

RNA-Mediated Neurodegeneration Caused by the Fragile X Premutation rCGG Repeats in *Drosophila*

Report

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Summary

Fragile X syndrome carriers have *FMR1* alleles, called premutations, with an intermediate number of 5' untranslated CGG repeats between patients (>200 repeats) and normal individuals (<60 repeats). A novel neurodegenerative disease has recently been appreciated in some premutation carriers. As no neurodegeneration is seen in fragile X patients, who do not express *FMR1*, we hypothesize that lengthened rCGG repeats of the premutation transcript may lead to neurodegeneration. Here, using *Drosophila melanogaster*, we show that 90 rCGG repeats alone are sufficient to cause neurodegeneration. This phenotype is neuron specific and rCGG repeat dosage sensitive. Although devoid of mutant protein, this neurodegeneration exhibits neuronal inclusion bodies that are *Hsp70* and ubiquitin positive. Overexpression of *Hsp70* could suppress the neurodegeneration. These results demonstrate that neurodegenerative phenotype associated with fragile X premutation is indeed caused by the lengthened rCGG repeats and provide the first in vivo experimental demonstration of RNA-mediated neurodegeneration.

Introduction

Fragile X syndrome, a common form of inherited mental retardation, is caused by a massive CGG trinucleotide repeat expansion in the 5' UTR of the *FMR1* gene that leads to transcriptional silencing and the absence of the encoded Fragile X Mental Retardation Protein (FMRP) (O'Donnell and Warren, 2002). Most affected individuals exhibit expansions of over 200 CGG repeats and are referred to as full mutations. Among normal individuals, the CGG repeat is highly polymorphic in length and content with numerous alleles <60 repeats that are occasionally interspersed with 1–3 AGG triplets (Kunst and Warren, 1994). Intermediate alleles between 60 and 200 repeats are referred to as premutations with an estimated prevalence of about 1 in 540 individuals (O'Don-

nell and Warren, 2002). Premutation alleles are extremely unstable during the germline transmission and may expand into full mutations upon maternal transmission (Sherman, 2002).

Premutation carriers have long been considered phenotypically normal. However, despite years of clinical experience with fragile X families, the recognition of a novel progressive neurodegenerative disorder has recently been made in several male premutation carriers (Hagerman et al., 2001). This tremor/ataxia syndrome is characterized by a progressive action tremor with ataxia, parkinsonism, and generalized brain atrophy (Jacquemont et al., 2003). More advanced cases are accompanied by memory and executive function deficits, anxiety, and eventual dementia (Hagerman and Hagerman, 2002). Postmortem examinations of the brains of four such males revealed neuronal degeneration in the cerebellum and the presence of ubiquitin-positive intranuclear inclusions in both neuronal and astrocytic nuclei of the cortex (Greco et al., 2002). The emerging picture suggests a previously unrecognized neurodegenerative phenotype in fragile X premutation carriers more prevalent in males than females. Strikingly, no evidence of neurodegeneration has been found in either full mutation males or in the *Fmr1* knockout mouse model, indicating that modulation of FMRP levels unlikely accounts for the neurodegeneration observed in these carriers. Since *FMR1* premutation alleles are distinguished from both normal and full mutation alleles by producing *FMR1* transcripts with lengthy rCGG repeats, it is possible that the neurodegeneration is RNA mediated. This notion, that rCGG abundance may be causal, is further supported by observations that the *FMR1* mRNA level is elevated in premutation carriers (Kenneson et al., 2001; Tassone et al., 2000). Thus, elevation of *FMR1* transcripts may work in concert with lengthy rCGG repeats to cause neurodegeneration.

To test this hypothesis, we have established a *Drosophila* model that ectopically expresses a portion of the human *FMR1* 5' UTR containing either normal or premutation-length rCGG repeats. We show that fragile X premutation rCGG repeats alone can cause neurodegeneration in a dosage- and repeat length-dependent manner. These results confirm the emerging clinical picture of a novel neurodegenerative phenotype in some premutation carriers and demonstrate that RNA alone can cause the neurodegeneration.

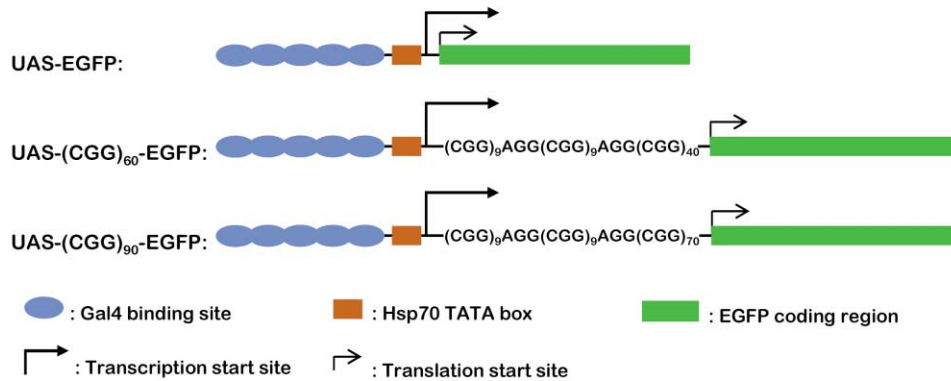
Results

Expression of Fragile X Premutation rCGG Repeats in *Drosophila*

To examine whether or not fragile X premutation-length rCGG repeats could cause neurodegeneration, we expressed human fragile X premutation rCGG repeats in *Drosophila melanogaster*. Control of transgene expression and tissue specificity was achieved by the *GAL4/UAS* (upstream activating sequence) system (Brand and Perrimon, 1993). A human genomic *FMR1* DNA fragment

