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Supplemental Information

Translation of Expanded CGG Repeats into FMRpolyG Is Pathogenic and May Contribute to Fragile X Tremor Ataxia Syndrome

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Figure S1

A

5'UTR *FMR1* (CGG)99x GFP (Gly frame)

```
...ACGGTGGGAGGTCTATATAAGCAGAGCTCtctggctaactagagaacccactgcttactggcttatcgaaattaatacgactcactataggg
agacccaagctggctagcgtttaaacttaagcttcgtcgacagggcccaccgcggaggccggccTCAGGCGCTCAGCTCCGTTTCGGTTTCACTT
CCGGTGGAGGGCCGCCTCTGAGCGGGCGGCGGGCCGACGGCGAGCGCGGGCGGCGGCGGTGACGGAGGCGCCGCTGCCAGGGGGCGTGCGGCAGC
GCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGC
GGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG
CGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCG
GCGGCGGCGGCGGCTGGGCCTCGAGCGCCCGCAGCCCACCTCTCGGGGGCGGGCTCCCGGCGCTAGCAGGGCTGAAGAGAAGATGGAGGAGCTGG
TGGTGGAAGTGCGGGGCTCCAATGGCGCTTTCTACAAGGCACCTAggaGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGT
CGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCT
GCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAG
CAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGA
GGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGT
ACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGC
AGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGC
CCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACA
AGTAActctagagcggccgcttcgagCAGACATGATAAGATA...
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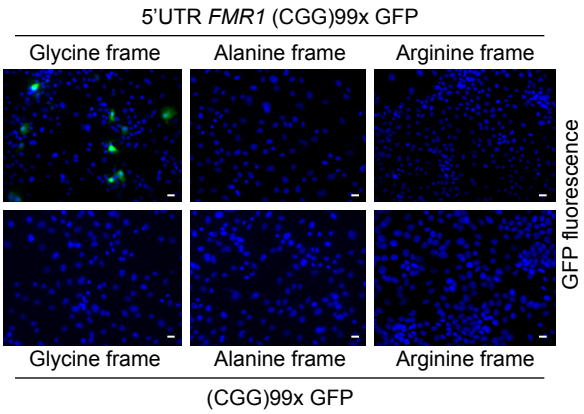
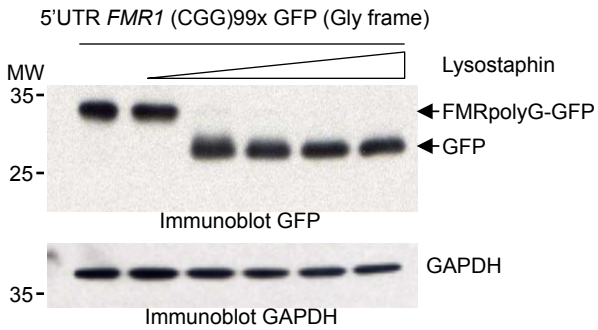
B



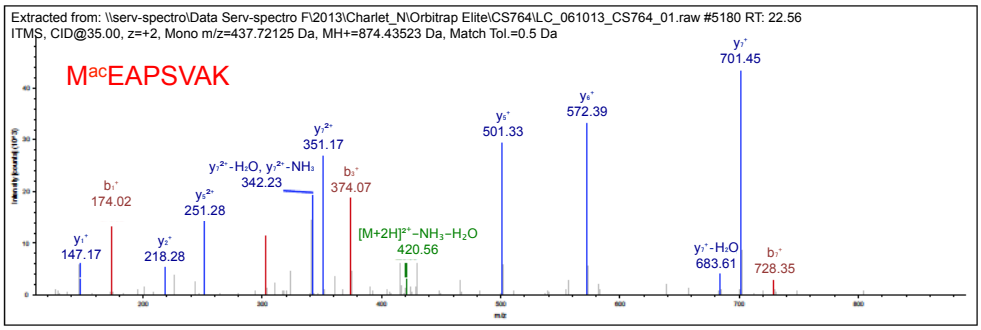
C



D

5'UTR *FMR1* LysC (CGG)99x GFP

```
...AGCGCGGGCGGCGGCGGTGACGGAGGCGCCgtccgtcgcaaaactagttccccgcggctcccgGGCGCCGCTGCCAGGGGGCGTGCGGCAGCGCGG...
                              M  E  A  P  S  V  A  K  L  V  P  R  G  S  R  A  P  L  P  G  G  V  R  Q  R  G
```



E

```
Homo Sapiens          ...CGGGCCGACGGCGAGCGCGGGCGGCGGCGGTGACGGAGGCGCCGCTGCCAGGGGGCGTGCGGCAGCGCGGCGGCGG...
Pan troglodytes       ...CGGGCCGACGGCGAGCGCGGGCGGCGGCGGTGACGGAGGCGCCGCTGCCAGGGGGCGTGCGGCAGCGCGGCGGCGG...
Macaca arctoides      ...CGGGCCGACGGCGAGCGCGGGCGGCGGCGGTGACGGAGGCGCCGCTGCCAGGGGGCGTGCGGCAGCGCGGCGGCGG...
Nomascus leucogenys   ...CGGGCCGACGGCGAGCGCGGGCGGCGGCGGTGACGGAGGCGCCGCTGCCAGGGGGCGTGCGGCAGCGCGGCGGCGG...
Otolemur garnettii    ...CGGGCCGACGGCtAGCGCGGGCGGCGaCGGTGACGGtGGCGCCGCTGCCgGGGGGCGTGCGGaAGCGCGGCGGCGG...
Sus scrofa            ...tGaGCCGACGGCGAGCGCaGGCGGCGGCGGTGACGGcGGCGCCGCTGCCgGGGGGCGTGCGGCAGCGCGGCGGCGG...
Canis familiaris      ...tGaGCCGACGGCGAGCGCGGGtGGCGGCGGTGACGGcGGCGCCGCTGCCgGGGGGCGTGCGaGAGCGCGGCGGCGG...
Rattus norvegicus     ...CCaaCgGACGGCGAGCGCGGGCGGtGtCaGTGACGGcGGCGCCGCTGCCgGGGGGCGTGCGGtAaCGCGGCGGCGG...
Mus musculus          ...CaaaCgGACGGCGAGCGCGGGCGGtGGCaGTGACGGcGGCGCCGCTGCCgGGGGGCGTGCGGtAaCGCGGCGGCGG...
Kozac consensus sequence                                RCCRCCATGG
```

Figure S1 (related to figure 1).
Translation of CGG repeats requires a near-cognate codon.

(**A**) Partial sequence of the 5'UTR FMR1 CGG99x GFP (glycine frame) plasmid (Addgene #63091). CMV promoter and sv40 polyadenylation sequences are indicated in black uppercase at the start and the end of the sequence, respectively. The plasmid multiple cloning site sequence is in black lowercase with Fse1 and Xba1 cloning site underlined. The 5'UTR sequence of human *FMR1* is in bold black uppercase with expanded CGG repeats in red. The ACG near cognate codon is also indicated in red. The stop codon of FMRpolyG and the ATG codon of GFP were deleted and replaced by a glycine codon (gga). The GFP sequence is in green uppercase.

(**B**) GFP fluorescence of HeLa cells transfected for 24 hours with expanded CGG repeats embedded or not in the 5'UTR of *FMR1* and fused in all three possible frames with the GFP deleted of its ATG.

(**C**) Immunoblotting against the GFP on the soluble lysate fraction of HeLa cells transfected for 24 hours with expanded CGG repeats embedded within the 5'UTR of *FMR1* and fused to the GFP in the glycine frame and treated with increasing quantity of lysostaphin, a glycine endopeptidase.

(**D**) Upper panel, sequence of the mutant *FMR1* 5'UTR including a LysC restriction site. Lower panel, LC-MS/MS spectra of the N-terminal part of the immunoprecipitated and LysC digested protein translated from expanded CGG embedded in the LysC mutant 5'UTR of *FMR1*.

(**E**) Sequence alignment of the 5'UTR of *FMR1* from human, chimpanzee, macaque, gibbon, galago, wild boar, dog, rat and mouse with Kozac consensus sequence. ACG translation start of FMRpolyG is indicated in red, the conserved nucleotides in the Kozac sequence are indicated in bold black.

Figure S2

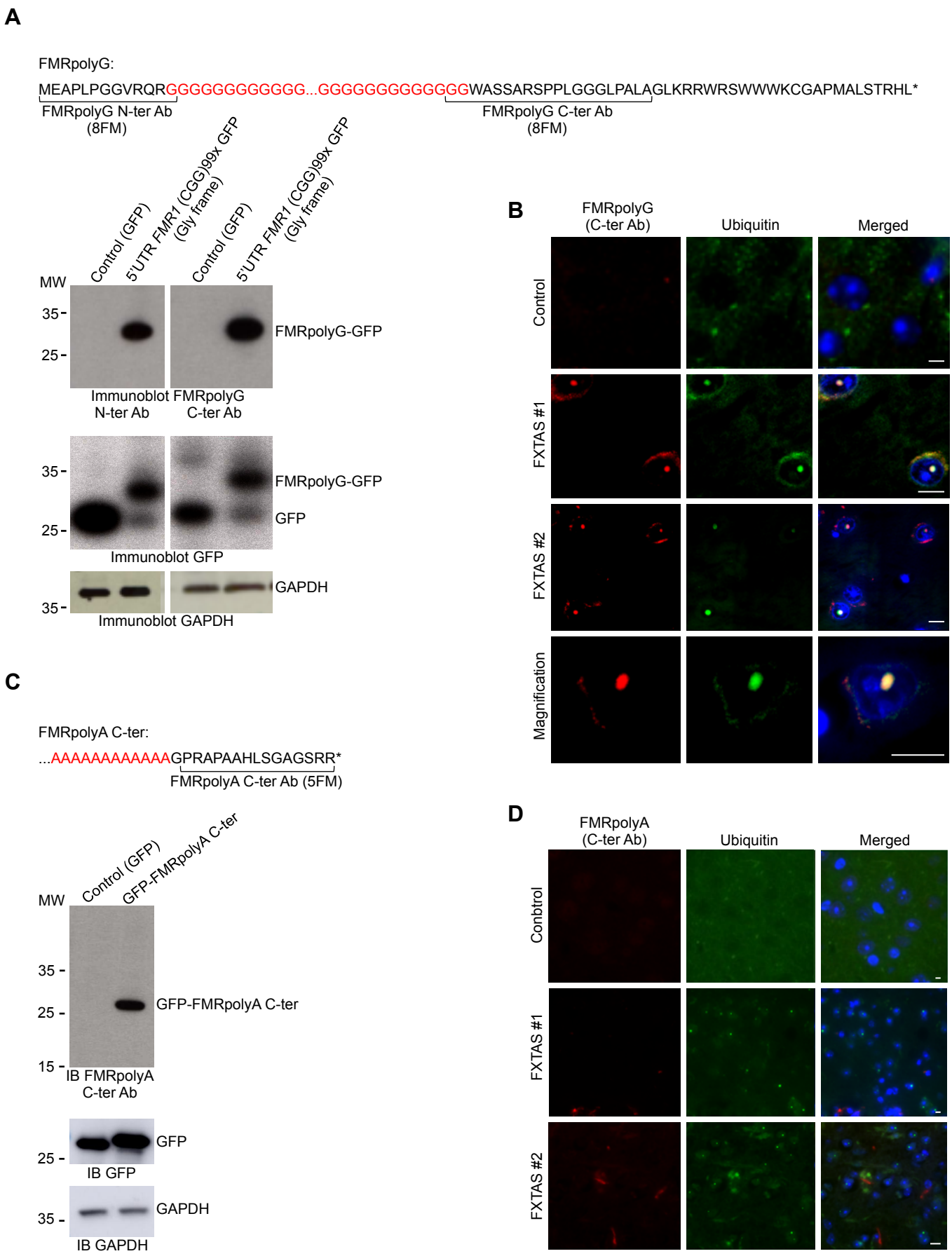

A
FMRpolyG:
MEAPLPGGVRQRGGGGGGGGGGGG...GGGGGGGGGGGGGGWASSARSPPLGGGLPALAGLKRRWRSWWWKCGAPMALSTRHL*
FMRpolyG N-ter Ab (8FM)
FMRpolyG C-ter Ab (8FM)
Control (GFP)
5'UTR FMR1 (CGG)99x GFP (Gly frame)
Control (GFP)
5'UTR FMR1 (CGG)99x GFP (Gly frame)
MW
35
25
FMRpolyG-GFP
Immunoblot FMRpolyG N-ter Ab
Immunoblot FMRpolyG C-ter Ab
35
25
FMRpolyG-GFP
GFP
Immunoblot GFP
35
GAPDH
Immunoblot GAPDH
B
FMRpolyG (C-ter Ab)
Ubiquitin
Merged
Control
FXTAS #1
FXTAS #2
Magnification
C
FMRpolyA C-ter:
...AAAAAAAAAAAAGPRAPAAHLSGAGSRR*
FMRpolyA C-ter Ab (5FM)
Control (GFP)
GFP-FMRpolyA C-ter
MW
35
25
15
GFP-FMRpolyA C-ter
IB FMRpolyA C-ter Ab
25
GFP
IB GFP
35
GAPDH
IB GAPDH
D
FMRpolyA (C-ter Ab)
Ubiquitin
Merged
Conbtrol
FXTAS #1
FXTAS #2

Figure S2 (related to figure 2).
