

# **Characterization of Fragile X mental retardation antibodies for use in cross-species immunoblotting, immunohistochemistry, and electron microscopy**

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## **Purpose of the document**

This information is provided on Cogprints for colleagues in the Fragile X field who have requested it directly in the past. It is also a companion work to the article "**Human Fragile X gene locus P1 artificial chromosome transgenic mice**" from our group (manuscript to be made available on Cogprints).

## **References**

The information presented here is summarized from the following sources:

- 1) **Analysis of *FMRI* PAC transgenic and *fmr1tm1cgr* knockout mice**  
Robert Bauchwitz M.D., Ph.D.  
The Banbury Center, Cold Spring Harbor Laboratory (2000)  
(Invited Lecture)
- 2) ***FMRI* Transgenesis: Animal Models and Therapeutic Considerations**  
Robert Bauchwitz M.D., Ph.D.  
Proceedings of the 7th International Fragile X Foundation Conference,  
National Fragile X Foundation, (2000) Sec.3  
(Invited Lecture and Published Summary)
- 3) **The *fmr1tm1cgr* knockout mouse is not a molecular null: implications for phenotype**  
Robert Bauchwitz M.D., Ph.D.  
The Banbury Center, Cold Spring Harbor Laboratory (2001)  
(Invited Lecture)

## **Acknowledgements**

This work was funded by grants from the FRAXA Research Foundation and the State of New York.

## Results and Discussion

### *Antibody characterizations*

Assessment of FMRP expression in the mouse initially employed the widely used monoclonal antibody **1a** (Devys et al., 1993), later known as **1C3** (Sittler et al., 1996) which was the first, and at least until the early part of the 21<sup>st</sup> century, the only anti-FMRP antibody commercially available (as **MAB2160**; Chemicon). MAB2160 was made against human FMRP and has been used to study mouse FMRP expression (Khandjian et al., 1995; Tamanini et al., 1997; Verheij et al., 1995).

Our Western immunoblotting with MAB2160 showed that adult Fragile X (FX) knockout mice (*fmr1<sup>tm1Cgr</sup>*, aka “ko”) produced what appeared to be FMRP-sized proteins of approximately 80 kd (Figure 1A, Figure 4, top), particularly when 60 ug or more of whole brain or other tissues were loaded<sup>1</sup>.

To investigate the identity of the seemingly FMRP-sized bands seen in FX mouse tissues, we used the MAB2160 to immunoprecipitate (IP) a testis protein extract (Figure 1A). Testis was chosen for the IP since less protein had to be loaded for comparable signal than from FX brain (e.g. Figure 4). The products of the FX IP were then analyzed by mass spectrometry. Among the peptides which appeared to be present were those from the mouse FMRP-like protein, FXR1P.

MAB2160 binding to FXR1P was further assessed by running whole brain lysate from 3-day-old C57BL/6J wild-type mice directly on two-dimensional gels. MAB2160-positive protein binding was identified by Western blotting and the comparable spots were excised from a duplicate gel which had been processed with a silver stain (Figure 1B). In this case, FMRP would have been expected to be prevalent. Nevertheless, FXR1P, and not FMRP, was identified from the peptide mass data (Figure 1C).

Therefore, ***MAB2160 had a strong affinity for mouse FXR1P***. That there could be paralog cross-reactivity using MAB2160 is not surprising since it has been shown that MAB2160 binds to the N-terminus of FMRP (Devys et al., 1993; Verheij et al., 1995), which is highly conserved among the FMRP paralogs. Indeed, this result confirms prior speculation of Khandjian et al. as to the likely identity of the potentially cross-reacting FMRP paralog (Khandjian et al., 1998).

What was surprising was that our data suggest that ***murine FXR1P in some conditions*** (such as in wild-type newborn mouse brain in which robust levels of FMRP are expected) ***could be detected preferentially over FMRP***. No FMRP was detected when Western blots of brain protein immunoprecipitated by MAB2160 were probed by another antibody against FMRP (EF8, characterized below)<sup>2</sup>. This may be related to the large difference in affinity of MAB2160 for human versus mouse FMRP (see following).

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<sup>1</sup> Khandjian et. al. (1998) first noted background staining in FX mouse extracts (40 ug) using the 1C3 antibody (Figure 3).

<sup>2</sup> EF8 itself was not able to immunoprecipitate mouse FMRP under the conditions used here.



Two additional anti-FMRP antibodies were obtained from other laboratories. The first, a rabbit polyclonal antibody produced by Andre Hoogeveen's laboratory, "1079" (Verheij et al., 1995), was made to a human FMRP C-terminal peptide which has little similarity to sequences in the FXRP paralogs. The second antibody, EF8 from Gideon Dreyfuss' laboratory, was a mouse monoclonal made against FMRP (first mentioned in Zhang et al., 1995 with antigen not specified).

