Cell Reports
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Cell PRESS
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Sequestration of DROSHA and DGCR8 by Expanded CGG RNA Repeats Alters MicroRNA Processing in Fragile X-Associated Tremor/Ataxia Syndrome

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http://dx.doi.org/10.1016/j.celrep.2013.02.004

SUMMARY

Fragile X-associated tremor/ataxia syndrome (FXTAS) is an inherited neurodegenerative disorder caused by the expansion of 55–200 CGG repeats in the 5′ UTR of *FMR1*. These expanded CGG repeats are transcribed and accumulate in nuclear RNA aggregates that sequester one or more RNA-binding proteins, thus impairing their functions. Here, we have identified that the double-stranded RNA-binding protein DGCR8 binds to expanded CGG repeats, resulting in the partial sequestration of DGCR8 and its partner, DROSHA, within CGG RNA aggregates. Consequently, the processing of microRNAs (miRNAs) is reduced, resulting in decreased levels of mature miRNAs in neuronal cells expressing expanded CGG repeats and in brain tissue from patients with FXTAS. Finally, overexpression of DGCR8 rescues the neuronal cell death induced by expression of expanded CGG repeats. These results support a model in which a human neurodegenerative disease originates from the alteration, in *trans*, of the miRNA-processing machinery.

INTRODUCTION

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a neurodegenerative disorder that affects older adult males who are carriers of an expansion of 55–200 CGG repeats in the 5′ UTR of the fragile X mental retardation 1 (*FMR1*) gene (Hagerman et al., 2001). The clinical features of FXTAS include progressive intention tremor and gait ataxia, frequently accompanied by progressive cognitive decline, parkinsonism, peripheral neuropathy, and autonomic dysfunctions (Jacquemont et al., 2003). Principal neuropathology of FXTAS includes mild brain atrophy and white matter lesions with the presence of ubiquitin-positive nuclear neuronal and astrocytic inclusions (Greco et al., 2002), which contain the expanded CGG RNA repeats (Tassone et al., 2004). In contrast to fragile X syndrome, where full mutations (>200 CGG repeats) result in hypermethylation and silencing of the *FMR1* gene, FXTAS carriers of shorter CGG expansions (55–200 CGG repeats) present increased expression of *FMR1* mRNA levels and normal, or near-normal, FMRP expression (Tassone et al., 2000). These observations suggest a toxic RNA gain-of-function model for FXTAS. In support of that model, cellular and transgenic *Drosophila* and mouse models demonstrate that the sole expression of a mutant RNA containing expanded CGG repeats is sufficient to induce the formation of ubiquitin-positive aggregates and to cause a pathology similar to human FXTAS (Willemsen et al., 2003; Jin et al., 2003; Arocena et al., 2005; Entezam et al., 2007; Hashem et al., 2009). A toxic RNA gain-of-function model predicts that expanded CGG repeats are pathogenic by sequestering specific RNA-binding proteins, resulting in loss of their normal functions and, ultimately, in neuronal cell dysfunction and death. Consistent with a titration model, various proteins were found to colocalize with CGG or ubiquitin-positive inclusions (Iwahashi et al., 2006; Jin et al., 2007; Sofola et al., 2007; Sellier et al., 2010); however, the pathological consequences of their recruitment are unclear, suggesting that the protein(s) sequestered within CGG RNA aggregates and responsible for the neuronal cell death, remains to be identified.

MicroRNAs (miRNAs) are small, conserved, noncoding RNAs that are key components of posttranscriptional gene regulation and are involved in the control of many fundamental processes, including both differentiation and survival of neurons (Schaefer et al., 2007; Davis et al., 2008; De Pietri Tonelli et al., 2008; Stark et al., 2008; Haramati et al., 2010; Hébert et al., 2010; Huang et al., 2010; Fénelon et al., 2011; Schofield et al., 2011). miRNAs are initially transcribed by the RNA polymerase II as primary miRNA (pri-miRNAs) transcripts, which are processed into precursor miRNAs (pre-miRNAs) by the type III RNase,

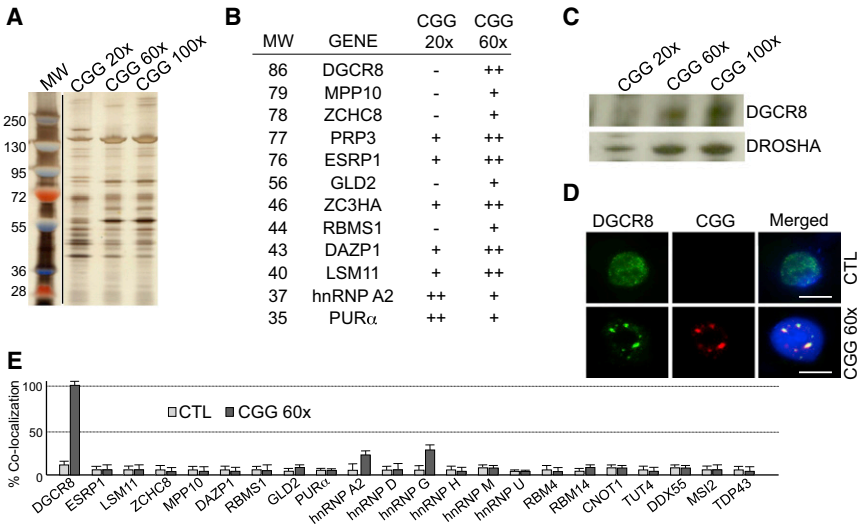


Figure 1. Identification of Proteins Associated with Expanded CGG Repeats

(A) Silver staining of proteins extracted from mouse brain and captured on streptavidin resin coupled to biotinylated in-vitro-transcribed RNA containing expanded CGG repeats of normal or pathogenic size. MW, molecular weight.

(B) List of the main proteins identified by nanoLS-MS/MS that are preferentially associated with 20 or 60 CGG repeats.

(C) Western blotting against DGCR8 or DROSHA of mouse brain proteins captured on 20 or 60 CGG RNA repeat columns.

(D) RNA FISH against CGG repeats, coupled to IF against DGCR8, of COS7 cells transfected with a plasmid expressing either no repeats (CTL) or 60 CGG repeats. Magnification, 630×. Scale bars, 10 μm.

(E) Percentage of colocalization of the endogenous-tested candidate proteins within CGG RNA aggregates analyzed by RNA FISH coupled to IF on COS7 transfected for 24 hr with a plasmid expressing either no repeats or 60 CGG repeats.

See also Tables S1, S2, and Figure S1.

DROSHA, and the double-stranded RNA-binding protein, DGCR8, which anchors DROSHA to the pri-miRNA transcript (Lee et al., 2003; Denli et al., 2004; Landthaler et al., 2004; Gregory et al., 2004; Han et al., 2004; Wang et al., 2007). Pre-miRNAs are then exported into the cytoplasm, where they are processed into mature miRNAs by the DICER enzyme. Global reduction of miRNA expression, for example through inactivation of DICER or DGCR8 in mice, results in embryonic lethality and if conditionally lost in brain, leads to neuronal dysfunction and cell death (Schaefer et al., 2007; Davis et al., 2008; De Pietri Tonelli et al., 2008; Stark et al., 2008; Haramati et al., 2010; Hébert et al., 2010; Huang et al., 2010; Fénelon et al., 2011; Schofield et al., 2011).

