CGG-repeat length threshold for FMR1 RNA pathogenesis in a cellular model for FXTAS

Gry Hoem†, Christopher R. Raske†, Dolores Garcia-Arocena, Flora Tassone, Eleonora Sanchez, Anna L. Ludwig, Christine K. Iwahashi, Madhur Kumar, Jane E. Yang and Paul J. Hagerman∗

Department of Biochemistry and Molecular Medicine, University of California, Davis, School of Medicine, Davis, CA 95616, USA

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Fragile X-associated tremor/ataxia syndrome (FXTAS) is a neurodegenerative disorder that affects carriers of premutation alleles (55–200 CGG repeats) of the fragile X mental retardation 1 (FMR1) gene. The presence of elevated levels of expanded mRNA found in premutation carriers is believed to be the basis for the pathogenesis in FXTAS, but the exact mechanisms by which the mRNA causes toxicity are not known. In particular, it is not clear whether there is a threshold for a CGG-repeat number below which no cellular dysregulation occurs, or whether toxicity depends on mRNA concentration. We have developed a doxycycline-inducible episomal system that allows us to study separately the effects of CGG-repeat number and mRNA concentration (at fixed CGG-repeat length) in neuroblastoma-derived SK cells. Our findings show that there is a CGG-repeat size threshold for toxicity that lies between 62 and 95 CGG repeats. Interestingly, for repeat sizes of 95 CGG and above, there is a clear negative correlation between mRNA concentration and cell viability. Taken together, our results provide evidence for an RNA-toxicity model with primary dependence on CGG-repeat size and secondary dependence on mRNA concentration, thus formally ruling out any simple titration model that operates in the absence of either protein-binding cooperativity or some form of length-dependent RNA structural transition.

INTRODUCTION

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a late-onset neurodegenerative disorder that affects carriers of premutation alleles (55–200 CGG repeats) of the fragile X mental retardation 1 (FMR1) gene (reviewed in 1–7). The primary clinical features of FXTAS include progressive intention tremor and gait ataxia, often with associated features of dementia and peripheral neuropathy. Larger expansions (>200 CGG repeats) are normally methylated, resulting in silencing of the gene and significantly reduced levels or absence of FMR1 mRNA and protein (FMRP) (8,9). The absence of FMRP causes the neurodevelopmental disorder fragile X syndrome.

In stark contrast to the general lack of FMR1 mRNA in individuals with full mutation alleles, premutation carriers have FMR1 mRNA levels that are increased by 2–8-fold relative to normal controls; whereas FMRP levels remain normal or slightly reduced (reviewed in 10–14), depending on the repeat size. These observations, coupled with the observation that FXTAS is limited to carriers of premutation FMR1 alleles, have given rise to a toxic RNA gain-of-function model for FXTAS (1,15). Additional support for a model of mRNA-induced toxicity comes from the observation that the expanded FMR1 mRNA is present within the intranuclear inclusions that are found in neurons and astrocytes of FXTAS patients, suggesting that the mRNA itself is important for the neurodegenerative phenotype (16–18). Furthermore, both animal and neural cell models have shown that expression of ~90 CGGs, outside of the context of the FMRP coding region, is sufficient to cause neurodegeneration and inclusion formation (19–21).

At present, the exact mechanism by which the expanded (premutation) CGG-repeat mRNA mediates toxicity remains unclear. In particular, it is not known whether there is a threshold CGG-repeat number below which no cellular
dysregulation occurs, or whether dysregulation depends also, or solely, on mRNA concentration. The latter alternative appears to be operating for the trinucleotide expansion disorder myotonic dystrophy 1 (DM1; OMIM #160900), whose pathogenesis is thought to arise through a toxic RNA gain-of-function of a CGG repeat element that lies within the 3′ non-coding region of the DMPK gene (22,23). For DM1, overexpression of an mRNA containing a normal size CUG repeat is sufficient to cause several of the main features of the disease (24); this observation is consistent with a protein-sequestration model in which a threshold molarity of the CUG repeat is sufficient for disease formation.

