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To cite this article: Alice Filippini, Daniela Bonini, Caroline Lacoux, Laura Pacini, Maria Zingariello, Laura Sancillo, Daniela Bosisio, Valentina Salvi, Jessica Mingardi, Luca La Via, Francesca Zalfa, Claudia Bagni & Alessandro Barbon (2017) Absence of the Fragile X Mental Retardation Protein results in defects of RNA editing of neuronal mRNAs in mouse, RNA Biology, 14:11, 1580-1591, DOI: [10.1080/15476286.2017.1338232](https://doi.org/10.1080/15476286.2017.1338232)

To link to this article: <https://doi.org/10.1080/15476286.2017.1338232>



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## Absence of the Fragile X Mental Retardation Protein results in defects of RNA editing of neuronal mRNAs in mouse

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### ABSTRACT

The fragile X syndrome (FXS), the most common form of inherited intellectual disability, is due to the absence of FMRP, a protein regulating RNA metabolism. Recently, an unexpected function of FMRP in modulating the activity of Adenosine Deaminase Acting on RNA (ADAR) enzymes has been reported both in *Drosophila* and Zebrafish. ADARs are RNA-binding proteins that increase transcriptional complexity through a post-transcriptional mechanism called RNA editing.

To evaluate the ADAR2-FMRP interaction in mammals we analyzed several RNA editing re-coding sites in the *fmr1* knockout (KO) mice. *Ex vivo* and *in vitro* analysis revealed that absence of FMRP leads to an increase in the editing levels of brain specific mRNAs, indicating that FMRP might act as an inhibitor of editing activity. Proximity Ligation Assay (PLA) in mouse primary cortical neurons and in non-neuronal cells revealed that ADAR2 and FMRP co-localize in the nucleus. The ADAR2-FMRP co-localization was further observed by double-immunogold Electron Microscopy (EM) in the hippocampus. Moreover, ADAR2-FMRP interaction appeared to be RNA independent.

Because changes in the editing pattern are associated with neuropsychiatric and neurodevelopmental disorders, we propose that the increased editing observed in the *fmr1*-KO mice might contribute to the FXS molecular phenotypes.

### ARTICLE HISTORY

Received 22 November 2016  
Revised 23 May 2017  
Accepted 31 May 2017

### KEYWORDS

ADAR2; FMRP; Fragile X syndrome; RNA editing

## Introduction

Fragile X syndrome (FXS) is one of the most common heritable form of intellectual impairment.<sup>1–5</sup> Estimates report that FXS affects approximately 1 in 2,500–5,000 men and 1 in 4,000 – 6,000 women.<sup>6,7</sup> The leading cause of the FXS is the expansion of the number of a polymorphic CGG triplet in the 5' UTR of *fmr1* gene, located on the X chromosome (Xq27.3). Through complex epigenetic modifications it results in hypermethylation of the chromosomal region and subsequent transcriptional silencing of *fmr1* gene, preventing expression of Fragile-X-Mental Retardation Protein (FMRP).<sup>8–10</sup>

FMRP is a RNA binding protein that shuttles between the nucleus and the cytoplasm, where it forms ribonucleoproteins.<sup>11–13</sup> FMRP regulates several characteristics of RNA metabolism like splicing, stability, subcellular transport and translation of mRNA encoding for proteins involved in synaptic structure and function.<sup>14–17</sup> However, the best known function for FMRP is as translational repressor, since its absence in *fmr1* KO mice leads to an increase of protein expression.<sup>18–23</sup>

Mouse models for FXS have revealed diverse phenotypes characterized by altered neuronal development and circuits formation (spine dysmorphogenesis represents the main marker of FXS) and by impairments in long-term synaptic plasticity underlying learning and memory.<sup>24,25</sup>

Recently, a new and unexpected nuclear function of FMRP has been reported, enhancing its role as post-transcriptional regulator.<sup>26</sup> Bhogal and collaborators reported that in *D. melanogaster*, dFMRP physically and biochemically interacts with dADAR (Adenosine Deaminase Acting on RNA) enzymes, a class of RNA binding proteins that catalyzes the peculiar post transcriptional mechanism called RNA editing.<sup>27,28</sup> RNA editing is a post-transcriptional hydrolytic deamination of an adenosine (A) to an inosine (I) which is read by the ribosomes as a guanosine (G). RNA editing might induce aminoacid substitutions contributing to a diversification of the information that is encoded by the genome.<sup>29</sup> RNA editing in mammals has been mainly described for genes expressed in the Central Nervous System (CNS) and changes in editing patterns are frequently

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 Supplemental data for this article can be accessed on the [publisher's website](#).

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found associated with neuropsychiatric and neurodevelopment disorders.<sup>30-33</sup> Three ADAR enzymes, ADAR1, ADAR2 and ADAR3 are expressed in mammals but only the first 2 are enzymatically active.<sup>28,34</sup> The enzymes are able to recognize specific double stranded RNA structures generated by the hybridization of complementary exon and intron sequences in the pre-mRNA of specific transcripts, and catalyze the nucleotide deamination.<sup>28,34</sup>

The report of Bhogal and collaborators,<sup>26</sup> for the first time, correlated the RNA editing pathway with FMRP (see also<sup>35</sup>). In *Drosophila*, dADAR and dFMRP interact in the nucleus, binding on several RNAs. dFMRP has been implicated in the modulation of the RNA editing levels of specific mRNAs encoding for proteins necessary for proper function of *Drosophila* Neuro-Muscular Junction (NMJ), including transcripts encoding a calcium channel (*Ca $\alpha$ 1D*), a potassium channel (*Shab*). Moreover, several ADAR target mRNAs have been shown to be associated with ADAR and FMRP in a messenger ribonucleoprotein complex.<sup>26</sup>

A recent study from Shamay-Ramot and collaborators,<sup>36</sup> using the zebrafish model for FXS, the *fmr1* *-/-* larvae, showed an interaction of ADAR2a with FMRP, also in vertebrates. Furthermore, a mild increase in RNA editing levels of mRNAs encoding neuronal and synaptic proteins like the Calcium Channel, Voltage-Dependent, L Type,  $\alpha$  1D Subunit (*cacna1d*), glutamate receptor ionotropic kainate type subunit 2 (*grik2*), glutamate receptor ionotropic AMPA type subunit 4 (*gria4b*), glutamate receptor ionotropic AMPA type subunit 3b (*gria3b*), was reported. These data in zebrafish further support a role of FMRP in inhibiting ADAR activity.

In this work, we wanted to elucidate the interplay between FMRP and ADAR enzymes in mammals, focusing mainly on ADAR2. We observed an increase in the level of RNA editing of specific neuronal transcripts in the cortex and hippocampus of *fmr1* KO mice. Furthermore, using *in vitro* and *ex vivo* approaches we show that ADAR2 and FMRP physically interact in neuronal and non-neuronal cells in an RNA-independent manner. Consistent with the nuclear localization of ADARs, we could detect FMRP in the nuclei. These data reveal a possible novel nuclear function of mammalian FMRP in modulating ADAR enzymes. Because its absence leads to an increase of RNA editing, we suggest that an upregulation of RNA editing might contribute to the synaptic dysfunctions observed in FXS patients.