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A



B

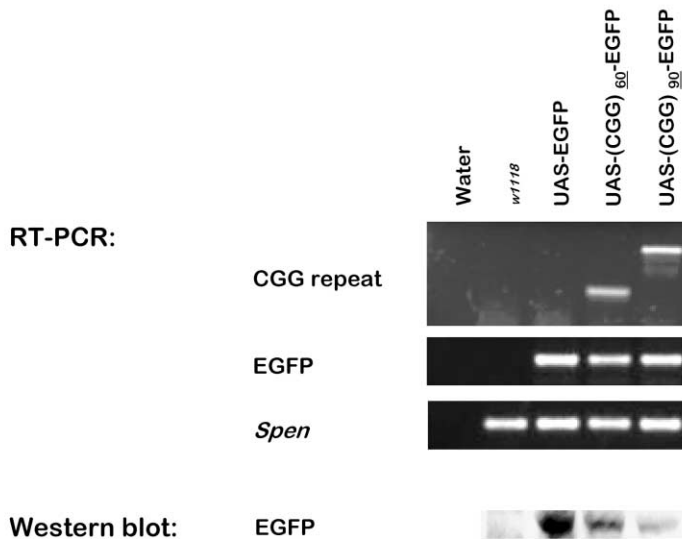


Figure 1. Expression of Fragile X Premutation rCGG Repeats

(A) Schematic representation of pUAST-(CGG)_n-EGFP constructs. A human genomic *FMR1* DNA fragment containing 90 CGG repeats was inserted upstream of the EGFP coding region between the transcription and translation start sites. Both the transcription and translation start sites are indicated. Two of the resulting transgenic lines did not maintain the repeat length of 90 but contracted the 3' end of the array, resulting in a tract of 60 CGG repeats (UAS-(CGG)₆₀-EGFP).

(B) Expression of (CGG)_n-EGFP transgenes. RT-PCR was performed using the primers specific for the CGG repeat fragment, EGFP or *Spen*. RNA and protein were isolated from the brains of flies: UAS-(CGG)_n-EGFP/*gmr*-GAL4. Parental fly stock *w*¹¹¹⁸ used to generate transgenic flies was used as a negative control.

from a premutation carrier, containing 90 CGG repeats (with a commonly observed interruption pattern of AGG triplets at repeat 10 and 20) and ~200 base pairs of flanking sequence, was subcloned into the *Drosophila* transformation vector pUAST-EGFP containing an enhanced green fluorescent protein (EGFP) reporter gene (Figure 1A). The CGG repeat fragment was inserted upstream of the EGFP coding region such that the transcriptional start site preceded and the first translational start site followed the CGG repeat. No alternative ATG translation start site is found between the transcription start site and the CGG repeat. Five transgenic lines were established with this construct, with all but two lines maintaining 90 repeats. In the remaining two lines, the 3' end of the repeat array contracted, resulting in a 60 CGG repeat tract that maintained the AGG interruption pattern (Figure 1A). By sequence analysis, none of the

transgenic lines showed additional sequence variation from the transforming construct. As a control, transgenic lines with the pUAST-EGFP vector alone were also generated.

To examine the expression of transgenes, we directed expression to the retina using the *gmr-gal4* driver (Freeman, 1996). As shown in Figure 1B, RT-PCR using primers flanking the CGG repeat clearly show the repeat is transcribed in all lines, as do primers specific for the EGFP coding sequence (with *Split ends [spen]* primers used as a positive control). In addition, the correct size of transcript for each genotype was detected by Northern blot using EGFP cDNA as probe (data not shown). Western analysis of the EGFP protein from all transgenic lines gave a product that appeared similar in size, indicating the use of the same translational start site in all lines (Figure 1B). Overexposure of this blot revealed no

Table 1. Effects of Targeted Expression Fragile X Premutation rCGG Repeats to Different Tissues of *Drosophila*

GAL4 line	Expression Pattern	EGFP	(CGG) ₆₀ -EGFP Moderate	(CGG) ₆₀ -EGFP Strong	(CGG) ₉₀ -EGFP Moderate	(CGG) ₉₀ -EGFP Strong
<i>gmr</i> -GAL4	All eye cells posterior to the furrow including photoreceptor neurons and pigment cells	No effect	No effect	Rough eye, loss of pigmentation, and holes in the tangential sections	Mild rough eye and holes in the tangential sections	Rough eye, loss of pigmentation, and severe cell death
<i>elav</i> -GAL4	All neurons of the peripheral and central nervous system	No effect	No effect	Reduced viability	Reduced viability	Lethal
<i>Act5C</i> -GAL4	Ubiquitous expression in embryo	No effect	No effect	Late larval lethal	Male lethality and reduced viability in female	Late larval lethal
<i>dpp</i> -GAL4	Along the anterior-posterior boundary of imaginal discs, epithelial cells	No effect	No effect	No effect	No effect	No effect

additional bands. Less EGFP was observed with increasing length of the CGG repeat, consistent with earlier observations that migration of the 40S ribosomal subunit is impeded by a structure formed by the CGG repeat and that there is a modest reduction in FMRP levels from premutation alleles in human cells (Feng et al., 1995; Kenneson et al., 2001; Primerano et al., 2002).

