SUPPLEMENTARY METHODS AND RESULTS

FMRP lysis buffer optimization

Initial lysis protocol – Frozen tissue powder was resuspended in RIPA lysis buffer (Boston Bioproducts, Ashland, MA), supplemented with Complete protease inhibitors (Roche Applied Science, Indianapolis, IN) at a final concentration of 1 mg tissue per 15 µl buffer. Cell lysis was allowed to proceed overnight at 4°C on an end-over-end rotator, then the lysates were cleared by centrifugation at 16,100 x g for 20 minutes at 4°C and supernatants were carefully removed and stored at -80°C. We determined the protein concentration of each sample using the BCA protein assay kit (Pierce, Rockford, IL), according to manufacturer's microplate instructions, and then separated 10 µg of each sample by electrophoresis.

Western blot complications – We noticed that, over time, mouse protein lysates became cloudy after freeze-thawing. While all of the FMRP Western blots had been performed with fresh frozen tissue, the large variation in FMRP levels led us to test whether FMRP was precipitating out of the lysis buffer. We therefore measured FMRP levels in the precipitation and soluble fractions of several lysates by pelleting at 16,100 x*g* for 15 min and resuspending the pellets in RIPA buffer at an equal volume as the supernatant. As can be seen in Supplementary Material, Fig. S6A, both FMRP and Gapdh were found in the pellet fractions.

We next tested the repeatability of our Western-blot method by measuring FMRP in a subset of mouse samples in 2 separate experiments, using fresh lysates from frozen brain samples each time. To account for experiment-to-experiment variation, the FMRP values were transformed into Z-scores and graphed as a Z-Z plot (Supplementary Material, Fig. S1A). The Z-score is a standard measure, representing an individual value's departure from the group mean (1). The Z-score of each FMRP measurement in a single experiment is obtained by the following formula:

$$\text{Z-score} = (\text{FMRP level}_{M} - \text{mean FMRP level}_{M1\ldots Mn})/\text{Standard Deviation}_{M1\ldots Mn}$$

where M refers to an individual mouse, and M1-Mn represents the entire group. As a result of Z-score transformation, the group's mean is 0, with the average standard deviation set to 1, and samples values are plotted as units of standard deviation away from the mean. Regardless of inter-experimental variations, a sample should maintain its *relative* value compared to the rest of the group; a perfect Z-Z plot has a regression line of y=x. It is clear from Supplementary Material, Fig. S1A that FMRP measurements were not consistent between replicate measurements; samples that had low FMRP expression in one experiment had a range of expression, from high to low FMRP levels, in the replicate experiment ($R^2 = 0.23$).

Simultaneously, in an effort to solubilize precipitation proteins, 3 replicate lysates of a mouse sample that had been stored at -80°C for between 1 month and 2 years were treated by either heat (95°C, 5 min), 2-mercaptoethanol, 1% SDS, or 2 M urea. Western blot quantification showed that none of the treatments equalized FMRP levels across the 3 replicates (data not shown), therefore we sought a new lysis treatment.

Protein lysis with high SDS and 2-mercaptoethanol –PEB lysis buffer followed by moderate heating (70°C) was tested for mouse-brain lysis, as it has been reported to result in 98% solubilization of brain tissue (2). Following this protocol, we saw little to no pellet during the lysis procedure. FMRP measurements after PEB lysis-buffer optimization were replicable, as can be seen in a Z-Z plot (Supplementary Material, Fig. S1B; $R^2 = 0.92$). Using this modified extraction method, we analyzed the variation of raw FMRP and GAPDH levels for our fiducial animal/sample (12 CGG repeats) for constant total protein loading (by BCA), in duplicate, across 7 Western blots (n=14 each for FMRP and GAPDH assays; 7 Westerns, samples run in duplicate). The results of this fiducial analysis (Supplementary Material, Fig. S2A) indicate that there is no difference in the variation in FMRP and GAPDH values (normalized sample means;

SDEV = 0.50 and 0.66 for FMRP and GAPDH, respectively). We extended this analysis to the distribution of FMRP and GAPDH values for all samples of normal genotype across the seven Western blots (n = 116 each for FMRP and GAPDH assays; 7 Westerns, samples run in duplicate; Supplementary Material, Fig. S2B). Again, there was no significant difference in the standard deviations for the two assays (SDEV = 0.45 and 0.32 for FMRP and GAPDH, respectively).

