correctly targeted to mitochondria in *Drosophila*.

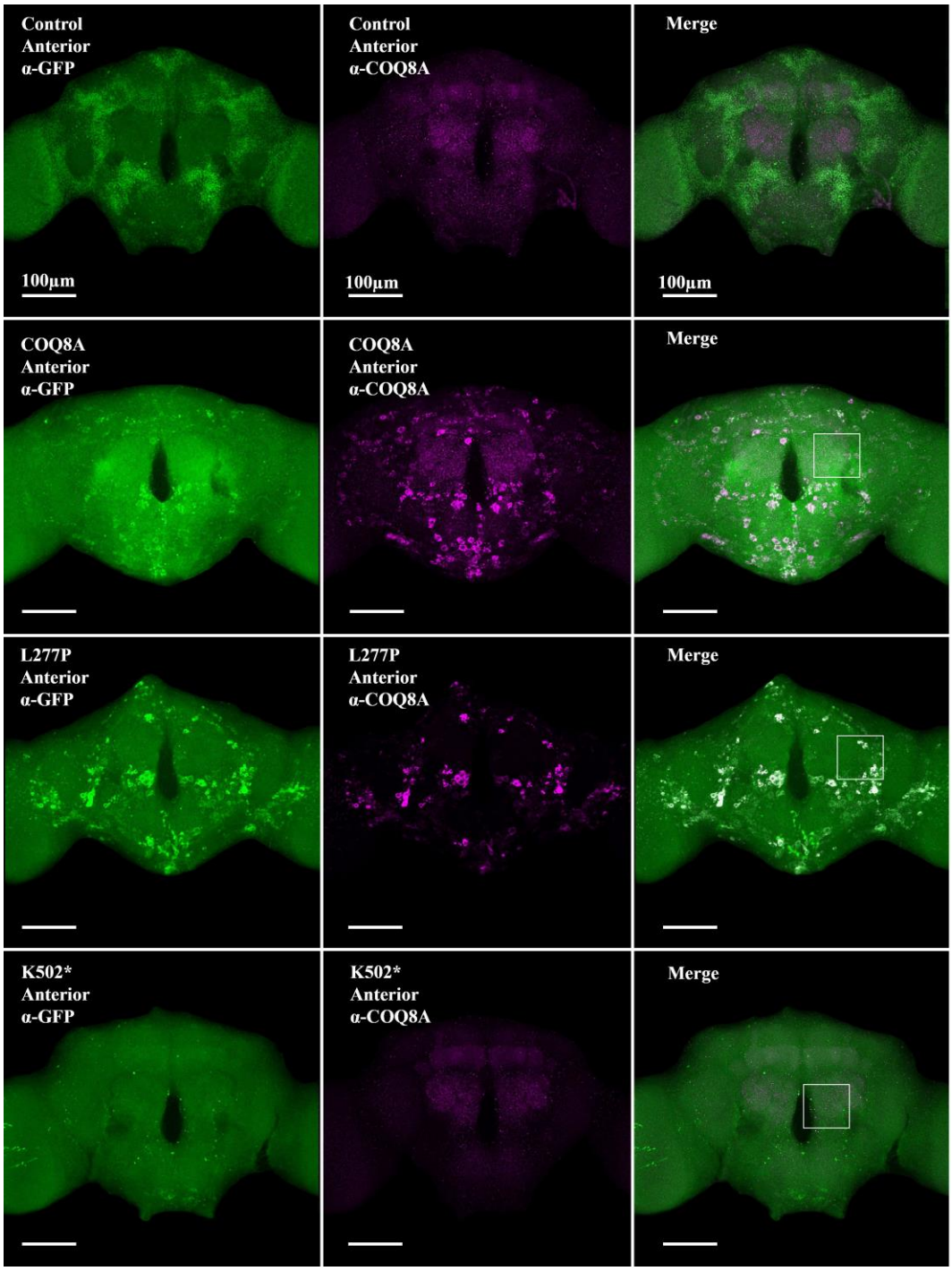


Figure 4.40 Immunohistochemistry of whole mount brains to characterise the expression of human COQ8A in Drosophila. *Brains are labelled with anti-COQ8A and anti-GFP to determine localisation of COQ8A expression. Control (top) indicated that expression of COQ8A was absent in the Drosophila brain. Boxes indicated the areas in which the zoom captures were taken in the following figure. Anterior view of the brain. Genotypes shown are w^{1118}/D42-GAL4; UAS-mito-GFP/+ (control); w^{1118}/D42-GAL4;COQ8A/+; UAS-mito-GFP, .(COQ8A), w^{1118}/D42-GAL4;L277P/+; UAS-mito-GFP, .(L277P) and w^{1118}/D42-GAL4;K502*/+; UAS-mito-GFP (K502*).*

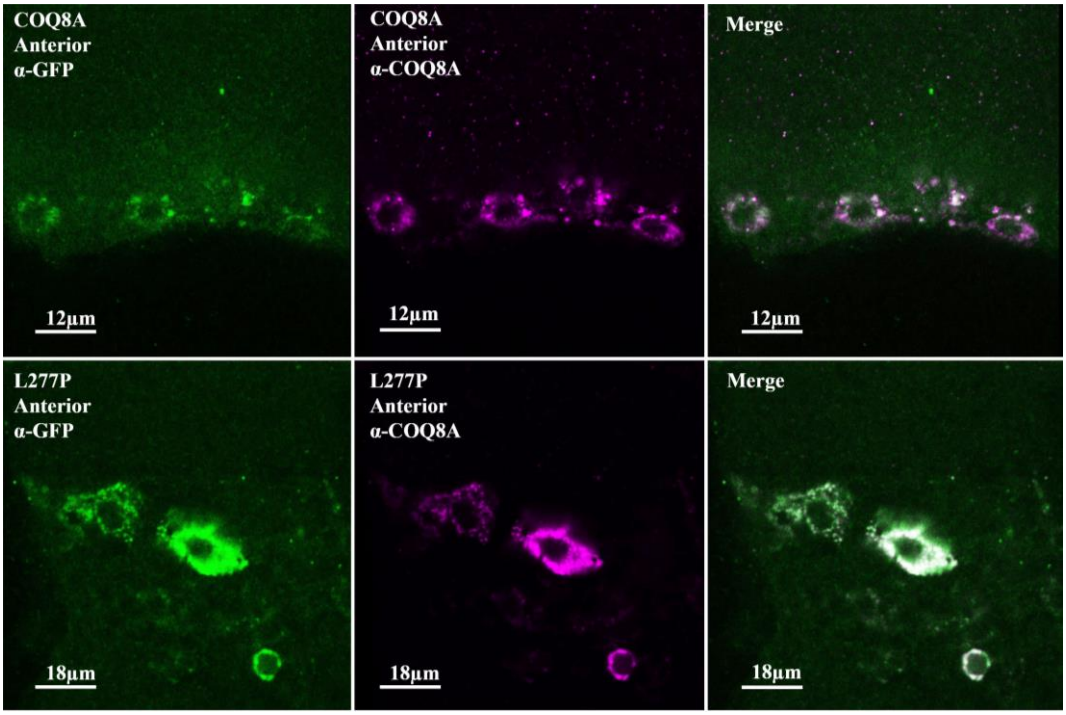


Figure 4.41 High magnification of optic slices to assess expression of human COQ8A and L277P. Immunohistochemistry using mito-GFP and anti-COQ8A. *Genotypes shown are w^{1118}/D42-GAL4; UAS-mito-GFP/+ (control); w^{1118}/D42-GAL4;COQ8A/+; UAS-mito-GFP, .(COQ8A), w^{1118}/D42-GAL4;L277P/+; UAS-mito-GFP, .(L277P) and w^{1118}/D42-GAL4;K502*/+; UAS-mito-GFP (K502*).*

4.7.4 Characterisation resulting from the expression of wild-type or mutant forms of human COQ8A in the *Drosophila* compound eye

The impact of expression of wild-type COQ8A on the developing eye was examined. Crosses were carried out as previously described (methods 3.2) with flies being raised at 25·C for 30 days. Flies were then anesthetised and imaged (methods 3.5). As previously observed, c*oq8* KD flies displayed black necrotic patches with the surface of the ommatidia somewhat smoothed showing a loss in cell shape and function. Expression of wild-type COQ8A, L277P and K502* did not affect normal eye development.

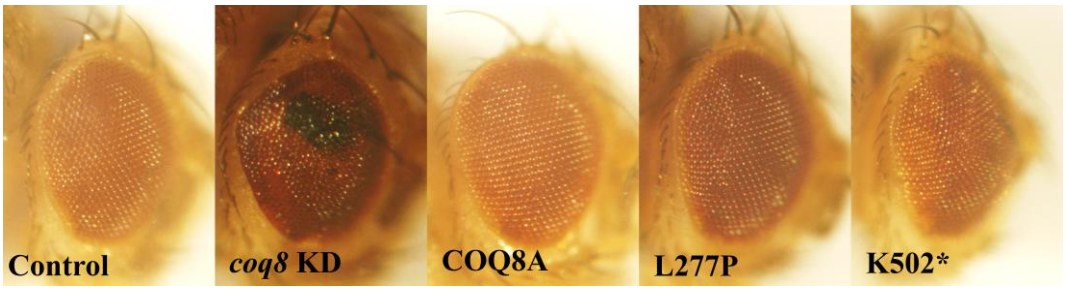


Figure 4.42 GMR-GAL4 characterisation experiments to determine the effect COQ8A variants have in the compound eye of Drosophila. *Necrosis is evident in the KD eye but is absent in the others. Genotypes shown are w^{1118}/+;GMR-GAL4/+ (control); GMR-GAL4/CG32649KD, (coq8 KD) GMR-GAL4/ COQ8A (COQ8A), GMR-GAL4/ L277P (L277P), GMR-GAL4/ K502* (K502*).*

4.8 Determination of whether wild-type or mutant human COQ8A could rescue the *coq8* knockdown phenotype

The ubiquitous knockdown of *Drosophila coq8* resulted in larval lethality and severe developmental delay (section 4.2.1). Reintroduction of wild-type *Drosophila coq8* into the knockdown background rescued larval lethality (section 4.4.3).

4.8.1 Generation of *Drosophila* fly lines that express wild-type or mutant forms of human COQ8A in a knockdown background

To assess whether the COQ8A, L277P or K502* transgenes could rescue the *coq8* KD phenotypes, they were first recombined into the same fly line as *coq8* KD. Unfortunately, due to time constrictions the only rescue experiment conducted was using the eye driver *GMR*-GAL4 with wild-type COQ8A and L277P.

4.8.2 Characterisation of the phenotypes resulting from the reintroduction of wild-type or mutant human COQ8A into a knockdown background expressed in the *Drosophila* compound eye

To determine whether the expression of wild-type COQ8A or L277P could rescue the necrotic phenotype resulting from knockdown of *coq8*, KD;COQ8A and KD;L277P

flies were crossed to *GMR*-GAL4 flies as previously described (methods 3.4). Imaging took place 30 days post cross (methods 3.5).

