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Perturbation of the Akt/Gsk3-β signalling pathway is common to *Drosophila* expressing expanded untranslated CAG, CUG and AUUCU repeat RNAs

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Recent evidence supports a role for RNA as a common pathogenic agent in both the 'polyglutamine' and 'untranslated' dominant expanded repeat disorders. One feature of all repeat sequences currently associated with disease is their predicted ability to form a hairpin secondary structure at the RNA level. In order to investigate mechanisms by which hairpin-forming repeat RNAs could induce neurodegeneration, we have looked for alterations in gene transcript levels as hallmarks of the cellular response to toxic hairpin repeat RNAs. Three disease-associated repeat sequences—CAG, CUG and AUUCU—were specifically expressed in the neurons of *Drosophila* and resultant common transcriptional changes assessed by microarray analyses. Transcripts that encode several components of the Akt/Gsk3-β signalling pathway were altered as a consequence of expression of these repeat RNAs, indicating that this pathway is a component of the neuronal response to these pathogenic RNAs and may represent an important common therapeutic target in this class of diseases.

INTRODUCTION

Despite the identification of expanded repeat sequences as disease-causing mutations two decades ago (1,2), the mechanisms by which this class of mutations exert their pathogenic effect still remain unclear. Repeat expansions that cause dominantly inherited diseases have been classified into two distinct groups of diseases, based upon the location of the expanded repeat tract within the gene. One group is the result of the expansion of trinucleotide repeat tracts within the coding regions of a number of unrelated genes. The most common such expansion is of a CAG repeat encoding glutamine, resulting in the 'polyglutamine diseases' which include Huntington's disease (HD), spinal bulbar muscular atrophy, dentatorubral-pallidoluysian atrophy and spinocerebellar ataxia (SCA) types 1, 2, 3, 6, 7 and 17 (Fig. 1). The polyglutamine diseases do not appear to be the result of a simple loss-of-function mechanism, since they show dominant inheritance and share a number of clinical features. This phenotypic overlap suggests that there are likely to be pathogenic mechanisms involved which are not gene-specific (3). There is a large amount of evidence to support a role for the polyglutamine peptides themselves in pathogenesis, including the demonstrated intrinsic toxicity of polyglutamine peptides in transfected cells (4–6) and *Drosophila* models (7,8).

There are another nine dominantly inherited expanded repeat diseases that are caused by the expansion of repeat tracts within the non-coding regions of genes (the 'untranslated repeat' diseases, Fig. 1). To date, expansions of tri-, tetra- and penta-nucleotide repeats of this class have been identified as the mutations causing myotonic dystrophy (DM) types 1 and 2 (DM1 and 2), HD like-2 (HDL-2), fragile X tremor ataxia syndrome (FXTAS) and spinocerebellar ataxia types 8, 10 and 12. Despite the apparent inability of these expanded repeat sequences to code for polyglutamine, in

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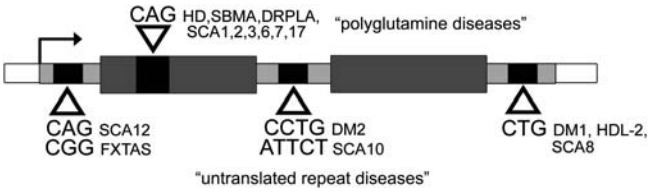


Figure 1. Schematic representation of the mutations currently known to cause dominant expanded repeat diseases. Expansions can occur either within the coding region of the gene, such as the CAG repeat expansions resulting in the 'polyglutamine' diseases (above), or within non-coding regions, resulting in the 'untranslated' repeat diseases (below). Expansions of tri-, tetra- and penta-nucleotide repeat sequences have been identified as pathogenic mutations in these diseases. While SCA8 has typically been considered an 'untranslated' repeat disease, polyglutamine aggregates have also been detected in both a mouse model and human autopsy tissue (51). DM, myotonic dystrophy; DRPLA, dentatorubral-pallidoluysian atrophy; FXTAS, fragile X tremor ataxia syndrome; HD, Huntington's disease; HDL-2, Huntington's disease like-2; SBMA, spinal bulbar muscular atrophy; SCA, spinocerebellar ataxia.

several cases there is significant phenotypic overlap with the polyglutamine diseases, suggesting that a common pathogenic mechanism may play a role in both classes of disease. Furthermore, given that there are diseases caused by the expansion of repeat sequences of differing sequence composition and that the expansions reside in functionally distinct genes, it seems likely that some common property of the expanded repeat tracts may be a component of pathogenesis in both sets of diseases.

One common property of disease-causing expanded repeat sequences is the predicted ability of their RNA transcripts to form strong hairpin secondary structures. In the case of CNG repeats, this structure is formed through binding between C and G residues, with a mismatch every third base (9,10). Trinucleotide repeats of this type account for the majority of the expanded repeat diseases, including all of the polyglutamine diseases. Expanded CCTG repeats, the mutation responsible for DM2, have been predicted to form a similar structure to CUG repeats *in vivo* (9), while the penta-nucleotide AUUCU repeat which is expanded in SCA10 is predicted to form an unusual anti-parallel hairpin structure, with a C-C mismatch every fifth base and an equal ratio of A-U/U-U mismatches (11).

A central role for hairpin RNA-mediated pathogenesis was first suggested in DM1 and 2. In both cases, the expanded repeat tract binds and sequesters the splicing factor muscleblind-like (MBNL) (12). Sequestration of MBNL is thought to be pathogenic via both the loss of MBNL-splicing activity and the associated mis-regulation of splicing by the antagonistic splicing factor CUG-binding protein (CUG-BP), since over-expression of human CUG-BP is sufficient to recapitulate a number of features of DM in a mouse model (13). A number of MBNL-splicing targets, including chloride channel 1, troponin T type 3 and insulin receptor, are mis-spliced in both DM1 and 2 individuals as a result of the inappropriate interaction of the expanded repeat-containing RNA and endogenous RNA-binding proteins (14,15).

A similar mechanism of pathogenesis has also been suggested for FXTAS, which is caused by a CGG expansion in the 5′UTR of the *Fragile X mental retardation 1 (FMR1)* gene within the pre-mutation range (55–200 repeats) for fragile X syndrome. Individuals with FXTAS also show inclusions which contain MBNL along with several intermediate filament proteins, including lamins A/C and internexin, and heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2) (16). In cells from SCA10 individuals and transgenic mice ectopically expressing the expanded intronic AUUCU repeat tract, inclusions containing both the expanded repeat RNA and the RNA-binding protein hnRNP K have been identified. The sequestration of hnRNP K within these aggregates is suggested to result in an increase in translocation of PKCδ to the mitochondria, resulting in induction of apoptosis (17). The localization of this repeat sequence with MBNL has not been demonstrated to date.