In the case of FMR1 premutation CGG-repeat expansions, the question of a CGG-repeat size threshold has not been resolved, although existing evidence tends to favor such a threshold. Greco et al. (25) have found a strong correlation between the number of CGG repeats and the number of inclusions found in post-mortem brain tissue. Furthermore, Sellier et al. (26) recently observed the formation of intranuclear CGG aggregates only in transgene-expressing cells harboring >40 CGG repeats. FMR1 mRNA levels are known to increase substantially over normal levels for premutation alleles (14), a process that appears to begin with allelic expansion within the gray zone (27). However, the distinction between the effects of repeat number and of mRNA concentration on cellular pathogenesis has not been established for the FMR1 gene.

In this report, we have used a doxycycline (DOX)-inducible episomal system, stably transfected into a human neuroblastoma-derived (SK-N-MC; ‘SK’) cell line, to study the effect of increasing levels of mRNA with differing CGG-repeat lengths. Importantly, the experimental system allows us to separately gauge the effects of the CGG repeat per se, and of mRNA concentration at a fixed CGG-repeat length. We found that expression of a normal (30 CGG) repeat enhanced green fluorescence protein (eGFP) reporter mRNA did not appear to adversely affect cells even at mRNA concentrations exceeding 100-fold the level of endogenous FMR1 mRNA in our transfected SK cells and patient blood. This finding argues against mechanisms for RNA toxicity that rely purely on the molarity of the CGG repeat. Toxic effects of the CGG repeat are observed for repeats that exceed a threshold value, consistent with the recent studies of Sellier et al. (26). Furthermore, we found that increasing expression of CGG repeats in the premutation range leads both to activation of the phosphorylated form of the variant histone H2AX (γH2AX), known to be important for DNA damage repair, and disruption of the nuclear lamin A/C architecture.

RESULTS

A DOX-inducible CGG-repeat/reporter expression system for studying the effects of mRNA concentration and CGG-repeat length on cellular dysregulation and viability

We have developed a set of non-integrating episomal expression plasmids that inducibly express an eGFP reporter downstream of an FMR1 5′ untranslated region (UTR), driven by a basal cytomegalovirus (CMV) promoter (i.e. replacing the endogenous FMR1 promoter region upstream of the transcription start site) under DOX control; the plasmids are designated pcep4-TreT-nCGG-eGFP (Fig. 1; see Materials and Methods). By varying the DOX concentration for each CGG-repeat number (n) in the FMR1 UTR (n = 30, 42, 54, 62, 95), we were able to independently study the effects of the mRNA concentration and of the CGG-repeat length on cellular dysregulation and on viability in SK cells.

The parent episomal plasmids (pcep4-invotrogen) contain the Epstein–Barr virus origin of replication (OriP) and express the EBNA1 (Epstein–Barr nuclear antigen 1) gene to allow for stable maintenance and replication of the episomes in mammalian cells. The parental episome also contains the hygromycin B resistance gene for selection of stably transfected cells. Stable extrachromosomal replication of the expression plasmid eliminates the cellular stress associated with transient transfections and ensures that all cells grown in hygromycin B have the potential to express the CGG-repeat-containing mRNA. The inducible episomes also contain an eGFP reporter and a chimeric intron downstream of the CGG element in the 5′ UTR to facilitate selective real-time polymerase chain reaction (RT-PCR) amplification of eGFP mRNA.

To achieve DOX-inducible expression, the episomes were stably transfected into the SK cell line (see Materials and Methods) bearing a stably integrated reverse tetracycline
transactivator (rtTA-62-1; 28). Multiple rtTA-SK lines were created by transfecting the cells with a plasmid, pCDNA4-rtTA-62-1, which expresses both rtTA-62-1 and Zeocin resistance genes (28), and then holding the lines under Zeocin selection until stable SK-rtTA transformants were obtained. One clonal line, SK-rtTA-15, was found to exhibit the highest eGFP expression after 24 h of DOX treatment, and was used for subsequent experiments.