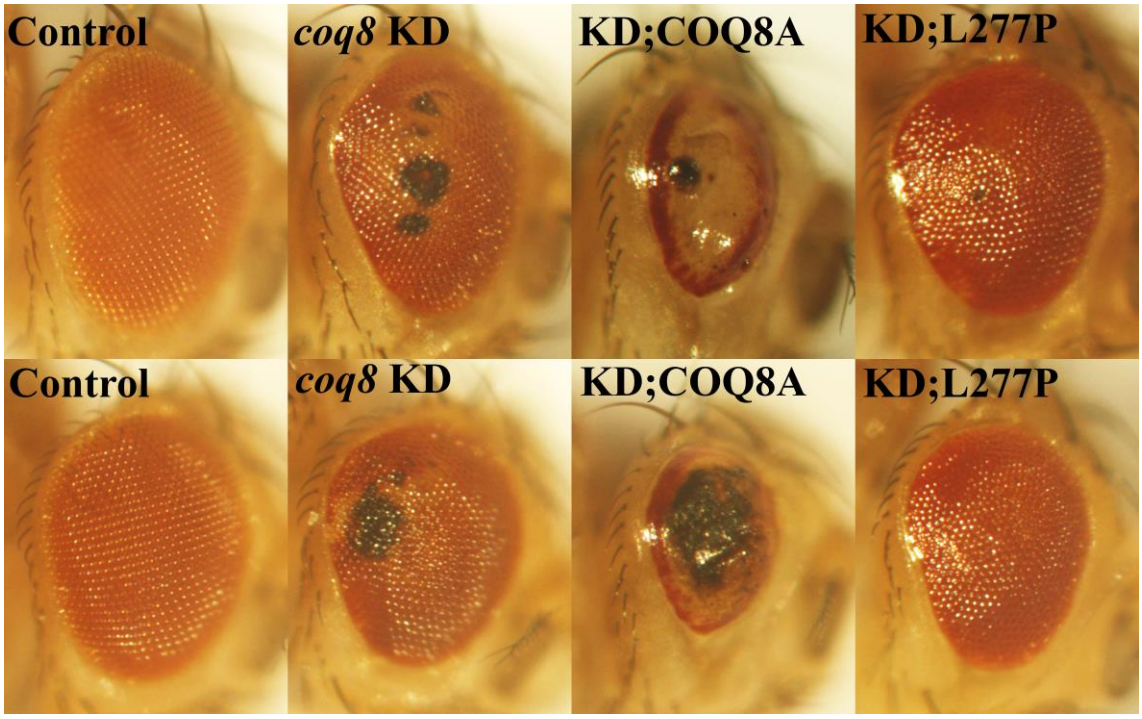


Figure 4.43 Rescue experiments using the highly specific eye driver GMR-GAL4 at 25°C. *Genotypes shown are w^{1118}/+;GMR-GAL4/+ (control); GMR-GAL4/coq8KD, (coq8 KD) GMR-GAL4/ coq8KD;COQ8A (KD;COQ8A), and GMR-GAL4/coq8KD; L277P (KD;L277P).*

Control and *coq8* KD flies presented with a similar phenotype to that seen in the initial characterisation experiments (Figure 4.13). Control flies displayed normal development in the eye while *coq8* KD flies contained black necrotic patches. Flies that expressed KD;COQ8A presented with an unanticipated glossy eye. This mutant phenotype was similar to the glossy eye phenotype seen in (Yarosh et al., 2008) resulting from loss of expression of the *Drosophila* optic atrophy 1 (*dOpa1*) gene, which is a homologue of human OPA1 a gene most commonly mutated in optic atrophy cases in humans. The dOpa1 gene is an essential gene in mitochondrial function (Yarosh et al., 2008). This could suggest that COQ8A is impairing normal mitochondrial function**.** In flies that expressed KD;L277P flies present with small spots of necrosis in the eye. This phenotype is more consistent with a rescue phenotype seen previously (section 4.4.7.1).

While these results are preliminary due to the absence of the KD;K502* construct to use as a comparison in these human rescue experiments, it was evident that wild-type

COQ8A may have a different or additional function in *Drosophila* than COQ8A and furthermore, human COQ8A expression appears to enhance the deleterious phenotype resulting from *coq8* KD, suggesting it may be further impairing mitochondrial function. These experiments will need to be explored further to gain a better understanding of the role and possible function of human COQ8A variants in *Drosophila*.

5 Discussion

Whole exome sequencing is increasingly being used to identify genetic mutations which may be responsible for a disease state. With many of these mutations currently uncharacterised, functional studies provide the means to characterise these mutations and determine whether the genetic mutations are responsible for the mutant phenotype. It has been previously determined that human COQ8A mutations can result in cerebellar atrophy, gait ataxia and CoQ_{10} deficiency. Mutations to COQ8A can occur in a number of different places in the gene resulting in highly variable disease phenotypes (Barca et al., 2016; Blumkin et al., 2014; Cullen et al., 2016; Gerards et al., 2010; Hikmat et al., 2016). Therefore, a model system such as *Drosophila* was needed to functionally characterise individual mutations. The project involved using, developing and characterising a model of *coq8* deficiency and then introducing both wild-type and variant forms of *Drosophila coq8* and human COQ8A to mimic the mutations found by Jacobsen et al. (2017) to determine whether they had impaired *coq8* function.

5.1 Ubiquitous knockdown of *coq8* is lethal

The ubiquitous knockdown of *coq8* in *Drosophila* resulted in severe developmental delay and larval lethality (section 4.2.1) when driven by *da*-GAL4 and the weaker *arm*-GAL4 driver line (section 4.2.2). The results suggested that *coq8* is a crucial gene for survival in *Drosophila* which is consistent with the role *coq8* plays in mitochondrial function (Barca et al., 2014). Reintroduction of wild-type *coq8* (myc-*coq8*) resulted in a partial rescue of the knockdown phenotype where an increase in the number of pupae was seen when compared to *coq8* KD (section 4.4.3). This was also the first indication that the knockdown phenotype was due to the loss of *coq8* and not an off-target effect due to RNAi expression. While it did not rescue the knockdown phenotype enough to result in viable adult progeny, progression to pupations was evident. Based on the loss of locomotor ability seen in the climbing assays (section 4.2.3.4, 4.3.4 and 4.4.6) it is possible that a decrease in coordination and muscle tone prevented adults from emerging from their pupal casings. In some instances, the pupal casings were opened, and heads were visible, but adult flies had died while emerging. However, most pupae had reached P12 which is the final stage of pupation.

5.2 Pan-neuronal knockdown of *coq8* was lethal in males and impaired locomotor activity in females

On restriction of the knockdown of *coq8* to neurons, a semi-lethal phenotype was observed which resulted in the pupal death of male progeny, again the pupae reached P12, suggesting that impaired neuronal function resulted in the inability to emerge (section 4.2.3). Having viable female progeny enabled the detection of a decrease in locomotor ability in *coq8* KD flies (section 4.2.3.4). This decrease in locomotor ability was reminiscent of the gait ataxia seen in humans afflicted with COQ8A mutations (Barca et al., 2014; Gerards et al., 2010; Jacobsen et al., 2017; Liu et al., 2014). Reintroduction of the wild-type *coq8* (myc-*coq8*) variant resulted in a partial rescue of the knockdown phenotype (section 4.4.6).

It is not clear why *elav*-driven expression of *coq8* RNAi was only lethal in males, but it is possible that it may have been a result of dosage compensation. Dosage compensation in *Drosophila* occurs on the male X chromosome (Gelbart & Kuroda, 2009; Lucchesi & Kuroda, 2015). *Drosophila* males transcribe their X chromosome genes two-fold to express X chromosome genes to a similar level as females who contain two X chromosomes. *Drosophila coq8* is located on the X chromosome (http://flybase.org/reports/FBgn0052649.html) as is *elav*-GAL4. If the level of expression of GAL4 or *coq8* RNAi differs between males and females, it is possible that the level of knockdown also differs, leading to an increase in male lethality. It has also been noted that some X chromosome genes are dosage-sensitive and that sensitivity is more likely to affect genes that are a part of a multiprotein complex (Pessia, Makino, Bailly-Bechet, McLysaght, & Marais, 2012) and this could be the case with *Drosophila coq8* as human COQ8A is thought to function in a regulatory capacity in the CoQ biosynthetic pathway(Quinzii & Hirano, 2010).

5.3 A reduction in *coq8* results in decreased ATP production but no noticeable effect on neuronal integrity or survival

ATP production, which occurs in the mitochondria of eukaryotes, was also assessed to determine whether the loss of *coq8* would affect ATP levels. It had been previously

determined that COQ8A was involved in electron shuttling from complex I and complex II to complex III in the MRC (Grant et al., 2010; Lagier-Tourenne et al., 2008; Stefely et al., 2015). Results indicated that in the absence of *coq8* in *Drosophila* there was a decrease in ATP production (section 4.2.5). This indicated that *coq8* is necessary for the production of ATP in *Drosophila*. As these experiments were carried out on one set of larvae and flies, it is necessary to repeat the experiment to determine reliability in the current results.

It was expected that *coq8* KD fly brains would exhibit some defects that would be easily distinguishable from those of wild-type flies. This idea was based on the assumption that human COQ8A results in cerebellar ataxia which often results in a neurodegenerative phenotype (Horvath et al., 2012; Jacobsen et al., 2017; Lagier-Tourenne et al., 2008; Mollet et al., 2008). Initial experiments looked at the gross anatomical structure of the brain (section 4.2.3.2), but no developmental nor neurodegenerative abnormalities (such as vacuoles) were seen. Due to the neurodegenerative phenotype evident in humans, apoptosis was assessed via analysis of caspase activation. This was with an anti-cleaved Dcp1 antibody (section 4.2.3.2) and Apoliner. (section 4.2.3.3). When compared to the severe phenotype seen in the eye, this suggests the brain is far more resistant to the effects of reduced CoQ production.

5.4 I295P substitution impairs *coq8* function within the KxGQ motif

The mutations of KxGQ, in the case of *Drosophila* I295P and human L277P, may affect the autoinhibitory function of the KxGQ domain which folds and occludes the substrate binding pocket in COQ8A (Stefely et al., 2015). As well as affecting the autoinhibitory function of KxGQ, the KxGQ residues are also a part of the QKE triad which aids in inhibiting protein kinase activity (Stefely et al., 2015). If the autoinhibitory function of KxGQ and the QKE triad are absent, due to the presence of the I295P and L277P mutations, the substrate binding pocket is able to bind substrates, and activation of protein kinase activity could occur. It has been previously determined that the QKE triad is essential for CoQ biosynthesis (Stefely et al., 2015). In addition to this *Drosophila* L520* mutation is located in regions that are capable of having active kinase function (Stefely et al., 2015). A salt bridge is present between K358 and E411

while cation-binding is present between N493 and D507. The L520* mutation results in a truncated protein product lacking a portion of the protein kinase domain. Due to the decrease in myc expression seen in L520* (section 4.5.1), it is possible that the loss of the C-terminus, due to the truncation at L520*, may be responsible for the decrease in expression. This could suggest that the C-terminus could be responsible for either mRNA or protein stability. As a result, mRNA could be degraded via nonsense-mediated decay. The G549S and E552K mutations mentioned previously (Table 1.2) result in decreased protein stability (Lagier-Tourenne et al., 2008) As this is integral for proper function of *coq8* L520* was non-functional (section 4.5). In addition, the results from these experiments suggest that the loss of the protein kinase domain in the L520* and K502 mutants is vital for *coq8* stability vs the loss of the autoinhibitory function of the KxGQ domain and the QKE triad as mutants harbouring the I295P and L277P mutations were present in similar patterns to those seen in the wild-type variants and also expressed to a greater extent than to that of the truncated protein products.

5.5 *coq8* is required for eye development

Knockdown of *coq8* in the *Drosophila* eye resulted in severe necrosis and neurodegeneration, resulting in a worsening phenotype over time (section 4.2.4). When the wild-type *coq8* was expressed in *coq8* KD eyes, there was an almost complete rescue of the necrotic phenotype with a small patch or a complete lack of necrosis. There also appeared to be some rescue in I295P which suggests it may retain some activity. However, there was a worsening of the knockdown phenotype in the presence of the L520* variant. The L520* mutant resulted in a truncated protein product and possibly the degradation of mRNA. This loss of protein production leads to a worsening of the *coq8* knockdown phenotype suggesting that *coq8* production is essential for the proper development of the *Drosophila* compound eye.

5.6 Human COQ8A exacerbated the *coq8* KD eye phenotype

The glossy eye phenotype present in KD;COQ8A flies (section 4.8.2) was unexpected and could be because human COQ8 is present in two isoforms, COQ8A and COQ8B, whereas *Drosophila* only has one isoform *coq8*. COQ8B mutations often result in nephrotic conditions and disruption of CoQ biosynthesis in humans (Ashraf et al., 2013)

and may be integral for proper function in *Drosophila*. The glossy eye phenotype was previously reported by Yarosh et al., (2008) where the *Drosophila* optic atrophy 1 (dOpa1) gene was mutated. It was determined that the dOpa1 is essential for mitochondrial function and could suggest human COQ8A functions in a similar capacity in *Drosophila*. However, due to the inability for COQ8A to rescue the mutant phenotype and also the presence of a more severe mutant phenotype, it is possible that COQ8A may impair eye function due to a dominant adverse effect.

