Investigation of the pathogenic agent in a *Drosophila* model of polyglutamine disease

A thesis submitted for the degree of Doctor of Philosophy, March 2006

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Chapter 4 – Investigation of alternative pathogenic pathways

Introduction

RNA pathogenesis ruled out – what is the pathogenic agent?

The results described in Chapter 3 provide evidence against RNA as the pathogenic agent in the polyglutamine diseases. One of the features of the RNA hypothesis is that it provides a unifying mechanism for the translated and untranslated repeat diseases, since in all cases the expanded repeat is present in the mRNA transcript. However, this is not their only common feature: the expanded repeat is also present in the DNA associated with these diseases. Therefore a pathogenic mechanism based around the effect of the expansion at the DNA level could also account for the common degenerative phenotypes observed in the translated and untranslated repeat diseases. Evidence exists in support of such a mechanism and thus investigation of a role in pathogenesis is warranted. The *Drosophila* model of repeat disease described in Chapter 3 provides an ideal system in which to investigate potential pathogenic pathways. A candidate gene approach can be used to identify genes, which upon removal of one copy, enhance or suppress the expanded repeat eye phenotype, suggesting the involvement in repeat pathogenesis of the pathway in which that gene product acts. This system was used to examine the possibility of pathogenesis at the DNA level. A recently proposed pathogenic pathway caused by blockage of axonal processes by cytoplasmic polyglutamine aggregates was also examined using this method.

A. DNA damage hypotheses

Association between SCA and DNA repair

In addition to the dominant SCAs caused by repeat expansion, there are several autosomal recessive forms of SCA. One of these is Friedreich's ataxia, caused by GAA repeat expansion and subsequent loss of expression of *Frataxin*, which leads to increased oxidative stress due to a defect in iron homeostasis [138]. There are also three forms of SCA that have been ascribed to mutations in genes involved in DNA damage repair. These are:

- ❖ Spinocerebellar ataxia with axonal neuropathy (SCAN1), a neurodegenerative disorder characterised by adolescent-onset ataxia and peripheral neuropathy, which is also accompanied by cerebellar atrophy. SCAN1 is caused by mutations in the gene encoding tyrosyl-DNA phosphodiesterase (TDP1) [232]. The normal role of TDP1 is to repair covalent topoisomerase-DNA complexes, which is essential for preventing the formation of single and then double stranded breaks that result when stalled topoisomerase complexes interfere with DNA replication [233,234]. TDP1 is a ubiquitously expressed DNA repair protein, yet the resulting phenotype manifests in the nervous system as neurodegeneration, rather than genetic instability or cancer.

- ❖ Ataxia ocular-motor apraxia (AOA1), also characterised by cerebellar atrophy and axonal motor neuropathy. AOA1 is caused by mutations in *Aprataxin* [235,236], the precise function of which is unknown. There is evidence that it is involved in the single stranded DNA break repair pathway, as it interacts with X-ray repair cross-complementing group 1 (XRCC1), a scaffold protein with an essential role in single stranded break repair [237]. In addition, cells from AOA1 patients show increased sensitivity to agents that induce single stranded breaks [238]. Like TDP1, Aprataxin in ubiquitously expressed yet the disease phenotype is neurological.

- ❖ Ataxia telangiectasia (AT), which shows a similar phenotype to AOA1, characterised by severe ataxia showing rapid progression, but also has additional extraneurological features including predisposition to cancer, telangiectases (dilated blood vessels) in the eyes and immune deficiency. It results from mutation in *ataxia telangiectasia mutated* (*ATM*), encoding a protein kinase involved in the response to double stranded breaks induced by ionising radiation [239]. Downstream targets of ATM include p53 and the histone 2A variant H2AX, the phosphorylation of which is an early response to double stranded breaks [240].

In addition to these, there are two other disorders resulting from mutations to the DNA repair pathway that feature prominent neurodegeneration but are also characterised by other multisystemic features. These are xeroderma pigmentosum (XP) and Cockayne syndrome (CS), both of which are caused by defects in nucleotide excision repair (NER) [241,242]. So far, mutations in eight different genes

involved in NER have been shown to cause XP, which is characterised by extreme sensitivity to UV light leading to skin cancers, as well as cortical and cerebellar degeneration in 20% of cases [241]. CS is caused by mutation in either of two genes encoding components of complexes that assist RNA polymerase II in dealing with transcription blocks. The features of CS are progressive neurodegeneration and developmental defects including severe physical and mental retardation, long limbs, pigmented retinopathy and gait defects [242].

The existence of the disorders outlined above supports a link between DNA repair and neurodegeneration, in particular the examples of SCAN1 and AOA1 where mutations in DNA repair genes result in pathology that is restricted to neurons. Further support for such a link comes from analysis of *ligase IV* knockout mice. Ligase IV accomplishes the final ligation step during repair of double stranded breaks, and is also required during V(D)J recombination in the immune system [243]. Mouse knockouts for *ligase IV* are embryonic lethal due to neuronal death rather than immunological problems. This death is caused by an increase in apoptosis of newly generated postmitotic neurons [244], which has been shown to be dependent on ATM [245] and p53 [246].

Together, these findings demonstrate that DNA repair is indispensable for neurons, both during neurogenesis and in later stages of terminal differentiation. Furthermore, it seems that defects in the response to, or repair of, DNA damage have a significant impact on neurons. It is unclear why this is so, partially because the DNA damage processing and repair mechanisms in neurons are not well understood [247]. Possibilities that have been suggested [248] are that neurons have a lower threshold for DNA damage, or that they sustain an intrinsically higher level of damage, possibly from reactive oxygen species generated at higher levels due to their high metabolic rate. Alternatively, the nervous system may be more sensitive to apoptosis following DNA damage than other tissues. There is some evidence to support each of these suggestions. Neurons do not proliferate and, if lost due to apoptosis, are not replaced by precursors, except for quite specific examples in localised regions of the brain. Also, there is evidence that DNA repair in terminally differentiated cells such as neurons does not occur efficiently due to attenuation of both NER and base excision repair [249]. However, a process known as transcription-coupled repair still functions to repair damage in expressed genes, which is detected when it blocks the RNA polymerase complex during transcription [250]. In addition, there is some evidence

that the non-transcribed strand of expressed genes is also repaired in neurons, in a process termed differentiation-associated repair [249]. This means that while mutations in the bulk of the genome build up, the integrity of active genes required by the neuron is maintained. This includes the non-transcribed strand so that it can be used as a template to correct damage to the transcribed strand. The fact that the majority of the genome accumulates damage may be related to the sensitivity of neurons to loss of certain components of the DNA repair pathway. In addition, DNA damage-induced death in terminally differentiated neurons is associated with re-entry into the cell cycle, whereas death induced by non genotoxic agents is not [251]. Increased levels of cell cycle proteins in neurons have been noted in many neurodegenerative disorders [252]. Thus in the expanded repeat disorders, activation of the DNA damage checkpoint by the presence of hairpin structures formed by the repeat tract may prompt cell cycle re-entry and subsequent apoptosis due to accumulated DNA damage which has mostly remained unrepaired in the genome of the terminally differentiated neuron.

Hypothesis 1: CAG expansions cause DNA damage

Long repeat tracts are known to be sites of DNA instability: AT-rich microsatellite repeats and CCG repeats are found at fragile sites, where they cause breaks to appear in chromosomes under certain conditions [253]. Long CAG and CTG repeats also display such instability: when present in yeast, these repeat tracts are sites of chromosome breakage. This breakage occurs in a repeat length-dependent manner, and is increased in the presence of mutation of *Rad2* (mammalian *Check1*), a DNA repair gene [254]. Mutations in replication proteins also increase breakage, suggesting that the fragility of repeat tracts is likely to be related to their propensity to form a hairpin secondary structure when the DNA is single stranded during transcription, repair or replication [255]. Indeed, CAG repeat tracts cause pausing at the replication fork in yeast and *E. coli* [256,257], and stalled replication forks are known to be sites of DNA breakage [258]. Furthermore, the presence of expanded CAG repeats in yeast activates the DNA damage checkpoint pathway, with components of the checkpoint machinery (*Mec1* (*mei41*), *Rad9*, *Rad53* (*Check2*)) playing an active role in maintenance of chromosomal integrity and stability of CAG repeats [259]. This evidence suggests that DNA damage occurs at the site of expanded repeats, stimulating the DNA damage response.

The evidence that expanded repeats induce DNA damage, considered in combination with the link between DNA repair and neurodegeneration established by SCAN1 and AOA1, suggests a mechanism of expanded repeat pathogenesis occurring at the DNA level, whereby the presence of an expanded repeat leads to DNA damage, to which neurons are particularly sensitive. DNA damage as a cause of neurodegeneration in expanded repeat diseases has also been proposed by others, and has been suggested to be "responsible for a portion of the neuronal death that occurs in repeat expansion diseases such as HD and the spinocerebellar ataxias. A similar mechanism could also operate in differentiated muscle cells affected in DM." [260].

Hypothesis 2: Polyglutamine proteins induce DNA damage

In addition to a pathogenic pathway caused by breakage at the repeat tract, DNA damage has been implicated in polyglutamine disease via a different mechanism. The expression of proteins containing an expanded polyglutamine repeat induced an increased sensitivity to stress-induced cell death in cultured cells. This sensitivity did not occur without expression of the polyglutamine protein, suggesting it is not caused by the presence of the repeat expansion in the DNA [261]. In these cells, the ATM-dependent DNA damage response was activated; this is also the case in fibroblasts from HD and SCA-2 patients [261]. Expanded polyglutamine containing proteins have also been shown to cause an increase in levels of reactive oxygen species [262]. Therefore an increase in DNA damage caused by the presence of expanded polyglutamine proteins, rather than the presence of the expanded repeat in the DNA, may be responsible for the increased susceptibility of neurons to apoptosis in the polyglutamine disorders. This mechanism has been proposed to account for the similarity in phenotypic outcome (i.e. spinocerebellar ataxia) caused by repeat expansion or mutations to components of the DNA damage pathway [261]. However, it cannot account for the diseases caused by untranslated repeats, where no polyglutamine protein is present yet neurodegeneration still occurs. According to this hypothesis, either DNA repair is not involved in these cases, or it is implicated via a different mechanism.

An important factor in analysis of the DNA damage hypotheses is whether they can account for the equal toxicity of CAG and CAA repeats observed in Chapter 3. The second mechanism involving damage induced by polyglutamine would predict equal toxicity of the two repeat tracts, as pathogenesis occurs at the protein level. In the

case of the first hypothesis, where the DNA damage occurs due to the presence of the expanded repeat in the genome, it is less clear. The toxicity of CAA in this case would depend on how the repeat expansions cause breakage, and whether this is related to the formation of a hairpin structure by the DNA. CAA repeats are not predicted to form a hairpin secondary structure as outlined in Chapter 3. Therefore, if the DNA damage is induced by the presence of a hairpin, CAA repeats would be predicted to be less toxic than CAG according to the DNA breakage hypothesis. However, in the case of Friedreich's ataxia, the expanded GAA repeat tract in the *frataxin* gene has been shown to form triplex structures [263] and act as a hot-spot for recombination [264]. This suggests that long GAA repeats, like CAG repeats, may be prone to DNA damage. Thus it is conceivable that CAA repeats could act in a similar manner, which may account for the phenotype resulting from expression of CAA if DNA breakage is involved in the pathogenic pathway.

Other observations that cannot be accounted for by the first DNA damage hypothesis include the recessive mode of inheritance of Friedreich's ataxia, and the lack of pathogenesis induced by an untranslated CAG repeat in the *Drosophila* model described in Chapter 3. In the case of Friedreich's ataxia, if this disease were caused by the same pathogenic pathway as the other repeat diseases and the CAA-induced pathogenesis in *Drosophila*, the same mode of inheritance as these diseases would be predicted (i.e. dominant). Also, according to the first hypothesis, a transcribed but untranslated CAG repeat would be expected to exhibit the same level of toxicity as a translated CAG repeat, as both repeats are present in the DNA and would be sites of DNA damage. However, these observations are accounted for by the second DNA damage hypothesis, where expanded polyglutamine proteins induce DNA damage. No toxicity would be predicted for an untranslated CAG repeat or an untranslated GAA repeat, and therefore Friedreich's ataxia would be predicted to be recessive, caused by loss of function of *Frataxin*. Therefore, whilst there is some evidence against each of the DNA damage hypotheses, there is also a body of evidence in support of each of them; thus they were further investigated.

Investigation of DNA damage hypotheses
To investigate the DNA damage hypotheses, experiments were undertaken to examine whether reducing the activity of genes involved in recognising or repairing DNA damage can modify the expanded repeat eye phenotype in *Drosophila*. A number of genes known to be involved in the DNA repair pathway were examined.

These were chosen based on the existence of a known ortholog in *Drosophila* and the public availability of a fly stock carrying a deficiency spanning this gene, targeted mutation disrupting it, or a P-element insertion. These flies and the corresponding genes are shown in Table 4.1.

Gene	Allele tested	Normal function (human disease)	Stock No.
Check1 (grapes)	*grapes[1]*	Regulates entry into mitosis following DNA damage	-
TDP1	*Df(2L)S2590*	tyrosyl phosphodiesterase, removes topoisomerase 1 covalently bound to DNA in stalled complexes (SCAN1)	4954
aprataxin (hint)	*P[265]CG2862[KG00798]*	Interacts with XRCC1 and p53 in DNA single stranded break repair (AOA1)	12893
aprataxin (hint)	*Df(2L)C144*		90
aprataxin and TDP1	*Df(2L)JS31*		1581
XRCC1	*Df(1)JC70*	Facilitates repair of single stranded breaks through interactions with repair proteins such as DNA ligase and polymerase	944
p53	*p53[5A-1-4]*	Responds to DNA damage by initiating repair or apoptosis	6815
H2AX (H2AvD)	*His2AvD[810]*	Histone variant phosphorylated in response to DNA breakage	-
ligase IV	*P[266]Lig4[EP385]*	Performs ligation step in repair of double stranded breaks	10116

Table 4.1 Mutations in DNA repair genes tested for modification of the polyglutamine eye phenotype. The relevant gene, allele used, normal function of the gene, and whether the gene is implicated in human disease are shown, as well as the Bloomington stock number (where relevant). Under the 'gene' column, the *Drosophila* ortholog is shown in brackets where applicable i.e. *hint* [267], *grapes* [268] and *histone 2A variant Drosophila* (*H2AvD*) [269]. For *ligase IV*, the P-element insertion site is 38 bp upstream of the ATG start codon; homozygous flies are viable, as are flies homozygous for a *ligase IV* deletion [270]. The *p53* allele is a 3.3 kb deletion in the coding region of *p53* [271]. The *grapes* and *H2AvD* alleles are both null alleles obtained from R. Saint [268] [272]. The P-element insertion in *hint* is viable. Other deficiencies map to the location of the gene of interest, but have not previously been characterised.

The rationale behind these experiments is that if DNA repair is involved in pathogenesis, either because expanded polyglutamine proteins cause DNA damage or because the expanded repeats in the genome are sites of DNA damage, then reducing the dose of a gene involved in sensing or repairing this damage by half may make pathogenesis worse. This would be seen as an enhancement of the expanded repeat eye phenotype. Modification of expanded repeat eye phenotypes in *Drosophila* in this way has been described previously. Removal of one copy of genes putatively involved in pathogenesis, such as molecular chaperones or components of the proteasome, resulted in enhancement of the polyglutamine eye phenotype [64]. In addition, this method has previously been used in genetic screens to identify novel modifiers of polyglutamine eye phenotypes in *Drosophila*. One of these screens used the ataxin-1 eye phenotype, and 1500 lethal P-element insertions, as well as 3000 enhancer/promoter (EP) lines overexpressing various genes due to the random

insertion of UAS sites, were screened to identify modifiers [64]. In another experiment, the eye phenotype used for the screen was caused by expression of a Q127 tract alone in the eye, and 7000 EP lines were screened [86]. Both screens identified a large number of modifiers. The Q127 screen identified 30 lines that suppressed the polyglutamine-dependent eye degeneration and 29 lines that enhanced it; except for two genes with similarity to *Hsp70*, the identity of these modifiers was not reported. The ataxin-1 screen identified seven P-element insertions that suppressed and 20 that enhanced the SCA1 eye phenotype; the EP screen identified 10 suppressors and 23 enhancers. The genes identified had various roles in the cell, including in the protein folding/heat-shock response pathway, transcriptional regulation, RNA binding and cellular detoxification [64].
None of the genes identified correspond to those in Table 4.1, and DNA repair was not listed as an area identified by the screen; however, it was not exhaustive of all known genes in the genome, and critical DNA repair genes may have been omitted.

B. Axon transport hypothesis

Polyglutamine disease and defects in axon transport
Recently, a new pathogenic pathway has been proposed for the polyglutamine diseases, whereby the presence of protein containing an expanded polyglutamine tract causes axonal blockage and defects in axon transport. Axon transport is a vital process for neurons, as newly synthesised proteins and anterograde signals must be carried down the axon to the synapse, sometimes a long distance, and retrograde signals and material for degradation must travel back the opposite way. Indeed, motor proteins such as kinesin and dynein have crucial roles in neurons, as well as essential roles in other cell types, and are required for development of the nervous system [273]. A mutation in kinesin has been linked to autosomal dominant hereditary spastic paraplegia type 10, a disease characterised by axonal degeneration of motor and sensory neurons [274]. Charcot-Marie-Tooth disease type 2A, characterised by progressive weakness and atrophy of muscles caused by axonal degeneration, is caused by a loss of function of the neuronal kinesin KIF1B [275]. Mutations in dynein have also been linked with motor neuron degeneration [276]. These disorders demonstrate a requirement for axon transport in the nervous system.

As well as nuclear aggregates, expanded polyglutamine proteins have been shown to form aggregates in the cytoplasm. The presence of cytoplasmic aggregates was hypothesised to perturb axon transport by causing a physical blockage of the axonal process, preventing the movement of motor proteins along the microtubules and ultimately leading to neuronal dysfunction and death [277]. Experimental evidence in support of this has been obtained in multiple systems including *Drosophila*, cultured cells and isolated squid giant axons.