Table S1. The effects of age, brain region, and CGG repeat on FMRP levels in mice of different ages. A three-way ANOVA was performed and the least square means calculated in order to assess the main effects of genotype, brain region, and age (**A**), as well as the interactions of these variables (**B**). Non-significant P-values are emphasized in red font.

A.

Comparison		***P* value**
wt	CGG	< 0.001
Cerebellum	Hippocampus	< 0.001
Cerebellum	Frontal Cortex	< 0.001
Hippocampus	Frontal Cortex	< 0.001
P0	P140	<0.001
P0	P21	<0.001
P0	P14	<0.001
P0	P35	<0.001
P35	P140	<0.001
P35	P21	<0.001
P35	P14	<0.001
P21	P140	0.005
P14	P140	0.006
P21	P14	0.961

B.

Comparison		***P* value**
Brain region within wt		
Cerebellum	Hippocampus	< 0.001
Cerebellum	Frontal Cortex	< 0.001
Hippocampus	Frontal Cortex	< 0.001
Brain region within expanded CGG		
Cerebellum	Hippocampus	< 0.001
Cerebellum	Frontal Cortex	0.009
Hippocampus	Frontal Cortex	0.012
Age within wt		

P0	P140	< 0.001
P0	P21	< 0.001
P0	P14	< 0.001
P0	P35	< 0.001
P35	P140	< 0.001
P35	P21	0.001
P35	P14	0.001
P21	P140	0.007
P14	P140	0.007
P21	P14	0.961
Age within expanded CGG		
P0	P140	< 0.001
P0	P21	< 0.001
P0	P14	< 0.001
P0	P35	< 0.001
P35	P140	< 0.001
P35	P21	0.050
P35	P14	0.049
P21	P140	0.433
P14	P140	0.358
P21	P14	0.984
Genotype within Cerebellum		
wt	CGG	<0.001
Genotype within Frontal Cortex		
wt	CGG	<0.001
Genotype within Hippocampus		
wt	CGG	0.079
Age within cerebellum		
P0	P140	< 0.001
P0	P21	< 0.001
P0	P14	< 0.001
P0	P35	< 0.001
P35	P140	0.959
P35	P21	0.010

P35	P14	< 0.001
P21	P140	0.002
P14	P140	< 0.001
P21	P14	0.234
Age within frontal cortex		
P0	P140	< 0.001
P0	P21	< 0.001
P0	P14	< 0.001
P0	P35	0.238
P35	P140	< 0.001
P35	P21	< 0.001
P35	P14	< 0.001
P21	P140	0.090
P14	P140	0.168
P21	P14	< 0.001
Age within hippocampus		
P0	P140	< 0.001
P0	P21	< 0.001
P0	P14	< 0.001
P0	P35	< 0.001
P35	P140	0.051
P35	P21	0.047
P35	P14	0.655
P21	P140	0.871
P14	P140	0.147
P21	P14	0.150
Brain Region within P0		
Hippocampus	Frontal Cortex	0.016
Cerebellum	Frontal Cortex	0.014
Hippocampus	Cerebellum	0.932
Brain Region within P14		
Hippocampus	Frontal Cortex	0.253
Cerebellum	Frontal Cortex	<0.001
Hippocampus	Cerebellum	<0.001