Generation of antibodies specific to FMRpolyG.

(**A**) Upper panel, amino acid sequence of FMRpolyG. The N- and C-terminal peptide sequences used to generate 8FM and 9FM mouse monoclonal antibodies are underlined. Lower panel, immunoblotting validation of 8FM and 9FM antibodies directed respectively against the N- and C-terminal parts of FMRpolyG on the soluble lysate fraction of HeLa cells transfected for 24 hours with expanded CGG repeats embedded in the 5'UTR of *FMR1* and fused to the GFP in the glycine frame.

(**B**) Immunofluorescence against the C-terminal part of FMRpolyG (red, 9FM antibody) and ubiquitin (green) on brain sections (hippocampal area) of FXTAS patients compared to a age-matched control individual.

(**C**) Upper panel, putative amino acid sequence of the C-terminal part of FMRpolyA. The peptide sequence used to generate the 5FM mouse monoclonal antibody is underlined. Lower panel, immunoblotting validation of the 5FM antibody on lysate of HeLa cells transfected for 24 hours with a construct expressing the GFP fused to the putative C-terminal part of FMRpolyA.

(**D**) Immunofluorescence against the C-terminal part of FMRpolyA (red, 5FM antibody) and ubiquitin (green) on brain sections (hippocampal area) of FXTAS patients. Scale bars, 10 μm. Nuclei were counterstained with DAPI.

Figure S3

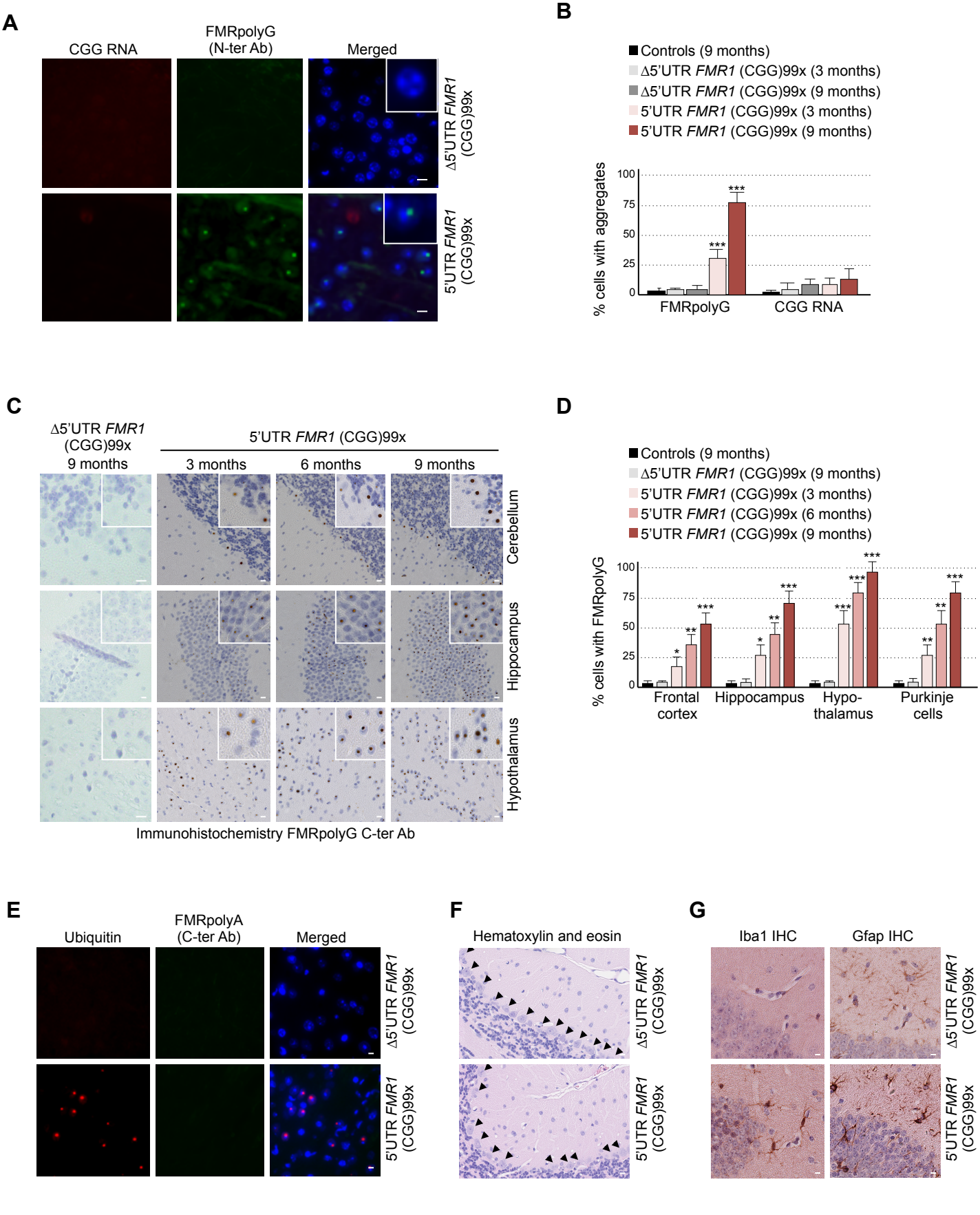

A
CGG RNA
FMRpolyG (N-ter Ab)
Merged
Δ5'UTR FMR1 (CGG)99x
5'UTR FMR1 (CGG)99x
B
Controls (9 months)
Δ5'UTR FMR1 (CGG)99x (3 months)
Δ5'UTR FMR1 (CGG)99x (9 months)
5'UTR FMR1 (CGG)99x (3 months)
5'UTR FMR1 (CGG)99x (9 months)
% cells with aggregates
100
75
50
25
0
***
FMRpolyG
CGG RNA
C
Δ5'UTR FMR1 (CGG)99x 9 months
5'UTR FMR1 (CGG)99x
3 months
6 months
9 months
Cerebellum
Hippocampus
Hypothalamus
Immunohistochemistry FMRpolyG C-ter Ab
D
Controls (9 months)
Δ5'UTR FMR1 (CGG)99x (9 months)
5'UTR FMR1 (CGG)99x (3 months)
5'UTR FMR1 (CGG)99x (6 months)
5'UTR FMR1 (CGG)99x (9 months)
% cells with FMRpolyG
100
75
50
25
0
*
**
***
Frontal cortex
Hippocampus
Hypo-thalamus
Purkinje cells
E
Ubiquitin
FMRpolyA (C-ter Ab)
Merged
Δ5'UTR FMR1 (CGG)99x
5'UTR FMR1 (CGG)99x
F
Hematoxylin and eosin
Δ5'UTR FMR1 (CGG)99x
5'UTR FMR1 (CGG)99x
G
Iba1 IHC
Gfap IHC
Δ5'UTR FMR1 (CGG)99x
5'UTR FMR1 (CGG)99x

Figure S3 (related to figure 3).
Expression of FMRpolyG is pathogenic in mice.

(**A**) RNA FISH against CGG RNA foci (red) coupled to immunofluorescence against FMRpolyG N-terminal part (8FM antibody, green) in brain of 6 months old bigenic CMV-cre/full-length or mutant FMR1 5'UTR transgenic mice. Nuclei were counterstained with DAPI.

(**B**) Quantification of FMRpolyG protein aggregates and CGG RNA foci in 3 or 9 months old control (n=3), bigenic CMV-cre/full-length (n=3) or mutant (n=3) FMR1 5'UTR transgenic mice (n=100 cells).

(**C**) Immunohistochemistry against FMRpolyG C-terminal part (9FM) of cerebellum, hippocampal and hypothalamic areas of 3, 6 or 9 months old bigenic CMV-cre/full-length or mutant FMR1 5'UTR transgenic mice. Sections were counterstained with Nissl staining.

(**D**) Quantification of FMRpolyG protein aggregates in control (n=3), bigenic CMV-cre/full-length (n=3) or mutant (n=3) FMR1 5'UTR transgenic mice.

(**E**) Immunofluorescence against ubiquitin (red) and the C-terminal part of FMRpolyA (5FM, green) on hippocampal areas of 6 months old bigenic CMV-cre/full-length or mutant FMR1 5'UTR transgenic mice. Nuclei were counterstained with DAPI.

(**F**) Hematoxylin and eosin staining of cerebellum of 9 months old bigenic CMV-cre/full-length or mutant FMR1 5'UTR transgenic mice. Arrowheads indicate Purkinje cells.

