Introduction

Trinucleotide repeat expansions within non-coding regions of RNA cause pathogenesis in a number of human diseases, including myotonic dystrophy type 1 (DM1), fragile X-associate tremor and ataxia syndrome (FXTAS), spinocerebellar ataxia type 8 (SCA8), and Huntington’s disease-like 2 (HDL2) [1-3]. The causative mutations of DM1, SCA8 and HDL2 are CTG repeat expansions. In DM1, the CTG expansion is located within the 3’ untranslated region (3’UTR) of the dystrophia myotonica-protein kinase (DMPK) gene [4,5]. The expanded CUG repeat RNA forms ribonuclear foci, and mislocalizes and misregulates RNA binding proteins such as CUG-BP1 and MBNL1 that influence alternative splicing [6-8]. Similarly, expanded CUG RNA also contributes to pathophysiology of SCA8 and HDL2 [9,10]. These findings indicate that CTG-based RNA expansion diseases may have the accumulation of RNA foci, sequestration of MBNL1, and disruption of alternative splicing as common components.

It is now recognized, however, that more than 70% of genomic loci show evidence of transcription from both sense and anti-sense strands in the mammalian genome [11], thus many of the trinucleotide repeat disease loci may display bidirectional transcription. Indeed, anti-sense transcripts have been detected for most trinucleotide repeat disease loci, including DM1 and SCA8 [12-16]. In DM1 cells, the sense and antisense transcripts can cause regional chromatin modification [15]. In SCA8, an antisense CAG transcript can be translated into a polyglutamine-encoding protein [14]. Given that anti-sense transcription occurs widely in the human genome [11,17,18], defining the range of potential roles and impact of anti-sense repeat transcripts on trinucleotide repeat diseases may provide novel insight into disease pathogenesis.

Drosophila has proven a powerful system to reveal insight into neurological and neurodegenerative disease with relevance to the human situation [19-22]. Thus, to gain insight into CUG RNA toxicity in the DM1 disease situation, we established transgenic flies that express pure, uninterrupted CTG repeat expansions in the 3’UTR of a control protein DsRed. These flies recapitulate major features of human CUG RNA expansion diseases. Given the finding that ~70% of genes show anti-sense transcription, we then tested the effect of co-expressing CTG and CAG disease transcripts in the 3’UTR of a control protein DsRed. These flies recapitulate major features of human CUG RNA expansion diseases. Given the finding that ~70% of genes show anti-sense transcription, we then tested the effect of co-expressing CTG and CAG disease transcripts. These data revealed dramatically enhanced toxicity upon co-expression of these transcripts; studies indicate this is due to the generation of triplet repeat-derived siRNAs which can target other repeat containing transcripts. These findings suggest that sense and anti-sense expanded repeat transcripts may interact in vivo to generate small RNAs that may dramatically enhance pathology in disease situations.

Results

Expression of expanded CUG RNA causes repeat-length dependent toxicity

To study CUG repeat RNA toxicity in flies, we generated UAS constructs with a pure CTG repeat expansion of 250 in length, (CTG)250, within the 3’UTR of DsRed (Figure 1A). Due to
Toxic Triplet Repeat-Derived siRNAs in DM1

Author Summary

Over 20 diseases are caused by the expansion of simple repeat sequences in the human genome. Among these are the polyglutamine protein diseases and other primarily RNA-based diseases like myotonic dystrophy (DM1) and spinocerebellar ataxia type 8 (SCA8). Recently, it has also become clear many genes are transcribed into RNAs from both strands. These include genes causing DM1 and SCA8. Small interfering RNAs (siRNAs) are short RNAs that are cleaved from double-stranded RNAs. These siRNAs can direct degradation of other complementary RNA sequences to reduce their expression. To study consequences of bidirectional transcription of pattern repeat sequences, we introduced repeat RNAs containing both CUG and CAG in a Drosophila model of DM1. We found that these repeat RNAs can be cleaved into repeat-derived siRNAs that are highly toxic to the animal. The mechanisms include targeting RNAs of other genes containing simple repeat sequence for degradation. These findings indicate that repeat-derived siRNAs generated from bidirectional transcription may, in a disease situation, contribute novel pathogenic components.