For individual experiments, SKrtTA-15 cell lines stably maintaining pcep4-TreT-nCGG-eGFP episomes were maintained on media with a range of DOX concentrations, ranging from 0 to 0.3 μg/ml, for 24 h. Cells were then harvested, total RNA was extracted and quantitative RT–PCR (qRT–PCR) was performed in order to measure the amount of FMR1 (CGG)n-eGFP mRNA expressed for each CGG repeat and at each DOX concentration. As can be seen in Figure 2, there is an increase in CGG-repeat mRNA expression with increasing DOX levels for each CGG repeat investigated, up to 0.3 μg/ml. We note that the DOX-induction curves as well as the maximum CGG-GFP mRNA levels differ among the constructs, with the 54-CGG-repeat construct having the highest levels. We suspect that these intrinsic differences relate to different numbers of episomal DNA molecules that are stably maintained following transfection and selection/propagation; however, these differences are not a function of CGG-repeat number. Finally, whereas the levels of episomal GFP mRNAs in the absence of DOX (promoter leakage) are comparable with the endogenous (FMR1) mRNA in the SK cells (Fig. 2, arrow on left axis), as well as to the levels found in peripheral blood leukocytes of individuals with normal CGG-repeat alleles, the levels of fully induced mRNA are more than 20–50-fold higher than the levels of FMR1 mRNA found in carriers of premutation alleles, which are elevated by 2–5-fold over normal levels (13,14).

Cell viability is reduced with increasing expression of reporter mRNAs containing 95 CGG repeats

To determine the effect of varying mRNA concentrations for a range of CGG-repeat lengths, episome-containing SK cells were treated with six DOX concentrations (0, 0.003, 0.01, 0.03, 0.1 and 0.3 μg/ml) for 24 h. The DOX-treated cells were then analyzed by fluorescence-activated cell scanning (flow cytometry) (FACS) to determine cell viability at increasing levels of CGG-repeat mRNA expression. A loss of cell viability was determined by measuring the fraction of all gated cells that incorporated propidium iodide (PI). For cells expressing the 95 CGG episme, decreased cell viability was observed at DOX concentrations above 0.03 μg/ml (Fig. 3A), a threshold at which the relative eGFP mRNA level (i.e. number of CGG-repeat eGFP mRNA molecules) is elevated by more than ~120-fold over the level of endogenous FMR1 mRNA in SK cells and, as noted above, more than 20–50-fold over the levels found in peripheral blood leukocytes of premutation carriers. At the two highest attainable mRNA concentrations, there is an average ~5% reduction in viable cells, consistent with previous studies (19). By comparison, there is no significant decrease in cell viability for the 54-CGG-repeat episome, representing the lower border of the premutation range (55 CGG repeats), even at mRNA concentrations much higher than are attainable for the 95-CGG-repeat episme. Similar studies with additional allele sizes showed no CGG-repeat-induced cell death for the 30-CGG-repeat allele, which is near the modal value in the general human population (29), or for either the 42- or 62-CGG-repeat alleles, where even higher mRNA levels are achieved compared with the 95-CGG-repeat allele (Fig. 3B). These results indicate that, at least for acute loss of cell viability, there must be a threshold number of CGG repeats, not simply increased molarity of the CGG repeat.

Expression of the 95-CGG-repeat allele leads to disruption of the lamin A/C architecture and activation of the DNA repair-associated histone variant, γH2AX

Arocena et al. (19) demonstrated that several days after transient transfection of SK cells with reporter plasmids bearing an 88-CGG-repeat FMR1 5′ UTR, the lamin A/C nuclear architecture became altered, with collapse of the normal nuclear lamina ring structure. Furthermore, these morphological changes were also observed in cultured fibroblasts and brain samples from patients with FXTAS (30). To investigate whether the lamin ring structure was altered in SK cells harboring episomes with variable CGG-repeat lengths within the observed threshold for CGG-repeat-induced cell death, we seeded cells harboring pcep4-TreT-nCGG-eGFP (n = 30, 42, 54, 62, 95) on glass cover slips and kept them on growth media with either 0 or 0.3 μg/ml of DOX for 72 h. Representative fluorescent microscopy images in Figure 4A show the lamin architecture of cells expressing either 30 or 95 CGG repeats. Note the round nuclei and complete lamina ring structures of cells over-expressing 30 CGG repeats, compared with the disrupted lamina architecture and misshapen nuclei in 95-CGG-repeat cells (19). As represented in Figure 4C, there is no significant reduction in the number of lamin rings...
in cells expressing 30, 42, 54 and 62 CGG repeats. However, we found a significant reduction ($P = 0.024$; Student’s $t$-test) in the number of nuclei with normal lamin ring structures between no DOX controls and 0.3 $\mu$g/ml DOX in cells expressing 95 CGG repeats.

