Fibroblast phenotype in male carriers of \textit{FMR1} premutation alleles

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Fragile X-associated tremor/ataxia syndrome (FXTAS) is an adult-onset neurodegenerative disorder among carriers of premutation expansions (55–200 CGG repeats) of the fragile X mental retardation 1 (\textit{FMR1}) gene. The clinical features of FXTAS, as well as various forms of clinical involvement in carriers without FXTAS, are thought to arise through a direct toxic gain of function of high levels of \textit{FMR1} mRNA containing the expanded CGG repeat. Here we report a cellular endophenotype involving increased stress response (\textit{HSP27}, \textit{HSP70} and \textit{CRYAB}) and altered lamin A/C expression/organization in cultured skin fibroblasts from 11 male carriers of premutation alleles of the \textit{FMR1} gene, including six patients with FXTAS and five pre-mutation carriers with no clinical evidence of FXTAS, compared with six controls. A similar abnormal cellular phenotype was found in CNS tissue from 10 patients with FXTAS. Finally, there is an analogous abnormal cellular distribution of lamin A/C isoforms in knock-in mice bearing the expanded CGG repeat in the murine \textit{Fmr1} gene. These alterations are evident even in mouse embryonic fibroblasts, raising the possibility that, in humans, the expanded-repeat mRNA triggers pathogenic mechanisms early in development, thus providing a molecular basis for the neurodevelopmental abnormalities observed in some children and clinical symptoms in some adults who are carriers of premutation \textit{FMR1} alleles. Cellular dysregulation in fibroblasts represents a novel and highly advantageous model for investigating disease pathogenesis in premutation carriers and for quantifying and monitoring disease progression. Fibroblast studies may also prove useful in screening and testing the efficacy of therapeutic interventions.

\textbf{INTRODUCTION}

Individuals who are carriers of premutation CGG-repeat expansions of the fragile X mental retardation 1 (\textit{FMR1}) gene (55–200 CGG repeats), although originally thought to be clinically uninvolved, are now known to experience a range of clinical phenotypes, including (i) the neurodegenerative disorder, fragile X-associated tremor/ataxia syndrome (FXTAS), seen in older adult carriers of the premutation (recent reviews on FXTAS: 1,2–4); (ii) developmental disorders occasionally seen in children with the premutation (5–8); (iii) neuropsychological deficits in adults without FXTAS (9–13); (iv) emotional difficulties including depression, anxiety and obsessive compulsive features (14–16); (v) loss of reproductive function in adult women (17,18) and (vi) autoimmune disorders in women including...
fibromyalgia and hypothyroidism (19). Many of these clinical features are thought to arise as a consequence of toxicity of the elevated levels of the expanded CGG-repeat FMR1 mRNA (20,21).

The principal features of FXTAS are action tremor and gait ataxia with associated, more variable features that include cognitive decline with disinhibition and executive function deficits, mild parkinsonism, peripheral neuropathy and autonomic dysfunction (21–28). The neuropathology of FXTAS includes significant white matter disease, spongiosis in both the cerebrum and cerebellum, prominent sub-cortical astrogial activation, Purkinje cell loss in the cerebellum and the presence of eosinophilic, intranuclear inclusions in neurons and astrocytes throughout the cortex, subcortical regions and brainstem (but rarely present in cerebellar Purkinje cells) (29,30). Studies of the composition of isolated inclusions reveal the presence of at least 30 proteins (31), including ubiquitin, stress response proteins and cytoskeletal proteins. Importantly, the inclusions contain FMR1 mRNA (32), in agreement with the proposed RNA-toxicity model for FXTAS (21,30,33,34) wherein the expanded CGG-repeat RNA itself triggers the pathogenic process leading to FXTAS. Among the proteins present in the inclusions are at least three RNA binding proteins, hnRNP A2, MBNL1 and puro (35). Although there is no evidence of functional importance for these proteins in FXTAS pathogenesis, the work of Jin et al. (35) in Drosophila suggests that the CGG-induced neurodegenerative phenotype can be rescued by overexpression of puro. Similarly, Sofola et al. (36) demonstrated at least partial rescue of an eye phenotype in Drosophila upon overexpression of hnRNP A2. We have previously reported the disruption of the nuclear lamin A/C architecture and the activation of the heat-shock protein (HSP) αB-crystallin in cultured neural cells expressing expanded CGG repeats (31,37). In the current report, we examine cultured skin fibroblasts from male premutation carriers with and without FXTAS clinical symptoms and study molecular parallels with findings in CNS tissue derived from 10 male post-mortem cases with FXTAS. In both tissues, we observe transcriptional upregulation of FMR1, LMNA and stress response genes. Protein products of the LMNA gene (OMIM*150330), principally the two isoforms lamin A and C (lamin A/C), provide a protein matrix juxtaposed to the inner nuclear membrane that is thought to stabilize the structure of the nuclear compartment. Lamin A/C also influences chromatin organization, indirectly affecting gene expression (38). On the basis of this observation, our current report studies putative alterations of the characteristic ring-like nuclear lamin A/C architecture in both fixed and cultured skin fibroblasts derived from skin biopsies of subjects with FMR1 premutations. Finally, we study the transcription patterns of several cellular stress response genes in both skin fibroblasts from premutation carriers and brain tissue samples derived from subjects with FXTAS, compared with controls. On the basis of the results of these four experimental approaches, we consider skin fibroblasts, derived from the same germ layer (ectoderm) as neural cells of the CNS, as a powerful and easily accessible cell model to study the key events in the cellular pathogenesis of diseases associated with expanded CGG repeats.

### RESULTS

**Human skin fibroblasts from patients with FXTAS reveal altered lamin A/C nuclear architecture**

A striking cytoarchitectural finding in cultured neural (SK) cells harboring expanded CGG-repeat plasmids is the collapse of the normal nuclear lamin ring morphology (37). Such changes have also been observed in fibroblasts from certain laminopathies, even under conditions where there is no evident clinical (skin) phenotype (39–44), raising the possibility that one or more features of such cellular phenotypes in FXTAS could also be present in a peripheral cell type from patients with FXTAS. To examine this possibility, we performed molecular studies on a panel of six controls, six FXTAS and five asymptomatic premutation carrier fibroblast cell lines (Table 1).