Brain Region within P21		
Hippocampus	Frontal Cortex	<0.001
Cerebellum	Frontal Cortex	<0.001
Hippocampus	Cerebellum	<0.001
Brain Region within P35		
Hippocampus	Frontal Cortex	<0.001
Cerebellum	Frontal Cortex	<0.001
Hippocampus	Cerebellum	0.017
Brain Region within P140		
Hippocampus	Frontal Cortex	0.010
Cerebellum	Frontal Cortex	<0.001
Hippocampus	Cerebellum	<0.001
Genotype within P0		
wt	CGG	<0.001
Genotype within P14		
wt	CGG	0.004
Genotype within P21		
wt	CGG	0.002
Genotype within P35		
wt	CGG	<0.001
Genotype within P140		
wt	CGG	0.173

Table S2. FMRP levels in mice with premutation repeats as reported in the literature. Two separate strains are reported; the strain used in the current study was engineered by Willemsen et. al (9)[1], while a second strain was developed by Entezam et al. (7)[2]. Data are depicted in **Supplementary Material, Fig. S4**.

Reference	n Animals	CGG repeat	% FMRP
Brouwer, 2008a[1]	1	70	110
	10	100-150	60
	5	151-200	42
	9	>200	38
Brouwer, 2007[1]	1	230	21
	1	230	5
	1	230	51
Brouwer, 2008b[1]	1	112	100
	1	129	100
	1	174	80
	1	184	70
Berman, 2012[1]	12	84-210 (138.6 ±12.0)	72
Willemsen, 2003[1]	2	105	~100
Entezam, 2007[2]	1	130	47
	1	190	14
	1	210	8
Qin, 2011[2]	3	132	15