## Results

### *Fmr1* KO mice show an increase in RNA editing of neuronal mRNAs

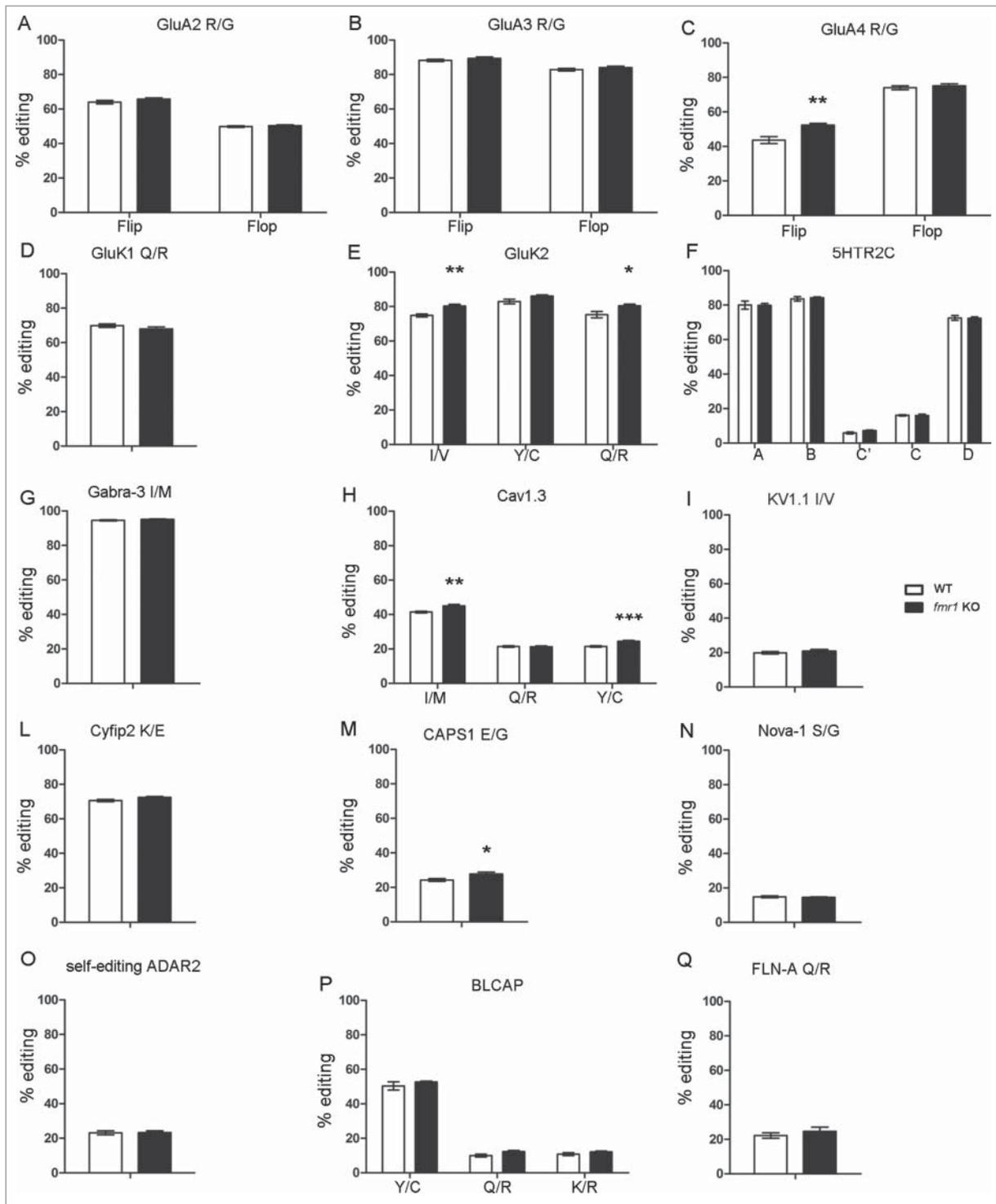
To determine whether FMRP might influence ADAR activity, the editing efficiency of different ADAR substrates was analyzed in frontal cortex (FC) and in the hippocampus (HC) of *fmr1* KO mice, using a gene candidate approach. Neuronal mRNAs involved in synaptic plasticity and/or known to be affected in FXS has been analyzed. In particular the re-coding editing sites of AMPA receptors subunits GluA2, GluA3, GluA4, Kainate receptor subunits GluK1–2, 5-hydroxytryptamine receptor 2C (5-HT<sub>2c</sub>), GABA(A) receptor subunit  $\alpha$ 3 (GABRA3), Calcium Channel, Voltage-Dependent, L Type,  $\alpha$

1D Subunit (Cav1.3), Potassium Channel, Voltage Gated Shaker Related Subfamily A, Member 1 (KV1.1), Cytoplasmic FMR1-interacting protein 2 (CYFIP2), Calcium-Dependent Activator Protein For Secretion 1 (CAPS1), Neuro-Oncological Ventral Antigen 1 (NOVA-1), ADAR2, Bladder Cancer Associated Protein (BLCAP), Filamin-a (FLN-A) were analyzed. The AMPA receptor R/G editing sites were analyzed in combination with the splicing variants called flip and flop.

In FC a mild but statistically significant increase of RNA editing level in the *fmr1* KO mice compared with the wild type was observed for: the GluA4 R/G site in the flip isoform (WT:  $43.6 \pm 1.99$ ; KO:  $52.4 \pm 1.00$ ,  $p < 0.01$  Fig. 1C), the GluK2 I/V site (WT:  $74.9 \pm 0.88$ ; KO:  $80.3 \pm 0.99$ ,  $p < 0.01$  Fig. 1E), the GluK2 Q/R site (WT:  $75.3 \pm 1.83$ ; KO:  $80.4 \pm 0.93$ ,  $p < 0.05$  Fig. 1E), the Cav 1.3 I/M site (WT:  $41.4 \pm 0.54$ ; KO:  $45 \pm 0.8$ ,  $p < 0.01$  Fig. 1H), the Cav 1.3 Y/C site (WT:  $21.5 \pm 0.4$ ; KO:  $24.5 \pm 0.5$ ,  $p < 0.001$  Fig. 1H) and CAPS1 E/G (WT:  $24.2 \pm 0.79$ ; KO:  $27.7 \pm 1.05$ ,  $p < 0.05$  Fig. 1M). In the HC an upregulation of RNA editing level for the GluA2 R/G site in the flop isoform (WT:  $36.4 \pm 0.78$ ; KO:  $39.0 \pm 0.82$ ,  $p < 0.05$  Fig. 2A), the GluK2 I/V site (WT:  $67.6 \pm 0.80$ ; KO:  $73.5 \pm 1.82$ ,  $p < 0.05$  Fig. 2E), the Cav 1.3 I/M site (WT:  $35 \pm 0.87$ ; KO:  $42.3 \pm 0.79$ ,  $p < 0.001$  Fig. 2H), the Cav 1.3 Y/C site (WT:  $18.4 \pm 0.71$ ; KO:  $23.7 \pm 1.16$ ,  $p < 0.01$  Fig. 2H) and CAPS1 E/G (WT:  $23.3 \pm 0.45$ ; KO:  $27.1 \pm 1.30$ ,  $p < 0.05$  Fig. 2M) was observed in the *fmr1* KO mice compared with control. Notably, while the level of editing is increased for GluK2 and Cav1.3 editing levels in both areas, a cortical specific upregulation was observed for GluA4 R/G and hippocampal upregulation for GluA2 R/G, suggesting a brain specific effect of FMRP. Because absence of FMRP in both in FC and HC leads to an increase of RNA editing, it is tempting to hypothesize that FMRP might function as an inhibitor of ADAR activity at specific editing sites. ADAR2, Bladder Cancer Associated Protein (BLCAP) and Filamin-A editing site (FLNA) were also analyzed, but no statistically significant variations were observed (Fig. 1O, P, Q and 2O, P, Q).