The first evidence of disrupted axon transport came from immortalised motoneuronal cells expressing the androgen receptor containing an expanded polyglutamine tract. Neuronal processes containing androgen receptor aggregates showed altered mitochondrial distribution corresponding with axonal swelling, and the distribution of kinesin was also altered [277]. In *Drosophila*, expression of human huntingtin exon 1 with an expanded polyglutamine tract led to organelle accumulations in larval neurons, which are characteristic of defects in axon transport. Accumulations were enhanced by a 50% reduction in kinesin heavy chain, and this reduction also enhanced neuronal apoptosis. Organelle accumulations and neuronal cell death were also seen in flies expressing ataxin-3 with an expanded polyglutamine tract, or a protein consisting of an expanded polyglutamine tract alone, implying that this effect is not specific to Huntingtin. Components of the axon transport machinery, such as kinesin light and heavy chains, dynein light and heavy chains and the dynactin complex component p150^{Glued} were reduced in level and showed colocalisation with polyglutamine aggregates, suggesting they are titrated away from their normal cargo by sequestration into the aggregates. These effects on the axon transport process and machinery were not observed when the polyglutamine proteins were targeted to the nucleus, suggesting that this represents a pathogenic pathway which is separate to, and may act in parallel with, nuclear toxicity [278].

Evidence supporting the axon transport hypothesis has also been gained from observing the effect of expanded polyglutamine proteins on transport in squid giant axons. In isolated axoplasm, both retrograde and anterograde transport was inhibited by Huntingtin or androgen receptor containing an expanded polyglutamine tract. However, this occurred without the presence of detectable aggregates, suggesting a direct action of the polyglutamine proteins on the transport machinery, rather than a physical blockage [279].

Thus there is considerable evidence from model systems that perturbation to axon transport results from the presence of expanded polyglutamine proteins. Such a perturbation seems likely to contribute to, if not cause, neuronal death, as mutations in components of the axon transport pathway have been shown to lead to neurodegeneration. Furthermore, a pathogenic mechanism based on defective axonal transport has been proposed to account for the major features of polyglutamine disease, such as toxic gain of function, preferential loss of neurons, and late onset of disease [279]. Expression of expanded polyglutamine proteins may compromise transport in neuronal processes such that it is reduced below normal levels. When this is combined with the decline in axonal transport that occurs due to the ageing process [280], transport may fall below the required minimum level, leading to death of neurons. The large size of neurons and their shape and organisation renders them particularly dependent on axonal transport; this is demonstrated by the rapid and severe effect that toxins targeting the transport machinery have on neurons [281]. Also, this mechanism accounts for the dominant toxic effect exerted by the expanded polyglutamine proteins, and disease-specific differences may be due to differences in the expression levels of the relevant proteins in different populations of neurons, or cell specific differences in other factors such as neurotrophins. Thus, whilst the axon transport hypothesis does not explain the diseases caused by untranslated repeats and therefore does not provide a unifying mechanism, it can account for many features of the polyglutamine diseases.

Investigation of axon transport hypothesis using the Drosophila eye

To investigate the axon transport hypothesis in the *Drosophila* model system, a candidate gene approach was again utilised to examine whether mutations that reduce the level of axon transport components by half can enhance the polyglutamine eye phenotype. This method has been used previously to show that a reduction in the level of kinesin heavy chain can enhance the organelle accumulation phenotype in *Drosophila* larval nerves caused by expression of Huntingtin containing an expanded polyglutamine domain. However, reduced levels of dynein light chain failed to show such an effect. Using larval death as an assay, a deficiency spanning *p150^glued* and a P-element insertion in *dynamatin* were shown to enhance the early larval lethality caused by neuronal expression of polyglutamine [278]. Therefore these alleles were tested for modification of the polyglutamine eye phenotype, along with other mutations in cellular transport components shown in Table 4.2.

Gene	Allele tested	Normal function	Stock No.
kinesin heavy chain	*KHC[8]* *	subunit of kinesin anterograde motor, contains motor domain and microtubule binding domain	1607
kinesin light chain	*Df(3L)8ex[25]*	subunit of kinesin, mediates binding to cargo	5090
dynein heavy chain	*Df(3L)GN24**	subunit of dynein retrograde motor, contains motor domain and microtubule binding domain	3686
roadblock	*Df(2R)robl-c*	cytoplasmic dynein light chain	5680
p150[glued]	*Df(3L)fz-GF3b**	component of dynactin (dynein activator complex) required for cytoplasmic dynein activity, binds to microtubules and dynein	3124
p150[glued]	*P{SUPor-P}Gl[kgo7739]*		14651
dynamitin	*P{w[+mc]=lacW} Dmn[k16109]* *	component of dynactin, links structural domains of dynactin	11159

Table 4.2 Alleles of axon transport pathway components tested for enhancement of polyglutamine eye phenotype. The allele, corresponding gene, normal function of the gene product and Bloomington stock number for that allele are shown. *KHC[8]* is a null allele of *kinesin heavy chain* [282]. The *robl-c* deficiency has been confirmed using molecular methods [283]. The P-element insertion in dynamitin is recessive lethal. Alleles marked with an asterisk have previously been shown to enhance polyglutamine phenotypes in *Drosophila* [278]. For other references describing the normal roles of these proteins in axon transport, see [284], [285] and [283].

In addition to demonstrating that axon transport mutations can enhance polyglutamine phenotypes in *Drosophila*, the previous study by Gunawardena *et al*. demonstrated that axon transport defects caused by polyglutamine proteins are specific to cytoplasmic aggregations, and are not induced by polyglutamine when it is targeted to the nucleus [278]. This suggests that the neuronal death induced by nuclear polyglutamine proteins occurs via an alternative mechanism that does not involve axon transport defects. Gunawardena *et al*. also demonstrated that expression of cytoplasmic polyglutamine can induce an eye phenotype in *Drosophila*. This implies that a non-nuclear pathway of pathogenesis occur in the *Drosophila* eye. This pathway may involve axon transport defects; there is some evidence in support of this, as the photoreceptor neurons that make up the ommatidium of the eye project axons back through the optic stalk into the lamina and medulla of the optic lobe of the brain. During development of the visual system, Huntingtin aggregates are transported along these axons and accumulate in the growth cones of the axons [286]. Thus the eye appears to be a suitable system to investigate axon transport defects and their contribution to polyglutamine pathogenesis in *Drosophila*.

Results

In order to test the DNA repair and axon transport hypotheses, a recombinant chromosome was generated carrying both *GMR-GAL4* and the UAS-repeat

construct. Flies carrying this chromosome were then crossed to stocks carrying the various mutations in the DNA repair or axon transport pathways. Progeny expressing the repeat and carrying one copy of the mutation were then examined, and their phenotype compared to flies expressing the repeat in a wild-type background.

A. No support for the involvement of the DNA repair pathway in pathogenesis

According to either of the DNA damage hypotheses, the involvement of the DNA damage response and repair mechanism in pathogenesis leads to the prediction that loss of one copy of a gene involved in this process would enhance the phenotype resulting from expression of polyglutamine in the *Drosophila* eye. To test this, flies expressing polyglutamine encoded by $(CAA)_{94}$ with a phenotype in the moderate-severe range were tested for modification by the loss of one copy of each of the genes shown in Table 4.1. This line shows approximately equal severity to the line used to screen for modifiers of the Q127 eye phenotype [86] and was chosen to test both the first and second DNA repair hypotheses, involving DNA breakage or polyglutamine-induced DNA damage respectively. The CAA repeat was chosen over the CAG repeat construct as CAA shows equal toxicity to CAG; therefore any hypothesis involving the nature of the pathogenic pathway must be able to account for the toxicity of CAA, and so putative modifiers involved in the pathogenic pathway should modify phenotypes induced by either repeat. Comparison of flies expressing $(CAA)_{94}$ in the eye with those expressing $(CAA)_{94}$ in addition to a mutation in the DNA repair pathway revealed no difference in the severity of the eye phenotype (Figure 4.1). Instead, loss of one copy of each gene shown in Table 4.1 had no effect on the $(CAA)_{94}$ eye phenotype, with the severity of the phenotype remaining unaffected.

These findings do not support the involvement of the DNA repair pathway in pathogenesis. However, there are other explanations for these results. It is possible that the first DNA repair hypothesis is correct, and that pathogenesis is due to repeat fragility, but that this only occurs for CAG repeats, which have been shown to be sites of DNA fragility, but does not occur for CAA repeats. In this case, the phenotype observed upon expression of the CAA repeat might be due to a different pathogenic pathway. Alternatively, as the phenotype of the $(CAA)_{94}$ line in Figure 4.1 is quite severe and the DNA repair mutations are predicted to enhance rather than suppress the phenotype, it is possible that increases in severity would not be visible. This seems unlikely, as lines showing a more severe polyglutamine phenotype were
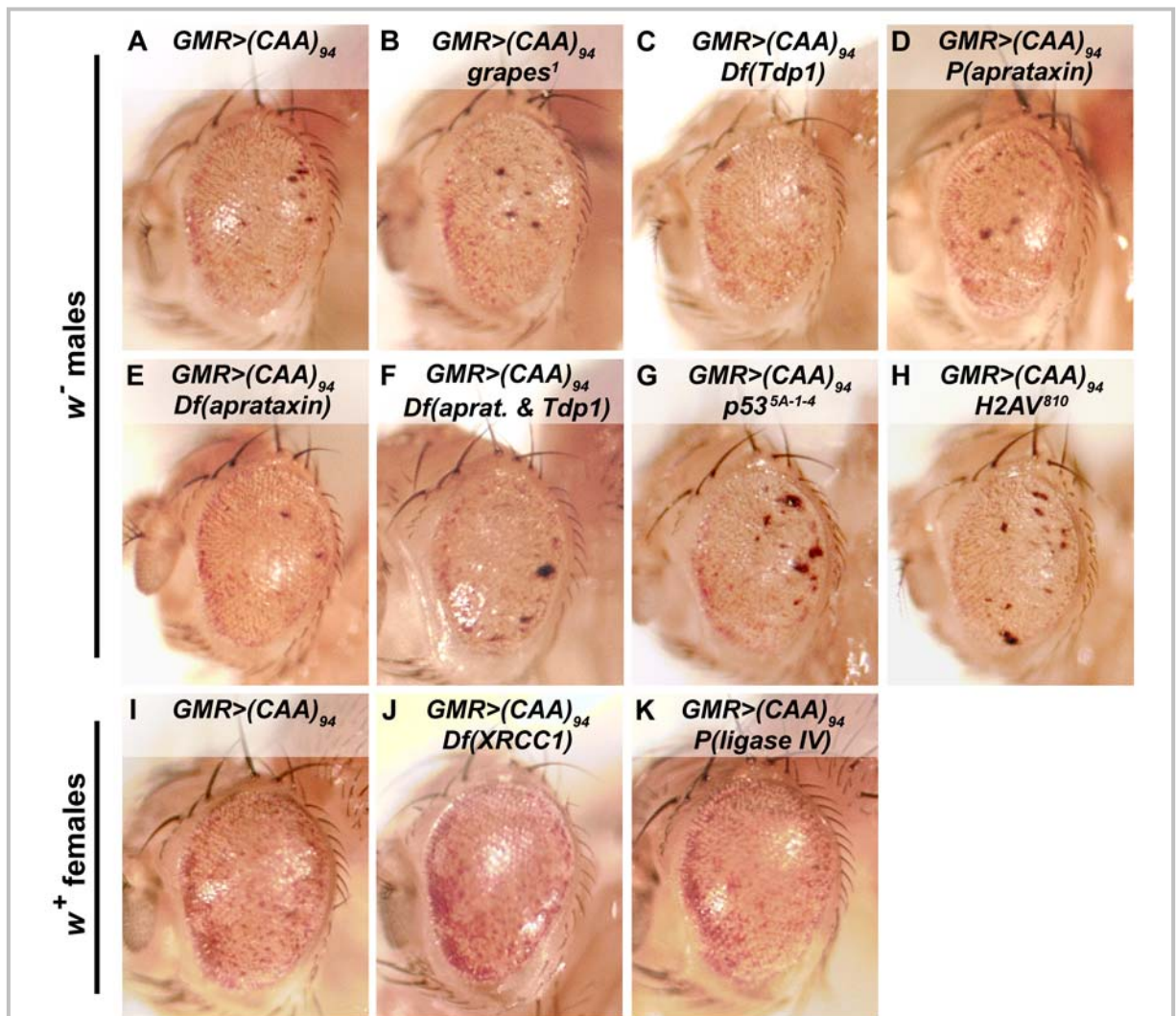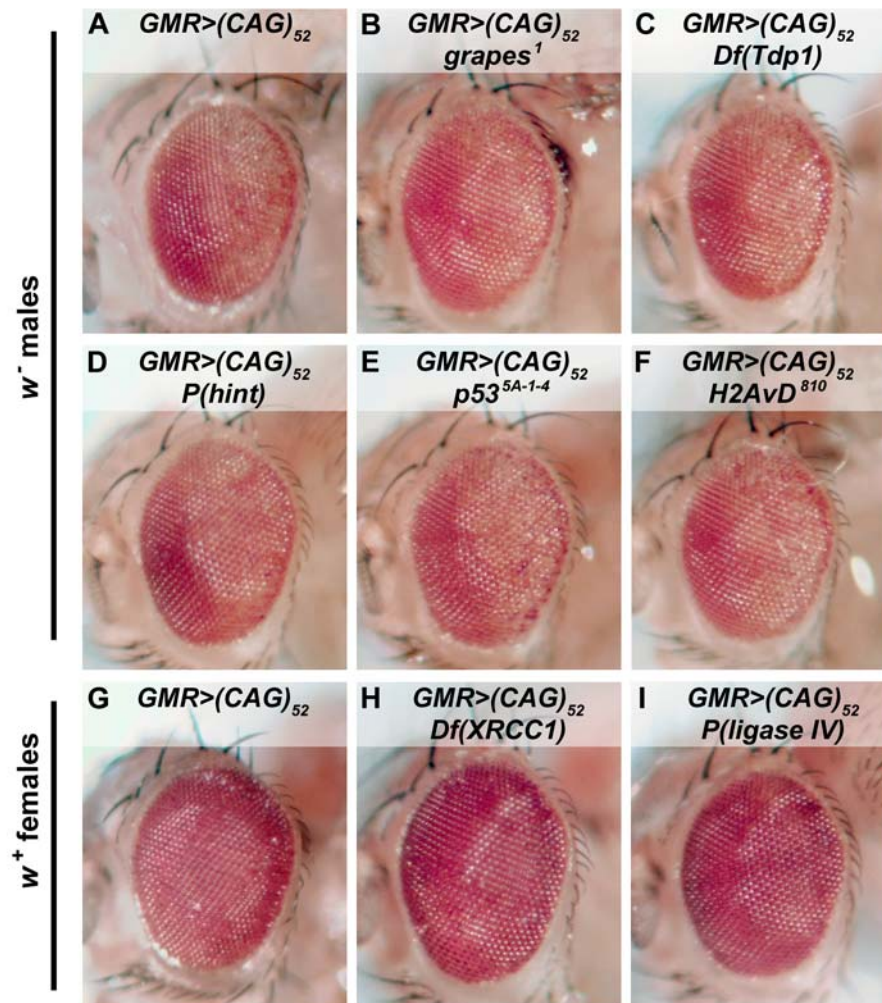
Figure 4.1 Mutation of genes involved in DNA repair does not visibly affect the polyglutamine eye phenotype. Flies from a moderate-severe line expressing polyglutamine encoded by $(CAA)_{94}$ driven by *GMR-GAL4* were crossed to lines with mutations in DNA repair genes as shown in Table 4.1. The eye phenotype of the resulting flies, expressing polyglutamine and heterozygous for the DNA repair mutation, was compared to flies expressing polyglutamine in a wild-type background. Most of the mutations were located on autosomes, and so the crosses were performed in a w^- background, and compared to w^- control flies (which contain pigment from a copy of *white* in the *GMR-GAL4* insertion). The control flies showed partial pigment loss throughout the eye, with more pigment remaining along the anterior edge of the eye. Dark patches of cells could be seen randomly scattered throughout each eye – these are presumably necrotic or dying cells (**A**). None of these mutations appeared to modify the polyglutamine phenotype, with flies carrying a DNA repair mutation (**B**-**H**) having eyes indistinguishable to that of the control, with pigment loss and dark patches. Two of the mutations examined, *Df(XRCC1)* and *P(ligase IV)*, were on the X chromosome and in a w^+ background. This necessitated the need for a w^+ control, as the presence of the endogenous *white* gene appears to increase the level of pigment in the eye (**A** vs **I**). Also, the location of the mutation on the X chromosome meant that females were examined in these cases. Again, the control eye showed pigment loss, with a stripe of pigment remaining in the anterior side (**I**). None of these mutations appeared to modify the polyglutamine phenotype, with the appearance of the resulting eyes (**J**,**K**) indistinguishable from the control.

generated (eg. severe phenotype shown in Figure 3.4), but it cannot be ruled out. To address both of these possibilities, a subset of the mutations were re-screened by crossing to flies expressing $(CAG)_{52}$. These flies demonstrated a milder eye phenotype, so that any enhancement should be easier to detect, and this also tested whether the lack of enhancement was due to use of a CAA rather than CAG repeat. However, no enhancement of the $(CAG)_{52}$ eye phenotype was caused by loss of expression of any of the DNA repair genes examined (Figure 4.2). Thus no evidence was obtained to support the involvement of the DNA repair pathway in pathogenesis.

It is possible that expression of the remaining wild-type allele of each gene could provide sufficient activity to sense and repair DNA damage in the fly. Therefore, while this pathway may be involved in pathogenesis, such an involvement is not detectable by removing only one copy of each gene. In order to address this possibility, further experiments could be done in which polyglutamine is expressed in the eye of flies homozygous for each mutation, removing all of the activity of each gene rather than half. This approach could be only be used for a subset of the mutations examined, as homozygous mutants for DNA repair genes can be frequently lethal (eg. *ligase IV* knockout mouse [287], and *Drosophila* homozygous for the *grapes[1]* allele [268]). However, in the expanded repeat disease state, the repeat expansions would not completely inactivate the DNA damage response, but rather the predicted pathogenic mechanism would be one where the response and/or repair mechanisms are overwhelmed. Such a mechanism would be expected to be sensitive to the levels of these critical DNA repair proteins, and so reducing the activity by half is predicted to have an effect on pathogenesis. Therefore, this explanation is unlikely to account for the lack of enhancement observed. Another possibility is that the phenotypes tested were not appropriate to reveal modification, perhaps because they were not sensitive to the removal of DNA repair genes, being either too mild or too severe. To test this, more lines with intermediate phenotypes could be used in the assay. However, it seems unlikely based on the results obtained so far that pathogenesis is caused by DNA damage at the site of the repeat, or by DNA damage induced by the presence of expanded polyglutamine.