Recent evidence also supports a role for RNA-mediated pathogenesis in the polyglutamine diseases. In a *Drosophila* model of SCA3 (18), altering expression levels of the *Drosophila* muscleblind (Mbl) splicing factor was found to modify phenotypes associated with expression of a pure CAG repeat tract, but not a mixed CAG/CAA repeat. This result suggests that an interaction between CAG repeats and Mbl is occurring at the RNA level and that the secondary structure of the RNA species is important for this interaction. Nevertheless, binding of MBNL to expanded CAG repeats has been shown not to elicit the same splicing defects as binding to CUG repeats in transfected cells and therefore the biological outcome of this interaction remains unclear (19). It does, however, suggest a pathogenic role for expanded repeat-containing RNA in the polyglutamine diseases. There is therefore mounting evidence of a role for expanded repeat-containing RNA species in pathogenesis of both the untranslated expanded repeat diseases and the polyglutamine diseases.

Drosophila is well established as a model for polyglutamine disease (7,20–22) and has also been demonstrated to exhibit neurodegenerative phenotypes resulting from expression of expanded repeat RNAs (18,23–25). In this study, we have used a *Drosophila* model of expanded repeat pathogenesis to investigate common transcriptional changes in neurons resulting from expression of different disease-associated RNA repeat sequences with the aim of identifying common cellular changes resulting from the intrinsic toxicity of repeat RNAs. The repeat sequences investigated—CAG, CUG and AUUCU—represent the mutations responsible for the majority of the dominant expanded repeat diseases, including all of the polyglutamine diseases as well as the untranslated repeat diseases DM1, HDL-2, SCA8, 10 and 12.

There are several unique components to the analyses performed in this study. First, it describes the only direct comparison of the transcriptional outcomes of expression of CAG and CUG expanded repeat RNAs. Furthermore, it is also the only study thus far to examine the general transcriptional response to neuronal expression of CUG repeat RNA. Since neuronal toxicity resulting from CUG repeat expansion is a feature of DM1 and may also play a role in HDL-2, defining the pathways involved in CUG repeat-mediated neuronal toxicity is of great importance. Similarly, while toxicity of CAG repeat RNA has now been described in several model systems (18,26,27), the cellular basis of this toxicity has not yet been extensively explored. Additionally, this is also the first report of the effects of expression of the SCA10-associated expansion in a *Drosophila* model.

RESULTS

We have previously reported a *Drosophila* expanded repeat disease model utilizing the *UAS-GAL4* system to drive expression of different expanded repeat sequences in a tissue-specific manner (8). In this model, repeats are expressed in the context of a short peptide sequence (7) which is unrelated to the context in any of the expanded repeat diseases (Fig. 2A). In contrast to most other models described in the literature, this model therefore allows investigation of the intrinsic toxicity of expanded repeat sequences, which is likely to account for common features observed in these diseases.

This *Drosophila* model was previously used to investigate the contribution of hairpin-forming RNA to toxicity of polyglutamine tracts (8) by comparing the phenotypes seen when CAG repeats, which encode polyglutamine and are able to form an RNA hairpin secondary structure, or CAA repeats, which encode polyglutamine but are unstructured at the RNA level, are expressed in the *Drosophila* eye. The results obtained in this investigation suggested that the majority of the phenotype seen in flies expressing polyglutamine is likely to be the result of the polyglutamine peptide itself and not the repeat-containing RNA. However, subtle cellular changes resulting from expression of RNA species, which may result in cell death over an extended period of time, were not further investigated (Fig. 2K). In this study, we look specifically at cellular changes resulting from neuronal expression of disease-causing untranslated expanded repeat sequences.

Expression of expanded, untranslated CAG, CAA, CUG and AUUCU repeat RNAs in *Drosophila*

In order to investigate the intrinsic toxicity of different disease-associated expanded repeat RNA sequences, a set of constructs were generated in which a termination codon is inserted ahead of the repeat sequence such that the repeat tract is effectively shifted into the 3′UTR of the transcript in each case (Fig. 2F). Expression of this construct containing a hairpin-forming CAG repeat RNA (rCAG) does not result in a phenotype when expressed in the *Drosophila* eye, as previously reported (8). Similarly, expression of untranslated hairpin-forming CUG repeat RNA (rCUG) or unstructured CAA repeat RNA (rCAA) does not alter the appearance of the *Drosophila* eye.

An identical construct was also generated with an insertion of 65 interrupted repeats of the penta-nucleotide AUUCU sequence responsible for the rare spinocerebellar ataxia, SCA10. The sequence of the repeat tract in this construct is $(ATTCT)_{20}$ ACTCT $(ATTCT)_{23}$ ATTCC $(ATTCT)_{15}$ ATTTT $(ATTCT)_{7}$, surrounded by 141 bp of sequence from the region of intron 9 of human *ataxin-10*. Although the repeat number in this construct is lower than in the CAG, CUG and CAA repeat constructs, in each case the total size of the repeat tract is ∼300 bp and therefore, in the cases where a secondary structure is predicted, the resulting hairpin RNAs will be of similar size.

In order to increase the expression level of expanded repeat RNA, *Drosophila* lines carrying four independent insertions of each of these repeat constructs were subsequently generated. Expression of four independent insertions of untranslated