To further understand the modifications in RNA editing induced by the absence of FMRP, we focused our attention on Cav1.3 mRNA that showed statistically significant variations in 2 out of 3 editing sites both in HC and in FC regions. Combinations of the edited nucleotides might generate different protein isoforms.<sup>37</sup> Interestingly, in HC a downregulation of the unedited IQDY isoforms could be detected with a parallel increase of the double edited MQDC isoforms (Supplemental Figure S1A); in FC the downregulation of IQDY isoforms was detected in parallel with the upregulation of the single edited MQDY and only partially of MQDC (Supplemental Figure S1B). Moreover, we tested also possible modulation in the frequency of 5-HT<sub>2c</sub> edited isoform, although no difference were present in the single editing sites. Editing at the 5 5-HT<sub>2c</sub> editing sites can generate up to 24 receptor isoforms, ranging from the completely unedited (INI) to the fully edited form (VGV). However, no statistically significant alteration were detected (Supplemental Table 1).

Although FMRP loss induced an upregulation of RNA editing level, this effect is not present on all editing site analyzed and it is slightly area specific. Next, we tested if FMRP absence might modulate the expression level of ADAR1 or ADAR2 mRNAs (Supplemental Figure S2). No statistical significant

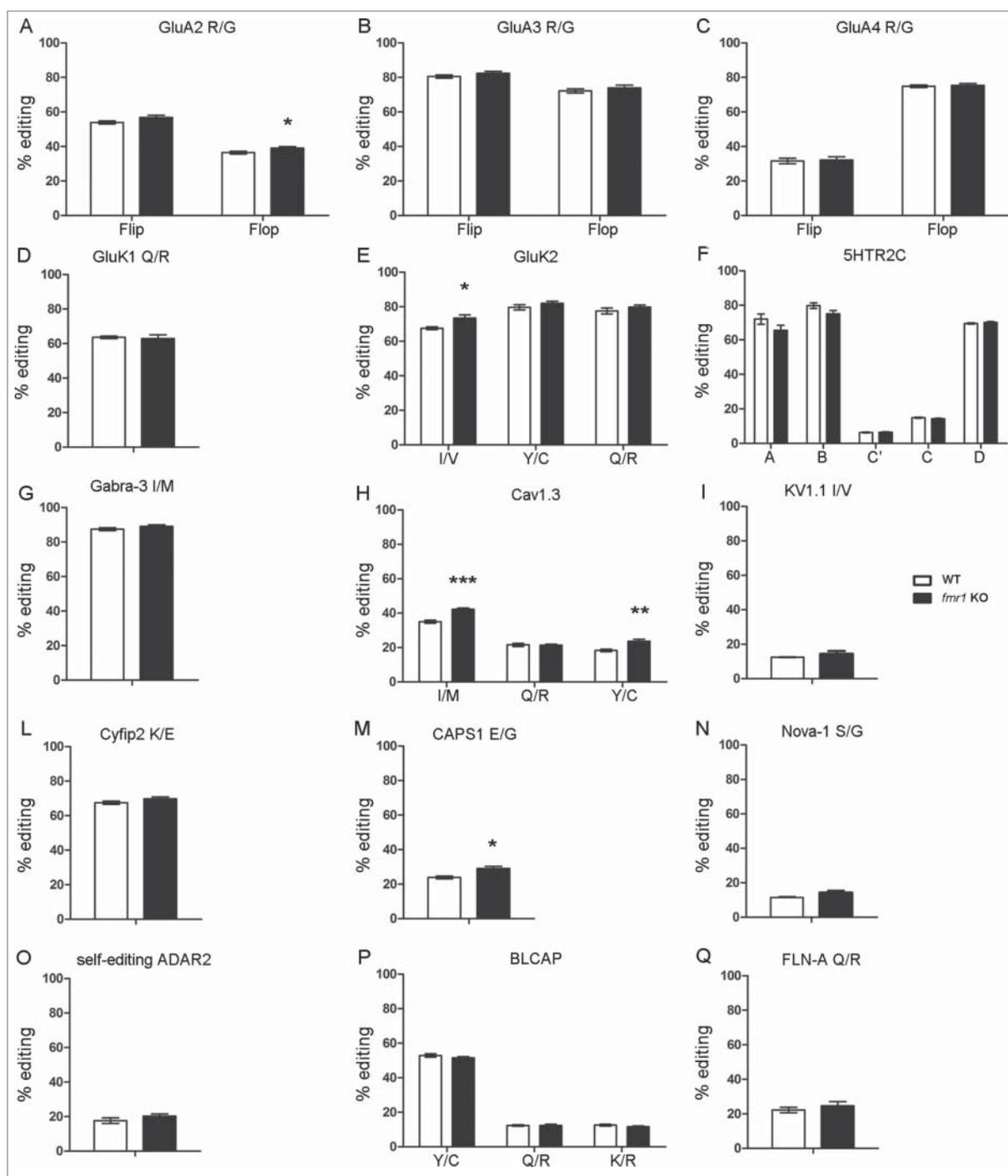


**Figure 1.** RNA-editing levels in Frontal Cortex of wild type (white bars) and *fmr1* KO (black bars) 3-week-old mice. The transcript and the edited site analyzed is reported above the graph. Data are presented as means  $\pm$  SEM ( $n = 8$ ). Statistical analysis was performed using unpaired t test with Welch's correction (\* $p < 0,05$ ; \*\* $p < 0,01$ ; \*\*\* $p < 0,001$ ).

expression variations were determined for either enzymes, although a trend for increase (up to 20%) could be detected for both enzymes in *fmr1* KO mice, both in FC and in HC. We then analyzed the expression pattern of several editing stimulatory factors such as Split Hand/Foot Malformation 1 (SHFM1), and the RNA binding protein hnRNP A2/B1 (hnRNPA2/B1), as well as inhibitory factors such as ribosomal protein S14 (RPS14) and Serine/Arginine-Rich Splicing Factor 9 (SRSF9)

(Supplemental Figure S3) which can modulate ADAR activity.<sup>38,39</sup> Only the inhibitory factor RPS14 mRNA showed a statistically significant decrease (Supplemental Figures S3A), only in the HC of the *fmr1* KO mice. These data might suggest that FMRP loss might influence other members involved in the regulation of RNA editing reaction.