Enhancement of CTG-repeat toxicity by CAG-repeat transcripts

Bi-directional transcription is prevalent in the mammalian genome and is thought to occur in DM1 [11,12,15,16]. We therefore asked whether expression of a non-coding CTG repeat transcript together with a CAG repeat transcript would have an effect distinct from that of the CTG repeat transcript alone. Toxicity of non-coding (CAG)/250 repeat transcripts has been previously characterized, as adult-stage late-onset neurological dysfunction and loss [26]. Expression of either (CAG)/250 or (CTG)/200 in the eye with gmr-gal4, however, causes minimal effects (Figure 1E) [26]. Co-expression of (CTG)/200 together with a (CAG)/250 transcript, however, resulted in dramatic toxicity: the eye was now severely rough with abnormal pigmentation, demonstrating severe loss of retinal integrity (Figure 2A). The effect was synergistic, as expression of either two copies of a (CAG)/250 or two copies of (CTG)/200 repeat alone had limited or no effects (Figure S3). Additional combinations of repeat lengths with gmr-gal4 indicated that co-expression of (CTG)/250 or (CTG)/270 with (CAG)/250 repeats caused lethality at pre-adult pupal stages and/or generated adults with severely disrupted eyes, depending upon the precise combination of transgenes (Figure 3A and 3D, and data not shown). The interaction was dependent on disease-length repeat expansions, since flies co-expressing small (CTG)/19 and (CAG)/34 repeats did not show toxicity (Figure 2A).

A toxic interaction between CTG/CAG transcripts was also seen using a heat shock driver in adults, and a muscle driver 24B. Adult flies with a 30 min heat shock induction of (CAG)/250 and (CTG)/250 transcripts started to die at ~24 h, with 90% flies dead at 30 h (Figure 3B). In contrast, flies expressing (CAG)/250 alone, (CTG)/250 alone or non-pathogenic (CTG)/19/(CAG)/34 transcripts were not affected. In muscle, co-expression of (CAG)/100 with (CTG)/130 caused developmental lethality, while (CTG)/19/(CAG)/34, (CAG)/100, and (CTG)/130 flies were viable (Figure 3C).

Taken together, these data indicate that co-expression of disease-length CTG repeat transcripts together with comparable CAG repeat transcripts causes synergistic pathogenesis.

CTG/CAG transcripts are processed into small RNAs

To define the basis of the enhanced toxicity upon co-expressed CTG and CAG transcripts, we reasoned that the stability of each transcript may become greater, such that each transcript then displays greater toxicity. However, northern analysis indicated that the levels of the full-length repeat mRNAs were reduced (Figure 2B and Figure S4A), arguing against increased transcript stability accounting for the enhanced toxicity.

A second possibility was that novel interactions between the two transcripts were taking place, causing an effect distinct from either transcript on its own. Small RNA northern analysis revealed that small RNAs of ~21 nt, detected with either (CAG)5 or (CUG)5 probes, were generated in the co-expression situation (Figure 2C and Figure S4B). These data indicated that expanded CAG and CTG transcripts are processed into triplet repeat-derived small RNAs when co-expressed.

Toxicity of co-expressed CTG/CAG transcripts is dependent on Dcr2 and Ago2

Given that the CTG/CAG transcripts produced small RNAs, we determined whether the enhanced toxicity as well as generation of the small RNAs were dependent on dcr2, the enzyme in flies that cleaves double-stranded RNA for siRNA biogenesis [27]. Flies expressing (CTG)/250/(CAG)/250 with gmr-gal4 were lethal at late developmental stages; dissection of animals from the pupal case revealed severely disrupted eye morphology (Figure 3A). Flies expressing the repeat transcripts, but homozygous mutant for dcr2 gene function, were now viable and displayed significantly restored eye structure (Figure 3A). Homozygous loss of dcr2 activity also rescued organosmal lethality with heatshock induction of (CTG)/250/(CAG)/250 transcripts, and rescued developmental lethality upon expression of (CTG)/130/(CAG)/100 transcripts in muscle (Figure 3B and 3C). Loss of dcr2 had a minimal or no effect on toxicity of (CTG)/250 alone, (CAG)/250 alone or a mutant tau protein, the latter being associated with frontotemporal dementia (Figure S5A and S5B and data not shown). This indicates that dcr2 function is required for the toxicity associated with CTG/CAG co-expression. The dcr2 null background also blocked the biogenesis of the triplet repeat-derived small RNAs generated upon co-expression of the two transcripts, restoring the full length repeat RNA levels, concomitant with mitigation of toxicity (Figure 3E and 3F).
To characterize the triplet repeat-derived small RNAs, we asked whether they were methylated at the 3' end, a modification specific to siRNAs loaded to Ago2-RISC [28]. They were resistant to oxidation/ß-elimination normally, but sensitive to ß-elimination in the hen1 mutant background which prevents methylation (Figure 3G and Figure S4C), arguing that the repeat-derived small RNAs are siRNAs and are assembled into the Ago2-RISC complex. Further, loss of ago2 also dramatically mitigated (CTG)200/(CAG)250 toxicity (Figure 3D and Figure S6). To address specificity, we determined whether genes that modulate the miRNA pathway contributed to the CTG/CAG interaction. Reduction in gene dosage of dcr1 or ago1 showed no effect (data not shown). In addition, whereas upregulation of dcr2 dramatically enhanced CTG/CAG toxicity with concomitant increase of the triplet repeat-derived small RNAs, there was no effect of dcr1 upregulation (Figure S7). These data suggest that the triplet repeat-derived small RNAs are siRNAs in nature and that their toxic effects are dependent on Dcr2 and Ago2 activity.