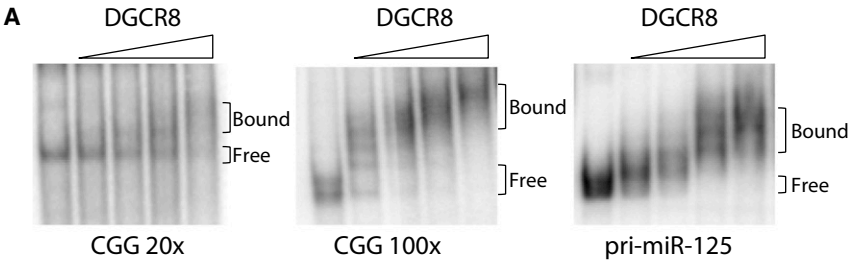
Here, we find that DGCR8 binds preferentially to expansions of CGG repeats of pathogenic length. This association results in titration of DGCR8 pri-miRNA-binding activity and in the partial sequestration of DGCR8 and its partner, DROSHA, within CGG RNA aggregates. Consequently, the processing of pri-miRNAs is reduced in cells expressing expanded CGG repeats, and in brain samples from patients with FXTAS, resulting in decreased levels of mature miRNAs. Finally, the expression of pathogenic-expanded CGG repeats in cultured mouse cortical neurons results in decreased dendritic complexity and reduced neuronal cell viability. Importantly, the sole overexpression of DGCR8 restored to normal both the dendritic morphological abnormalities and the loss of neuronal cells, demonstrating that titration of DGCR8 by expanded CGG repeats is a leading event to CGG-induced neuronal cell death.

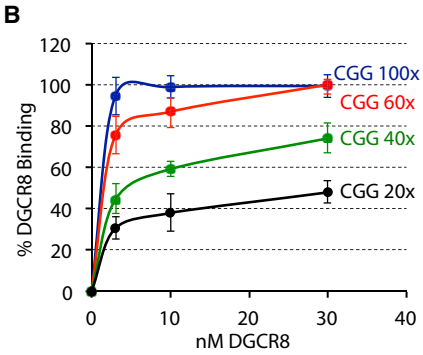
RESULTS

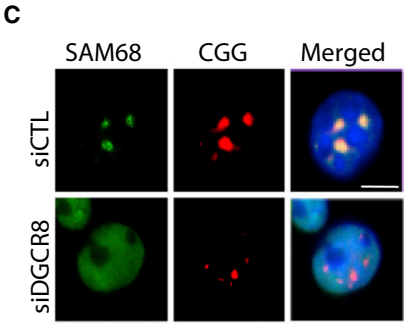
Identification of Proteins Associated with Expanded CGG Repeats

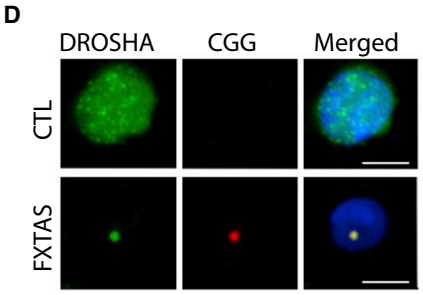
To identify proteins involved in FXTAS physiopathology, proteins extracted from mouse brain nuclei were captured on streptavidin resin coupled to biotinylated RNA composed of nonpathogenic (20 CGG repeats) or pathogenic expansions (60 or 100 CGG repeats), eluted, separated on SDS-PAGE, and identified by nano-LC-MS/MS analysis (Figure 1A). More than 30 RNA-binding proteins were identified (Figure 1B; Tables S1 and S2), including hnRNP A2/B1, hnRNP G, and PURα, which were also identified in previous studies (Iwahashi et al., 2006; Jin et al., 2007; Sofola et al., 2007; Sellier et al., 2010). However, these proteins were recruited preferentially onto short CGG repeats compared to long pathogenic CGG stretches (Figure 1B). In contrast, ten proteins (ESRP1, PRP3, ZC3HA, LSM11, ZCHC8, MPP10, DAZP1, RBMS1, GLD2, and DGCR8) were found preferentially associated with expanded CGG repeats of pathogenic size (Figure 1B). We confirmed the preferential binding of DGCR8 on long CGG stretches by western blotting on the protein eluted from the RNA affinity columns (Figure 1C). The partner of DGCR8, DROSHA, was also preferentially recruited to long expanded CGG repeats (Figure 1C).

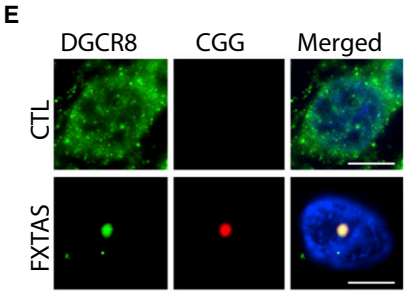
Next, to discard nonspecific RNA-binding proteins, we tested whether these candidate proteins colocalize with the aggregates formed by expanded CGG repeats in transfected COS7 cells (Figures S1A and S1B). Among the ten candidates tested, only three (PRP3, ZC3HA, and DGCR8) colocalized with CGG RNA aggregates (Figures 1D and S1A). However, we noted that PRP3 and ZC3HA were naturally localized in speckles, which are normal nuclear structures enriched in some splicing factors and that were found previously to be associated with CGG RNA aggregates in transfected cells (Sellier et al., 2010). Thus, neither PRP3 nor ZC3HA was specifically recruited within CGG RNA aggregates (Figure S1A). In contrast, DGCR8 presented a diffuse pattern within the nucleoplasm, but upon expression of 60 expanded pathogenic CGG repeats, DGCR8 changed localization and was recruited within CGG RNA inclusions (Figure 1D). As further controls, we also tested the colocalization of proteins, such as hnRNP A2/B1 and PURα, which were found mostly associated with 20 biotinylated CGG RNA repeats in vitro. Consistent with a

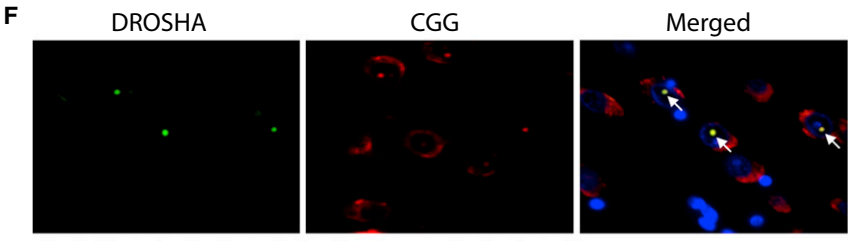












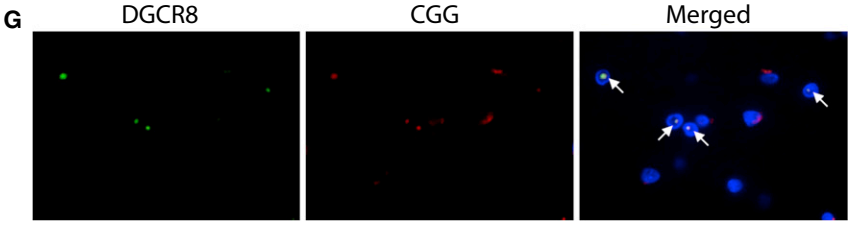


Figure 2. DGCR8 and DROSHA Bind to Expanded CGG RNA Repeats

(A) Gel shift assays of purified bacterial recombinant His-DGCR8Δ with 100 nM (30,000 cpm) of uniformly α-[^{32}P]CTP internally labeled in-vitro-transcribed RNA containing 20 or 100 CGG repeats or the pri-miR-125.

(B) Quantification of DGCR8 binding to 20, 40, 60, or 100 CGG RNA repeats.