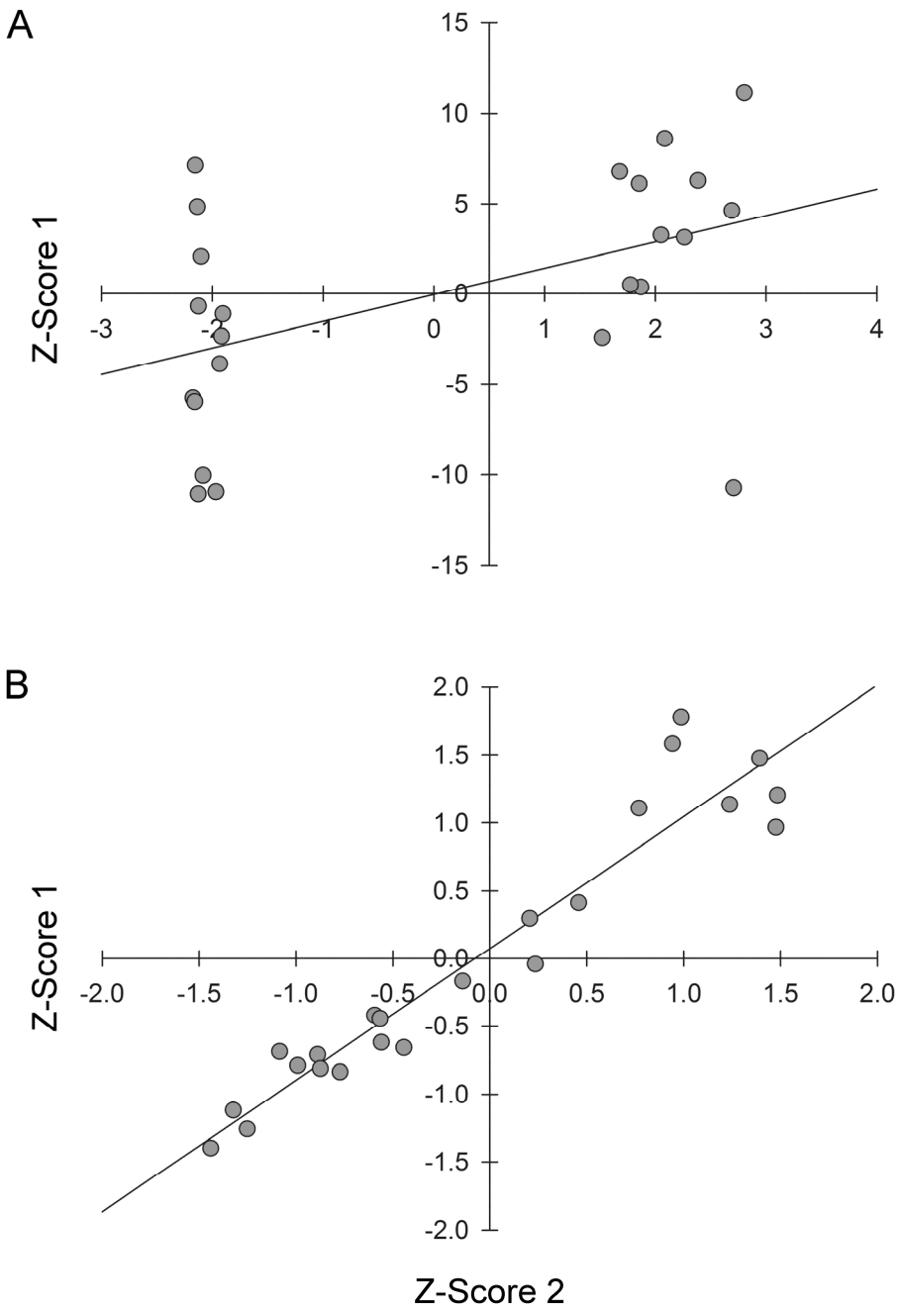

Figure S1. Z-Z plots of mouse FMRP levels before (A) and after (B) lysis buffer optimization. Z-scores of replicate experiments were plotted against each other in order to determine the experimental repeatability of FMRP Western blots. (**A**) Original lysis buffer. FMRP levels were measured in 12 wt, 10 low-repeat (<120 CGG), and 2 large-repeat (>200 CGG) mice in two separate experiments starting with frozen brain tissue. Linear regression: Z-Score 1 = 0.0376 + (0.160 * Z-Score 2). R^2 = 0.23. (**B**) PEB lysis buffer. FMRP levels were measured in 12 wt, 3 low-repeat (<120 CGG), 2 mid-repeat (120-200), and 6 high-repeat (>200) mice. Linear regression: Z-Score 1 = -0.0763 + (0.944 * Z-Score 2). R^2 = 0.92.