Triplet repeat derived siRNAs compromise the expression of genes containing short CAG stretches

We tested whether the siRNAs may be competing with endogenous small RNAs for the biogenesis machinery, and thus by a titration mechanism causing toxicity. However, up-regulation of dcr2, which should suppress according to a titration mechanism, instead enhanced toxicity of expanded CTG/CAG (see Figure S7). Moreover, generation of miRNAs, with analysis of miR-277 and miR-8, and generation of endogenous small RNAs, with analysis of hsp-CG4068B and esiRNA-sl-1 (dependent on dcr2 activity [29]), were not affected upon co-expression of the CTG/CAG repeats.

Table 1. Length-dependent toxicity of CTG repeats in different tissues.

| Tissue type | CTG repeat number |
|-------------|------------------|
|             | 19   | 130  | 200  | 230  | 250  | 270  |
| muscle      | –    | –    | +/−  | +/−  | +/−  |
| neuronal     | –    | –    | +    | ++   | ++   | ++   |
| ubiquitous  | –    | ++   | ++   | ++   | ++   |

Transgenic lines bearing the specific repeat lengths noted were outcrossed to driver gal4 lines that selectively express in specific tissues. Muscle expression was with 248-gal4, neural expression with elav-gal4, and ubiquitous expression with da-gal4.

Key: −, viable; +/−, semi-lethal; +, pupal lethal; ++, larval lethal. doi:10.1371/journal.pgen.1001340.001
transcripts (Figure 4A). Transcription of retrotransponon 412 was also not affected in flies expressing expanded CAG/CAG repeats (Figure 4B), suggesting that the RNA interference pathways in these flies were largely intact. Together, these data argue that overwhelming endogenous RNA interference pathways cannot account for the enhanced toxicity.

In light of the requirement for ago2 and dcr2, we then asked whether the triplet repeat-derived siRNAs targeted other transcripts with small CAG or CUG stretches, such that disruption or loss of the activity of those genes may subsequently caused the deleterious effects. We selected two endogenous fly genes which, like their human counterparts, contain CAG repeat stretches, atx2 (containing CAG5) and tub (containing CAG5;CAA/CAG3), and analyzed their expression levels by realtime PCR. In flies expressing expanded CAG/CAG transcripts, the levels of these CAG-containing mRNAs were downregulated ~60–70%; transcripts without such repeats and control transcripts such as tubulin and appl were unaffected (Figure 4B). Further analysis revealed that the atx2 and tub transcripts were being cleaved within their CAG repeat stretches in flies expressing expanded CAG/CAG repeats in a dcr-2 dependent manner (Figure 4C and 4D). In contrast, we did not observe down-regulation of CUG containing transcripts by realtime PCR nor did we detect cleavage of these transcripts by RLM-RACE in flies expressing expanded CAG/CAG (data not shown; see Discussion). These data indicate that co-expression of CAG/CAG repeat transcripts generates triplet repeat-derived siRNAs that target other CAG-containing transcripts within the genome; deleterious effects on the levels and activity of these genes may contribute to the pathogenic effects of genes with expanded repeats that are bi-directionally transcribed.