Figure 4.2 Mutation of genes involved in DNA repair does not visibly affect a milder polyglutamine eye phenotype. A line showing a moderate phenotype expressing polyglutamine encoded by $(CAG)_{52}$ driven by *GMR-GAL4* was crossed to a subset of the lines with mutations in DNA repair genes shown in Table 4.1. The eye phenotype of the resulting flies, expressing polyglutamine and heterozygous for the DNA repair mutation, was compared to flies expressing polyglutamine in a wild-type background. In w^- control flies, the phenotype was visible as a loss of pigment in the posterior half of the eye (**A**). None of the mutations appeared to modify this phenotype, with the appearance of the eyes of flies carrying DNA repair mutations and expressing polyglutamine in a w^- background (**B-F**) indistinguishable from the w^- control expressing polyglutamine alone. Again, two of the mutations examined were on the X chromosome and in a w^+ background, necessitating the need for a control expressing polyglutamine in a w^+ background and examination of the phenotype in females. The eye phenotype of w^+ females expressing $(CAG)_{52}$ was milder than that of w^- males (**G** vs **A**), and is characterised by mild pigment loss all over the eye, especially in the posterior region. The introduced mutations in DNA repair genes did not alter the appearance of the eye phenotype (**H**,**I**) compared to the control (**G**).

B. No support the involvement of the axon transport pathway in pathogenesis

To investigate polyglutamine-induced axon transport defects using the *Drosophila* eye, flies carrying a chromosome expressing polyglutamine encoded by $(CAA)_{94}$, driven by *GMR-GAL4* were crossed to flies carrying the mutations shown in Table 4.2. As for the DNA repair hypotheses, progeny expressing polyglutamine in the eye and carrying a mutation causing a 50% reduction in an axon transport component were then compared to flies expressing polyglutamine alone. According to the axon transport hypothesis, enhancement of the eye phenotype would be the predicted outcome. Therefore the crosses were performed at 18 °C, which results in a milder phenotype due to a lower level of expression of the polyglutamine protein, as the activity of GAL4 in *Drosophila* is temperature-dependent [288]. However, despite this milder phenotype, which should make enhancement easier to detect, the eye phenotype in flies carrying the axon transport mutations was no more severe than in flies expressing polyglutamine alone (Figure 4.3). These results do not support the axon transport hypothesis, and suggest that this pathway is not contributing to polyglutamine pathogenesis in the *Drosophila* eye, at least in this model.
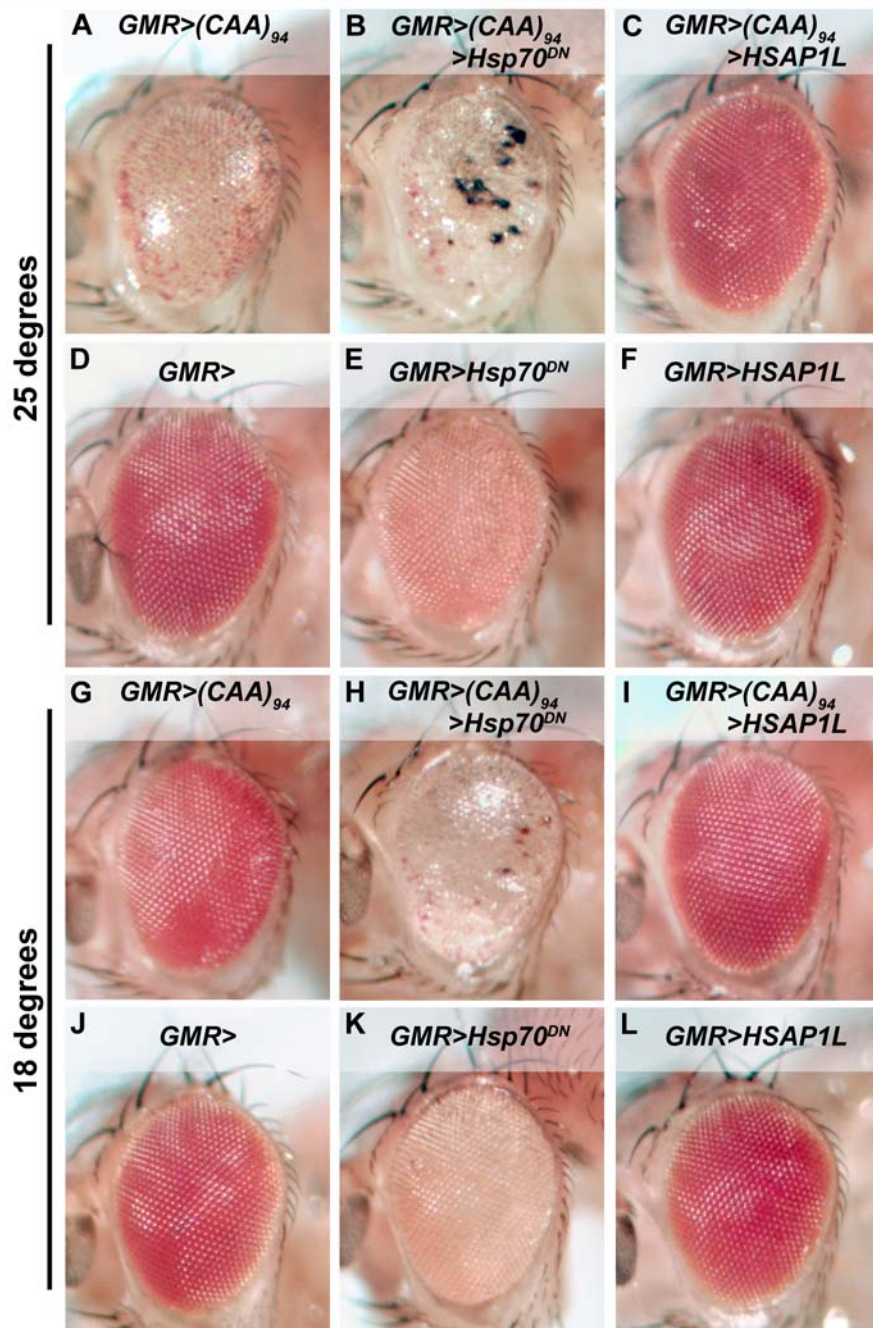
Can the polyglutamine eye phenotype be modified?

Since no enhancers of the polyglutamine phenotype were identified using the candidate gene approach, experiments were undertaken to determine whether this phenotype is sensitive to modification. Previous studies have used modification of polyglutamine eye phenotypes in *Drosophila* to identify interacting pathways [64,86]; however none of these used this specific polyglutamine construct. To investigate whether the eye phenotypes used to examine the DNA damage and axon transport hypotheses can be modified, two *Hsp70* constructs that have previously been shown to modify polyglutamine eye phenotypes in *Drosophila* were tested. Expression of the dominant-negative allele *HSC70.K71S* (referred to here as *Hsp70^{DN}*), containing a point mutation in the ATP binding domain [289], enhances the polyglutamine eye phenotype, while expression of wild-type human Hsp70 encoded by *HSPA1L* suppresses polyglutamine eye phenotypes [107,290,291]. In order to test whether expression of these Hsp70 constructs can modify the polyglutamine eye phenotypes used in this study, flies expressing these alleles under UAS control were crossed to the polyglutamine lines used to investigate the DNA repair and axon transport hypotheses. Both a mild phenotype resulting from polyglutamine expression at 18 °C and a severe phenotype caused by expression at 25 °C showed enhancement when
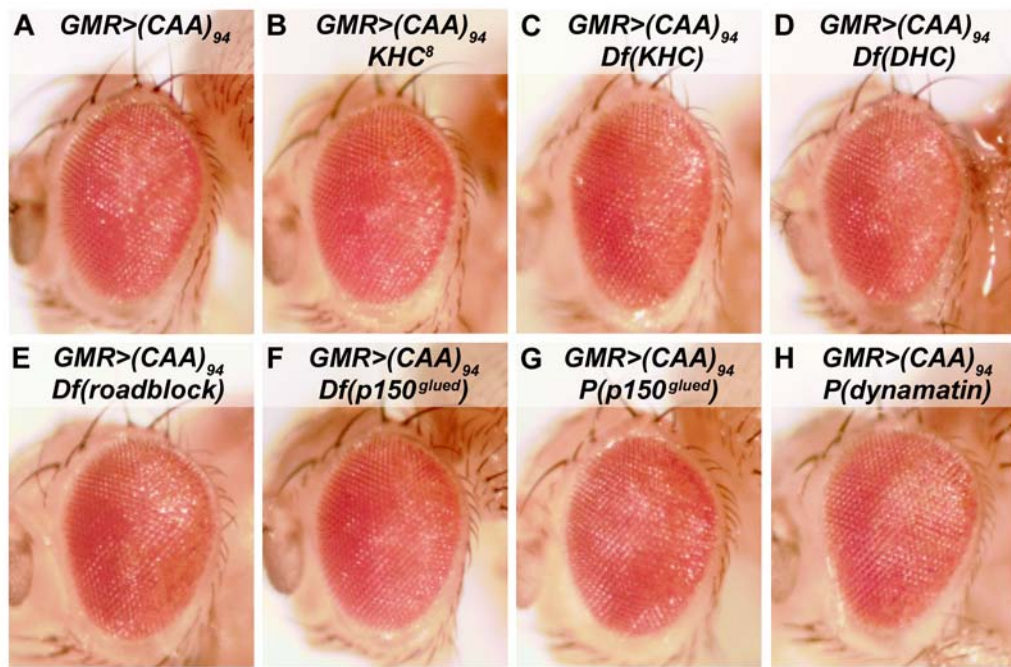
Figure 4.3 Mutation of genes involved in axon transport does not visibly affect the polyglutamine eye phenotype. A mild-moderate line expressing polyglutamine encoded by $(CAA)_{94}$ driven by *GMR-GAL4* was crossed to lines with mutations in axon transport genes as shown in Table 4.2. These crosses were performed at 18 °C to decrease the severity of the polyglutamine eye phenotype (at 25 °C this line gives the moderate-severe phenotype shown in Figure 4.1), and thus make any potential enhancement of the phenotype more apparent. Eye phenotypes of flies expressing polyglutamine and heterozygous for the axon transport mutations were compared to flies expressing polyglutamine in a wild-type background. The eye of the control flies expressing $(CAA)_{94}$ in a wild-type background shows pigment loss, making the eye paler in colour, which is more apparent in the posterior region of the eye (**A**). No enhancement of the polyglutamine eye phenotype was detected, with the eyes of flies carrying axon transport mutations (**B**-**H**) having an appearance indistinguishable from that of the control.
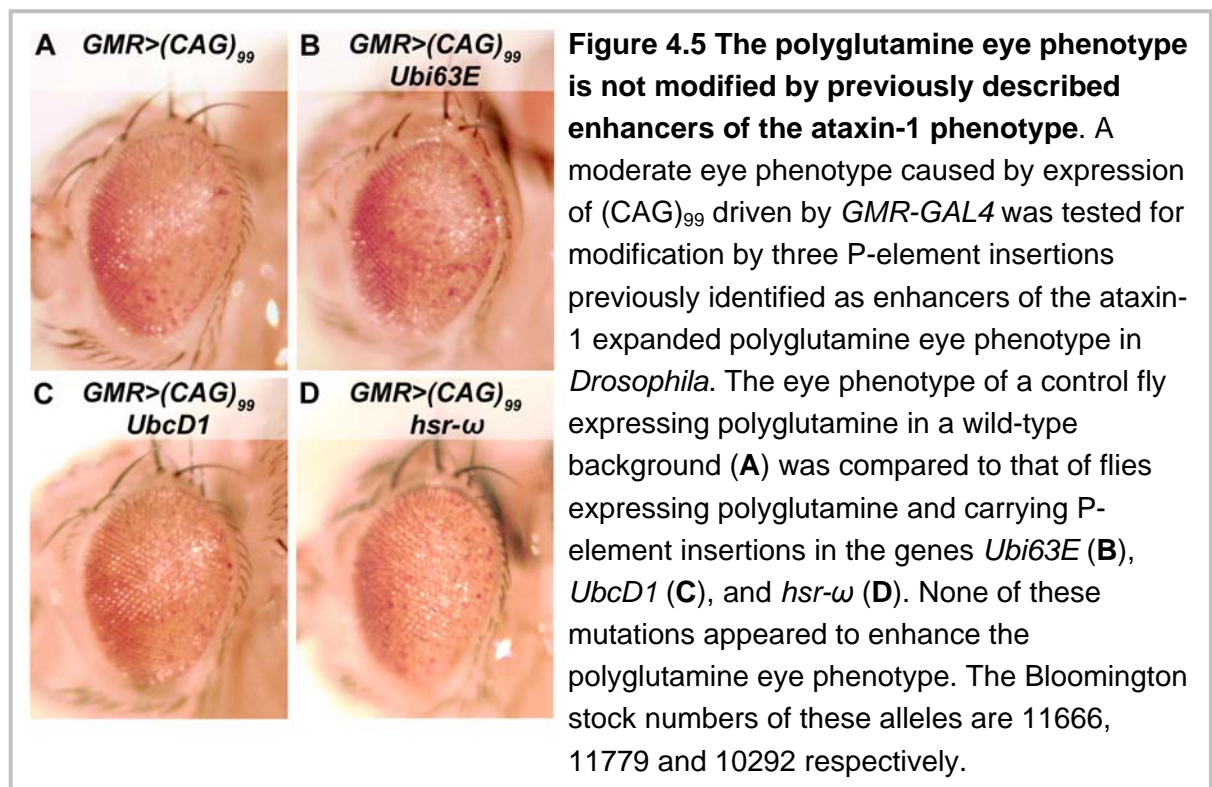
$Hsp70^{DN}$ was co-expressed, and suppression upon co-expression of *HSAP1L* (Figure 4.4). The *HSC70.K71S* allele has previously been reported to cause "no effect on the eye" [107] upon expression driven by GMR-GAL4; the control crosses performed here, using the same temperature, suggest that this is not the case, with a mild effect on the eye observed. However, expression of this allele causes a striking increase in severity of the polyglutamine phenotypes, which appears to be greater than the mild effect it has on the eye when expressed alone, suggesting that there is an interaction between polyglutamine toxicity and *Hsp70* as has been previously reported [107,291]. Thus it is possible to modify these polyglutamine phenotypes, both at the mild and severe end of the range, either by increasing or decreasing their severity.

Figure 4.4 Severe and mild polyglutamine eye phenotypes can be modified by manipulation of Hsp70 activity. Both the polyglutamine construct and the *Hsp70* wild-type and mutated constructs were downstream of UAS sites and expression in the eye was driven by *GMR-GAL4*. A relatively severe eye phenotype caused by expression of $(CAA)_{94}$ (**A**) is enhanced when a dominant negative allele of *Hsp70* (*HSC70.K71S*, referred to as $Hsp70^{DN}$) is also expressed in the eye. This enhancement is seen as a further loss of pigment and the appearance of black patches in the eye (**B**). In contrast, overexpression of human *HSAP1L* appears to completely suppress the polyglutamine eye phenotype (**C**). The *GMR-GAL4* driver alone (**D**) or *HSAP1L* driven by *GMR-GAL4* (**F**) has no discernable effect on the eye, while the expression of $Hsp70^{DN}$ causes a mild loss of pigment (**E**). These crosses were repeated at 18 °C, causing a milder polyglutamine phenotype (**G**), which was also enhanced by expression of $Hsp70^{DN}$ (**H**) and suppressed by *HSAP1L* (**I**). Again, the presence of the driver alone (**J**) or expression of *HSAP1L* (**L**) had no detectable effect on the eye; however, in contrast to the polyglutamine phenotype, expression of $Hsp70^{DN}$ had a slightly stronger effect on the eye at 18 °C (**K**) than at 25 °C; the reason for this is unclear.

To further test for modification of the polyglutamine phenotypes, enhancers identified in the screen for modifiers of the expanded polyglutamine ataxin-1 eye phenotype were also tested. This screen identified 20 enhancers and 7 suppressors from 1500 lethal P-element insertions tested. Three of these enhancers were P-elements in the genes *Ubi63E, UbcD1* and *hsr-ω* encoding ubiquitin, ubiquitin conjugase and a heat shock response factor [64]. These P-element insertions were tested for enhancement of the $(CAA)_{94}$ polyglutamine eye phenotype used to test the DNA repair hypothesis, and also a slightly less severe $(CAG)_{99}$ phenotype. In both cases, no enhancement of the eye phenotype was observed, with the presence of the P-element insertion appearing to have no effect on the severity of either eye phenotype (Figure 4.5 and data not shown). Thus whilst the polyglutamine phenotype can be modified by manipulation of Hsp70 activity, previously identified ataxin-1 enhancers failed to affect the phenotype in a visible manner.

Figure 4.5 The polyglutamine eye phenotype is not modified by previously described enhancers of the ataxin-1 phenotype. A moderate eye phenotype caused by expression of $(CAG)_{99}$ driven by *GMR-GAL4* was tested for modification by three P-element insertions previously identified as enhancers of the ataxin-1 expanded polyglutamine eye phenotype in *Drosophila*. The eye phenotype of a control fly expressing polyglutamine in a wild-type background (**A**) was compared to that of flies expressing polyglutamine and carrying P-element insertions in the genes *Ubi63E* (**B**), *UbcD1* (**C**), and *hsr-ω* (**D**). None of these mutations appeared to enhance the polyglutamine eye phenotype. The Bloomington stock numbers of these alleles are 11666, 11779 and 10292 respectively.

Discussion

An important consideration when interpreting the results described here is whether the lack of enhancement observed reflects a real lack of interaction between the DNA repair and axon transport pathways and the pathogenic process, or rather is due to a failure of this model system to show such an interaction. There is some evidence that this second possibility may be correct. Firstly, some of the alleles tested in these experiments have previously been shown in other studies to modify polyglutamine

phenotypes in *Drosophila*. For example, many of the axon transport alleles had been tested in a previous study, and modified polyglutamine phenotypes in *Drosophila* even in the presence of one wild-type allele. However, different phenotypes (larval lethality or the presence of accumulations in axons) were assayed in this study, and these may be more sensitive than the external appearance of the eye and therefore more likely to reveal an interaction.