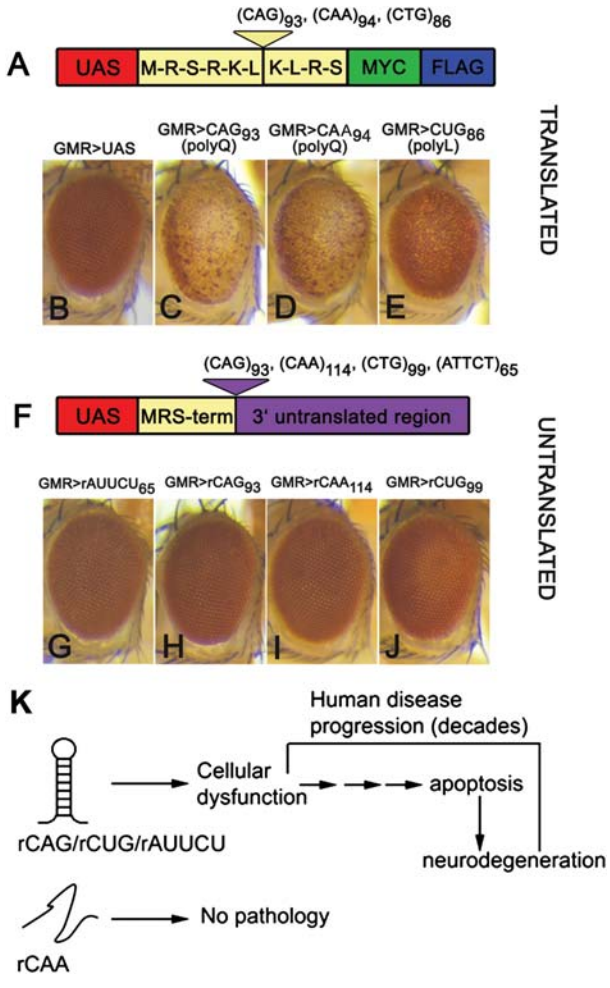


Figure 2. Schematic representation of repeat constructs and effect of expression of translated and untranslated expanded repeat constructs on the external appearance of the *Drosophila* eye. (**A**) Translated repeat constructs: similarly sized expanded repeat tracts are inserted downstream of UAS sites to allow expression under the control of a GAL4 driver. The repeats are flanked by six amino acids on the N-terminal side and four on the C-terminal, and a myc/flag epitope tag is located downstream. Repeat sequences are CAA and CAG, both of which encode polyglutamine, and CUG which encodes polyleucine. CAG and CUG repeat RNAs are predicted to form a hairpin secondary structure, while the CAA repeat RNA is not. (**B**) Expression of UAS sequences alone results in an eye of wild-type appearance. (**C** and **D**) Expression of polyglutamine encoded by either a CAG or CAA repeat tract results in an indistinguishable loss of pigment phenotype in the *Drosophila* eye (8). (**E**) Expression of polyleucine encoded by a CUG repeat tract results in a mild disruption to the patterning of the eye. (**F**) Untranslated repeat constructs: constructs in which the repeat tract is not translated were generated by insertion of a stop codon upstream of the repeat tract. (**G**–**J**) Expression of four transgene insertions of any of the untranslated expanded repeat sequences does not cause a visible disruption to the external patterning of the *Drosophila* eye. (**K**) Proposed mechanism of RNA-mediated pathogenesis in the expanded repeat diseases. Expression of expanded, untranslated CAG, CUG or AUUCU repeat-containing RNAs, all of which are predicted to form a hairpin secondary structure, results in cellular dysfunction which, over an extended period of time, leads to apoptosis and neurodegeneration. The expanded rCAA repeat RNA used in this study is not able to form a hairpin secondary structure and therefore should not induce cellular dysfunction in this manner.

CAG, CUG, CAA or AUUCU repeat RNAs in the *Drosophila* eye does not result in any external phenotype (Fig. 2G–J). This is in contrast to the phenotypes observed when a single

transgene insertion of a translated CAG or CAA repeat tract, both of which encode polyglutamine, or a translated CUG repeat tract, encoding polyleucine, are expressed in the *Drosophila* eye (Fig. 2B–E). Similarly, no obvious defects were observed in newly eclosed flies when any of the untranslated repeat RNA constructs were expressed pan-neuronally.

Detection of neuronal transcriptional changes resulting from expression of expanded repeat RNA

While no phenotype was observed when rCAG, rCUG or rAUUCU repeat RNAs were expressed in the nervous system of newly eclosed flies, the presence of expanded repeat transcripts was detected in all cases (Supplementary Material, Fig. S1A and C). To investigate the transcriptional effects of expression of different expanded repeat sequences in the neurons of *Drosophila*, microarray analyses were performed using Affymetrix *Drosophila* Genome 2.0 arrays. A summary of lines used in this analysis can be found in Supplementary Material, Figure S1 and a summary of analyses performed can be found in Figure 3A and B. For flies expressing rCUG and rCAG repeat sequences, separate microarray experiments were performed using two alternative *elav-GAL4* driver lines (depicted in Supplementary Material, Fig. S1B) in order to increase the robustness of these analyses. Multiple independent lines carrying either two (experiment 1) or four (experiment 2) transgene insertions of the repeat constructs were also tested, in order to minimize any transcriptional effects related to the insertion sites or expression levels of the transgenes. A single four-transgene-insertion line was also included in experiment 2 for the rAUUCU repeat RNA.

For flies expressing each hairpin repeat sequence, transcript ratios were calculated compared with flies expressing rCAA repeat RNA, which cannot form a hairpin secondary structure, as well as to flies heterozygous for the *elav-GAL4* driver, but not expressing any repeat construct. Flies heterozygous for *elav-GAL4* were used as a control for genetic background. However, since GAL4 has been reported to be toxic in *Drosophila* neurons (28), flies expressing rCAA repeat RNA were included as an additional control. These flies carry the same number of transgene insertions as the rCAG, rCUG and rAUUCU repeat expressing flies, thus minimizing the contribution of GAL4 toxicity to the transcriptional read-out. The inclusion of flies carrying *elav-GAL4* alone should also identify any effects resulting from expression of CAA repeat RNA, which are therefore not the result of secondary structure of the RNA species.

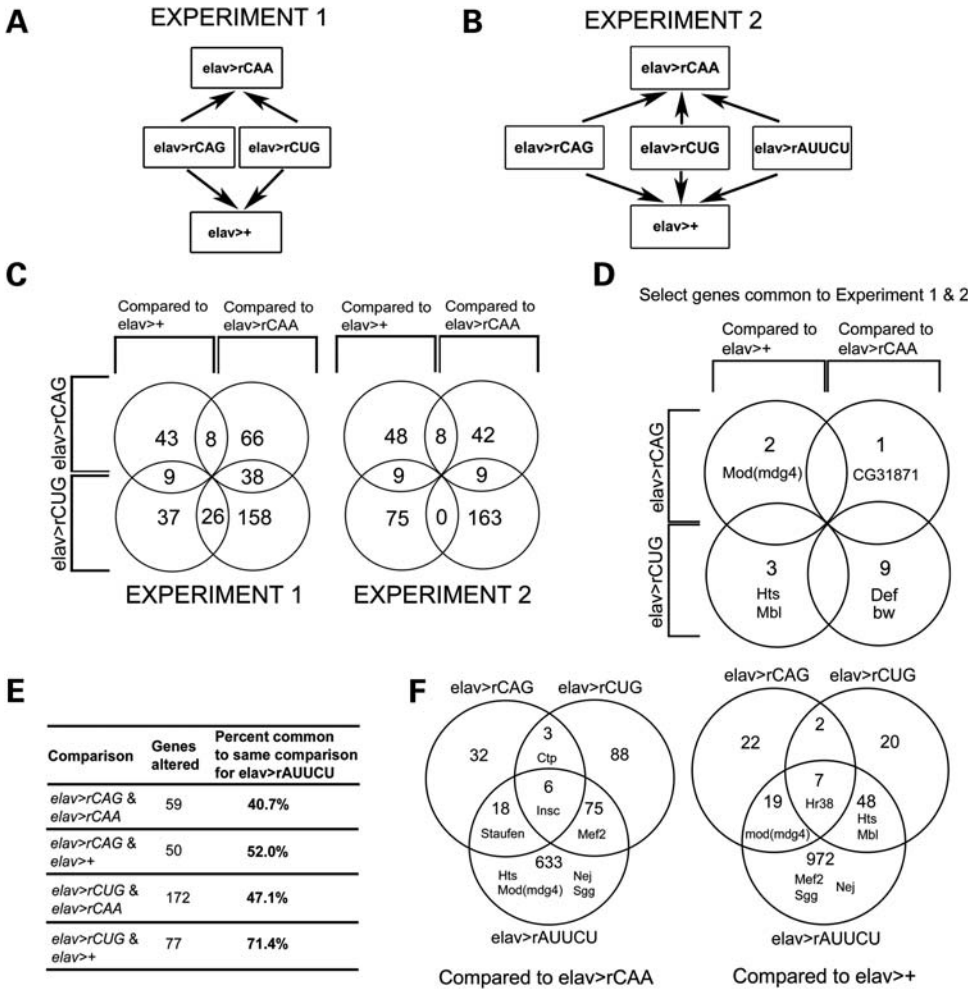
Some variation in expression levels of expanded repeat sequences was observed, both between independent lines for the same repeat and different repeat sequences. The most striking difference was seen in flies expressing the rCAA repeat, which showed consistently low steady-state levels compared with other repeat sequences, possibly as a result of the inability of this sequence to form a stable secondary structure. These lines were included in analysis primarily as a control for GAL4 toxicity. Additionally, with the exception of the rAUUCU repeat, several independent lines were analysed for each repeat sequence so as to minimize the impact of variation in repeat expression levels on the transcriptional outcome.