(**G**) Immunohistochemistry against Iba1 and Gfap of the hippocampal areas of 9 months old bigenic CMV-cre/full-length or mutant FMR1 5'UTR transgenic mice. Sections were counterstained with H&E staining. Scale bars, 10 µm. Error bars indicate s.e.m. Student t-test, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

Figure S4

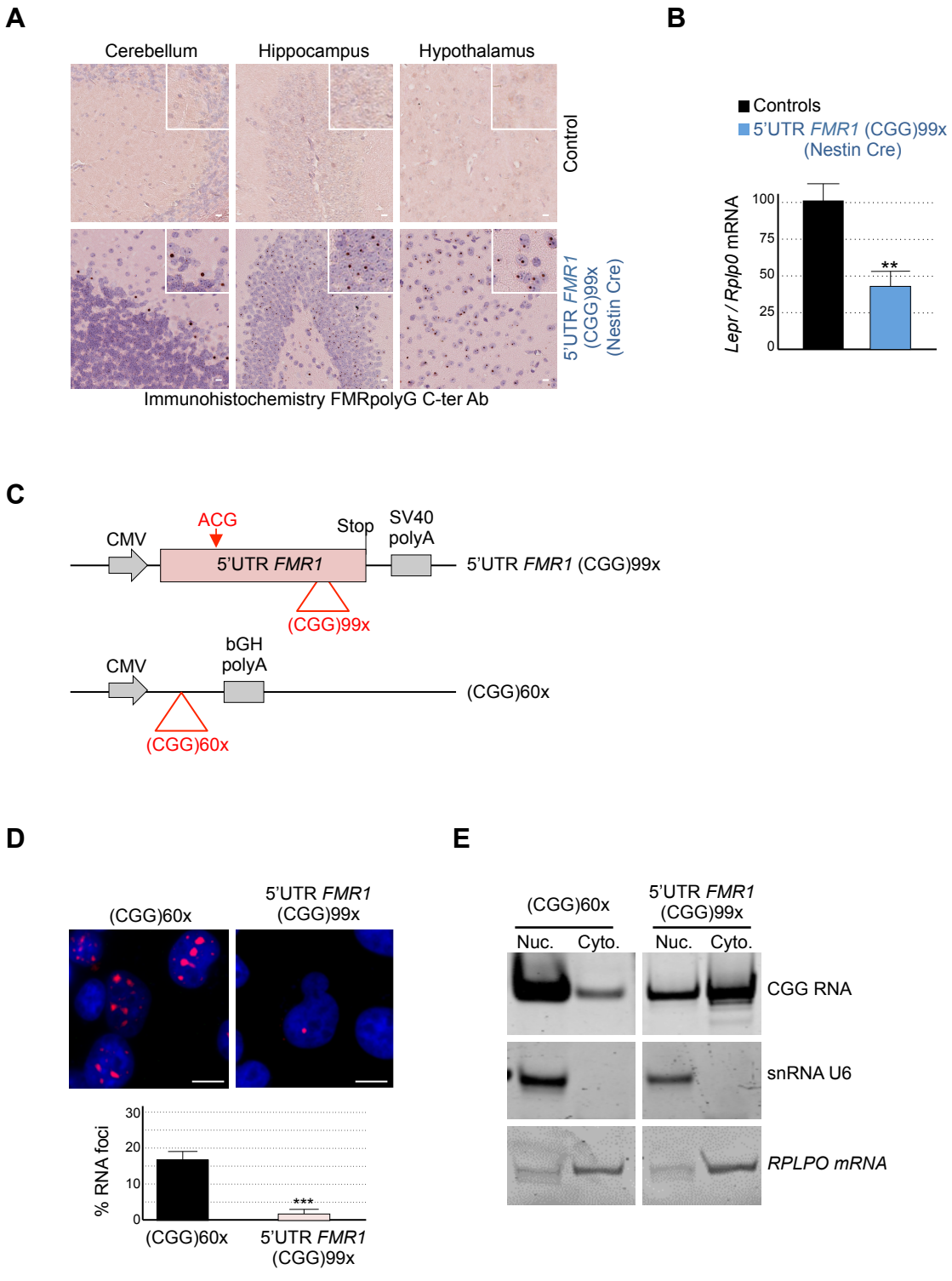

A
Cerebellum
Hippocampus
Hypothalamus
Control
5'UTR FMR1 (CGG)99x (Nestin Cre)
Immunohistochemistry FMRpolyG C-ter Ab
B
Controls
5'UTR FMR1 (CGG)99x (Nestin Cre)
Lepr / Rplp0 mRNA
100
75
50
25
0
**
C
CMV
ACG
5'UTR FMR1
Stop
SV40 polyA
5'UTR FMR1 (CGG)99x
(CGG)99x
CMV
bGH polyA
(CGG)60x
(CGG)60x
D
(CGG)60x
5'UTR FMR1 (CGG)99x
% RNA foci
30
20
10
0
***
(CGG)60x
5'UTR FMR1 (CGG)99x
E
(CGG)60x
5'UTR FMR1 (CGG)99x
Nuc.
Cyto.
Nuc.
Cyto.
CGG RNA
snRNA U6
RPLPO mRNA

Figure S4 (related to figure 4).
The 5'UTR sequence of FMR1 impairs formation of RNA foci.

(**A**) Immunohistochemistry against FMRpolyG C-terminal part (9FM antibody) in the cerebellum, hippocampus and hypothalamus of 6 months old bigenic Nestin-cre/full-length FMR1 5'UTR mice. Scale bars, 10 µm. Sections were counterstained with H&E staining.

(**B**) Quantitative RT-PCR analysis of Leptin receptor mRNA expression relative to the *RplpO* mRNA in the hypothalamic brain areas of 9 months old control (n=3) or Nestin-cre/full-length FMR1 5'UTR bigenic (n=3) mice.

(**C**) Schematic description of the expanded CGG repeats constructs embedded in the human 5'UTR sequence of FMR1 or cloned without any FMR1 sequence. The 99 CGG repeats embedded in their natural 5'UTR sequence is deposited at Addgene, #63089. The construct with 60 CGG repeats inserted into the multiple cloning site of pcDNA3.1 is described in Sellier et al, 2010.

(**D**) Upper panel, RNA FISH against CGG RNA foci in cells transfected for 24 hours with expanded CGG repeats embedded or not in the 5'UTR of FMR1 (untagged). Scale bars, 10 µm. Nuclei were counterstained with DAPI. Lower panel, quantification of cells (n=50) with CGG RNA foci (n=3 transfection). Error bars indicate s.e.m. Student t-test, *** indicates $p<0.001$.

(**E**) RT-PCR analysis of nuclear and cytoplasmic fractions of neuronal cells transfected for 24 hours with expanded CGG repeats embedded or not in the 5'UTR of FMR1 (untagged). Correct nuclear and cytoplasmic fractionation was controlled by RT-PCR for nuclear U6 snRNA and for cytoplasmic RPLP0 mRNA.

Figure S5

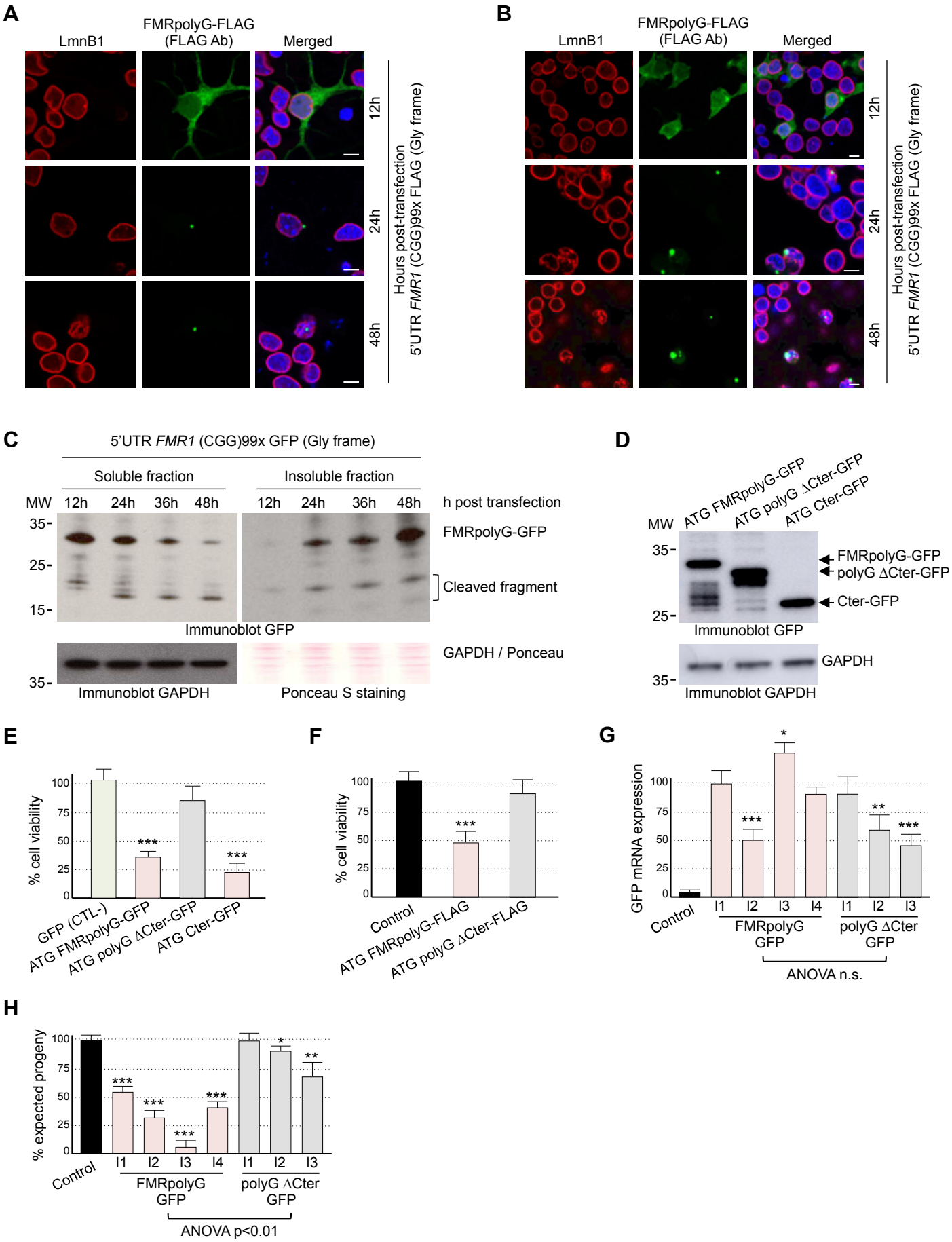

A
LmnB1
FMRpolyG-FLAG (FLAG Ab)
Merged
12h
24h
48h
Hours post-transfection
5'UTR FMR1 (CGG)99x FLAG (Gly frame)
B
LmnB1
FMRpolyG-FLAG (FLAG Ab)
Merged
12h
24h
48h
Hours post-transfection
5'UTR FMR1 (CGG)99x FLAG (Gly frame)
C
5'UTR FMR1 (CGG)99x GFP (Gly frame)
Soluble fraction
Insoluble fraction
MW
12h
24h
36h
48h
12h
24h
36h
48h
h post transfection
35-
25-
15-
FMRpolyG-GFP
Cleaved fragment
Immunoblot GFP
GAPDH / Ponceau
35-
Immunoblot GAPDH
Ponceau S staining
D
MW
ATG FMRpolyG-GFP
ATG polyG ΔCter-GFP
ATG Cter-GFP
35-
25-
FMRpolyG-GFP
polyG ΔCter-GFP
Cter-GFP
Immunoblot GFP
GAPDH
35-
Immunoblot GAPDH
E
% cell viability
100
75
50
25
0
***
***
GFP (CTL-)
ATG FMRpolyG-GFP
ATG polyG ΔCter-GFP
ATG Cter-GFP
F
% cell viability
100
75
50
25
0
***
Control
ATG FMRpolyG-FLAG
ATG polyG ΔCter-FLAG
G
GFP mRNA expression
100
75
50
25
0
*
***
**
***
Control
I1
I2
I3
I4
I1
I2
I3
FMRpolyG GFP
polyG ΔCter GFP
ANOVA n.s.