Discussion

Like many genes within the mammalian genome [11], the DM1 gene displays bi-directional transcription, generating an anti-sense CAG repeat transcript in addition to the disease-associated CTG transcript [15]. These transcripts have been shown to interact in human cells to generate small RNAs, with one effect being local gene silencing [15]; however additional ways in which this may contribute to pathogenicity in disease is largely unknown. In order to provide new insight into DM1, we generated a Drosophila model by expressing pure, uninterrupted CTG repeat expansions; fly models for various disorders have revealed critical insight into a number of human disease situations (Clark et al., 2006; Fernandez-Funez et al., 2000; Jin et al., 2003; Meulener et al., 2005; Warrick et al., 1998). Interestingly, targeted expression of the long CTG repeats in the fly eye caused a variable toxic effect (see Figure S1). This was also observed in a fly model of SCA8, which carries an uninterrupted CTG repeat expansion [30]. In contrast, fly models generated using uninterrupted CTG repeats were not reported to show variable phenotype [24,25]. It is thus possible that phenotypic variability may be a feature of pure repeat sequences, which is in line with the fact that DM1 is among the most variable human disorders. To define potential effects of bi-directional transcription, we then co-expressed expanded CAG repeat transcripts with the DM1 CTG repeats. This resulted in dramatically enhanced toxicity concomitant with the generation of triplet repeat-derived siRNAs. Our results are in striking contrast with previous findings that co-expression of CGG and CCG expansions in flies leads to mitigated toxicity in a ago2-dependent manner [31], suggesting that toxicity derived from interactions between sense and anti-sense repeat transcripts may be specific to CTG/CAG situations. Both CAG and CUG strands can be loaded into mature, holo-RISCs presumably due to the symmetrical thermodynamic properties of the repeat small RNA duplex [28,32-34]. In our studies, we detected direct cleavage of
Figure 3. Toxicity and small RNA biogenesis of co-expressed CTG and CAG transcripts are dependent on dcr2 and ago2. A. Loss of dcr2 rescues the toxicity caused by co-expression of (CAG)250 and (CTG)250. With normal dcr2 gene function (wildtype), (CAG)250(CTG)250 caused lethality at the pre-adult pupal stage, with dissected animals showing severely disrupted eyes externally and internally. In the dcr2 null background, these flies were now viable and displayed a dramatically improved retinal structure. Genotypes: gmr-gal4 in trans to UAS-DsRed-(CTG)250 UAS-DsRed-(CAG)250 in normal or homozygous dcr2 null background. Age of flies: 1d. B. Mutation of dcr2 also rescued lethality of flies co-expressing expanded...
repeat transcripts. Survival of adult flies was scored 50 hr after 30 min heat shock induction of transgenic expression with hs-gal4. *p<0.01 when compared to dcr2 null background. ANOVA and Newman-Keuls post-test. Genotypes: hs-gal4 in trans to UAS-DsRed-(CAG)34 (UAS-DsRed-(CTG)19), 2xUAS-DsRed-(CAG)250, 2xUAS- DsRed-(CTG)250, and UAS-DsRed-(CAG)130/UAS-DsRed-(CTG)250 in normal or homozygous dcr2 background. C. Co-expression of (CAG)100 and (CTG)130 in muscle with 24B-gal4 leads to developmental lethality, which is rescued by dcr2 mutation. Genotype of parental flies: 24B-gal4: UAS-DsRed-(CAG)34 UAS-DsRed-(CTG)19/TM6B, Tb. 24B-gal4: 2xUAS-DsRed-(CAG)100 UAS-DsRed-(CTG)130/TM6B, Tb. dcr2[141-9]; 24B-gal4/ TM6B, Tb. *: p<0.05 when compared to flies in wildtype background. ANOVA and Newman-Keuls post-test. D. Homozygous loss ago2 suppressed the toxicity caused by co-expression of (CAG)250 and (CTG)200, with flies showing improved external eye. Flies were raised at 29 °C. Age of flies: 1d. Genotypes: gmr-gal4 in trans to UAS-DsRed-(CAG)250 UAS-DsRed-(CTG)200 in normal or homozygous ago2 null background. E. Loss of dcr2 restored levels of full-length repeat transcripts. Head RNA was subject to Northern blot. 18S rRNA, loading control. Genotypes: E: hs-gal4 in trans to UAS-DsRed-(CAG)250 UAS-DsRed-(CTG)250 in wildtype or dcr2 null background. G. Triplet repeat- derived small RNAs were methylated at the 3’ end by Hen1 shown by oxidation and β-elimination reactions. Small RNA from heads was analyzed by Northern blot and probed with (CAG5) oligonucleotide.

CAG containing transcripts, and we were unable to detect cleavage of CUG containing transcripts mediated by CAG small RNAs (see Figure 4B–4D). Although underlying reasons for this differential effect remain unclear, CUG and CAG transcripts may have differential expression levels or translation efficiencies, and/or CUG-containing and CAG-containing transcripts may be associated with different RNA binding proteins of various affinities, making CUG-transcripts less accessible to the RISC complex than CAG-containing transcripts [35]. A number of CUG-binding proteins have been defined, such as MBNL1, CUGBP1 and PKR [36-38]. Interestingly, in-vitro gel retardation analysis indicated that MBNL1 has a much lower affinity for CAG repeat RNA than CUG repeat RNA [36]. Moreover, expanded CAG transcripts, although co-localizing with MBNL1 in ribonuclear foci similarly to expanded CUG transcripts, do not appear to cause mis-regulation of alternative splicing in cells [39], further highlighting differential properties of these repeats in interacting with RNA binding proteins.