In addition, the deletion within *p53* tested here has previously been used to modify a polyglutamine phenotype in the *Drosophila* eye [292]; however, again there were some differences in the model (the polyglutamine tract was in the first exon of Huntingtin) and assay (modification was measured by examining tangential sections of the eye or counting rhabdomeres). Importantly, both copies of *p53* were removed, rather than only one, suggesting that homozygosity for the mutant allele was required to reveal modification. Interestingly, deletion of *p53* resulted in suppression of the polyglutamine phenotype, rather than the enhancement predicted here in accordance with the DNA repair hypothesis. However, this result may still provide support for the DNA repair hypothesis, as it suggests an interaction between repeat expansion and *p53*. It is possible that detection of DNA damage induced by the repeat expansion could lead to inappropriate and excessive apoptosis, which is reduced by decreasing *p53* activity.

These observations raise the possibility that interactions between polyglutamine pathogenesis and the pathways tested here may not have been detected due to a lack of sensitivity of the assays used to detect modification. If two mutant alleles of each gene had been tested, removing all of the protein in question rather than reducing its levels by half, modification may have been detected. However, this is not possible for many of the genes tested, as a complete knockout results in lethality. More sensitive methods of detecting modification of the eye phenotype could alternatively be used, such as tangential sectioning or counting rhabdomeres; screening at the light microscope level is only a gross assay, and relies on the introduced mutations affecting the phenotype to the degree that the external appearance of the eye is affected. However, this is what makes the *Drosophila* eye an appealing system to use to screen for modifiers, and even using the candidate gene approach rather than an unbiased screen, which narrows the numbers of candidates, examining phenotypes other than external appearance requires a more significant investment of time. Furthermore, reducing protein levels by half and

examining the external appearance of the eye is a technique used frequently in *Drosophila* to perform unbiased screens, and has revealed many modifiers of polyglutamine eye phenotypes [64,86]. Therefore, in general this method is sensitive enough to detect interactions; perhaps stronger or more significant interactions are required than those tested in this study. More in-depth experiments to further investigate the DNA repair or axon transport hypotheses could be performed to determine whether there is more subtle modification of the eye phenotype occurring by examining the structure of the eye at the cellular level.

The reason behind the lack of modification observed in this study could also be that the phenotype used for screening is resistant to modification. The severity of the phenotype is one possible aspect of this; it may be that it is too severe to be noticeably enhanced. This seems unlikely based on two observations: 1) the severe phenotype was enhanced by expression of a *Hsp70* dominant-negative allele, and therefore can be made worse, and 2) polyglutamine phenotypes of equal or more severity have been used in screens to identify modifiers, and many were identified including enhancers [64,86]. To account for this possibility, milder phenotypes were also used in modification assays described here, which show modification by *Hsp70*, although they revealed no modification with candidates from the DNA repair and axon transport pathways. However, it is possible that a phenotype between these would be more sensitive. Further experiments to test this could utilise the modifiers previously identified in the ataxin-1 screen, testing these with a wider range of lines to determine whether there is a phenotype within the range that is sensitive to modification by these alleles.

Another possibility is that increased toxicity of isolated polyglutamine tracts compared to those in the context of a gene leads to a phenotype that is less susceptible to modification. In the model described here, an isolated polyglutamine tract was overexpressed in the *Drosophila* eye. It is well established that in the human disease state and in models of polyglutamine disorders, smaller fragments containing polyglutamine tracts or polyglutamine tracts alone are more toxic than those located in the context of a full-length protein. This enhanced toxicity may be due to pathogenesis occurring via alternative pathways that are not invoked by longer, less toxic proteins, or it may suggest that the normal mechanisms that the cells use to deal with the expanded polyglutamine are more rapidly overwhelmed. This may also be brought about by the high level of expression of polyglutamine proteins in the

Drosophila eye in this system. Supporting this possibility are other cases where overexpression has caused pathologies distinct from the normal pathogenic pathway [293-295]. For this reason, the model described here may not be suitable for investigation of polyglutamine pathogenesis by testing for modification of the phenotype. This suggestion is contradicted by a previous study where the identification of enhancers and suppressors was performed using an isolated polyglutamine tract [86]. However, it is possible that these represent the most significant modifiers, and that polyglutamine tracts in gene context are required to identify more subtle interactions. The lack of enhancement observed in this study using previously identified ataxin-1 enhancers supports the notion that the eye phenotype used here is less sensitive to enhancement by the P-element insertions identified in the ataxin-1 screen. This suggests that a different model, where the polyglutamine tract is located in a full-length gene involved in one of the disorders, may be more sensitive to modification than the isolated polyglutamine tract, and may be better suited to addressing the axon transport and DNA repair hypotheses.

Thus to continue the investigation of the DNA repair and axon transport hypotheses, an alternative model where the polyglutamine tract is located in a full-length human protein could be used. In addition, the modified eye phenotypes could be examined at the cellular level by staining tangential sections to reveal a more subtle enhancement of the phenotype. From the results obtained here, none of the hypotheses can be definitively ruled out, although it can be stated that no evidence to support them has been found. It is possible that this is due to the shortcomings of the model and approach used. However, in the case of either hypothesis, titration of components of the affected pathways (i.e. DNA repair proteins or axon transport proteins) together with overwhelming of the pathway induced by the repeat expansion would mean that the resulting phenotypes are predicted to be sensitive to the levels of these proteins. Therefore the reduction by half of these vital components would be expected to alter the phenotype, if indeed these pathways are involved. Further investigation of these hypotheses is required to reach a definitive conclusion, but these are beyond the scope of this project. However, within the limitations of the experimental approaches used here, it appears as though CAG repeat expansion does not bring about pathogenesis by disrupting the DNA repair or axon transport pathways.

Chapter 5 – RNA Pathogenesis Revisited

Introduction

In addition to the results described in Chapter 3, in particular that rCAG repeats are not toxic in *Drosophila*, other studies of RNA toxicity in *Drosophila* have been published recently, and report mixed findings. As described in Chapter 1, *Drosophila* models of FXTAS and SCA8 have been generated, in which rCGG and rCUG repeats respectively exhibit toxicity in the fly [187,188]. However, in the case of the SCA8 model, there is some uncertainty regarding the pathogenic entity, as the *SCA8* transcript containing CUG repeats in the normal range also exhibits toxicity in this system. Contributing to this uncertainty are the results of a recent unsuccessful attempt to create a *Drosophila* model of DM1 [296]. In this case, the 3' UTR of the *DMPK* gene containing a $(CTG)_{162}$ repeat was fused to GFP and expressed in various tissues of the fly, including muscle, which is the major site of pathogenesis in DM, and in the eye using *GMR-GAL4*. However, under these conditions the expanded rCUG repeat did not induce toxicity, with no effect observed in any tissue. What did result from expression of the repeat, however, was the formation of RNA foci, detectable using a $(CAG)_{10}$ probe. Furthermore, in some tissues these foci showed colocalisation with aggregates of muscleblind. Thus, some aspects of DM pathogenesis, such as RNA foci formation and sequestration of muscleblind, appear to be present in *Drosophila* but do not induce toxicity. These results conflict with the SCA8 model, where expanded rCUG repeats did appear to exhibit toxicity, which was enhanced by a mutation in *muscleblind* [187]. Therefore it is unclear whether expanded rCUG repeats are intrinsically toxic in the *Drosophila* system, and what role, if any, muscleblind plays in the pathogenic pathway.
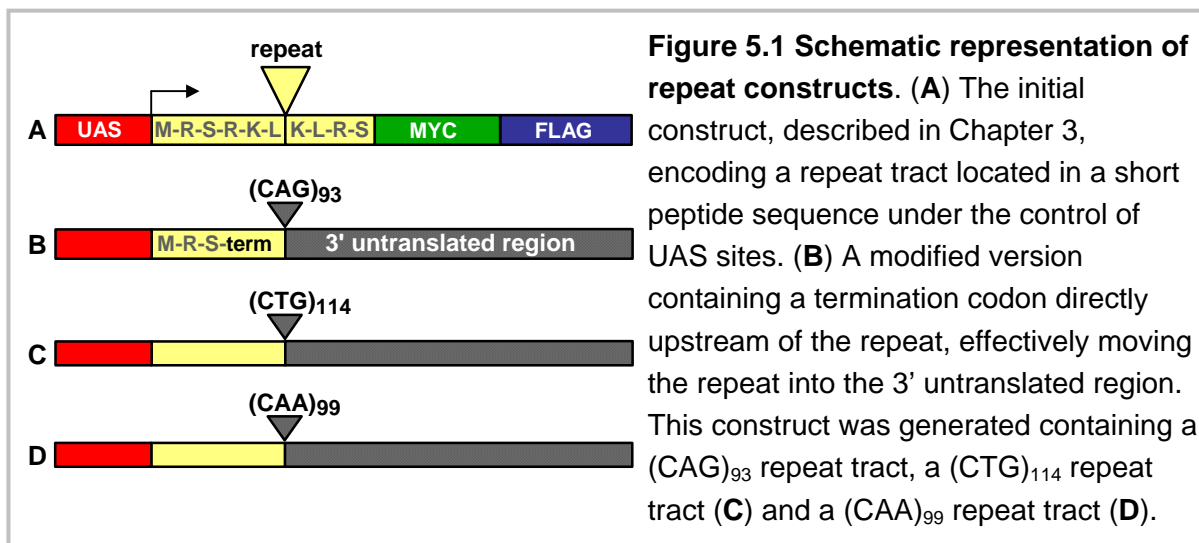
Generation of a *Drosophila* model of rCUG repeat diseases in which the CTG repeat is separated from gene context would allow pathways of pathogenesis in rCUG repeat diseases such as SCA8 and DM1 to be compared with other pathways of repeat pathogenesis. Modifiers of polyglutamine pathogenesis that have previously been identified could be tested on an rCUG phenotype to identify common and distinguishing features of these pathways. These could also be compared in other *Drosophila* models of repeat disease, such as the FXTAS fly model, to identify similarities and differences in the rCGG and rCUG pathogenic pathways.

Results

RNA phenotype from CUG repeats

To investigate the inherent toxicity of expanded rCUG repeats, independent of gene context, a $(CTG)_{114}$ repeat was expressed in *Drosophila* in the 3' UTR of a construct encoding a small peptide. This is the same as the term$(CAG)_{93}$ construct described in Chapter 3, but the CAG repeat tract has been replaced with $(CTG)_{114}$ (Figure 5.1). The peptide coding sequence and repeat tract are downstream of UAS sites, allowing expression in all cells of the eye driven by *GMR-GAL4*.

Figure 5.1 Schematic representation of repeat constructs. (**A**) The initial construct, described in Chapter 3, encoding a repeat tract located in a short peptide sequence under the control of UAS sites. (**B**) A modified version containing a termination codon directly upstream of the repeat, effectively moving the repeat into the 3' untranslated region. This construct was generated containing a $(CAG)_{93}$ repeat tract, a $(CTG)_{114}$ repeat tract (**C**) and a $(CAA)_{99}$ repeat tract (**D**).

In total, 14 independent lines were generated carrying term$(CTG)_{114}$. Upon expression in the eye, the rCUG repeat appeared to have no effect in 12 out of the 14 lines, with the external appearance of the eye indistinguishable from a *GMR-GAL4* heterozygous control fly. However, in two lines, expression of the rCUG repeat caused a very mild rough eye phenotype (Figure 5.2). The colour of the eye was unaffected, but the organization of the ommatidia appeared to be disrupted, with a less-ordered appearance than that of the control eye. This phenotype was very mild, and more variable than the polyglutamine eye phenotype, with flies of the same genotype displaying varying severities. The phenotype was stronger in females than in males.

The reason behind the lack of phenotype in 12 of the 14 lines is unknown. A likely possibility is that a high level of expression of the rCUG repeat is required to induce a phenotype in the fly; thus the two lines with the highest level of expression are those which display a phenotype, while in the remaining 12 lines the level of expression is below the threshold level required to cause a visible phenotype. To investigate this
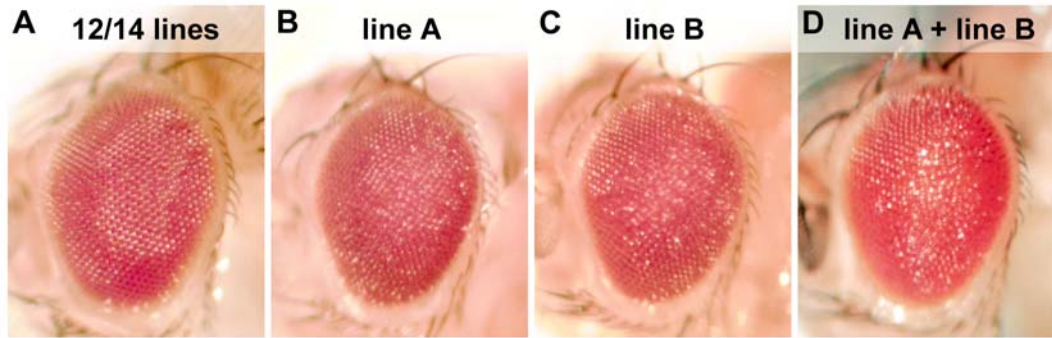
Figure 5.2 Expression of rCUG in the *Drosophila* eye causes a rough eye phenotype in two out of 14 lines. Expression in the eye was driven by *GMR-GAL4*. (**A**) In 12 out of 14 independently generated lines, no effect on the external appearance of the eye is observed upon expression of term(CTG)$_{114}$. (**B**) In one line, referred to here as 'line A', expression of term(CTG)$_{114}$ causes a very mild rough eye phenotype. This is seen as a disruption to the normal ordered array of ommatidia in the eye. The phenotype is variable, and more severe in females. (**C**) In a second line, 'line B', a slightly more severe rough eye phenotype is observed. Again it is variable and stronger in females. (**D**) Flies expressing both line A and line B show a more severe rough eye phenotype. Disorganisation of the ommatidia is visible, in a patch roughly in the middle of the eye. The disorganisation of cells leads to a shiny appearance in this area. The phenotype is still variable, and more evident in females. In each case, the example shown is at more severe end of the range.

possibility, flies containing two independent copies of the term(CTG)$_{114}$ construct were generated. The purpose of this was to increase the level of rCUG in these flies when they are crossed to *GMR-GAL4*, compared to flies carrying a single copy of the construct. This reasoning depends on the level of transcription of the CTG repeat being limiting, rather than the amount of GAL4 protein. There is evidence supporting this being the case, as phenotypes in *Drosophila* resulting from GAL4-driven expression are not decreased in the presence of a second UAS construct [192]. This suggests that GAL4 is not limiting, and that there is sufficient GAL4 for expression of the second construct without affecting expression of the first.

In total, six independent lines were used to generate three lines of flies carrying two copies of the term(CTG)$_{114}$ construct. One of these lines contained both insertions that independently gave a mild phenotype upon expression in the eye; expression of both together in the eye of a single fly resulted in a slightly more severe phenotype (Figure 5.2 D). The phenotype was one of disorganization of the ommatidia, which is more apparent in SEM images (Figure 5.3 B and B'). No loss of pigment was observed. The rough eye phenotype was still variable; flies that shared the same genotype, expressing the same two copies of term(CTG)$_{114}$, showed variation in

severity of the phenotype, and again the phenotype was stronger in females. A similar phenotype, but milder, was seen in another of the lines expressing two copies of rCUG in the eye. The remaining line containing two insertions did not show an eye phenotype when crossed to *GMR-GAL4*. In further experiments, the term$(CTG)_{114}$ line expressing two copies showing the more severe phenotype was used.

RNA phenotype from CAG repeats

The results described for rCUG repeats suggest that their ability to induce toxicity is indeed dependent on the level of expression of the transcript, with a higher level (generated by the presence of two copies of the construct) having a more severe effect. This raises the possibility that the same is true for rCAG repeats, and therefore the reason for the lack of phenotype upon expression of term$(CAG)_{93}$ in Chapter 3 is that the level of expression was insufficient. To address this possibility, the same approach used for the rCUG repeat was utilised, and three lines of flies each containing two independent copies of term$(CAG)_{93}$ were generated. None of these insertions gave a phenotype when expressed individually in the eye, as described in Chapter 3 (Figure 3.4). However, when two copies were simultaneously expressed in the eye, a phenotype was observed in one of the three lines containing two copies (Figure 5.3). This phenotype was again one of disorganisation, with disruption to the ordered array of ommatidia and bristles, and some ommatidia appeared to be fused. This roughening was more apparent in SEM images. No loss of pigment was observed, and like the eye phenotype induced by rCUG, the rCAG rough eye was variable and stronger in females. The nature of the phenotype was the same as that induced by the rCUG repeat, and comparable in severity to the weaker of the two rCUG lines expressing two copies of the repeat construct. It was clearly distinct from the polyglutamine phenotype, which is characterised by pigment loss apparent before disorganisation of the ommatidia is visible (Figure 3.5). Thus it appears that rCAG can induce toxicity if expressed at a sufficiently high level. In the following experiments, the line containing two copies of term$(CAG)_{93}$ that induced a phenotype was used.