In addition to the altered lamin A/C architecture, we have observed the phosphorylated DNA repair-associated histone variant, γH2AX, in the intranuclear inclusions of FXTAS patients (17). As shown in Figure 5A, γH2AX co-localizes with ubiquitin-positive inclusions in nuclei extracted from brain tissue from a subject with FXTAS. In a previous mass-spectrometric analysis of the inclusions of FXTAS cases (17), two proteins with significant coverage were classified as members of the histone 2A family. Further inspection of the amino acid sequences of the peptides for those proteins revealed significant overlap with H2AX, with sequence coverage for the 143 amino acid protein of 53.8% (Fig. 5B). Based on recent observations (reviewed in 31–33) of activation of the DNA repair machinery in mice with premutation-expanded CGG repeats, we asked whether γH2AX was also activated when SK cells containing expanded CGG repeats are induced to express high levels of CGG-repeat mRNA. In particular, we used western blots to quantify the amount of γH2AX in SK cells containing expanded CGG repeats are induced to express high levels of CGG-repeat mRNA. In particular, we used western blots to quantify the amount of γH2AX in SK cells containing expanded CGG repeats. Following a 24 h incubation period with varying DOX concentrations, there was a significant increase in γH2AX levels (relative to no DOX) only in SK cells expressing 95 CGG repeats.

![Figure 4. Altered lamin A/C nuclear architecture in cells expressing an expanded CGG-repeat element. (A) Lamin A/C architecture in cells harboring 30 or 95 CGG repeats following 72 h of DOX treatment (DOX; 0.3 $\mu$g/ml) or in the absence of DOX (noDOX); in descending order: lamin A/C, 4',6-diamidino-2-phenylindole (DAPI), merged image, respectively. (B) Example of a lamin A/C-positive intranuclear inclusion following 72 h induction of 95 CGGs in the presence of DOX (0.3 $\mu$g/ml). (C) Ratio of lamin A/C rings, DOX (0.3 $\mu$g/ml) versus no DOX as a function of the number of CGG repeats in SK cells. All staining experiments used rabbit anti-lamin A/C primary and Alexa 555 goat anti-rabbit (see Materials and Methods).](https://academic.oup.com/hmg/article-abstract/20/11/2161/634954/Downloaded-from?version=1)
Although γH2AX is often used as a marker for DNA repair, we cannot rule out other mechanisms that may also lead to increases in this histone variant.

**Timing of the expression of γH2AX in SK cells expressing 95 CGG repeats**

To determine the temporal relationship between expression of γH2AX and DOX-induced transcriptional activation of the 95-CGG-repeat mRNA, we designed a time-course experiment that followed both CGG-eGFP mRNA production and γH2AX phosphoprotein level over a 24 h period. Cells were cultured for 24 h prior to DOX induction, after which the cells were cultured for an additional 24 h in culture medium containing DOX (0.0125 μg/ml). RNA and protein samples were collected at 0, 5, 15, 45, 90, 180, 360 and 1440 min post-induction. As shown in Figure 6A, significantly elevated levels of 30- and 95-CGG mRNA are observed after 45 min of DOX induction. However, the upregulation of γH2AX is only apparent in cells expressing 95-CGG-repeat mRNA and is only significant at the 24 h time point (1.78-fold, $P = 0.029$; Student’s $t$-test). In contrast, levels of γH2AX remain unchanged over the 24 h period in DOX-induced cells expressing the 30-CGG-repeat mRNA (Fig. 6B).

**DISCUSSION**

We have developed a human neural cell culture system that allows independent variation of CGG-repeat number and CGG-repeat/reporter mRNA level for the purpose of determining their relative contributions to induced cellular toxicity. Using this system, we have demonstrated that, within a 24 h period, expression of 95-CGG-repeat mRNA leads to reduced cell viability, which is more pronounced with increasing mRNA expression. In contrast, expression of a normal (30 CGG repeats) reporter mRNA does not manifest acute cellular toxicity even at the highest attainable levels of expression—more than 100 times higher than the concentration of endogenous FMR1 mRNA in both SK cells and patient blood. For the largest CGG repeat, we have observed additional signs of cellular dysregulation in surviving (attached) cells, including disruption of the lamin A/C nuclear structure and...
the induction of the DNA repair response protein, γH2AX (34). These results thus provide direct evidence for an absolute CGG-repeat threshold for toxicity between 62 and 95 CGG repeats, such that there is no detectable phenotypic dysregulation, at least acutely, even for CGG-repeat mRNA levels that exceed the endogenous levels of FMR1 mRNA by more than 100-fold. For repeat sizes above the threshold, we demonstrate an mRNA-concentration-dependent decrease in viability.