The toxicity caused by co-expression of expanded CAG and CTG was associated with deleterious effects on transcripts of other CAG containing genes within the genome; additional mechanisms that contribute to toxicity may also exist. A large number of genes contain CAG stretches in fly and human genomes (Table S1 and Table S2). The enhanced toxicity we observed in flies expressing expanded CAG and CTG may therefore be reflecting an additive effect of knockdown of multiple CAG-containing genes, with each individual gene contributing only partially to the overall outcome. Although further reducing atx2 dosage did not enhance toxicity of co-expressed CTG/CAG expansions (ZY and NB, unpublished observations), the compromised activities of many target genes may be involved and further compromising any single one has minimal impact. The toxic effects seen of the CAG/CTG situation may also be complicated by the later-onset and progressive nature of the toxicity. Further study will clarify the contribution of this mechanism, and key targets among all possible transcripts, to the overall phenotype of the disease. Moreover, the deleterious effects caused by triplet repeat derived small RNAs may be further exacerbated by the wide prevalence of CAG stretches in the human transcriptome (Table S2) and the relative low specificity of RNA interference when siRNAs and/or RNA targets contain simple repeats like CAG [40,41]. Such interactions may represent a novel activity of endo-siRNAs that characterize disease situations where bi-directional transcription spanning the repeat region occurs (Figure 4E).

We confirmed that two of CAG containing genes, atx2 and thp, are targets of the triplet repeat-derived siRNAs. Interestingly, CAG repeat expansions in ATXN2 (the human Ataxin-2 gene) and TBP define two of the CAG-repeat expansion diseases (SCA2 and SCA17, respectively). In such diseases, the expanded polyglutamine domain is thought to confer toxicity [1,2]; however, increasing evidence suggests that the loss-of-function of gene activity, and not just dominant activities of the protein with an expanded polyglutamine repeat region, occur in disease [42,43]. Our findings raise the possibility that bi-directional transcription of the repeat region in diseases like DM1 may confer additional components of pathogenicity due to deleterious interactions between the two overlapping repeat-containing transcripts through the generation and activity of triplet repeat-derived siRNAs.

Studies indicate that bi-directionally transcribed RNAs, and presumably resultant endogenous double-stranded RNAs, are processed into ~21–23 nt small RNAs in human cells [44,45]. This is despite the fact that in most mammalian cells, long exogenous double-stranded RNAs can elicit the interferon response [46,47]. That response presumably occurs in a threshold-dependent manner; cells may also respond differentially to long exogenous double-stranded RNAs versus endogenous double-stranded RNAs. Thus, these findings suggest that the biogenesis pathway of small RNAs from endogenous double-stranded RNAs is conserved in mammalian cells. Many loci are bi-directionally transcribed throughout the mammalian genome [11,17,18], and among these are a number of human trinucleotide disease genes, including SCA8 and DM1 [12,16]. In SCA8, an anti-sense transcript is proposed to encode a polyglutamine protein, which itself may have deleterious actions [14]. In DM1, the two transcripts interact to produce small RNAs that can have local effects on gene silencing [15]. Our findings raise another possibility, that processing of co-expressed transcripts containing CUG/CAG expansions into triplet repeat-derived siRNAs in vitro, may contribute to toxicity with widespread deleterious effects. These effects may include downregulating the expression of other genes containing CAG repeats. Among the genes that could be targets are the polyglutamine disease genes themselves, one of which is TBP. Expansion of the TBP polyglutamine repeat underlies SCA17 [48]; intriguingly, general transcriptional compromise has been shown to be a component of repeat expansion diseases [49,50]. Our studies raise the possibility that perhaps another reason why these diseases share transcriptional compromise may be that they share bi-directional transcript interactions that compromise common elements like TBP. This possibility underscores the idea of shared therapeutic targets and mechanisms in repeat expansion diseases.

It has been proposed that siCAG and siCUG may be used for therapy of triplet repeat expansion diseases based on findings in cell culture that these siRNAs seem to specifically target mutant transcripts with expanded repeats [51]. Our data suggest caution in designing such siRNA-based therapy, as in the intact organismal situation, pathogenic activities may be noted.

Although
previous findings suggest that expanded CUG alone can be processed into small RNAs, [51], our data suggest that both expanded CAG and CTG are required for triplet repeat-derived siRNA generation and toxicity in vivo (see Figure 2C and Figure S5). Thus, co-expressed CAG and CTG expansions may contribute to DM1 pathogenesis through a fundamentally different mechanism from that of CTG expansions alone.

Although our studies were conducted in fly models, the findings may apply to human trinucleotide expansion diseases. Targeting these diseases at the transcriptional level may therefore be a...
promising therapeutic approach that would minimize not only the effects of single expanded repeat transcripts, but deleterious interactions between sense and anti-sense repeat domains.