We then tested if a difference in the expression level of FMRP and ADAR2 in HC and FC brain areas might correlate



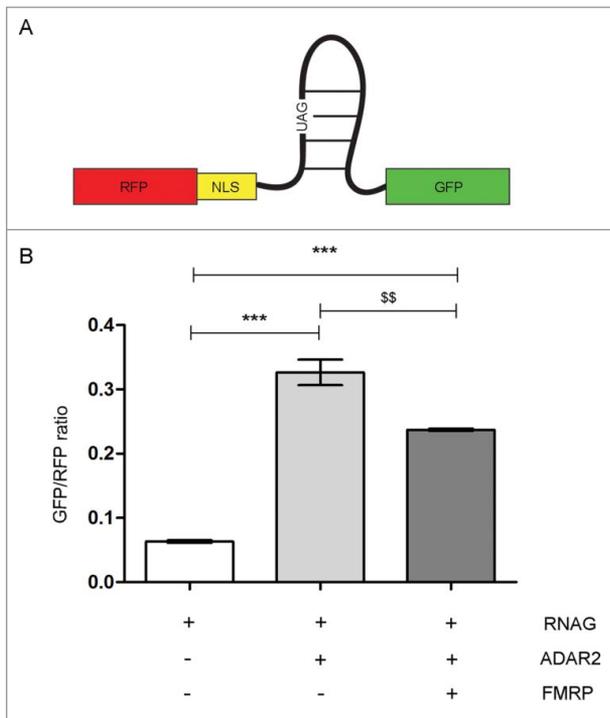
**Figure 2.** RNA-editing levels in Hippocampus of wild type (white bars) and *fmr1* KO (black bars) 3-week-old mice. The transcript and the edited site analyzed is reported above the graph. Data are presented as means  $\pm$  SEM ( $n = 8$ ). Statistical analysis was performed using unpaired t test with Welch's correction (\* $p < 0,05$ ; \*\* $p < 0,01$ ; \*\*\* $p < 0,001$ ).

with the specific RNA editing modulation; however no clear variations in the mRNA expression level were reported. (Supplemental Figure S4).

### FMRP inhibits ADAR2 activity

To explore a possible role of FMRP in inhibiting ADAR activity, we performed an *in vitro* editing assay (Tariq et al., 2013) in HEK293T cells stably expressing ADAR2 and transiently expressing FMRP

and an editable target. The target is generated by a vector (RNAG) expressing RFP and GFP proteins separated by an editable stop codon (Fig. 3A, see methods for details). Upon editing, the stop codon is converted to a tryptophan codon, allowing the expression of GFP. The ratio between GFP and RFP fluorescence indicated the level of editing (Fig. 3B). HEK293T cells transiently transfected only with the construct RNAG showed a GFP/RFP ratio of  $0.06 \pm 0.019$ ; this is consistent with the low levels of editing previously observed in this cell line.<sup>40</sup> HEK293T cells expressing stably



**Figure 3.** Editing Assay in HEK293T cells. (A) Scheme of an editing reporter construct (RNAG) constitutively expressing RFP and GFP only after editing of an amber-stop codon to a tryptophan codon (UAG→UGG). (B) FACS analysis of transfected HEK293T. Increase of the green to red fluorescence ratio indicates increase in editing level. All cells are transfected with the reported construct RNAG. White bar: HEK293T cells transfected with RNAG vector alone; Gray bar: HEK293T cells transfected with RNAG and ADAR2 vectors; Dark gray bar: HEK293T cells transfected with RNAG, ADAR2 and FMRP vectors. Data are presented as means ± SEM. Statistical analysis was performed using one-way ANOVA (\*\**p* < 0,001; <sup>§§</sup>*p* < 0,01). NLS: nuclear localization signal.

ADAR2 (HEK293T/ADAR2) with showed an increase GFP/RFP ratio ( $0.32 \pm 0.019$ , *p* < 0.001). In HEK293T/ADAR2 cells expressing exogenous FMRP, a downregulation of the levels of editing was observed, as shown by a reduction of the GFP/RFP ratio ( $0.24 \pm 0.002$ , *p* < 0.001 vs only RNAG transfected cells, *p* < 0.01 vs HEK293T/ADAR2 cells). This result supports a role for FMRP as inhibitor of ADAR2 activity.

## FMRP interacts directly with ADAR2

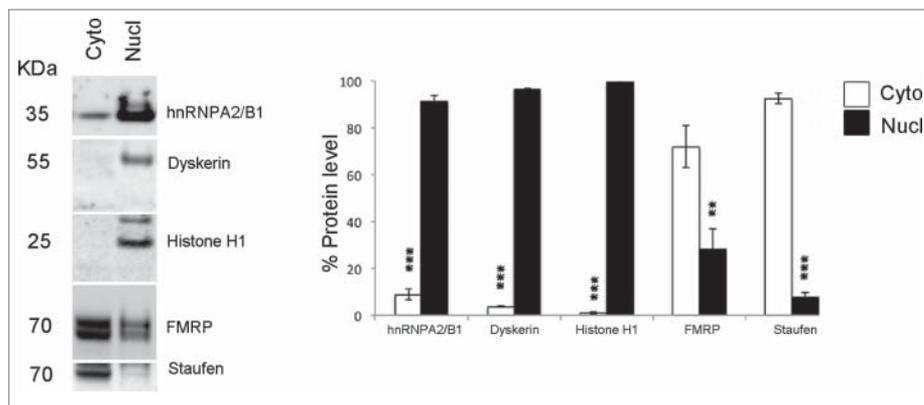
ADARs are mainly nuclear proteins<sup>28</sup> while FMRP localization has been largely described to be cytoplasmic.<sup>3,41</sup> To test a possible physical interaction between FMRP and ADAR, we first studied the cytoplasmic and nuclear distribution of FMRP. As reported in Fig. 4, FMRP is present in both cytoplasmic (high levels) and nuclear (low levels) fractions. The enrichment of the 2 fractions is shown by the presence of the RNA binding protein Staufen mainly in the cytoplasmic fraction and Dyskerin and Histone H1 in the nuclear fraction.

The ADAR2-FMRP co-localization was at first analyzed *ex vivo* with double-immunogold Electron Microscopy (EM) (Fig. 5 and Supplemental Figure 5) in hippocampal sections. The co-localization of ADAR2 and FMRP occurs mainly in the nucleus (red boxes in panels A and C and red arrows in panels B and D) and only a few FMRP-ADAR2 dots were detected in the cytoplasm, where the majority of FMRP (smaller dots) is localized.