(C) RNA FISH against CGG repeats, coupled to IF against SAM68, on COS7 cells cotransfected with a plasmid expressing 60 CGG repeats and a siRNA against the luciferase (siCTL) or against DGCR8.

(D and E) RNA FISH of CGG repeats coupled to IF of DROSHA or DGCR8 on brain sections (hippocampal area) of age-matched control or patients with FXTAS. Magnification, 630×. Nuclei were counterstained with DAPI.

(F and G) Larger fields of CGG RNA FISH coupled to IF of DROSHA or DGCR8 on brain sections of patients with FXTAS. White arrows indicate CGG RNA aggregates. Note some nonspecific perinuclear background inherent to RNA FISH of autopsied human brain samples.

Scale bars, 10 μm.

See also Figure S2.

preferential binding to normal but not to expanded CGG repeats, we found no significant colocalization of these candidates with CGG RNA aggregates at early time points (Figure 1E). Thus, we pursued our study on the one protein found specifically associated with expanded CGG repeats of pathogenic size, DGCR8.

DGCR8 Binds to Mutated RNA Containing Expanded CGG Repeats

Recruitment of DGCR8 to biotinylated CGG RNA questions whether DGCR8 binds directly, or through indirect protein-protein interactions, to expanded CGG repeats. Gel shift assays showed that purified recombinant HIS-tagged DGCR8 protein binds directly to RNAs containing expanded CGG repeats of pathogenic size (60 and 100 CGG repeats; Figures 2A, S2A, and S2B). In contrast, DGCR8 binds weakly to RNAs containing CGG stretch of nonpathogenic size, such as 20 or 40 CGG repeats (Figures 2A, S2A, and S2B). Furthermore, DGCR8 binds to expanded CGG repeats with an affinity similar to control pri-miRNAs, such as pri-miR-124, pri-miR-125, or pri-Let-7 (Figures 2A and S2A). UV-cross-linking assays confirmed that purified recombinant DGCR8 binds directly to control pri-miR-124 and pri-miR-125, as well as to expanded CGG repeats of pathogenic size, but does not bind to short CGG stretch (Figure S2B).

The in vitro binding of DGCR8 to long expanded CGG repeats prompted us to investigate whether DROSHA and DGCR8 are

recruited within the nuclear aggregates found in cell models of FXTAS. CGG RNA aggregates are dynamic nuclear structures that accumulate various proteins in a time-dependent manner (Sellier et al., 2010), which raises the question of the timing of DROSHA and DGCR8 recruitment. Analysis of the formation of CGG RNA aggregates at various time points after transfection of COS7 cells with a plasmid expressing 60 CGG repeats revealed that DROSHA and DGCR8 colocalized within CGG aggregates from the time of their formation (6–8 hr posttransfection; data not shown). Furthermore, depletion of either DROSHA or DGCR8 by siRNA reduced the recruitment of SAM68, one of the earliest proteins found to colocalize with CGG aggregates (Figures 2C and S2C). Next, coimmunoprecipitation experiments demonstrated that DROSHA and DGCR8 interact with SAM68, suggesting that the recruitment of SAM68 within CGG RNA aggregates is mediated through protein-protein interactions with DROSHA or DGCR8 (Figure S2D). These results suggest that in transfected cells, DROSHA and DGCR8 are among the first proteins to be recruited within the CGG RNA aggregates and are essential for the further aggregation of other proteins, such as SAM68. These results were obtained in cells expressing large amounts of expanded CGG RNA repeat. Thus, to rule out any overexpression bias, we tested the localization of endogenous DROSHA and DGCR8 in brain sections from patients with FXTAS. RNA FISH coupled to immunofluorescence (IF) experiments showed that both DROSHA and DGCR8 consistently colocalized with endogenous CGG nuclear RNA aggregates in brain sections of patients with FXTAS (Figures 2D and 2E; larger fields in Figures 2F and 2G), whereas DROSHA and DGCR8 were diffusely localized within the nucleoplasm of age-matched non-FXTAS controls. Finally, we tested the localization of endogenous DROSHA, DGCR8, and CGG aggregates in brain sections of a knockin mouse model, in which endogenous CGG repeats had been replaced with an expansion of 98 CGG repeats (Willemsen et al., 2003). RNA FISH coupled to IF labeling showed the presence of rare nuclear CGG RNA aggregates that colocalized with both endogenous DROSHA and DGCR8 in mice expressing expanded CGG repeats (Figures S2E and S2F). By contrast, DROSHA and DGCR8 were diffuse throughout the nucleoplasm in control mice. We noted that the CGG RNA aggregates were larger and much more frequent in patients with FXTAS than in knockin mice, which is consistent with the milder neurological disturbances observed in knockin mice compared to patients with FXTAS (Willemsen et al., 2003).

The direct binding of DGCR8 to expanded CGG trinucleotide repeats raises the question as to how specific this interaction is and, notably, whether DGCR8 can also recognize other trinucleotide repeats such as expanded CUG repeats, the mutation responsible of myotonic dystrophy of type 1 (DM1). UV-crosslinking assays showed that DGCR8 binds weakly to RNAs containing 20 or 100 CUG repeats, compared to an RNA containing 100 CGG repeats (Figure S2G). Similarly, both DROSHA and DGCR8 from mouse brain extract were captured by RNA affinity columns composed of expanded CGG repeats, yet neither was recruited by expanded CUG repeats (Figure S2H). Finally, RNA FISH coupled to IF demonstrated that both endogenous DROSHA and DGCR8 were recruited within CGG RNA repeat aggregates that formed in COS7 cells transfected with a plasmid expressing 60 CGG repeats (Figures S2I and S2J). In contrast, DROSHA and DGCR8 were not recruited within RNA aggregates of similarly transfected cells expressing either expanded CUG or AUUCU repeats, which are involved in DM1 and spinocerebellar ataxia of type 10 (SCA10), respectively (Figures S2I and S2J). Identical results were obtained in neuronal PC12 or GT17 cells (data not shown). These results indicate that DGCR8 binds preferentially expanded CGG repeats compared to CUG repeats, which is consistent with the enhanced stability of the double-stranded helical structure formed by expanded CGG repeats compared to CUG repeats (Mooers et al., 2005; Sobczak et al., 2003; Zumwalt et al., 2007; Kiliszek et al., 2009, 2011; Kumar et al., 2011). Alternatively, the presence of U:U mismatches versus noncanonical G:G base pairing, or other structural differences that are significant, between CUG and CGG hairpins, may impair the binding of DGCR8. Overall, these results suggest that DROSHA and DGCR8 are specific components of the CGG RNA aggregates in FXTAS.

DROSHA Does Not Cleave Expanded CGG Repeats

The binding of DROSHA and DGCR8 to the expanded CGG RNA repeat raises the possibility that DROSHA may cleave these repeats into shorter CGG hairpins, which is an attractive hypothesis considering that DICER was reported, in vitro, to partially process expanded CGG repeats into potentially toxic miRNA-like CGG RNA repeats (Handa et al., 2003), an observation also reported for expanded CUG and CAG repeats (Krol et al., 2007; Bañez-Coronel et al., 2012). Accordingly, we tested whether DROSHA and DGCR8 can process an RNA containing 60 expanded CGG repeats. However, no cleavage products were observed, although DROSHA correctly processed a control pri-miR-125 (Figure S3A). Furthermore, we found no trace of small miRNA-like CGG RNA after massive parallel sequencing of RNA extracted from cells transfected with a plasmid expressing 60 CGG repeats (data not shown). We propose that structural differences between pri-miRNAs and CGG expanded repeats, such as noncanonical G:G base pairing, impair the cleavage activity of DROSHA. Overall, the recruitment of DROSHA and DGCR8, without the processing of CGG repeats and the subsequent release of the proteins, suggests that the aggregates of CGG repeats may act as molecular sink, titrating DROSHA and DGCR8 away from their normal functions.