In this way, candidate genes were identified which were significantly altered (two-tailed *t*-test value of $P < 0.05$) in flies expressing rCAG and rCUG expanded repeat sequences compared with either flies carrying *elav-GAL4* alone or expressing rCAA repeat RNA for each experiment. The total number of genes identified in each comparison is shown in Figure 3C. Genes found to be significantly altered in more than one comparison are also listed in Supplementary Material, Table S1, while select candidates identified in both experiments 1 and 2 are shown in Figure 3D. In experiment 2, a remarkably high overlap in transcriptional changes between flies expressing rCAG or rCUG repeats and those expressing rAUUCU repeats was observed (between 40.7 and 71.4%, Fig. 3E), suggesting that there is a considerable common component to cellular perturbation in flies expressing each of these repeat sequences. Select candidates identified in experiment 2, which included the rAUUCU repeat expressing flies, are also listed in Figure 3F.

Genetic validation of effects of expanded repeat expression

Disruptions to the ordered patterning of ommatidia in the *Drosophila* eye can be readily observed in adult flies and for this reason have been frequently used as a basis for genetic screens. This approach has also been successfully used to identify mechanisms of toxicity in models of expanded repeat disease (24,29–33). We have tested candidate genes identified by microarray analysis for their ability to alter the patterning of the *Drosophila* eye. While expression of untranslated expanded repeat RNAs does not result in a phenotype in the eye (Fig. 2G–J), expression of expanded repeats encoding polyglutamine or polyleucine causes an easily visualized perturbation to the exterior patterning of the eye (Fig. 2B–E) and therefore these phenotypes were initially used as a screening tool. Flies expressing a translated CAG repeat tract express both polyglutamine peptide and hairpin-forming RNA, while flies expressing a translated CAA repeat express polyglutamine peptide in the absence of any hairpin-forming RNA. Similarly, flies expressing the translated CUG repeat construct express both polyleucine peptide and hairpin-forming RNA. Therefore, the ability of candidate genes to alter either the polyglutamine or polyleucine phenotype in a manner that was dependent upon the sequence composition of their encoding RNAs would be indicative of this functional interaction occurring at the RNA level. A similar methodology has been used recently to identify *Drosophila mblA* as important in pathogenesis in a model of SCA3 (18). In this study, the authors demonstrate the ability of *mblA* to modify phenotypes in flies expressing truncated Ataxin-3 containing a pure CAG repeat but not a mixed CAG/CAA repeat, suggesting that this interaction is occurring at the RNA level.

Using this approach, two genes which were able to induce toxicity in flies expressing untranslated expanded repeats were identified (Fig. 4). One of these genes, *mef2*, showed a significant interaction with translated CUG repeats, but no interaction with translated CAG or CAA repeats (Fig. 4D–F). This may suggest that Mef2 is a unique modifier of either polyleucine or CUG repeat RNA pathogenesis (or both), or that a critical pathogenic threshold was not reached in the flies expressing expanded CAG repeats. The other



Comparison	Genes altered	Percent common to same comparison for elav>rAUUCU
elav>rCAG & elav>rCAA	59	**40.7%**
elav>rCAG & elav>+	50	**52.0%**
elav>rCUG & elav>rCAA	172	**47.1%**
elav>rCUG & elav>+	77	**71.4%**

Figure 3. Experimental design of microarray experiments. (**A**) Experiment 1: microarray analyses were performed on flies expressing two insertions of the rCAG, rCUG and rCAA repeat sequences driven by the pan-neuronal *elav^{c155} −GAL4* driver and flies heterozygous for the *elav^{c155} −GAL4* driver (elav>+). For each repeat sequence, three lines with independent insertion sites were tested and candidates were selected which showed altered transcription in all three lines. Microarray analysis of *elav^{c155} −GAL4* heterozygotes was performed in duplicate. Comparisons were performed between elav>rCAG or elav>rCUG and each of the controls, which were elav>+ and elav>rCAA. (**B**) Experiment 2: microarray analysis was performed on flies expressing four insertions of the rCAG, rCUG, rCAA and rAUUCU repeat sequences driven by the pan-neuronal *elavII−GAL4* driver and flies heterozygous for the *elavII−GAL4* driver (elav>+). For rCUG, rCAG and rCAA, two lines with independent insertion sites were tested. A single four-transgene-insertion rAUUCU line was tested. Microarray analysis of *elavII −GAL4* heterozygotes was performed in duplicate. Comparisons were performed between elav>rCAG, elav>rCUG or elav>rAUUCU and each of the controls, which were elav>+ and elav>rCAA. (**C**) Number of transcripts significantly altered in flies expressing rCAG and rCUG repeats compared with flies expressing rCAA repeats or heterozygous for the driver line. In each case, transcripts were selected which met the criteria: log2(ratio)>0.5 or <−0.5, where the ratio was calculated using an average of all independent lines tested, with a two-tailed *t*-test value of $P < 0.05$. Transcripts common to more than one comparison are listed in Supplementary Material, Table S1. (**D**) Select genes which were detected as altered in flies expressing rCAG or rCUG in both microarray experiments. (**E**) Percent of genes altered in experiment 2 for rCAG and rCUG repeat expressing flies which were also altered in flies expressing the rAUUCU repeat construct. These transcripts are listed in Supplementary Material, Table S2. (**F**) Number of transcripts significantly altered in experiment 2, selected for log2(ratio)>0.5 or <−0.5, where the ratio was calculated using an average of all independent lines tested, with a two-tailed *t*-test value of $P < 0.05$ for flies expressing rCAG or rCUG. *T*-tests were not performed for rAUUCU repeat expressing flies, since a single four-transgene-insertion line was available for this repeat. Genes of particular interest are listed.

gene, *mod(mdg4)*, demonstrated an interaction with both translated CAG and CUG repeats (Fig. 4G and I). In flies expressing polyglutamine encoded by expanded CAG, reduction in expression of mod(mdg4) by RNAi resulted in nearly complete lethality, with the small number of flies that did eclose showing a significant enhancement of the eye phenotype compared with flies expressing the CAG repeat alone (Fig. 4G). In contrast, flies expressing polyglutamine encoded by expanded CAA with reduction in levels of *mod(mdg4)* showed only a mild enhancement of the loss of pigment eye phenotype (Fig. 4H). It is likely that the observed lethality in flies expressing CAG repeats with reduced *mod(mdg4)* is the result of the expression of both the expanded repeat and the mod(mdg4) RNAi construct in tissues other than the eye, as has been previously reported for the GMR-GAL4 driver (7).