**Materials and Methods**

**Fly lines**

General fly lines were ordered from public stock centers and maintained at 25 °C on standard medium unless otherwise indicated. CGT repeats of various length (DNA templates kindly provided by C. Thornton, University of Rochester) were inserted into the 3’UTR of DsRed2 gene (Clontech) in pUAST. All transgenic constructs were confirmed by sequencing. Fly lines were generated by P-element mediated transformation. Repeat lengths were determined by Southern blot and confirmed by Genscan for select lines showing variability. dcr2<sup>L116S</sup>, ago2<sup>414</sup>, Hen1 mutant Pme<sup>00010</sup>, UAS-dcr1, UAS-dcr2, UAS-DsRed-(CAG)100 and UAS-DiRed-(CAG)250 lines are described [26,27,52-54].

**Western, Southern, and Northern blots**

Standard techniques were used. Primary antibodies for Westerns were anti-DsRed (1:400, anti-rabbit, Clontech), anti-actin (1:4,000, anti-mouse, Abcam). HRP conjugated secondary anti-mouse (1:4000, Chemicon) and anti-rabbit antibodies (1:4000, Zymed) were used with ECL+ reagents (Amerham). For Southern blots, genomic DNA was extracted from ~50 flies using the Gentra Puregene Cell Kit (Qiagen) and 5 μg of genomic DNA was fully digested with 200 units of EcoRI and Xbal. DsRed DNA was PCR amplified using primers: forward 5’-GCCCCTCTGGCC-CTTGCCC-3’ and reverse 5’-CTACAGAAACAGGTGGTG-GGC-3’, purified using QIAquick Gel Extraction Kit (Qiagen) and labeled using the High Prime DNA Labeling Kit (Roche Applied Science). For Northern blots, flies were heatshocked at 37 °C for 30 min and allowed to recover at 25 °C for 20 h. Total RNA was extracted from Trizol Reagent (Invitrogen) and treated with Turbo DNase (Ambion) and labeled using the mirVana miRNA Isolation Kit (Ambion). cDNA was synthesized in a 20 μl reaction volume from 0.2 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 0.2 μl of cDNA was used as the template in a 20 μl reaction volume diluted from the Power SYBR Green PCR Master Mix Kit (Applied Biosystems). Real-time PCR was performed in triplet or quadruplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems). Data were analyzed using the ΔΔCt method. Endogenous control was rp49. Each experiment was repeated at least three times on independent RNA preparations.

**Radioactive PCR**

Radioactive PCR for the alternative splicing assay was performed as described [55]. Total RNA was extracted from fly heads using RNeasy Mini Kit (Qiagen) and cdna was synthesized using the SuperScript First-Strand Synthesis for RT-PCR (Invitrogen). Primers used for radioactive PCR reaction were: forward 5’-GCCATGCTCATCGGAGAT-3’, reverse 5’-GCCATTTGACCATTCACCACATTGGTGTG-3’. The forward primer was labeled using the T4 polynucleotide kinase (New England Biolabs). PCR reactions were run 21–23 cycles and separated on 5% non-denaturing TBE polyacrylamide gels. The gel was then dried and exposed to Storage Phosphor Screens (GE Healthcare). Band densitometry was quantified using Image J (NIH).

**Real-time PCR**

0-3d flies were heatshocked at 37 °C for 30 min and recovered at 25 °C for 9 h. Total RNA was extracted from fly heads using Trizol Reagent (Invitrogen), treated with Turbo DNase (Ambion) and further purified using the mirVana miRNA Isolation Kit (Ambion). cDNA was synthesized in a 20 μl reaction volume from 0.2 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed in triplet or quadruplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems). Data were analyzed using the ΔΔCt method. Endogenous control was rp49. Each experiment was repeated at least three times on independent RNA preparations.

**In situ hybridization**

In situ hybridization was performed as described [26].

**RNA ligase mediated amplification of cdna ends (RLM-RACE)**

To detect cleavage of atx2 and tbl transcripts, RLM-RACE and cloning of RLM-RACE products were carried out using the GeneRacer Kit (Invitrogen, Carlsbad, CA). Briefly, 3-4d flies were heatshocked at 37 °C for 30 min and then maintained at 25 °C for 14 hr. Total RNA was extracted from fly heads using the RNAeasy kit (Qiagen, Valencia, CA). The 5’ GeneRacer RNA adaptor molecule was ligated onto the total RNA population. Ligated products were reverse transcribed using random primers and nested PCR was performed using primers derived from the 5’ GeneRacer adaptor and gene specific primers, respectively, to detect RISC cleavage products. PCR products were analyzed on 1% agarose TAE gel. To analyze cleavage sites, PCR products were gel purified using the gel purification kit (Qiagen, Valencia, CA).