DROSHA and DGCR8 Are Partially Sequestered by Expanded CGG Repeats

To test a potential sequestration of DROSHA and DGCR8, we first analyzed the effect of expanded CGG repeats on the RNA-binding activity of DGCR8. Gel shift experiments demonstrated that addition of increasing amounts of unlabeled expanded CGG RNA repeat progressively competed with the binding of recombinant purified DGCR8 to radioactively labeled pri-miR-125 (Figure 3A). As a control, addition of unlabeled expanded CUG repeats had little or no effect. We confirmed these results by UV-crosslinking assays, which demonstrated that addition of increasing amounts of expanded CGG repeats competed with the binding of DGCR8 to radioactively labeled pri-miR-124 and pri-miR-125, whereas expanded CUG repeats had no effects (Figure S3B). Next, we tested the effect of

expanded CGG repeats on the processing activity of DROSHA. COS7 cells were cotransfected with a plasmid expressing an ectopic pri-miRNA under the expression of a CMV promoter, allowing the detection of the primary, precursor, and mature miRNAs by northern blotting (Figure 3B). Importantly, coexpression of increasing amounts of expanded CGG repeats reduced the processing of ectopic pri-miR-124 into pre-miR-124. This inhibition is specific because expression of control expanded CUG repeats had no effect on the biogenesis of pri-miR-124 (Figure 3B). We confirmed these results using ectopically expressed pri-miR-206, pri-miR-146, and pri-miR-26 and found that expression of expanded CGG repeats inhibited the DROSHA cleavage of pri-miRNA transcripts into pre-miRNAs (Figure S3C). As a control, expression of expanded CUG repeats had no or little effects on miRNA biogenesis (Figure S3D). These results suggest that expanded CGG repeats compete with the binding of DGCR8 to pri-miRNAs, reducing the quantity of free DROSHA and DGCR8 available to process pri-miRNAs, which leads to reduced processing of pri-miRNAs into pre-miRNAs.

Theoretically, the titration of free DROSHA and DGCR8 may result in reduced formation of mature miRNAs. To test this hypothesis, we quantified the expression of endogenous mature miRNAs upon expression of expanded CGG repeats in neuronal GT17 cells. Microarray profiling demonstrated that most of the miRNAs, which presented a modified expression, were reduced upon transfection of a plasmid expressing 60 CGG repeats (Figure 3C). Decreased levels of mature miRNAs were confirmed by quantitative real-time RT-PCR (Figure 3D). The limited number (56) of miRNAs presenting an expression change, as well as the limited decrease (~20%–50%) of miRNA levels, are consistent with a progressive titration of DROSHA and DGCR8 and with the early time point (24 hr after transfection) chosen for analysis. Also, we noted that the expression of a minority of miRNAs was upregulated upon expression of expanded CGG repeats. However, a similar upregulation occurred at early time point (24 hr after transfection) in neuronal GT17 or COS7 cells depleted of DGCR8 by siRNA, suggesting the existence of rescue mechanisms to transiently increase the expression of some miRNAs in response to DGCR8 reduction (Figures S3E and S3F).

Importantly, a decrease in miRNA expression could be triggered by a specific alteration of the miRNA-processing machinery but may also reflect a global alteration of RNA transcription due to reduced cell viability. To discriminate between these two hypotheses, we quantified the levels of the primary transcripts hosting the decreased miRNAs. Quantitative RT-PCR demonstrated that, whereas mature miRNAs presented reduced levels, expression of their corresponding pri-miRNAs was not altered or, even, increased (Figure 3E). Similarly, the expression of various mRNAs containing pri-miRNAs within their introns was not altered (Figure 3F). Comparable decrease of mature miRNA levels with no alterations of the expression of their corresponding pri-miRNAs was observed in a second cell model: COS7 cells expressing 60 CGG repeats (Figures S3G and S3H). These results demonstrate that expanded CGG repeats alter specifically the processing of pri-miRNAs, without affecting their transcription. As a further control, we tested the expression of mirtrons, which are miRNAs processed by the splicing machinery, thus independent of the processing by DROSHA (Okamura et al., 2007; Ruby et al., 2007). Quantitative RT-PCR analysis of mirtron-877, mirtron-1224, mirtron-1225, and mirtron-1226 levels in neuronal GT17 cells expressing expanded CGG repeats demonstrated that their expression was not altered (Figure 3G). Similarly, mirtron expression was not altered in COS7 cells expressing expanded CGG repeats (Figure S3I). Also, the mRNA and protein expression levels of DROSHA and DGCR8 were normal in cells expressing expanded CGG repeats, indicating that pathogenic CGG repeats affect the activity, but not the expression, of DROSHA and DGCR8 (Figures S3J and S3K). Finally, the transfection of a plasmid encoding DGCR8 in neuronal cells expressing expanded CGG repeats rescued the decreased expression of miRNAs, whereas expression of an inactive form of DGCR8, which was deleted for its double-stranded RNA-binding domains, presented no rescue activity (Figure 3H). Overall, these data suggest that expanded CGG repeats specifically reduced the activity of DROSHA and DGCR8, without affecting other mechanisms such as general transcription.

The Processing of miRNAs Is Altered in Brain Samples of Patients with FXTAS

The altered processing of miRNAs in cells overexpressing expanded CGG repeats raises the question whether a similar alteration occurs in patients with FXTAS. Microarray analysis of cerebellar samples from patients with FXTAS revealed that mis-regulation of miRNAs involved predominantly decreased expression compared to age-matched controls (Figure 4A). Quantitative RT-PCR analysis confirmed reduced quantities of various mature miRNAs in patients with FXTAS relative to controls (Figure 4B). Note that the expression of mature miRNAs was not fully abolished but decreased by 20%–50%, indicating a partial titration of DROSHA and DGCR8 by the expanded CGG repeats. In contrast, the quantity of mirtron-877, whose biogenesis depends on the splicing machinery and bypasses DROSHA, was normal in FXTAS brain samples (Figure 4B). As a control, quantification of the primary transcripts hosting the downregulated mature miRNAs demonstrated no changes or increased expression in patients with FXTAS (Figure 4C). We confirmed these results in frontal cortex samples of patients with FXTAS compared to age-matched controls. Quantitative RT-PCR confirmed that expression of mature miRNAs was decreased in patients with FXTAS (Figure 4D). Consistent with a specific alteration of the activity of DROSHA, the quantities of their corresponding pri-miRNAs were normal or increased in FXTAS samples compared to age-matched control samples (Figure 4E). As further controls, expressions of various mirtrons, whose biogenesis is independent of DROSHA, were normal in patients with FXTAS (Figure 4F). Overall, these results suggest that transcription and splicing of pri-miRNAs are not globally altered in patients with FXTAS but that their processing by DROSHA is reduced.