5.7 Mitochondrial localisation of wild-type *coq8* and COQ8A

It is well known that COQ8A functions in the MRC and is located in the inner mitochondria membrane (Jacobsen et al., 2017; Lagier-Tourenne et al., 2008; Liu et al., 2014). Cullen et al. (2016) determined that there was an MTS present on the N-terminal of COQ8A. Similar experiments were carried out in this project where adult fly brains expressing each *Drosophila coq8* or human COQ8A variant were stained using primary antibodies targeted to either COQ8A (human) or myc (*Drosophila*). Mito-GFP was also expressed in the brains to determine *Drosophila coq8* or human COQ8A localisation. Confocal microscopy determined colocalisation in most cases with the intensity of staining extremely faint in the L520* and K502* variants (section 4.5.2 and 4.7.3). Colocalisation occurred in human COQ8A, L277P and *Drosophila coq8*-myc. Colocalisation was not seen in *Drosophila* myc-*coq8* due to the myc tag being present on the N-terminal of the protein. This indicated that similar to the findings of Cullen et al. (2016) there may be an MTS present on the N-terminal of *Drosophila coq8*. When the amino acid sequence for *Drosophila coq8* was run through a number of websites to identify the presence of an MTS the results indicated a possibility that there may be a targeting sequence present, but there were mixed results (section 4.6.4). For two of the five websites used it was predicted that there was a 56 aa sequence that is likely to be a MTS. Further assays will need to be conducted in *Drosophila* to determine whether this is true.

6 Future work

The rescue of lethality, climbing ability and necrotic eye phenotype by wild-type *coq8* suggests that the *coq8* RNAi was explicitly targeting *coq8* mRNA. However, RT-qPCR

should be performed to confirm that there is a significant decrease in the expression levels of coq8 in *coq8* KD flies. It would be beneficial to compare the expression levels of *coq8* KD and *coq8* KD2 to ensure that *coq8* KD did indeed have a stronger knockdown than *coq8* KD2 as suspected in section 4.2.1. It may also be beneficial to do qRT-PCR on flies expressing wild-type and mutant forms of *Drosophila coq8* and human COQ8A as well as all KD;*coq8* variants and KD;COQ8A variants this is particularly important for the L520* mutant in order to determine whether the lack of protein expression is a result of the loss of mRNA or protein instability. Expression levels would give a quantitative measure for the knockdown phenotypes seen during the project. Barca et al. (2016) determined that a mutation (c.1511_1512delCT) in COQ8A, although it showed no change in mRNA levels, saw a severe decrease in protein stability suggesting that post-translational degradation occurred in the mutant.

Due to the possibility of an MTS present in *Drosophila coq8* using MTS prediction websites, it may be helpful to perform western blotting for both myc-*coq8* and *coq8*-myc to determine the presence or absence of the myc-tag. In the presence of a MTS, it would be expected that myc-*coq8* would not appear when using an anti-myc antibody due to the N-terminal cleavage of the MTS. In contrast, it would be expected that a band would be present for *coq8*-myc which would occur at the appropriate band size of that for *coq8*.

Preliminary results were obtained during the characterisation experiments for both *Drosophila coq8* (section 4.3) and human COQ8A (section 4.7) variants when expressed in a wild-type background. More experiments are needed to obtain statistical significance. Once these are obtained an interpretation of the results will be supported statistically.

It is necessary to complete the characterisation experiments for the climbing assays and all of the rescue experiments for the human COQ8A variants in *Drosophila* (section 4.8) to assess whether the knockdown phenotype can be rescued by either wild-type COQ8A or either mutant variant. The difference in COQ8A expression in the wild-type background (section 4.7.2) is of interest as a more severe mutant phenotype emerged (section 4.8.2) suggesting that COQ8A has a different function to *Drosophila coq8*. Human COQ8 exists as two isoforms COQ8A (also known as ADCK3) and COQ8B (ADCK4) (Ashraf et al., 2013) both resulting in very distinctive mutant phenotypes.

The KD;K502* rescue construct must be completed for characterisation. Once all three constructs, KD;COQ8A, KD;L277P and KD;K502*, are completed rescue experiments can be completed as done so for the *Drosophila* variants (section 4.4).

Expression of the wild-type and mutant forms of *Drosophila coq8* in a wild-type background indicated that both myc-*coq8* (wild-type) and I295P did not have a negative impact of *Drosophila* survival while L520* resulted in a decrease in progeny numbers, and decreased locomotor ability (section 4.3.4). To mimic the mutant phenotype seen in the case study by Jacobsen et al. (2016) it would be interesting to combine both *Drosophila* mutant variants into the same fly line and characterise the effects of compound heterozygous inheritance in *Drosophila*. This would aid in the understanding of the working model that *Drosophila* provided in this project and also shed some light on the homology and efficacy associated with *Drosophila* as a functional model for human disease.

Loss of *coq8* in the eye resulted in impaired formation of the ommatidial lattice (section 4.2.4) it may be beneficial to delve deeper into the eye by looking at individual ommatidia, which contain secreting cone cells, photoreceptor neurons, and bristle cells in order to determine the effect the loss of *coq8* has on the compound eye. Both adult and larval eye dissections (Williamson & Hiesinger, 2010) would be able to further characterise the role *coq8* plays eye development. Immunohistochemistry could be implemented to determine the presence or absence of specific cells present in each ommatidium (Hsiao et al., 2012). As ommatidia are arranged in a regular and consistent array, any changes would be due to the presence of absence of *Drosophila coq8* or human COQ8A in *Drosophila*. Another possibility would be the use of antibodies targeting to specific caspase initiators and effectors to determine the role *coq8* plays in the eye (Yu et al., 2002).

An additional parameter that could be introduced into the project could be the impact age has on climbing ability and survival in flies expressing *coq8* RNAi. As there was a noticeable disparity seen in climbing ability of flies expressing *coq8* RNAi and the mutant variants (section 4.2.3.4, 4.3.4, and 4.4.6) it may be beneficial to conduct climbing assays at specified time points to see if age influences climbing ability over time. Morbidity experiments could also be conducted to assess the impact *coq8* RNAi has on lifespan in *Drosophila*. In flies that are heterozygous for the *small boy* (*sbo*)

gene, which is the *Drosophila* homologue of human COQ2, a 12.5% increase in male lifespans and a 30.8% increase in females was observed (Liu et al., 2011).

It has been previously described that an increase in oxidative stress and a decrease in ATP production is correlated with the decrease of CoQ biosynthesis (Quinzii et al., 2010). During this project, it was established that there was a decrease in ATP production due to the loss of *coq8* in *Drosophila* (section 4.2.5). It would be beneficial to assess the levels of reactive oxygen species (ROS) in the *Drosophila* brain. Dihydroethidium (Thermo-Fisher), which is a superoxide detection mechanism, could be used to assess the presence of ROS in the brain. Blue fluorescence is present in the cytosol of the cell until it is oxidised, at this point the stain intercalates with DNA and results in the presence of a red stain in the nucleus. In the presence of ROS, there would be an increase red fluorescence seen in the cells.

While *Drosophila coq8* rescue experiments were able to partially rescue some of the knockdown phenotypes (section 4.4) CoQ levels were not evaluated. The most common and also most treatable phenotypes in human COQ8A mutations is a decrease in CoQ levels. To determine the effect, the loss of *coq8* had in *Drosophila* it would be beneficial to determine the CoQ levels of CoQ_8 (5%), CoQ_9 (89%), CoQ_{10} (13%) which are the most common forms of CoQ in *Drosophila,* via HPLC. By identifying the amount of CoQ present in *coq8* KD flies, CoQ supplementation amounts could be determined and administered.

7 Conclusion

Drosophila is an excellent model for biomedical research due to its genetic tractability and the ability to use a number of genetic tools to investigate the inner working of a disorder that is often not possible in humans. Whole exome sequencing can determine the identity of genetic mutations responsible for a disease state. As the output of this information increases, a model system is needed to functionally characterise them.

Drosophila provides the means to characterise the effects of *Drosophila coq8* variants, and human COQ8A variants had on *Drosophila*. The loss of *Drosophila coq8* resulted in larval lethality, developmental delay, a decrease in locomotor ability, a decrease in ATP production as well as necrosis and neurodegeneration. Reintroduction of wild-type *Drosophila coq8* resulted in a rescue in locomotor ability and partial rescue in necrosis

in the eye. This indicated that the loss of *coq8* was responsible for the knockdown phenotype. Reintroduction of I295P and L520* did not rescue the knockdown phenotypes, although there was a slight improvement with I295P indicating it may retain some function. Reintroduction of COQ8A into the knockdown background resulted in a new phenotype in the compound eye. The glossy eye phenotype was previously described in the dOpa1gene which is involved in mitochondrial function. This suggests that human COQ8A impairs mitochondrial function.

Here, we have shown that *Drosophila* can be used to investigate and functionally characterise the effects of the loss of coq8 on a range of neuronal phenotypes by aiding in the understanding of *coq8* function in humans. Due to the highly conserved nature of essential genes *Drosophila* aid in our understanding of how a gene functions and in which instances they can be compared to genes involved in other cellular functions or networks.

8 Bibliography

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9 Appendices

9.1 Fly lines

Table 9.1 Transgenic fly lines used during the project.

Name	Genotype	Phenotype	Source
w^{1118}	*White eyes wild-type strain*	White eyes	R.Davis
CD8::GFP	*y1w*;P{UAS-mCD8::GFP.L}LL5*	Yellow body, red eyes	Bloomington Stock Center 5137
Myc-*coq8*	*y[1]w[67c23]; PBac{y+-attP-3B}VK37, UAS-coq8 WT Insert into VK37(2L) 22A3*	Red eyes	Genetivision
I295P	*y[1]w[67c23]; PBac{y+-attP-3B}VK37, UAS-coq8 I295P Insert into VK37(2L) 22A3*	Red eyes	Genetivision
L520*	*y[1]w[67c23]; PBac{y+-attP-3B}VK37, UAS-coq8 L520* Insert into VK37(2L) 22A3*	Red eyes	Genetivision
***coq8*-myc**	*y[1]w[67c23]; PBac{y+-attP-3B}VK37, UAS-coq8-myc WT. Insert into VK37(2L) 22A3*	Red eyes	Helen Fitzsimmons
COQ8A	*y[1]w[67c23]; PBac{y+-attP-3B}VK37, UAS-hCOA8A WT* Insert into VK37(2L) 22A3*	Light Orange eyes	Genetivision
L277P	*y[1]w[67c23]; PBac{y+-attP-3B}VK37, UAS-hCOQ8A L277P Insert into VK37(2L) 22A3*	Orange eyes	Genetivision
K502	*y[1]w[67c23]; PBac{y+-attP-3B}VK37, UAS-hCOQ8A K502mut. Insert into VK37(2L) 22A3*	Light orange eyes	Genetivision
EGFP-KASH	*y[1]w[67c23]; PBac{y+-attP-*	Red eyes	Genetivision

	3B}VK37, UAS-EGFP::Msp-300[KASH]. Insert into VK37(2L) 22A3		
MEF2-VP16	*y[1]w[67c23]; PBac{y+-attP-3B}VK37, UAS-MEF2DBD-VP16. Insert into VK37(2L) 22A3*	Red eyes	Genetivision
w[CS10];CyO/Sco	*W[CS10];CyO,Sco*	White eyes, curly wings	R. Davis
VK37	*Y[1]M{vas-int.Dm}ZH-2A w[*]; PBac{y[+]-attP-3B}VK00037 22A3 2L*	White eyes	BDSC 24872

9.2 RNAi lines

Table 9.2 RNAi lines obtained from VDRC to reduce Drosophila coq8 expression in order to characterise the knockdown phenotype.

RNAi Name	Genotype	Phenotype	Source
***coq8* RNAi 1**	w[1118];P[GD11306]v26534 CG32649	Red eyes	VDRC
***coq8* RNAi 2**	y,w[1118];P[attP,y[+],w[3'],KK107966 CG32649	Red eyes	VDRC

9.3 Driver lines

Table 9.3 GAL4 driver lines used to drive transgenic expression in Drosophila.