The current observations therefore appear to rule out, for the CGG repeat, a pure molarity-based model for RNA toxicity of the type evidenced by myotonic dystrophy, where elevated levels of normal CGG-repeat mRNA were capable of recapitulating several aspects of the cellular phenotype, presumably through sequestration of the protein, muscleblind-like 1 (MBNL1) and possibly additional proteins, by the CUG element (24). Thus, our data suggest that whether or not (or to what extent) RNA molarity effects contribute to toxicity, there appears to be a threshold CGG repeat below which molarity alone cannot induce cellular toxicity.

The presence of a threshold for RNA toxicity does not rule out a sequestration model for CGG-repeat-induced pathogenesis. Indeed, Sellier et al. (26) recently provided evidence for a threshold in their study on the sequential sequestration of the proteins Sam68, MBNL1 and hnRNP G, to the expanded CGG mRNA. They found that in both CGG-transfected cells and in FXTAS cases, sequestration of Sam68 altered RNA splicing activity for several mRNAs, suggesting that sequestration of Sam68 can play a part in the pathogenesis of FXTAS. However, they observed that substantial nuclear aggregates formed only in cells with 60 or 100 CGG repeats, consistent with the CGG-repeat size threshold between 62 and 95 CGG repeats observed in the current study. More recently, Sellier et al. (35) reported that the primary RNA processing complex (microprocessor), comprising Drosha and DGCR8 proteins, interacts directly with the CGG-repeat mRNA, resulting in protein sequestration and consequent reduced ability to process pri-miRNA to pre-miRNA. Although speculative at this point, it is possible that the threshold effect is itself a consequence of the highly cooperative binding of DGCR8, or the DGCR8–Drosha complex to a hairpin structure formed within the CGG-repeat region (36, 37).

For higher levels of expression of the 95-CGG-repeat allele, two additional features of the abnormal cell phenotype were observed; namely, disruption of the lamin A/C nuclear architecture, observed previously in transient transfection experiments and in fibroblasts from FXTAS patients (19, 30), and γH2AX induction. γH2AX is a DNA damage/repair protein that is involved in both signaling and recruitment of other proteins involved in the repair of DNA double-strand breaks (34), and is also found within the inclusions of FXTAS cases (17). Taken together with the earlier observation that ataxia telangiectasia mutated, and ataxia telangiectasia- and Rad3-related checkpoint kinases are both activated in premutation carriers (38, 39), we speculate that a DNA repair process is activated in FXTAS (or more generally in carriers), perhaps in response to the elevated burden of oxidative stress and mitochondrial dysfunction in cells expressing the premutation allele (40); however, we cannot formally rule out a direct co-transcriptional response to the production of FMR1 CGG-GFP mRNA.

The increased γH2AX level observed for the 95-CGG-repeat mRNA might also be a consequence of the altered lamin A/C structure, which can trigger the DNA repair machinery (41). Activation of γH2AX may also be a specific response to DNA damage caused by shortened telomeres (42), consistent with the findings of Jenkins et al. (43) of reduced telomere length in older male carriers of premutation CGG-repeat expansions. Interestingly, short telomeres can compromise cell viability as has been noted in a number of age-related diseases and premature aging syndromes (reviewed in 44).

Although disruption of the lamin A/C nuclear architecture and γH2AX induction were only observed in SK cells expressing 95 CGGs, more subtle effects may be revealed in long-term culture for slightly smaller CGG repeats. Clearly, additional studies are necessary to reveal both the exact mechanisms by which the expression of expanded elements mediates cellular toxicity; indeed, the current study is limited by the lack of clear markers for cellular dysfunction for alleles in the gray zone and lower premutation range, where there is some epidemiological evidence of co-morbidity associated with such alleles in individuals with Parkinson disease (45). This limitation notwithstanding, the current investigation has
demonstrated that cellular toxicity is not simply due to a CGG-repeat molarity effect, but that a threshold CGG repeat is necessary for cellular dysregulation. Our observations are also consistent with the clinical and pathological findings in FXTAS patients, where the formation of intranuclear neural cell inclusions only becomes significant for CGG repeats above \( \sim 70 \) CGG repeats (25), and where significant clinical involvement is predominantly associated with alleles with at least 70 CGG repeats (46).