A comparison of the binding of 1079, EF8, and MAB2160 is shown in Figure 2. FMRP isoforms run as multiple bands in the 75 kd region. Bands in the 50 kd region, which are evident in some samples, are produced in the absence of primary antibody and are therefore likely due to binding of the anti-mouse secondary antibody to endogenous murine immunoglobulin heavy chains (see also Figure 1A). 1079, EF8, and MAB2160 all recognize common bands. In particular, the intensity of the human (P+ transgenic/ko) and mouse FMRP-sized bands in the brain using 1079 and EF8 were comparable (Figure 2). The human FMRP shown in Figure 2 is from P1 artificial chromosome (PAC) transgenic animals<sup>3</sup>. In these experiments, there were additional bands seen in the 1079 bound samples which were not observed using the two mouse monoclonal antibodies.

In addition to the cross-reactivity for FXR1P by MAB2160 raised earlier, the data in Figure 2 illustrate another issue: ***MAB2160 has a dramatically enhanced affinity for human compared to mouse FMRP***. By contrast, the EF8 antibody, which showed very little background binding of any kind by immunoblotting, produced similar binding to human and mouse FMRP (Figure 2).

Peier et. al. (2000) described a large overexpression of human FMRP in all tissues of their human *FMRI* YAC transgenic animals. In mice with what they estimated to have approximately two to three copies of *FMRI*-bearing YACs, a greater than 10-fold enhancement of FMRP staining was observed on MAB2160-stained immunoblots (13-17x by slot blotting). They proposed that human FMRP translation was not regulated in the mouse in the same way as mouse FMRP. Such a finding could have had a significant inhibitory effect on attempts to study human FMRP in mice.

It is conceivable that attempts to microinject relatively large YACs may have led to their breakage and misregulation at the RNA level. The high likelihood of breaking microinjected YACs of greater than 200 kb has been demonstrated (Bauchwitz and

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<sup>3</sup> A bacterial P1 artificial chromosome (PAC) with an insert spanning the human *FMRI* gene locus was isolated (see Cogprints manuscript "Human Fragile X gene locus P1 artificial chromosome transgenic mice"). The approximately 90 kb insert was purified as described (Bauchwitz and Costantini, 1998) and microinjected into the pronuclei of DBA x C57BL/6J ("F1") embryos. Several *FMRI* transgenic lines were obtained. The only one discussed in the context of this work will be referred to as "PAC+" or "P+". Southern analysis using a cDNA probe with sequences common to murine and human *FMRI* indicated that the PAC+ insert was present as a single copy. mRNA expression of this transgene was equivalent in most tissues to that from the endogenous *Fmr1*, consistent with genomic measurements suggesting a single copy insertion into the mouse genome.

**Figure 1. FMRP antibody MAB2160 can immunoprecipitate mouse FXR1P**  
**(A)** 6-month-old wild-type (11.5 mg) and 8-month-old FX mutant (“ko”, *fmr1-tm1Cgr*, 15.3 mg) mouse testis NP-40 lysates were immunoprecipitated (“IP” lanes) in a volume of 500 ul with 1 ul MAB2160 and 10 ul packed Protein G agarose. 150 ug of each lysate was also immunoblotted with the same monoclonal antibody (1:2000). “Mock IP” contained antibody in buffer but no protein lysate. The secondary antibody recognized endogenous mouse immunoglobulin heavy chains (arrow at 55 kd), as well as a higher molecular weight band. The most prominent testis FMRP bands are indicated by an arrow at 80 kd. **(B)** A two-dimensional gel of a 3-day-old wild-type brain whole cell lysate (100 ug ) was probed with MAB2160. Arrows indicate spots on a silver stained duplicate gel (50 ug lysate loaded) that corresponded to binding by MAB2160. **(C)** Mass spectrogram of tryptic digests of one of the spots indicated by arrows in (B). The peaks are consistent with the presence of peptides from FXR1P. Peptides from FXR1P were also identified from FX lysates immunoprecipitated with MAB2160 as shown in (A).

Costantini, 1998). However, the most likely explanation for the perceived overexpression is that the antibody employed to measure FMRP levels (1C3/MAB2160) had a substantial bias (>10x) for human over mouse FMRP, as discussed above (Figure 2)<sup>4</sup>. This may seem surprising given that there are few amino acid differences between human and mouse FMRP at the N-terminal to which the MAB2160 antibody has been shown to bind. However, since the MAB2160 antibody was made by injecting human FMRP into mice, the mouse immune system would have primarily targeted those antigens which are different from its own.

Neither antibody 1079 nor EF8 bound the human and mouse FMRPs equally; the difference in binding was simply less dramatic than for MAB2160. For example, we showed that the 1079 rabbit polyclonal anti-FMRP antibody had an approximately two-fold preference for the human FMRP peptide to which it was made compared to the murine counterpart, which had a single amino acid difference (Figure 3). Furthermore, a peptide common to the human and mouse immunogen showed almost no binding.