Driver Lines	Genotype	Phenotype	Source
***arm*-GAL4**	w[*];P{w[+mW.hs]=GAL4-arm.S}4a P{w[+mW.hs]=GAL4-arm.S}4b/TM3,Sb[1]Ser[1]	Red eyes, stubble bristles, serrate wings	BDSC 1561
***D42*-GAL4; Mito-GFP**	w[1118], P{w[+mW.hs]=GawB}, P{w[+mC]=UAS-mito-HA-GFP.AP}3 E[1]/TM6B,Tb[1]	Red eyes	BDSC 42737
***da*-GAL4**	w[*];P{w[+mW.hs]=GAL4-da.G32}2; P{w[+mW.hs=GAL4-da.G32}UH1	Red eyes	BDSC 55849
***Elav^{C155}*-GAL4**	w[CS10];P{w[+mW.hs]=GawB]elav[C155]	Red eyes	BDSC 458
***GMR*-GAL4**	w[*];P{w[+mC]=GAL4-ninaE.GMR}12	Red eyes	BDSC1104
***Elav^{C155}*-GAL4; UAS Apoliner**	P{w[+mW.hs]=GawB}elav[C155]; P{w[+mC]=UAS-Apoliner	Red eyes	In house cross between H243 and BDSC 32122
***ey*-GAL4**	w[*];P{w[+m*]=GAL4-ey.H}4-8/CyO	Orange eyes, curly	BDSC 5535

9.4 Restriction enxymes

Table 9.4 List of restriction enzymes used during this cloning experiments including the restriction cutting sequences, the buffer used for each restriction enzyme, the incubations and deactivation temperatures and also the supplier.

Restriction enzymes	Sequence	Buffer	Incubation Temperature	Deactivation Temperature	Supplier
XhoI	C/TCGAG	Cutsmart	37°C	65°C	BioLabs
XbaI	T/CTAGA	Cutsmart	37°C	65°C	BioLabs
BamHI	G/GATCC	3.1	37°C	No	BioLabs
SalI	G/TCGAC	3.1	21°C	65°C	BioLabs
PstI	CTGCA/G	3.1	37°C	80°C	BioLabs
SmaI	CCC/GGG	Cutsmart	25°C	65°C	BioLabs

9.5 Crossing schemes

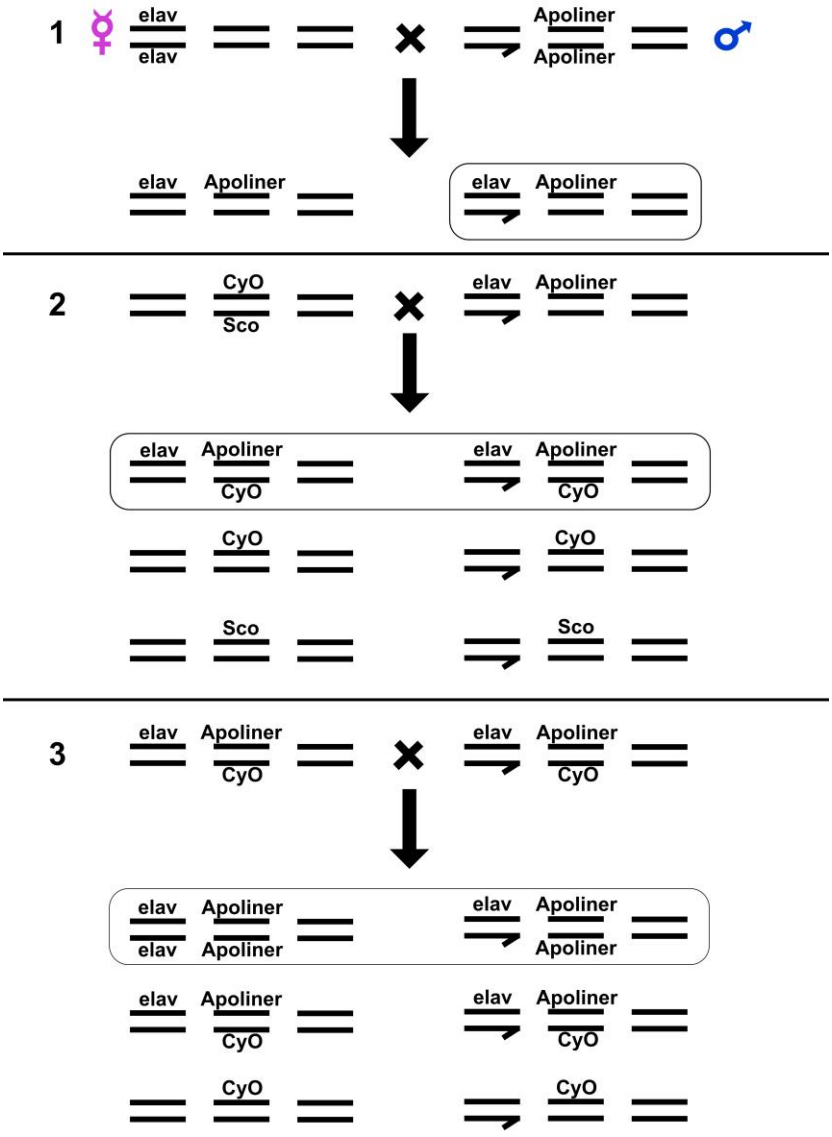

Figure 9.1 Crossing scheme used to create the elav-GAL4/Apoliner driver line for the visualisation of apoptosis in Drosophila.

The pairs of horizontal lines represent pairs of chromosomes in Drosophila. Females are displayed on the left with males on the right. The male Y chromosome is depicted as a half arrow. Each cross begins with a virgin female crossed to a male of the appropriate genotype.

elav-GAL4 virgin females are crossed to UAS-Apoliner males.
Male progeny produced from cross 1 are crossed to virgin females that contain the Cyo/Sco *balancer chromosome on the 2nd chromosome.*
Curly progeny produced from cross 2 that contain elav-GAL4;UAS-Apoliner (glowing under fluorescent microscope) were crossed together to create a homozygous stock. Progeny that emerge from this cross with normal wings are selfed to create a homozygous stock.

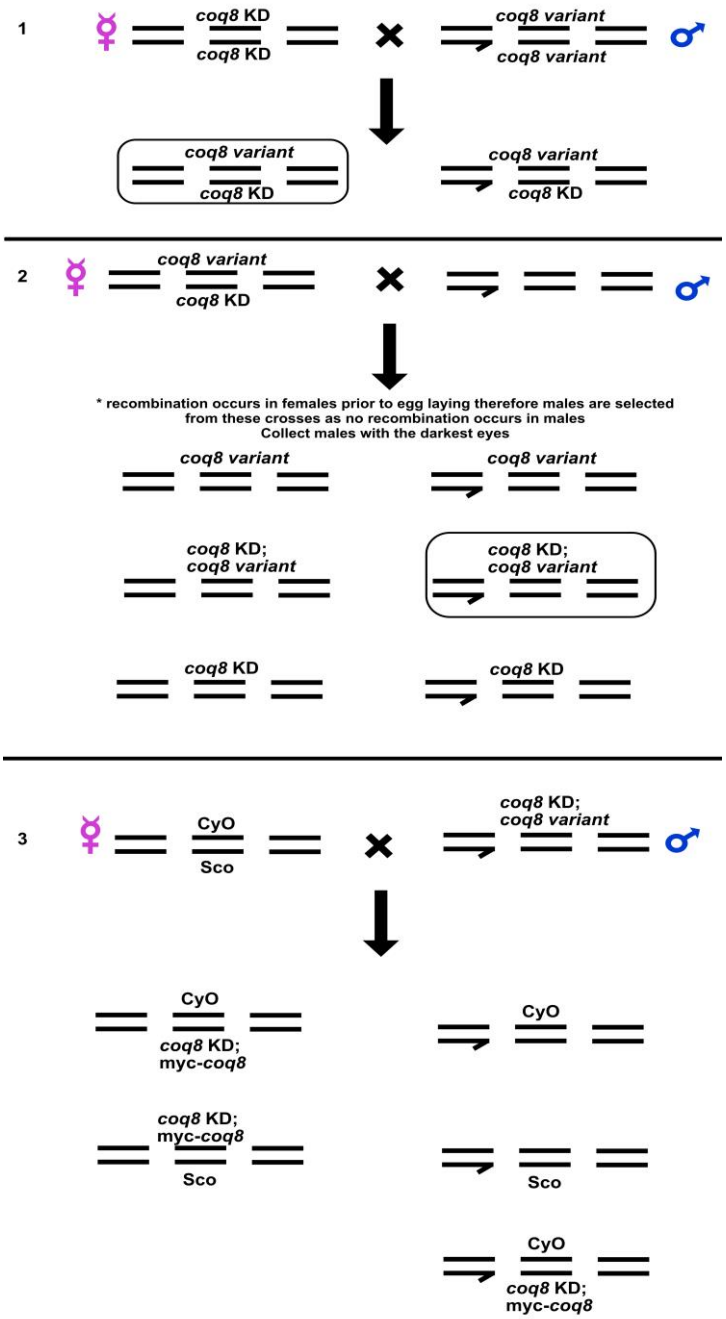

Figure 9.2 Crossing scheme for the generation of Drosophila coq8 variants expressed in a knockdown background. *Flies of the desired genotype from each cross (boxed), were selected for the following cross. All fly lines are in a w- (white) genetic background. Both coq8 KD and each coq8 transgene construct also contain the w+ minigene which confers orange eyes, allowing flies carrying the transgenes to be selected. The presence of both coq8 KD and the coq8 transgene results in red eyes, which allows for selection of male flies in which both constructs have recombined onto the same chromosome in cross #2. The balancer chromosome CyO prevents recombination in females. Cy is a dominant mutation resulting in curly wings. After cross #3, red eyed, curly winged males and females were selfed to generate a homozygous stock.*

9.6 Sequence Alignment

```
CLUSTAL O(1.2.4) multiple sequence alignment

Yeast          ------------------------------------------------------------    0
Drosophila     MSRSAQEAAALLRGLRILLEACGREHL---AHGRHLWSN-SSIRELIAENVAHTQQLVRS    56
Human          MAAILGDTIMVAKGLVKLTQAAVETHLQHLGIGGELIMAARALQSTAVEQIGMF--LGKV    58

Yeast          ------------------------------------------------------------    0
Drosophila     SSQNPTEELKKLQQTVKETGERGYVVAKGICSLLETKIKMSREESAISEPAQVSSIRKST    116
Human          QGQDKHEEYFA-ENFGGPEGEFHFSVPHAA--------GASTDFSSAS-----------A    98

Yeast          ----------------MVTNMVKLRNLRRLYC--------SSRLLRTIQNGRI------S    30
Drosophila     PTAQNPASWDAANLDIS---SITLQEFEEILSRRNKDRSVSLRTPATKSNQTMPKKETST    173
Human          PDQSAPPSLGHAHSEGPAPAYVASGPFREAGF------PGQASSPLGRANGRLFANPRDS    152
                                     :    :..           .       *  :      :

Yeast          SVSSISLSKKYTTK------------------SAKEGEENVERKHEEEKKDTLKSSSVPT    72
Drosophila     NPGIITQDTEYVNNVLRFVAGAKVEEQPPDAQPKKKSD--QPAIEVPELSKVAKQRKVPS    231
Human          F-SAMGFQRRFFHQDQSPVGGLTAEDIEK-ARQAKARP--ENKQHKQTLSEHARERKVPV    208
                 . :  . .:  :                     *         .    ..  :. .**

Yeast          SRISRLFHYGSLAAGVGMNAAAKGISEVAKGNSPT------WKSLILSDSNIDRITNKFS    126
Drosophila     SRIGRMASFGGLFAGLGLGTLNELTKGALGLGGSTSM-----REALLSPANAERIVDTLC    286
Human          TRIGRLANFGGLAVGLGFGALAEVAKKSLRSEDPSGKKAVLGSSPFLSEANAERIVRTLC    268
               :**.*:  :*.* .*:*:.:  :  .      . :        . :** :* :**. .:.

Yeast          KMRGVALKIGQLLSFQDEKVLPKELYEILSRVQNSANHMPQRQLEKVMAKELGANWKTKF    186
Drosophila     KVRGAALKIGQILSIQDSSVVSPQLAKAFERVRQAADYMPDWQVERVMNTQLGADWRQRL    346
Human          KVRGAALKLGQMLSIQDDAFINPHLAKIFERVRQSADFMPLKQMMKTLNNDLGPNWRDKL    328
               *:**.***:**:**:**. .:  .* : :.**:::*:.**  *: :.: .:** :*: ::

Yeast          SKFDKIPMAAASIGQVHAAELPSGQRVVVKIQYPGVKESIDSDLNSLLMLLTASSLLPKG    246
Drosophila     KSFEDKPFAAASIGQVHRATLSDGMDVAIKIQYPGVAQSIESDIDNLVGMLKVWDVFPQG    406
Human          EYFEERPFAAASIGQVHLARMKGGREVAMKIQYPGVAQSINSDVNNLMAVLNMSNMLPEG    388
               . *:. *:********* * : .*  *.:******* :**:**::.*: :*.  .::*:*

Yeast          LFLDKTIANARTELKWECDYNREARALQKFEALLKDDPAFEVPHVFPEYTTDNIITMTRM    306
Drosophila     FFIDNVVRVAKRELQWEVDYDREAEYTEKFREMIAPYPEYYVPRVVRDLTTSSVLTTELV    466
Human          LFPEHLIDVLRRELALECDYQREAACARKFRDLLKGHPFFYVPEIVDELCSPHVLTTELV    448
               :* :: :   : **  * **:***   .**. ::   * : **.:. :  :  ::*   :

Yeast          EGTEIMKLPKASQETKNFISENIMRLCLEEIATFKYMQTDPNWANFLYNGRTKKIELLDF    366
Drosophila     PGVPLDKCFDLSYEHRRHIAASVLKLCLRELFEIECMQTDPNWSNFLYDAPSRRLMLIDF    526
Human          SGFPLDQAEGLSQEIRNEICYNILVLCLRELFEFHFMQTDPNWSNFFYDPQQHKVALLDF    508
                *  : :    * * :. *. .:: ***.*:  :. *******:**:*:   ::: *:**

Yeast          GASRPFAEDFILKYRKLLTYATLRDRKGAYEMSVQLGYLTGLESQSMKDAHVDSVLTLGE    426
Drosophila     GSTRFYRHEFIRNYRRVIMSAAENNRQGVLEMSREMGFLTGYETKQMEQAHVDAVMILGE    586
Human          GATREYDRSFTDLYIQIIRAAADRDRETVRAKSIEMKFLTGYEVKVMEDAHLDAILILGE    568
               *::* : ..*   * :::  *: .:*: .   * :: :*** * : *:.**:*::: ***

Yeast          PFRGDVDKSFDFKDQTVSDRIRGNIGLMLNERLCPPPEETYSLHRKFSGIFLLCARMGAS    486
Drosophila     IFRYD--GDFDFGRQNTTERLAALVPTMVAHRLCPPPEEIYSIHRKLSGIFLLCARLNVR    644
Human          AFASD--EPFDFGTQSTTEKIHNLIPVMLRHRLVPPPEETYSLHRKMGGSFLICSKLKAR    626
                *  *    ***  *..::::   :  *: .** ***** **:***:.* **:*::: .

Yeast          VHCAKLFKEIFAYKV------   501
Drosophila     MNCVPFYKDIVLGKFKD----   661
Human          FPCKAMFEEAYSNYCKRQAQQ   647
               . *  ::::
```