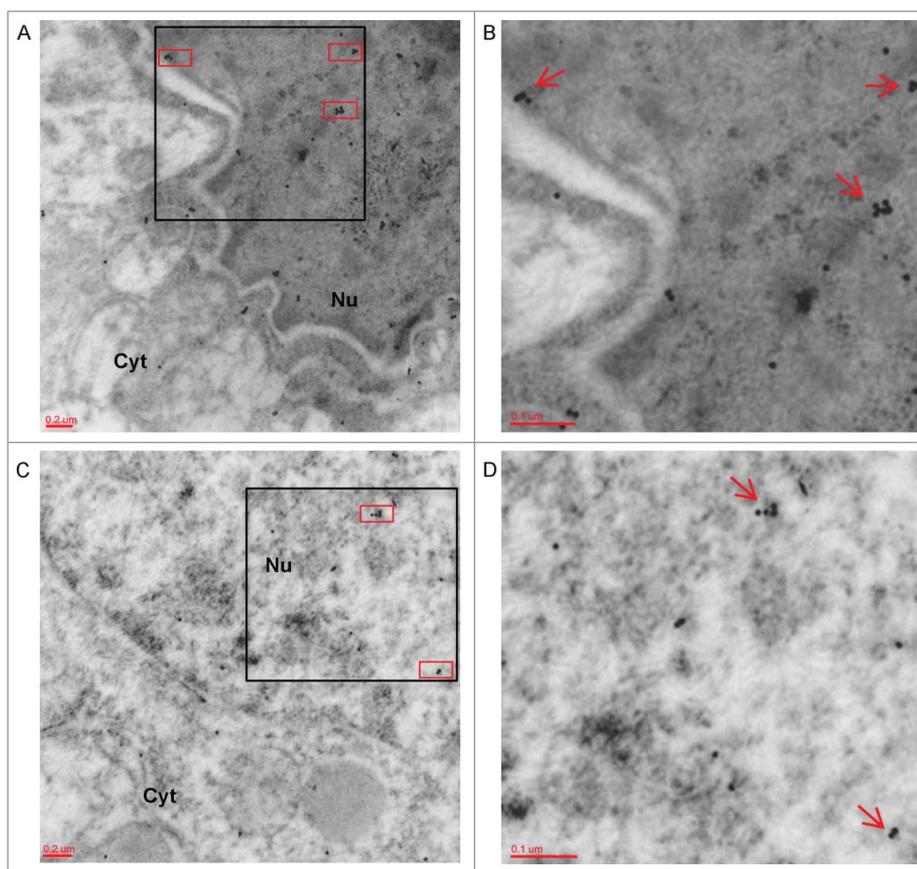
Moreover, the endogenous ADAR2-FMRP interaction was analyzed in primary neurons at DIV 14 using the Proximity Ligation Assay (PLA)<sup>42</sup> (Fig. 6). Several dots that represent the interaction of FMRP with ADAR2 were observed mainly in the nuclei (Fig. 6A-B). The PLA for a known FMRP interacting protein FXR1P,<sup>43</sup> showed several dots mainly in the cytoplasm (Fig. 6C); without primary ADAR2 antibody processed samples didn't show any dot (Fig. 6D).

These findings were further confirmed in HEK293T non-neuronal cells expressing stably ADAR2-HA and upon the transient exogenous expression of FMRP-Myc. PLA fluorescent signals, using primary antibody against HA and Myc, were detected mainly in the nuclei (Supplemental Figure 6A-B) indicating FMRP-ADAR interaction. A few rare dots were also observed in the cytoplasm possibly due to the overexpression of both proteins. On the other hand, no signals were detected in not-transfected HEK293T cells (Supplemental Figure 6C-D) or in HEK293T/ADAR2 cells overexpressing  $\alpha$ -synuclein (Supplemental Figure 6E-F), that does not interact with ADAR2.

To further corroborate the interaction of ADAR2 and FMRP, we performed co-immunoprecipitation experiments



**Figure 4.** Cytoplasm/nucleus fractionation of FMRP. Cytoplasmic and nuclear fractions were prepared from the P21 mouse cortex. (Left) Western blotting showing the distribution of FMRP in cytoplasmic (Cyto) and nuclear (Nucl) fractions. hnRNPA2/B1, Dyskerin, histone H1 proteins (markers of nucleus) are present only or mainly in nuclear fraction, while the cytoplasmic protein Staufen is present in the cytoplasmic fraction. (Right) The histogram shows the quantification of 4 independent experiments. FMRP is present in both cytoplasmic (high levels) and nuclear (low levels) fractions. Data are presented as means ± SEM. Statistical analysis was performed using Student's t test (\*\**p* < 0,01; \*\*\**p* < 0,001).



**Figure 5.** Co-localization of FMRP and ADAR2 in hippocampal sections by electron microscopy. (A-C) Two representative electronic micrographs of a double-immunogold for FMRP (labeled by 10 nm gold particles) and ADAR2 (labeled by 15 nm gold particles) performed on hippocampal ultra-thin sections of adult male mice. The red boxes highlight the co-localization of FMRP and ADAR2 proteins inside the nucleus (Nu). Magnification: 20000 x (B) 30000 x magnification of black box showed in panel A. Red arrows show FMRP/ADAR2 co-localization. (D) 30000 x magnification of black box in panel C. Red arrows show FMRP/ADAR2 co-localization. Nu: Nucleus; Cyt: Cytoplasm.

from cortical tissues of WT and *fmr1* KO mice (Fig. 7). FMRP can be clearly detected in ADAR2 immunoprecipitates (Fig. 7 A) in WT but not in *fmr1* KO extracts, confirming the specificity of the interaction. Moreover, the result was not altered by treatment with RNaseA showing that ADAR2 and FMRP interact in a RNA independent manner (Fig. 7 B).

## Discussion

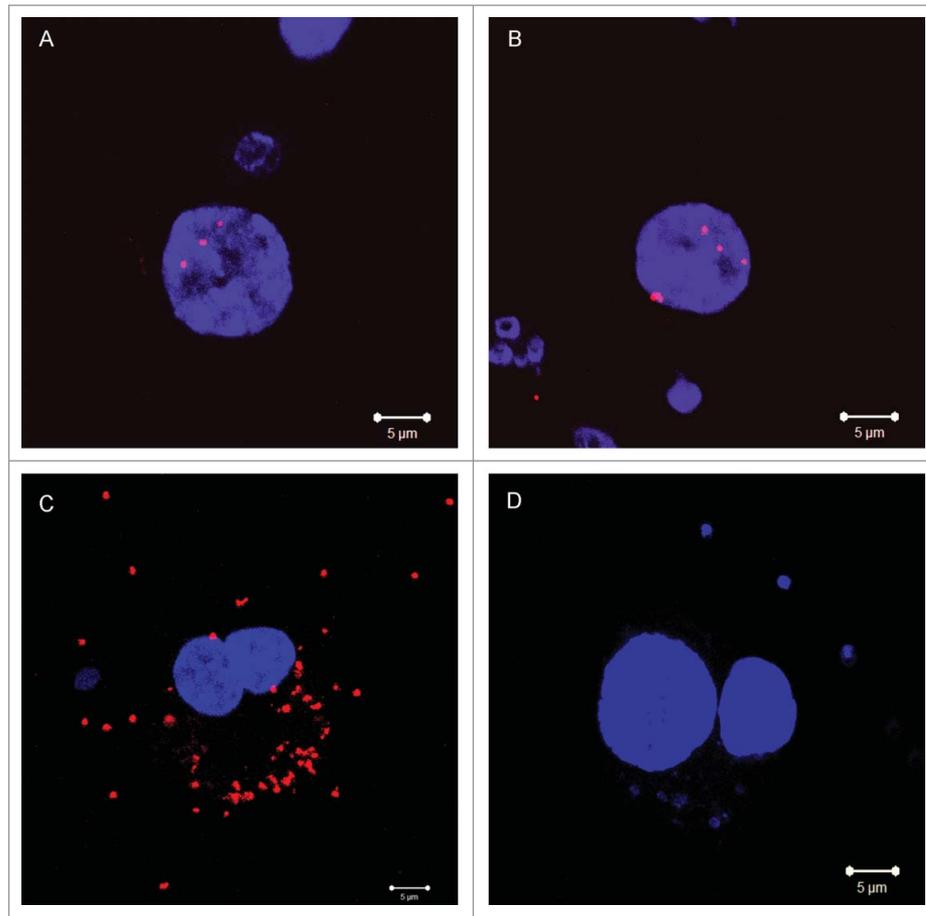
Here we report, for the first time in mammals, that the absence of FMRP increases ADAR enzymes activity both in FC and HC, as measured by enhanced editing level of specific sites. These changes, although of limited magnitude, might be of interest since the edited mRNAs encode for proteins involved in the synaptic structure and function.