Dosage- and Repeat Length-Dependent Toxicity of Fragile X Premutation rCGG

To test whether or not the premutation rCGG repeat produced a phenotype, we directed transgene expression to several tissues by crossing the lines to different GAL4 drivers. Transgene expression level was assessed by quantitative RT-PCR, and transgenic lines with both moderate and strong expressions were used for the following studies. In no case did expression of EGFP alone have an observed phenotypic effect (Table 1, Figure 2, column 1). In contrast, the expression of the premutation rCGG repeat, (CGG)₉₀, had deleterious consequences (Table 1). Strong expression of (CGG)₉₀-EGFP severely disrupted eye morphology when expression was directed to the retina using *gmr*-GAL4 drivers (Table 1, Figure 2, column 5). The fly retina is composed of approximately 800 virtually identical unit eyes, the ommatidia, which are arranged in a precise hexagonal array that makes up the adult eye (Figure 2, UAS-EGFP; Hsiung and Moses, 2002). Expression of (CGG)₉₀-EGFP led to dramatic cell death, loss of pigmentation, and ommatidial disruption (Figure 2, column 5). When expression was targeted to all developing cells of the peripheral and central nervous system using *elav*-GAL4, strong expression of (CGG)₉₀-EGFP caused lethality (Table 1; Lin and Goodman, 1994). Ubiquitous expression of (CGG)₉₀-EGFP in the embryo using the *Act5C*-GAL4 line also led to lethality at the late larval stage (Ito et al., 1997). However, no phenotype was observed in any transgenic line when expression was targeted to epithelial cells using the *dpp*-GAL4 line. To determine if this apparent different tissue sensitivity is due to different GAL4 activity among driver lines, we crossed different driver lines to UAS-EGFP. Compared to *gmr*-GAL4 and *elav*-GAL4,

we did not observe significant difference of promoter activity with *dpp*-GAL4 (data not shown). This indicates that with similar expression level, rCGG appears more toxic in neuronal than epithelial cells.

The severity of phenotype also appeared to be rCGG repeat dosage and length dependent. When (CGG)₆₀-EGFP was ubiquitously expressed at moderate levels in the embryo by *Act5C*-GAL4, there was no effect, while moderate expression of (CGG)₉₀-EGFP led to male lethality and reduced viability in females despite the fact that both (CGG)₆₀-EGFP and (CGG)₉₀-EGFP were transcribed at a similar level (Table 1). Under the control of *gmr*-GAL4, moderate expression of (CGG)₆₀-EGFP appears to have little effect on eye morphology and histology, while moderate expression of (CGG)₉₀-EGFP can lead, in either sex, to mild rough eye and small holes in tangential sections of the eye (Figure 2, columns 2 and 4). Thus, similar to the observations in humans, moderate expression of a large normal CGG repeat (CGG)₆₀ in *Drosophila* does not result in any phenotypes seen with premutation allele (CGG)₉₀. However, overexpression of either the 60 or the 90 CGG repeats always led to a more severe phenotype, indicating that dosage as well as repeat length is important (Figure 2, columns 3 and 5). Compared to strong (CGG)₆₀-EGFP expression, strong expression of (CGG)₉₀-EGFP had more severe consequences, using both *gmr*-GAL4 and *elav*-GAL4, despite the fact that (CGG)₉₀-EGFP was expressed at a lower level and overall rCGG repeat expression per se was lower (Table 1, Figure 2). Taking these results together, we demonstrated that, similar to the human observation, the fragile X premutation rCGG repeat indeed is pathogenic in *Drosophila*.

Fragile X Premutation rCGG Repeats Cause Progressive Neurodegeneration in the Eye

To determine if the rCGG-induced neurodegeneration is progressive with age in *Drosophila*, we examined the phenotypes caused by rCGG expression in the eyes of the aged flies, which has been extensively used to model other human neurodegeneration diseases (Muqit and Feany, 2002; Warrick et al., 1998). We observed the

