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Correcting deregulated *Fxyd1* expression ameliorates a behavioral impairment in a mouse model of Rett Syndrome

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Abstract

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder caused by mutations in the *MECP2* gene. Several genes have been shown to be MECP2 targets. We previously identified *FXYD1* (encoding phospholemman; a protein containing the motif phenylalanine-X-tyrosine-aspartate), a gene encoding a transmembrane modulator of the Na,K-ATPase (NKA) enzyme, as one of them. In the absence of MECP2, *FXYD1* expression is increased in the frontal cortex (FC) of both RTT patients and *Mecp2*^{Bird} null mice. Here, we show that *Fxyd1* mRNA levels are also increased in the FC and hippocampus (HC) of male mice carrying a truncating mutation of the *Mecp2* gene (*Mecp2*^{308}). To test the hypothesis that some of the behavioral phenotypes seen in these *Mecp2* mutants could be ameliorated by genetically preventing the *Fxyd1* response to MECP2 deficiency, we crossed *Fxyd1* null male mice with *Mecp2*^{308} heterozygous females and behaviorally tested the adult male offspring. *Mecp2*^{308} mice had impaired HC-dependent novel location recognition, and this impairment was rescued by deletion of both *Fxyd1* alleles. No other behavioral or sensorimotor impairments were rescued. These results indicate that reducing FXYD1 levels improves a specific cognitive impairment in MECP2-deficient mice.

Keywords

Fxyd1; *Mecp2*; learning and memory; Rett syndrome; novel object recognition; novel object location; mouse model

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1. Introduction

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder that ranks as the second most prevalent cause of mental retardation in girls (Hagberg, 1995; Naidu, 1997; Sekul and Percy, 1992). RTT has been shown to be primarily caused by loss of function of the DNA binding protein methyl CpG binding protein 2 (MECP2) (Amir et al., 1999; Bienvenu et al., 2000; Wan et al., 1999)

Because MECP2 is a transcriptional regulator of gene expression, intense efforts have been devoted in recent years to the identification of genes regulated by this protein. The underlying premise of these efforts is that altered expression of MECP2 target genes is responsible for specific aspects of RTT neuropathology, and that identifying these genes will pave the way for efficacious therapeutic intervention. Several genes have been shown to be targets for MECP2, including brain-derived neurotrophic factor (*BDNF*) (Chang et al., 2006; Chen et al., 2003; Martinowich et al., 2003), distal-less homeobox 5 (*DLX5)* (Horike et al., 2005), the Inhibitors of Differentiation genes (*ID1-ID4*) (Peddada et al., 2006), members of the glucocorticoid signaling pathway (Nuber et al., 2005), a component of mitochondrial respiratory complex III (Kriaucionis et al., 2006), corticotropin releasing hormone (McGill et al., 2006), and several others (Bienvenu and Chelly, 2006; Chahrour and Zoghbi, 2007; Gibson et al., 2010). An additional gene is *FXYD1*, which encodes phospholemman, a modulator of the sodium-potassium ATPase (NKA) pump (Crambert and Geering, 2003). *FXYD1* is overexpressed in the frontal cortex (FC) of both RTT patients and $Mecp2^{Bird}$ null mice (Deng et al., 2007), and was shown by two different groups to be a direct MECP2 target gene (Banine et al., 2011; Deng et al., 2007; Jordan et al., 2007).

Despite the identification of an ever increasing number of target genes, the involvement of these genes in the neuropathology of RTT is just beginning to be unraveled [reviewed in (Cobb et al., 2010)]. In general, it appears that activation of MECP2 expression is the most efficacious method of rescuing the neurological impairments of RTT (Cobb et al., 2010; Gadalla et al., 2011; Giacometti et al., 2007; Guy et al., 2007; Luikenhuis et al., 2004; Tropea et al., 2009). Manipulating the abundance of putative MECP2 target gene products, such as BDNF (Chang et al., 2006) and IGF1 (Tropea et al., 2009), and modifying GABAergic/serotoninergic (Abdala et al., 2010) or noradrenergic (Roux et al., 2007) neurotransmission or the glucocorticoid system (Braun et al., 2012) have been shown to rescue some impairments, but not others, suggesting that deregulation of additional, not yet recognized, genes must contribute to these RTT phenotypes.