No RNA phenotype from CAA repeats

So far, all of the repeats capable of inducing RNA toxicity in *Drosophila* (rCAG and rCUG described here, rCGG described previously [188]) have in common the ability to form a hairpin secondary structure, at least *in vitro*. To investigate whether this is related to their ability to induce toxicity, a term$(CAA)_{99}$ construct was generated to
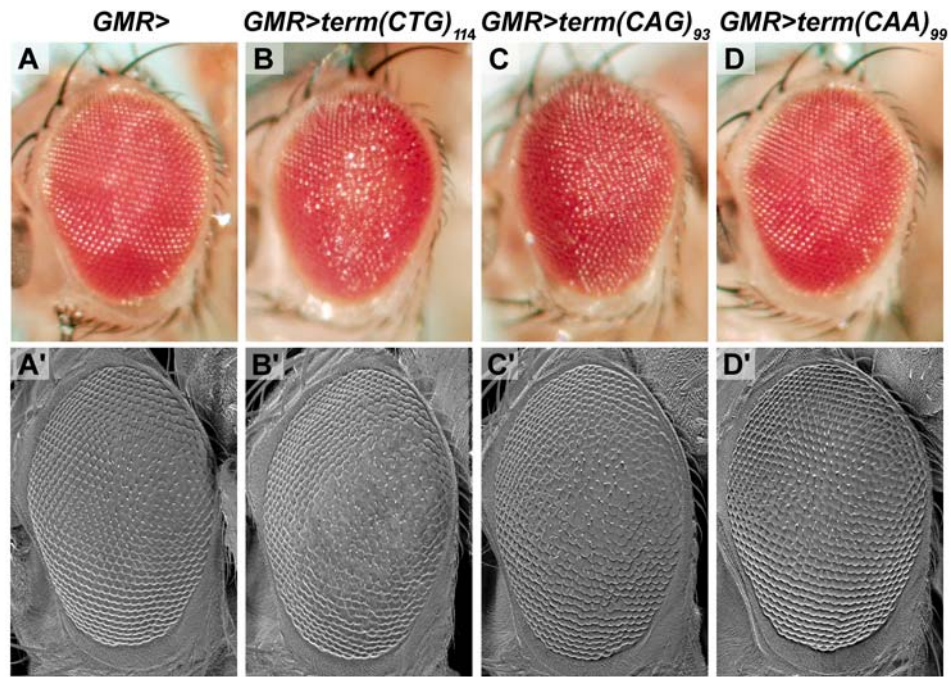
Figure 5.3 Effect of expressing two copies of untranslated repeat transcripts in the *Drosophila* eye. Expression of all repeat transcripts was driven in the eye by *GMR-GAL4.* For each repeat construct, two independent lines were combined and expressed together in the eye to increase the level of the repeat transcript. Control flies carrying *GMR-GAL4* alone show a regular array of ommatidia in light microscope (**A**) and scanning electron microscope (SEM) (**A'**) images. In contrast, expression of two copies of term(CTG)$_{114}$ causes a mild rough eye phenotype (**B**) which is visible as a disorganisation of the ommatidia in a large area of the eye (**B'**). A milder rough eye results from expression of two copies of term(CAG)$_{93}$ (**C**). Again, SEM images reveal a disorganisation of the ommatidia in a large area of the eye (**C'**). Expression of two copies of term(CAA)$_{99}$ has no effect on the eye, with the ordered array of ommatidia visible in light microscope (**D**) and SEM (**D'**) images. For term(CTG)$_{114}$ and term(CAG)$_{93}$, the rough eye phenotypes were variable; the most severe examples are shown here.

express in the fly, to compare with the effect of expression of term(CAG)$_{93}$ and term(CTG)$_{114}$. As described in Chapter 3, the CAA repeat is not predicted to form a hairpin structure, and so if it were unable to induce pathogenesis, this would suggest that formation of a hairpin is vital in the pathogenic pathway of CAG and CTG. A (CAA)$_{99}$ repeat was inserted into the construct containing the premature termination codon. The resulting construct was identical to that containing the untranslated CTG and CAG repeats, except that it contains a CAA repeat (Figure 5.1). Unexpectedly, some difficulty was experienced in generating transgenic flies carrying this construct. The *white* gene was carried on the plasmid containing the repeat construct, and once inserted into the genome, was used to identify flies that had been transformed. 50 independent lines were generated which express *white* and therefore presumably carry the insertion containing the repeat construct; however, the repeat insertion could only be amplified by PCR in three of these 50 lines. This difficulty had not been

encountered previously for any of the other constructs, including the $(CAA)_{94}$ repeat construct, which is identical to the term$(CAA)_{99}$ construct apart from 1 base pair which is altered in the term$(CAA)_{99}$ construct to encode a termination codon, and the slight difference in repeat length. Therefore it seems unlikely that toxicity of the repeat construct is the reason for the difficulty in obtaining transgenic lines, as if this construct was toxic the $(CAA)_{94}$ construct would be predicted to also exhibit toxicity. The three independent lines in which the inserted repeat construct could be amplified by PCR were analysed by sequencing and appeared to contain no mutations or deletions, with the repeat copy number around the same length as in the plasmid used for microinjection. Therefore the reason for the difficulty in obtaining more lines is unknown.

When expression of term$(CAA)_{99}$ was driven in the eye using *GMR-GAL4*, no phenotype was observed in any of the three lines. To increase the level of expression so that this repeat could be compared to the rCAG and rCUG repeats, a line of flies carrying two copies of the term$(CAA)_{99}$ construct was generated. When these flies carrying two copies of term$(CAA)_{99}$ were crossed to *GMR-GAL4*, a phenotype was not observed, with the appearance of the eye indistinguishable from the control fly (Figure 5.3). This suggests that rCAA is not toxic in *Drosophila*, supporting the notion that formation of a hairpin secondary structure is involved in the pathogenesis of the rCUG and rCAG repeats. However, as the phenotype induced by two copies of rCAG was only apparent in one of three lines, and for two copies of rCUG in two of three lines (Figure 5.4), more than a single line of flies expressing two copies of rCAA is required before a definite conclusion regarding toxicity of the rCAA transcript can be reached.

Are the phenotypes due to translation of the repeats?
Despite the presence of a termination codon directly upstream of the repeat, it is possible that read-through was occurring, meaning that some of the repeat transcripts were being translated into either polyglutamine or polyleucine protein, and it is this protein responsible for the phenotypes observed, rather than RNA. This explanation seems unlikely based on two observations. Firstly, in the case of the rCAG repeat, the eye phenotype observed was clearly different to that caused by polyglutamine, even at the milder end of the polyglutamine phenotype spectrum. As shown in Chapter 3 (Figure 3.5), the mildest polyglutamine phenotype is characterised by pigment loss in the posterior part of the eye, while the order of the

| Eye Phenotype:	None	Rough eye	Total
term(CAG)$_{93}$	16	0	16
term(CAG)$_{93}$ 2 copies	2	1	3
term(CAA)$_{99}$	3	0	3
term(CAA)$_{99}$ 2 copies	1	0	1
term(CTG)$_{114}$	12	2	14
term(CTG)$_{114}$ 2 copies	1	2	3

Figure 5.4 Effect of expressing RNA repeat transcripts in the *Drosophila* eye driven by *GMR-GAL4* varies between lines. For each repeat construct, the total number of independently generated lines is indicated, as well as the number of lines generated carrying two independent insertions. The number of these lines showing a rough eye phenotype upon expression in the eye is indicated. Examples of these phenotypic categories are shown, which do not correspond to any of the particular lines.

ommatidia and bristles of the eye is unaffected. In contrast, the rCAG phenotype shows disorganisation of the bristles and ommatidia, but no pigment loss is observed. This suggests that the phenotype is not due to the presence of even low levels of polyglutamine protein. Secondly, if low levels of polyglutamine protein caused the phenotype, then the rCAA flies should display the same phenotype as the rCAG flies, as both of these constructs encode the same polyglutamine protein. However, this is not what was observed, again suggesting that the phenotype is RNA-mediated.

To confirm that no read-through was occurring, western analysis was used to determine whether any read-through protein products were detectable. As a termination codon was introduced into the construct by altering one base pair, the reading frame of the transcript downstream of the termination codon was unaltered. Therefore, if read-through were occurring, a polyglutamine or polyleucine protein tagged with a myc/flag tag on the C terminus would result. These proteins would be identical to those encoded by the (CAG)$_{99}$ or (CTG)$_{86}$ constructs described in Chapter 3. Therefore, eye discs from larvae expressing these constructs were used as controls in the western analysis, and compared to larvae expressing term(CAG)$_{93}$ or term(CTG)$_{114}$. A polyglutamine protein was detectable in larvae expressing (CAG)$_{99,}$ however the polyleucine protein encoded by (CTG)$_{86}$ was not detected. No proteins were detected by the myc antibody in eye discs from larvae expressing term(CAG)$_{93}$

or term(CTG)$_{114}$ (although some polyglutamine protein from the next lane has run into the lane containing term(CTG)$_{114}$). (Figure 5.5). This suggests that there is no read-through occurring in flies expressing term(CAG)$_{93}$, and that the phenotype in the rCAG flies is indeed RNA mediated.
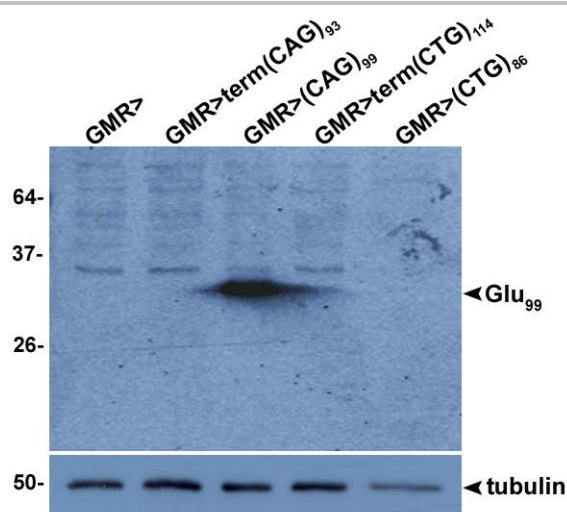
Figure 5.5 Read through products containing an expanded repeat cannot be detected by western blot. *GMR-GAL4* was used to drive expression of UAS repeat constructs. Eye discs from third instar larvae were run on a 15% polyacrylamide gel, which was transferred to nitrocellulose and probed with an antibody against the C-terminal myc tag present on any translated repeat products (top panel). Lane one shows control larvae expressing GAL4 alone. Lane two shows larvae expressing two copies of the term(CAG)$_{93}$ transcript; no polyglutamine proteins are visible. Lane three shows larvae expressing a translated (CAG)$_{99}$ construct, encoding a protein consisting of a 99 glutamine tract, which is detected by the myc antibody at approximately 33 kDa. Lane four shows larvae expressing two copies of the term(CTG)$_{114}$ transcript; some of the polyglutamine protein from lane 3 is visible in this lane, but the protein product encoded by read-through of the term(CTG)$_{114}$ transcript is predicted to be slightly larger that this; no larger proteins are visible (apart from background bands present also in the GAL4 control larvae). Lane five shows larvae expressing the (CTG)$_{86}$ transcript. The encoded polyleucine protein is not visible. The membrane was also probed with anti-α tubulin as a loading control (bottom panel). This shows a slightly lower level of protein in the fifth lane.

In the case of the rCUG flies, the evidence that the phenotype is RNA-mediated is not as conclusive. The polyleucine eye phenotype is closer in appearance to the rCUG eye phenotype, as both are characterised by disruption to the ommatidia without pigment loss (compare Figure 3.8 C and Figure 5.3 B). Furthermore, the polyleucine protein could not be detected by western blot (Figure 3.11 and Figure 5.5). Therefore, if read-through of the term(CTG)$_{114}$ construct was occurring, it seems likely that the encoded polyleucine protein would also not be detected on the western blot. However, the fact that the termination codon was sufficient to terminate transcription in the rCAG transcript suggests that it is likely to be sufficient in the

rCUG transcript as well, as the transcripts are identical up to the termination codon. Therefore, whilst the presence of a low level of polyleucine protein is impossible to rule out, it seems likely that the term(CTG)$_{114}$ phenotype is also RNA mediated.

Characterisation of the RNA phenotypes.

The finding that rCAG and rCUG repeats can induce a phenotype reopens the possibility that RNA may be contributing to the polyglutamine diseases, as well as to the diseases caused by untranslated repeats, and that there is a pathogenic pathway common to both groups of diseases. To analyse whether the RNA phenotypes observed in *Drosophila* may be relevant to the human disease process, the nature of these phenotypes was examined further. Two striking characteristics of the human diseases are their degenerative nature, and their cell-type specificity. The rCAG and rCUG phenotypes observed in the fly appear to be cell type-specific, as pigment cells are spared, compared to the phenotype induced by polyglutamine expression where eye colour is affected. To examine the cellular basis of the rough eye phenotype further, tangential sections of the eye were cut and stained, to allow visualisation of the cells of the eye in flies expressing *GMR-GAL4* and two copies of term(CTG)$_{114}$, term(CAG)$_{93}$, or term(CAA)$_{99}$. These were compared to a relatively mild polyglutamine eye phenotype caused by expression of (CAG)$_{52}$, to reveal similarities and differences between the phenotypes (Figure 5.6). In the eyes of flies expressing rCAG and rCUG, a mild disorganisation of the regular arrangement of the ommatidia was revealed, and some ommatidia contained only 6 visible photoreceptors instead of 7. This was also occasionally seen in the *GMR-GAL4* control eye, but at a very low frequency. Flies expressing rCAA showed no disruption, with the organisation of the cells in the eye indistinguishable to the control. In contrast, the flies expressing polyglutamine in the eye showed a severe phenotype at the cellular level. Externally, the only visible defect was a loss of pigment in the posterior part of the eye, with the order of the ommatidium apparently unaffected. However, the tangential sections reveal extensive loss of photoreceptor cells and complete disorganisation, despite the external appearance. This effect was observed uniformly across the eye, despite the posterior appearing to be more affected externally. Therefore even the apparently mild polyglutamine phenotype caused by expression of (CAG)$_{52}$ gives a much more severe effect at the cellular level than rCAG or rCUG, even in newly eclosed flies, with more cell types affected.

The second aspect of the RNA phenotypes examined was whether they demonstrate age-related degeneration. Previous investigations into the nature of the polyglutamine eye phenotype in *Drosophila* have revealed progressive degeneration with age [80,81]; this was also shown to occur upon expression of rCGG in the eye [188]. To investigate this aspect of the rCUG and rCAG phenotypes, flies expressing two copies of term(CTG)$_{114}$ or term(CAG)$_{93}$ were aged for 30 days, and their eyes compared to one-day-old flies. The effect of age on the phenotype was also compared in flies expressing two copies of term(CAA)$_{99}$, flies with a mild polyglutamine phenotype caused by expression of (CAG)$_{52}$, and control flies carrying *GMR-GAL4* alone (Figure 5.6).

In the control flies and in flies expressing term(CAA)$_{99}$, no age-related degeneration was observed. Externally, neither eye showed disorganised ommatidia at day one or day 30. *Drosophila* eyes darken with age; this could be seen in the control and rCAA flies, and no pigment loss was observed at either time point. At the cellular level, no disorganisation was observed after ageing in the control flies or the rCAA flies. Again, ommatidia with only 6 visible photoreceptors were infrequently observed; these were apparent in both rCAA and control flies, at roughly the same frequency, and therefore are either a normal feature of age in the *Drosophila* eye, or due to the presence of GAL4. No ommatidia containing fewer than 6 visible photoreceptors were observed. Therefore at the cellular level, expression of rCAA appears to have no effect on the *Drosophila* eye, even after ageing.

In contrast, the mild polyglutamine eye phenotype showed dramatic deterioration with age. Externally, whilst the eyes darkened with age, pigment loss was evident over time, although no disruption to the order of the ommatidia was visible externally, even after 30 days. However, at the cellular level, the phenotype was dramatic, with age causing a complete loss of photoreceptor cells. Gaps were seen in the tissue and the remaining cells were disorganised and unrecognisable. This degeneration is in line with what has previously been reported for polyglutamine eye phenotypes in *Drosophila* [80,81].

In flies expressing rCAG and rCUG, no age-related degeneration was evident externally: no pigment loss was visible after 30 days, and the mild roughness of the eye did not appear to be more severe. However, tangential sectioning revealed significant degeneration at the cellular level. An increase in the disorganisation of the

arrangement of the ommatidia was evident after 30 days, with large gaps in the tissue present, and ommatidia with only four or five visible photoreceptors were frequently apparent. Concordant with the external appearance of the eyes, at the cellular level the rCUG phenotype was slightly more severe than the rCAG phenotype; an age-related increase in severity was observed in both (Figure 5.6). Thus it appears that the rCUG and rCAG eye phenotypes described here in *Drosophila* are degenerative.

A second feature of the rCUG and rCAG eye phenotypes investigated was the possible involvement of Hsp70. In the FXTAS *Drosophila* model, where degeneration of cells in the eye induced by rCGG lead to a rough eye phenotype, Hsp70-positive aggregates were present in sections of the eye. Furthermore, modulation of Hsp70 activity could modify the eye phenotype [188], suggesting a possible involvement of Hsp70 in pathogenesis or at least a pathogenic pathway that can be affected by Hsp70. Presumably, this was initially investigated because aggregates staining positively for cellular components such as ubiquitin and the proteasome have been detected in neurons in the brains of FXTAS patients [154]. Furthermore, similar aggregates detected in a FXTAS mouse model contained Hsp40 [297]. In the *Drosophila* FXTAS model, in addition to the Hsp70-positive aggregates, staining with antibodies against ubiquitin or a subunit of the proteasome also revealed the presence of aggregates, showing that they share similarities with the aggregates detected in patient tissue. How these aggregates are involved in the pathogenic pathway is unclear; as FXTAS appears to be RNA mediated, it is not obvious how the ubiquitin/proteasome pathway or Hsp70 would be involved.