H
% expected progeny
100
75
50
25
0
***
***
***
***
*
**
Control
I1
I2
I3
I4
I1
I2
I3
FMRpolyG GFP
polyG ΔCter GFP
ANOVA p<0.01

Figure S5 (related to figure 5).
The C-terminal of FMRpolyG contributes to its toxicity.

(**A**) Immunofluorescence against the FLAG tag (green) and lamin B1 (Lmnb1, red) in primary cultures of E18 mouse cortical neurons transfected for the indicated time period with expanded CGG repeats embedded within the 5'UTR of FMR1 and fused to a FLAG tag in the glycine frame. Scale bars, 10 μm. Nuclei were counterstained with DAPI.

(**B**) Immunofluorescence against the FLAG tag (green) and lamin B1 (Lmnb1, red) in HEK293 cells transfected for the indicated time period with expanded CGG repeats embedded within the 5'UTR of FMR1 and fused to a FLAG tag in the glycine frame. Scale bars, 10 μm. Nuclei were counterstained with DAPI.

(**C**) Immunoblotting against the GFP of the soluble and insoluble fractions of neuronal cells transfected for the indicated time period with expanded CGG repeats embedded within the 5'UTR of FMR1 and fused to the GFP in the glycine frame.

(**D**) Immunoblotting against the GFP of the soluble fractions of N2A cells transfected for 24 hours with ATG-driven FMRpolyG-GFP full-length or deleted of its N- or C-terminal part.

(**E**) Cell viability of N2A cells transfected for 24 hours with ATG-driven FMRpolyG-GFP full-length or deleted of its N- or C-terminal part (5 independent transfections).

(**F**) Cell viability of N2A cells transfected for 24 hours with ATG-driven FMRpolyG-FLAG full-length or deleted of its C-terminal part (3 independent transfections).

(**G**) Transgene mRNA expression in independent Drosophila lines expressing either FMRpolyG-GFP full-length or deleted of its C-terminus. RNA levels are significantly different from one line to another by student t-test. However, this is independent of the construct as there is no significant difference of transgene expression between FMRpolyG-GFP and polyG ΔCter-GFP lines by ANOVA. FMRpolyG-GFP line #2 and polyG ΔCter-GFP line #2 correspond to the Drosophila lines analyzed in figure 5C and present similar transgene expression.

(**H**) Progeny eclosion ratio (n=100, 3 independent crosses) of independent Drosophila lines expressing FMRpolyG full-length or deleted of its C-terminus compared to control driver line (Actin5C-Gal4/+). Fly lines expressing FMRpolyG-GFP present a decrease eclosion rate compared to Drosophila lines expressing polyG ΔCter-GFP (one way ANOVA, $P<0.01$). Error bars indicate s.e.m. Student t-test, * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

Figure S6

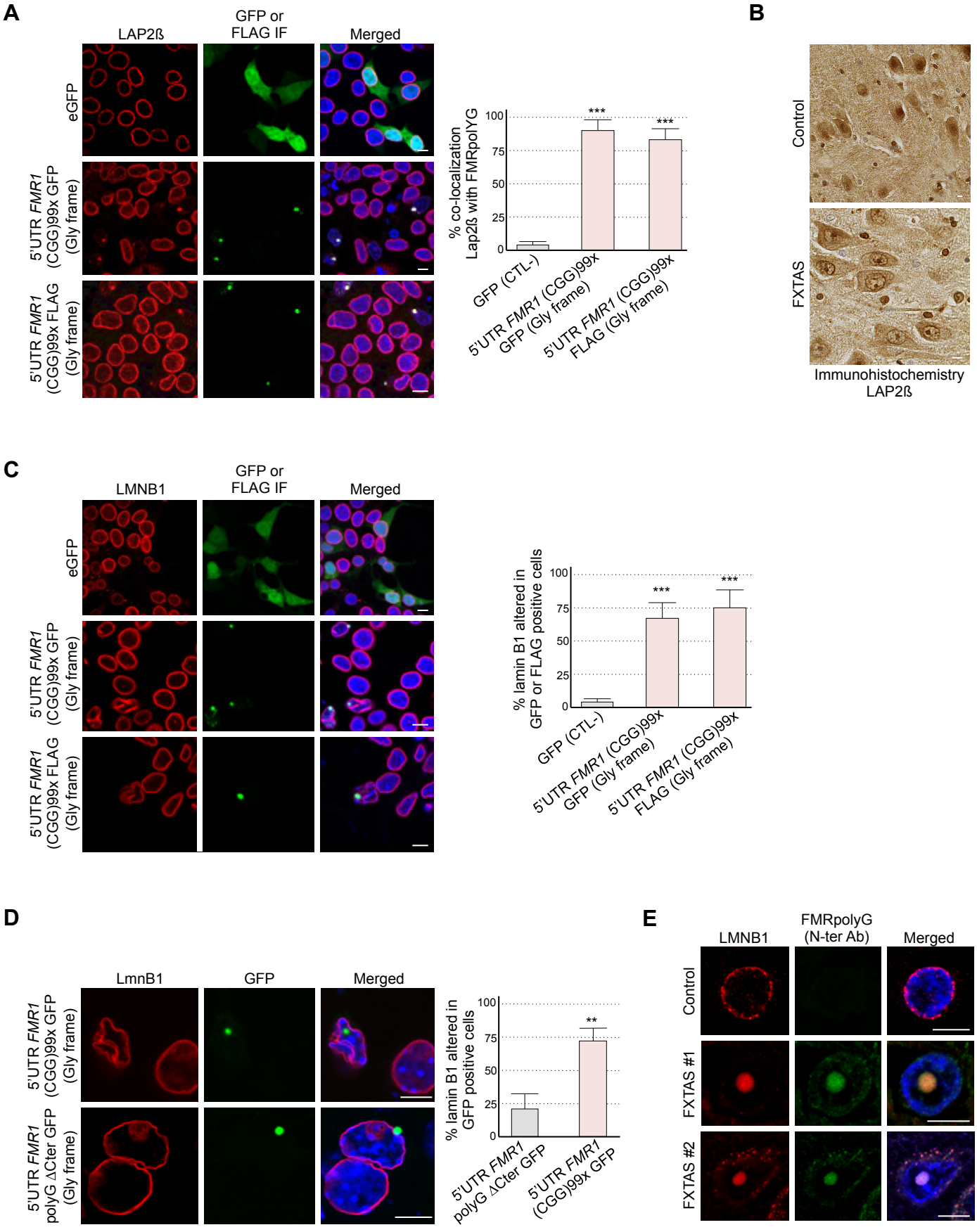

A
LAP2ß
GFP or FLAG IF
Merged
eGFP
5'UTR FMR1 (CGG)99x GFP (Gly frame)
5'UTR FMR1 (CGG)99x FLAG (Gly frame)
% co-localization Lap2ß with FMRpolyG
100
75
50
25
0
***
***
GFP (CTL-)
5'UTR FMR1 (CGG)99x GFP (Gly frame)
5'UTR FMR1 (CGG)99x FLAG (Gly frame)
B
Control
FXTAS
Immunohistochemistry LAP2ß
C
LMNB1
GFP or FLAG IF
Merged
eGFP
5'UTR FMR1 (CGG)99x GFP (Gly frame)
5'UTR FMR1 (CGG)99x FLAG (Gly frame)
% lamin B1 altered in GFP or FLAG positive cells
100
75
50
25
0
***
***
GFP (CTL-)
5'UTR FMR1 (CGG)99x GFP (Gly frame)
5'UTR FMR1 (CGG)99x FLAG (Gly frame)
D
LmnB1
GFP
Merged
5'UTR FMR1 (CGG)99x GFP (Gly frame)
5'UTR FMR1 polyG ΔCter GFP (Gly frame)
% lamin B1 altered in GFP positive cells
100
75
50
25
0
**
5'UTR FMR1 polyG ΔCter GFP
5'UTR FMR1 (CGG)99x GFP
E
LMNB1
FMRpolyG (N-ter Ab)
Merged
Control
FXTAS #1
FXTAS #2

Figure S6 (related to figure 6).
FMRpolyG alters LAP2ß and lamin B1 nuclear organization.

(**A**) Left panel, GFP fluorescence and immunofluorescence against the FLAG tag (green) and LAP2ß (red) in HEK293 cells transfected for 24 hours with GFP as control or with expanded CGG repeats embedded within the 5'UTR of FMR1 and fused either to the GFP or to a FLAG tag in the glycine frame. Right panel, quantification of LAP2ß co-localization with FMRpolyG (n=50 cells, 3 independent transfections).

(**B**) Immunohistochemistry against LAP2ß of hippocampal areas of FXTAS individual or age-matched control. Sections were counterstained with H&E staining.

(**C**) Left panel, GFP fluorescence and immunofluorescence against the FLAG tag (green) and lamin B1 (LMNB1, red) in HEK293 cells transfected for 24 hours with GFP as control or with expanded CGG repeats embedded within the 5'UTR of FMR1 and fused either to the GFP or to a FLAG tag in the glycine frame. Right panel, quantification of lamin B1 alterations in FMRpolyG positive cells (n=50 cells, 3 independent transfections).

(**D**) Left panel, GFP fluorescence and immunofluorescence against lamin B1 (LmnB1, red) in primary cultures of E18 mouse cortical neurons transfected with expanded CGG repeats embedded within the 5'UTR of FMR1 full-length or deleted of FMRpolyG C-terminus and fused to the GFP in the glycine frame. Right panel, quantification of lamin B1 alteration in GFP-positive transfected neurons (n=100 neurons, 3 independent transfections).

(**E**) Immunofluorescence against the N-terminal part of FMRpolyG (green, 8FM antibody) and lamin B1 (LMNB1, red) on brain sections (hippocampal area) of FXTAS patients or age-matched control individual. Scale bars, 10 µm. Nuclei were counterstained with DAPI. Error bars indicate s.e.m. Student t-test, ** indicates $p<0.01$, *** indicates $p<0.001$.