Here, we considered the possibility that some of the behavioral impairments observed in MECP2 mutant mice are related to *Fxyd1* overexpression. We chose FXYD1 as a potentially relevant candidate, because of several earlier observations suggesting an involvement of FXYD1 in a diversity of neuronal functions, such as maintaining neuronal excitability (Garcia-Rudaz et al., 2008), responding to MECP2 deficiency with increased expression in the FC of RTT patients and $Mecp2^{Bird}$ null mice, and reducing dendritic arborization and spine formation, hallmarks of RTT neuropathology (Deng et al., 2007), when overexpressed in cerebro-cortical neurons. To test this hypothesis we bred *Fxyd1* null male mice (Jia et al., 2005) to heterozygote female mice carrying a mutated *Mecp2* allele, termed $Mecp2^{308}$ (Shahbazian et al., 2002). Using the resulting male offspring we sought to determine if partial and/or total loss of *Fxyd1* expression can rescue the cognitive and sensorimotor impairments detected in $Mecp2^{308}$ mice (De Filippis et al., 2010; McGill et al., 2006; Moretti et al., 2006; Shahbazian et al., 2002).

2. Results

2.1. Deleting Fxyd1 alleles rescues a hippocampus-dependent cognitive impairment in *Mecp2*^{308} mutant mice

Because it is not known if *Fxyd1* mRNA levels are increased in the FC of *Mecp2*^{308} mice as previously shown (Deng et al., 2007) for *Mecp2*^{Bird} null mice, we addressed this issue before performing the behavioral studies. We measured *Fxyd1* mRNA abundance in the FC, HC, and cerebellum (CB) by real-time PCR in adult *Mecp2* WT and *Mecp2*^{308} littermates (Fig. 1A–B). While *Fxyd1* mRNA levels were significantly increased in the FC of *Mecp2*^{308} mice at both 4 and 6 months of age ($p<0.01$ and 0.05, respectively) a significant increase in the HC was only seen at 6 months of age ($p<0.05$). As in *Mecp2*^{Bird} null mice (Deng et al., 2007), the CB of *Mecp2*^{308} mice showed no significant change in *Fxyd1* mRNA abundance. We also examined *Fxyd1* mRNA levels in the male offspring from a cross between *Mecp2*^{308} heterozygous (HT) female mice with *Fxyd1* KO males. As expected, *Fxyd1* mRNA levels were decreased by half in *Fxyd1* HTs and to undetectable levels in *Fxyd1* KOs, regardless of the presence or absence of a normal *Mecp2* allele (Fig. 1C). These changes in gene expression were confirmed by western blotting of the FXYD1 protein (Fig. 1D).

2.2. General health

None of the five mutant genotypes resulting from crossing females *Mecp2*^{308} HT with males *Fxyd1* KOs had any discernible morphological abnormalities of the brain or other organs. Their lifespan was similar to that of WT littermates (data not shown). With regard to body weights, there was a week x genotype interaction ($F = 1.809$, $p = 0.001$). However, when potential effects of genotype effects were analyzed in different weeks, there was a trend towards an effect of genotype in week 13 but this trend did not reach statistical significance ($F = 2.132$, $p = 0.08$) (Table 1).

2.3 Anxiety-related behavior

We examined adult males (6–9 month old) in the open field and elevated zero maze tests to determine if they exhibited genotype differences in anxiety-related behavior. In the elevated zero maze, there was a significant effect of genotype on time spent in the open areas with *Mecp2*^{308}/*Fxyd1* KO mice spending more time in these more anxiety-provoking area than *Mecp2* WT/*Fxyd1* HT, *Mecp2*^{308}/*Fxyd1* WT and *Mecp2*^{308}/*Fxyd1* HT mice (Table 2). There was also an effect of genotype on distance moved, with *Mecp2* WT/*Fxyd1* WT moving less than *Mecp2*^{308}/*Fxyd1* HT mice (Table 2). There was no significant effect of genotype on measures of anxiety in the open field (Table 2).

2.4. Novel location and novel object recognition

In the HC-dependent novel location recognition test, WT animals (*Mecp2* WT/*Fxyd1* WT group) spent significantly more time (one-tailed t-test, $t=-2.621$, $p = 0.016$) exploring an object moved to a new location than exploring the same object left in a familiar location (Fig. 2A). This behavior was impaired in *Mecp2*^{308} mice and deletion of one *Fxyd1* allele failed to rescue it. However, deletion of both *Fxyd1* alleles fully rescued the impairment ($t=-3.291$, $p=0.017$, Fig. 2A). Interestingly, deletion of both *Fxyd1* alleles in *Mecp2* WT mice (*Mecp2* WT/*Fxyd1* KO) resulted in a phenotype similar to that of *Mecp2*^{308} mice with an intact *Fxyd1* gene (Fig. 2A).