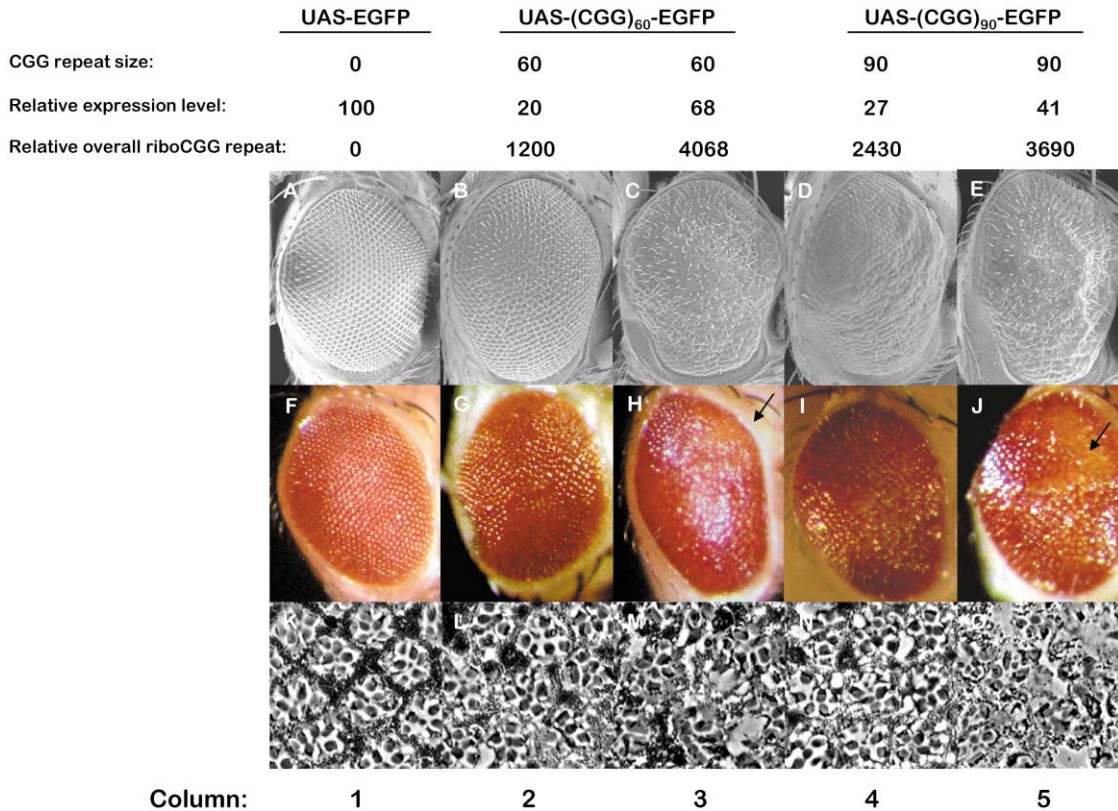


Figure 2. Expressions of Fragile X Premutation rCGG Repeats Disrupt Eye Morphology in a Dosage- and Length-Dependent Manner
Column 1, flies expressing EGFP only; column 2, moderate expression of (CGG)₆₀-EGFP; column 3, strong expression (CGG)₆₀-EGFP; column 4, moderate expression of (CGG)₉₀-EGFP; column 5, strong expression (CGG)₉₀-EGFP. Genotypes are UAS-(CGG)_N-EGFP in *trans* to *gmr*-GAL4. All the flies shown here are 4 days old. Relative transgene expression levels determined by quantitative RT-PCR (see Experimental Procedures) are indicated at the top, and relative overall rCGG repeats were calculated by rCGG repeat size times relative transgene expression level.

(A–E) SEM eye images.

(F–J) Light microscopic eye images. The areas with loss of pigmentation are indicated with arrow.

(K–O) Tangential sections through the eyes expressing different transgenes.

increased disruptions of eye morphology with the aged transgenic flies expressing (CGG)₉₀-EGFP from day 1 to day 30 (data not shown). Since *gmr*-GAL4 line used here drives expression in all cells of the developing and adult eyes, including the photoreceptor neurons as well as accessory pigment cells, we further examined whether the progressive cell death we observed is truly neurodegenerative rather than developmental (Ellis et al., 1993). Using the UAS/GAL4 system allowed us to conditionally modulate the promoter activity/transgene expression level by shifting fly cultures to different temperatures. We used a moderate expression line of (CGG)₉₀-EGFP to set up the cross with *gmr*-GAL4 at 18°C and maintain it at this temperature until after eclosion (Figure 3A). No abnormal eye structure was found at day 0 after eclosion or 30-day-old flies that were maintained at 18°C (Figure 3B and data not shown). The adult flies were transferred to 29°C to increase transgene expression level and aged (Figure 3A). Similar to the previous results, over the time, the eyes showed progressive loss of pigmentation and increased disruptions of ommatidia (Figure 3C). Indeed, by day 30, similar phenotype was observed as compared to the strong expression lines that were kept at

29°C (Figure 3D). No abnormality was observed with flies expressing EGFP only (data not shown). These results suggest that the phenotype that we observed is indeed degenerative. Further, we tested the toxicity of fragile X premutation rCGG repeats using a neuron-specific GAL4 driver, *elav*-GAL4. Because high-level expression of (CGG)₉₀-EGFP led to lethality, we performed similar cross to the above experiment using a moderate expression line of (CGG)₉₀-EGFP. No eye abnormality was found with flies at day 1 after eclosion or 4-day-old flies that were maintained at 18°C (Figures 3E and 3G). However, the flies shifted to 29°C displayed the loss of pigmentation and disruption of eye structures (Figure 3F). Based on these results, we conclude that, similar to human male premutation carriers, expression of the rCGG repeat in *Drosophila* leads to progressive neurodegeneration.