![Table 1. Cultured fibroblast samples derived from male subjects](image)

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>EXP ID</th>
<th>Gender</th>
<th>Age&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CGG repeats&lt;sup&gt;b&lt;/sup&gt;</th>
<th>FXTAS score&lt;sup&gt;c&lt;/sup&gt;</th>
<th>FXTAS stage&lt;sup&gt;d&lt;/sup&gt;</th>
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<td>M</td>
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<tr>
<td>FXTAS</td>
<td>F1</td>
<td>M</td>
<td>71 122</td>
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<td>F6</td>
<td>M</td>
<td>61 95</td>
<td>49</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Age at skin biopsy.
<sup>b</sup>In cultured fibroblasts.
<sup>c</sup>FXTAS Rating Scale for major motor features in subjects who underwent videotaping sessions. The ranges for subdomains scored are: Tremor (0–53), ataxia (0–73) and parkinsonism (0–100) (1).
<sup>d</sup>Bourgeois et al. (16) and Hagerman et al. (21).
controls and six subjects with FXTAS (Supplementary Material, Fig. S1). We performed 2–6 technical replicates per subject with the majority involving three replicates. The mean fraction of complete lamin rings in the group of subjects with FXTAS was reduced from 77.44 ± 5.99% (controls) to 35.19 ± 5.65% (FXTAS) (P < 0.01) (Fig. 1C). Similar results are observed for lamin A/C architecture in fibroblasts derived from patients with FXTAS and from controls, regardless of whether fetal bovine serum was present in or absent from the cell culture media. In fact, asynchronized cultured fibroblasts from patients with FXTAS display a more significant reduction in the percentage of cells displaying normal ring-like organization of lamin A/C, compared with controls (data not shown). However, neither LMNA mRNA (Supplementary Material, Table S2A and B) nor protein levels were significantly altered in the fibroblast cultures compared with controls. We did not observe intranuclear inclusions in our panel of cultured fibroblast lines using antibodies directed to lamin A/C (0/4731 cells).

The mean CGG-repeat number in the control group was 26.2 ± 5.0. In the groups of premutation carriers with FXTAS and those without FXTAS, the mean number of CGG repeats was 100.1 ± 14.7 and 71.8 ± 5.9, respectively (P = 0.0002). As expected, FMR1 mRNA levels were significantly elevated in both groups of premutation carriers: FXTAS and asymptomatic carriers collectively over-expressed FMR1 mRNA by 2.58 ± 1.15-fold (P < 0.01) compared with controls. Interestingly, although the average expression of FMR1 mRNA is higher in premutation carriers with FXTAS relative to carriers with no clinical involvement, this difference was not statistically significant (Fig. 2).

**Human skin fibroblasts from patients with FXTAS show increased mRNA levels of heat-shock genes, αB-crystallin, HSP27 and HSP70**

To determine whether there is transcriptional upregulation of the stress response genes, CRYAB, HSP27 and HSP70, in fibroblast cultures corresponding to their accumulation in intranuclear inclusions in frontal cortex tissue of patients who died with FXTAS (31), we quantified the levels of these mRNAs in the panel of fibroblast cell lines (Table 1). We found that the HSP mRNAs from premutation carriers were all upregulated with respect to controls (Fig. 2 and Table 2). Similar results are observed for mRNA levels in fibroblasts derived from patients with FXTAS and from controls, regardless of whether the cells were synchronized or not (data not shown). As additional controls for the anti-lamin A/C (green) and anti-beta tubulin (red) antibodies to define the contour of the cells. Control fibroblasts derived from C2 display normal ring-like lamin A/C architecture (upper panel); whereas fibroblasts derived from F4 (lower panel) exhibit distorted lamin A/C organization and overall decreased staining (magnification ×400). Arrows indicate uneven distribution of lamin A/C; arrowheads indicate non-fusiform fibroblasts and abnormal morphology of the nuclei. (C) Dot plot representing the percentage of skin fibroblasts with complete lamin A/C rings in cultured fibroblasts derived from five premutation carriers with no clinical signs of FXTAS (asymptomatic; Asym Pre) and six subjects with FXTAS (black dots, premutation carriers) compared with six age-matched controls (white dots) described in Table 1. Horizontal lines represent the mean value per phenotypic group.

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RT–PCR method, and in particular for the integrity of the RNA samples, we quantified the mRNA expression levels of two mRNAs, coding for tuberin (TSC2) and oncogene Jun-B (JUNB), that were not expected to be influenced by FMR1 gene expression. Neither of these control mRNAs was overexpressed in the panel of premutation carriers compared with controls (Fig. 2). Moreover, unlike the greater variation in FMR1 and HSP mRNA levels in the premutation subgroups, there was no increase in variation for either JUNB or TSC2 (see Discussion section).

Western blot analysis of the panel of fibroblast cell lines showed no significant increases in αB-crystallin, HSP27 or HSP70 protein levels in total cell, enriched cytoplasmic or nuclear protein extracts from subjects with FXTAS compared with controls (P > 0.05). We found no intranuclear inclusions after immunostaining cultured fibroblast lines from three

Figure 2. Box plots demonstrating elevated mRNAs for FMR1 and three HSPs relative to GUS in cultured fibroblasts from carriers of FMR1 premutation alleles relative to controls. The sample groups are controls (C), premutation carriers with FXTAS (F) and asymptomatic premutation carriers (AsP). Genes are as indicated: FMR1, CRYAB, HSP27, HSP70 (upper and middle panels). The levels of mRNA expression of control genes JUNB and TSC2 are displayed in the lower panels. We found no significant differences in the mRNA levels of JUNB and TSC2 relative to GUS among the three groups of subjects considered in this study (neither intra-subject nor inter-subject variability). The horizontal box lines are 25, 50 (median) and 75% of the distribution with whiskers marking 1.5 times the interquartile range. The ‘+’ symbol indicates the mean of the distribution. Open circles indicate potential outliers (in CRYAB—control group and HSP70—non-FXTAS group). The results of our analysis with or without these two data points (outliers) do not differ.
subjects with FXTAS (F4, F5, F6, Table 1), including a patient with FXTAS clinical stage 5, using antibodies directed to αB-crystallin, HSP27, HSP70 and ubiquitin proteins typically found in FXTAS subjects with intranuclear inclusions in brain.