Assessment of the ability to recognize a novel object, which is an FC-dependent function, showed that only the WT group spent more time exploring the novel objects than the two familiar objects (effect of novel object; $F = 7.634$, $p = 0.002$; $p < 0.05$ versus object 2 and $p < 0.001$ versus object 1) (Fig. 2B). *Mecp2*^{308} mice failed to recognize a novel object and this

impairment was not rescued by deletion of either one or both *Fxyd1* alleles. This cognitive behavior was almost normal in animals with an intact *Mecp2* gene and lacking only one *Fxyd1* allele (*Mecp2* WT/*Fxyd1* HT; effect of novel object: F=5.721, $p = 0.012$; $p = 0.179$ versus object 2 and $p < 0.01$ versus object 1), but was obliterated by the lack of both *Fxyd1* alleles in animals carrying an intact *Mecp2* gene (Fig. 2B). These results indicate that the *Fxyd1* gene is required for both FC and HC-dependent behaviors.

To determine if the animals used in these behavioral tests exhibited the expected genotype-dependent changes in MECP2 and FXYD1 content in the regions of interest, we measured by western blot the abundance of both MECP2 and FXYD1 in the FC of the same animals used for behavioral testing. MECP2 was detected only in the *Mecp2* WT group (Fig. 3A), an expected result because the antibody used targets amino acids 465-478, a region that is downstream from the point mutation that interrupts the MECP2 open reading frame. In keeping with the results presented in Fig. 1, the abundance of FXYD1 increased approximately 1.2-fold ($p<0.05$) in the absence of MECP2, and decreased by about 50% in animals with one deleted *Fxyd1* allele (Fig. 3B; $p<0.01$). No FXYD1 was detected in *Fxyd1* KOs (Fig. 3B).

2.5. Sensorimotor function

We finally assessed sensorimotor function using three different tests. On day 1 of the rotorod test, the *Mecp2*^{308}/*Fxyd1* WT group fell earlier ($p = 0.009$) than the WT group (Fig. 3A). However, this difference disappeared when the performance of each group in 3 days of testing was averaged (Fig. 3B). In the inclined test, the latency to the first misstep tended to be shorter in the *Mecp2*^{308}/*Fxyd1* WT group when comparing it to the WT group (Fig. 3C), but this difference did not achieve statistical significance (one way ANOVA, F=1.722, p=0.146). Deletion of one or two *Fxyd1* alleles did not alter the performance of *Mecp2*^{308} or WT mice (Fig. 3C). Finally in the wire hang test, the *Mecp2*^{308} mice fell significantly faster (one-way ANOVA, $F = 4.442$, $p = 0.002$ by Newman-Keuls pairwise comparison test) than WT mice (Fig. 3D), and this deficit was not rescued by deletion of either one or both *Fxyd1* alleles (Fig. 3D). *Mecp2* WT mice carrying either one or both deleted *Fxyd1* alleles behaved like double WT controls (Fig. 3D).

3. Discussion

FXYD1 is a gene that becomes deregulated in the absence of MECP2 in the FC of both humans affected by RTT and male mice lacking MECP2 (Deng et al., 2007). An increased FXYD1 expression is detected in the FC, but not the CB, of *Mecp2*-null mice and appears to be the direct consequence of a loss of MECP2-dependent transcriptional repression (Banine et al., 2011; Deng et al., 2007). In the present study we found that *Fxyd1* expression is also increased in the FC (and the HC, but not the CB) of *Mecp2*^{308} mice, indicating that in both mouse models of RTT, loss of MECP2 function leads to a region-specific de-repression of the *Fxyd1* gene.

Employing *Mecp2*^{308} mice (Shahbazian et al., 2002), an animal model frequently used for behavioral studies, we set out to determine if correcting *Fxyd1* overexpression is able to rescue or ameliorate some of the behavioral and cognitive impairments affecting these animals. We found that deletion of both *Fxyd1* alleles, instead of only one, rescued the ability to recognize a familiar object placed in a novel location, an HC-dependent cognitive behavior. Interestingly, animals with an intact *Mecp2* gene, but lacking *Fxyd1*, exhibited impairments in both novel location and novel object recognition tests similar to those seen in *Mecp2*^{308} animals with an intact *Fxyd1* gene. This suggests that the *Fxyd1* gene itself is required for these FC and HC-dependent cognitive behaviors. Manipulating *Fxyd1* dosage did not influence other behavioral and sensorimotor impairments affecting these animals.