Different RNA repeat sequences demonstrate distinct interactions with candidate genes in our *Drosophila* model

While expression of rCAG, rCUG or rAUUCU repeats in the *Drosophila* eye does not result in a phenotype, reducing

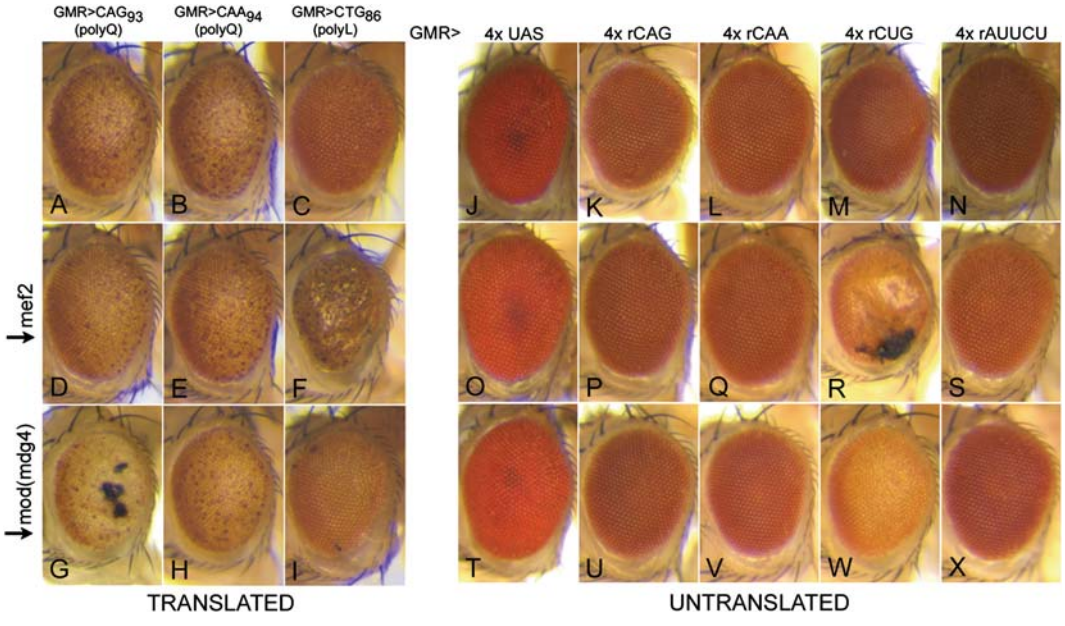


Figure 4. Altering levels of Mef2 and Mod(mdg4) can modify the effect of expression of expanded repeat RNAs in the *Drosophila* eye. (**A**–**C**) Eye phenotypes resulting from expression of expanded translated CAG, CAA and CUG repeats, as shown in Figure 2. (**D** and **E**) Co-expression of an RNAi construct targeting mef2 with polyglutamine encoded by either a CAG or CAA repeat tract does not alter the appearance of the eye. (**F**) Reducing expression of mef2 in the eye of flies expressing a translated CUG repeat causes an enhancement of the polyleucine eye phenotype, resulting in flies with smaller eyes which have a glazed appearance and necrotic patches. (**G**) Reducing expression of mod(mdg4) in flies expressing polyglutamine encoded by a CAG repeat results in an enhancement of the eye phenotype and a reduction in viability of the flies. (**H**) Expression of this same RNAi construct with polyglutamine encoded by a CAA repeat results in a milder enhancement of the loss of pigment phenotype and no reduction in viability. (**I**) An enhancement of the polyleucine phenotype was also observed with this RNAi construct. (**J**–**N**) Expression of four insertions of any of the untranslated repeat constructs alone does not cause an alteration to the appearance of the *Drosophila* eye, as described in Figure 2; however co-expression of either the RNAi construct targeting mef2 or mod(mdg4) with four insertions of the rCUG repeat construct was able to induce a strong eye phenotype (**R** and **W**). This effect was not seen with any of the other untranslated repeat constructs (**O**–**Q**, **S**, **T**–**V**, **X**).

expression of genes that are components of pathogenic pathways involved in RNA-mediated toxicity may result in the appearance of a phenotype, through pushing cells beyond a critical pathogenic threshold. Therefore, candidates identified by microarray analysis were also directly assayed for functional interaction at the RNA level with untranslated expanded repeat RNAs.

Co-expression of RNAi constructs targeting mef2 or mod(mdg4) with expanded rCUG repeats in the *Drosophila* eye resulted in a marked disruption of the pigmentation and patterning of the eye (Fig. 4R and W) supporting a role for these candidates in CUG repeat pathogenesis. No disruption to the eye was observed when these RNAi constructs were co-expressed with any other repeat sequence, supporting the conclusion that these candidates may either be unique to CUG repeat pathogenesis in this model, or that a critical threshold of toxicity has not been reached in flies expressing the other expanded repeat sequences.

Common perturbation of downstream effectors of Akt/Sgg signalling

A number of candidate genes identified in these microarray analyses of different disease-associated expanded repeat sequences were downstream effectors or regulators of the *Drosophila* Gsk-3β (Sgg) signalling pathway (Fig. 5). Therefore, we also investigated a role for this pathway in RNA-mediated pathogenesis. Investigation of genetic interaction between expanded translated repeat sequences revealed a reciprocal effect of over-expressing or reducing Sgg expression in flies expressing translated CUG repeats (Fig. 6C, G, K). The effect was less clear for CAG and CAA repeat-encoded polyglutamine expressing flies. While there was a clear enhancement when Sgg was over-expressed with either CAG or CAA (Fig. 6A, B, I, J), there was little effect in either case when Sgg expression levels were reduced (Fig. 6A, B, E, F). Furthermore, no difference in the strength of interaction with Sgg was observed between flies expressing CAG or CAA, and therefore it was not possible to determine whether there was any contribution of the hairpin-forming repeat RNA to this interaction.