Premutation rCGG Repeats Induce the Formation of Inclusion, and the Molecular Chaperone *Hsp70* Modifies the Neurodegeneration Phenotype

Since postmortem examination of the brains of four fragile X premutation male patients with tremor/ataxia re-

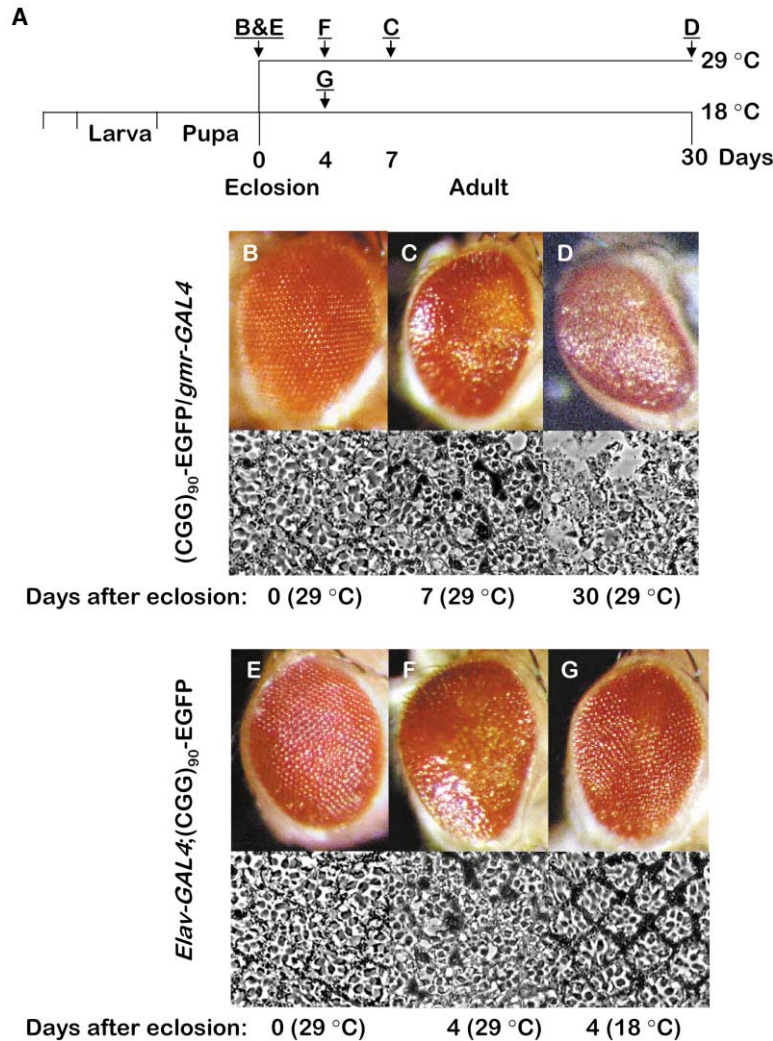


Figure 3. Fragile X Premutation rCGG Repeats Cause Neurodegeneration in Adult Eyes

(A) The scheme used to study the effect of rCGG repeats on adult eye. Flies were first crossed and grown at 18°C. After eclosion, the flies were shifted to 29°C to increase the expression of rCGG repeats.

(B) The fly eye from day 0 after eclosion is shown at left as control.

(C and D) Light microscopic eye images and tangential sections from the aged (7 and 30 days) flies of (CGG)₉₀-EGFP in *trans* to *gmr*-GAL4.

(E and G) Light microscopic eye images and tangential sections from the aged (4 days) flies of *elav*-GAL4; (CGG)₉₀-EGFP. The eyes from day 0 after eclosion and the aged fly at 18°C were shown as well.

vealed the presence of ubiquitin-positive intranuclear inclusion, we examined the *Drosophila* eye strongly expressing (CGG)₉₀-EGFP by immunohistochemistry using a panel of antibodies (Greco et al., 2002). Indeed, we found the inclusions in the retina of 7-day-old flies, with (CGG)₉₀-EGFP in *trans* to *gmr*-GAL4. The inclusions were positive for ubiquitin, *Hsp70* chaperone, and the proteasome, but negative for EGFP (Figure 4A and Supplemental Data at <http://www.neuron.org/cgi/content/full/39/5/739/DC1>). We further examined the subcellular localization of these inclusions. Using both DAPI (stain for chromatin) and wheat germ agglutinin (label the nuclear membranes), we found inclusions in both nuclei and cytoplasm, which is different from postmortem analysis of the four premutation brains examined to date (Figure 4B; Greco et al., 2002). To further examine the structure and formation of inclusions, we performed transmission electron microscopy using 7-day-old flies. Small and morphologically distinct nuclear aggregates were found in the nuclei of eye cells (Figure 4C). These small nuclear aggregates were extremely electron dense and localized closely with each other. In some cells, large nuclear inclusions could also be found, surrounded by small aggregates. Since these electron-dense structures were

not found in control flies (expressing EGFP only), they likely represent the nuclear inclusions observed in the above immunohistochemistry experiments (Figure 4C). These observations suggested that the premutation rCGG repeat could induce the formation of nuclear inclusions, as observed in humans.