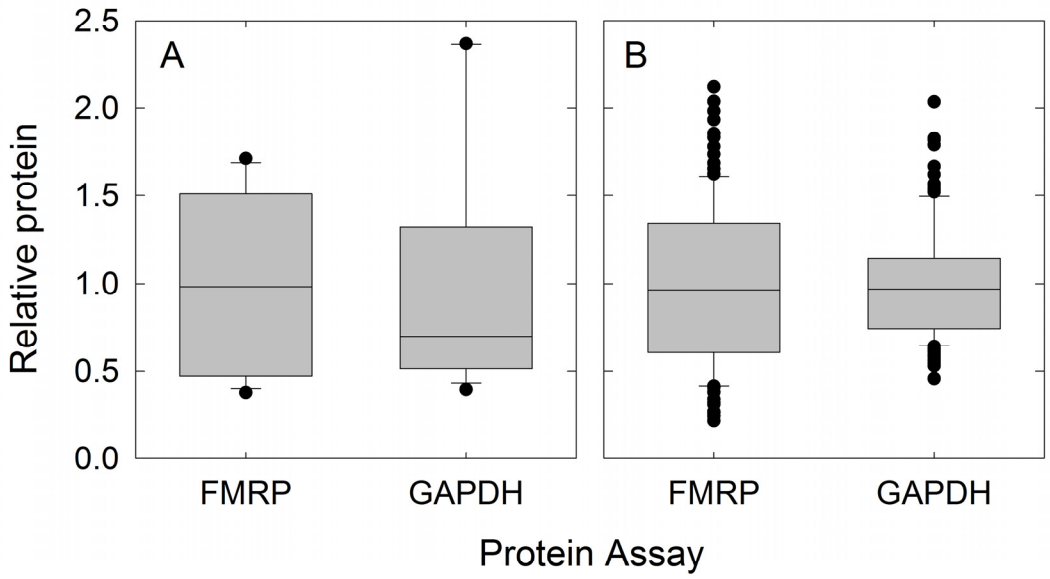

Figure S2. (**A**) Box plots of the distributions of raw FMRP and GAPDH levels for a single fiducial animal/sample (12 CGG repeats) for constant total protein loading (by BCA), in duplicate, across 7 Western blots (n=14 each for FMRP and GAPDH assays; 7 Westerns, samples run in duplicate); for normalized sample means = 1.0; SDEV = 0.50 and 0.66 for FMRP and GAPPH, respectively). (**B**) Analysis to the distribution of FMRP and GAPDH values for all samples of normal genotype across the 7 Western blots (n = 116 each for FMRP and GAPDH assays; 7 Westerns, samples run in duplicate); for normalized sample means = 1.0; SDEV = 0.45 and 0.32 for FMRP and GAPDH, respectively.

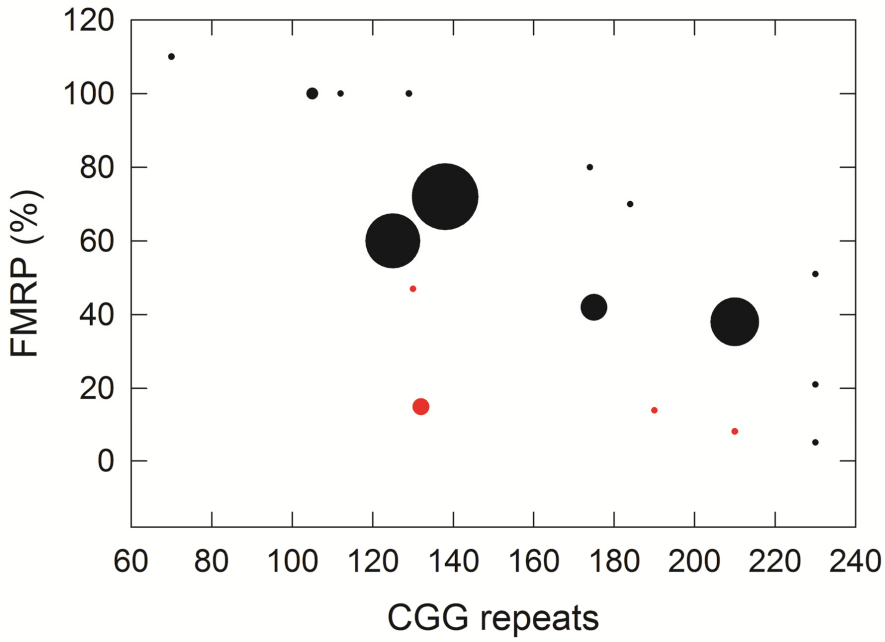

Figure S3. Approximate FMRP levels from 2 premutation CGG-repeat KI mouse lines, as reported in the literature (3-9). FMRP measurements from fresh brain tissue (uncultured), from either the line used in the current study (black circles; (10)), or the Entezam et al. (7) mouse line (red circles), were included. As some FMRP and CGG-repeat values were reported as averages, the size of each circle represents the number of animals included in each point.