Finally, it is important to recognize that FXTAS is not simply a late-onset consequence of the expanded CGG-repeat \( FMR1 \) mRNA, but now appears to be the end-stage of a process that starts in early development (31,47,48), with behavioral problems, cognitive impairment and seizure activity in children (49–51). Therefore, premutation alleles are likely to give rise to both neurodevelopmental and neurodegenerative phenotypes throughout the life.

**MATERIALS AND METHODS**

**Construction of the tet-inducible \( FMR1-5' \) UTR episomes**

The episomal plasmid pcep4 (Invitrogen, Carlsbad, CA, USA) was modified to express eGFP with an \( FMR1 \) 5' UTR under the control of the tetracycline responsive promoter element (TRE). Utilizing oligonucleotide poly-linkers, the CMV promoter in pcep4 was replaced with the TRE promoter from the pTRE-TIGHT vector (Clontech Laboratories Inc., Mountain View, CA, USA) to drive tet-inducible expression. Immediately downstream of the TRE promoter, \( FMR1 \) 5' UTRs containing 30, 42, 54, 62 and 95 CGG repeats were cloned upstream of a chimeric intron from the pRL-CMV plasmid (Promega Corp., Madison, WI, USA). The eGFP reporter from the eGFPn-1 vector (Clontech) was cloned downstream of the chimeric intron followed by an SV40 poly A signal from the pRL-CMV vector (Fig. 1A). The resulting plasmids are pcep4-TreT-30CGG-eGFP, pcep4-TreT-42CGG-eGFP, pcep4-TreT-54CGG-eGFP, pcep4-TreT-62CGG-eGFP and pcep4-TreT-95CGG-eGFP. The \( FMR1 \) 5' UTRs containing 30, 42, 62 and 95 CGG repeats were cloned from the parent plasmid CMV-FMR1-FL (52) between BpiI and NheI restriction sites (all restriction enzymes from New England Biolabs Inc., Ipswich, MA, USA). The \( FMR1 \) 5' UTR containing 54 CGG repeats was amplified by PCR from patient genomic DNA, followed by a BpiI–XhoI restriction enzyme digestion, and cloned into pcep4-TreT-95CGG-eGFP. An additional tet-inducible episome, pcep4-TreT-D-eGFP, which does not contain the \( FMR1 \) 5' UTR sequence, was also constructed to serve as a calibrator for relative eGFP mRNA quantification.

**Construction of the rtTA expression plasmid for creation of stable SK-MC-rtTA cell line**

The modified reverse transactivator, rtTA-62-1 (kindly provided by H Bujard), is constitutively expressed downstream of the CMV promoter in pcDNA4 His Max A (Invitrogen). A polylinker containing EcoRI and BamHI sites was cloned between the CMV promoter and BHG polyadenylation sequence to allow for the insertion of rtTA-62-1. The resulting plasmid is pcDNA4-rtTA-62-1. Additionally, pcDNA4 His Max A contains a Zeocin resistance gene driven by the EM-7 promoter to allow for mammalian selection.

**Creation of stable SK-rtTA cell lines**

Stable neural cell lines expressing the rtTA-62-1 transactivator were derived by transfecting the pcDNA4-rtTA-62-1 plasmid into human neuroblastoma-derived SK-N-MC cells (SK). SK cells were plated into \( 5 \times 10 \) cm tissue culture dishes and grown in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (GIBCO) and 1 \( \times \) antibiotic antimycotic solution (penicillin-G, streptomycin solution, amphotericin-B) (Gemini Bio-Products, West Sacramento, CA, USA). Cells were transfected at 80% confluency with 15 \( \mu \)g of pcDNA4-rtTA-62-1 plasmid mixed with 40 \( \mu \)l of Lipofectamine 2000 (Invitrogen). Twenty-four hours post-transfection, cells from each plate were equally divided into \( 5 \times 10 \) cm dishes and maintained for 48 h, and subsequently cultured in media containing 0.5 mg/ml Zeocin (Invitrogen) to select for cells stably expressing the Zeocin resistance gene in pcDNA4-rtTA-62-1. The Zeocin concentration was determined using kill curve analysis. After 4–6 weeks of Zeocin selection, resistant colonies of cells were transferred to six-well plates using cloning discs (VWR, Bridgeport, NJ, USA), and individually expanded.