Figure S7

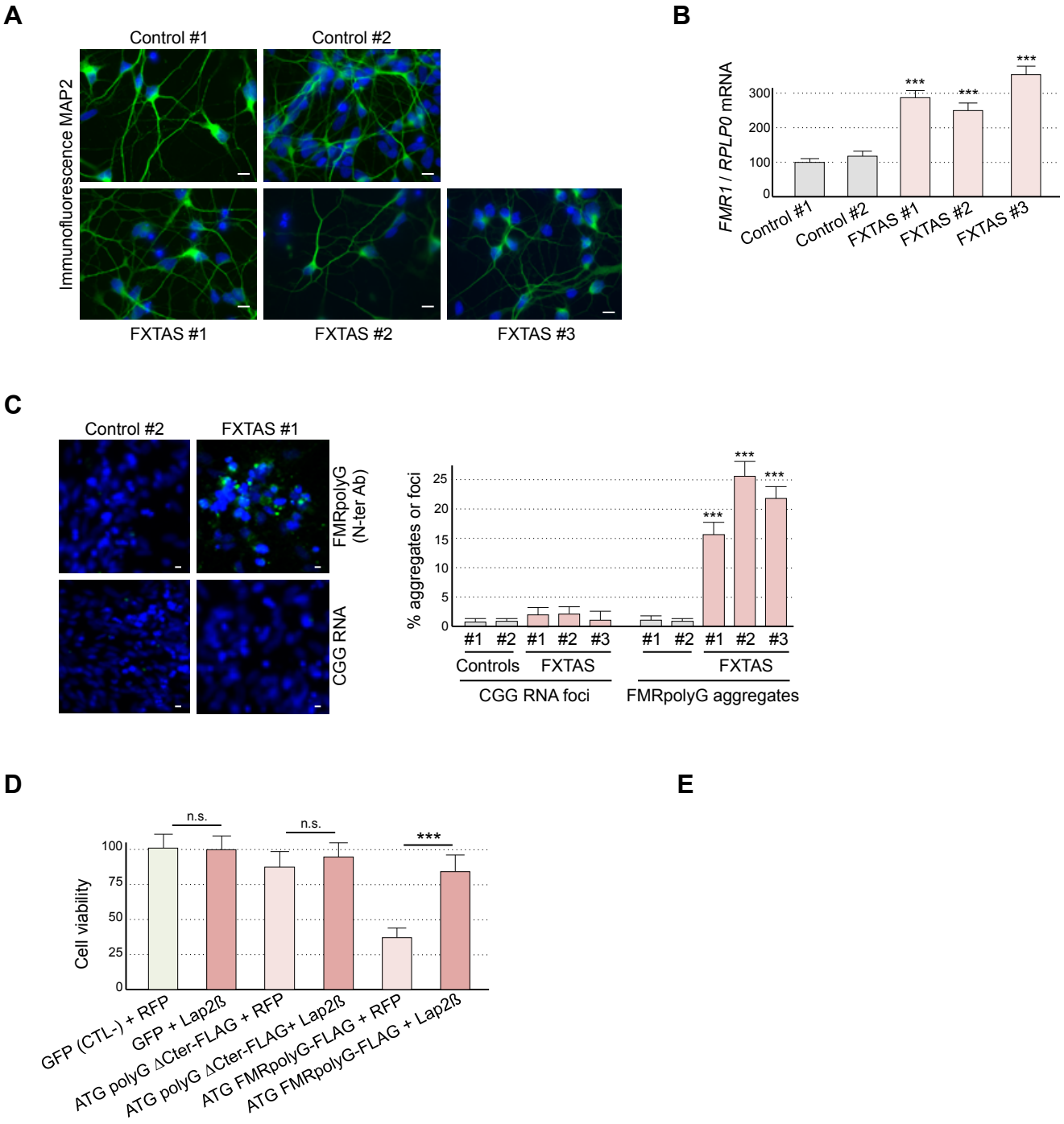

A
Control #1
Control #2
Immunofluorescence MAP2
FXTAS #1
FXTAS #2
FXTAS #3
B
FMR1 / RPLP0 mRNA
300
200
100
0
***
***
***
Control #1
Control #2
FXTAS #1
FXTAS #2
FXTAS #3
C
Control #2
FXTAS #1
FMRpolyG (N-ter Ab)
CGG RNA
% aggregates or foci
25
20
15
10
5
0
***
***
***
#1 #2 #1 #2 #3 #1 #2 #1 #2 #3
Controls
FXTAS
FXTAS
CGG RNA foci
FMRpolyG aggregates
D
Cell viability
100
75
50
25
0
n.s.
n.s.
***
GFP (CTL-) + RFP
GFP + Lap2ß
ATG polyG ΔCter-FLAG + RFP
ATG polyG ΔCter-FLAG+ Lap2ß
ATG FMRpolyG-FLAG + RFP
ATG FMRpolyG-FLAG + Lap2ß
E

Figure S7 (related to figure 7).
Neurons from iPS cell of FXTAS express FMRpolyG.

(**A**) Immunofluorescence against MAP2 on neuronal cultures differentiated 40 days from iPS cells of FXTAS or control individuals.

(**B**) *FMR1* mRNA expression relative to RPLP0 in neuronal cultures (n=3 independent cultures) differentiated 40 days from iPS cells of FXTAS or control individuals.

(**C**) Left panel, RNA FISH against expanded CGG repeats (red) coupled to immunofluorescence against FMRpolyG N-terminus (8FM antibody, green) in neurons differentiated 40 days from iPS cells originating from FXTAS or control individuals. Right panel, quantification of CGG nuclear RNA foci and FMRpolyG nuclear aggregates in neurons differentiated 40 days from iPS cells originating from FXTAS or control individuals (n=100 neurons, 3 independent cell cultures).

(**D**) Cell viability of neuronal N2A cells transfected with ATG-driven FMRpolyG-FLAG full-length or deleted of its C-terminus and with a plasmid expressing RFP as control or Ha-tagged LAP2ß (n=3 transfections). Scale bars, 10 µm. Nuclei were counterstained with DAPI. Error bars indicate s.e.m. Student t-test, *** indicates $p<0.001$.

Table S1

Accession	Description	Score	Coverage	# Peptides	# PSM	# AAs	MW [kDa]
Q61029-3	Lamina-associated polypeptide 2, isoforms beta/delta/epsilon/gamma OS=Mus musculus GN=Tmpo - [LAP2B_MOUSE]	48	16	13	15	412	46
P57780	Alpha-actinin-4 OS=Mus musculus GN=Actn4 PE=1 SV=1 - [ACTN4_MOUSE]	23	7	5	7	912	105
Q9CPW4	Actin-related protein 2/3 complex subunit 5 OS=Mus musculus GN=Arpc5 PE=2 SV=3 - [ARPC5_MOUSE]	20	31	3	4	151	16
Q5SW19-2	Isoform 2 of Clustered mitochondria protein homolog OS=Mus musculus GN=Cluh - [CLU_MOUSE]	17	8	5	5	818	92
Q61033	Lamina-associated polypeptide 2, isoforms alpha/zeta OS=Mus musculus GN=Tmpo PE=1 SV=4 - [LAP2A_MOUSE]	15	7	4	5	693	75
Q9WUI1	Mitogen-activated protein kinase 11 OS=Mus musculus GN=Mapk11 PE=1 SV=2 - [MK11_MOUSE]	15	10	3	5	364	41
Q9QUR6	Prolyl endopeptidase OS=Mus musculus GN=Prep PE=2 SV=1 - [PPCE_MOUSE]	7	4	2	2	710	81
P61290	Proteasome activator complex subunit 3 OS=Mus musculus GN=Psme3 PE=1 SV=1 - [PSME3_MOUSE]	7	4	1	2	254	29
Q08189	Protein-glutamine gamma-glutamyltransferase E OS=Mus musculus GN=Tgm3 PE=1 SV=2 - [TGM3_MOUSE]	7	2	1	2	693	77
O70250	Phosphoglycerate mutase 2 OS=Mus musculus GN=Pgam2 PE=2 SV=3 - [PGAM2_MOUSE]	6	10	2	2	253	29
P08032	Spectrin alpha chain, erythrocytic 1 OS=Mus musculus GN=Spta1 PE=2 SV=3 - [SPTA1_MOUSE]	6	1	2	2	2415	280
P08752	Guanine nucleotide-binding protein G(i) subunit alpha-2 OS=Mus musculus GN=Gnai2 PE=1 SV=5 - [GNAI2_MOUSE]	6	7	2	2	355	40
Q9R0Q6	Actin-related protein 2/3 complex subunit 1A OS=Mus musculus GN=Arpc1a PE=1 SV=1 - [ARC1A_MOUSE]	6	7	2	2	370	42
Q922D4-2	Serine/threonine-protein phosphatase 6 regulatory subunit 3 OS=Mus musculus GN=Ppp6r3 - [PP6R3_MOUSE]	6	2	1	2	827	93
Q8C6G8	WD repeat-containing protein 26 OS=Mus musculus GN=Wdr26 PE=2 SV=3 - [WDR26_MOUSE]	6	4	2	2	641	70
Q4KML4	Costars family protein ABRACL OS=Mus musculus GN=Abracl PE=3 SV=1 - [ABRAL_MOUSE]	6	16	1	2	81	9
P10853	Histone H2B type 1-F/J/L OS=Mus musculus GN=Hist1h2bf PE=1 SV=2 - [H2B1F_MOUSE]	5	7	1	2	126	14
Q8C0D7-4	Isoform 4 of Inhibitor of growth protein 4 OS=Mus musculus GN=Ing4 - [ING4_MOUSE]	5	8	1	2	166	19
P0C0S6	Histone H2A.Z OS=Mus musculus GN=H2afz PE=1 SV=2 - [H2AZ_MOUSE]	5	7	1	2	128	14
Q9D7X8	Gamma-glutamylcyclotransferase OS=Mus musculus GN=Ggct PE=1 SV=1 - [GGCT_MOUSE]	5	6	1	2	188	21
P04117	Fatty acid-binding protein, adipocyte OS=Mus musculus GN=Fabp4 PE=1 SV=3 - [FABP4_MOUSE]	4	9	1	1	132	15
Q8VBT0	Thioredoxin-related transmembrane protein 1 OS=Mus musculus GN=Tmx1 PE=1 SV=1 - [TMX1_MOUSE]	4	4	1	1	278	31
P15532	Nucleoside diphosphate kinase A OS=Mus musculus GN=Nme1 PE=1 SV=1 - [NDKA_MOUSE]	3	11	1	1	152	17
Q8BQP8	Rab11 family-interacting protein 4 OS=Mus musculus GN=Rab11fip4 PE=1 SV=1 - [RFIP4_MOUSE]	3	2	1	1	635	72
Q8BH74	Nuclear pore complex protein Nup107 OS=Mus musculus GN=Nup107 PE=2 SV=1 - [NU107_MOUSE]	3	1	1	1	926	107
O70456	14-3-3 protein sigma OS=Mus musculus GN=Sfn PE=1 SV=2 - [1433S_MOUSE]	3	4	1	1	248	28
O70435	Proteasome subunit alpha type-3 OS=Mus musculus GN=Psma3 PE=1 SV=3 - [PSA3_MOUSE]	3	5	1	1	255	28
O70166	Stathmin-3 OS=Mus musculus GN=Stmn3 PE=1 SV=1 - [STMN3_MOUSE]	3	9	1	1	180	21
Q9JM76	Actin-related protein 2/3 complex subunit 3 OS=Mus musculus GN=Arpc3 PE=1 SV=3 - [ARPC3_MOUSE]	3	6	1	1	178	21
P41969	ETS domain-containing protein Elk-1 OS=Mus musculus GN=Elk1 PE=2 SV=3 - [ELK1_MOUSE]	3	4	1	1	429	45
Q07643	Collagen alpha-2(IX) chain OS=Mus musculus GN=Col9a2 PE=2 SV=1 - [CO9A2_MOUSE]	3	2	1	1	688	65
P68510	14-3-3 protein eta OS=Mus musculus GN=Ywhah PE=1 SV=2 - [1433F_MOUSE]	3	6	1	1	246	28
O89094-2	Isoform 2 of Caspase-14 OS=Mus musculus GN=Casp14 - [CASPE_MOUSE]	3	5	1	1	159	18
Q61176	Arginase-1 OS=Mus musculus GN=Arg1 PE=1 SV=1 - [ARGI1_MOUSE]	3	3	1	1	323	35
Q91UZ4	Egl nine homolog 3 OS=Mus musculus GN=Egln3 PE=2 SV=1 - [EGLN3_MOUSE]	3	5	1	1	239	27
P97372	Proteasome activator complex subunit 2 OS=Mus musculus GN=Psme2 PE=2 SV=4 - [PSME2_MOUSE]	3	4	1	1	239	27
Q61941	NAD(P) transhydrogenase, mitochondrial OS=Mus musculus GN=Nnt PE=1 SV=2 - [NNTM_MOUSE]	3	1	1	1	1086	114
P50580-2	Isoform 2 of Proliferation-associated protein 2G4 OS=Mus musculus GN=Pa2g4 - [PA2G4_MOUSE]	3	3	1	1	340	38
Q64331	Unconventional myosin-VI OS=Mus musculus GN=Myo6 PE=1 SV=1 - [MYO6_MOUSE]	3	1	1	1	1265	146
Q91WD9	Secretagogin OS=Mus musculus GN=Scgn PE=2 SV=1 - [SEGN_MOUSE]	3	7	1	1	276	32
P31695	Neurogenic locus notch homolog protein 4 OS=Mus musculus GN=Notch4 PE=1 SV=2 - [NOTC4_MOUSE]	3	1	1	1	1964	207
O54818	Tumor protein D53 OS=Mus musculus GN=Tpd52l1 PE=2 SV=1 - [TPD53_MOUSE]	3	7	1	1	204	23
O35685	Nuclear migration protein nudC OS=Mus musculus GN=Nudc PE=1 SV=1 - [NUDC_MOUSE]	3	4	1	1	332	38
Q9CWP6-2	Isoform 2 of Motile sperm domain-containing protein 2 OS=Mus musculus GN=Mospd2 - [MSPD2_MOUSE]	3	3	1	1	481	55
Q7TSH3	Zinc finger protein 516 OS=Mus musculus GN=Znf516 PE=1 SV=1 - [ZN516_MOUSE]	3	2	1	1	1157	125