Therefore, monoclonal antibody EF8 demonstrated much less difference in binding human and mouse FMRP than did MAB2160. Of further note, EF8 also did not show significant binding of FMRP-sized bands in Fragile X knockout mouse brain tissue (Figure 4). Therefore, we conducted all subsequent protein analyses in the mouse using EF8.

### ***Immunohistochemistry***

Immunohistochemistry of *fmr1<sup>tm1Cgr</sup>* (exon 5 disrupted) Fragile X mouse brains using anti-FMRP antibody EF8 repeatedly demonstrated light or modest staining of some neurons and dendrites compared to an absence of staining of other cells and axonal/white matter in the same section. (There was no obvious staining in sections in which the EF8 primary antibody was omitted.) This light EF8 staining of neurons was seen in the FX mouse cerebellum (Figure 5)<sup>5</sup>, as well as in the neocortex and hippocampus (Figures 6 and 7). However, there was essentially no EF8 staining in the FX mouse testis (Figures 6 and 7), a location in which FXR1P levels were strong and therefore cross-reaction would have been most expected (Tamanini et al., 1997)<sup>6</sup>.

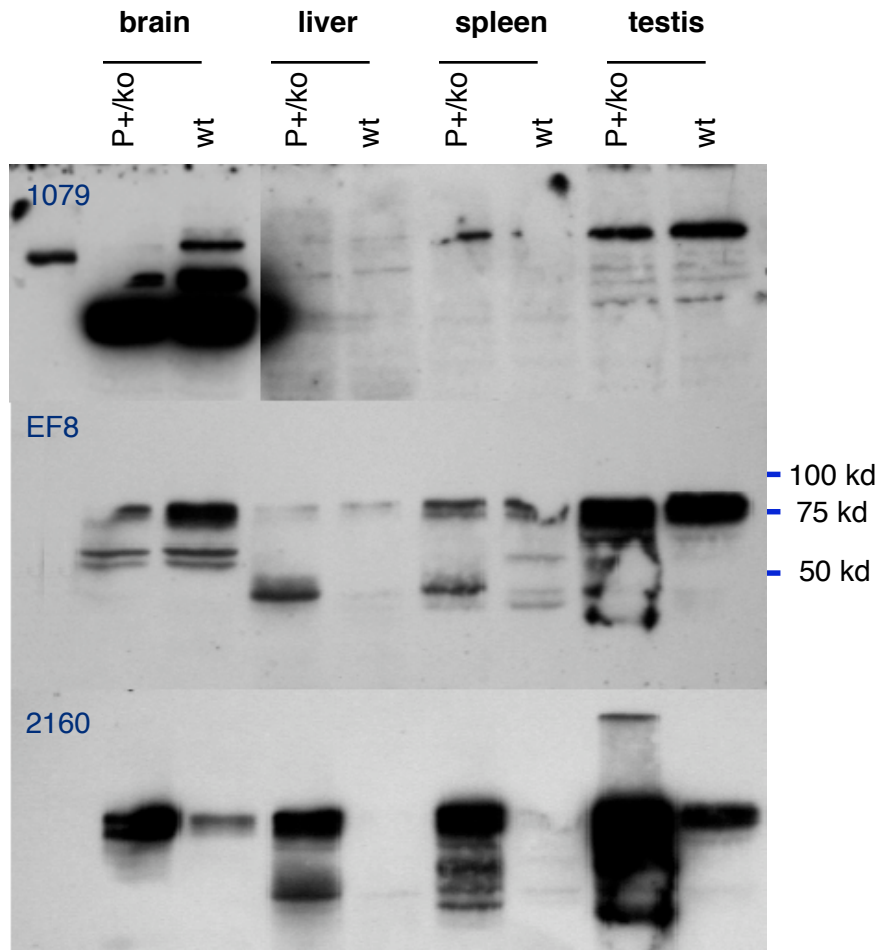
Our ability to see some modestly well stained neurons in the brains of *fmr1<sup>tm1Cgr</sup>* mice by immunohistochemistry (IHC) was greater than our ability to definitively identify FMRP protein bands by immunoblotting, though light FMRP-bands were seen in the latter with increased protein loads (beginning at about 60 ug of whole brain lysate). One

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<sup>4</sup> Peier et. al. noted this possibility but stated that they did not see a difference in FMRP expression between normal human and mouse tissues as assessed by the 1C3/MAB2160 antibody (data was not shown).

<sup>5</sup> For example, in the enlarged image of FX mouse cerebellum stained with EF8 antibody shown in the bottom panel of Figure 5, Purkinje cell bodies are well stained, as are their dendrites, in the molecular layer, but no staining is seen in the cytoplasm of basket cells in that same area. There is also no visible staining of the axons of the Purkinje cells in the folium, nor for many cells in the granular layer.

<sup>6</sup> Interestingly, Tamanini et. al. did see FMRP staining in adult spermatogonia from FXS patients with the 1C3/MAB2160 antibody. They ascribed this result to the presence of *FMRI* premutations in human sperm.