To further investigate a role for this pathway in RNA-mediated pathogenesis, we over-expressed Sgg in the presence of each of the different untranslated repeat sequences. Over-expression of Sgg in the presence of rCAG, rAUUCU or rCAA expanded repeat sequences resulted in a decrease in the amount of pigmentation in the eye compared with over-expression of Sgg in flies carrying four transgene insertions in the absence of a repeat sequence (Fig. 6R–U compared with Q). However, there was a marked increase in the degree of roughness of the surface of the eye in flies expressing the rCAG or rAUUCU repeat sequences compared with those expressing the rCAA repeat (Fig. 6R and U compared with S). Since the steady-state levels of rCAA RNA are

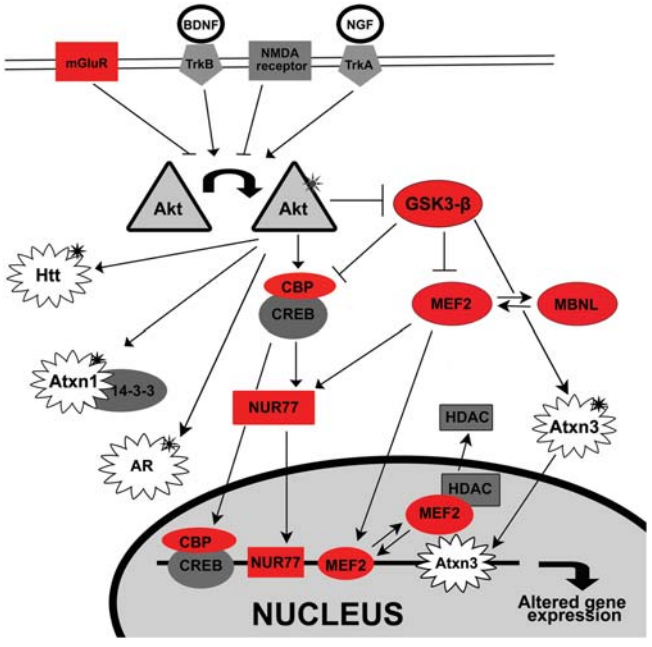


Figure 5. Alteration to activity of the Akt/GSK3-β signalling pathway can explain a number of the changes observed in microarray analysis of flies expressing rCAG, rCUG and rAUUCU repeats in the nervous system. Genes for which a *Drosophila* orthologue showed altered transcript levels in microarray analysis of flies expressing at least one of the untranslated repeat constructs are shown in red. Activation of Akt can be regulated by a number of different signals, including glutamate (52) or neurotrophic (53,54) signals and Ca^{2+} signalling (55). Activated Akt is in turn involved in down-regulation of GSK3-β activity which is involved in regulation of a number of transcription factors, including MEF2 (56) and CREB (57), which is an orthologue of *Drosophila* Nej. Both CREB and MEF2 have been demonstrated to play a role in the regulation of expression of the nuclear receptor NUR77, an orthologue of *Drosophila* Hr38, in a calcium-dependent manner (58). Activation of NUR77 can also be regulated directly by Akt (59). The Akt/GSK3-β signalling pathway is therefore able to have broad downstream transcriptional effects. A number of links between Akt activity and expanded repeat-containing proteins themselves have also been demonstrated. Akt phosphorylates HTT, ataxin-1 and the androgen receptor (AR) (stars), altering their interactions with other proteins (60–62). Phosphorylation of ataxin 3 by GSK3-β (star) has also been recently demonstrated to regulate nuclear entry and therefore may play a role in SCA3 (63). Expression of expanded CUG repeats has also been demonstrated to alter activation of the Akt/Gsk3-β pathway in PC12 cells (64).

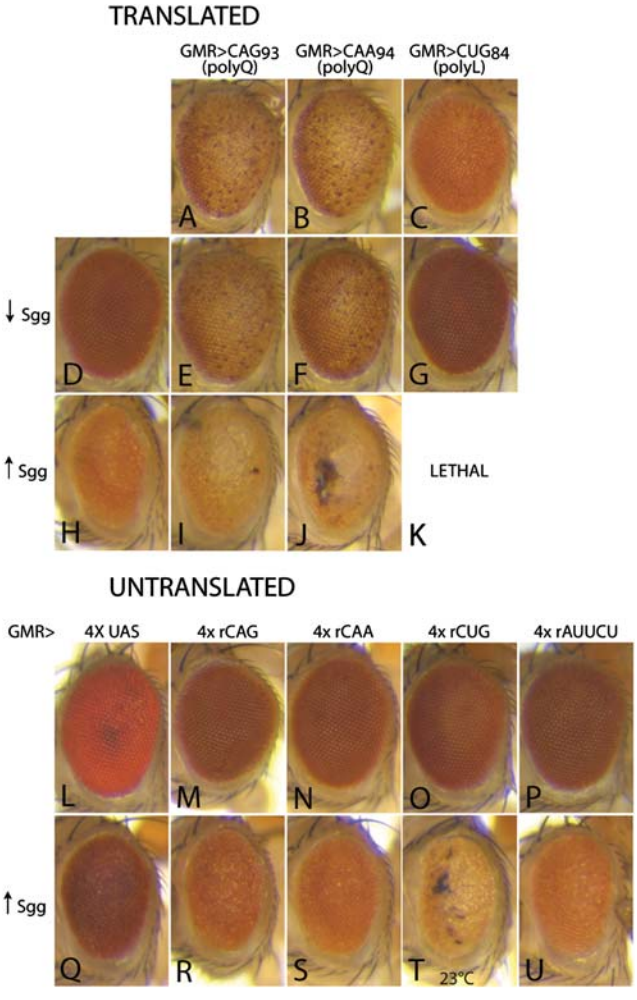


Figure 6. Altering expression of GSK3-β can modify the effect of expression of expanded repeat RNAs in the *Drosophila* eye. (**A**–**C**) Phenotypes resulting from expression of translated CAG, CAA and CUG repeat expression, as shown in Figure 2. (**D**) Expression of RNAi construct targeting *Drosophila sgg* does not alter the exterior appearance of the eye. (**E** and **F**) Co-expression of an RNAi construct targeting *sgg* with polyglutamine encoded by either CAG or CAA does not dramatically alter the exterior appearance of the eye. (**G**) Co-expression of the *sgg* RNAi with polyleucine results in eyes of wild-type appearance. (**H**) Ectopic expression of Sgg in the eye results in a severe rough eye phenotype with a dramatic reduction in the size of the eye and the amount of pigmentation. (**I** and **J**) Co-expression of polyglutamine encoded by either a CAG or CAA repeat with ectopically expressed Sgg results in an increase in the size of the eye compared with ectopic expression of Sgg alone. There appears to be a reduction in the amount of pigment in the eye and in most cases there are necrotic patches and nearly complete loss of the ommatidial array structure. (**K**) Ectopic expression of Sgg in the eye of flies co-expressing polyleucine is completely lethal. (**L**–**P**) Expression of four insertions of any of the untranslated expanded repeats alone does not result in a visible phenotype in the eye, as shown in Figure 2. (**Q**) Driving expression of four insertions of the UAS portion of the transgene without the remainder of the construct in flies ectopically expressing *sgg* in the eye results in a milder rough eye phenotype than expression of *sgg* alone. (**S**) Co-expression of four transgene insertions of the rCAA construct with the Sgg overexpression construct does not significantly alter the organization of the eye compared with co-expressing the UAS sites alone. (**R** and **T**) Co-expression of the rCAG or rAUUCU repeat constructs with the Sgg overexpression construct results in eyes which are consistently rougher than those of flies co-expressing either rCAA or the UAS construct with Sgg. (**U**) Co-expression of rCUG with the Sgg overexpression construct results in complete lethality at 25°C. At 23°C, the few flies which survive to eclosion have a strong loss of pigment phenotype, with the loss of ommatidial structures and the appearance of necrotic patches.