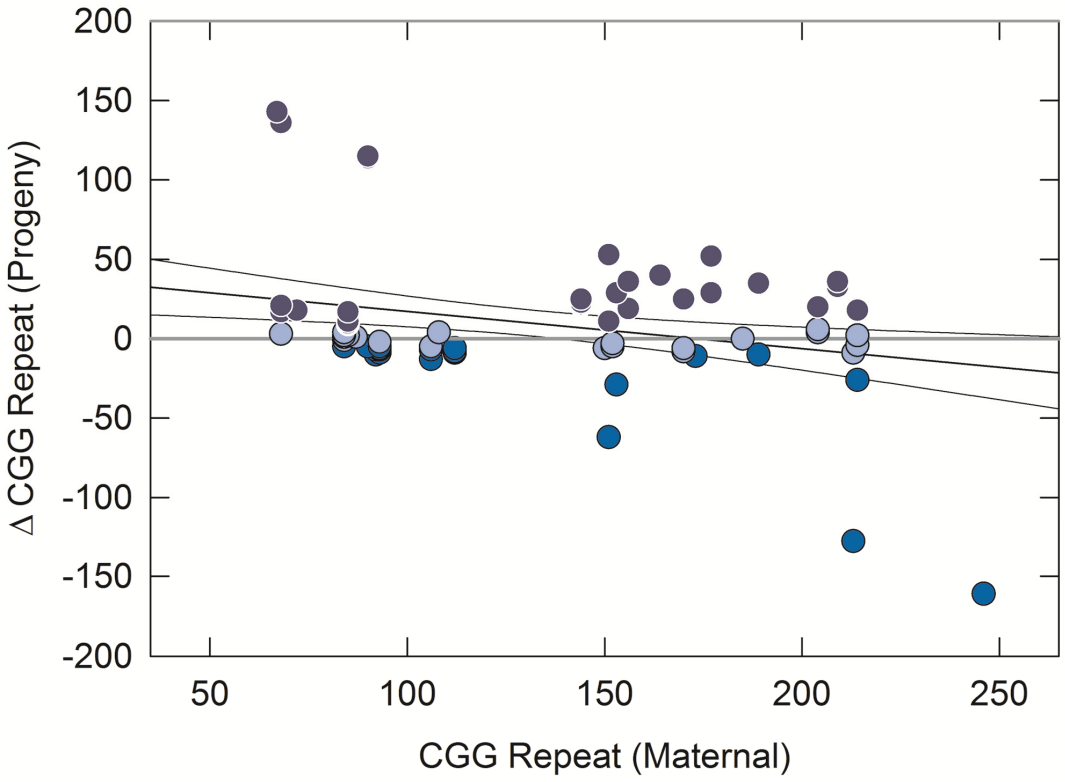

Figure S4. Expansion, contraction, or retention of the CGG repeat in the progeny versus the dam's repeat length. Thirty-nine mice with CGG-repeat expansions (dark blue), 29 with contractions (medium blue), and 27 with repeat-length changes that were within 5% of maternal CGG repeats (light blue) are contrasted to the repeat of each animal's mother. There is no significant relationship between maternal CGG repeat and offspring CGG repeat (R^2 = 0.0831).