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CA), cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced. Primers used for nested PCR were:
dEBch5RACEst3: 5′-GGGCCCCATCTGTTGATGT-3′
dEBch5RACEst2: 5′-TGGTTGATGTTGCTCAGG GC-AC TT-3′
dAx2ch5RACEst1: 5′-TGTGGC CGGCGCATTTGATG- GAAA-3′
dAx2ch5RACEst2: 5′-TGTGGCGCGGCGCTGTCGAC TT-3′
GeneRacer5′ Primer: 5′-CGACACTGAGCAGGACGACGAC AGGAGTA-3′
GeneRacer5′ nested Primer: 5′-GGACACTGACATGGACT GAAGGAGUAGAAA-3′

Heat shock survival assay
0-3d flies were heat shocked at 37 °C for 30 min and then maintained at 25 °C for 50 hr. Numbers of dead/living flies were recorded. At least ~100 flies were scored for each genotype and the experiments were repeated three times.

Viability assay
24B-gal4 driver flies were outcrossed to flies of appropriate genotype and progeny flies were scored for viability. At least 100 progeny flies were scored for each cross and experiments were repeated three times.

Supporting Information
Figure S1 Variable effect of flies expressing (CTG)270 repeat transcripts in the eye. (A) Representative images of mild, medium and severe eyes of the flies expressing the (CTG)270 repeat in the eye with gmr-gal4. Severe were eyes that were smaller in size with more pigmentation loss, less organized and rougher eye surface than flies with mild effects. Retinal sectioning confirmed that the internal retinal structure correlated with the degree of disruption of the external eye. 1d flies of genotype gmr-gal4/UAS-DsRed-(CTG)270. (B) Percentage of flies in each category. 740 female flies were scored. Repeat length of parental males was 265–273 by genescan (data not shown). Both male and female flies showed variability; shown here are females.

Figure S2 Expanded CUG repeat RNA accumulation in the nuclei and affect alternative splicing. (A) RNA foci in flies expressing expanded CTG repeat transcript. Body-wall muscles of 3rd instar larvae were stained with propidium iodide to highlight nuclei (left panel), and FAM-labeled (CAG)7 probe for CUG RNA accumulation (right panel). CTG transcripts accumulate in (CTG)230 but not in control (CTG)19 larvae. Foci were sensitive to RNase A but resistant to DNase I treatment. Genotype: 24B-gal4/UAS-DsRed-(CTG)230, 24B-gal4/UAS-DsRed-(CTG)19 and 24B-gal4/UAS-DsRed-(CTG)270 (B) Structure of the minigene construct for the splicing assay. The human cTNT minigene reporter (kindly provided by T Cooper) was subcloned into pUAST vector for transgenesis. In fly photoreceptor neurons, exon 2 of sTNI was alternatively spliced, resulting in either 140 bp or 140 bp RT-PCR product using the primers indicated. (C) Expression of expanded CUG RNA promoted exclusion of exon 2 of sTNI as indicated by the ratio of 140 bp/110 bp. A representative radioactive gel image was shown on top right corner of the chart. Genotypes: Rhl-gal4 UAS-heTNT in trans to (lane 1) w1118, (lane 2) UAS-DsRed-(CTG)19, (lane 3) UAS-DsRed-(CTG)270; UAS-DsRed-(CTG)230 and (lane 4) 2xUAS-DsRed-(CTG)250. Quantification of 3 independent experiments is shown. (*p<0.05, **p<0.01 comparing to (CTG)19; ANOVA and Dunnett’s post test).

Figure S3 Expression of two copies of (CAG)250 or (CTG)200 transcripts alone did not cause an effect as severe as that of co-expression of (CAG)250 together with (CTG)200. External eyes and internal retinal structure. Genotypes: gmr-gal4 in trans to (left) 2xUAS-DsRed-(CAG)250 and (right) 2xUAS-DsRed-(CTG)200. Compare to eyes in Figure 3A, which shows that co-expression of short CAG and CTG transcripts has no effect, while co-expression of long (CAG)250 with (CTG)200 transcripts is severely toxic. Expression of a single copy of the (CAG)250 transcript in the eye has minimal effects, expression of a single copy of the (CTG)200 transcript is shown in Figure 1E.