**Cultured skin fibroblasts from asymptomatic premutation carriers display features of molecular dysregulation similar to those observed in patients with FXTAS**

As noted above, *FMR1* mRNA levels were significantly elevated in fibroblasts from five premutation carriers without the core features (intention tremor, gait ataxia) of FXTAS, relative to six controls described in Table 1. This pattern of overexpression among groups of premutation carriers (six FXTAS, five asymptomatic carriers and both groups combined) is also observed for mRNA levels of all HSPs considered in this study (Table 2). However, we found no significant differences in mRNA levels of these four genes between FXTAS and asymptomatic premutation groups (Fig. 2). No significant differences in the levels of these proteins were observed for FXTAS or asymptomatic premutation carrier groups relative to controls.

Analyses of immunofluorescent staining of cultured skin fibroblasts from five asymptomatic premutation carriers with an antibody directed to lamin A/C also revealed an unexpected mean reduction (36.58%) in the percent of cells displaying normal lamin A/C rings relative to controls. Asymptomatic premutation carriers had 49.1 ± 6.6% normal lamin A/C rings (*P < 0.01*) relative to the control group (77.4 ± 6.0% normal lamin A/C rings). Although the group of fibroblasts derived from subjects with FXTAS displayed a 28.3% reduction in the number of normal lamin rings relative to the asymptomatic premutation group, this difference was not significant (*P = 0.13*; Fig. 1C).

**CNS tissue from a panel of 10 patients with FXTAS reveals elevated LMNA mRNA levels**

To quantify the post-mortem *FMR1* mRNA levels in frontal cortex samples of patients with FXTAS, a panel of 10 male subjects who died with FXTAS and three controls were analyzed via real-time (TaMan) qRT–PCR (Table 3). As expected (32), subjects with FXTAS had significantly elevated levels of *FMR1* mRNA relative to controls, 2.53 ± 1.06-fold (*P < 0.01*) (Fig. 3). Using the same panel of post-mortem brain samples, *LMNA* mRNA levels were found to be increased 2.02 ± 0.43-fold (*P < 0.01*) in FXTAS samples relative to controls (Fig. 3). Despite the elevated levels of *LMNA* mRNA in the CNS panel, there was no corresponding elevation in lamin A/C protein levels. In fact, western blot analysis of the RIPA-soluble fraction of protein extracts from the CNS panel showed trends toward reduced levels of lamin A/C isoforms, though only lamin C demonstrated a significant reduction: lamin A (0.69 ± 0.57-fold decrease; *P = 0.45*) and lamin C (0.44 ± 0.41-fold decrease; *P = 0.04*).

One of the 10 subjects (Case 10, Table 3) displayed normal levels of lamin A/C protein by western blot. Next, we analyzed the levels of lamin A/C proteins in the RIPA-insoluble fractions from the CNS panel (Table 3) to determine if the reduction of lamin A/C levels observed in most of the samples reflected decreased solubility of lamin A/C protein isoforms. Relative to the normalized control mean value of 1, FXTAS cases showed an increase of both lamin isoforms (lamin A = 2.40 ± 2.27-fold increase and lamin C = 1.53 ± 0.87-fold increase), although these increases were not statistically significant.

Parallel immunofluorescent analysis of lamin A/C in frontal cortex sections of Case 10 indicated the presence of normal levels of these isoforms, though the nuclear distribution of these filaments was abnormal. This patient displayed a ~3-fold decrease in the percentage of neural cells in frontal cortex sections with ring-like organization of lamin A/C (15.18 ± 12.77%, relative to Controls 1 and 2 (50.82 ± 2.25%; *P = 0.04*).

**Expression of heat-shock genes, αB-crystallin, HSP27 and HSP70 in CNS tissue**

In addition to the lamin A/C protein isoforms, the HSPs, αB-crystallin, HSP27 and HSP70 were also found within the intranuclear inclusions characteristic of FXTAS (31). To determine whether the transcription of these HSPs was also dysregulated in CNS tissue of patients with FXTAS, the relative mRNA levels for the three HSPs were quantified in the same CNS panel of FXTAS and control patients (Table 3), using qRT–PCR. Our results indicate that mRNA levels were elevated (~1.5–1.8-fold) for all three HSPs in the frontal cortex samples of 10 subjects affected with FXTAS compared with the controls (Fig. 4). Note that only two of three control samples were used to quantify mRNA levels of HSP27 between phenotypic groups (Supplementary Material, Table S2A and B). Therefore, although the HSP27 levels are observed to be elevated (1.8-fold measured increase) in brain samples from subjects with FXTAS, the increase does not reach the level of significance due to small sample size.

As in the fibroblast studies, we also quantified the mRNA expression levels of two control genes, *TSC2* and *JUNB*, relative to GUS, which were not expected to be influenced by *FMR1* gene expression. Neither of these control mRNAs was overexpressed in the panel of premutation carriers compared with controls (Fig. 4).

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**Table 2. Increased steady state mRNA levels of *FMR1* and of stress response genes in cultured skin fibroblasts from premutation carriers relative to controls**

<table>
<thead>
<tr>
<th>Relative mRNA levels</th>
<th>Asymptomatic carriers*</th>
<th>Carriers with FXTAS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Meanb (SD)</td>
<td>P-value</td>
</tr>
<tr>
<td><em>FMR1</em></td>
<td>2.25 (1.05)</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td><em>CRYAB</em></td>
<td>5.89 (7.09)</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td><em>HSP70</em></td>
<td>1.71 (0.54)</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td><em>HSP27</em></td>
<td>2.07 (0.87)</td>
<td>0.022*</td>
</tr>
<tr>
<td><em>JUNB</em></td>
<td>0.99 (0.22)</td>
<td>&gt;0.95</td>
</tr>
<tr>
<td><em>TSC2</em></td>
<td>1.13 (0.17)</td>
<td>&gt;0.36</td>
</tr>
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</table>

*Carriers of premutation *FMR1* alleles.

*Mean values of asymptomatic premutation cases relative to the normalized control mean values of 1.

*Significant at level *P < 0.05.*
As with FMR1 and LMNA genes, elevated mRNA levels for HSP70 and CRYAB were not accompanied by increases in their respective protein levels. Western blot analysis of the RIPA-soluble fraction of CNS protein extracts from the panel indicated that HSP70 protein is reduced in patients with FXTAS (0.76 ± 0.18-fold decrease; P = 0.03) relative to controls. The levels of soluble αB-crystallin and HSP27 proteins were not significantly altered in FXTAS compared with controls, although all three stress proteins were increased in the RIPA-insoluble (SDS-soluble) protein fraction of brain extract from subjects with FXTAS (Table 4 and Fig. 5).