Overexpression of DGCR8 Rescues Neuronal Cell Death

A model of titration of DGCR8 by expanded CGG repeats in patients with FXTAS predicts that depletion of DGCR8 would enhance any phenotype caused by expanded CGG repeats. To test that hypothesis, we took advantage of

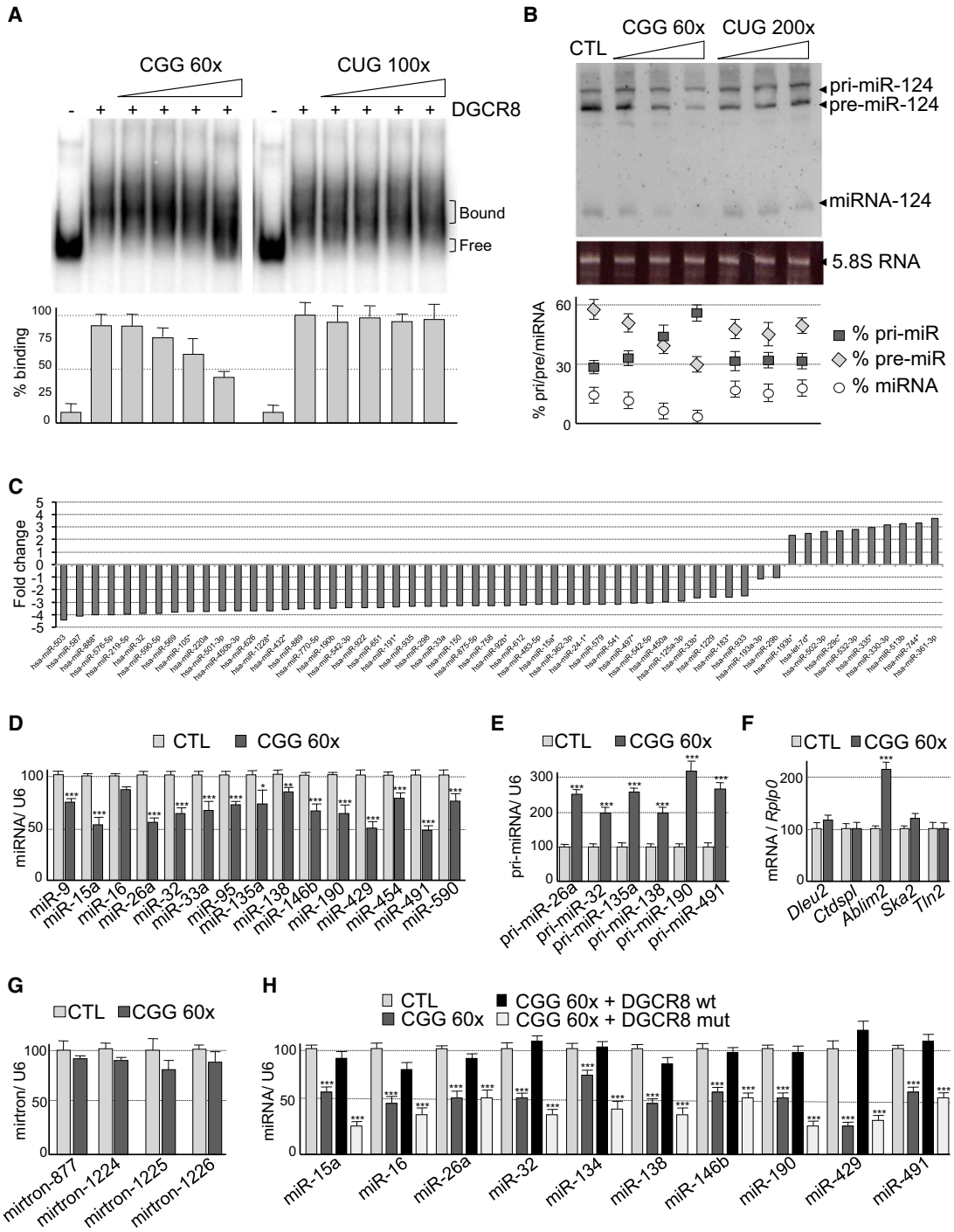


Figure 3. DROSHA and DGCR8 Activities Are Reduced in CGG-Expressing Cells

(A) Gel shift assays of recombinant His-DGCR8Δ binding with 100 nM (30,000 cpm) of uniformly α-[^{32}P]CTP internally labeled in-vitro-transcribed pri-miR-125 in presence of increasing amounts of nonlabeled in-vitro-transcribed RNA containing either 60 CGG repeats or 100 CUG repeats.

(B) A total of 20 μg of total RNA extracted from COS7 cells cotransfected with a pri-miR-124-2 minigene and increasing amounts of a plasmid expressing either 60 CGG or 200 CUG repeats were analyzed by northern blotting using a miR-124 γ-[^{32}P]ATP-labeled antisense probe. The mean of at least three independent transfections is depicted as the percentage of pri-, pre-, and mature miR-124-2. Error bars indicate SD.

(C) Microarray profiling of mature miRNAs expressed in GFP-positive FACS-isolated GT17 neuronal cells cotransfected with a plasmid expressing GFP and either a plasmid expressing no repeats (n = 3) or 60 CGG repeats (CGG, n = 3). Ordinate is in Log2 scale.

(D–G) Quantitative RT-PCR analysis of the expression of mature (D), pri-miRNAs (E), mRNAs (F), and mirtrons (G) relative to the U6 snRNA or to the *RPLPO* mRNA in GFP-positive FACS-isolated GT17 neuronal cells cotransfected with a plasmid expressing GFP and either a plasmid expressing no repeats (n = 3) or a plasmid

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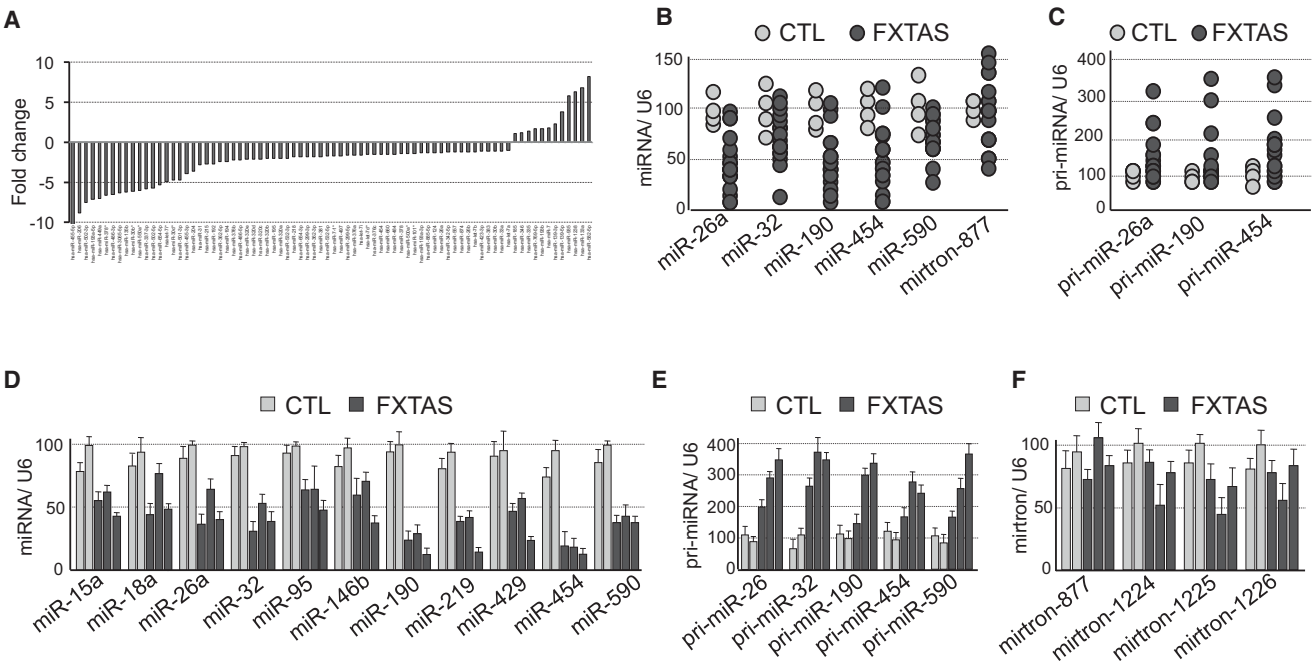

Figure 4. miRNA Levels Are Reduced in FXTAS Brain Samples

(A) Microarray profiling of mature miRNAs expressed in cerebellum samples of FXTAS and age-matched control patients.