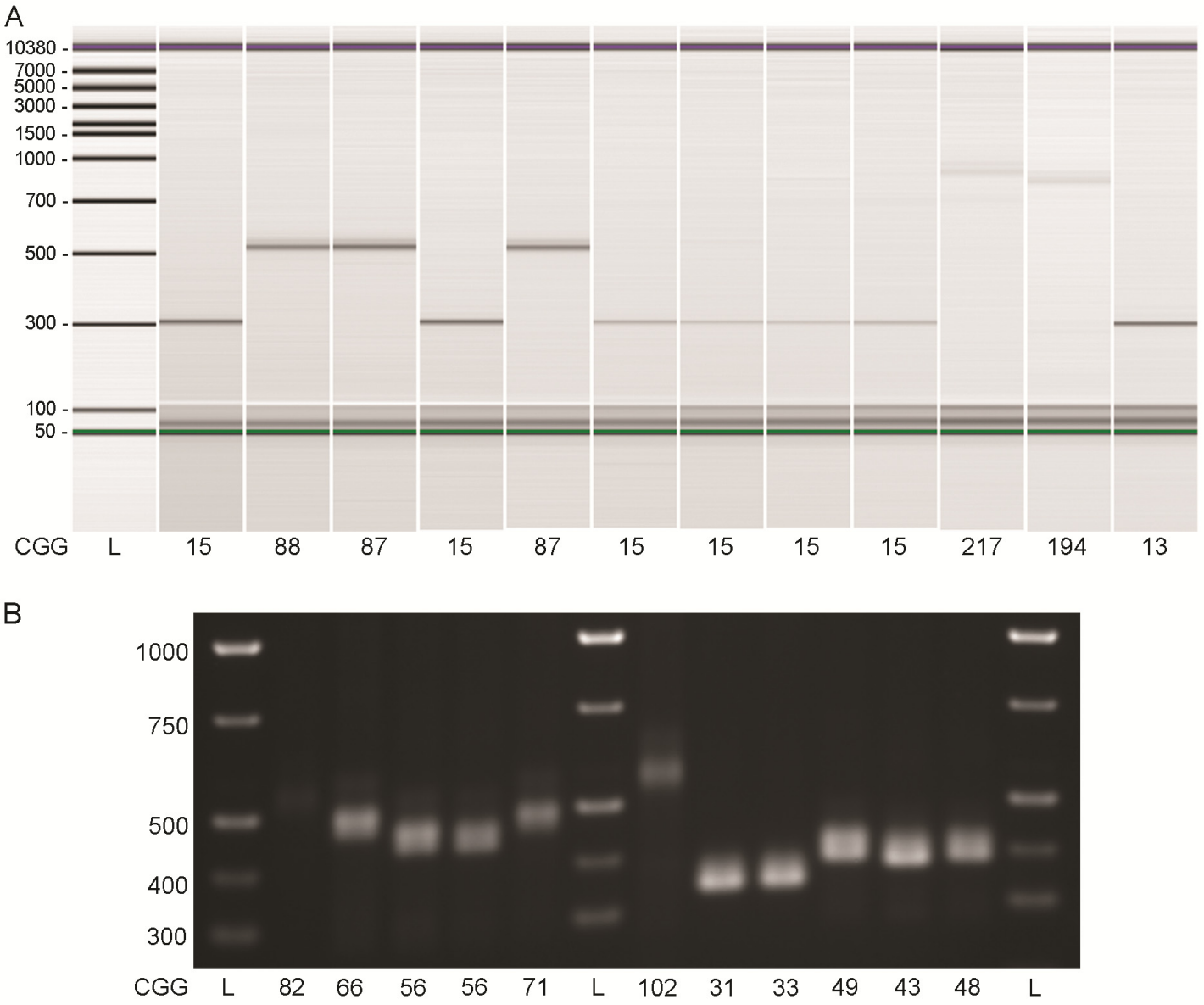

Figure S5. CGG-repeat sizing of amplicons that span the CGG repeat in mouse and human DNA samples. (**A**) A digital gel trace from a Bioanalyzer on-chip flow cytometer showing Asuragen-amplified mouse PCR products. The amplicon is 263 bp plus the CGG repeat. (**B**) Agarose gel of human PCR amplifications, which are 264 bp plus the CGG repeat. L, DNA ladder; numbers are calculated CGG-repeat lengths.