Mouse embryonic fibroblasts harboring large premutation expansions also display nuclear lamin A/C dysregulation

To address the possibility that there may be a manifestation of RNA toxicity early in development when levels of FMR1 mRNA are highest, we utilized two mouse embryonic fibroblast (MEF) lines derived from a knock-in (KI) mouse line (45) with expanded unmethylated Fmr1 alleles. In the current study, we quantified the levels of Fmr1 mRNA in cultured mouse fibroblasts derived from two embryos and two adult mice, both with ~200 CGG repeats, relative to control murine fibroblasts. MEFs derived from expanded CGG-repeat mice express ~7-fold elevation in Fmr1 mRNA levels compared with MEFs derived from wild-type (wt) controls (6.9 ± 0.89 and 1.0 ± 0.2, respectively; P < 0.01). Moreover, Fmr1 expression in embryonic fibroblasts is substantially higher compared with fibroblasts derived from adult KI mice (6.9 ± 0.89 and 0.98 ± 0.30, respectively; P < 0.01). We observed no significant difference in Fmr1 mRNA levels between expanded repeat and wt cultured fibroblasts from adult mice.

Remarkably, cultured MEFs from premutation mice already display evidence of lamin A/C abnormalities. In particular, we observed a 4.6-fold decrease (85.10 ± 5.00%, wt; 18.43 ± 1.40%, KI; P < 0.01) in the percentage of complete lamin rings relative to wt MEFs (Fig. 6). Fibroblasts derived from adult mice with the expanded CGG repeat display a smaller difference in the percentage of complete lamin rings than their MEF counterparts (38.60 ± 14.1%, adults; 18.43 ± 0.81%, MEF; P < 0.01).

DISCUSSION

The nuclear intermediate filament, lamin A/C (A and C protein isoforms), was originally associated with the pathogenesis of FXTAS by virtue of its presence in the intranuclear inclusions found in the post-mortem brain tissue of a patient with FXTAS (31). Additional studies suggested that lamin A/C disorganization and aggregation may participate in the pathogenesis of FXTAS, and led us to hypothesize that in FXTAS a broad dysregulation of lamin A/C may result in (or at least be highly correlated with) CNS pathology and may impact other tissues where FMR1 is expressed, even in the absence of intranuclear inclusion formation (37). The intranuclear inclusions found in CNS tissue in FXTAS also contain several heat-shock response proteins, including αB-crystallin, HSP27 and HSP70.
suggesting that there may be a cellular stress response as a downstream consequence of expression of the expanded CGG-repeat mRNA. There is an extensive literature on the role of HSPs in stabilizing cytoskeletal proteins (46, reviewed in 47, 48–52). In this regard, the upregulation of HSP mRNAs observed both in frontal cortex and in cultured fibroblasts could reflect a stress response to the disordered architecture of the lamin A/C network, rather than to the elevated FMR1 mRNA.

We analyzed the nuclear lamin A/C architecture and cellular stress response in skin fibroblasts of male carriers of premutation FMR1 alleles and explored the possibility that this cellular phenotype would be aggravated in patients with FXTAS. Consistent with these expectations, we observe in subjects with FXTAS a significant reduction in the percentage of cultured fibroblasts with normal ring-like lamin A/C patterns (Fig. 1). A similar pattern of dysregulation, involving upregulation of mRNA levels of three HSPs and a decrease in the percentage of fibroblasts displaying normal lamin A/C rings (Figs 1D and 2), was observed in cultured skin fibroblasts from asymptomatic male carriers of premutation alleles. We did not find a significant correlation between the percentage of cells displaying lamin A/C rings and the number of CGG repeats in all premutation carriers (data not shown); however, the absence of a significant correlation could be due to the small sample size available for this study.

The levels of lamin A/C expression were also quantified in post-mortem frontal cortex from 10 patients with FXTAS and from three controls. Whereas the group of patients with FXTAS expressed increased levels of LMNA mRNA (Fig. 3), the levels of lamin A/C protein in the RIPA-soluble cellular fraction was reduced or undetectable in eight of 10 cases with FXTAS. Part of this reduction in soluble lamin levels appears to reflect repartitioning of the protein isoforms to less soluble, possibly aggregated forms of lamin (data not shown). These changes may contribute to inclusion formation in a small number of cells.

Associated with the elevated levels of the three HSPs and the decrease in the percentage of fibroblasts displaying normal lamin A/C rings (Figs 1D and 2), was observed in cultured skin fibroblasts from asymptomatic male carriers of premutation alleles. We did not find a significant correlation between the percentage of cells displaying lamin A/C rings and the number of CGG repeats in all premutation carriers (data not shown); however, the absence of a significant correlation could be due to the small sample size available for this study.

The levels of lamin A/C expression were also quantified in post-mortem frontal cortex from 10 patients with FXTAS and from three controls. Whereas the group of patients with FXTAS expressed increased levels of LMNA mRNA (Fig. 3), the levels of lamin A/C protein in the RIPA-soluble cellular fraction was reduced or undetectable in eight of 10 cases with FXTAS. Part of this reduction in soluble lamin levels appears to reflect repartitioning of the protein isoforms to less soluble, possibly aggregated forms of lamin (data not shown). These changes may contribute to inclusion formation in a small number of cells.

Associated with the elevated levels of the three HSP and of FMR1 mRNAs in FXTAS subjects (Figs 2 and 4; Table 3 and Supplementary Material, Table S2A), it is evident in the box plots (Fig. 2) that the range of mRNA levels for each of the four genes is substantially greater in premutation carriers (with or without FXTAS) than for the corresponding controls; this is true for either normalized data (to account for potential systematic experimental effects) or non-normalized data. This variation arises from at least two sources. First, within the premutation groups, we observe upward trends in mRNA levels with increasing CGG-repeat number (data not shown), although these trends do not reach significance, such variation is all captured as intra-group variation. The positive correlation

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Table 3. Characteristics of individuals contributing autopsy material