Given that *Hsp70* chaperone is part of the inclusions induced by fragile X premutation rCGG repeats and *Hsp70* has been shown to be a universal suppressors of multiple human neurodegenerative models caused by mutant proteins, we tested whether overexpression of *Hsp70* could alter the toxicity of rCGG repeats by generating the flies carrying both (CGG)₉₀-EGFP and UAS-HSC70-4.WT transgenes along with *gmr*-GAL4 (Elefant and Palter, 1999). To our surprise, coexpression of fly *Hsp70* could suppress rCGG-induced degeneration, even though no mutant protein is present (Figure 4D). In addition, the coexpression of a dominant-negative form of *Hsp70*-4.K71S with an amino acid substitution in the ATP binding domain could enhance the phenotype caused by rCGG repeats (Figure 4D; Elefant and Palter, 1999). Very modest effect on the eye expressing *Hsp70*-4.K71S only was observed (Figure 4D). This data suggests that besides its role in the refolding of mis-

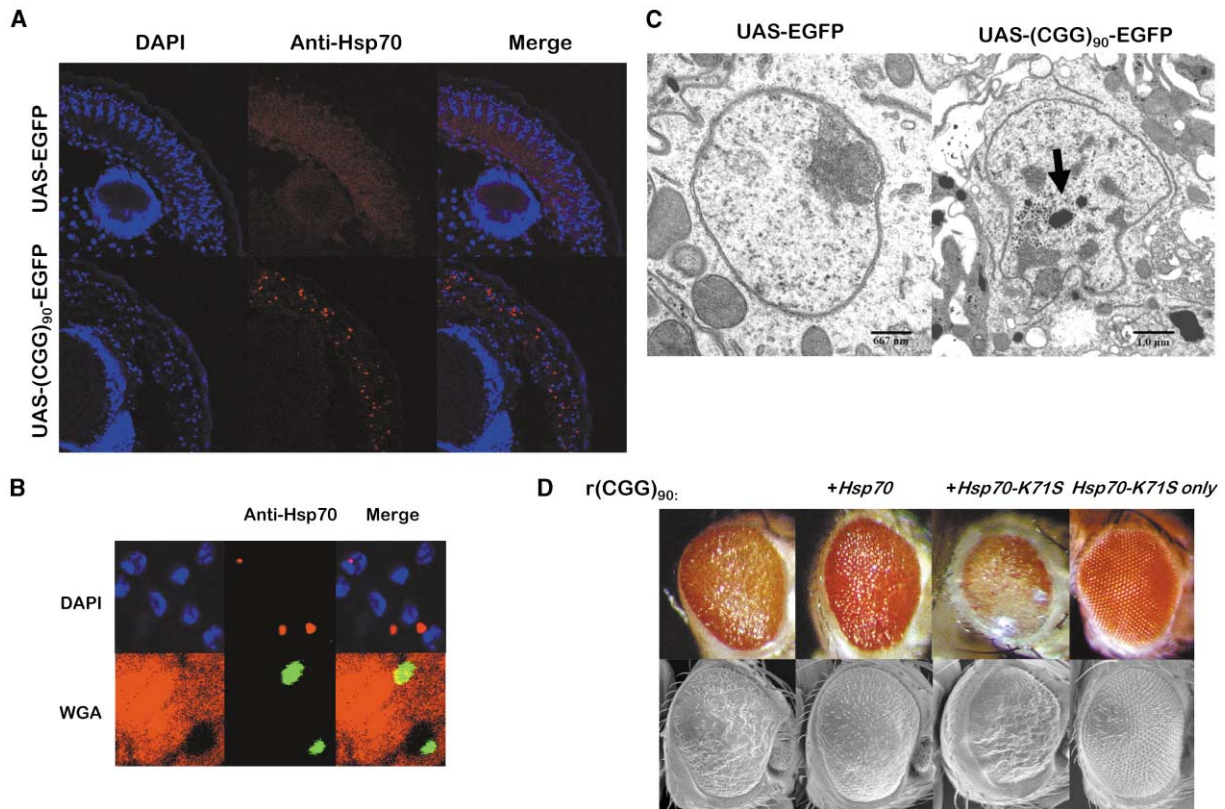


Figure 4. Fragile X Premutation rCGG Repeats Induce the Formation of Inclusions, and the Molecular Chaperone *Hsp70* Suppresses rCGG Repeat-Induced Neurodegeneration In Vivo

(A) Confocal images are shown of the brain sections from 7-day-old flies of either UAS-EGFP only or (CGG)₉₀-EGFP in trans to *gmr*-GAL4, stained with an antibody against *Hsp70* (red). The nuclei were stained with DAPI (blue).

(B) Higher magnification of individual cells from the retina expressing (CGG)₉₀-EGFP. The nuclei were stained with DAPI, and nuclear envelope was labeled with wheat germ agglutinin-tetramethylrhodamine. *Hsp70*-positive inclusions are both nuclear and cytoplasmic.

(C) The transmission electron micrographs of 7-day-old fly eyes expressing EGFP only (left) or (CGG)₉₀-EGFP (right). The nuclear inclusions are indicated with an arrow.

(D) Shown are light level and SEM pictures of the eyes of 7-day-old flies expressing (CGG)₉₀-EGFP only (left), with *Hsp70*-4.WT, with *Hsp70*-4.K71S, and *Hsp70*-4.K71S only. The *gmr*-GAL4 driver was used.

folded protein, as a molecular chaperone, the activity of *Hsp70* is a critical modulator of toxicity caused by not only mutant proteins but also pathogenic RNAs, although perhaps indirectly via unfolded proteins associated with the rCGG repeat.

Discussion

A progressive neurodegenerative syndrome has recently been described in some fragile X premutation carriers. However, the molecular basis of this neurodegenerative disorder is unclear and indeed the link between the *FMR1* premutation and neurodegeneration has yet to be formally established by a prospective study. Here we presented a *Drosophila* model and demonstrated that a fragile X premutation rCGG repeat alone is sufficient to cause neurodegeneration and induce the formation of inclusions. These findings strongly support the emerging clinical picture of a novel neurodegenerative disorder in humans carrying *FMR1* premutation alleles. This *Drosophila* model, besides shedding insight on the molecular basis of this neurodegeneration, pro-

vides the first experimental demonstration of RNA-mediated neurodegeneration.