**Figure 2. Monoclonal antibody MAB2160 has a significant preference for human over mouse FMRP**

Western blots of mouse tissue extracts were developed with three anti-FMRP antibodies: rabbit polyclonal “1079” (1:1000) and mouse monoclonals “EF8” (1:500) and MAB2160 (1:2000, also known as “1a” or “1C3”). “P+/ko” were made from the tissues of mutant mice with a disruption in exon 5 of the *Fmr1* gene and also carried a human genomic *FMR1* transgene. “wt” indicates tissues from wild-type mice. 75 ug of protein were loaded in each lane of this 10% acrylamide SDS gel.

explanation for this would be that any cells expressing FMRP at levels at which they would be visible by IHC in the *fmr1<sup>tm1Cgr</sup>* brain would be very diluted by non- or lowly-expressing cells in a whole brain lysate. Therefore, it is possible that we were often at or past our limit of detection for *fmr1<sup>tm1Cgr</sup>* FMRP using the standard immunoblotting technique. It would be expected that low and possibly variable expression of FMRP could be found in the *fmr1<sup>tm1Cgr</sup>* brain since some novel splice isoforms allowing full RNA open reading frames had been observed (and subsequently published in Yan et al., 2004).

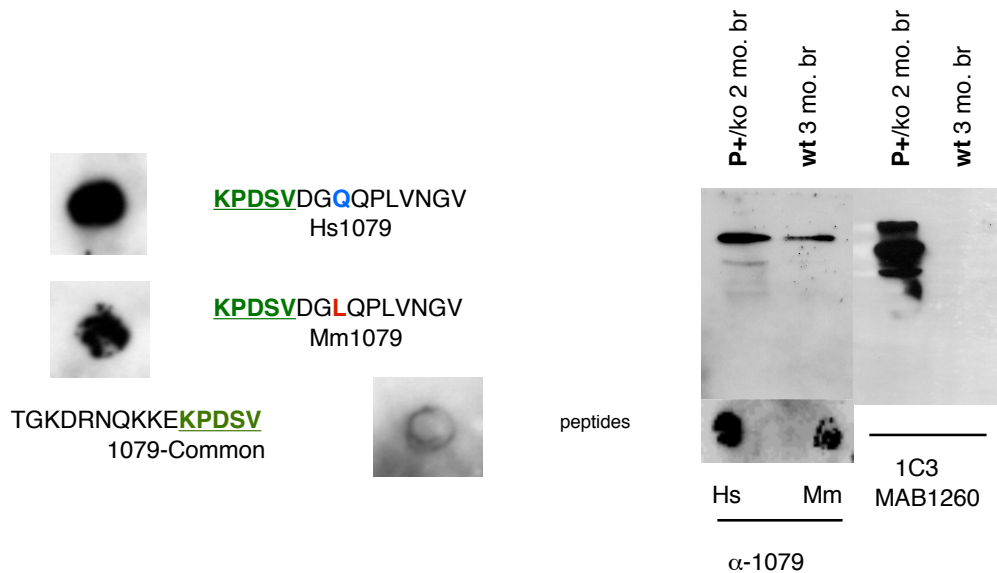
By contrast, binding of human FMRP expressed in transgenic mouse tissue (“P+”), was equivalent to that of the endogenous wild-type mouse FMRP expression in the cerebellum (Figure 5), neocortex and hippocampus (Figure 6). The amount of FMRP staining varied among neurons and among regions of the brain, i.e. it was reproducibly the case that even for neurons close to each other in the same section, the intensity of FMRP staining could be noticeably different. One interpretation of this phenomenon is that individual neurons were differentially active and therefore expressing different levels of FMRP.

Immunohistochemical staining of *FMR1* transgenic (“P+”) testes using the EF8 monoclonal antibody was not identical to that of wild-type testes. FMRP in transgenic testes was observed throughout the various stages of sperm development (Figure 6). This phenomenon has been previously attributed to *Fmr1* normally being inactivated along with the rest of the X chromosome during male spermatogenesis (Vandeberg, 1983), but not as an autosomally linked transgene (Peier et al., 2000). Elevated testicular expression of transgenic FMRP was also evident by Western blotting as an increased signal intensity (for a given weight of tissue), most obvious among usually lesser staining FMRP isoforms (Figure 2).

For the reasons noted above, we would characterize elevated testicular FMRP from transgenic mice as ***misexpression rather than overexpression***. Use of a mouse *Fmr1* transgene would be predicted to suffer the same misexpression beyond the spermatogonial stage in the testis as the human transgene employed here and in the YAC by Peier et. al. (2000), unless the *Fmr1* transgene were placed on the X chromosome. It should be noted from a practical perspective that mice expressing FMRP in advanced stages of spermatogenesis were fertile. This is consistent with the fertility observed in male animals carrying an inactive XIST gene on their X chromosome (Marahrens et al., 1997), without which the X chromosome is not inactivated during spermatogenesis.