<table>
<thead>
<tr>
<th>Group</th>
<th>Gender</th>
<th>Age of death</th>
<th>CGG repeats</th>
<th>FXTAS clinical stage</th>
<th>CNS neural inclusions</th>
<th>Prior case</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>M</td>
<td>69</td>
<td>30</td>
<td>0</td>
<td>No</td>
<td>None</td>
</tr>
<tr>
<td>Control 2</td>
<td>M</td>
<td>57</td>
<td>28</td>
<td>0</td>
<td>No</td>
<td>None</td>
</tr>
<tr>
<td>Control 3</td>
<td>M</td>
<td>53</td>
<td>21</td>
<td>0</td>
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<td>None</td>
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<tr>
<td>FXTAS</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Case 1</td>
<td>M</td>
<td>70</td>
<td>113</td>
<td>6</td>
<td>Yes</td>
<td>Case 1c</td>
</tr>
<tr>
<td>Case 2</td>
<td>M</td>
<td>72</td>
<td>62</td>
<td>6</td>
<td>Rare</td>
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<tr>
<td>Case 3</td>
<td>M</td>
<td>75</td>
<td>78</td>
<td>6</td>
<td>Yes</td>
<td>None</td>
</tr>
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<td>Case 4</td>
<td>M</td>
<td>87</td>
<td>65</td>
<td>4</td>
<td>Yes</td>
<td>Case 11c</td>
</tr>
<tr>
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<td>M</td>
<td>66</td>
<td>105</td>
<td>5</td>
<td>Yes</td>
<td>Case 5c</td>
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<tr>
<td>Case 6</td>
<td>M</td>
<td>77</td>
<td>77</td>
<td>6</td>
<td>Yes</td>
<td>Case 6c</td>
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<td>Case 7</td>
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<td>76</td>
<td>88</td>
<td>6</td>
<td>Yes</td>
<td>Case 7c</td>
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<td>Case 8</td>
<td>M</td>
<td>75</td>
<td>92</td>
<td>5</td>
<td>Yes</td>
<td>Case 8c</td>
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<tr>
<td>Case 9</td>
<td>M</td>
<td>81</td>
<td>88</td>
<td>5</td>
<td>Yes</td>
<td>Case 9c</td>
</tr>
<tr>
<td>Case 10</td>
<td>M</td>
<td>68</td>
<td>98</td>
<td>6</td>
<td>Yes</td>
<td>Case 10c</td>
</tr>
</tbody>
</table>

aFXTAS clinical stages: stages 0/1, uninvolved/equivocal involvement; stage 2, definite tremor or ataxia not interfering with activities of daily living (ADL); stage 3, tremor or ataxia interfering with ADLs; stage 4, use of cane or walker; stage 5, use of a wheelchair; stage 6, bedridden (22).
bFrom Maryland Brain Bank.
cGreco et al. (29, 30).
dBourgeois et al. (16) and Hagerman et al. (21).
of FMRI mRNA levels with increasing CGG-repeat number has been reported previously (53–56). A second source of added variation in the premutation subgroups is the greater biological variation than that observed for controls. Although the origin of this biological variation is not understood, repeat measurements based on separate blood draws typically display less than ~20% variation (53,54). As expected, the expression levels of control genes JUNB and TSC2 are not elevated in either fibroblast or brain samples from premutation groups, nor do they display increased variation within the premutation groups relative to the corresponding control groups (Figs 2 and 4). Therefore, the greater variation of HSP mRNA levels in the premutation subgroups appears to be a biological effect of the premutation FMRI allele.

Despite increased FMRI and HSP mRNA levels in individuals with premutation alleles, the levels of the soluble forms of the corresponding proteins were not elevated in either CNS tissue or the skin fibroblasts samples analyzed. At present, we cannot explain the absence of significant, parallel increases in protein levels; however, these observations may reflect either redistribution to insoluble forms of these proteins or a marginal shift toward decreased stability. Interestingly, we did find a significant increase in accumulation of the three HSPs in the RIPA-insoluble (SDS-soluble) protein fraction of brain extracts from the FXTAS group relative to the controls (Table 4 and Fig. 5). These observations in samples from patients with FXTAS may also reflect changes in protein translocation into the nucleus. Lamin A/C protein levels were also quantified in the soluble protein fraction of brain extracts from patients with FXTAS. However, as with the HSPs, we did not observe any increases in the levels of lamin A or C isoforms that would correspond to the elevated LMNA mRNA levels.

Expression of the expanded CGG-repeat FMRI mRNA in cultured human neural cells is capable of inducing cellular pathology within a very short time frame (~1 week) (37), raising the possibility that early in development, when levels of FMRI mRNA are highest, there may be evidence of FMRI mRNA-induced cellular dysregulation. To explore this possibility, MEFs were derived from embryos of a female mouse homozygous for the expanded CGG repeat (~180 and ~210 CGG repeats) that was previously reported to have elevated Fmr1 mRNA levels and neuropathological characteristics of FXTAS, including the presence of inclusions in the CNS and non-CNS organs (45,57–59). We observed that cultured MEFs harboring large premutation Fmr1 alleles have both elevated levels of Fmr1 mRNA and altered morphology of the lamin A/C network (Fig. 6). Fibroblasts derived from adult KI mice with the expression of normal levels of expanded CGG-repeat mRNA display a lesser degree of lamin dysregulation than their MEF counterparts. This last result suggests that the fundamental processes leading to lamin dysregulation may be at least partially reversible and that severity seems to be correlated with Fmr1 mRNA levels.

Although the mechanisms that lead to the cytoskeletal abnormalities in FXTAS and some asymptomatic male premutation carriers remain unknown, the impact that lamin A/C has on other cytoskeletal components is well documented (60–63). Moreover, the consequences of abnormal cytoskeletal organization in neural cells include impaired neural cell migration during neurogenesis (64–67) and impaired neural plasticity (68). For example, impaired functional connectivity has been demonstrated in fMRI studies of the amygdala in response to fearful faces in young adult men with the premutation who do not have neurological symptoms (7). This fits with the elevated rates of psychopathology and social deficits that have been reported in the previous studies of premutation carriers, particularly the males, though the females have elevated rates of depression and anxiety (6,14,15,69–71).

Our findings provide evidence for a model in which increased expression of FMRI expanded CGG mRNA results in a range of molecular consequences, including altered expression and disruption of the nuclear lamin A/C architecture and induction of the expression of stress response genes. Whether these molecular/cellular changes, also observed in asymptomatic carriers, are ultimately useful for early diagnosis and intervention will depend on whether signs of molecular pathology are predictive of clinical involvement, including incipient neurodegenerative disease; proper assessment of any such predictive associations will require both larger sample sizes and/or longitudinal studies. Nevertheless, the current study provides the experimental underpinnings (fibroblast cellular dysregulation) for such future investigations.