Figure 9.3 sequence alignment between yeast COQ8, Drosophila coq8 and human COQ8A with mutants identified by Jacobsen et al. (2017) highlighted. *The KxGQ motif is also highlighted (outlined) to indicate the highly conserved nature the motif. Sequence alignment constructed using Clustal Omega https://www.ebi.ac.uk/Tools/msa/clustalo/.*

9.7 Sequences for fly lines created during the project

Wild-type myc-*coq8*

Myc tag is highlighted in bold

atggagcagaagctgattagtgaggaggacctgggaacaagccgcagtgcccaggaggcc
M E Q K L I S E E D L G T S R S A Q E A
gccgccctgctgcgcggactgcgcattctgctggaggcctgcggacgcgagcacctggcc
A A L L R G L R I L L E A C G R E H L A
cacggccgccacctgtggagcaacagctccatccgcgagctgatcgccgagaatgtggcc
H G R H L W S N S S I R E L I A E N V A
cacacccagcagctggtgcgcagcagcagccagaacccaaccgaggagctgaagaagctg
H T Q Q L V R S S S Q N P T E E L K K L
cagcagaccgtgaaggagacgggagagcgcggatacgtggtggccaagggcatctgcagc
Q Q T V K E T G E R G Y V V A K G I C S
ctgctggagacgaagatcaagatgagccgcgaggagtcggccatctcggagccagcccag
L L E T K I K M S R E E S A I S E P A Q
gtgtcgagcatccgcaagtccacccccaccgcccagaaccccgcctcgtgggacgccgcc
V S S I R K S T P T A Q N P A S W D A A
aacctggacatcagcagcatcaccctgcaggagttcgaggagatcctgtcccgccgcaat
N L D I S S I T L Q E F E E I L S R R N
aaggaccgcagcgtgtccctgcgcacccccgccaccaagagcaaccagaccatgcccaag
K D R S V S L R T P A T K S N Q T M P K
aaggagacgagcaccaatccaggcatcatcacccaggataccgagtacgtgaacaatgtg
K E T S T N P G I I T Q D T E Y V N N V
ctgcgcttcgtggccggagccaaggtggaggagcagcccccagatgcccagcccaagaag
L R F V A G A K V E E Q P P D A Q P K K
aagagcgatcagccagccatcgaggtgcccgagctgtcgaaggtggccaagcagcgcaag
K S D Q P A I E V P E L S K V A K Q R K
gtgccaagctcccgcattggacgcatggcctcgttcggcggcctgttcgccggcctgggc
V P S S R I G R M A S F G G L F A G L G
ctgggcaccctgaacgagctgaccaagggcgccctgggcctgggcggctcgacctccatg
L G T L N E L T K G A L G L G G S T S M
cgcgaggccctgctgtcccccgccaatgccgagcgcatcgtggacaccctgtgcaaggtg
R E A L L S P A N A E R I V D T L C K V
cgcggcgccgccctgaagattggacagatcctgtccatccaggattcgagcgtggtgtcg
R G A A L K I G Q I L S I Q D S S V V S
ccacagctggccaaggccttcgagcgcgtgcgccaggccgccgactacatgcccgattgg
P Q L A K A F E R V R Q A A D Y M P D W
caggtggagcgcgtgatgaacacccagctgggagccgactggcgccagcgcctgaagtcg
Q V E R V M N T Q L G A D W R Q R L K S
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F E D K P F A A A S I G Q V H R A T L S
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D G M D V A I K I Q Y P G V A Q S I E S
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D I D N L V G M L K V W D V F P Q G F F
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I D N V V R V A K R E L Q W E V D Y D R
gaggccgagtacaccgagaagttccgcgagatgatcgccccatacccggagtactacgtg
E A E Y T E K F R E M I A P Y P E Y Y V
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P R V V R D L T T S S V L T T E L V P G
gtgccactggacaagtgcttcgatctgtcgtacgagcaccgccgccacattgccgccagc
V P L D K C F D L S Y E H R R H I A A S

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tggagcaatttcctgtacgatgccccgtcccgccgcctgatgctgattgacttcggcagc
 W  S  N  F  L  Y  D  A  P  S  R  R  L  M  L  I  D  F  G  S
acccgcttctaccgccacgagttcatccgcaactaccgccgcgtgatcatgtcggccgcc
 T  R  F  Y  R  H  E  F  I  R  N  Y  R  R  V  I  M  S  A  A
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 E  N  N  R  Q  G  V  L  E  M  S  R  E  M  G  F  L  T  G  Y
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 E  T  K  Q  M  E  Q  A  H  V  D  A  V  M  I  L  G  E  I  F
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 R  Y  D  G  D  F  D  F  G  R  Q  N  T  T  E  R  L  A  A  L
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 V  P  T  M  V  A  H  R  L  C  P  P  P  E  E  I  Y  S  I  H
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 R  K  L  S  G  I  F  L  L  C  A  R  L  N  V  R  M  N  C  V
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 P  F  Y  K  D  I  V  L  G  K  F  K  D  -
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myc-*coq8* I295P

Myc tag is highlighted in bold

I295P mutation is highlighted in red

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A A L L R G L R I L L E A C G R E H L A
cacggccgccacctgtggagcaacagctccatccgcgagctgatcgccgagaatgtggcc
H G R H L W S N S S I R E L I A E N V A
cacacccagcagctggtgcgcagcagcagccagaacccaaccgaggagctgaagaagctg
H T Q Q L V R S S S Q N P T E E L K K L
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Q Q T V K E T G E R G Y V V A K G I C S
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L L E T K I K M S R E E S A I S E P A Q
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V S S I R K S T P T A Q N P A S W D A A
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K D R S V S L R T P A T K S N Q T M P K
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K E T S T N P G I I T Q D T E Y V N N V
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L R F V A G A K V E E Q P P D A Q P K K
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K S D Q P A I E V P E L S K V A K Q R K
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V P S S R I G R M A S F G G L F A G L G
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L G T L N E L T K G A L G L G G S T S M
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R E A L L S P A N A E R I V D T L C K V
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R G A A L K **P** G Q I L S I Q D S S V V S
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Q V E R V M N T Q L G A D W R Q R L K S
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F E D K P F A A A S I G Q V H R A T L S
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D G M D V A I K I Q Y P G V A Q S I E S
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D I D N L V G M L K V W D V F P Q G F F
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E A E Y T E K F R E M I A P Y P E Y Y V
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P R V V R D L T T S S V L T T E L V P G
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V P L D K C F D L S Y E H R R H I A A S
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 W  S  N  F  L  Y  D  A  P  S  R  R  L  M  L  I  D  F  G  S
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 T  R  F  Y  R  H  E  F  I  R  N  Y  R  R  V  I  M  S  A  A
gagaacaatcgccagggagtgctggagatgtcccgcgagatgggcttcctgaccggctac
 E  N  N  R  Q  G  V  L  E  M  S  R  E  M  G  F  L  T  G  Y
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 E  T  K  Q  M  E  Q  A  H  V  D  A  V  M  I  L  G  E  I  F
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 V  P  T  M  V  A  H  R  L  C  P  P  P  E  E  I  Y  S  I  H
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myc-*coq8* L520*

Myc tag is highlighted in bold

L520* mutation is highlighted in red

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A A L L R G L R I L L E A C G R E H L A
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H G R H L W S N S S I R E L I A E N V A
cacacccagcagctggtgcgcagcagcagccagaacccaaccgaggagctgaagaagctg
H T Q Q L V R S S S Q N P T E E L K K L
cagcagaccgtgaaggagacgggagagcgcggatacgtggtggccaagggcatctgcagc
Q Q T V K E T G E R G Y V V A K G I C S
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L L E T K I K M S R E E S A I S E P A Q
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V S S I R K S T P T A Q N P A S W D A A
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K E T S T N P G I I T Q D T E Y V N N V
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L R F V A G A K V E E Q P P D A Q P K K
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K S D Q P A I E V P E L S K V A K Q R K
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V P S S R I G R M A S F G G L F A G L G
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L G T L N E L T K G A L G L G G S T S M
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R E A L L S P A N A E R I V D T L C K V
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R G A A L K I G Q I L S I Q D S S V V S
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F E D K P F A A A S I G Q V H R A T L S
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P R V V R D L T T S S V L T T E L V P G
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V P L D K C F D L S Y E H R R H I A A S
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V L K L C L R E L F E I E C M Q T D P N
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W S N F L Y D A P S R R -
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Wild-type *coq8*-myc

Myc tag is highlighted in bold

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 A  C  G  R  E  H  L  A  H  G  R  H  L  W  S  N  S  S  I  R
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 E  L  I  A  E  N  V  A  H  T  Q  Q  L  V  R  S  S  S  Q  N
ccaaccgaggagctgaagaagctgcagcagaccgtgaaggagacgggagagcgcggatac
 P  T  E  E  L  K  K  L  Q  Q  T  V  K  E  T  G  E  R  G  Y
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cccccagatgcccagcccaagaagaagagcgatcagccagccatcgaggtgcccgagctg
 P  P  D  A  Q  P  K  K  K  S  D  Q  P  A  I  E  V  P  E  L
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 S  K  V  A  K  Q  R  K  V  P  S  S  R  I  G  R  M  A  S  F
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 D  W  R  Q  R  L  K  S  F  E  D  K  P  F  A  A  A  S  I  G
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 Q  V  H  R  A  T  L  S  D  G  M  D  V  A  I  K  I  Q  Y  P
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 R  R  V  I  M  S  A  A  E  N  N  R  Q  G  V  L  E  M  S  R
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 V M I L G E I F R Y D G D F D F G R Q N
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 T T E R L A A L V P T M V A H R L C P P
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 P E E I Y S I H R K L S G I F L L C A R
ctgaatgtgcgcatgaattgcgtgccgttttacaaggacatcgtgctgggaaagtttaag
 L N V R M N C V P F Y K D I V L G K F K
gat**gagcagaagctgattagtgaggaggacctg**taa
 D **E Q K L I S E E D L** -