### ***Immunogold Electron Microscopy***

The EF8 antibody was used to stain FMRP for an electron microscopic examination of wild-type and FX mutant (exon 5 disrupted) mouse brain tissues. After exposing brain sections to EF8, the antibody was bound with immunogold-labeled particles. Signals were seen bound to what appeared to be actin cytoskeleton (microfilaments) of dendrites in wild-type sections (Figure 8, left) and also in FX ko brain sections (Figure 8, right).



**Figure 3. Differential binding of antibodies 1079 and MAB2160 (1C3) to human and mouse FMRP**

A single amino acid difference between human and mouse FMRP antigens produced a two-fold difference in binding by antibody 1079. **(Left)** Binding of 1079 antibody to peptides spotted on nitrocellulose filters. The 1079 antibody was made against human FMRP amino acids 607-631 (Verheij et. al., 1995). A single amino acid difference between the human and mouse sequences is shown in blue (“Q”) and red (“L”). The C-terminal amino acids used as the immunogen are very different from those found in the FXRPs. Therefore, these peptides would be expected to be FMRP specific; the amino acid sequences common to FXR1P and FXR2P are shown as “NGV” and “VNGV”. **(Right)** Western blots comparing transgenic and wt FMRP staining with 1079 and MAB2160 (aka 1C3).

## Conclusions

Using a monoclonal antibody (EF8) that showed little to no FX (*fmr1<sup>tm1Cgr</sup>*) background on Western blots even at relatively high protein loads, light but notable cytoplasmic staining of some neuronal populations was observed in the FX mouse brain, in particular in Purkinje cells and some cortical neurons<sup>7</sup>. It was also found that human FMRP originating from a single copy genomic transgene was expressed in the same way in the brain as wild-type FMRP. However, human FMRP from the P1 artificial chromosome was expressed throughout spermatogenesis, as had been observed for human FMRP expression from transgenic YACs (Peier et al., 2000). The primary contrast with the earlier study was not with the distribution of expression, but rather that this work did not observe a seeming overexpression of human FMRP relative to its transgene copy number. An explanation for this discrepancy was provided in this study from the Western blot analysis of anti-FMRP antibodies, which indicated that the MAB2160 (1a, 1C3) antibody had a large preference for human over mouse FMRP. Moreover, it was shown here that MAB2160 could bind and immunoprecipitate mouse FXR1P from both wild-type and FX brains and testes. Therefore, the results of this study indicate that antibody EF8 has some clear advantages over MAB2160 in quantifying and comparing FMRP levels in the mouse.

## Methods

### Protein Preparation

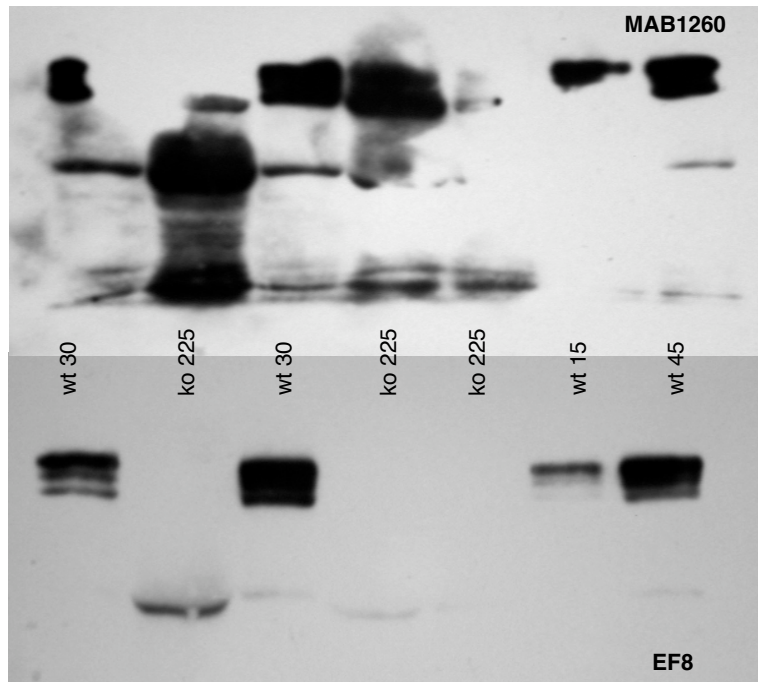
Tissues were briefly soaked in 4°C PBS to remove serum and blood. Lysis buffers used were “SDS-denaturation” (2% SDS, 50 mM Tris 7.5), “TX-100” (1% TX-100, 50 mM NaCl, 50 mM Tris 8.0), or “NP-40” (150 mM NaCl, 1% NP-40, 0.5% deoxycholate/”DOC”, 0.1% SDS, 50 mM Tris 8.0), with 1 ml buffer for approximately 100 mg tissue. A protease inhibitor cocktail (Sigma P2714) was used as necessary for tissues showing evidence of protease activity. SDS-denaturation lysates were heated at 85-95°C for 10 minutes, followed by several bursts of sonication to shear DNA and then a 10,000 x g, 10 minute spin. TX-100 and NP-40 lysates were prepared without heating or sonication but with the same centrifugation. Supernatants were quantitated by optical density at 206 nm (up to 20 ul of SDS lysate or 5 ul TX-100 were used; the latter produced significant background above 10 ul). The extinction coefficient for 1 mg/ml protein at 206 nm was 29. OD206 with a titration of fresh 10 mg/ml BSA-V showed that the scale was linear to 50-100 ug/ml. Prior to immunoprecipitation, SDS-denaturation lysates were diluted 20x in 50 mM Tris 7.5/2% BSA to reduce the SDS concentration.