(B and C) Quantitative RT-PCR analysis of the expression of mature (B) and pri-miRNAs (C) relative to the U6 snRNA in cerebellum samples of FXTAS (n = 13) and age-matched control (n = 4) patients.

(D–F) Quantitative RT-PCR analysis of the expression of mature (D), pri-miRNAs (E), and mirtrons (F) relative to the U6 snRNA in frontal cortex samples of FXTAS (n = 3) and age-matched control (n = 2) patients. Error bars indicate SD.

the neurodegenerative phenotype observed in transgenic *Drosophila melanogaster* expressing 90 CGG repeats (Jin et al., 2003). As previously described, expression of 90 CGG repeats decreases adult *Drosophila* viability (Figure S4A). Interestingly, decreased expression of Pasha (the *Drosophila* homolog of DGCR8) by RNAi in CGG transgenic flies resulted in enhanced early lethality (Figure S4A). A similar aggravated phenotype was observed with Drosha RNAi lines (Figure S4B). In contrast, overexpression of Drosha or Pasha in CGG flies had little or no effect and did not rescue fly lethality (data not shown). These data suggest that several pathological mechanisms may coexist in the fly model of FXTAS, a model consistent with the observation of cytoplasmic inclusions containing PURα in *Drosophila* (Jin et al., 2007), but not in human cells (Sellier et al., 2010). Accordingly, we tested mammalian neuronal cells, in which expression of expanded CGG repeats leads to formation of CGG RNA nuclear aggregates without formation of PURα-positive cytoplasmic aggregates. Organotypic cultures of E18 mouse cortex neurons were transfected at 7 days in vitro (DIV) with a plasmid expressing 60 CGG repeats and a plasmid expressing the GFP marker for 1 day (8 DIV). As reported previously by Chen et al. (2010), expression of expanded CGG repeats resulted in shorter dendrites and an ~40% decrease in dendritic branchpoints (Figures 5A and 5B). As a control, expression of expanded CUG repeats had no or little effect on neuronal dendritic complexity, indicating a specific deleterious effect of the expanded CGG repeats (Figure 5B). Importantly, the sole expression of a plasmid expressing DGCR8 restored normal dendritic growth and branching in neurons expressing the expanded CGG repeats (Figures 5A and 5B). In contrast, expression of an inactive form of DGCR8, which was deleted for its double-stranded RNA-binding domains, presented no rescue activity (Figure 5B). Similarly, expression of a control plasmid expressing MBNL1 had no significant effect and did not alleviate the dendritic alterations, indicating a specific role for DGCR8 (Figure 5B). We also tried to rescue the neuronal cell death induced by expression of expanded CGG RNA repeats by transfecting either miR-124, miR-9 or miR-125, which are important miRNAs for neuronal cell function, but we failed to observe a full rescue, suggesting that more than one miRNA is

expressing 60 CGG repeats (CGG, n = 3). *DLEU2* is the host gene of pri-miR-16-1, *CTDSPL* of pri-miR-26a1, *ABLIM2* of pri-miR-95, *SKA2* of pri-miR-454, and *TLN2* of pri-miR-190. Error bars indicate SD.

(H) Quantitative RT-PCR analysis of the expression of mature miRNAs relative to U6 snRNAs in GFP-positive FACS-isolated GT17 neuronal cells cotransfected with a plasmid expressing GFP, a plasmid expressing 60 CGG repeats, and either a vector expressing Flag-tagged wild-type (WT) DGCR8 or mutant (mut) DGCR8, deleted for its double-stranded RRM. Error bars indicate SD.

***p < 0.001; **p < 0.01; *p < 0.1.

See also Figure S3.

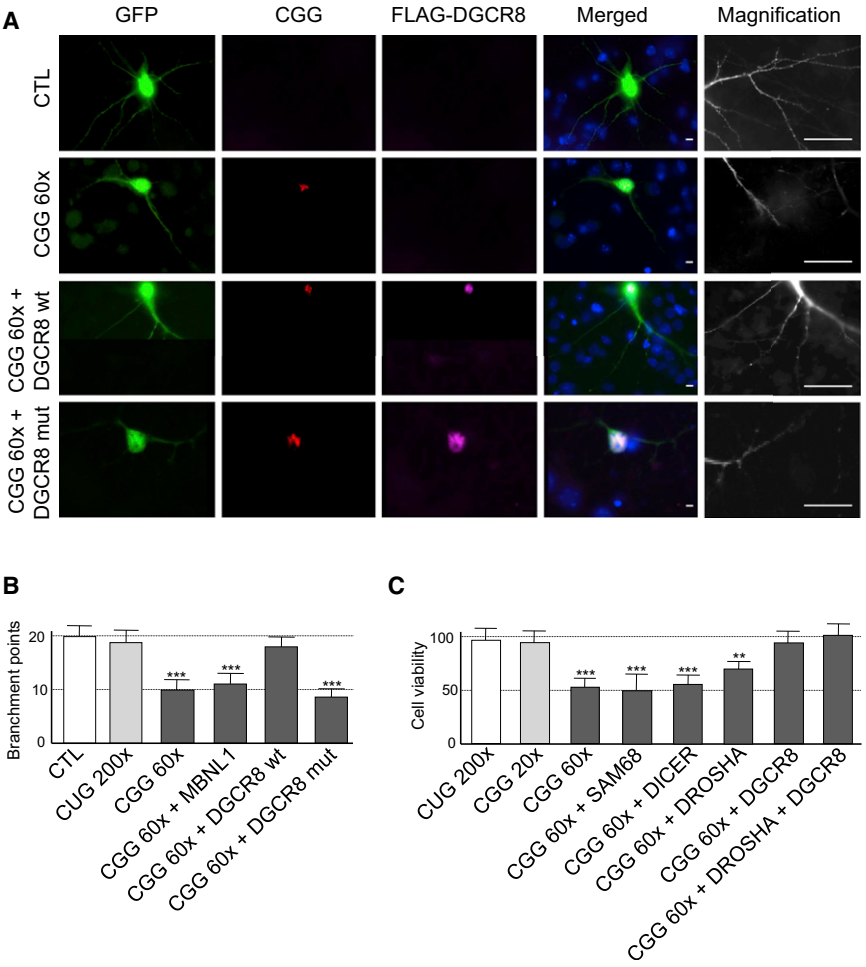

Figure 5. DGCR8 Is Sufficient to Rescue Expanded CGG Toxicity

(A) Primary cultures of cortex neurons from E18 mouse embryos were cotransfected with a plasmid expressing GFP, a vector expressing either no or 60 CGG repeats, and a plasmid expressing either a Flag-tagged wild-type DGCR8 or a mutant Flag-DGCR8 deleted for its double-stranded RRM. Neurons were analyzed 24 hr after transfection by RNA FISH using a CCG_{8x}-Cy3 DNA probe coupled to IF using an antibody directed against the FLAG tag. Magnification, 360×. Magnification of the insets, 2,500×. Scale bars, 20 μm.

(B) Quantification of the number of dendritic branchpoints of individual neurons transfected as in (A). Error bars indicate SD.