P30658	Chromobox protein homolog 2 OS=Mus musculus GN=Cbx2 PE=1 SV=2 - [CBX2_MOUSE]	3	3	1	1	519	55
P62305	Small nuclear ribonucleoprotein E OS=Mus musculus GN=Snrpe PE=3 SV=1 - [RUXE_MOUSE]	3	13	1	1	92	11
Q9WTX8	Mitotic spindle assembly checkpoint protein MAD1 OS=Mus musculus GN=Mad1l1 PE=2 SV=1 - [MD1L1_MOUSE]	3	2	1	1	717	83
Q791T5-2	Isoform 2 of Mitochondrial carrier homolog 1 OS=Mus musculus GN=Mtch1 - [MTCH1_MOUSE]	3	5	1	1	372	40
Q9CXF0	Kynureninase OS=Mus musculus GN=Kynu PE=2 SV=3 - [KYNU_MOUSE]	3	3	1	1	464	52
Q60770	Syntaxin-binding protein 3 OS=Mus musculus GN=Stxbp3 PE=1 SV=1 - [STXB3_MOUSE]	3	2	1	1	592	68
Q61687	Transcriptional regulator ATRX OS=Mus musculus GN=Atrx PE=1 SV=3 - [ATRX_MOUSE]	3	1	1	1	2476	278
Q8BKT8-2	Isoform 2 of HAUS augmin-like complex subunit 7 OS=Mus musculus GN=Haus7 - [HAUS7_MOUSE]	3	4	1	1	248	28
Q62407-2	Isoform 2 of Striated muscle-specific serine/threonine-protein kinase OS=Mus musculus GN=Speg - [SPEG_MOUSE]	3	2	1	1	861	94
O88508	DNA (cytosine-5)-methyltransferase 3A OS=Mus musculus GN=Dnmt3a PE=1 SV=2 - [DNM3A_MOUSE]	3	1	1	1	908	102
O54774	AP-3 complex subunit delta-1 OS=Mus musculus GN=Ap3d1 PE=1 SV=1 - [AP3D1_MOUSE]	3	1	1	1	1199	135
Q9WUM3	Coronin-1B OS=Mus musculus GN=Coro1b PE=1 SV=1 - [COR1B_MOUSE]	3	2	1	1	484	54
Q8BQM8	Echinoderm microtubule-associated protein-like 5 OS=Mus musculus GN=Eml5 PE=2 SV=2 - [EMAL5_MOUSE]	3	1	1	1	1977	220
Q9JIT1	RING finger protein 32 OS=Mus musculus GN=Rnf32 PE=2 SV=1 - [RNF32_MOUSE]	3	4	1	1	368	42
Q925I7-2	Isoform 2 of Platelet-derived growth factor D OS=Mus musculus GN=Pdgfd - [PDGFD_MOUSE]	3	3	1	1	364	42
Q5DU28-3	Isoform 3 of Pecanex-like protein 2 OS=Mus musculus GN=Pcnxl2 - [PCX2_MOUSE]	3	1	1	1	1121	121
P28741	Kinesin-like protein KIF3A OS=Mus musculus GN=Kif3a PE=1 SV=2 - [KIF3A_MOUSE]	3	1	1	1	701	80
Q99PL5-5	Isoform RRp16.8 of Ribosome-binding protein 1 OS=Mus musculus GN=Rrbp1 - [RRBP1_MOUSE]	3	3	1	1	398	42
Q6NZL8-3	Signal peptide, CUB and EGF-like domain-containing protein 1 OS=Mus musculus GN=Scube1 - [SCUB1_MOUSE]	3	1	1	1	961	104
A2AM05-2	Isoform 2 of Centlein OS=Mus musculus GN=Cntln - [CNTLN_MOUSE]	3	3	1	1	318	36
Q9D952	Envoplakin OS=Mus musculus GN=Evpl PE=2 SV=3 - [EVPL_MOUSE]	3	0	1	1	2035	232

Table S1 (related to figure 6).
Proteins interacting with FMRpolyG.

Proteins associated with HA-FLAG-tagged FMRpolyG expressed in mouse N2A neuronal cells were captured through consecutive anti-FLAG and anti-HA affinity purification steps and identified by orbitrap ion trap mass analyzer.

Supplemental videos 1 and 2 (related to figure 3).
Mice expressing FMRpolyG present gait instability.

Video recording of ledge test of 3 months old bigenic CMV-cre/full-length or mutant FMR1 5'UTR transgenic mice indicates that FMRpolyG-expressing mice present some gait instability and increase foot slippage compared to CGG RNA only-expressing mice.

Supplemental videos 3 to 6 (related to figure 5).
Time laps recoding of neurons expressing FMRpolyG-GFP.

24 hours recording of primary cultures of E18 mouse cortical neurons transfected with RFP and with control GFP or FMRpolyG-GFP full-length or deleted of its N- or C-terminus. Expression of either FMRpolyG-GFP or its C-terminus fused to the GFP leads to neuronal cell death. In contrast, expression of the polyglycine stretch deleted of FMRpolyG C-terminal part leads to aggregates formation with no overt cell toxicity.

Supplemental Experimental Procedures

Fibroblasts were successfully reprogrammed using retroviruses expressing Oct4, Sox2, Nanog and Lin28 (Jung et al., 2014). Briefly, primary dermal fibroblasts were maintained on gelatin-coated dishes in DMEM 1 g/l glucose with antibiotic, antimycotic and 0.1 mM non-essential amino acids (Invitrogen) and 10% FBS for 5 passages. On day 1, 1x10exp5 fibroblasts were transduced by lentivirus carrying cDNAs of Oct4, Sox2, Nanog and Lin28 with 8 mg/ml of polybrene (Sigma). On day 2, medium was replaced with fresh medium and on day 3, infected cells were transferred onto a 100-mm dish containing 1x10exp6 feeder cells (passage 3 mitomycin-C treated mouse embryonic fibroblasts). From day 6 to 9, fibroblast medium was progressively switched to human induced pluripotent stem cell medium (KO-DMEM, 20% KOSR, 2 mM L-glutamine, 0.1 mM non-essential amino acids, Penicillin-Streptomycin, 0.1 mMb-mercaptoethanol supplemented with 10 ng/ml of bFGF (R&D Systems). Human iPSC clones were picked at week 4 and expanded on matrigel coated 35 mm dishes (BD Biosciences) in mTeSR1 medium (Stemcell Technologies). For embryoid body formation, hiPSC were dissociated with dispase solution (1 mg/ml, Stemcell Technologies), resuspended in 1 ml of Aggrewell medium (Stemcell Technologies) containing 2 mM Y27632 (Stemgent), centrifuged in Aggrewell plates for 3 min at 80g and further incubated at 37°C for 24 h. The next day, embryoid bodies were transferred in 3 ml of Aggrewell medium. The following days, medium was progressively switched to KO-DMEM, 20% FBS, 2 mM L-glutamine, 0.1 mM Non-Essential Amino Acids, penicillin–streptomycin and after 30 days, embryoid bodies were collected. Of interest, expanded CGG repeats were stable with no contraction or expansion in iPS clones compared to fibroblasts. karyotype analyses were normal for all cell lines and retroviral silencing as well as stemness and pluripotency were confirmed by classic RT-qPCR and teratoma assays. For karyotype analysis, hiPSC cells were treated with colchicine (Sigma) for 4 h and cells were shocked with hypotonic KCl 0.075 M solution for 20 min at 37°C. Cells were fixed in methanol:acetic acid solution (3:1) and conventional cytogenetic was performed applying RHG banding on metaphase chromosomes. Expression of pluripotent surface markers of hIPSC was analyzed by FACS using anti-Tra-I-60, anti-Tra-I-81 and anti-SSEA4 antibodies (Millipore). Expression of 90 validated genes associated with stem cell pluripotency and differentiation to all three germ layers of hiPSC and corresponding embryoid bodies were analyzed using the Human Stem Cell Pluripotency Array (Applied Biosystems) according to manufacturer instructions. For in vivo teratoma formation, cells from one Matrigel coated 60 mm-dish were collected by dispase treatment and resuspended in 75 ml of KO-DMEM, mixed with 75 ml of Matrigel (BD Biosciences) and the hiPSC-Matrigel mixture was injected subcutaneously in 8-week-old NOD/SCID female mice (Charles River Laboratory, 2 mices injected for each hiPSC clone). After two to three months, teratomas were dissected and fixed in formalin, embedded in paraffin and processed with hematoxylin and eosin staining at the histology laboratory of the Institute Clinique de la Souris (ICS).