Overexpression or downregulation of *Drosophila* FMRP has been shown to change the editing efficiency of specific dADAR targets involved in synaptic transmission.<sup>26</sup> Moreover, zebrafish FMRP was shown to regulate RNA editing, synaptic density and locomotor activity in zebrafish.<sup>36</sup> If confirmed also in mammals, these data will clearly indicate the existence of a new and evolutionarily conserved FMRP activity that might be dysregulated in FXS pathogenesis.

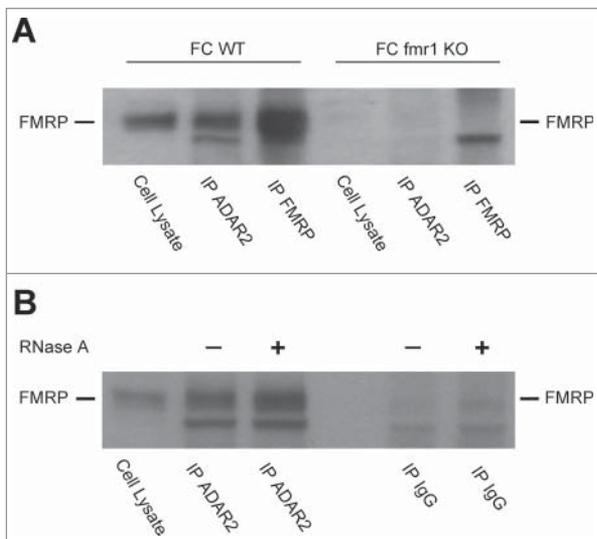
We investigated the ADAR-FMRP interaction in mammals and we showed that loss of FMRP leads to a mild increase of

ADAR activity both in FC and HC of *fmr1* KO mice, resulting in increased editing level. Although the changes in editing are moderate, these could result in considerable functional consequences.<sup>32,44,45</sup> In particular, the increased editing levels of the R/G site for GluA2 in flop isoform in HC and for GluA4 in flip isoform in FC, imply a faster recovery rate from desensitization compared with the wild type mice.<sup>46</sup> Thus, the increased editing in these sites could enhance synaptic strength.<sup>46</sup> Moreover, we found increased editing levels for the I/V site of GluK2 in both tissues. This editing site is located in the first transmembrane domain of the subunit and the editing process seems to be involved in a finely tuned regulation of ion permeability of the channel,<sup>47</sup> together with the modulation of editing levels for GluK2 Q/R site, which is increased in both FC of KO mice. Of note, the zebrafish *fmr1* *-/-* shows changes in editing levels of glutamate receptor transcripts, leading to altered synaptic strength and morphology and ultimately defects in locomotor activity. Taken together, these data indicate that loss of mammalian FMRP might alter glutamate receptor excitability via modification of RNA editing efficacy.

Furthermore, we detected increased editing levels for Cav 1.3 subunit, in the I/M and Y/C editing sites both in FC and HC. The editing sites are all ADAR2 specific and they are located in the so called IQ domain of the subunit, a calmodulin-binding site mediating inhibitory Ca<sup>2+</sup>-feedback (CDI) on



**Figure 6.** Detection of endogenous ADAR2 and FMRP interaction by Proximity Ligation Assay (PLA) in mouse primary cortical neuron cultures. (A-B) PLA dots for ADAR2 and FMRP show mainly a nuclear localization. (C) PLA dots for FMRP and FXR1P interaction are in the cytoplasm; (D) Without-primary-ADAR2 antibody processed samples do not show any dot in PLA experiment. Scale bars 5 $\mu$ m.



**Figure 7.** ADAR2-FMRP interaction determined by co-immunoprecipitation experiments. (A) FMRP western blot on frontal cortex total cell lysate prior and after immunoprecipitation with ADAR2 and FMRP antibodies. Both WT and *fmr1* KO murine FC were analyzed. (B) ADAR-FMRP interaction is RNA independent. FMRP western blot of frontal cortex total cell lysate from WT mice prior and after immunoprecipitation with ADAR2 and rabbit IgG antibodies treated or not with RNase A.

channels. The increase of editing levels in these 2 sites leads to downregulation of the IQDY isoform expression, with a parallel increase of the double edited MQDC isoform in HC and of both the single edited MQDY and MQDC edited isoforms in FC. Edited channels exhibit a strong reduction of the CDI,<sup>37</sup> leading to a prolonged activation of the channel itself and increased load of calcium. This feature might be linked to the imbalance of the excitatory and inhibitory synaptic pathways described in patients with FXS.<sup>3</sup>

In addition, E/G editing level of CAPS1 was found to be increased both in FC and HC; the encoded protein is a cytosolic and peripheral membrane protein required for Ca<sup>2+</sup> regulated exocytosis of dense-core vesicles carrying neurotransmitters and neuropeptides.<sup>48</sup> A recent paper<sup>49</sup> for the first time, investigated the functional role of RNA editing at this site, generating mutant mice expressing solely the edited CAPS1. The mutant mice were leaner and exhibited increased physical activity due to augmented neurotransmitters release, most probably dopamine.<sup>49</sup> It is intriguing to speculate that FXS patient hyperactivity behavior might be in part due to increased RNA editing at CAPS1 site. Moreover a recent paper<sup>50</sup> demonstrates a reduced BDNF release following exocytosis in dendrites of CAPS1-deficient neurons; accordingly, it is possible to speculate that dysregulation in CAPS1 editing could also be linked to the neuronal

spine dysmorphogenesis found on postmortem brains from patients lacking FMRP.<sup>51</sup>

Our data indicate a role of FMRP in the inhibition of the editing process, a mechanism that participate in the physiologic modulation of synapses in the CNS. The way in which ADAR enzymes and FMRP might interact in modulating RNA editing process is not known in mammals. dFMRP and dADAR interact in the nucleus possibly to regulate RNA editing, and dADAR acts downstream FMRP to modulate synaptic morphology of *Drosophila* neuromuscular junction.<sup>26</sup> Similarly, in zebrafish ADAR and FMRP seem to biochemically interact as shown by co-immunoprecipitation experiments, but it is not known if this interaction occurs in the nucleus or in the cytoplasm.<sup>36</sup>

Using *in vitro* PLA experiments and double-immunogold Electron Microscopy in brain slices we showed that mammalian FMRP and ADAR2 interact in the nucleus. Moreover, a functional interaction has resulted from the *in vitro* RNA Editing Assay, confirming an inhibiting role of FMRP on ADAR2 activity. In addition, co-immunoprecipitation experiment and RNase treatment, showed that ADAR2 and FMRP interact in an RNA-independent manner.