Figure S4 Repeat derived siRNAs are generated when CTG is co-expressed with CAG. (A) Northern blot. The expression level of the (CAG)250 transcript is reduced when co-expressed with the (CTG)250 transcript. The blot was also probed with DsRed to compare the relative level between (CAG)250 and (CUG)250. (B) ~21 nt small repeat RNAs were generated as probed by (CUG)5 when expanded CAG and CTG repeats were co-expressed. Genotype: hs-gal4 in trans to UAS-DsRed-(CAG)19 UAS-DsRed-(CAG)34, w1118, UAS-DsRed, UAS-DsRed-(CAG)250, UAS-DsRed-(CTG)250 and UAS-DsRed-(CAG)250 UAS-DsRed-(CTG)250 (C) Triplet repeat-derived small CAG RNAs were methylated at the 5′ end by Hn1 shown by oxidation and β-elimination reactions. Small RNA from heads was analyzed by Northern blot and probed with (CUG)5. 28 sRNA blot served as the control. Genotype: hs-gal4 in trans to UAS-DsRed-(CAG)250 UAS-DsRed-(CTG)270.

Figure S5 Mutations of dcr2 or ago2 have minimal effects on toxicity of the expanded CAG transcript and tauR106W. (A) Loss of dcr2 does not block eye degeneration caused by expression of (CTG)250 transcripts. Arrows highlight black necrotic patches on the eye surface, age of flies: 14d animals. Genotypes: gmr-gal4, UAS-DsRed-(CTG)250 in normal or homozygous dcr2 background. (B) Loss of dcr2 has a minimal effect on toxicity due to expression of mutant tau R106W. Age of flies: 1d. Genotypes: gmr-gal4, UAS-DsRed in normal or homozygous dcr2 background. (C) Loss of ago2 does not prevent eye degeneration caused by expression of (CTG)270. Loss of pigmentation and disorganization of ommatidia were similar between wildtype and ago2 null flies expressing the (CTG)270 transcript. Shown are eyes with severe phenotype in both genotypes. Genotypes: gmr-gal4, UAS-DsRed-(CTG)270 in normal or homozygous ago2 background.

Figure S6 Loss of ago2 suppresses CAG/CTG toxicity. (A) Survival rate of flies expressing various transgenes by hs-gal4 was scored 50 h after heat shock induction of transgene expression. *: p<0.05 when compared to flies in null ago2 background. Statistics: ANOVA and Newman-Keuls post-test. Genotype of flies: hs-gal4 in trans to: UAS-DsRed-(CAG)34 UAS-DsRed-(CTG)19, UAS-DsRed-(CAG)250/ UAS-DsRed-(CTG)250, UAS-DsRed-(CTG)270/UAS-DsRed-(CTG)250, UAS-DsRed-(CTG)250/UAS-DsRed-(CAG)250 in either wildtype or ago2 null background. (B) Internal eye degeneration of (CTG)200/(CAG)250 is suppressed in ago2 null flies. 1d animals. Genotypes: gmr-gal4 in trans to UAS-DsRed-(CAG)250 UAS-DsRed-(CTG)200 in normal or homozygous ago2 null background. 29 °C.

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Figure S7  Uregulation of dcr2 enhances toxicity of (CTG)200/(CAG)250 and increases levels of triplet repeat derived small RNAs in flies expressing (CTG)130/(CAG)100. (A) Flies coexpressing expanded (CTG)/CTG transcripts with added dcr2 activity showed severe eye disruption. In contrast, uregulation of dcr1 had little effect. Genotypes: from left to right: gmr-gal4 UAS-DsRed- (CTG)200; UAS-DsRed- (CTG)/250 in trans to w1118, UAS-dcr2 and UAS-dcr1. (B) Dcr2 upregulation increases levels of triplet repeat derived small RNAs. Genotype of flies: hs-gal4/UAS-DsRed- (CAG)100 UAS-DsRed- (CTG)130 either with or without UAS-dcr2. Found at: doi:10.1371/journal.pgen.1001340.s007 (1.88 MB TIF)

Table S1  Fly genes that contain CAG or CUG repeats. List of fly genes that contain 7 or more CAG or CUG repeats in at least one of the splicing variants was obtained by performing a BLAST search of the Drosophila melanogaster Refseq RNA database using (CAG)7 as the query sequence. Gene accession numbers were converted to gene names using the Gene ID Conversion Tool (http://david.abcc.ncifcrf.gov/conversion.jsp). Found at: doi:10.1371/journal.pgen.1001340.s008 (0.23 MB DOC)

Table S2  Human genes that contain CAG or CUG repeats. List of human genes that contain 7 or more CAG or CUG repeats in at least one of the splicing variants was obtained by performing a BLAST search of the Homo sapiens Refseq RNA database using (CAG)7 as the query sequence. Gene accession numbers were converted to gene names using the Gene ID Conversion Tool (http://david.abcc.ncifcrf.gov/conversion.jsp). Found at: doi:10.1371/journal.pgen.1001340.s009 (0.19 MB DOC)

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Author Contributions

Conceived and designed the experiments: ZY NM. Performed the experiments: ZY XT. Analyzed the data: ZY NM. Wrote the paper: ZY NM.

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