To determine whether the rCUG and rCAG phenotypes described here share similarities in pathogenesis with the rCGG phenotype, the possible involvement of Hsp70 in the rCUG and rCAG phenotypes was investigated. To determine whether Hsp70-positive aggregates were present, the same process was undertaken as has been described previously for rCGG flies [188]. This involved cutting horizontal sections of the eye and staining with an anti-Hsp70 antibody, from the same source as the antibody used to detect aggregates previously, and under the same conditions. This revealed a diffuse, uniform pattern of staining in the *GMR-GAL4* control fly, which may correspond to the normal distribution of Hsp70, previously
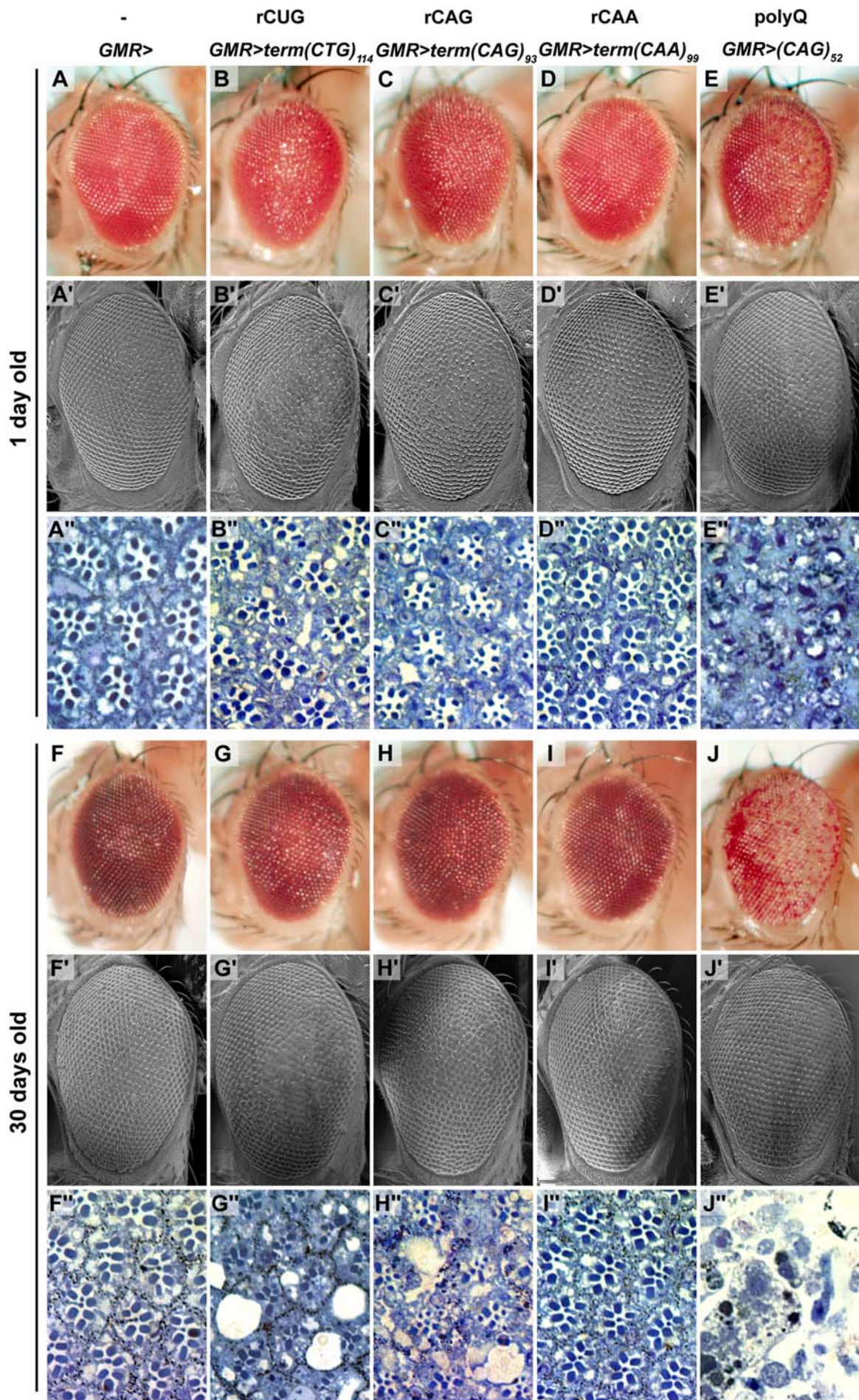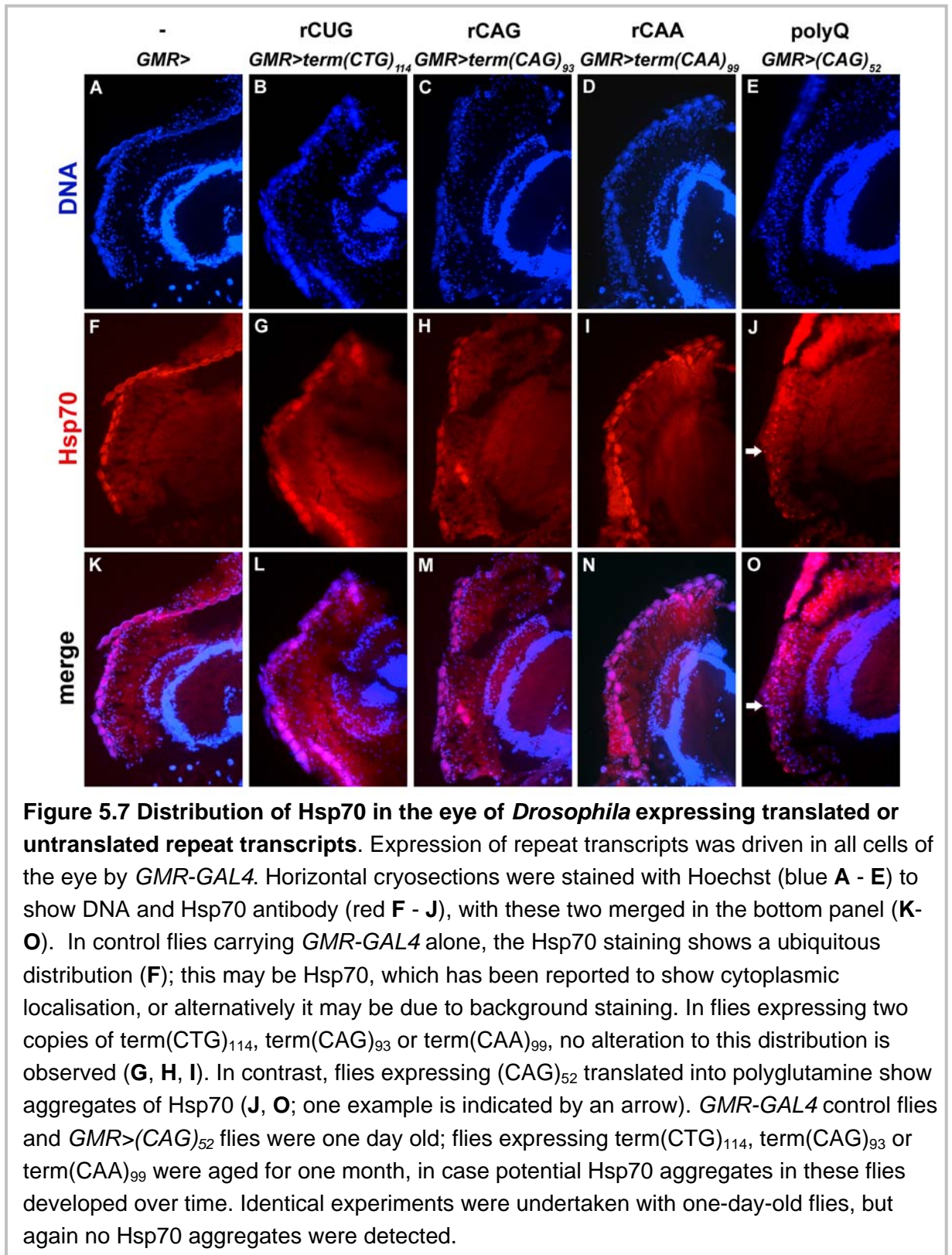
Figure 5.6 Effect of expression of repeat-containing transcripts on the *Drosophila* eye before and after ageing. Expression of repeat transcripts was driven in all cells of the eye by *GMR-GAL4*. Light microscope images (**A** - **J**) and scanning electron microscope (SEM) images (**A'** - **J'**) show the external appearance of the eye, whilst tangential sections stained with methylene blue (**A''** - **J''**) show the underlying cellular structure. One-day-old flies (**A** - **E**) and flies of the same genotype that have been aged for 30 days (**F**- **J**) are shown. Control flies carrying *GMR-GAL4* alone show a regular array of ommatidia and bristles, which is evident in SEM images (**A'**). Tangential sectioning also reveals the regular array of ommatidia, each of which contains seven visible photoreceptor cells arranged in a hexagonal pattern, and is surrounded by pigment granules (**A''**). After ageing, the organisation of the cells of the eye of the control flies is preserved, both externally (**F**, **F'**) and internally, although ommatidia containing only six visible photoreceptors are occasionally observed (**F''**). In contrast, disorganisation of the cells of the eye can be seen in flies expressing two copies of term(CTG)$_{114}$. Some of the ommatidia appear to be fused, and the arrangement of bristles is disordered. This is visible externally in light (**B**) and SEM images (**B'**), and the external appearance is unaffected by ageing (**G** and **G'**). At the cellular level, only a very mild disruption to the order of the cells can be detected in one-day-old flies (**B''**), and some photoreceptors are smaller or missing. After 30 days, this phenotype is more severe, with many ommatidia containing only four or five visible photoreceptors, and large holes in the tissue visible (**G''**). A similar effect is seen in flies expressing two copies of term(CAG)$_{93}$. At day one, a mild disruption to the order of the ommatidia and bristles can be seen externally (**C** and **C'**); this does not appear to be any worse after 30 days (**H** and **H'**). At the cellular level, mild disorganisation and some missing photoreceptors can be seen at day one (**C''**). After 30 days this is more severe, with many ommatidia showing fewer than six visible photoreceptors and gaps in the tissue evident (**H''**). In flies expressing two copies of term(CAA)$_{99}$, no differences from the control flies can be detected. At day one and day 30, the external appearance of the eye is unaffected (**D** and **D'**, **I** and **I'**), and the tangential sections reveal no differences from the control, with ommatidia containing only six photoreceptors occasionally visible in the aged flies, to the same extent as the control (**I''**). Unlike the term(CTG)$_{114}$ and term(CAG)$_{93}$ flies, no photoreceptors were observed that contain fewer than six ommatidia, even after ageing. In comparison, flies with a mild polyglutamine phenotype caused by expression of one copy of (CAG)$_{52}$ show a phenotype characterised by pigment loss in the posterior part of the eye (**E**) that worsens visibly with age (**J**). However, no disorganisation of the ommatidia is observed at either time point (**E'**, **J'**). Examination of the cells of the eye reveals extensive degeneration of the photoreceptor cells in one-day-old flies (**E''**), which worsens considerably such that at 30 days, there are no recognisable cells remaining (**J''**).

shown to be cytoplasmic in *Drosophila* eye discs [107], or it could be due to background (Figure 5.7). In a fly with a mild polyglutamine phenotype, the anti-Hsp70 staining revealed aggregates in the eye; polyglutamine aggregates have previously been shown to contain Hsp70 [107]. This acts as a positive control, as it demonstrates that the antibody is indeed able to detect Hsp70. In flies expressing 2 copies of term(CTG)$_{114}$, term(CAG)$_{93}$, or term(CAA)$_{99}$, no aggregates were detected by the anti-Hsp70 antibody; instead, the same uniform staining as that seen in the control was observed. The same level of background staining is not evident in the previously published results [188]. For this reason, the experiment was repeated, but with a lower concentration of anti-Hsp70 antibody (1/3 of the concentration previously described). However, similar results were obtained, with a relatively high level of uniform staining present in the control flies as well as in flies expressing rCUG or rCAG (results not shown). Thus it seems that Hsp70 aggregates are not present in flies expressing rCUG, rCAG or rCAA.

The second aspect of the involvement of Hsp70 investigated was whether it modifies the rCUG and rCAG eye phenotypes. In the FXTAS *Drosophila* model, overexpression of human Hsp70 completely suppressed the rCGG eye phenotype, and it was enhanced by expression of a dominant negative form of Hsp70 [188]. The same *Hsp70* constructs used in the rCGG study were therefore co-expressed with rCAG or rCUG to determine whether the rCAG and rCUG phenotypes show the same modification. These *Hsp70* constructs, *HSPA1L* and *Hsp70.K71S* (*Hsp70^{DN}*) have previously been described in Chapter 4, where they were used to modify the polyglutamine eye phenotype. To test whether they also modify the RNA phenotypes, they were co-expressed with two copies of either term(CAG)$_{93}$ or term(CTG)$_{114}$ in the *Drosophila* eye, and the resulting phenotype compared to expression of the repeat transcript alone. Co-expression of human Hsp70 (*HSPA1L*) with either two copies of term(CAG)$_{93}$ or two copies of term(CTG)$_{114}$ resulted in complete suppression of the rough eye phenotype caused by expression of the repeat transcripts (Figure 5.8). Expression of rCAG or rCUG alone results in a variable rough eye phenotype, but when *HSPA1L* was also expressed, no flies were observed with this phenotype and instead all flies had an eye indistinguishable in appearance from the control fly. Expression of *HSPA1L* alone driven by *GMR-GAL4* had no noticeable effect on the. eye. Therefore, the eye phenotype induced by rCAG or rCUG can be completely suppressed by Hsp70.

Figure 5.7 Distribution of Hsp70 in the eye of *Drosophila* expressing translated or untranslated repeat transcripts. Expression of repeat transcripts was driven in all cells of the eye by *GMR-GAL4*. Horizontal cryosections were stained with Hoechst (blue **A** - **E**) to show DNA and Hsp70 antibody (red **F** - **J**), with these two merged in the bottom panel (**K-O**). In control flies carrying *GMR-GAL4* alone, the Hsp70 staining shows a ubiquitous distribution (**F**); this may be Hsp70, which has been reported to show cytoplasmic localisation, or alternatively it may be due to background staining. In flies expressing two copies of term(CTG)$_{114}$, term(CAG)$_{93}$ or term(CAA)$_{99}$, no alteration to this distribution is observed (**G**, **H**, **I**). In contrast, flies expressing (CAG)$_{52}$ translated into polyglutamine show aggregates of Hsp70 (**J**, **O**; one example is indicated by an arrow). *GMR-GAL4* control flies and *GMR>(CAG)$_{52}$* flies were one day old; flies expressing term(CTG)$_{114}$, term(CAG)$_{93}$ or term(CAA)$_{99}$ were aged for one month, in case potential Hsp70 aggregates in these flies developed over time. Identical experiments were undertaken with one-day-old flies, but again no Hsp70 aggregates were detected.

In the case of *Hsp70^{DN}*, the results are less clear-cut. When expressed in the eye under the control of *GMR-GAL4*, *Hsp70^{DN}* alone caused an eye phenotype characterised by pigment loss and disorganisation of the ommatidia (Figure 5.8). When co-expressed with two copies of term(CAG)$_{93}$ or two copies of term(CTG)$_{114}$, the rough eye phenotypes caused by the repeat-containing RNA were significantly worse, with an increase in the rough appearance of the eye due to increased

disorganisation of the ommatidia. However, it is difficult to determine whether this increase in severity is greater than the sum of the two phenotypes individually. Therefore it is unclear whether there is an interaction occurring between *Hsp70^DN* and rCAG/rCUG, or whether the combination of Hsp70^DN and rCAG/rCUG results in a worse phenotype simply due to the combined effects of Hsp70^DN and the repeat transcript on the eye. However, it can be concluded is that *Hsp70^DN* is not having a strong effect on the rCAG or rCUG phenotypes.

In addition to Hsp70, the effect of expressing p35 on the rCAG and rCUG eye phenotypes was also examined. p35 is a baculovirus inhibitor of apoptosis that prevents programmed cell death in *Drosophila* by inhibiting caspase activation [298,299]. Mixed effects of p35 on *Drosophila* models of polyglutamine disease have been reported previously: it suppressed the phenotype caused by polyglutamine expression in the eye in a SCA3 model [80], but did not modify the eye phenotype in a Huntington's disease model [81]. In a separate study, the *Drosophila* eye phenotype caused by expression of expanded polyglutamine located in the ataxin-1 or ataxin-3 proteins was suppressed by p35, but the phenotype caused by expression of a polyglutamine tract alone was not suppressed [300]. These findings suggest the possibility that different pathways of cell death may be involved in some of the different polyglutamine fly models.