Differentiation of iPSC in neurons was performed according Marteyn et al., 2011 and Boissart et al., 2013. Biefly, for differentiation in Neuronal Stem cell (NSC), one B6 dish of IPS (60-80 % confluence) was washed with NFS medium (N2B27 supplemented with FGF2 5 ng/ml (Peprotech, AF-100-18B), hNoggin 260 ng/ml (R&D120-10C), SB431542 8,7 µg/ml (Tocris, 1614) containing ROCK inhibitor 3,5 µg/ml (Y-27632, Calbiochem, 688000) before to cut clumps. Clumps were collected and incubated overnight at 37°C in B3 UltraLow Attachment Dish (Corning). The next day, the clumps were transferred to a dish pre-coated with poly-ornithine 0,1% (Sigma, p4957) and laminine at 1 mg/ml (Sigma, L2020) and maintained with medium. After 24 hours, the medium was changed to NFS medium without rock inhibitor and medium was changed every two days during 8-10 days. After the appearance of neural rosette, the medium was replaced with NSC (N2B27 supplemented with FGF2 10 ng/ml, EGF 10 ng/ml (R&D, 263-EG-200RD), hBDNF 20 ng/ml (R&D, 248-BD-025CF). At confluence, cells were passaged 1:3 in NSC medium. For differentiation of NSC in neurons, confluent cells were dissociated with trypsin and plated on pre-coated with polyornithine (Sigma) and laminin (Sigma) in 24-well plate (50000 cells/well) in neuron medium (N2B27 supplemented with hBDNF 20 ng/ml and laminine 2 µg/ml). Media change was performed every 2 days and cells were differentiated during 55 days.

Mouse models, genotyping and phenotyping.

All mouse procedures were done according protocols approved by the Committee on Animal Resources of the ICS animal facility and under the French and European authority guidelines.

For transgene construction, human 5'UTR FMR1 fused in the glycine frame to the GFP (Addgene) was cloned between the FseI and SmaI sites of the Rosa26 5' arm – CAG promoter – LOXP - SV40 polyA 3x - LOXP – Rosa26 3' arm vector Ai2 (Addgene). Deleted transgene for the ACG near-cognate codon was constructed by deleting 100 nucleotides from FseI to KasI sites within the FMR1 5'UTR plasmid. Both constructs are driven by the strong chimeric ubiquitous CAG promoter, and inserted by homologous recombination within the neutral Rosa26 mouse locus. To control expression of the transgenes, three upstream SV40-polyadeneylation sites bordered by loxP sites limit transcription of the expanded CGG repeats. Hence, expression and potential pathogenicity of the expanded CGG repeats is permitted only in offspring of the transgenic CGG mice crossed with mice expressing Cre recombinase.

Both mutant mouse lines were established at the ICS (Mouse Clinical Institute; http://www-mci.u-strasbg.fr). Both linearized constructs were electroporated separately in C57Bl/l6N mouse embryonic stem (ES) cells. After G418 selection, targeted clones were identified by long-range PCR using external primers and further confirmed by Southern blot with an internal probe against Neomycin and 5' external probe against Rosa26. We confirmed by southern blot and PCR the correct insertion of the transgenes into the Rosa26 locus, the absence of concatamerization at the locus and the presence of 99 CGG repeats, which were stably transmitted with no obvious contraction or expansion. Two positive ES clones for each future transgenic mice were injected into Balb/cN blastocysts. Resulting male chimeras were bred with wild type C57Bl/6N females to obtain germline transmission. Deletion of the floxed STOP cassette were performed by breeding F1 males with CMV-cre deleter females (Birling et al., 2012) or Nestin-cre delete mice (Isaka et al., 1999). Genotyping across the expanded CGG repeats was performed using the Expand High Fidelity PCR System (Roche, 11-732 -650 001) according manufacturer instructions and supplemented with 2,5 M Betaine (B0300 Sigma, 12,5 µl of 5 M Betaine for a final PCR volume of 25 µl) with one denaturation step at 94 °C for 2 min, 30 cycles of amplification 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min and a final step at 72 °C for 5 min using the forward primer 5'-TCGACCTGCAGCCCAAGCTAGATCG and the reverse primer 5'-TCCTTGAAGAAGATGGTGCGCTCC. Rotarod test (Bioseb, Chaville, France) was performed with three testing trials during which the rotation speed accelerated from 4 to 40 rpm in 5 min. Trials were separated by 5-10 min interval. The average latency was used as index of motor coordination performance. Grip test: this test measures the maximal muscle strength (g) using an isometric dynamometer connected to a grid (Bioseb). Mice were allowed to grip the grid with all its paws then they were pulled backwards until they released it. Each mouse was submitted to 3 consecutive trials immediately after the modified SHIRPA procedure. The maximal strength developed by the mouse before releasing the grid was recorded and the average value of the three trials adjusted to body weight. The string test consisted of 3 trials separated by 5-10 min interval. On each trial the forepaws of the animal were placed on the thread that is a wire stretched horizontally 40 cm above the bench. The latency the animal took to catch the wire with its hindpaws was recorded. Open field test: mice were tested in automated open fields (Panlab, Barcelona, Spain), each virtually divided into central and peripheral regions. The open fields were placed in a room homogeneously illuminated at 150 Lux. Each mouse was placed in the periphery of the open field and allowed to explore freely the apparatus for 30 min, with the experimenter out of the animal's sight. The distance traveled, the number of rears, and time spent in the central and peripheral regions were recorded over the test session. The number of entries and the percent time spent in center area are used as index of emotionality/anxiety

***Drosophila* models of FMRpolyG.**

All Drosophila lines were maintained on standard culture and food conditions at 25oC, while all crosses and experiments were performed at 29oC. Control and driver lines used in this study are w1118 (control) from Bloomington, Actin5C-GAL4/CyO driver (ubiquitous driver line) as a gift from Zhe Han's lab, and RU486-inducible Geneswitch tubulin driver line Tub5-GAL4 (ubiquitous expression) as a gift from Scott Pletcher's lab. DNA fragments containing FMRpolyG-GFP or polyG-GFP without the C-terminal sequence were PCR amplified from counterpart of mammalian transfection vectors described elsewhere and inserted to a pUAST vector between EcoRI and XbaI sites. All constructs were sequence verified by Sanger sequencing and transgenic flies with these constructs were made via standard p-element insertion (BestGene, CA). Transgene expression levels of GFP gene were analyzed 3 days after induction with RU486 in flies from the individual lines crossed to the Tub5-GAL4 driver, and those with comparable RNA expression levels were used for this study. The fly eclosion assay has been described elsewhere (Todd et al, 2013). Briefly, homozygous UAS transgenic lines or control lines were crossed to a Actin5C-GAL4 ubiquitous driver line balanced over a marker chromosome (CyO), on standard food at 29oC, if the transgene elicited no toxicity, then the number of progeny bearing the GAL4 driver would be expected to be equivalent to those bearing the CyO marker. Over 100 flies of each genotype were scored from multiple crosses. The ratio of expected progeny carrying the transgene compared to those carrying the CyO marker was expressed as a percentage of the expected ratio of one. These percentages were then compared using a Fischer exact test to determine statistical significance. For the fly survival assay, The UAS transgenic lines or control lines were crossed to Tub5-Gal4 geneswitch driver flies on standard food absent of RU486 at 29oC. Adult offspring of the desired genotypes were collected 2-3 days after eclosion and transferred to standard fly food containing 200 µM RU486 without yeast granules. The flies were transferred to fresh food with drug every 2-3 days. Each genotype started with at least 4 vials of 25 flies/vial and the survival was determined daily or every other day for 3 weeks.

Monoclonal antibody production.

To generate monoclonal antibodies directed against FMRpolyG or FMRpolyA, 8 week old female BALB/c mice were injected intraperitoneally with KLH conjugated peptides (FMRpolyA C-ter 5FM: PRAPAAHLSGAGSRR, FMRpolyG N-ter 8FM: MEAPLPGGVRQRG or FMRpolyG C-ter 9FM: GGWASSARSPPLGGGLPALA) with 200ug of poly(I/C) as adjuvant. Three injections were performed at 2 weeks intervals and four days prior to hybridoma fusion, mice with positively reacting sera were re-injected. Spleen cells were fused with Sp2/0.Agl4 myeloma cells. Supernatants of hybridoma cultures were tested at day 10 by ELISA for cross-reaction with peptides. Positive supernatants were then tested by Immunofluorescence and western blot on transfected HeLa cells. Specific cultures were cloned twice on soft agar. Specific hybridomas were established and ascites fluid was prepared by injection of 2x106 hybridoma cells into Freund adjuvant-primed BALB/c mice. All animal experimental procedures were performed according to the French and European authority guidelines.

Cell cultures, viability assays and transfections.

Neuro2A, HEK293 or HeLa cells were plated in 6 well tissue culture plates in DMEM 1 g/l glucose with 5% FCS and gentamycin. After 24 hours, cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) according to manufacturer instructions. Primary cortical neurons were prepared from C57Bl/6 mice embryos of day E18 and grown on polylysine coated 24-well plates in Neurobasal Medium (NBM) supplemented with 1xB27, 0.5 mM L-glutamine and 100 IU/ml penicillin/streptomycin at 37° C with 5% CO2. Neurons were transfected at day 3 with Lipofectamine 2000 (Invitrogen) in 400 µl NBM. Medium was replaced after 3h with a 1:1 (v:v) mixture of conditioned and fresh NBM. For cell viability, cells were detached by scraping and resuspended in PBS. TO-PRO-3 iodide (Fisher scientific, T-3605) was added at 20 nM to each sample and gently mix just prior to analysis on the FACS and 30 000 cells were counted.

Western blotting.
For the small FMRpolyG-FLAG tagged or endogenous FMRpolyG (<15kDa), 20 to 50 µg of proteins were resolved by 12% bis-Tris Gel (NuPAGE) and transferred onto PVDF 0,2 µm membrane. The membrane was blocked with 5% non-fat dry milk in TBS-Tween 1% and incubated with FLAG (rabbit PA1-984B), 8FM or 9FM antibody (1:100) overnight at 4°C. Membrane was washed 3 times and incubated with secondary peroxidase antibody (1:3000, Cell Signaling) 1 hour in TBS-Tween 1%, followed by washing and ECL chemoluminescence revelation (Amersham ECL Prime). Concerning FMRpolyG-GFP tagged (>30kDa) and other large proteins analyzes, 20 µg of proteins were homogenized in 1x laemmli sample loading buffer, denatured 3 min at 95°C, separated on 4-12% bis-Tris Gel (NuPAGE), transferred on nitrocellulose membranes (Whatman Protan), blocked with 5% non-fat dry milk in TBS-Tween 1% (Tris Buffer Saline buffer), incubated with anti-FMRpolyG (8FM or 9 FM, 1/100), Lap2b (BD Biosciences 611000), GFP (Abcam ab290), GAPDH (ab125247, Abcam), HA (ThermoFisher Scientific 26183) in TBS-Tween 1%, washed 3 times and incubated with anti-rabbit or mouse Peroxidase antibody (1:3000, Cell Signaling) 1 hour in TBS-Tween 1%, followed by washing and ECL chemoluminescence revelation (Amersham ECL Prime). Concerning human brain tissue preparation, small pieces of lyophilized frozen brain tissue were homogenized in 100 µl of Tris-SDS buffer (100 mM Tris pH 9, 5 % SDS 20%, 5 % β-mercaptoethanol), boiled at 100°C during 5 min then centrifuged at 13 000 rpm for 20 min at 4 °C. The supernatant was removed. The pellet was washed twice with water and homogenized in 20 µl of formic acid and incubated at 37°C during 30 min. Next, the homogenate was dried in speed-vac and resuspended in 40 µl Laemmli loading buffer prior to western blot analysis.