To test the stable SK-rtTA cell line’s ability to induce expression from a TRE promoter in the presence of DOX, the cells were cultured in six-well plates and transfected with 1 \( \mu \)g of pTRE-11CGG-eGFP (unpublished data) and 2 \( \mu \)l Lipofectamine 2000. After 24 h, the transfected cells were treated with and without 1 \( \mu \)g/ml DOX; after an additional 24 h, levels of eGFP were quantified with a flow cytometer, based on FACS (BD FACS, Becton Dickson, Mountain View, CA, USA). The cell line, SK-rtTA-15, was found to exhibit the highest expression of eGFP upon DOX induction, and was therefore used for expression of the inducible \( FMR1 \) episomes.

**Creation of inducible \( FMR1 \) SK-rtTA-15 lines**

SK cells were plated to reach 80% confluency in a T25 flask at the time of transfection. Four hours prior to transfection, the media were changed to antibiotic-free media. Ten micrograms of pcep4-TreT-30CGG-eGFP, pcep4-TreT-42CGG-eGFP, pcep4-TreT-54CGG-eGFP, pcep4-TreT-62CGG-eGFP, pcep4-TreT-95CGG-eGFP and pcep4-TreT-D-eGFP were each separately mixed with 25 \( \mu \)l of Lipofectamine 2000 and incubated for 20 min. Each construct was transfected into an individual T25 flask. After 24 h, the cells were transferred into T75 flasks, and after a further 24 h the media were changed to contain 0.1 mg/ml hygromycin (Invitrogen). The cells were passaged every 2–3 days into media containing 0.1 mg/ml hygromycin for 2–3 weeks. Once a population of hygromycin-resistant SK cells was established, they were distributed into six-well plates and tested by treatment with 1 \( \mu \)g/ml DOX for 24 h. To confirm DOX-inducible expression of the \( FMR1 \) 5' UTR-nCGG-eGFP, protein levels of the reporter were quantified by FACS.
Genotyping and verification of episomal genotype and stability

Total DNA was purified from cells (passage number 5–12) using the Qiagen Gentra Purigene kit (Qiagen, Valencia, CA, USA). Ten nanograms of DNA from each episomal cell line were PCR-amplified using primers c and f (53). The following PCR primers that specifically amplify the CGG repeat within the episome were used: forward: 5′-TCAGCGCTAGCTC
CGTTTCGGT; reverse: 5′-CTTACCTGCCCAGTGCCTC
ACACCAG. Amplicons were loaded in a 2% agarose gel and CGG-repeat numbers were calculated relative to a molecular weight marker (Fig. 1B).

Cell growth

All SK cells harboring episomes with varying CGG-repeat lengths were maintained at 37°C in DMEM, supplemented with 10% fetal bovine serum (GIBCO) and 1× antibiotic antimycotic solution (penicillin-G, streptomycin solution, amphotericin-B) (Gemini Bio-Products), 0.1 µg/ml of hygromycin B and 0.5 µg/ml of Zeocin. For each set of experiments, cells were grown in both 12-well plates (for RNA extraction and viability assay) and 6-well plates (for protein extraction) with growth media (see above) containing varying concentrations of DOX. Three technical replicates were performed for each DOX concentration, and experiments were repeated a minimum of three times (biological replicates).

mRNA reverse transcription and quantification

Total RNA was extracted using the RNeasy Miniprep Kit (Qiagen) and quantified on a NanoDrop 1000 (Nanodrop Products, Wilmington, DE, USA) by total absorbance at 260 nm. Total DNA was purified from cells (passage number 5–12) using the Qiagen Gentra Purigene kit (Qiagen, Valencia, CA, USA). Ten nanograms of DNA from each episomal cell line were PCR-amplified using primers c and f (53). The following PCR primers that specifically amplify the CGG repeat within the episome were used: forward: 5′-TCAGCGCTAGCTC CGTTTCGGT; reverse: 5′-CTTACCTGCCCAGTGCCTC ACACCAG. Amplicons were loaded in a 2% agarose gel and CGG-repeat numbers were calculated relative to a molecular weight marker (Fig. 1B).