It is tempting to hypothesize that FMRP might inhibit the recognition of specific RNA secondary structures by ADAR. Furthermore, ADAR2 mRNA has been reported as a FMRP target<sup>14,52</sup> indicating that FMRP might modulate also ADAR activity by regulating its RNA metabolism; however, the global mRNA expression level of ADARs enzymes in *fmr1* KO mice is not altered.

Because ADAR2-FMRP interaction is RNA-independent, RNA editing inhibition might occur through protein-protein interaction.

We speculate that the ADAR2- FMRP complex might be assembled on specific editing sites repressing the editing reaction or that FMRP might sequester ADAR2 inhibiting its capability to bind and edit target RNAs, as already proposed for other ADAR co-factors.<sup>39</sup> Further studies are needed to address these possibilities.

ADAR enzymes have a main role in the maintenance of the central nervous system homeostasis, given that their absence results in severe neurologic defects or it could be lethal, as shown by animal models.<sup>53,54</sup> Although the changes in RNA editing reported in the absence of FMRP in *Drosophila*, zebrafish and, in the present work, in the mouse are moderated, they occur in evolutionally conserved transcripts that are stringently regulated. Accordingly, only mild alterations in RNA editing level have been reported in patients suffering of neurologic disorders such as ALS, epilepsy, schizophrenia and depression.<sup>55</sup>

In conclusion, our data support the presence of a nuclear function of FMRP in the regulation of RNA editing and suggest that a dysregulation of this mechanism might contribute to the FXS pathogenesis.

## Materials and methods

### Animal care

Animal care was conducted conforming to the institutional guidelines that are in compliance with Italian national (DL

N116, GU, suppl 40, 18–2–1992) and international laws and policies (European Community Council Directive 86/609, OJ L 358, 1, December 12, 1987; National Institutes of Health Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996). The *fmr1*-FBV KO and FVB wild type mice were used.

### RNA extraction and retro-transcription reaction

Total RNA was extracted from frontal cortex (FC) and hippocampus (HC) of P21 mice (n = 8 per group) using the TRIzol<sup>®</sup> Reagent (Thermo Fisher Scientific), according to the manufacturer instructions. Reverse transcription was performed using Moloney Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT) (Thermo Fisher Scientific). Briefly, 2  $\mu$ g of total RNA was mixed with 2.2  $\mu$ l of 0.2 ng/ $\mu$ l random hexamer (Thermo Fisher Scientific), 10  $\mu$ l of 5  $\times$  buffer (Thermo Fisher Scientific), 10  $\mu$ l of 2 mM dNTPs, 1  $\mu$ l of 1 mM DTT (Thermo Fisher Scientific), 0.4  $\mu$ l of 33 U/ $\mu$ l RNasin (Promega, Madison, WI, USA) and 2  $\mu$ l MMLV-RT (200 u/ $\mu$ l) in a final volume of 50  $\mu$ l. The reaction mixture was incubated at 37°C for 2 h, and then the enzyme was inactivated at 75°C for 10 min.

### RNA editing quantification

The levels of editing in re-coding sites of AMPA receptors subunits GluA2, GluA3, GluA4, Kainate receptor subunits GluK1–2, 5-hydroxytryptamine receptor 2C (5-HTR2c), GABA(A) receptor subunit  $\alpha$ 3 (GABRA3), Calcium Channel, Voltage-Dependent, L Type,  $\alpha$  1D Subunit (Cav1.3), Potassium Channel, Voltage Gated Shaker Related Subfamily A, Member 1 (KV1.1), Cytoplasmic FMR1-interacting protein 2 (CYFIP2), Calcium-Dependent Activator Protein For Secretion 1 (CAPS1), Neuro-Oncological Ventral Antigen 1 (NOVA-1), ADAR2 self editing site, Bladder Cancer Associated Protein (BLCAP), Filamin-a (FLN-A) were analyzed. The quantification was performed by sequence analysis as described previously<sup>56</sup> using Discovery Studio (DS) Gene 1.5 (Accelrys Inc., San Diego, CA, USA).

### In vitro RNA editing assay

For RNA editing assay a vector expressing RFP and GFP proteins separated by an editable stop codon, called RNAG, was used (gift from Prof. Jantsch). Briefly, the stem-loop containing the R/G editing site of glutamate receptor subunit B was modified to contain an amber stop codon at the editing site.<sup>39,38</sup> The substrate stem-loop was cloned between the red fluorescent protein (RFP) and green fluorescent protein (GFP) ORFs. The transient transfection of this construct induces the constitutive expression of RFP. The stop codon in the loop prevents GFP translation without editing process; otherwise, the increase of editing levels leads to a conversion of the stop codon to a tryptophan codon inducing the production of GFP. The ratio between the GFP and RFP fluorescence indicates the editing levels in the cell population: if the editing activity increases, the GFP expression increases as well. After 24h from the transfection of the HEK293T cell lines with the RFP/GFP vector, the

samples were collected and maintained in PBS EDTA 2 mM until the FACS analysis was performed.

The samples were read on a MACSQuant flow cytometer (Miltenyi Biotec) and analyzed with FlowJo (Tree Star Inc., Ashland, USA). Editing efficiencies were determined by calculating the ratio of green to red arithmetic mean fluorescence of cells with solid RFP expression as described previously.<sup>57</sup> At least 10000 events were collected for each sample. Statistical analysis has been performed on triplicate experiments.

### Nuclear and cytoplasmic fractionation

Cortices from P21 mice were resuspended in fractionation buffer (75 mg in 600  $\mu$ l). Cytoplasmic and nuclear fractions were prepared using the Paris kit (Ambion).

### Western blotting

For Western blot analysis standard methodologies were used. Protein samples were separated by SDS-PAGE electrophoresis and blotted on a PVDF membrane (Millipore). Membranes were incubated using specific antibodies: mouse anti-hnRNP A2B1 antibody (1:1000 Abcam), Dyskerin (1:1000, kindly provided by Yves Henry, University Paul Sabatier Toulouse), Mouse Anti-Histone H1 (1:1000 SIGMA), Rabbit anti-Staufen (1:1000 Abcam), rabbit anti-FMRP (1:1000, Ferrari et al. 2007).

HRP-conjugated anti-rabbit, anti-mouse antibodies (1:10000) were purchased from Promega or Chemicon. Proteins were revealed using an enhanced chemiluminescence kit (GE Healthcare) and the imaging system LAS-4000 mini (GE). Quantification was performed using the IQ ImageQuant TL software (GE Healthcare). The amount of analyzed proteins was normalized by Coomassie blue staining.