consistently lower than the other repeats (Supplementary Material, Fig. S1), it is not possible to determine whether there is generally a stronger interaction between Sgg and hairpin-forming RNA repeats. However, there does appear to be a degree of sequence-dependence to the interaction with Sgg, since the rAUUCU repeat RNA is expressed at similar levels to rCUG, while the rCAG repeat RNA is expressed at much higher levels than rCUG and yet there is a weaker interaction observed with Sgg in both cases. Co-expression of rCUG repeat RNA with Sgg was completely lethal at 25°C. When flies were grown at 23°C to reduce expression levels of the UAS constructs, a severe phenotype involving loss of pigment, necrotic patches and a loss of ommatidial organization of the eye was observed, indicating a strong interaction between this repeat sequence and Sgg (Fig. 6T). Therefore, while there appears to be a mild interaction between all

expanded repeat sequences tested and Sgg in this model, the strength of this interaction is affected by the sequence of the repeat.

DISCUSSION

Identification of the molecular pathway(s) from mutation to clinical symptoms in the dominantly inherited neurodegenerative diseases has proved extremely difficult. A contributing factor to this is that the sensitive cells of affected individuals are lost in the course of the disease. Animal models therefore afford the opportunity to access cells in which the pathogenic pathways are active and also to explore alternative hypotheses as to the nature of the pathogenic agent(s) responsible for these diseases. RNA is such a potential pathogen in the dominantly inherited expanded repeat neurodegenerative diseases. The use of animal models such as *Drosophila* enables the identification of pathways through which such potential pathogens act and the identification of biomarkers of the responsible pathways. These biomarkers can subsequently be tested in the respective human diseases to validate the role of the pathway and its contribution to the disease. Using this approach, we have modelled repeat RNA pathology in *Drosophila* and have identified common pathways perturbed by the expression of expanded repeat RNAs.

Analyses of transcriptional changes in a number of models of expanded repeat disease have previously been reported (34–40). These studies have largely modelled toxicity of polyglutamine, which induces severe, early phenotypes in both mouse and *Drosophila* models, and therefore transcriptional changes are likely to partially reflect downstream effects of cell death. More recently, evidence for a role of RNA-mediated pathogenesis in the polyglutamine diseases has been reported (18). Since expression of each of the repeat sequences as untranslated RNA either in the *Drosophila* eye or throughout the nervous system does not result in gross developmental or degenerative phenotypes, this model can be used to investigate markers of cellular dysfunction attributable to these repeat sequence RNAs which precede cell death and are therefore more likely to represent causative changes in disease progression.

Given the ability of all of the disease-associated repeat sequences to form hairpin secondary structures at the RNA level and the phenotypic overlap seen in the expanded repeat diseases, despite the presence of the repeat tracts within unrelated genes, we predicted that there are likely to be common, intrinsic, sequence-independent cellular effects of expression of expanded repeat sequences. In support of this prediction, pan-neuronal expression of rCAG, rCUG and rAUUCU expanded repeat RNAs was found to elicit a number of common transcriptional changes. Strikingly, a comparison of transcripts showing altered expression in flies expressing rAUUCU repeat RNA revealed a minimum of 40.7% and maximum of 71.4% overlap with genes altered in flies expressing either rCAG or rCUG repeats (Fig. 3E). This result is strongly suggestive of common mechanisms of toxicity of expanded repeat RNAs.

In the untranslated expanded repeat diseases where there is no toxic peptide expressed, RNA-mediated pathogenesis is presumably sufficient to induce all of the cellular changes leading to neurodegeneration and disease progression. This *Drosophila* model of RNA repeat pathogenesis investigates some components of pathogenesis in these diseases, but there are also likely to be specific effects of expression of the repeat-containing transcript in each disease which are dependent on the context of the repeat tract. Nevertheless, at least one candidate which showed a strong interaction with context-independent repeat RNAs used in this study, *mod(mdg4)*, has been previously identified as transcriptionally altered in another *Drosophila* model which used repeats within the context of the SCA8 transcript (24).

In addition, changes identified in these microarray analyses support a role for more generalized transcriptional dysregulation in toxicity of expanded repeat RNAs. In particular, altered transcript levels of histones (H3 and H1), histone acetylating enzymes (*msl-2* and *Atac1*), chromatin modifiers (*mod(mdg4)*), a number of transcription factors (including *mef2*, *lola*, *cut*, *hr38*, a member of the SP1/SP3-like transcription factor family and a *Drosophila* orthologue of PAX5) and transcriptional co-regulators (*tna* and *med24*) were detected in flies expressing more than one of the repeat sequences (Supplementary Material, Tables S1 and S2). This is consistent with observations in several models of polyglutamine pathogenesis in which wide-spread transcriptional dysregulation has been reported (35,40,41) and suggests that this sort of effect may be an intrinsic property of expanded repeat sequences.

Examination of common transcriptional changes detected in this model also revealed changes to a number of other components of the cell that have been previously implicated in polyglutamine pathogenesis, including several cellular transport and cytoskeletal components. For example, the actin-binding proteins *hu li tai shao*, which is an orthologue of mammalian Adducin 1, and *cut up*, a component of the dynein complex, both showed altered expression in flies expressing more than one of the expanded repeat sequences (Fig. 3F). *Hu li tai shao* has been previously demonstrated to suppress a phenotype associated with expression of an expanded N-terminal fragment of Huntingtin in the *Drosophila* eye (29), while *cut up* and its human orthologue, *DYNLL1*, showed altered expression in a comparison of *Drosophila* and human cell culture models of polyglutamine pathogenesis (37). We therefore predict that some of the pathogenic pathways previously identified in models of expanded repeat disease may be common to both polyglutamine and untranslated repeat diseases and therefore some of these effects may be at least partially mediated through RNA pathogenesis.