RNA-Mediated Neurodegeneration

Neurodegenerative diseases are a heterogeneous group of disorders that usually strike in mid-life and include the polyglutamine diseases, the tauopathies, and Parkinson's disease (Zoghbi and Botas, 2002). Most of these mutations are found within the coding region of the relevant loci and share a common feature of misfolding of the mutant proteins. However, several neurodegenerative disorders, including Spinocerebellar ataxia type 8, 10, 12, and Huntington's disease-like type 2, have been linked to noncoding repeat expansions (Ranum and Day, 2002). While the underlying mechanisms for these disorders remains obscure, a toxic RNA-mediated gain-of-function has been suggested along with other possibilities. Apparently similar to these disorders is the recently described neurodegenerative syndrome characterized by progressive intension tremor and ataxia in fragile X premutation carriers (Hagerman et al., 2001). The common feature, besides neurodegenera-

tion, is the hypothetical link of a noncoding RNA-mediated neurodegeneration. Since full mutation patients with fragile X syndrome, who do not express *FMR1* message, do not show evidence of neurodegeneration, the causal focus has fallen upon the premutation message. Using *Drosophila* as a model system, we demonstrate here that indeed a portion of the human *FMR1* 5' UTR of a premutation allele containing 90 rCGG repeats is alone sufficient to cause neurodegeneration. We further show that a normal CGG repeat of 60 triplets, when moderately expressed, has little phenotype, and this same allele, when overexpressed, does lead to neurodegeneration, supporting the notion that overall rCGG abundance is critical. Therefore, it is likely for the human disorder that a combination of CGG repeat length and *FMR1* message abundance together may define a threshold for the clinical phenotype.

Formation of Inclusions Caused by rCGG Repeats

The intriguing observation from the neuroanatomical studies on fragile X premutation carrier males with neurodegenerative phenotype is the presence of ubiquitin-positive intranuclear neuronal inclusions (Greco et al., 2002). The origin of the intranuclear inclusions is unknown; however, some features are also observed with the polyglutamine disorders. In this study, we showed that fragile X premutation rCGG repeats not only cause neurodegeneration but also induce the formation of inclusions. The presence of ubiquitin and proteasome complex within the inclusions suggests a role of the protein degradation pathway in the pathogenesis of this tremor/ataxia syndrome associated with fragile X premutation carriers. Interestingly, recent neurohistological studies on expanded-CGG repeat mouse also showed the presence of ubiquitin-positive inclusions (Willemsen et al., 2003). These results suggest that high level of rCGG repeat can lead to the formation of inclusions. One discrepancy between our fly model and human pathological study is the presence of inclusion in both nuclei and cytoplasm in our fly model. However, in the expanded CGG repeat mouse model, both nuclear and cytoplasmic inclusions were also observed. This difference may be human specific; however, in some polyglutamine diseases, such as Huntington disease, inclusions were also found present in both nuclei and cytoplasm (Li et al., 2000). Alternatively, it might be an age-related phenomenon or disease state-dependent, since human pathological study was done using postmortem brains.

We also found that molecular chaperone, *Hsp70*, is a constituent of the inclusions, and variable expression of *Hsp70* could modify the degenerative phenotype in the eye. Studies in both flies and mice show that overexpression of chaperones or HSPs, particularly *Hsp70*, which help fold proteins or target them for degradation, increase resistance to polyglutamine-induced toxicity (Chan et al., 2000, 2002; Cummings et al., 2001; Fernandez-Funez et al., 2000; Kazemi-Esfarjani and Benzer, 2000; Warrick et al., 1999). However, in our fly model, the CGG repeats were only transcribed but not translated, there is no mutant protein to misfold and be a chaperone target. (We note that CGG, in any reading frame, cannot code for polyglutamine nor is any polyglutamine detected immunohistochemically in the inclusions, thereby

ruling out the trivial explanation of our results by upstream promiscuous translation.) It has been well known that long triplet repeats can form stable hairpin structure, and it is possible that the protein(s) interacting with long rCGG repeats (possible double-stranded RNA) may fold into a stable alternative conformation, which results in aggregation, and become the target for protein degradation (O'Donnell and Warren, 2002). In addition, *Hsp70* may also confer the protection by inhibiting signal transduction pathways leading to cell death, by preventing activation of stress kinases, or by blocking pro-caspase processing or caspase activation (Gabai et al., 1997; Mosser et al., 2000; Zhou et al., 2001). Finally, this data implicating the role of protein degradation in RNA-mediated neurodegeneration links this form of neurodegeneration to the larger class of neurodegenerative diseases exhibiting features of protein misfolding (Bonini and Fortini, 2003; Soto, 2003; Zoghbi and Botas, 2002). By inference of this *Drosophila* model, we might now speculate that the human disorders linked to noncoding repeat loci are likely to involve RNA-mediated neurodegeneration and to share this overall feature of protein misfolding, thus linking all human neurodegenerative diseases together.