(C) Cell viability (tetrazolium) assay of neuronal GT17 cells transfected for 24 hr with a plasmid expressing either 200 CUG repeats or 20, 40, or 60 CGG repeats alone, or 60 CGG repeats plus a plasmid expressing either GFP-SAM68, Flag-DICER, Flag-DROSHA or Flag-DGCR8. Error bars indicate SD.

***$p < 0.001$; **$p < 0.01$.

See also Figure S4.

probably necessary to restore normal neuronal functions. Next, we tested whether DGCR8 can also rescue the neuronal cell death caused by expressing expanded CGG repeats. Transfection of a plasmid expressing 60 CGG repeats in neuronal GT17 cells resulted in a 50% decrease of the cell viability, whereas expression of either expanded CUG repeats or 20–40 control CGG repeats was not toxic (Figure 5C). Importantly, the overexpression of DGCR8 alone or of DGCR8 plus DROSHA alleviated the cell death due to the expanded CGG repeats (Figure 5C). As a control, transfection of control plasmids expressing either DROSHA alone, DICER or SAM68 had no or little effect. Similar results were obtained in COS7 cells (Figure S4C). Overall, these results suggest that a reduced quantity of free DROSHA and DGCR8 is the principal cause of the decreased dendritic complexity and reduced cell viability observed in neuronal cells expressing pathogenic-expanded CGG repeats.

DISCUSSION

Our results suggest a model for the cellular dysfunction induced by the expanded CGG repeats present within the 5′ UTR of the *FMR1* mRNA. Expanded CGG repeats form a double-stranded RNA hairpin (Sobczak et al., 2003; Zumwalt et al., 2007; Kumar et al., 2011; Kiliszek et al., 2011), which mimics the structure of pri-miRNAs recognized by DGCR8 (Zeng and Cullen, 2005; Han et al., 2006). We propose that DGCR8 interacts with the expanded pathogenic CGG repeats located within the 5′ UTR of the *FMR1* mRNA, thus sequestering itself and its partner, DROSHA (Figure 6). As a consequence, the level of free DROSHA-DGCR8 microprocessor is decreased, reducing the expression of mature miRNAs and ultimately resulting in neuronal cell dysfunction and degeneration. In that aspect, our work is reminiscent of the abnormal miRNA biogenesis and phenotypic abnormalities caused by the genetic haploinsufficiency of *Dgcr8* in the 22q11-deletion mouse model (Stark et al., 2008; Fénelon et al., 2011; Schofield et al., 2011).

A DROSHA-DGCR8 titration model has several predicted consequences for FXTAS pathology. First, a model based on DGCR8 titration predicts that the expansion of CGG repeats must exceed a minimal threshold size to accommodate DGCR8 binding (Zeng and Cullen, 2005; Han et al., 2006). This is consistent with the CGG repeat dependence of clinical involvement and degree of severity observed in patients with FXTAS (Tassone et al., 2007; Hoem et al., 2011), in whom it is estimated that "normal" CGG polymorphic repeat lengths are below 40 repeats, "gray zone" alleles contain 40–55 repeats, and patients with FXTAS are defined by premutation alleles containing 55–200 CGG repeats. Second, we observed that miRNA expression was decreased but not fully abolished, which is consistent with a partial and progressive sequestration of DROSHA-DGCR8 and, consequently, a progressive worsening of the disease symptoms. Third, previous studies have identified various

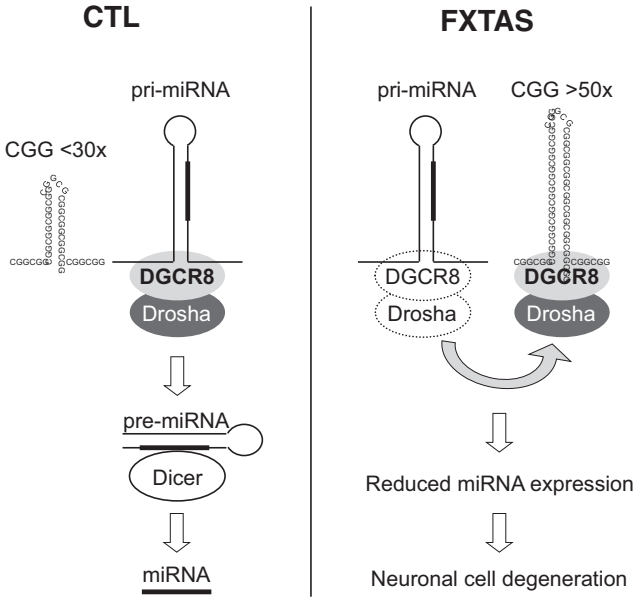

Figure 6. Model of DROSHA and DGCR8 Titration by Expanded CGG Repeats

Expanded CGG repeats fold into a double-stranded RNA hairpin that recruits DGCR8, resulting in the titration and immobilization of DROSHA and DGCR8. The reduced levels of free DROSHA and DGCR8 available to normally process pri-miRNAs into pre-miRNAs result in reduced levels of mature miRNAs, and ultimately, in neuronal cell dysfunctions.

proteins (e.g., PURα, hnRNP A2/B1, SAM68, etc.) as candidates for transduction of the CGG-expanded repeat toxicity (Iwahashi et al., 2006; Jin et al., 2007; Sofola et al., 2007; Sellier et al., 2010). However, the presence of cytoplasmic inclusions recruiting PURα in fly, but not in mammalian neuronal cells expressing expanded CGG repeats, raises the question of the superimposition of two different pathological mechanisms in *Drosophila*: one involving PURα in cytoplasmic inclusions, and another involving the sequestration of specific RNA-binding protein(s) by the expanded CGG repeats within nuclear aggregates. Such superimposition of pathogenic mechanisms may explain why overexpression of Pasha has no evident rescue effect in CGG-transgenic *Drosophila*, whereas expression of DGCR8 rescues the toxicity induced by expression of expanded CGG repeats in primary cultures of mouse neurons. Also, this superimposition model would explain the presence of ubiquitin-positive aggregates that do not colocalize with CGG RNA aggregates in knockin mouse models expressing expanded CGG repeats, as well as the recent report that only a subset of miRNAs is mis-regulated in *Drosophila* expressing expanded CGG repeats (Tan et al., 2012). Whether such a superimposition of pathological mechanisms also exists in patients with FXTAS remains an open question, yet to be determined. Finally, we found previously that SAM68 is sequestered within CGG RNA aggregates and that SAM68 rescues some of the splicing alterations observed in CGG-expressing cells (Sellier et al., 2010). However, we now show that the sequestration of SAM68 into CGG RNA aggregates requires DGCR8 and that restoration of SAM68 function is not sufficient to recover all normal neuronal cell functions. By contrast, expression of DGCR8 alone is sufficient to restore to normal both the dendritic morphological abnormalities and the loss of neuronal viability induced by expression of pathogenic-expanded CGG repeats in cultured mouse neurons.

In conclusion, this work raises the possibility that other human diseases could operate through a similar sequestration mechanism, provided that the mutant RNA forms a secondary structure that is recognized by the double-stranded RNA-binding protein, DGCR8. An appealing hypothesis in light of the recent finding that noncoding RNAs exceed coding transcripts by more than 5-fold and that an increasing number of potentially structured G-rich expanded repeats are found associated with pathologies, such as the expanded CCGGGG repeats in the *C9ORF72* gene that cause ALS-FTD (DeJesus-Hernandez et al., 2011). The current observations should also facilitate the development of novel model systems to better understand the molecular and cellular mechanisms underlying FXTAS. Finally, from the perspective of FXTAS treatment, identification of a key interaction between DGCR8 and the expanded CGG repeats represents an attractive target for therapeutic intervention. Indeed, if DROSHA and DGCR8 are sequestered, they are nevertheless present and potentially functional; hence, a strategy based on CGG-antisense oligonucleotides or pharmacological compounds (Disney et al., 2012) able to release the trapped DGCR8 would presumably return to normal the expression of miRNAs altered in FXTAS.