### Electron microscopy

Transverse hippocampal sections from 3-month-old mice were fixed 3 hours at 4°C in a mixture of 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6, dehydrated in alcohol at progressively higher concentrations and embedded in Bioacryl resin (British Biocell, Cardiff, United Kingdom), followed by UV polymerization, according to standard procedures.<sup>58</sup> Consecutive thin and ultrathin sections were cut using a diamond knife (Diatome, Biel, Switzerland) on a Reichert ultramicrotome (Depew, NY, USA). Ultrathin sections (80–90 nm) were collected on 300 mesh nickel grids. To block non-specific binding sites, these grids were treated with a blocking buffer made of phosphate buffer saline supplemented with 0.1% Tween-20, 0.1% bovine serum albumin and 4% normal goat serum and incubated overnight at 4°C with a rabbit polyclonal anti-FMRP<sup>59</sup> and a rabbit anti-ADAR2 (Abcam; cod. ab64830) primary antibodies. The grids were then incubated for 1 hour with goat anti-rabbit IgG conjugated with 10 and 15 nm colloidal-gold particles (British Biocell, Cardiff, United Kingdom), counterstained for 5 min in 4% uranyl acetate (in 70% ethanol) to evidentiate the cell morphology, and observed with EM 109 Zeiss (Oberkochen, Germany). A without-primary-antibody negative control was processed in parallel. Photographs were taken with GATAN Orius SC200 TEM CCD camera.

### Primary neuronal and HEK293T cell cultures

Mouse primary cortical cultures were prepared as described previously.<sup>60</sup> Briefly, mouse cerebral cortices from day 13.5 mouse embryos were mechanically dissociated in cold HBSS containing 10mM HEPES (Invitrogen); the cell suspension was re-suspended in serum-free Neurobasal medium (Invitrogen) supplemented with B-27 (Invitrogen), 30 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), 30  $\mu$ g/ml streptomycin (Sigma-Aldrich) and 0.5 mM Glutamax (Invitrogen). The neurons were then plated at a density of 30,000 cells/cm<sup>2</sup> on a poly-D-lysine coating (Sigma-Aldrich) in multi-well plates. Three days after plating, 50% of the medium was replaced with fresh medium; subsequently, half of the medium was replaced once a week for a maximum of 4 weeks.

Stable HEK293T expressing HA tagged ADAR2 enzyme, generated after viral infection with modified pRRLSIN.cPPT.PGK-GFP.WPRE vector (addgene: 12252), were obtained after cloning selection of HA-positive colonies.

HEK293T cell lines were cultured at 37°C and 5% CO<sub>2</sub> in DMEM medium (Invitrogen) supplemented with 10% of heat-inactivated fetal bovine serum (FBS), 30 U/ml penicillin (Sigma-Aldrich), 30  $\mu$ g/ml streptomycin (Sigma-Aldrich), 1% minimum Eagle's medium nonessential amino acids, 1 mM sodium pyruvate.

### Transient Transfection

HEK293T cells are plated 24h before transfection at a density of 30'000 cell/cm<sup>2</sup> in 6-well plates; the medium was changed 2h before transfection. 2.5 $\mu$ g of the plasmid of interest (pcDNA3.1-FMR1-myc-his<sup>43</sup>; pcDNA3.1- $\alpha$ -synuclein<sup>61</sup>; RNAG<sup>39</sup>) were mixed with 0.1  $\times$  TrisEDTA (TE 0.1  $\times$ )/dH<sub>2</sub>O (2:1) and 2.5M CaCl<sub>2</sub>; the mixture is maintained 5 min at RT. The precipitate is formed by adding dropwise 2  $\times$  HBS solution to the mixture, then the suspension should be added immediately to the cells. The calcium-phosphate plasmid DNA mixture should be allowed to stay on the cells for 14–16h, after which the medium should be replaced with fresh medium.

### Proximity Ligation Assay (PLA)

The Duo-link<sup>®</sup> using PLA Technology<sup>®</sup> kit (Sigma-Aldrich) was used for the proximity ligation assay, accordingly to the manufacturer instructions with minor modifications. Briefly, the cells were fixed with paraformaldehyde 4% (PFA); in particular neurons were fixed at DIV14. Each sample was permeabilized with PBS-Triton 0.3% and then incubated with the blocking solution (Roche<sup>TM</sup>) for about 45 min at room temperature; the primary antibodies incubation was performed overnight at 4°C with mouse anti-FMRP (Merck Millipore cod. MAB2160), rabbit anti-FXR1P (Abcam; cod. ab129089) and rabbit anti-ADAR2 (Abcam; cod. ab64830) for endogenous PLA experiments in neuronal cultures; rabbit anti-HA (SIGMA; cod. H6908) and mouse c-Myc (Santa Cruz Biotechnology; cod. SC40), recognizing the HA tag for ADAR2 and the Myc tag for FMRP respectively, mouse anti  $\alpha$ -synuclein (Santa Cruz cod. sc-12767), for exogenous PLA experiments on transfected HEK293T cells (Supplemental results). On the following

day, the samples were washed 3 times in PBS at room temperature and then the cells were incubated 1h at 37°C with the PLA probe containing the secondary antibodies conjugated with the DNA probes. After PLA probe removal, the samples were washed 4 times x 10' with the Buffer A at 37°C. After a brief wash with Buffer A at 37°C, the samples were incubated with the ligation buffer containing oligonucleotides that hybridize to the PLA probe and the DNA ligase which allows the annealing between probe and oligonucleotides to form a rolling circle DNA strand. This reaction was incubated for 30 min at 37°C. Subsequently the cells were washed with Buffer A at 37°C and then incubated with the amplification-detection solution containing the DNA polymerase for rolling circle amplification (100 min at 37°C). Next, the samples were washed 4 times with Buffer B at room temperature; then the coverslips were incubated for 10 min with mounting buffer containing DAPI and analyzed with a confocal microscope.

### Co-immunoprecipitation experiments

FC tissues for WT and *fmr1* KO mice at P21 were lysed by sonication in immunoprecipitation buffer (Tris-HCl 50 mM pH 7.4, NaCl 300 mM, 1% Triton X-100, Protease inhibitor Roche® 1x). The extracts were added to 60 µl of Protein G Dynabeads™ (10007D Invitrogen® by Thermo Fisher Scientific) coupled with 5 µg of rabbit anti-FMRP (Abcam ab17722) or rabbit anti-ADAR2 (Abcam ab64830). After 2h of incubation at 4°C on a rotating wheel, 5 washes with immunoprecipitation buffer were performed. The elution step was performed with 50 µl of sample buffer 2x and DTT 10x; then, the samples were denatured at 75°C for 10 min for Western Blot procedure.

This step was performed using rabbit anti FMRP (Abcam ab17722) 1:500 in 5% non-fat dry milk in TBST 0.2%; the Alkaline Phosphatase (AP)- conjugated anti-rabbit secondary antibody was used 1:10000 in TBST 0.2% (Promega cod. S373B); for both the antibodies the incubation was performed 1h at RT. The RNase A treatment was performed by adding to the extracts 340 µg of enzyme in each sample. Then the above mentioned procedure for the co-IP was followed.

### Statistical analysis

Statistical analysis of the editing data was performed using unpaired t test with Welch's correction while FACS data were analyzed by one-way ANOVA followed by Bonferroni's post-test. Graph pad software was used to perform the analysis and create the graphs

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

### Acknowledgment

This work was supported by MIUR (PRIN 2012 prot.2012A9T2S9\_004) to AB and Fondazione Cariplo, Associazione Italiana Sindrome X Fragile, Telethon GGP15257, Lejeune Foundation to CB. We thank Prof. Keith Murai for the gift of FXRIP vectors; Dr. Arianna Bellucci for the gift of  $\alpha$ -synuclein vector; Prof. Michel Jantsch for the gift of RNAG vector; Prof. Rosa Alba Rana for the support with EM experiments.

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