### Immunoprecipitation (IP)

For each IP, two tubes containing 20 ul Protein G agarose (Sigma P4691) or

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<sup>7</sup> Nevertheless, without protein sequence identification of what was being bound at the cellular level, as seen by IHC and EM, it remains to be confirmed that the positive signals seen in the FX *fmr1<sup>tm1Cgr</sup>* tissues were actually FMRP, or rather another potential target such as FXR1P.



**Figure 4. Comparison of EF8 and MAB2160 antibody binding of polypeptides from Fragile X mouse brain**

Western blots of 16-day-old wild-type (“wt”) and 11-day-old *fmr1-tm1Cgr* (“ko”) FX knockout mouse brain protein extracts probed with anti-FMRP antibodies EF8 (1:500) or MAB2160 (1:2000) followed by HRP-conjugated secondary antibody (1:15,000). Numbers on the images indicate the total amount of protein loaded in micrograms onto 7.5% acrylamide SDS gels and then blotted onto nitrocellulose (4.5  $\mu$ m). The proteins in lanes 3 and 4 were extracted from tissue with Triton X-100 buffer; all other lanes were extracted with NP-40 buffer.

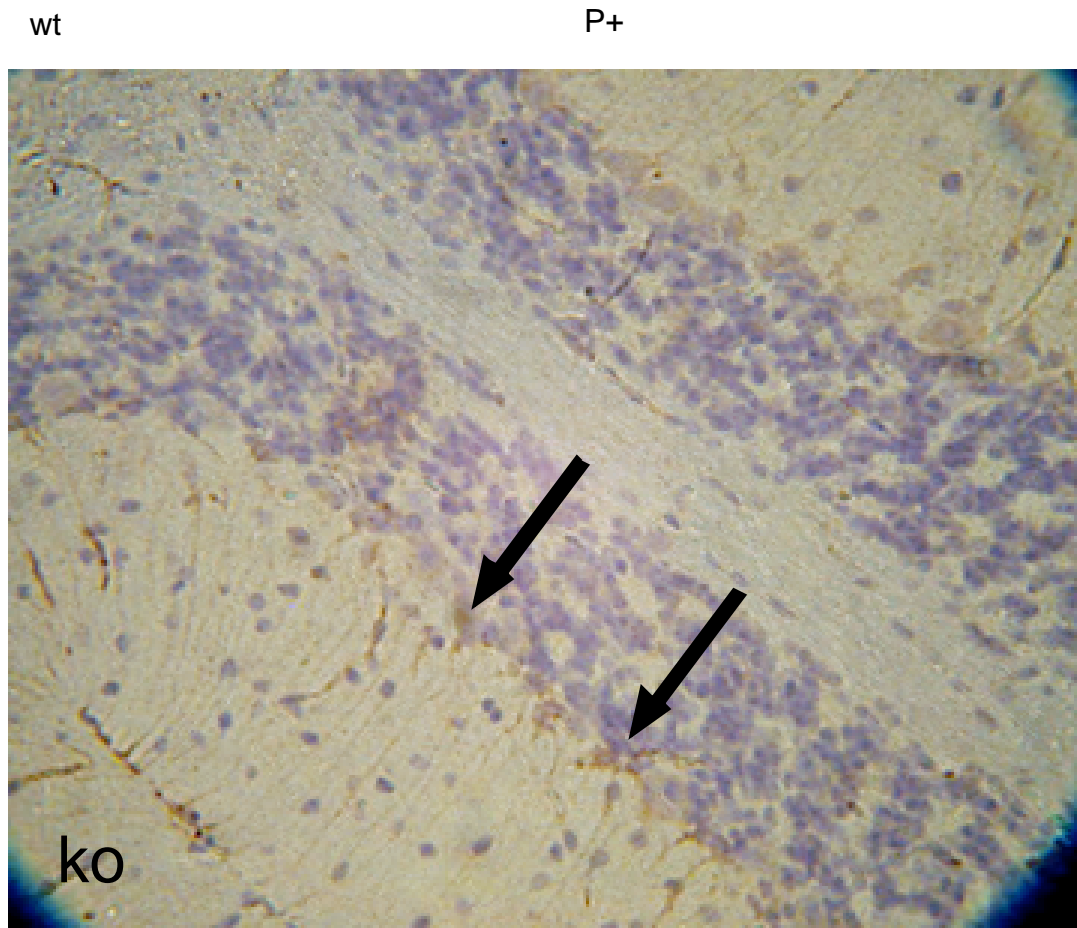
acrylamide (Pierce) were washed by centrifugation at 10,000 x g in a microfuge (“spinning”) for 15 seconds with 1 ml IP buffer (0.1%SDS/0.1% DOC), using a pipet tip made wide bore with a razor blade to draw up the beads. Supernatant was aspirated with a 26-gauge needle on a Pasteur pipet (beads were lost with a normal 20 ul pipet tip). Each lysate (0.5 - 1 ml) was pre-cleared of antibodies which could bind to Protein G by adding the lysate to one of the tubes of beads, rocking 4<sup>0</sup>C, 45 minutes, and then spinning 15 seconds at room temperature. The cleared supernatant was transferred to a fresh 1.5 ml tube, and 1 ul monoclonal antibody (MAB2160, Chemicon) added, followed by rocking at 4<sup>0</sup>C for 1 hour. After incubation, the antibody-supernatant mixture was transferred to the remaining tube of washed beads, which had been left on ice. The supernatant-antibody-Protein G mixture was rocked at 4<sup>0</sup>C for 1 hour and then spun 15 seconds, room temperature. The resulting supernatant was aspirated (or saved as “post-IP sup” for further analysis). The protein G-agarose pellet was washed 3 times with 1 ml IP buffer. Aspiration was to the surface of the beads for the first two washes and into the beads for the last. Laemmli buffer (20 ul) containing 100 mM DTT was added and heated to 85-95<sup>0</sup>C for 5 minutes. After a brief centrifugation, the denatured supernatant (amounts as given in the figure legends) was loaded directly onto an SDS-polyacrylamide gel.