To investigate whether apoptosis is involved in the rCAG and rCUG eye phenotypes, p35 was co-expressed in the eye along with two copies of term(CAG)$_{93}$ or term(CTG)$_{114}$, and compared to the effect of expressing the repeat transcripts alone. The effect of p35 expression on a mild polyglutamine eye phenotype was also examined. Expression of p35 alone in the eye has a very mild effect on the structure of the eye, due to the prevention of apoptosis that normally functions to remove excess cells during development [299]. When co-expressed with (CAG)$_{52}$ in the eye, p35 partially suppressed the mild polyglutamine phenotype, restoring pigment to the posterior part of the eye (Figure 5.9). Suppression of the rCAG phenotype by p35 was less obvious. Co-expression of p35 and two copies of term(CAG)$_{93}$ resulted in an eye that showed mild disorganisation of the ommatidia; SEMs revealed that this disorganisation was slightly milder than that caused by term(CAG)$_{93}$ alone. Some suppression by p35 of the rCUG phenotype was also evident. Expression of two copies of term(CTG)$_{114}$ alone gives a rough eye phenotype that is variable in nature,
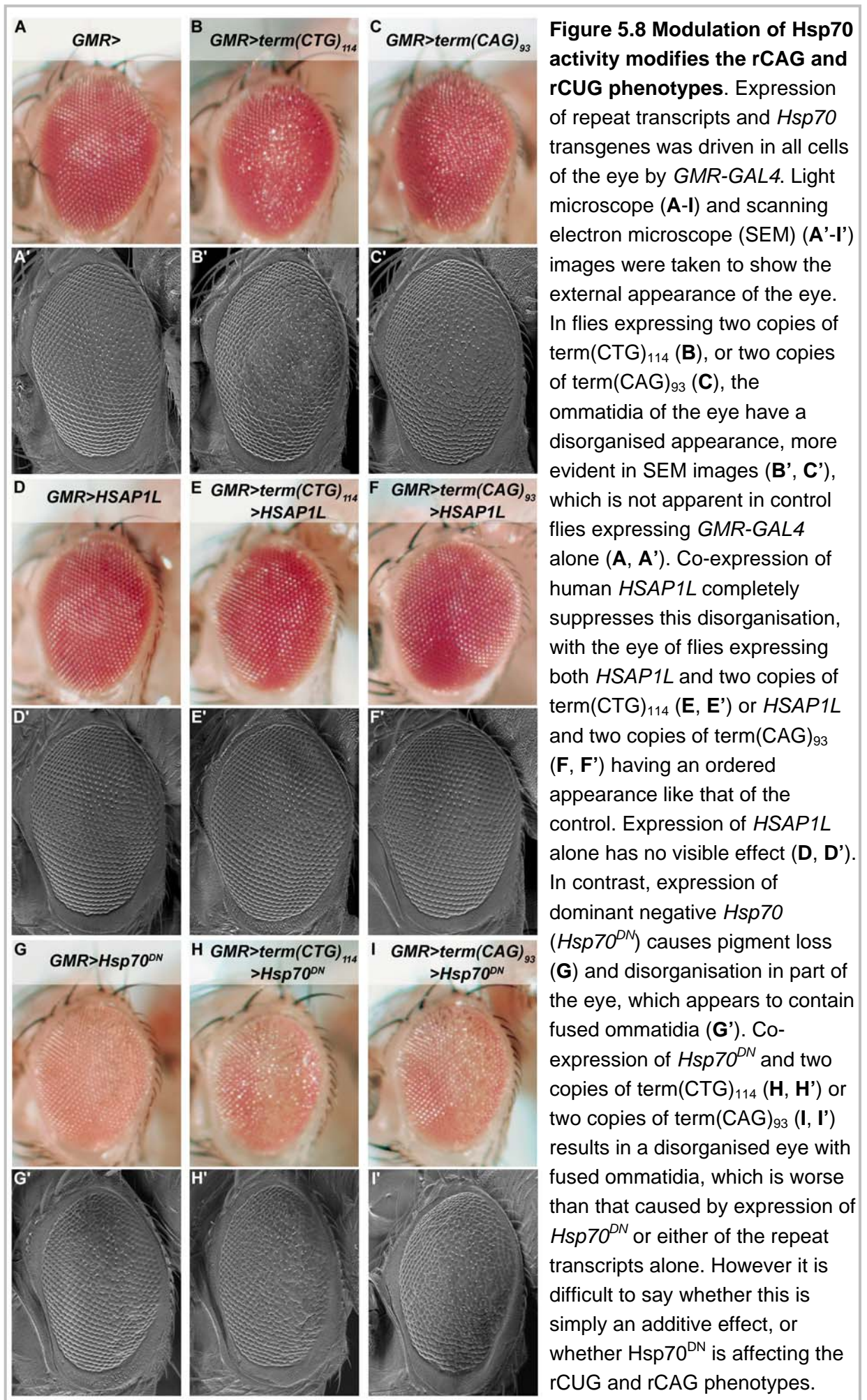
Figure 5.8 Modulation of Hsp70 activity modifies the rCAG and rCUG phenotypes. Expression of repeat transcripts and *Hsp70* transgenes was driven in all cells of the eye by *GMR-GAL4*. Light microscope (**A**-**I**) and scanning electron microscope (SEM) (**A'**-**I'**) images were taken to show the external appearance of the eye. In flies expressing two copies of term(CTG)$_{114}$ (**B**), or two copies of term(CAG)$_{93}$ (**C**), the ommatidia of the eye have a disorganised appearance, more evident in SEM images (**B'**, **C'**), which is not apparent in control flies expressing *GMR-GAL4* alone (**A**, **A'**). Co-expression of human *HSAP1L* completely suppresses this disorganisation, with the eye of flies expressing both *HSAP1L* and two copies of term(CTG)$_{114}$ (**E**, **E'**) or *HSAP1L* and two copies of term(CAG)$_{93}$ (**F**, **F'**) having an ordered appearance like that of the control. Expression of *HSAP1L* alone has no visible effect (**D**, **D'**). In contrast, expression of dominant negative *Hsp70* (*Hsp70^{DN}*) causes pigment loss (**G**) and disorganisation in part of the eye, which appears to contain fused ommatidia (**G'**). Co-expression of *Hsp70^{DN}* and two copies of term(CTG)$_{114}$ (**H**, **H'**) or two copies of term(CAG)$_{93}$ (**I**, **I'**) results in a disorganised eye with fused ommatidia, which is worse than that caused by expression of *Hsp70^{DN}* or either of the repeat transcripts alone. However it is difficult to say whether this is simply an additive effect, or whether Hsp70^{DN} is affecting the rCUG and rCAG phenotypes.
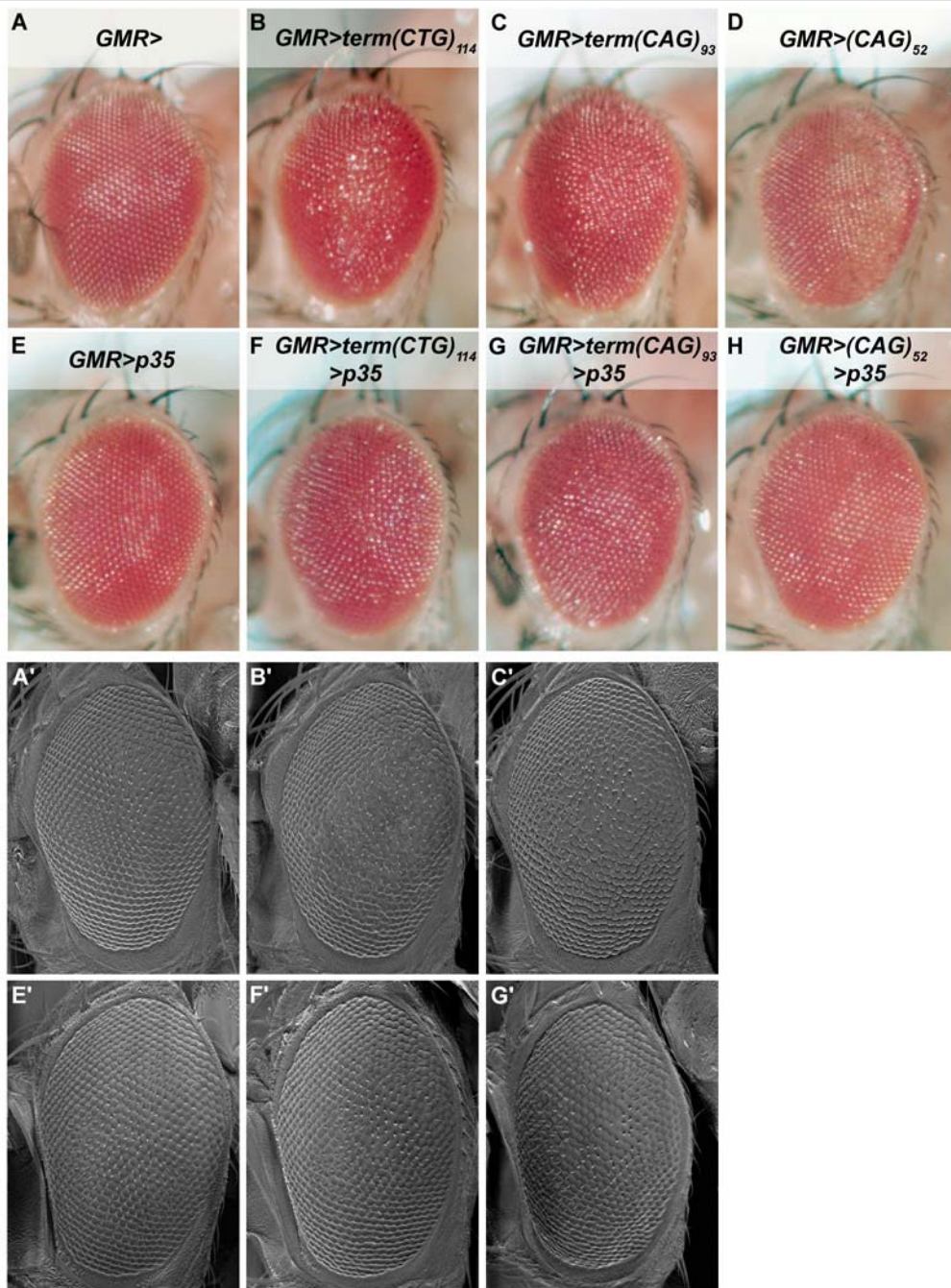
Figure 5.9 Expression of the apoptotic inhibitor p35 has a modest effect on the rCAG and rCUG phenotypes. Expression of repeat transcripts and p35 was driven in all cells of the eye by *GMR-GAL4*. Light microscope (**A**-**H**) and scanning electron microscope (**A'**-**C'**, **E'**-**G'**) images were taken to show the external appearance of the eye. In flies expressing two copies of term(CTG)$_{114}$ (**B**), or two copies of term(CAG)$_{93}$ (**C**), the ommatidia of the eye have a disorganised appearance, more apparent in SEM images (**B'**, **C'**), which is not apparent in control flies expressing *GMR-GAL4* alone (**A**, **A'**). Expression of UAS-p35 has a very mild effect on the organisation of ommatidia in the eye (**E**, **E'**). Upon co-expression with term(CTG)$_{114}$, the disorganisation of the ommatidia is decreased slightly (**F**,**F'**), compared to expression of term(CTG)$_{114}$ alone. The effect of this was to reduce the overall severity of the term(CTG)$_{114}$ eye phenotype, most notably reducing the number of flies with phenotypes at the severe end of the spectrum. A similar mild suppression of the term(CAG)$_{93}$ phenotype was also observed upon co-expression of p35; this was not obvious at the light microscope level (**G**), but can be seen in SEM images (**G'**). A mild polyglutamine phenotype caused by expression of (CAG)$_{52}$, characterised by pigment loss in the posterior of the eye (**D**) was more noticeably suppressed by co-expression of p35, with pigment restored to the eye (**H**).

with some flies showing a stronger phenotype than others. Co-expression of p35 appeared to suppress the stronger phenotypes most noticeably, shifting the range so that the average phenotype is slightly milder. However, this was a very mild effect, particularly in comparison to the results observed upon co-expression of Hsp70, which completely suppressed the rCUG and rCAG phenotypes. Thus it appears that whilst apoptosis is involved in the polyglutamine phenotype, it plays only a minor role in the rCUG and rCAG phenotypes.

Why do only some lines show a phenotype?
It seems likely that so few lines expressing term(CTG)$_{114}$ and term(CAG)$_{99}$ display a phenotype because a relatively high level of expression is required in *Drosophila* to induce toxicity. This notion is supported by the observation that when two lines that do not display a phenotype alone are combined, a phenotype then results upon expression of both copies simultaneously. To test whether those lines showing a phenotype are indeed expressing the repeat transcript at a higher level than those without a phenotype, quantitative (real-time) PCR could be used to compare the levels of repeat transcript in various lines. Also, varying the temperature can modulate the activity of the GAL4-UAS system. This could be used to increase the level of repeat transcript in lines which do not show a phenotype at 25 °C, to determine whether they do so when the expression level is increased. However, caution is required using this approach, as GMR-GAL4 alone causes a severe rough eye phenotype when the flies are exposed to a higher temperature (29 °C) throughout development. Performing crosses at 25 °C then shifting the adult flies to 29 °C to increase expression may prevent this effect; these experiments would need to be performed on control flies to determine the effect on the eye before further experiments involving the repeat transcripts were undertaken.

Discussion

The results described here demonstrate that rCUG and rCAG can induce a phenotype in *Drosophila* that is degenerative and cell-type specific. This is a significant finding, as earlier studies have not discovered any effect of rCUG repeats in *Drosophila*: in the DM1 model, no toxic effect was exerted by (CTG)$_{162}$ located in the *DMPK* 5' UTR [296], and in the SCA8 model, it was *SCA8* transcript itself that induced toxicity, independent of the presence of an expanded CUG repeat [187]. In

the case of rCAG, the only previously published attempts to investigate pathogenesis are the results obtained here, described in Chapter 3 and published [226]. Again, no phenotype was found, which in hindsight seems likely to be due to insufficient expression of the rCAG repeat transcript. It seems that a relatively high level of expression of the repeat transcripts is required to induce toxicity, as for rCUG, a phenotype was only observed in two lines out of 14 expressing one copy of term$(CTG)_{114}$, whilst for rCAG, a phenotype was only observed upon simultaneous expression of two copies of the term$(CAG)_{93}$ transcript. Whether this suggests that rCUG is more toxic than rCAG is unclear; it may simply be due to chance that the two lines with a phenotype expressing rCUG do so at a higher level than any of the rCAG lines. Further investigation into the relative levels of the repeat transcripts in each of the lines would reveal whether this is the case.

Is there a common pathogenic pathway?

From the results obtained here, it is difficult to determine whether there is a common pathogenic pathway shared by rCAG and rCUG-induced pathogenesis. The modifiers of the rCAG and rCUG phenotypes described so far seem to affect both phenotypes in the same manner and to roughly the same extent: Hsp70 overexpression can completely suppress both, Hsp70^{DN} had only a moderate effect, if any, on both phenotypes, and p35 had a very mild effect on both. In addition, Hsp70 aggregates were not detected in either case. Therefore none of the results obtained so far have suggested a difference between rCUG and rCAG pathogenesis.

Further investigation of modifiers of the rCAG and rCUG phenotypes may reveal differences between them. It will be particularly interesting to see whether *muscleblind* can modify one or both phenotypes. Muscleblind is implicated in DM1 pathogenesis via its binding to expanded CUG repeats in the *DMPK* transcript, and it modifies the *SCA8(CTG)$_{112}$ Drosophila* eye phenotype. Based on these observations, it would be predicted to be involved in rCUG pathogenesis and to modify the rCUG eye phenotype described here. Whether it will have the same effect on rCAG is less clear. Recent findings in cultured cells expressing interrupted $(CTG)_{960}$ or $(CAG)_{960}$ repeats demonstrated that both the rCUG and rCAG repeat transcripts formed nuclear foci, and that muscleblind binds to and is sequestered by transcripts containing either repeat. Furthermore, fluorescent recovery after photobleaching (FRAP) analysis revealed that the sequestration by CUG or CAG was roughly equal in strength, but only CUG expression resulted in splicing

alterations in the two downstream targets of muscleblind tested in the study [200]. The results of this are difficult to interpret, as they separate the sequestration of muscleblind by the repeat transcripts from the downstream effects on muscleblind targets; nonetheless, formation of nuclear foci and binding of muscleblind to rCAG repeats was clearly demonstrated. This suggests that rCAG pathogenesis could share parallels with DM1 pathogenesis, which appears to involve rCUG foci formation and sequestration by muscleblind. Therefore, future investigation into rCAG and rCUG pathogenesis in the *Drosophila* model described here may involve investigation of the presence of RNA foci, and modification of the rCAG and rCUG eye phenotypes by *muscleblind* alleles. In addition, the involvement of muscleblind could be investigated by staining eye sections with anti-muscleblind antibodies. This would be predicted to reveal muscleblind foci in flies expressing rCUG, and also possibly in flies expressing rCAG if muscleblind is also involved in this phenotype.

According to the RNA hypothesis outlined in Chapter 3, both rCAG and rCUG repeats are predicted to form a hairpin secondary structure, and this may be related to pathogenesis. In line with this prediction, rCAA repeats do not exhibit toxicity in this model system; however, more independent lines of flies expressing two copies of term(CAA)$_{99}$ and a quantitative assessment of RNA levels are required before a definite conclusion regarding the toxicity of rCAA can be reached.

Additional questions regarding the nature of the rCAG and rCUG phenotypes that warrant further investigation relate to the previous findings of rCGG toxicity in FXTAS. In this case, foci of the transcript containing the expanded rCGG repeat have not been described in patient tissue or in fly or mouse models of the disease; it is unclear whether this is because they are not present, or simply because experiments have not been undertaken to detect them. However, the presence of aggregates containing ubiquitin and components of the proteasome has been described in this disease [154,297]. It will therefore be interesting to see whether such aggregates are also present in flies expressing rCAG or rCUG. In the experiments described here, Hsp70 aggregates were not detected in flies expressing rCAG or rCUG as they are in flies expressing rCGG [188]. However, it is possible that this is due to technical reasons rather than because such aggregates are not present, as the background staining found here was significantly higher than that previously published. This may be due to differences in the batch of the anti-Hsp70 antibody used, or some difference in technique. Future experiments could be undertaken using the anti-

ubiquitin or anti-proteasome subunit antibodies previously described, with the aim of clarifying whether a similar pathogenic pathway operates in rCGG and rCAG/rCUG pathogenesis.

What do these results suggest about polyglutamine pathogenesis?
The question at the heart of this investigation is whether RNA contributes to polyglutamine pathogenesis in the human diseases. The findings described in Chapter 3 (and published [226]) appeared to rule out this possibility; however, the subsequent findings described here raise the possibility once more. The rCAG phenotype in *Drosophila* is clearly distinct from the polyglutamine phenotype and it appears to require a significantly higher level of expression before pathogenesis is evident. However, this does not necessarily mean that it is insignificant. The fly has a relatively short lifespan, and the human diseases require decades before pathology is evident; therefore it makes sense that a much higher level of expression of the pathogenic agent would be required in *Drosophila* before effects are observed. The rCAG phenotype appears to be restricted to a smaller range of cell-types than the polyglutamine phenotype, as pigment cells are unaffected. It is also a degenerative phenotype, as photoreceptor loss is more evident after 30 days than at day one. Therefore, these results raise the possibility that rCAG mediates the human polyglutamine diseases, and that the polyglutamine eye phenotype observed in *Drosophila* is not representative of the human phenotype. It may be that overexpression of toxic polyglutamine protein overwhelms the protective mechanisms of the cells in the *Drosophila* eye, whereas human cells are able to cope with chronic expression of a lower level of polyglutamine, sequestering it in protective aggregates. rCAG may be the true toxic agent, perhaps acting via the formation of foci as occurs in DM1 and DM2, sequestering vital RNA binding proteins. It is difficult to test this theory. One approach could be to express rCAG and polyglutamine in the nervous system of the fly to determine whether the relative toxicity of these two entities is preserved. It may be that rCAG shows increased toxicity when expressed only in neurons, whereas the effect of polyglutamine may be reduced, as its effect on other cell types would be lacking. Polyglutamine expression has been shown to affect pigment cells and photoreceptor neurons in the eye, whilst leaving the mechanosensory bristle cells unaffected [84]. rCAG expression does not appear to affect pigment cells, but photoreceptor neurons undergo degeneration. It is unknown whether the relative toxicities of polyglutamine and rCAG would be conserved if their expression were restricted to only one cell type.

An additional aspect of the rCAG phenotype that requires investigation is whether it is length-dependent. As the polyglutamine diseases show severity and age-of-onset dependent on repeat length, the hypothesis that RNA is the pathogenic agent requires confirmation that the rCAG phenotype is also dependent on repeat length. To investigate this aspect, flies expressing rCAG repeat transcripts containing a range of repeat copy numbers above the pathogenic threshold, and one below, could be generated. This could be examined using the eye, and tangential sections of the eyes of flies cut at varying ages to see when pathology is first apparent. Such experiments may reveal that age of onset of pathogenesis is indeed related to repeat length, which would support a role in the human diseases.

The nature of the rCAG and rCUG eye phenotypes also requires further investigation. The rough eye phenotype observed suggests that disruption to development is occurring, as the patterning of the cells of the eye is disordered. This potential effect on development may be distinct from the degeneration observed at the cellular level after ageing – indeed, the fact that no worsening of the rough eye phenotype is observed upon external examination suggests that this is the case. It may be that rCAG or rCUG expression is both affecting the development of the eye and inducing degeneration, and that there are separate pathways involved in these two effects. Further experiments to investigate these possibilities could involve examination of the patterning of the eye in flies expressing rCAG and rCUG to determine whether development is affected. It may also be informative to examine the two lines of flies expressing rCAG and one line expressing rCUG where no external phenotype is evident, to see whether degeneration is occurring at the cellular level. Temperature affects the activity of the GAL4-UAS system [288], and this aspect of the system could therefore be used to modulate expression levels of the repeat transcripts at various stages. In the FXTAS *Drosophila* model, crosses were performed at 18 °C to decrease the expression of the repeat transcript, and then adult flies shifted to 29 °C to increase expression. This removed the effect of the rCGG transcript on development, allowing its degenerative effect to be observed in isolation [188]. A similar approach could be used for the rCAG and rCUG flies, to reduce the effect of the repeat transcripts on development and allow examination of the degenerative phenotype alone.