Wild-type human COQ8A

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 Q  S  A  P  P  S  L  G  H  A  H  S  E  G  P  A  P  A  Y  V
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 A  V  G  L  G  F  G  A  L  A  E  V  A  K  K  S  L  R  S  E
gatccatcgggcaagaaggccgtgctgggctcctcgcccttcctgtcggaggccaatgcc
 D  P  S  G  K  K  A  V  L  G  S  S  P  F  L  S  E  A  N  A
gagcgcattgtgcgcaccctgtgcaaggtgcgcggcgccgccctgaagctgggccagatg
 E  R  I  V  R  T  L  C  K  V  R  G  A  A  L  K  L  G  Q  M
ctgtcgatccaggatgacgccttcatcaatccacacctggccaagatcttcgagcgcgtg
 L  S  I  Q  D  D  A  F  I  N  P  H  L  A  K  I  F  E  R  V
cgccagtcggccgacttcatgcccctgaagcagatgatgaagaccctgaacaatgatctg
 R  Q  S  A  D  F  M  P  L  K  Q  M  M  K  T  L  N  N  D  L
ggccccaactggcgcgacaagctggagtacttcgaggagcgcccgttcgccgccgccagc
 G  P  N  W  R  D  K  L  E  Y  F  E  E  R  P  F  A  A  A  S
atcggccaggtgcacctggcccgcatgaagggcggccgcgaggtggccatgaagatccag
 I  G  Q  V  H  L  A  R  M  K  G  G  R  E  V  A  M  K  I  Q
tacccgggcgtggcccagtccatcaattcggatgtgaacaatctgatggccgtgctgaac
 Y  P  G  V  A  Q  S  I  N  S  D  V  N  N  L  M  A  V  L  N
atgtccaatatgctgccggagggcctgttcccagagcacctgattgatgtgctgcgccgc
 M  S  N  M  L  P  E  G  L  F  P  E  H  L  I  D  V  L  R  R
gagctggccctggagtgcgactaccagcgcgaggccgcctgcgcccgcaagttccgcgat
 E  L  A  L  E  C  D  Y  Q  R  E  A  A  C  A  R  K  F  R  D
ctgctgaagggccacccccttcttctacgtgccggagatcgtggacgagctgtgctcgccg
 L  L  K  G  H  P  F  F  Y  V  P  E  I  V  D  E  L  C  S  P
cacgtgctgaccaccgagctggtgagcggattcccactggatcaggccgagggcctgtcc
 H  V  L  T  T  E  L  V  S  G  F  P  L  D  Q  A  E  G  L  S
caggagatccgcaacgagatctgctacaatatcctggtgctgtgcctgcgcgagctgttc
 Q  E  I  R  N  E  I  C  Y  N  I  L  V  L  C  L  R  E  L  F
gagttccacttcatgcagaccgatcccaactggagcaatttcttctacgacccgcagcag
 E  F  H  F  M  Q  T  D  P  N  W  S  N  F  F  Y  D  P  Q  Q
cacaaggtggccctgctggacttcggagccacccgcgagtacgatcgctccttcaccgac
 H  K  V  A  L  L  D  F  G  A  T  R  E  Y  D  R  S  F  T  D
ctgtacatccagatcattcgcgccgccgccgatcgcgaccgcgagacggtgcgcgccaag
 L  Y  I  Q  I  I  R  A  A  A  D  R  D  R  E  T  V  R  A  K
tccatcgagatgaagttcctgacaggctacgaggtgaaggtcatggaggatgcccacctg
 S  I  E  M  K  F  L  T  G  Y  E  V  K  V  M  E  D  A  H  L
gacgccatcctgattctgggagaggccttcgcctcggatgagccgttcgacttcggcacc
```

```
 D  A  I  L  I  L  G  E  A  F  A  S  D  E  P  F  D  F  G  T
cagtcgaccaccgagaagatccacaacctgattccagtgatgctgcgccaccgcctggtg
 Q  S  T  T  E  K  I  H  N  L  I  P  V  M  L  R  H  R  L  V
ccacccccggaggagacgtacagcctgcaccgcaagatgggcggctcgttcctgatctgc
 P  P  P  E  E  T  Y  S  L  H  R  K  M  G  G  S  F  L  I  C
agcaagctgaaggcccgcttcccctgcaaggccatgtttgaggaggcctattccaactat
 S  K  L  K  A  R  F  P  C  K  A  M  F  E  E  A  Y  S  N  Y
tgcaagcgccaggcccagcagtag
 C  K  R  Q  A  Q  Q  -
```

Human COQ8A L277P
L277P mutation highlighted in red

```
atggccgccattctgggagatacgattatggtggccaagggactggtgaagctgacacag
 M  A  A  I  L  G  D  T  I  M  V  A  K  G  L  V  K  L  T  Q
gccgccgtggagacacatctgcagcatctgggaattggcggcgagctgatcatggccgcc
 A  A  V  E  T  H  L  Q  H  L  G  I  G  G  E  L  I  M  A  A
cgtgccctgcagtccaccgccgtggagcagatcggcatgttcctgggcaaggtgcagggc
 R  A  L  Q  S  T  A  V  E  Q  I  G  M  F  L  G  K  V  Q  G
caggataagcacgaggagtacttcgccgagaacttcggcggacccgagggagagttccac
 Q  D  K  H  E  E  Y  F  A  E  N  F  G  G  P  E  G  E  F  H
ttctccgtgccacacgccgccggagcctcgaccgatttcagctccgcctccgccccagac
 F  S  V  P  H  A  A  G  A  S  T  D  F  S  S  A  S  A  P  D
cagagcgccccaccctccctgggccacgcccacagcgagggcccagcccccgcctacgtg
 Q  S  A  P  P  S  L  G  H  A  H  S  E  G  P  A  P  A  Y  V
gcctccggcccgttccgcgaggccggcttcccaggacaggccagcagcccccctgggacgc
 A  S  G  P  F  R  E  A  G  F  P  G  Q  A  S  S  P  L  G  R
gccaatggacgcctgttcgccaatccccgcgacagcttctccgccatgggcttccagcgc
 A  N  G  R  L  F  A  N  P  R  D  S  F  S  A  M  G  F  Q  R
cgcttcttccaccaggatcagtccccggtgggcggcctgaccgccgaggacattgagaag
 R  F  F  H  Q  D  Q  S  P  V  G  G  L  T  A  E  D  I  E  K
gcccgccaggccaaggcccgcccagagaacaagcagcacaagcagaccctgtcggagcac
 A  R  Q  A  K  A  R  P  E  N  K  Q  H  K  Q  T  L  S  E  H
gcccgcgagcgcaaggtgccagtgacccgcattggacgcctggccaatttcggcggcctg
 A  R  E  R  K  V  P  V  T  R  I  G  R  L  A  N  F  G  G  L
gccgtgggcctgggattcggagccctggccgaggtggccaagaagtcgctgcgcagcgag
 A  V  G  L  G  F  G  A  L  A  E  V  A  K  K  S  L  R  S  E
gatccatcgggcaagaaggccgtgctgggctcctcgcccttcctgtcggaggccaatgcc
 D  P  S  G  K  K  A  V  L  G  S  S  P  F  L  S  E  A  N  A
gagcgcattgtgcgcaccctgtgcaaggtgcgcggcgccgccctgaagCCCggccagatg
 E  R  I  V  R  T  L  C  K  V  R  G  A  A  L  K  P  G  Q  M
ctgtcgatccaggatgacgccttcatcaatccacacctggccaagatcttcgagcgcgtg
 L  S  I  Q  D  D  A  F  I  N  P  H  L  A  K  I  F  E  R  V
cgccagtcggccgacttcatgcccctgaagcagatgatgaagaccctgaacaatgatctg
 R  Q  S  A  D  F  M  P  L  K  Q  M  M  K  T  L  N  N  D  L
ggccccaactggcgcgacaagctggagtacttcgaggagcgcccgttcgccgccgccagc
 G  P  N  W  R  D  K  L  E  Y  F  E  E  R  P  F  A  A  A  S
atcggccaggtgcacctggcccgcatgaagggcggccgcgaggtggccatgaagatccag
 I  G  Q  V  H  L  A  R  M  K  G  G  R  E  V  A  M  K  I  Q
tacccgggcgtggcccagtccatcaattcggatgtgaacaatctgatggccgtgctgaac
 Y  P  G  V  A  Q  S  I  N  S  D  V  N  N  L  M  A  V  L  N
atgtccaatatgctgccggagggcctgttcccagagcacctgattgatgtgctgcgccgc
 M  S  N  M  L  P  E  G  L  F  P  E  H  L  I  D  V  L  R  R
gagctggccctggagtgcgactaccagcgcgaggccgcctgcgcccgcaagttccgcgat
 E  L  A  L  E  C  D  Y  Q  R  E  A  A  C  A  R  K  F  R  D
ctgctgaagggccacccccttcttctacgtgccggagatcgtggacgagctgtgctcgccg
 L  L  K  G  H  P  F  F  Y  V  P  E  I  V  D  E  L  C  S  P
cacgtgctgaccaccgagctggtgagcggattcccactggatcaggccgagggcctgtcc
 H  V  L  T  T  E  L  V  S  G  F  P  L  D  Q  A  E  G  L  S
caggagatccgcaacgagatctgctacaatatcctggtgctgtgcctgcgcgagctgttc
 Q  E  I  R  N  E  I  C  Y  N  I  L  V  L  C  L  R  E  L  F
gagttccacttcatgcagaccgatcccaactggagcaatttcttctacgacccgcagcag
 E  F  H  F  M  Q  T  D  P  N  W  S  N  F  F  Y  D  P  Q  Q
cacaaggtggccctgctggacttcggagccacccgcgagtacgatcgctccttcaccgac
 H  K  V  A  L  L  D  F  G  A  T  R  E  Y  D  R  S  F  T  D
ctgtacatccagatcattcgcgccgccgccgatcgcgaccgcgagacggtgcgcgccaag
 L  Y  I  Q  I  I  R  A  A  A  D  R  D  R  E  T  V  R  A  K
tccatcgagatgaagttcctgacaggctacgaggtgaaggtcatggaggatgcccacctg
```

```
 S  I  E  M  K  F  L  T  G  Y  E  V  K  V  M  E  D  A  H  L
gacgccatcctgattctgggagaggccttcgcctcggatgagccgttcgacttcggcacc
 D  A  I  L  I  L  G  E  A  F  A  S  D  E  P  F  D  F  G  T
cagtcgaccaccgagaagatccacaacctgattccagtgatgctgcgccaccgcctggtg
 Q  S  T  T  E  K  I  H  N  L  I  P  V  M  L  R  H  R  L  V
ccacccccggaggagacgtacagcctgcaccgcaagatgggcggctcgttcctgatctgc
 P  P  P  E  E  T  Y  S  L  H  R  K  M  G  G  S  F  L  I  C
agcaagctgaaggcccgcttcccctgcaaggccatgtttgaggaggcctattccaactat
 S  K  L  K  A  R  F  P  C  K  A  M  F  E  E  A  Y  S  N  Y
tgcaagcgccaggcccagcagtag
 C  K  R  Q  A  Q  Q  -
```

Human COQ8A K502*

The wildtype protein ends at K502 (red). Due to the mutation in the splice donor site, 60 nucleotides in the intron are predicted to be translated (20 amino acids blue) until a stop codon is reached