### **Western blotting**

Proteins were separated on standard Tris/Glycine/SDS polyacrylamide gels of 6.5 – 10%. (The acrylamide percentage and amounts of protein loaded are given in the main text or in the figures.) Proteins were transferred to nitrocellulose in Tris/Glycine/SDS running buffer containing 20% methanol at 30V (constant) overnight, 4<sup>0</sup>C. The membranes were air dried for 30 minutes (m.), and then re-wetted and rinsed several times (2 x 2 m.; 2 x 5 m.) with TBST (500 mM NaCl, 20 mM Tris 7.4, 0.05% Tween 20), following which the filter was blocked with TBSTC (TBST containing 1% casein – Sigma C-8654) for 2 hours at room temperature (or overnight in a sealed bag at 4<sup>0</sup>C) with rocking. The blocked filter was air dried 30 minutes, after which it was re-wet with TBST. Primary antibody was diluted and applied in TBST (with antibody dilutions as given in the figure legends). Antibody binding was performed in sealed polypropylene bags with rocking overnight at 4<sup>0</sup>C. After application of the primary antibody, filters were washed four times in TBST, each for 1 – 2 minutes. Secondary antibodies were added in TBSTC at a dilution previously determined by titration to produce no background with strong signal. For horseradish peroxidase conjugated secondary antibodies, a dilution of 1/15,000 was employed. Chemiluminescent detection was performed using the Pierce SuperSignal West PICO reagent (Pierce, Rockford, IL).

### **Two-dimensional electrophoresis**

Two-dimensional electrophoresis was performed by Kendrick Laboratories of Madison, WI, according to the method of O’Farrell (JBC 250: 4007-4021, 1975). In brief, duplicate samples were first separated on two isoelectric focusing (2%, pH 3.5 – 10 ampholines) for 9600 volt-hours. The isoelectric focusing gels were then loaded onto 8% acrylamide gels. One of the gels was blotted onto a PVDF filter, which was then blocked and probed with antibody MAB2160. The second gel was processed with a silver stain



**Figure 5. Cerebellar FMRP expression in FMR1-PAC transgenic and *fmr1-tm1Cgr* Fragile X mutant mice**

FMRP expression in the mouse cerebellum (brown staining; arrows) was assessed by immunohistochemistry using antibody EF8. “wt” indicates tissue from wild-type mice. “P+” shows tissue from mice with an *FMR1* transgene on the *fmr1-tm1Cgr* mutant background. “ko” is an enlarged view of “knockout” mouse tissue (*fmr1-tm1Cgr* allele, exon 5 disrupted).

that allowed subsequent MALDI-TOF mass spectrometric analysis (below). Stained spots from the antibody-probed filter were matched to silver stained spots for excision and processing.

### **Mass spectrometry**

Endoproteinase Lys C digestion of excised 2-D gel spots and mass spectrometry of the resulting peptides was performed by Mary Ann Gawinowicz of the Protein Chemistry Core Facility, Howard Hughes Medical Institute, Columbia University, using a Voyager RP/DE MALDI mass spectrometer (Perseptive Biosystems). PeptideMass and ProFound software packages were used to determine probable identities for the peptide masses.