Molecular Pathogenesis of Neurodegeneration Caused by rCGG Repeats

Pathogenic RNAs that alter cellular functions have been previously associated with several human diseases (Ranum and Day, 2002). In myotonic dystrophy type 1 (DM1), a CTG expansion in the 3' UTR sequesters CUG binding proteins from their normal cellular functions, leading to abnormal RNA splicing of several genes (Ranum and Day, 2002). It is likely that fragile X premutation rCGG may behave similarly. In the fragile X premutation carriers with elevated *FMR1* mRNA, the long rCGG tract may attract and sequester rCGG binding protein(s) from its normal functions, affect RNA metabolism, increase cellular toxicity, and lead to progressive cell death, particularly in the brain since it has highest expression of *FMR1* gene. Indeed, it has been shown that rCGG repeats could be bound by the proteins from mouse brain (Rosser et al., 2002). Identification of these rCGG binding proteins will be important to test this hypothesis and understand the pathogenesis of this novel disorder.

In conclusion, we demonstrated that RNA alone is sufficient to cause neurodegeneration and that this form of neurodegeneration shares the feature of protein misfolding involvement common to most other forms of genetic neurodegeneration. These data also strongly support the emerging clinical picture of a specific neurodegenerative disease associated with fragile X premutation carriers and suggest this disorder may exhibit a clinical threshold based upon total rCGG abundance. Finally, based upon the data presented here, the power of *Drosophila* genetics can now be used to dissect the molecular basis of RNA-mediated neurodegeneration through enhancer and suppressor screens and to test novel therapeutic approaches.

Experimental Procedures

Drosophila Genetics

The pUAST-EGFP construct was generated by cloning EGFP cDNA in the pUAST transformation vector. The pUAST-(CGG)₉₀-EGFP was

then generated by inserting a genomic DNA fragment (between primer C and F) from a fragile X premutation carrier upstream of the EGFP cDNA (EcoRI site) in the pUAST-EGFP transformation vector. The configuration of inserted CGG repeats is (CGG)₉AGG(CGG)₉AGG(CGG)₇₀. These constructs were confirmed by DNA sequencing and then injected in a *w¹¹¹⁸* strain by standard methods. The CGG repeat size from each transgenic line was determined by PCR and ALF sequencer. All the UAS and GAL4 lines used in this study were obtained from the Bloomington *Drosophila* stock center, including *gmr-GAL4*, *elav-GAL4*, *Act5C-GAL4*, and *dpp-GAL4*. Fly lines were grown on standard medium with yeast paste added. Adult flies were aged in standard medium after eclosion.

Histology, Immunofluorescence, and Electron Microscopy

For sections of adult fly eyes, adult heads were fixed, dehydrated, and embedded in Epon for vertical semithin sections. For immunofluorescence, dissected fly heads were fixed for 1 hr in 4% paraformaldehyde, rinsed in PBS, and then saturated in 20% sucrose overnight at 4°C. Tissues were embedded in OCT (Tissue Tech) and frozen by emersion in liquid nitrogen. Sections were cut at -20°C and then stained with different antibodies. Primary antibodies: mouse anti-ubiquitin (ZYMED), mouse anti-Hsp70/hsc70 (1:100; Strees-Gen), and mouse anti-19S regulator ATPase subunit 6b(Tbp7) (1:100; AFFINITI). Secondary fluorochrome-conjugated antibodies: Cy3 (1:500; Jackson ImmunoResearch) or Cy5 (1:500; Jackson ImmunoResearch). Chromatin was stained with DAPI (Molecular Probe), while nuclear envelope was labeled with WGA-tetramethylrhodamine (1:1000, Molecular Probe). Confocal microscopy was carried out on a Zeiss LSM 510 NLO system. For scanning electron microscopy (SEM) images, whole flies were dehydrated in ethanol, dried with hexamethyldisilazane (Sigma), and analyzed with an ISI DS-130 LaB6 SEM/STEM microscope. Standard transmission electron microscopy was performed on brains from 7-day-old experimental (UAS-(CGG)₉₀-EGFP/*gmr-GAL4*) and control (UAS-EGFP/*gmr-GAL4*) flies.

RT-PCR, Quantative PCR, and Western Blot

To test the expression of transgenes, UAS-EGFP, UAS-(CGG)₆₀-EGFP, and UAS-(CGG)₉₀-EGFP transgenic lines were crossed with *gmr-GAL4*. The fly heads of the following progenies were collected: UAS-EGFP/*gmr-GAL4*, UAS-(CGG)₆₀-EGFP/*gmr-GAL4*, and UAS-(CGG)₉₀-EGFP/*gmr-GAL4*. Trizol (Invitrogen) was used to isolate total RNA and protein from each genotype. For RNA analysis, RNA was reverse-transcribed with oligo(dT)₁₂₋₁₈ and SuperScript II (Invitrogen). The regular PCR was carried out using CGG repeat-specific primers (Primer C and F), EGFP-specific primers, and *Spent1*-specific primers (control). The transgene expression level was quantified using a LightCycler (Roche Molecular Biochemicals) while *Split ends (spen)* was used as internal control. Western analysis was performed as described (Kenneson et al., 2001). Anti-EGFP (Abcam) was used at a dilution of 1:2000. Anti-rabbit secondary antibodies were horseradish peroxidase (HRP) conjugated (Amersham Pharmacia) and detected by Enhanced ChemiLuminescence (Amersham Pharmacia Biotech).

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