```
atggccgccattctgggagacacaattatggtggccaagggactggtgaagctgacacag
 M  A  A  I  L  G  D  T  I  M  V  A  K  G  L  V  K  L  T  Q
gccgccgtggagacgcatctgcagcacctgggaattggcggcgagctgatcatggccgcc
 A  A  V  E  T  H  L  Q  H  L  G  I  G  G  E  L  I  M  A  A
cgtgccctgcagtccaccgccgtggagcagatcggcatgttcctgggcaaggtgcagggc
 R  A  L  Q  S  T  A  V  E  Q  I  G  M  F  L  G  K  V  Q  G
caggataagcacgaggagtacttcgccgagaacttcggcggacccgagggagagttccac
 Q  D  K  H  E  E  Y  F  A  E  N  F  G  G  P  E  G  E  F  H
ttctccgtgccacacgccgccggagcctccaccgatttcagctccgcctcggcccccgac
 F  S  V  P  H  A  A  G  A  S  T  D  F  S  S  A  S  A  P  D
cagagcgccccaccctccctgggccacgcccactccgagggcccagcccccgcctacgtg
 Q  S  A  P  P  S  L  G  H  A  H  S  E  G  P  A  P  A  Y  V
gcctcgggcccattccgcgaggccggcttcccaggacaggccagcagccccctgggacgc
 A  S  G  P  F  R  E  A  G  F  P  G  Q  A  S  S  P  L  G  R
gccaatggacgcctgttcgccaatccgcgcgacagcttctccgccatgggcttccagcgc
 A  N  G  R  L  F  A  N  P  R  D  S  F  S  A  M  G  F  Q  R
cgcttcttccaccaggatcagtcccccgtgggcggcctgaccgccgaggacattgagaag
 R  F  F  H  Q  D  Q  S  P  V  G  G  L  T  A  E  D  I  E  K
gcccgccaggccaaggcccgcccagagaacaagcagcacaagcagaccctgtcggagcac
 A  R  Q  A  K  A  R  P  E  N  K  Q  H  K  Q  T  L  S  E  H
gcccgcgagcgcaaggtgccagtgacccgcattggacgcctggccaatttcggcggcctg
 A  R  E  R  K  V  P  V  T  R  I  G  R  L  A  N  F  G  G  L
gccgtgggcctgggattcggagccctggccgaggtggccaagaagtcgctgcgcagcgag
 A  V  G  L  G  F  G  A  L  A  E  V  A  K  K  S  L  R  S  E
gatccatccggcaagaaggccgtgctgggctcctcgcccttcctgtcggaggccaatgcc
 D  P  S  G  K  K  A  V  L  G  S  S  P  F  L  S  E  A  N  A
gagcgcattgtgcgcaccctgtgcaaggtgcgcggcgccgccctgaagctgggccagatg
 E  R  I  V  R  T  L  C  K  V  R  G  A  A  L  K  L  G  Q  M
ctgtccatccaggatgacgccttcatcaatccacacctggccaagatcttcgagcgcgtg
 L  S  I  Q  D  D  A  F  I  N  P  H  L  A  K  I  F  E  R  V
cgccagtccgccgacttcatgcccctgaagcagatgatgaagaccctgaacaatgatctg
 R  Q  S  A  D  F  M  P  L  K  Q  M  M  K  T  L  N  N  D  L
ggccccaactggcgcgacaagctggagtacttcgaggagcgcccgttcgccgccgccagc