### **Immunohistochemistry (IHC)**

Mice were anesthetized with isoflurane and then 0.9% saline infused into the left atrium of the heart. The entire brain (or other organ) was quickly removed and placed in 4% fresh EM grade paraformaldehyde at 4<sup>0</sup>C for 22 hours. The tissue was transferred to cold 30% sucrose in 0.1 M TBS. When the tissue had dropped to the bottom of the sucrose solution, it was sectioned on a cryostat, then floated on 0.1M TBS and mounted to slides. Sections were washed in 0.1 M TBS (2 x 5 minutes) and then transferred to a pressure cooker containing boiling antigen unmasking solution (Vector Laboratories). The cooker was allowed to pressurized for 1 minute, after which it was cooled under cold running water, opened, and the slides removed to TBS (500 mM NaCl , 20 mM Tris 7.4). Two 3-minute washes in TBS were followed by incubation for 1 hour in mouse IgG blocking solution (M.O.M., Vector Laboratories). After further TBS washing (3 x 5 minutes), antibody staining was performed using primary antibody EF8 (1:100 dilution) and a secondary antibody conjugated to horseradish peroxidase according to manufacturer's instructions (VectaStain Elite ABC and diaminobenzidine tetrahydrochloride, Vector Laboratories).

### **Electron microscopy (EM)**

#### Mice

Wild-type cerebellum was from the brain of a one month old male FVB/NJ inbred mouse. The brain was split midsagittally and parasagittaly. A small block of cerebellum adjacent to the midsagittal plane of the brain was immediately fixed in 4<sup>0</sup>C 4% paraformaldehyde (see immunogold electron microscopy, below). The *fmr1<sup>tm1Cgr</sup>* (“knockout”) brain sample was obtained from a newborn, FVB/NJ *fmr1<sup>tm1Cgr</sup>* mouse and processed as for the wild type.

#### Antibodies

EF8 antibody, a mouse monoclonal produced against human FMRP, was provided by Gideon Dreyfuss of the University of Pennsylvania. For the wild-type samples, the antibody was diluted 1:100. For the newborn (“NB”) knockout samples, the antibody was diluted 1:50. As a result of the differing concentrations of antibody used, the density of

gold particles is not directly comparable in the two specimens. A higher concentration of EF8 was employed because the level of FMRP in the knockout tissue was lower than in the wild type, and therefore gold particles were not observed in comparable density as in the wild type at 1:100 dilution.

#### Immunogold-electron microscopy

For preparation of cryosections, tissues were fixed in 4% EM grade paraformaldehyde (EM Sciences) in 200 mM Sorensen's phosphate buffer pH 7.2 (EM Sciences) for approximately 24 hours at 4°C. They were then soaked in 200 mM Sorensen's phosphate buffer pH 7.2 without paraformaldehyde for approximately 16 hours. Prior to freezing in liquid nitrogen, the tissue was washed in 0.12% glycine in PBS for 15 minutes (to quench free aldehyde groups) and infiltrated with 2.3 M sucrose in PBS for 15 minutes. Frozen samples were sectioned at -120°C, the sections were transferred to formvar/carbon coated copper grids and floated on PBS until the immunogold labeling was carried out.

The immunogold labeling was carried out at room temperature on a piece of parafilm. All antibodies and protein A gold were diluted in 1% BSA in PBS. The diluted antibody solution was centrifuged 1 minute at 14,000 rpm prior to labeling to avoid possible aggregates. Grids were floated on drops of 1% BSA for 10 minutes to block unspecific labeling, transferred to 5 µl drops of primary antibody and incubated for 30 minutes at room temperature or overnight at 4°C. The grids were then washed in 4 drops of PBS for a total of 15 minutes, transferred to 5 µl drops of rabbit anti-mouse IgG (diluted 1:200, from Dako) for 30 minutes, washed in 4 drops of PBS for 15 minutes, transferred again to 5 µl drops of Protein A gold for 20 minutes, washed again in 4 drops of PBS for 15 minutes followed by 6 drops of double distilled water.

Contrasting/embedding of the labeled grids was carried out on ice in 0.3% uranyl acetate in 2% methylcellulose for 10 minutes. Grids were picked up with metal loops (diameter slightly larger than the grid) and the excess liquid was removed with a filterpaper (Whatman #1), leaving a thin coat of methylcellulose (bluish interference color when dry).

The grids were examined in a JEOL 1200EX transmission electron microscope at 80 kV and images were recorded at a primary magnification of 25,000x. The prints were magnified 3.4x relative to the negatives (85,000x final).

The above immunogold EM method was based upon Griffiths, G. 1993. *Fine Structure Immunocytochemistry*. Springer Verlag, Heidelberg, Germany, and was performed by Maria Ericsson of the Harvard Medical School EM Facility.

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