EXPERIMENTAL PROCEDURES

Nano-LC-MS/MS Analysis

Nuclear extract was prepared from mouse brain as described by Dignam et al. (1983). A total of 300 μg of nuclear extract was passed over an in-vitro-transcribed and -biotinylated RNA (Biotin 11 CTP; PerkinElmer) bound to streptavidin-coated magnetic beads (Dynabeads M-280 streptavidin; Invitrogen) in the presence of 20 mM HEPES, 300 mM NaCl, 8 mM $MgCl_2$, 0.01% NP40, 1 mM DTT, and protease inhibitor (PIC; Roche). The magnetic beads with immobilized RNA and its bound proteins were washed three times with the binding buffer, and bound proteins were eluted by boiling 3 min in the sample buffer prior to 4%–12% SDS-PAGE (NuPAGE 4%–12% bis-Tris Gel; Invitrogen) separation and silver staining (SilverQuest; Invitrogen). The protein bands were excised, digested, and identified using NanoESI_Ion Trap (LTQ XL; Thermo Fisher Scientific).

Cell Cultures and Transfections

Primary cortical neurons were prepared from C57Bl/6 mouse embryos at E18 and grown on polylysine-coated 24-well plates in neurobasal medium (NBM) supplemented with 1×B27, 0.5 mM L-glutamine, and 100 IU/ml penicillin/streptomycin at 37°C with 5% CO_2. Neurons were transfected at day 7 with Lipofectamine 2000 (Invitrogen) in 400 μl NBM. Medium was replaced after 3 hr with a 1:1 (v:v) mixture of conditioned and fresh NBM. After 30 hr, the neurons were fixed for FISH/ IF. COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum, and gentamicin at 37°C in 5% CO_2. PC12 cells were cultured in DMEM, 10% horse serum, 5% fetal calf serum, and penicillin at 37°C, 5% CO_2. GT17 cells were grown in 10% fetal bovine serum, gentamicin, and penicillin at 37°C in 5% CO_2 and transfected 24 hr after plating in DMEM and 0.1% fetal bovine serum to block cell divisions, using either FuGENE HD (Roche) for COS7 cells or Lipofectamine 2000 for PC12 or GT17 cells. For RNA FISH/IF, GT17 cells were plated in 24-well plates on glass coverslips precoated with a solution of 1% collagen type I (BD Biosciences).

RNA FISH Combined with IF

Patients with FXTAS have been described previously (case 6, 7, and 9 of Greco et al., 2006). Mouse or human brain sections were deparaffinized

two times for 20 min in Histosol Plus (Shandon) and dehydrated as follows: twice in ethanol 100% (5 min), twice in ethanol 95% (5 min), once in ethanol 80% (5 min), once in ethanol 70% (5 min), and rinsed in PBS before RNA FISH. Glass coverslips containing plated cells or brain sections treated as described above were fixed in cold acetone during 20 min at −20°C and washed three times with PBS. The coverslips or slides were incubated for 10 min in PBS plus 0.5% Triton X-100 and washed three times with PBS before prehybridization in 40% DMSO, 40% formamide, 10% BSA (10 mg/ml), 2× SCC for 30 min. The coverslips or slides were hybridized for 2 hr in 40% formamide, 10% DMSO, 2× SCC, 2 mM vanadyl ribonucleoside, 60 μg/ml tRNA, and 30 μg/ml BSA plus 0.75 μg (CCG)8×-Cy3 DNA oligonucleotide probe (Sigma-Aldrich). The coverslips or slides were washed twice in 2× SCC/50% formamide and twice in 2× SCC. Following FISH, the coverslips or slides were washed twice successively in 2× SCC/50% formamide, in 2× SCC, and in PBS. The coverslips or slides were incubated 2 hr with primary antibody against Drosha (1:100 dilution, AB12286; Abcam) or DGCR8 (1:100 dilution, HPA019965; Sigma-Aldrich). Slides or coverslips were washed twice with PBS before incubation with a goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (1:500 dilution; Thermo Fisher Scientific) for 60 min, incubated for 10 min in 2× SCC/DAPI (1:10,000 dilution), and rinsed twice in 2 × SSC before mounting in Pro-Long media (Molecular Probes). Slides were examined using a fluorescence microscope (Leica), and identical exposure or microscope setting was used for control or FXTAS brain section analyses.

Quantitative Real-Time PCR

Total RNA from cells or patient brains, the latter obtained under approved IRB protocols (University of California, Davis), was isolated by TriReagent (Molecular Research Center). cDNAs were generated using the miScript II RT Kit (QIAGEN) for quantification of miRNAs or the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics) for quantification of mRNAs. qPCR of miRNAs was realized using the miScript Primer Assay (QIAGEN) and miScript Sybr Green PCR Kit (QIAGEN) in a LightCycler 480 (Roche) with 15 min at 94°C followed by 50 cycles of 15 s at 94°C, 20 s at 55°C, and 20 s at 72°C. U6 snRNA was used as standard. qPCR of mRNAs was realized using the LightCycler 480 SYBR Green I Master (Roche) in a LightCycler 480 with 15 min at 94°C followed by 50 cycles of 15 s at 94°C, 20 s at 58°C, and 20 s at 72°C. The primers are listed in Table S1. *RPLPO* mRNA was used as standard, and data were analyzed using the LightCycler 480 analysis software (2ΔCt method).

For additional details, please see the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.02.004.

LICENSING INFORMATION

ACKNOWLEDGMENTS

We thank Tom Cooper (Baylor College of Medicine, Houston) and Joelle Marie (CNRS, Gif-sur-Yvette, France) for the gift of the CUG-expressing plasmids, Karen Usdin (NIH, Bethesda, MD, USA) for the gift of the CGG 65x-expressing plasmid, Narry Kim (University of Seoul, Seoul, Korea) for the gift of the Flag-DROSHA and Flag-DGCR8 WT or mutant plasmids, Peng Jin (Emory School of Medicine, Atlanta), who provided the FXTAS model flies, Scott Pletcher (University of Michigan, Ann Arbor, MI, USA), who provided the Geneswitch flies, and Claudio Sette (University of Tor Vergata, Roma) for the gift of the GFP-SAM68 construct. This work was supported by ANR GENOPAT grant P007942 (to N.C.-B.), AFM and Jérôme Lejeune funding (to N.C.-B.), Collège de France (to C.S.), NIH grant K08NS069809 (to P.K.T.), NIH-NINDS grant NS062411 (to R.W.), Netherlands Brain Foundation (F2012(1)-101) (to R.W.), NIH grant 1R01GM079235-01A2 (to M.D.D.), and the NIH Roadmap Initiative grants DE019583 and AG032119 (to P.J.H.). Experiments were performed by C.S., F.F., R.T., F.H., T.T., F.R., and V.A. Samples and patient data were obtained from F.T., P.J.H., and R.W. Data were analyzed by C.S., C.T., A.P., M.D.D., P.K.T., H.M., R.W., N.C.-B., and P.J.H. The study was designed and coordinated by N.C.-B.

Received: June 26, 2012
Revised: November 30, 2012
Accepted: February 1, 2013
Published: March 7, 2013

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