Another aspect of the rCUG and rCAG phenotypes that requires examination is their cell-type specificity. The GAL4-UAS system lends itself to investigation of this issue, as different drivers can be used to express the repeat transcripts in different tissues to compare their effects on different cell types. Previous investigations into the cell type-specificity of polyglutamine toxicity in *Drosophila* [80] or the specificity or rCGG-mediated degeneration [188] used *dpp-GAL4* to drive expression in epithelial cells of the imaginal discs or *elav-GAL4* to drive expression in all neurons, to demonstrate that both polyglutamine and rCGG-mediated toxicity is neuron-specific. A similar approach using the same drivers could be used to determine whether this is also the case for rCAG and rCUG.

Chapter 6 – Discussion

Summary of Results

The primary aim of this thesis was to investigate the hypothesis that RNA contributes towards polyglutamine pathogenesis by inducing toxicity in a similar manner to the rCUG repeat that causes DM1. Initial experiments were designed to investigate this by comparison of a CAA and a CAG repeat, as an RNA transcript containing a CAG can form a hairpin like the rCUG repeat in DM1, but a CAA repeat is not predicted to do so. In addition, an untranslated CAG repeat was also tested. Results from these experiments suggested that RNA does not contribute to polyglutamine pathogenesis in the *Drosophila* system. However, further investigation revealed that rCAG can induce pathogenesis in *Drosophila*, as can rCUG. In this model system, a higher level of expression of rCAG than translated CAG is required to induce a phenotype in the fly. Pathogenesis induced by rCAG in this model does not occur when the repeat is translated into polyglutamine because the resulting polyglutamine expression causes cell loss before the threshold level of expression required for rCAG pathogenesis to occur is reached. Thus, rCAG is not contributing to the polyglutamine phenotype in *Drosophila*, but it can induce pathogenesis. In addition to being able to induce pathogenesis, the phenotype induced by rCAG is degenerative, and shares some similarities with rCGG-mediated pathogenesis (i.e. Hsp70 suppression). These observations support the notion of a contribution by RNA to pathogenesis in the human repeat expansion diseases, as well as the possibility that there is a pathogenic pathway common to all of the diseases caused by repeat expansion, both translated and untranslated.

Secondary avenues of investigation undertaken in this thesis included preliminary analysis of polyleucine and polyalanine toxicity in *Drosophila*, and also a preliminary investigation into the possibility of DNA repair or axon transport defects in the polyglutamine disorders. No evidence in support of the involvement of these pathways in pathogenesis was obtained; however, further experiments are required before they can be discounted. In the case of polyalanine, expression of polyalanine protein in the *Drosophila* eye was not achieved; despite expression of a GCA repeat-transcript, no protein could be detected on a western blot or using immunostaining. Furthermore, a subsequent report of polyalanine toxicity in *Drosophila* has revealed

that polyalanine does induce a phenotype when expressed in the *Drosophila* eye [229], supporting the conclusion that no protein was expressed in this study. Expression of a CTG repeat encoding polyleucine did appear to induce toxicity in the eye; however, as an untranslated CTG repeat was also found to induce toxicity, and cause a similar phenotype, pathogenic RNA rather than polyleucine toxicity cannot be ruled out as the cause.

Implications for polyglutamine pathogenesis

Further investigation is required to determine whether RNA does contribute towards, or is responsible for, pathogenesis in the polyglutamine diseases. It may be that the *Drosophila* eye is an accurate representation of the relative toxicities of rCAG and polyglutamine, and as in the fly eye, polyglutamine kills human neurons before RNA toxicity can occur. Alternatively, human cells may be more resistant to polyglutamine-induced toxicity, perhaps handling the toxic protein by sequestering it into aggregates, and the real pathogenic insult may be RNA-mediated. In the *Drosophila* eye the rCAG phenotype is milder and restricted to a narrower range of cell types than the polyglutamine phenotype (pigment cells are unaffected), features that suggest the rCAG phenotype may better represent the human disease state than the polyglutamine phenotype does. These findings suggest that RNA warrants further investigation as a pathogenic entity in the polyglutamine diseases. Such investigations could include characterisation of the RNA phenotype in *Drosophila* as well as investigation of RNA pathogenesis in vertebrates.

Recent studies into polyglutamine toxicity have provided evidence in support of protein context playing an important role in pathogenesis. Examples of this include ataxin-1 phosphorylation at a residue outside of the polyglutamine repeat, which results in 14-3-3 stabilisation and accumulation of ataxin-1. This phosphorylation and interaction with 14-3-3 appears to be important for polyglutamine pathogenesis in mouse and fly models of SCA1 [125,126]. In addition, other studies have identified brother of ataxin-1 (Boat) and the transcription factor senseless/Gfi-1 as ataxin-1 binding partners [127,192]. Interactions with these proteins, which normally play important roles in Purkinje cells, may help account for the Purkinje-cell specificity of SCA1. Insight into cell-type specificity has also been attained for SCA7, which features retinal degeneration characterised by loss of photoreceptor rods, cone cells

and ganglion cells not seen in the other polyglutamine disorders. ataxin-7 interacts with CRX, a transcription factor that regulates several eye-specific genes. Expansion of the polyglutamine tract in ataxin-7 alters CRX function, causing a decrease in levels of CRX-responsive genes. Mutations in CRX cause cone-rod degeneration similar to that seen in SCA7 mice, suggesting that the alteration in CRX function induced by polyglutamine expansion in ataxin-7 is responsible for the retinal phenotype in SCA7 [301]. A normal binding partner of huntingtin also appears to be important for HD pathology. p53, which binds to huntingtin with an expanded polyglutamine tract in an enhanced manner, appears to be activated in HD, which may mediate mitochondrial dysfunction. Deletion of p53 in *Drosophila* and mouse models of HD suppresses the polyglutamine phenotypes, suggesting that p53 is a critical mediator of pathogenesis. This effect appeared to be specific to huntingtin, as a similar interaction was not found for ataxin-1 [292]. In addition to these examples, an interaction between the androgen receptor and androgen is required for SBMA pathogenesis. In a *Drosophila* model of SBMA, a phenotype was only apparent in flies exposed to androgen receptor ligands [203]. Similar results were obtained from a mouse model, where significant pathology was not observed in females or castrated males [204]. Thus it seems that androgen is required, causing translocation of the AR into the nucleus, for AR pathology to occur – the presence of a polyglutamine expansion in the AR is not sufficient to cause disease in this model.

These studies provide evidence that protein sequences outside of the polyglutamine tract, as well as protein-protein interactions, are critical for pathology in the polyglutamine diseases. In contrast, a recent mouse model of SBMA revealed that an aspect of the SBMA phenotype previously presumed to be related to the normal function of the protein is actually caused by the polyglutamine expansion. An SBMA knock-in mouse model, in which $(CAG)_{113}$ was inserted into the mouse *AR* gene, displayed androgen insensitivity as is seen in the human disease. However, in these mice, testicular pathology was distinct from that caused by an *AR* loss-of-function mutation in *testicular feminisation mutant* mice, suggesting it is mediated by expanded polyglutamine rather than loss of AR function [302]. In support of this conclusion, a yeast artificial chromosome (YAC) transgenic mouse model of Huntington's disease displayed similar testicular pathology [303], suggesting a common mechanism of toxicity based around the polyglutamine expansion.

Thus, whilst it is clear that the primary cause of disease is the CAG repeat expansion, and some aspects of pathology common to all of the diseases are polyglutamine-mediated, the above examples provide verification of the importance of protein-specific interactions in polyglutamine pathogenesis. It is difficult to reconcile these findings with an RNA-based model of pathology. One possibility is that both RNA and protein are responsible for the phenotypes and they are an additive effect of polyglutamine toxicity and RNA-mediated toxicity. Some of the common properties of the disorders may be RNA mediated, whilst the differences may be due to effects mediated at the protein level. In addition, disease-specific differences could be caused by protein-RNA interactions that are specific for the disease-related transcripts. There is no direct evidence in support of protein and RNA-mediated pathology; however, if RNA alone cannot account for the above observations, but was proven to play a role in pathogenesis, this explanation may account for them. Other proposed pathways of polyglutamine pathogenesis such as transcriptional disruption could be partially accounted for by an RNA mechanism; for example, reported decreases in global transcription [266,304-306] may be due to the sequestration of transcription factors by the repeat-containing RNA, in addition to the polyglutamine protein. Indeed, the DM1 example supports this suggestion, as in this disorder, alteration in transcription caused by sequestration of proteins by the expanded repeat-containing RNA transcript appear to be responsible for pathogenesis. Sequestration of the splicing factor Muscleblind and the transcription factor Sp1 have been implicated in RNA pathogenesis in DM1 [182,307]; there is evidence that Sp1-mediated transcription is also disrupted in polyglutamine pathogenesis [111], and that muscleblind can bind to rCAG repeats [200], and so could plausibly play a role. However, evidence that RNA plays a role in polyglutamine pathogenesis is required before investigation into potential mechanisms such as these is undertaken.

Is *Drosophila* an appropriate model for polyglutamine disease?

An important question raised by these studies is whether the *Drosophila* model described here represents an accurate model of the human polyglutamine disease state. Polyglutamine appears to be quite toxic when expressed in the fly eye, compared to rCAG and rCUG, which have milder effects. For a given amount of CAG transcript, the threshold for toxicity is lower for the encoded polyglutamine than it is

for the rCAG transcript itself, meaning that in this system, polyglutamine appears to kill the cells before the transcript encoding it can induce toxicity. It is not known whether this is also the case in human neurons.

The results described in Chapter 4, where modifiers of the *Drosophila* SCA1 eye phenotype failed to modify the eye phenotype in the polyglutamine model described here, suggest that different pathways of toxicity are present in this model compared to expression of polyglutamine in the context of a full-length protein. Indeed, previous studies have shown that protein context can dramatically alter the effects of polyglutamine expression in the fly, with the addition of a short peptide sequence decreasing toxicity of a given protein and expression of an expanded polyglutamine tract in an unrelated protein having no toxic effect [84]. These observations suggest that a polyglutamine tract in the context of a full-length, disease-related gene may better represent the human disease state; it is possible that the reduced toxicity of such a protein would allow RNA pathogenesis to occur in cells expressing the repeat transcript before they succumb to polyglutamine toxicity. The same events may also be occurring in the mouse: many mouse models of polyglutamine disease use short protein fragments containing long polyglutamine tracts, expressed at high levels, to induce a phenotype within the lifespan of the mouse. For both mouse and fly models, it is possible that polyglutamine toxicity is killing the cells via a pathway that is not invoked in the human disease state, and therefore the study of these models may not provide information relevant to the human diseases.

Another issue regarding the *Drosophila* model used here is whether the eye is an appropriate tissue in which to study pathogenesis. A recent investigation into this question examined genes previously identified as modifiers of the polyglutamine eye phenotype in *Drosophila*. These were tested to determine whether they modify polyglutamine toxicity in the postmitotic neurons of the brain in the same manner. Modifiers from the major classes previously identified using the eye, namely molecular chaperones and antiapoptotic proteins, also modified the phenotype in the fly brain, suggesting that common pathways mediate polyglutamine toxicity in the eye and brain. However, three enhancers of the polyglutamine eye phenotype had no effect on polyglutamine toxicity in the brain, suggesting that there are differences in the two systems [300]. Therefore, findings in the eye such as those described here for RNA should be verified using brain-specific drivers to determine whether similar effects are induced in the postmitotic neurons of the brain.

A *Drosophila* model of RNA-mediated neurodegeneration

In addition to providing support for the RNA hypothesis, the finding that rCAG and rCUG can induce pathogenesis in the fly is significant, as it provides a useful tool to investigate RNA-mediated neurodegeneration in those disorders where the repeat is untranslated. Currently there is no mouse model of SCA8, and the SCA8 *Drosophila* model reported equal toxicity with a CTG repeat in the non-pathogenic range, suggesting the *SCA8* transcript itself is responsible for pathogenesis. In the model described here, the transcript itself does not appear to induce toxicity, as so far no toxicity has been observed with an expanded CAA repeat in the same context. This suggests that in this model, the rCAG or rCUG repeat is responsible for the phenotypes observed, rather than some other property of the transcript.

In contrast to the findings for rCUG in this study, a previous attempt to generate a *Drosophila* model of DM1 was unsuccessful, as no toxicity was observed upon expression of an rCUG repeat. The results obtained here, particularly the observation of a phenotype in only two lines out of 14, suggest that the reason for this lack of phenotype may simply be that the level of expression was insufficient. Alternatively, there may be some property of the transcript containing the repeat in the DM1 model that modulated its toxicity in the fly. In the model described here, repeat toxicity is separated from gene context, allowing the intrinsic toxicity of the repeat to be investigated. The rCUG phenotype in the fly provides a model with which to study mechanisms of pathogenesis of expanded CTG repeats that will be relevant for SCA8, DM1, and possibly HDL2. The rCAG phenotype will allow investigation of SCA12 pathogenesis, in addition to any relevance for the polyglutamine disorders. Thus the fly models of rCAG and rCUG pathogenesis described here may be useful for investigation of pathogenesis in disorders caused by untranslated repeats.

In addition to providing a tool with which to investigate rCAG and rCUG pathogenesis, use of the *Drosophila* system described here will allow the amount of overlap in pathogenesis in the disorders caused by different untranslated repeats to be investigated. Modifiers of either phenotype, including those previously identified in the SCA8 study, can be tested on the rCAG and rCUG phenotypes to determine the extent of similarity between the two. This will allow the identification of common and distinguishing pathways in these two groups of disorders. Furthermore, aspects of pathogenesis shared with other repeat diseases, including FXTAS and polyglutamine disease, could also be examined using this approach.

The complete suppression of the rCAG and rCUG phenotypes by Hsp70 is intriguing. Hsp70 is also able to suppress the rCGG eye phenotype in *Drosophila* [188], and this suppression of RNA-mediated phenotypes is puzzling, as the expanded repeat is not translated and no mutant protein is produced to misfold. This suppression suggests that protein misfolding plays a role in RNA pathogenesis; Jin *et al.* and others [188,209] have suggested that the expanded repeat induces misfolding of other proteins associated with the transcript, triggering the cellular response to misfolding. In addition, it was pointed out that molecular chaperones also play a role in the normal trafficking of proteins within the cell, and this may be disrupted by the repeat expansion. It has also been suggested that the Hsp70 and ubiquitin-positive inclusions found in flies expressing rCGG may correspond to an enhanced accumulation of these proteins at a site related to normal function of the protein or RNA–protein complex [209]. These suggestions may also apply to rCAG- and rCUG-mediated pathogenesis. It will be interesting to determine whether ubiquitin/proteasome components form aggregates in the rCAG and rCUG flies, which may provide evidence in support of this suggestion. Such aggregates would also suggest that the pathogenic pathways in the rCAG, rCUG and rCGG flies are common, or contain common components.

Future Experiments

The results described here open up the possibility that RNA might contribute to polyglutamine pathogenesis by demonstrating that rCAG repeats can induce degeneration. However, more information must be obtained to determine whether RNA does contribute to pathogenesis. It will be important to validate any *Drosophila* rCAG pathogenic pathways as relevant to the human diseases by identifying as-yet unknown aspects of this pathway that can then be screened for in the human diseases.

The fly model described here presents a useful tool that can be used to investigate RNA pathogenesis. There are many issues that remain to be addressed. Further characterisation of the RNA phenotypes described here is required: more term(CAA)$_{94}$ lines need to be generated to confirm that rCAA has no effect in *Drosophila*, and the cell-type specificity of rCAG and rCUG should be investigated by

expression of term(CAG)$_{93}$ and term(CTG)$_{114}$ specifically in neurons and also non-neuronal cells. Further experiments suggested at the end of Chapter 5 should be undertaken to determine whether expression of rCAG or rCUG is affecting development of the *Drosophila* eye, and whether any developmental effects are separate to the degenerative effects of the repeat transcripts. Whether the rCAG and rCUG phenotypes are dependent on repeat length also requires further investigation.

An additional aspect of the phenotypes that remains to be examined is whether RNA foci containing the repeat transcripts and also aggregates of ubiquitin and/or proteasome components are present. Furthermore, if both of these are found, it would be interesting to see whether they co-localise. So far, RNA foci containing rCUG have been described in DM1, and ubiquitin/proteasome aggregates have been described in FXTAS, but it is unknown whether foci are present in FXTAS and aggregates present in DM1. The presence of both foci and aggregates in the rCAG and rCUG flies would suggest that there is a common pathway of pathogenesis in the RNA-mediated disorders such as DM1 and FXTAS, of which foci and aggregate formation is a feature. The parallels between these disorders and polyglutamine toxicity, which also features ubiquitin/proteasome positive aggregates, would further strengthen the case for a unifying pathogenic pathway in the translated and untranslated repeat disorders.

An advantage of the *Drosophila* system is the ease with which unbiased genetic screens can be performed to identify novel pathways involved in a given phenotype. This approach could be utilised to identify modifiers of the RNA phenotypes described here. Initially, candidates identified in the SCA8 screen could be tested [187]. These are neuronally expressed RNA binding proteins encoded by *muscleblind*, *staufen*, *split ends*, and *CG3249*. As they modified the SCA8 eye phenotype, it would be interesting to see whether they also modify the rCUG eye phenotype described here. In addition, they could be tested to see whether they also modify the rCAG phenotype; this may provide information regarding the extent of similarity between the pathogenic pathways mediated by the repeats. In addition, the rCUG phenotype could be used in a screen to identify novel modifiers, as it is more severe (and therefore easier to visualise) than the rCAG phenotype. Modifiers identified could be relevant in DM and SCA8 pathogenesis. In addition, these modifiers could be tested on the rCAG phenotype, and also the rCGG phenotype, to determine whether they have the same effects on all RNA phenotypes in the fly. This

would provide further information regarding the existence of a common pathogenic pathway in the RNA-mediated diseases. Furthermore, such modifiers could also be tested on a polyglutamine fly model to identify commonalities in pathogenesis. However, a milder polyglutamine phenotype, perhaps one in which the polyglutamine tract is in the context of a full-length disease gene, would be required for this purpose. RNA may possibly contribute towards the phenotype in such a model, whereas it appears not to contribute towards pathogenesis in the polyglutamine model used in this study.

In conclusion, the model described here has been utilised to address the possibility that RNA contributes towards polyglutamine pathogenesis. The results obtained suggest that this could be the case, and provide a useful tool to investigate pathways involved in RNA pathogenesis and how they pertain to polyglutamine pathogenesis.