Whereas it may be argued that the lack of significant differences between premutation carriers with and without FXTAS is due to the small sample sizes employed in the current study, it is also possible that the basis of incomplete penetrance is due to second gene effects and/or environmental factors (e.g. general anesthesia). Thus, the findings of at least some level of molecular dysregulation in all premutation carriers suggest that premutation status may be a strong risk factor, albeit not sufficient, for developing FXTAS. Similar findings at the cellular level prior to disease onset have been reported for other neurodegenerative disorders including Alzheimer disease, where neuropathology exists many years before the onset of clinical symptoms (72). In the current instance, the identification of a fibroblast phenotype provides us with a powerful tool to look for additional genetic and/or environmental factors that may give rise to clinical involvement.

As noted above, an important limitation of the current study is small sample size. Moreover, although the ages of the subjects and controls donating tissue lie within a narrow range, it was not possible to precisely age-match the subgroups. An additional limitation of this study is the variability in the length of post-mortem intervals (PMI) of the subjects.

### Table 4. Increased levels of aggregated HSP in frontal cortex from 10 patients with FXTAS relative to three controls, quantified by western blot

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mean ± SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-B-crystallin</td>
<td>2.34 (1.22)</td>
<td>0.0461*</td>
</tr>
<tr>
<td>HSP70</td>
<td>1.30 (0.25)</td>
<td>0.0056*</td>
</tr>
<tr>
<td>HSP27</td>
<td>5.69 (3.40)</td>
<td>0.0009**</td>
</tr>
</tbody>
</table>

*Mean values of two replicate analyses of insoluble protein extracts from 10 FXTAS cases relative to the normalized mean values (1.0) of three controls. **Significant at level $P = 0.05$. 
CONCLUSION

We have demonstrated that the several key features of the pathogenesis of FXTAS, namely dysregulation of the nuclear lamin A/C and transcriptional induction of several stress response genes, are manifest in both CNS tissue and skin fibroblasts derived from male premutation carriers. Together, these findings suggest that the underlying cellular response to the expanded CGG-repeat mRNA is not limited to cells of the CNS and that such responses may be taking place early in development. As part of a common underlying pathogenic (RNA toxicity) mechanism, our observations provide a framework for considering the developmental involvement occasionally seen in children with the premutation (5–8); emotional difficulties including depression, anxiety and obsessive compulsive features (14,15); and neuropsychological deficits in adults without FXTAS (9–13). Indeed, the presence of cellular dysregulation in older adults who do not manifest clinical features of FXTAS may reflect the participation of additional genetic or environmental protective factors. Future studies will be directed towards proteins binding the expanded CGG-repeat mRNA and their pathogenic downstream effects that underlie the clinical symptoms seen in FXTAS.

The foregoing studies have underscored the potential strengths of the fibroblast as a peripheral cell model to investigate the basis of FXTAS and other premutation-associated disorders. Moreover, from a clinical perspective, further understanding of the similarities of form and extent of cellular dysregulation between the fibroblast and neural cells in FXTAS should allow the fibroblast to serve as a gauge of the progression of clinical involvement in this disorder, and possibly as an early indicator of incipient disease and/or as a monitor of the efficacy of targeted therapeutic interventions.

MATERIALS AND METHODS

Subjects

All studies of post-mortem and fibroblast (biopsy) tissue samples were performed with approved protocols and informed consent in accordance with the Institutional Review Boards of the University of California, Davis or Rush University Medical Center.

The subjects included in this study were referred to our centers because they presented with FXTAS symptoms, including tremor and/or ataxia, were carriers ascertained through families with a fragile X syndrome proband or were controls in similar age group range. There was no attempt to recruit subjects of any particular ethnicity. The ethnic back-ground of the subjects participating in this study is white Caucasian for skin fibroblast studies and mainly white Caucasian subjects for brain studies, (two control subjects and 10 cases with FXTAS were white Caucasian, control subject C1 was African-American), and all were non-Hispanic.

We studied cultured skin fibroblasts from 11 male premutation carriers: six with FXTAS and five without FXTAS symptoms (asymptomatic) and six controls (Table 1). The control group had a similar age range (mean 67.00 ± 11.80) to the groups of asymptomatic premutation carriers and carriers with FXTAS (mean 70.20 ± 3.60 and 69.70 ± 8.10, respectively). There was no significant difference in age between the control group and the two groups of premutation carriers that donated skin fibroblasts (P = 0.81) through punch biopsy.

The current investigation also included post-mortem frontal cortex samples from 10 men who died between 2002 and 2005 with symptoms of FXTAS and from three controls (Table 2). The PMIs for cases affected with FXTAS were shorter (~12 h) than control cases (between 12 and 17 h). For post-mortem samples, although the FXTAS group had a higher mean age than the control group (74.7 ± 6.1 versus 60.0 ± 8.3 years, respectively), the difference did not reach significance (P = 0.068).

The brain samples and fibroblast samples were not from the same subjects.

Cultured human fibroblasts

All subjects contributing skin biopsies were participants in a multicenter study to characterize neurological findings in premutation carriers (Table 1).

Full thickness skin biopsies were performed with a 3 mm punch under local anesthesia with lidocaine. The biopsy was placed in culture media and later diced under sterile conditions on a culture plate and then plated in T25 flasks in AmnioMAX™-C100 Basal Medium (Gibco, Grand Island, NY, USA) containing 15% AmnioMAX™-C100 Supplement (Gibco). After cells grew out, longer-term cultures were maintained in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1× penicillin–streptomycin (pen–strept) media (100 U/ml penicillin G sodium and 100 µ/ml streptomycin sulfate; Gibco). Synchronization of fibroblast cultures was achieved by contact inhibition and serum starvation using a modified protocol (73); briefly, confluent fibroblast cultures were cultured in RPMI-1640 media without fetal bovine serum, at 37°C, 5% CO2, for 2 days. The ability to synchronize cultured fibroblasts in the G1–G0 phase of the cell cycle was monitored by processing for flow cytometric (FACS) analysis of DNA content of fixed cells with propidium iodide, the level of enrichment of fibroblasts in G0–G1 –G0 was 83 ± 2%. The rationale for synchronizing the cultured fibroblasts is based on the fact that abnormalities in lamin A/C organization in FXTAS were previously reported using a neural model for FXTAS (37), as well as the fact that lamin A/C organization varies with stages of the cell cycle. The assembly of the lamin A/C network is increased during G1–S, and it is visualized as a ring around the inner nuclear membrane. This organized structure, together with the nuclear envelope, is disassembled during mitosis—resulting in the loss of lamin A/C rings—in order for the condensed
chromosomes to gain access to the mitotic spindle. In telophase, the dispersed lamins are recycled to form nuclear envelopes in each daughter cell (reviewed in 74). Moreover, the expression of HSPs is also thought to oscillate throughout different stages of the cell cycle (75,76). Taken together, in order to evaluate the effects that premutations in FMR1 have on the expression of lamin A/C and HSPs, we decided to analyze fibroblasts synchronized in the G₁ stage of the cell cycle.