Recently published data (42) demonstrate that expanded CAG repeat alleles are able to be translated internally in all three reading frames, irrespective of whether or not they are located in coding regions and without requiring an initiation AUG, through a mechanism known as repeat-associated non-ATG translation (RAN translation). It is thought that the hairpin structure formed by the expanded repeat RNAs is acting as an Internal Ribosome Entry Site (IRES). In our model, expression of up to four transgene insertions of untranslated CAG and CUG repeat sequences does not result in a phenotype, while expression of a single polyglutamine

or polyleucine-encoding transgene is sufficient to induce a visible phenotype in the *Drosophila* eye (Fig. 2; 8). Therefore, RAN translation does not appear to play a major role in RNA toxicity in this model. However, as a consequence of these recent findings, homopolymeric amino acid sequences have emerged as a potential mediator of repeat RNA toxicity in the 'untranslated' repeat diseases.

Altered transcription of components of the Akt/GSK3-β regulatory pathway was consistently observed in flies expressing rCAG, rCUG and rAUUCU repeat RNAs by microarray analysis, suggesting that this is a key component of cellular dysfunction in this *Drosophila* model of untranslated repeat disease pathogenesis. While the ability of CUG repeat RNA to disrupt Akt/GSK3-β signalling has been described, this is the first evidence that expression of other hairpin-forming RNA species can also influence activity of this pathway. The initial stimulus resulting in the disruption of Akt/GSK3-β signalling in this model is unclear; however, there is precedent for similar effects in fragile X syndrome where increased levels of stimulation of the mGluR5 receptor have been demonstrated to increase GSK3-β activity (43). A disruption to mGluR5 signalling has also been described in a presymptomatic model of HD (44), and in other HD models alterations to *N*-methyl-*D*-aspartate receptor (45), brain-derived neurotrophic factor (46,47) and nerve growth factor (48) signalling, all of which are associated with activation of the Akt/GSK3-β pathway, have also been observed. Our observations indicate that expression of expanded repeat RNA is sufficient to cause transcriptional changes to the Akt/GSK3-β pathway, and therefore that the hairpin RNAs expressed in the disease situation might also interact with components of this pathway to disrupt normal signalling.

MATERIALS AND METHODS

Drosophila strains and husbandry

CAG and CUG repeats were generated by ligating together shorter repeat oligonucleotides and expanded using polymerase chain reaction (PCR)-based techniques as described previously (8). The AUUCU repeat construct was generated from a PCR product containing the region in intron 9 of human ataxin-10 containing the AUUCU repeat, plus 141 bp of surrounding sequence which was amplified from HeLa cell DNA. This repeat tract was expanded from an original size of 13 repeats by a PCR-based method, based on a previously outlined protocol (49).

Each repeat sequence was subcloned into the *Drosophila* transformation vector pUAST, which had been modified to contain the amino acid sequence surrounding the repeat, including the MYC and FLAG epitope tags. For the untranslated repeat constructs, a stop codon was inserted in front of the repeat tract by PCR mutagenesis as described previously (8). The length and integrity of repeat constructs were confirmed by DNA sequencing and then each construct was microinjected into the w^{1118} strain (Stock #3605) by standard methods to obtain germline transformants. Multiple transgenic lines were obtained for each construct, and the repeat length from each line was confirmed by PCR and sequencing.

The *GMR-GAL4* (Stock #9146), $elav^{c155}$*-GAL4* (Stock #458) and P{*GAL4-elav.L*}*2* (designated *elavII*) (Stock #8765) strains used in this study were obtained from the Bloomington Drosophila stock centre (Indiana University, Indiana, PA, USA). *GMR-GAL4* drives expression of UAS constructs in all cells posterior of the morphogenetic furrow in the developing eye. Both *elav-GAL4* insertions drive expression of UAS constructs throughout the central and peripheral nervous system.

RNAi strains tested for genetic interaction with expanded repeats were obtained from the Vienna *Drosophila* RNAi centre (50). VDRC strains shown in figures are: Stock #15550 (*mef2*), 52268 (*mod(mdg4)*) and 101538 *(Sgg)*. UAS-Sgg was obtained from the Bloomington *Drosophila* stock centre (Stock #5361). All *Drosophila* stocks were maintained in vials containing Fortified (F1) medium and kept at either 18 or 25°C. The F1 medium was composed of 1% agar, 18.75% compressed yeast, 10% polenta, 10% treacle, 1.5% acid mix and 2.5% tegosept. Crosses were performed at 25°C unless otherwise indicated.

RNA extraction and purification

Approximately 100 male *Drosophila* heads from newly eclosed flies were collected and snap frozen for each genotype, before homogenization in Trizol (Invitrogen). Total RNA was extracted using chloroform and precipitated with isopropanol, and then further purified using the RNeasy mini kit (Qiagen). RNA to be used for microarrays was precipitated in sodium acetate and ethanol and shipped under ethanol on ice.

Quantitative real-time PCR

One microgram of total RNA per sample was treated with DNAse I (Invitrogen) and reverse-transcribed with oligo(dT)18 and SuperScript III (Invitrogen). Quantitative real-time PCR was performed in a LightCycler (Roche Molecular Biochemicals) using Power SYBR green mix (Applied Biosystems) and either GAL4-specific primers (forward: 5′-CACTGACCCCGTCTGCTTTG-3′, and reverse: 5′-GGTTCGGACCGTTGCTACTG-3′) or primers specific for the repeat-containing transcript. The transgene expression level was quantified using the ΔCt method for relative quantitation and expressed relative to the level of GAL4 transcript for each line.

Microarrays

Total RNA was processed using the One-Cycle Target Labelling and Control Reagents Kit, as per manufacturer's instructions (Affymetrix Inc.). Briefly, 2 μg of total RNA was converted to cDNA (Superscript II, Invitrogen) and an overnight *in vitro* transcription reaction performed to generate a pool of cRNA carrying a biotin tag (MEGAscript T7 Kit, Ambion, Inc). The Drosophila Genome 2.0 Array was hybridized for 16 h and washed/stained on a FS 450 Fluidics Station using the Midi euk2 v3 script. Data were acquired on a 7G GeneChip Scanner 3000 and data extraction performed in GCOS v1.2. Candidates were selected from the pool of transcripts which showed a 'present' call in either all independent lines for a particular repeat sequence, or in all samples for the *elav-GAL4/+* control in that experiment.

Where possible, two-tailed Student's *t*-tests were performed on raw values to identify significantly altered transcripts (P-value < 0.05). The microarray data have been deposited on the NCBI database (GEO accession number GSE27178).

Microscopy

Image preparation was performed using Adobe Photoshop 6.0. Light photos were taken with an Olympus SZX7 dissection microscope fitted with an SZX-AS aperture. Images were captured with a Colorview IIIu camera and AnalysisRuler image acquisition software. In all cases, anterior is to the left. Flies were photographed at 24–48 h post-eclosion.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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