 G  P  N  W  R  D  K  L  E  Y  F  E  E  R  P  F  A  A  A  S
atcggccaggtgcacctggcccgcatgaagggcggccgcgaggtggccatgaagatccag
 I  G  Q  V  H  L  A  R  M  K  G  G  R  E  V  A  M  K  I  Q
taccccggcgtggcccagtccatcaattcggatgtgaacaatctgatggccgtgctgaac
 Y  P  G  V  A  Q  S  I  N  S  D  V  N  N  L  M  A  V  L  N
atgtccaatatgctgccggagggcctgttcccagagcacctgattgatgtgctgcgccgc
 M  S  N  M  L  P  E  G  L  F  P  E  H  L  I  D  V  L  R  R
gagctggccctggagtgcgactaccagcgcgaggccgcctgcgcccgcaagttccgcgat
 E  L  A  L  E  C  D  Y  Q  R  E  A  A  C  A  R  K  F  R  D
ctgctgaagggccacccccttcttctacgtgccggagatcgtggacgagctgtgcagcccg
 L  L  K  G  H  P  F  F  Y  V  P  E  I  V  D  E  L  C  S  P
cacgtgctgaccaccgagctggtgtccggattcccactggaccaggccgagggcctgtcc
 H  V  L  T  T  E  L  V  S  G  F  P  L  D  Q  A  E  G  L  S
caggagatccgcaacgagatctgctacaatatcctggtgctgtgcctgcgcgagctgttc
 Q  E  I  R  N  E  I  C  Y  N  I  L  V  L  C  L  R  E  L  F
gagttccacttcatgcagaccgatcccaactggagcaatttcttctacgacccgcagcag
 E  F  H  F  M  Q  T  D  P  N  W  S  N  F  F  Y  D  P  Q  Q
cacaagatgtcgccccgcgtgggagcccccgccgcccgcccggagtccttctcggagtgg
 H  K  M  S  P  R  V  G  A  P  A  A  R  P  E  S  F  S  E  W
ggaacctaa
```

G T –

9.8 Plasmid maps

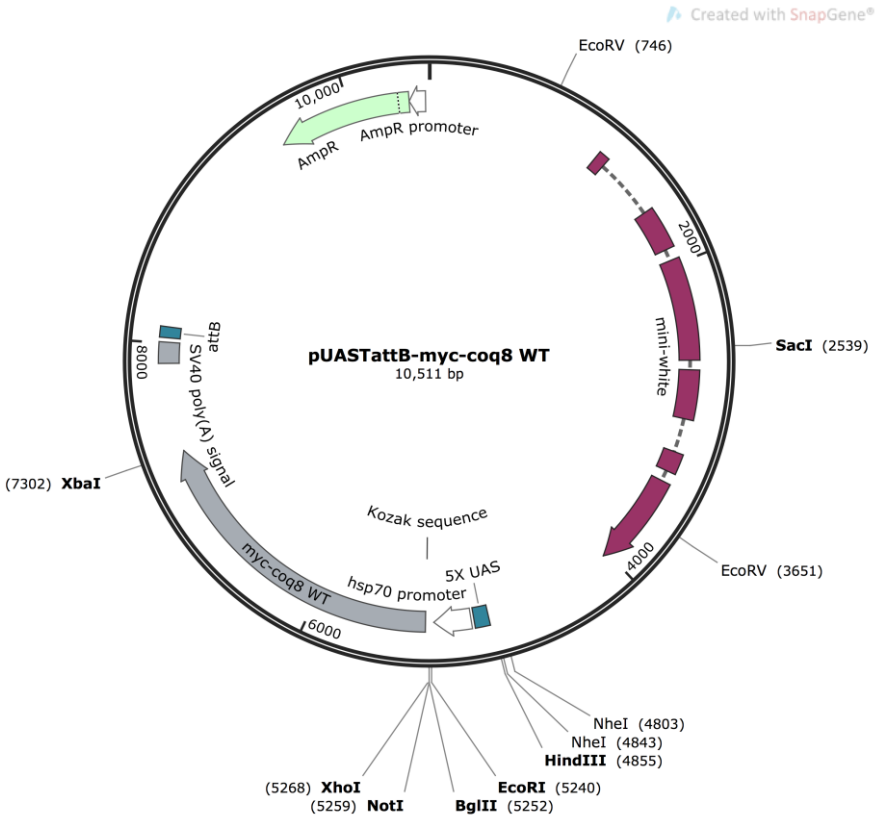

Figure 9.4 Physical map of pUASTattB-myc-coq8 WT.

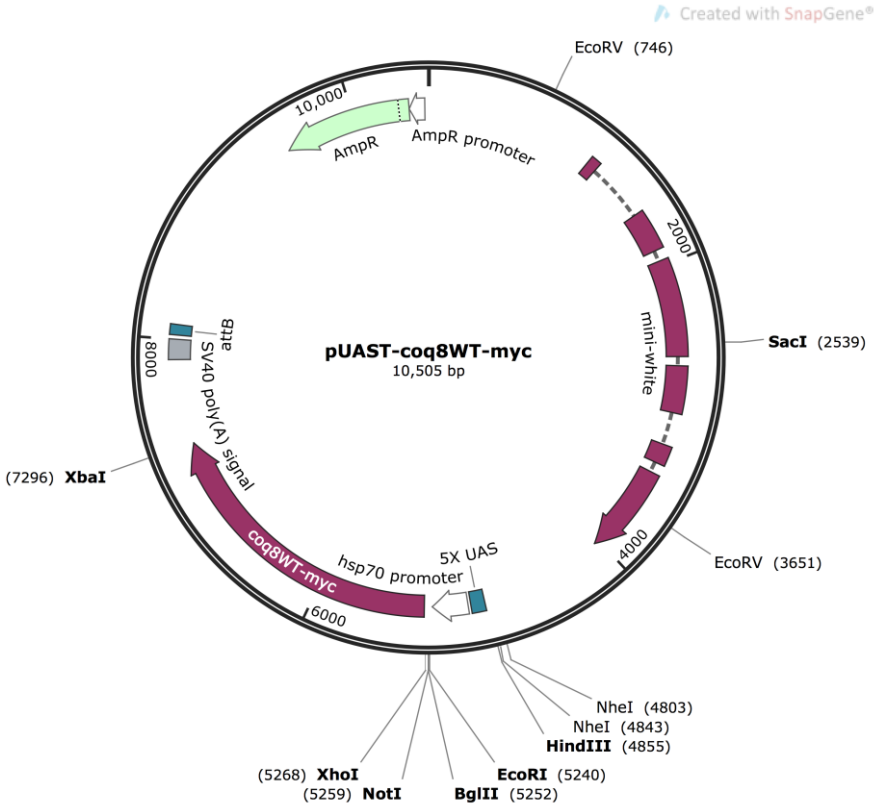

Figure 9.5 Physical map of pUAST-coq8WT-myc.

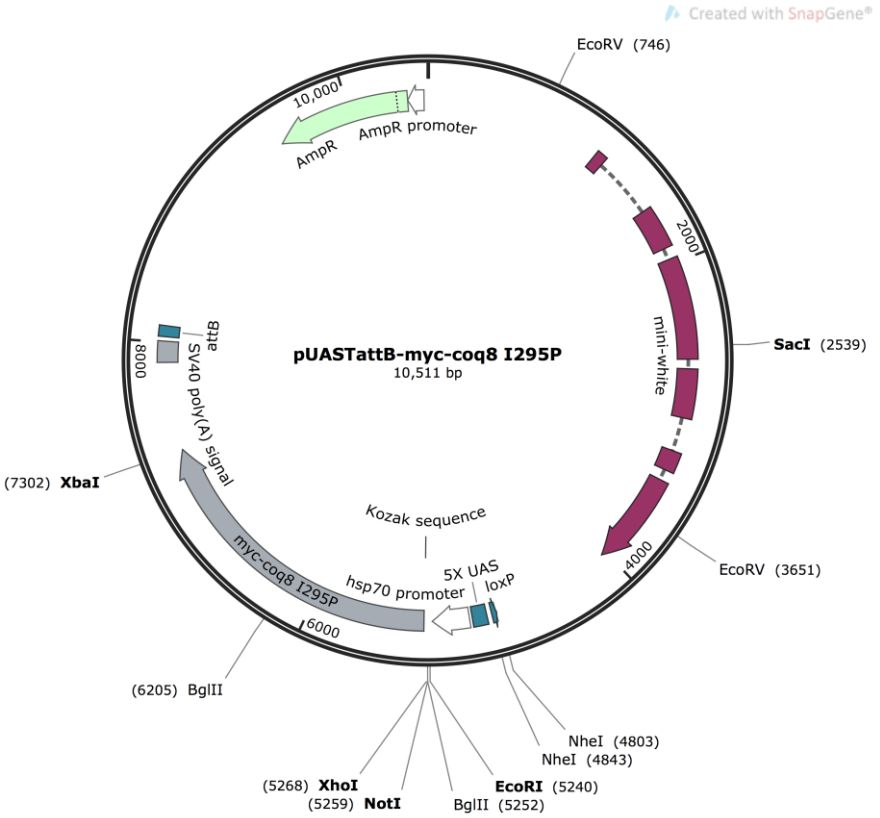

Figure 9.6 Physical map of pUASTattB-myc-coq8 I295P.

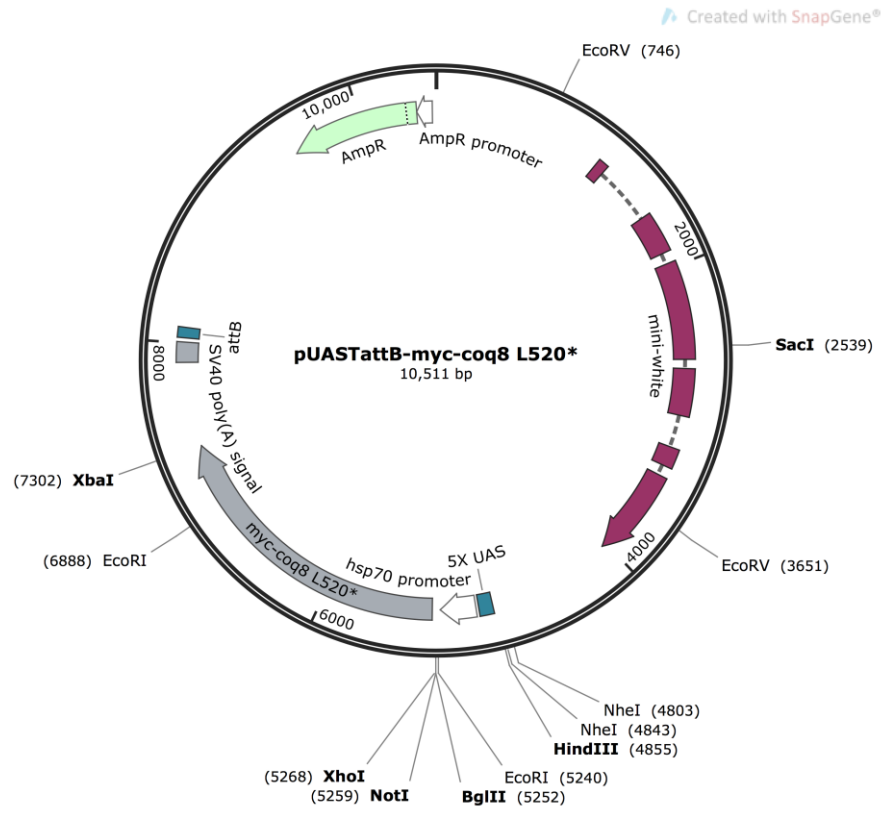

*Figure 9.7 Physical map of pUASTattB-myc-coq8 L520**

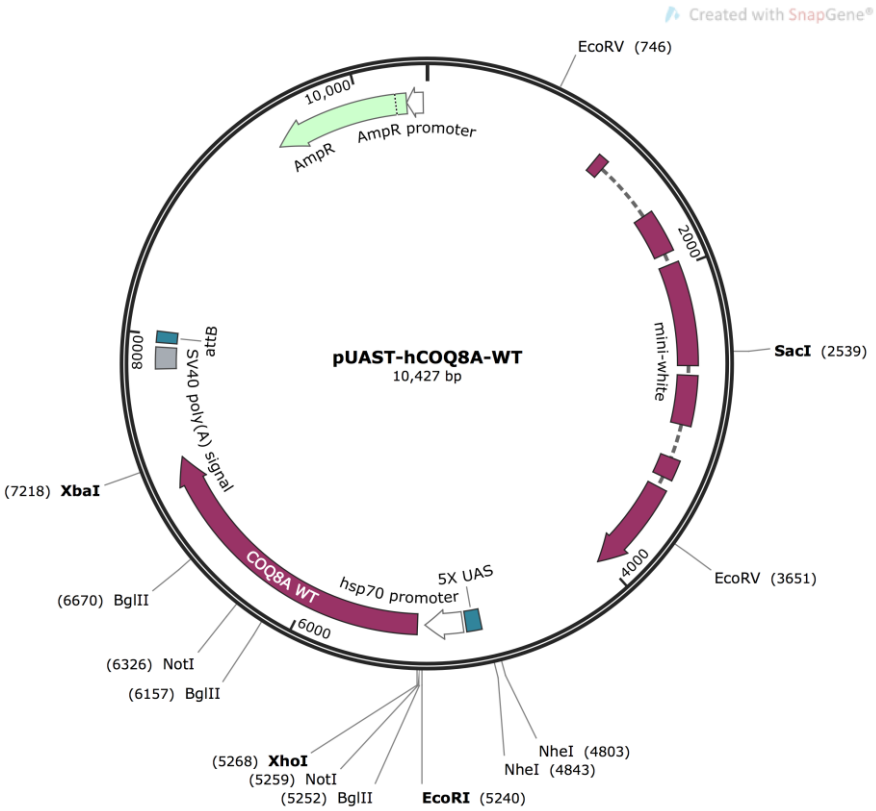

Figure 9.8 Physical map of pUAST-hCOQ8A-WT

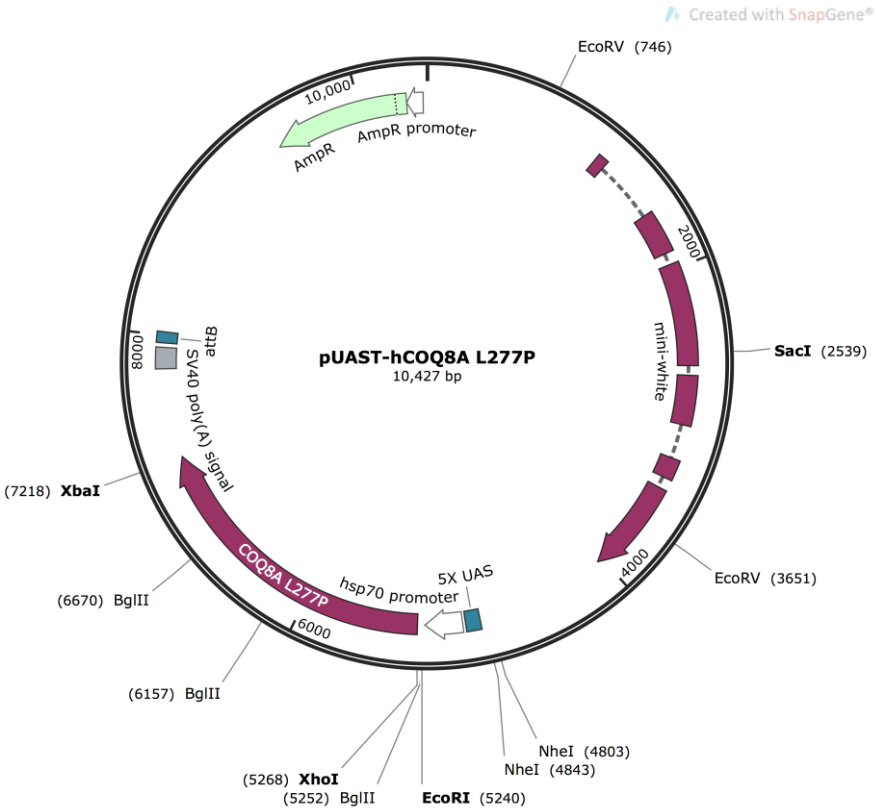

Figure 9.9 Physical map of pUAST-hCOQ8A L277P

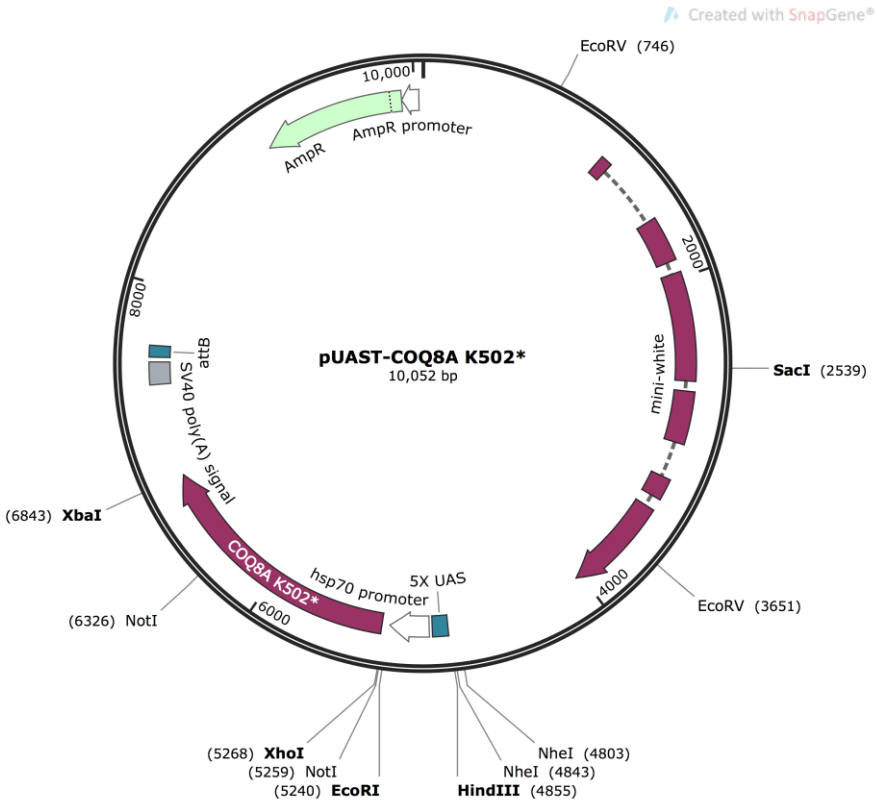

*Figure 9.10 Physical map of pUAST-COQ8A K502**

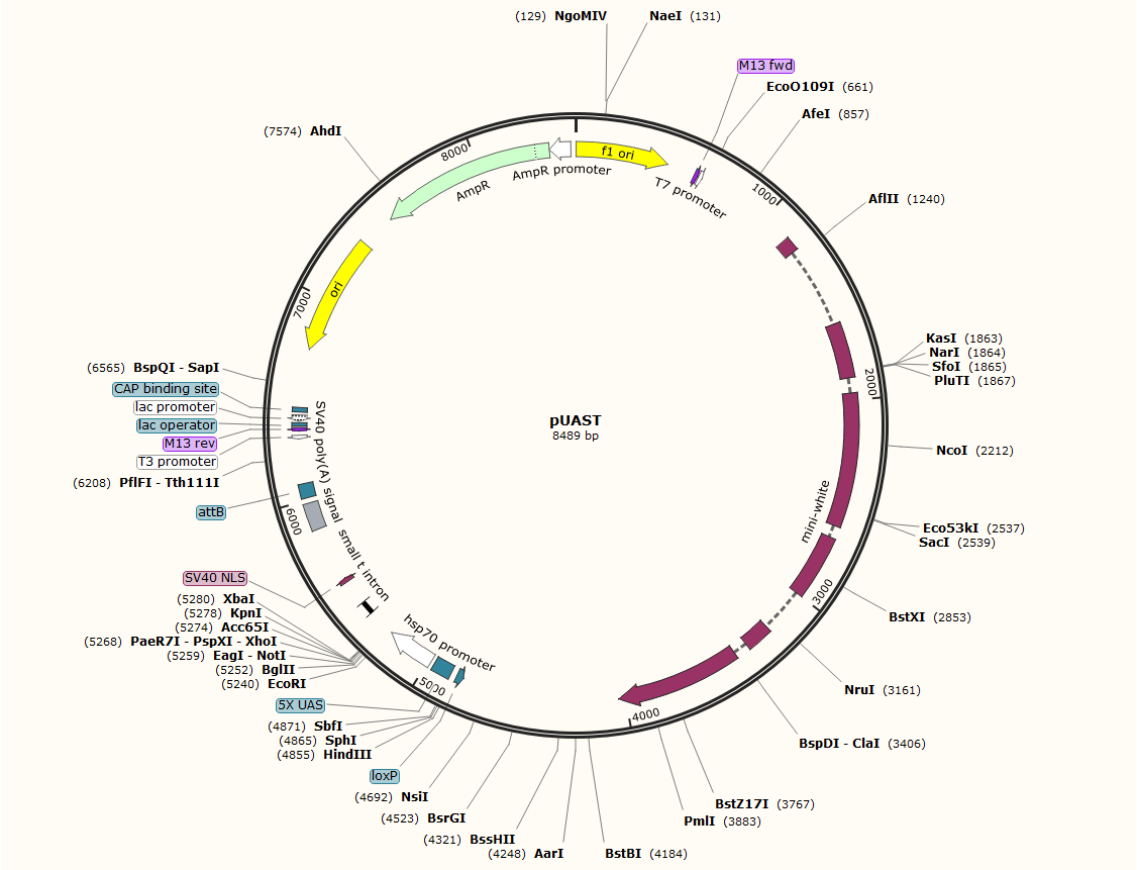

Figure 9.11. PUAST cloning vector. The map indicates the multiple cloning region (MCR) which is susceptible to restriction digest and insertion of transgene.