**FXTAS Rating Scale**

Videotapes from the structured neurological examination, designed to capture the major motor features of FXTAS: tremor, cerebellar dysfunction and parkinsonism, were scored by a movement disorder neurologist blinded to the subject’s premutation status, utilizing the FXTAS Rating Scale, Version 1.0 (77).

**FXTAS clinical staging scale**

Subjects included in this study were also assigned a rating on a six-point FXTAS clinical staging scale (Tables 1 and 2) based on the description of the degree of movement and gait problems, as previously described (22,78).

**Post-mortem brain tissue**

Brain autopsies were performed as described (29). Characteristics of individuals contributing autopsy materials are listed in Table 2. The clinical and neuropathological features of FXTAS Cases 1, and 4–9 have been described previously (29,30), and correspond in case number to those presented in Greco et al. (29). Clinical features have also been described for Case 10 (21,79). The following cases were not previously reported.

**Case 2** was characterized by family members as a brilliant professional who had life-long social anxiety, with alcoholism and obsessive thinking. At age 60, he had a thalamic stroke, followed by neurological deterioration with increasing difficulty swallowing. He had also experienced severe neuropathic pain in both legs for a number of years. Ataxia began at age 65; intention tremor was evident but sporadic. He developed cognitive decline and dementia over several years prior to his death. At autopsy, his left hemisphere showed vascular hyalinization, and scattered cystic infarcts (remote ischaemic nature) in cerebral white matter, basal ganglia and pons. Rare intranuclear inclusions were identified only in astrocytes of the hippocampal endplate.

**Case 3** had a long history of ataxia, frequent falling and deterioration in his handwriting. His MRI demonstrated moderate volume loss and severe white matter disease in basal ganglia, anterior limb of the internal capsule and subcortical regions, as well as a prominent MCP sign, which confirmed his clinical diagnosis of FXTAS before his death at age 76. Post-mortem examination of his brain (right cerebrum) showed moderate fronto-parietal atrophy. Histopathological evaluation showed pallor and spongiosis of cerebral white matter. Eosinophilic intranuclear inclusions were identified in neurons and astrocytes of the hippocampal endplate.

**Molecular data**

**Determination of CGG-repeat length.** Genomic DNA from either cultured fibroblasts or brain samples was amplified using an enhanced PCR technique as described (80), followed by sizing using the Qiaccel Genetic Analyzer (Qiagen, Valencia, CA, USA) (81). CGG-repeat sizes used in analyses for whole dermal mounts were determined from peripheral blood lymphocytes.

**Quantification of mRNA levels.** Measurements of FMR1 mRNA levels utilized qRT–PCR, using the 7900 Sequence Detector (Applied Biosystems, Foster City, CA, USA) as described (82). *FMR1, LMNA, CRYAB, HSP27* and *HSP70* mRNA levels were determined by qRT–PCR using gene-specific probe/primer sets by Applied Biosystems, Assays on Demand. These mRNAs of interest were quantified relative to *GUS* mRNA expression. The mRNA levels of tuberin (*TSC2*) and of oncogene Jun-B (*JUNB*) genes (gene-specific probe/primer sets by Applied Biosystems, Assays on Demand) relative to *GUS* were also quantified as additional internal controls. Each individual data point consists of 12 measurements: two independent primary fibroblast cultures and mRNA extractions from either primary cultured fibroblasts or brain tissue, in duplicate, analyzing three mRNA concentrations per sample, including technical replicate values (same mRNA preparations measured twice). Mouse-specific sets of primer/probe were used to quantify *Fmr1* mRNA relative to mouse *Gus* mRNA levels (Applied Biosystems, Assays on Demand). Quantification of mRNA levels in human fibroblasts, MEFs and adult mouse fibroblasts was performed on cell cultures that were synchronized using the same conditions described for cultured human fibroblasts.

**Generation of stable, expanded CGG MEF cell lines**

The KI mice harboring large expansions (~200 CGG repeats) of the CGG-repeat element in the *Fmr1* gene have been described (45,58,59). A timed mating was set up between a male mouse (~220 CGG repeats) and a female mouse homozygous for the expanded CGG repeat (~180 and ~210 CGG repeats). The pregnant female was sacrificed at day 14 postcoitum. Two embryos were processed to make two MEF lines. The embryonic sacs and placenta were removed from each embryo. The embryos were placed in PBS. After removal of PBS, the remainder of the embryo was minced. Five milliliter of trypsin/EDTA (TE) was added per embryo and incubated for 10 min at 37°C while shaking. The cell-TE suspension was removed and added to DMEM (Bio-Whittaker, Walkersville, MD, USA) with 10% fetal calf serum (FCS) and 1% pen–streptomycin to neutralize the trypsin. After incubation for 30 min at 37°C with shaking, the cells were filtered and spun down, resuspended in DMEM with 10% FCS and 1% pen–streptomycin and plated onto a culture dish for each embryo. Cells were split upon reaching 100% confluency.

Adult mouse skin fibroblast lines were generated by cutting a piece of shaved skin from a mouse harboring a CGG repeat of ~200 CGGs, and from a wt control, which were sacrificed at 20 weeks of age. The pieces of skin were minced and left to
proliferate in DMEM with 10% FCS and 1% pen–strept. Cells were split upon reaching confluence and cultured until they became established fibroblast lines.