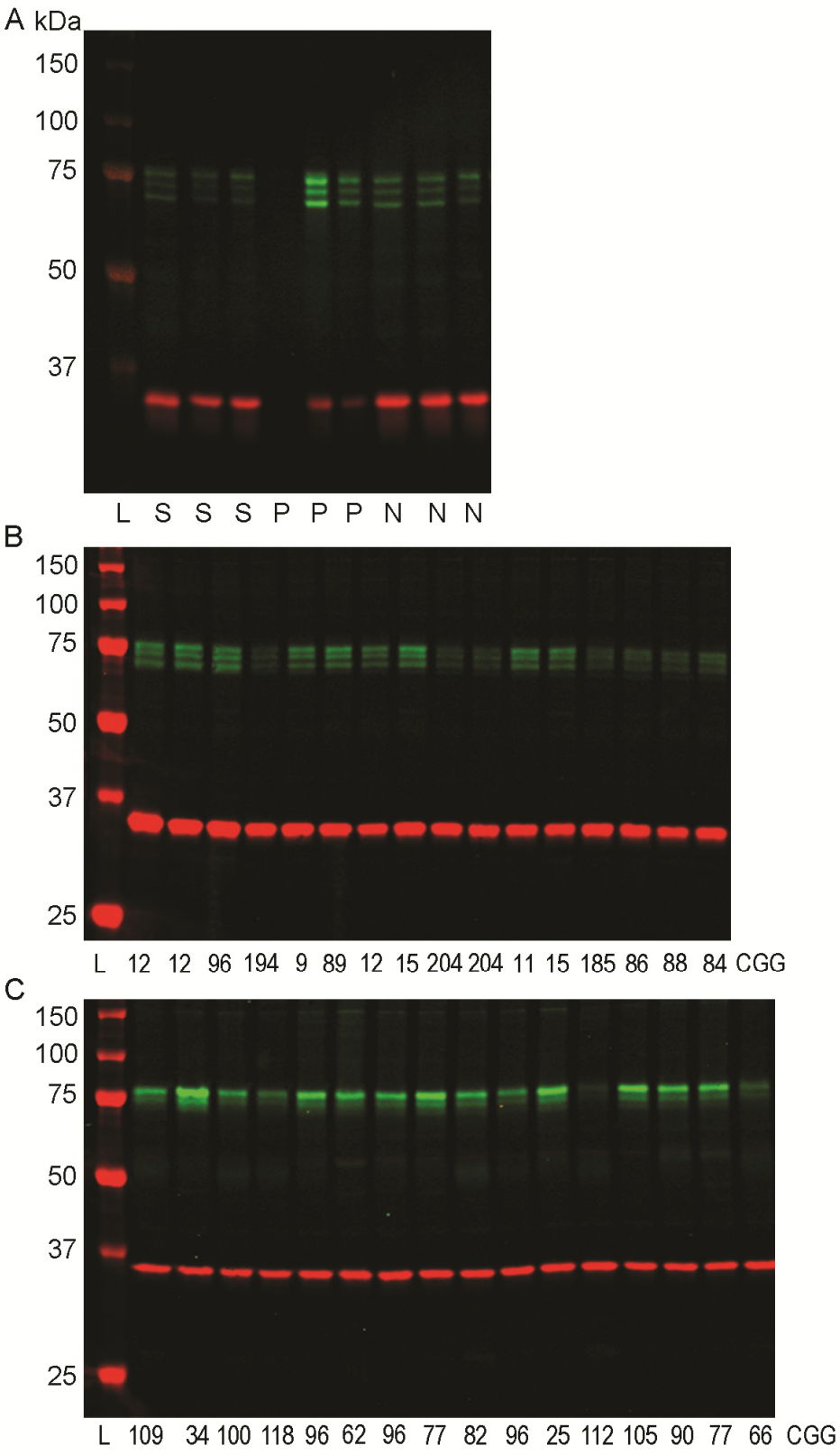

Figure S6. Western blots for FMRP expression in mice and humans of varying CGG repeats. (**A**) Western blot of a single mouse brain that was extracted on 3 separate occasions in RIPA

buffer and then stored at -80°C for up to a year, showing FMRP (green) and Gapdh (red) in the insoluble, pelleted fraction. L, ladder; S, Supernatant; P, Pellet; N, No treatment. Representative Western blot of FMRP and Gapdh in mouse brain hemispheres (**B**) and human frontal cortex (**C**) after lysis-buffer optimization. Numbers across the bottom are CGG repeat lengths. Note the differences in FMRP expression with CGG repeat, as well as the number of FMRP splice isoforms. Blots were imaged using the LI-COR Odyssey for IRDye-secondary antibodies.

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