Western blot analysis

Twenty-four hours after seeding in six-well plates (in DMEM) with specified DOX concentrations between 0 and 0.3 µg/ml, SK cells were rinsed with Dulbecco’s phosphate-buffered saline (PBS) (GIBCO), frozen on dry ice and detached in radioimmuno precipitation assay (RIPA) buffer (50 mM Tris–HCl, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 0.5% IGEPAL, 0.1% sodium dodecyl sulfate, 0.012% deoxycholate, 0.5% Triton X-100, pH 7.4) with protease and phosphatase inhibitors (Protease Inhibitor cocktail set 3, Phosphatase Inhibitor cocktail set 3, Calbiochem, Gibbstown, NJ, USA). Cell lysates were rocked overnight at 4°C followed by centrifugation (16 000 g for 30 min at 4°C). Protein concentrations derived from the RIPA-soluble fraction (supernatant) were quantified using a protein assay kit (#23225, BCA Protein Assay Kit, Pierce Biotechnology, Rockford, IL, USA). Following electrophoresis of 10 µg per sample on 10–20% linear gradient Criterion Tris–HCl gels (BioRad, Hercules, CA, USA), proteins were transferred to nitrocellulose membranes. The membranes were subsequently blocked with BLOTTO [5% non-fat dry milk in 100 mM Tris–HCl, 0.9% NaCl, 0.1% polyoxyethylene (20) sorbitan monolaurate, pH 7.5], followed by overnight incubation with primary antibodies: rabbit anti-γH2AX (#2577, Cell Signaling, Danvers, MA, USA) diluted in 5% bovine serum albumin (Fisher BioReagents, Pittsburgh, PA, USA), and β-tubulin (Millipore, Billerica, MA, USA) diluted in BLOTTO. Following washes, incubation with secondary antibodies, detection of antibodies and quantifications were performed as described by Garcia-Arocena et al. (30).

Cell viability assay

Cells were seeded in 12-well plates with DMEM containing the specified DOX concentrations 24 h prior to FACS analysis. Both floating and attached cells were collected, pelleted at 1600 rcf for 6 min and resuspended in 200 µl Dulbecco’s PBS (DPBS; GIBCO) together with 7 µl of 50 µg/ml PI.

Lamin A/C architecture

SK cells were grown on individual glass cover slips in DMEM supplemented with 10% fetal calf serum and 1× pen-strep, and incubated at 37°C for 72 h. Cells were fixed and blocked as previously described by Arocena et al. (19). To evaluate the lamin A/C structure, blocked cover slips were stained at room temperature overnight with rabbit antibody against lamin A/C (Cell Signaling # 2032, 1:500) followed by washes and incubation with 1:4000 Alexa 555 goat anti-rabbit (Invitrogen). The presence of the normal lamin A/C structure was evaluated (blinded to the ID of the sample) via fluorescent microscopy (Leica Microsystems, Bannockburn, IL, USA) by manually counting the fraction of nuclei with normal lamin rings in multiple randomly selected fields as previously described (30).
Immunohistochemical staining for γH2AX

Isolated nuclei from the frontal cortex of a subject with FXTAS were prepared as described by Iwahashi et al. (17), and spread on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA) and fixed in 70% methanol. Slides were washed in PBS-T [10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4, 0.1% polyethylene (20) sorbitan monolaurate] and blocked for 2 h with 5% goat serum in PBS-T. A mouse anti-ubiquitin antibody (Novus Biologicals, Littleton, CO, USA) and a rabbit anti-γH2AX antibody (Cell Signaling) were diluted at 1:400 (v/v) and 1:100 (v/v), respectively, in blocking buffer, and incubated on slides overnight at 4°C. Slides were washed and incubated with 1:500 (v/v) Alexa 555 anti-rabbit IgG, or Alexa 488 anti-mouse IgG. Nuclei were stained with 2 μM 4′,6-diamidino-2-phenylindole (DAPI), and the slides were preserved with cover slips in the presence of ProLong Gold (Invitrogen).

Conflict of Interest statement. None declared.

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