Immunofluorescence microscopy

Cultured cells. Primary cultures of human dermal fibroblasts and mouse embryonic and adult skin fibroblasts (10 000 cells/coverslip) were grown on individual glass coverslips in RPMI-1640 supplemented with 10% FCS and 1 × pen–strept and incubated for 48 h at 37°C and 5.0% CO₂. When cells reached 70% confluency, they were synchronized via serum starvation (grown in media without FBS for 48 h), followed by fixation and blocking (37). Intranuclear inclusion formation of lamin A/C and HSPs was evaluated by co-staining blocked coverslips overnight (4°C) with rabbit anti-lamin A/C (BD Biosciences, San Jose, CA, USA; 1:1000) and mouse anti-oB-crystallin (Stressgen, Ann Arbor, MI, USA; 1:1000 dilution) antibodies, followed by washes with incubation with Alexa 488 goat anti-rabbit (Molecular Probes, Carlsbad, CA, USA; 1:1000) and Alexa 555 goat anti-mouse. The cellular distributions of HSP27 and HSP70 relative to lamin were studied by incubating fibroblasts with a combination of rabbit anti-lamin C (GeneTex Inc., Irvine, CA, USA; 1:1000) and mouse anti-HSP27 (Stressgen; 1:1000) antibodies, or mouse anti-lamin A/C (BD Biosciences; 1:1000) and rabbit anti-HSP70 (Stressgen; 1:1000) antibodies, respectively. Coverslips were washed in PBS-T, and then incubated with second-antibodies: Alexa 488 goat anti-rabbit (Molecular Probes; 1:1000) and Alexa 555 goat anti-mouse (Molecular Probes; 1:1000) and Alexa 488 goat anti-rabbit (Molecular Probes, Carlsbad, CA, USA; 1:1000) and Alexa 555 goat anti-mouse (Molecular Probes; 1:1000) for HSP70. All samples were counterstained with DAPI (Sigma-Aldrich, St Louis, MO, USA; 1:1000) and mouse anti-HSP70 (Stressgen; 1:1000) antibodies, respectively. Coverslips were subsequently blocked with BLOTTO (5% non-fat dry milk in 100 mM Tris–HCl, 0.9% NaCl, 0.1% polyoxyethylene (20) sorbitan monolaurate (20) sorbitan monolaurate, pH 7.5) followed by overnight incubation with primary antibodies: mouse monoclonal anti-lamin A/C (BD Biosciences), rabbit polyclonal anti-HSP70 (SPA-812, Stressgen), mouse monoclonal anti-HSP27 (SPA-800, Stressgen), mouse monoclonal anti-oB-crystallin (SPA-222, Stressgen) or mouse monoclonal anti-GAPDH (GTX29484, GenTex, Inc.), diluted in BLOTTO. Following washes with TBS-T (100 mM Tris, 150 mM NaCl, 0.1% polyoxyethylene (20) sorbitan monolaurate) blots were incubated with horseradish peroxidase conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Detection of antibodies was accomplished with SuperSignal West Dura Extended Duration Substrate (#34075, Pierce Biotechnology). Autoradiograms were quantified by densitometry using ImageJ (83).

Frozen frontal cortex from three controls and 10 cases with FXTAS were powdered in the presence of liquid N₂ and homogenized (Dounce) in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 0.5% IGEPA, 0.1% SDS, 0.012% deoxycholate, 0.5% Triton X-100, pH 7.4) with protease and phosphatase inhibitors. Homogenates were spun at 16 000g at 4°C. RIPA-insoluble fractions (protein pellets) and soluble protein fractions were quantified and diluted in Laemmli sample buffer and heated for 5 min at 95°C before loading 20 μg per sample. The protocol described above to quantify proteins of interest in fibroblast samples was also used to quantify lamin A/C and HSPs (αB-crystallin, HSP27 and HSP70) in protein extracts from human brain.

Altogether, the levels of these proteins were quantified from (i) total protein extracts (RIPA-soluble fractions) from asynchronous tissue from subjects listed in Tables 1 and 2; (ii) enriched nuclear protein extracts and enriched cytoplasmic protein extracts from synchronized cultures (from subjects listed in Table 1), isolated with NePer kit (Pierce Biotechnology) and (iii) total insoluble protein fractions in RIPA from subjects listed in Table 2.

Statistical analysis

Comparison of the ages and CGG-repeat numbers of the participants that provided skin samples to this study was based on...
the analysis of variance (ANOVA). The sample groups for cultured skin fibroblasts (Table 1) are premutation carriers without FXTAS (asymptomatic), carriers with FXTAS and controls. We also compared all premutation carriers (with and without FXTAS) to the control group for fibroblast data. Fibroblast mRNA and protein quantification analyses included replicate measurements: two replicates for mRNA measures (FMR1, CRYAB, HSP27, HSP70, JUNB and TSC2); four replicates for nuclear protein measures (lamin A/C, αB-crystallin, HSP27, HSP70); two replicates for cytoplasmic protein, two replicates for total protein and a single measurement for nuclear insoluble protein. Thus, comparisons of expression among groups were also based on the ANOVA method for nuclear, insoluble lamin A/C protein. For replicated measurements, the repeated measures ANOVA method was used to account for within- and between-replicate variability in comparisons among groups. Prior to fitting repeated measures ANOVA models, each measurement was rescaled via normalization by dividing each variable (e.g. lamin A) by the mean of the corresponding variable in the control group and analyses were performed on log-transformed data to stabilize the variance and potential skewness of the data.

The sample groups for the brain studies (Table 2) are subjects who died with FXTAS and controls. Due to the smaller sample size of the control group in brain samples, we also performed non-parametric tests comparing differences in mRNA and protein expression in brain between control and premutation. The conclusions based on this latter analysis were unchanged from those of the ANOVA. Independent analyses of brain samples were performed for relative mRNA levels of FMR1, HSP27, HSP70, LMAFA and CRYAB. In qRT–PCR assays for mRNA levels, three RNA concentrations per subject were utilized as described in Tassone et al. (82). Protein and mRNA quantification in brain samples included technical replicate measurements: two replicates for mRNA measures (FMR1, CRYAB, HSP27, HSP70); four replicates for total soluble protein measures (lamin A/C, HSP27, HSP70) and two measurements for the insoluble fraction of total protein extracts. The expression of mRNAs and proteins are reported as the mean levels per group ± SD, unless specified otherwise.

For the analysis of the numbers of normal lamin A/C rings in cultured human fibroblasts or in the MEF cell lines, nuclei were scored for the presence or absence of a uniform ring-like structure (a complete/closed ring of lamin staining the inner nuclear membrane). The percentages of human fibroblasts with complete lamin rings were grouped according to clinical phenotype: FXTAS (stages 3 or greater), asymptomatic premutation carriers (stage 0) and controls; and for mouse-derived fibroblasts according to wt controls, and KI mice with ~200 CGG repeats. Pairwise inter-group comparisons of the average percent of rings in (2–6) independent staining experiments were based on repeated measures ANOVA.

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Conflict of Interest statement. None declared.

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REFERENCES


SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.