Lysostaphin treatment
3x10exp5 transfected HeLa cells were scrapped in PBS 1X and centrifuged during 10 min at 3000 rpm at 4°C. The pellet was resuspended in 800 µl of RIPA. 16 µl of cell extract was incubated with 1 µg of lysostaphin (Prospec, ENZ-269) during 10 to 30 minutes at 37°C. Laemmli buffer was add to the mix and proteins were analyze by western blot.

Immunofluorescence and immunohistochemistry.
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. Glass coverslips containing plated cells or brain sections treated as described above were fixed in PFA during 10 min 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 incubation during 1 hours with primary antibody against FMRpolyA (5FM, 1/100), FMRpolyG (8FM or 9FM, 1/50 to 1/100), ubiquitin (DAKO, Z0458), GFP (Abcam ab1218), Lap2 (Millipore 06-1002; Abcam ab185718; Abcam ab189993), Lamin B1 (Abcam ab16048). Slides or coverslips were washed twice with PBS before incubation with a goat anti-rabbit or goat anti-mouse secondary antibody conjugated with Cyanine 3 (1/500 dilution; Fisher) for 60 min; incubated for 2 min in PBS 1X-DAPI (1/10 000 dilution) and rinsed twice with PBS 1X before mounting in Pro-Long media (Molecular Probes). Slides were examined using a fluorescence microscope (Leica). For immunochemistry, brain sections were deparaffinized followed by antigen retrieval using microwave treatment in 0.01 m sodium citrate and treatment with 10 µg/ml protein kinase for 20 min at 37°c. Endogenous peroxidase activity was blocked, and immunostaining was performed overnight at 4°C using antibody against Iba1 (Abcam ab15690), Gfap (Abcam ab7260), ubiquitin (Dako Z0458; 1:250), FMRpolyG (8FM or 9FM, 1:10 to 1/50) or Lap2 (Millipore 06-1002; Abcam ab185718; Abcam ab189993). Antigen–antibody complexes were visualized by incubation with DAB substrate (Dako) and slides were counterstained with hematoxylin and eosin.

RNA FISH coupled to immunofluorescence.
Mouse brain sections were deparaffinized and rehydrated. Coverslips containing primary culture of E18 mouse cortical neurons cells or brain sections were fixed in PFA during 10 min 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 pre-hybridization in 40% DMSO, 40% formamide, 10% BSA (10 mg/ml), 2 × SCC for 30 min. The coverslips or slides were hybridized for 2 h in 40% formamide, 10% DMSO, 2 × SCC, 2 mM vanadyl ribonucleoside, 60 µg/ml tRNA, 30 µg/ml BSA plus 0.75 µg (CCG)8x-Cy3 DNA oligonucleotide probe (Sigma). The coverslips or slides were washed twice in 2 × SCC/50% formamide and twice in 2 × SCC. The coverslips were incubated for 2 min in 2 × SCC/DAPI (1/10 000 dilution) and rinsed twice in 2 × SSC before mounting in Pro-Long media (Molecular Probes). Coverslips were examined using a fluorescence microscope (Leica). For FISH followed by immunofluorescence, after 2 × SCC wash, the slide were washed twice in PBS 1X. The slides were incubated 1 hour with primary antibody against FMRpolyGly antibody (8FM, 1/50). Slides were washed twice with PBS before incubation with a goat anti-mouse secondary antibody conjugated with cyanine-3 (1/500 dilution; Fisher) for 60 min; incubated for 2 min in PBS 1X-DAPI (1/10 000 dilution) and rinsed twice in PBS 1X before mounting in Pro-Long media (Molecular Probes). Slides were examined using a fluorescence microscope (Leica).

Mass spectrometry analysis of FMRpolyG interactant and FMRpolyG N-terminus.
5x10exp6 HeLa cell were transfected with 18 µg of HA-FLAG tagged plasmid using Fugen HD (Promega) for 24h hours. Proteins were purified by HA-FLAG tandem purification kit according to the manufacturer's instruction (Sigma-Aldrich), separated on 4-12% bis-Tris Gel (NuPAGE) and visualized by silver staining (SilverQuest, Invitrogen). Gel bands were excised and subjected to manual in-gel reduction in 10 mM DTT in 100 mM NH4HCO3 (Sigma Aldrich) for 1 h at 57°C, alkylated for 45 min in the dark with 55 mM iodoacetamide in 100 mM NH4HCO3 (Sigma Aldrich), washed in 25 mM NH4HCO3, dehydrated with acetonitrile and dried in SpeedVac 5301 Concentrator (Eppendorf). Then the gel pieces were rehydrated with 12.5 ng/µL trypsin or LysC solution (Promega) in 50 mM NH4HCO3 and incubated overnight at 37°C. The peptides were extracted twice with acetonitrile/water/formic acid-45/45/10-v/v/v followed by a final extraction with acetonitrile /formic acid (FA)-95/05-v/v. Extracted peptides were then analyzed using an Ultimate 3000 nano-RSLC (Thermo Scientific) coupled in line with an Orbitrap ELITE (Thermo Scientific). Peptides were separated on a C18 nano-column with a linear gradient of acetonitrile and analyzed with in a Top 20 CID (Collision-induced dissociation) data-dependent mass spectrometry with an inclusion list. Data were processed by database searching using SequestHT (Thermo Fisher Scientific) with Proteome Discoverer 1.4 software (Thermo Fisher Scientific) against a homemade database of all potential three frames translated proteins or peptides from the 5'UTR of FMR1. Precursor and fragment mass tolerance were set at 7 ppm and 0.5 Da respectively. Oxidation (M) and Nterminal Acetylation were set as variable modification, and Carbamidomethylation (C) as fixed modification. Peptides were filtered with the Fixed value node of Proteome Discoverer 1.4. Similarly, for identification of protein interactants, 5x10exp6 Neuro2A cell were transfected with 15 µg of FMRpolyG FLAG-HA double-tagged plasmid using Fugen HD (Promega) for 24h hours. Proteins were purified by HA-FLAG tandem purification kit according to the manufacturer's instruction (Sigma-Aldrich). Proteins were visualized by silver staining (SilverQuest, Invitrogen) after separation of 4-12% bis-Tris Gel (NuPAGE) and identified using NanoESI_Ion Trap (LTQ XL Thermo Fisher).

Constructs.
PCMV6 containing C-terminally FLAG-tagged human cDNAs of LAP2ß was purchased from OriGene. Plasmids containing the 5'UTR of human FMR1 fused in the glycine frame with the FLAG tag or GFP are deposited at Addgene (Plasmid #63089, #63090 and #63091). The FLAG tag is fused to the C-terminal end of FMRpolyG with a two amino acids (glycine valine) linker, and the GFP tag is fused to the C-terminus of FMRpolyG with one amino acid (glycine) linker. Mutations of the ACG into ATG or deletions of the 5'UTR of FMR1 were achieved by oligonucleotide ligations. To insure stability of the expanded CGG repeats, all CGG plasmids were transformed into STBL3 bacterial strain (Invitrogen) and growth at room temperature (22°c).

Quantitative real time RT-PCR.
Total RNAs from mouse tissues or cells were isolated by TriReagent (Molecular Research Center). cDNAs were generated using the Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics) for quantification of mRNAs. qPCR of mRNAs were realized using the LightCycler 480 SYBR Green I Master (Roche) in a Lightcycler 480 (Roche) with 15 min at 94°C followed by 50 cycles of 15 sec at 94°C, 20 sec at 58°C and 20 sec at 72°C. RPLPO mRNA was used as standard and data were analyzed using the Lightcycler 480 analysis software (2ΔCt method).

Subcellular fractionation and PCR.
Cells were scraped in PBS, cells were pelleted by centrifugation at 3000 rpm for 10 min minutes. The pellet was resuspended in Dautry Buffer (Tris HCl pH 7,8, 10 mM; NaCl 140 mM, MgCl2 1,5 mM; EDTA 10 mM, NP40 0,5%) and kept on ice 5 minutes. The homogenate was centrifuged at 3000 rpm for 15 minutes during 5 min at 4°C, pellet corresponding to nuclear fraction and supernatant to cytosolic fraction. Cytosolic fraction was centrifuged at 13000 rpm for 15 minutes to remove potential nucleus and 1 ml TriReagent (Molecular Research Center) was added. The pellet was washed with 400 µl of Dautry Buffer, centrifuged at 3000 rpm for 5 minutes at 4°C. Supernatant was removed and the pellet was homogenized in 400 µl of Dautry Buffer and 1 ml TriReagent (Molecular Research Center) was added. Total RNA from nuclear or cytosolic fraction was isolated as described in the manufacturer's protocol of TriReagent (Molecular Research Center). cDNAs were generated using the Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics) for quantification of mRNAs. PCR was performed with Taq polymerase (Roche), one denaturation step at 94 °C for 2 min, 25 cycles of amplification 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min and a final step at 72 °C for 5 min using the primer described below. The PCR products were precipitated, analyzed by electrophoresis on a 6.5% polyacrylamide gel, stained with ethidium bromide and quantified with a Typhoon scanner.

Oligonucleotides.

RPLO_FW	GAAGTCACTGTGCCAGCCCA
RPLO_REV	GAAGGTGTAATCCGTCTCCA
U6_FW	CTCGCTTCGGCAGCACATATA
U6_REV	GGAACGCTTCACGAATTTGCG
FMR1_FW	GAAAACAACTGGCAGCCTGA
FMR1_REV	AGCTAACCACCAACAGCAAG
GFP_FW	ACGTAAACGGCCACAAGTTC
GFP_REV	AAGTCGTGCTGCTTCATGTG
(CGG)60x_FW	GAACCCACTGCTTACTGGCTTA
(CGG)60x_REV	AACGCTAGCCAGTTGGGTC
Transgene mouse FMRpolyG_FW	GCAAGCTGACCCTGAAGTTC
Transgene mouse FMRpolyG_REV	GTCTTGTAGTTGCCGTCGTC

Supplemental references

Birling MC, Dierich A, Jacquot S, Hérault Y, Pavlovic G. (2012). Highly-efficient, fluorescent, locus directed cre and FlpO deleter mice on a pure C57BL/6N genetic background. Genesis. 50(6):482-9.

Boissart C, Poulet A, Georges P, Darville H, Julita E, Delorme R, Bourgeron T, Peschanski M, Benchoua A. (2013). Differentiation from human pluripotent stem cells of cortical neurons of the superficial layers amenable to psychiatric disease modeling and high-throughput drug screening. Transl Psychiatry. Aug 20;3:e294.

Jung L, Tropel P, Moal Y, Teletin M, Jeandidier E, Gayon R, Himmelspach C, Bello F, André C, Tosch A, et al. (2014). ONSL and OSKM cocktails act synergistically in reprogramming human somatic cells into induced pluripotent stem cells. Mol Hum Reprod. 20(6):538-49.

Isaka F, Ishibashi M, Taki W, Hashimoto N, Nakanishi S, Kageyama R. (1999). Ectopic expression of the bHLH gene Math1 disturbs neural development. Eur J Neurosci. 11:2582-8

Marteyn A, Maury Y, Gauthier MM, Lecuyer C, Vernet R, Denis JA, Pietu G, Peschanski M, Martinat C. (2011). Mutant human embryonic stem cells reveal neurite and synapse formation defects in type 1 myotonic dystrophy. Cell Stem Cell. 8(4):434-44.