In the open field test, *Mecp2*^{308} animals did not show an increase in anxiety-related behavior, which is in agreement with a previous report showing that such an increase is subtle and can only be observed during a second and third 10 min sessions (Shahbazian et al., 2002). In addition, it was recently shown that animals of an age similar to our mice do not display this delayed increase in anxiety-related behavior (De Filippis et al., 2012). Although *Mecp2*^{308} mutant mice were reported to exhibit more anxiety-related behavior in the elevated plus maze (McGill et al., 2006) and the zero maze (De Filippis et al., 2010), we did not observe this behavioral change when testing these animals in the elevated zero maze. This discrepancy could be due to differences in the experimental paradigms and testing conditions used, as well as the age of the mice tested [3–5 months in (De Filippis et al., 2010; McGill et al., 2006) vs. 6–9 months in this study] and/or different genetic background (Moretti et al., 2006; Shahbazian et al., 2002).

Although *Mecp2*^{308} mice show an interest in exploring similar to WT animals when presented with a single novel object (De Filippis et al., 2012; Moretti et al., 2005), their performance in a more complex setting, i.e. remembering the location of 3 different objects, their specific location, and identifying any new object has not been formally assessed. The novel location recognition task assesses the ability of a mouse to discriminate between a familiar object placed in a familiar or new location, and in the experimental design used here is considered to be mostly HC-dependent (Ennaceur et al., 1997; Lenck-Santini et al., 2005). On the other hand, the novel object recognition task assesses the ability of a mouse to identify a new object placed among familiar ones (new object recognition test) and the version used in this study is considered FC-dependent (Akirav and Maroun, 2006; Kamei et al., 2006; Mitchell and Laiacona, 1998) and HC-independent (Ennaceur et al., 1997). These tasks rely mostly on the rodent's innate exploratory behavior, without the need for reinforcement, and can therefore be considered as a "pure" working-memory test (Ennaceur and Delacour, 1988). Our study shows that *Mecp2*^{308} mice are impaired in both the HC/FC-dependent novel location recognition and FC-dependent novel object recognition tests. These results are consistent with the findings of Schaevitz et al. (Schaevitz et al., 2010) who showed that *Mecp2*-deficient males (*Mecp2*^{1lox} strain) exhibited a deficit in novel object preference. Using this mouse model, the same laboratory reported earlier that both young adult (4–12 weeks) *Mecp2*^{1lox}-deficient males and females exhibited a deficit in the novel location recognition task, while only *Mecp2*^{1lox} females were impaired in the novel object recognition task (Stearns et al., 2007). The use of a slightly different experimental procedure and another mouse model of RTT (*Mecp2*^{1lox}) (Stearns et al., 2007) could account for the absence of impairment in *Mecp2*^{1lox} males in the novel location recognition test.

Because total deletion of the *Fxyd1* gene, but not that of only one allele, rescues the impairment in the novel location test, our results suggest that an increase in FXYD1 expression is involved in the process by which a HC-dependent cognitive function deteriorates as a consequence of MECP2 deficiency. Consistent with a role for FXYD1 in hippocampus-dependent cognitive impairments, FXYD1 levels in the CA1 region of the hippocampus are upregulated in male rats in mid-life, the same time at which cognitive impairments start to appear (Kadish et al., 2009).

While no improvement in the novel object recognition test was observed in *Mecp2*^{308} mice after deleting one or two *Fxyd1* alleles, both manipulations in *Mecp2*-intact mice resulted in a deficit similar to that seen in *Mecp2*^{308} animals. These findings suggest that a normal level of FXYD1 is required for the maintenance of FC-dependent cognitive function, and that an excess of *Fxyd1* expression is not solely responsible for the impairment in novel object recognition caused by MECP2 deficiency.

To assess sensorimotor function, which is mostly CB-dependent (Nadler et al., 2006; Walter et al., 2006), we tested all six mouse genotypes on the rotorod, inclined screen, and wire-hang tests. As reported by others (De Filippis et al., 2010; Shahbazian et al., 2002), *Mecp2*^{308} mice showed a slight impairment in the first, but not subsequent, trials of the rotorod test. The mutants showed a mild, not significant, deficit in the inclined screen test, and a significant impairment in the wire hang test, as previously reported (Shahbazian et al., 2002), Modulating *Fxyd1* levels did not alter motor function performance. As there was no change in *Fxyd1* expression in the CB of *Mecp2*^{308} mutants, these data suggest that expression of Fxyd1 in the CB is not required for CB-dependent sensory-motor function.

The present results indicate that the *Fxyd1* gene needs to be expressed at the right level to sustain normal function. This feature of *Fxyd1* biology is of great interest, because of its similarity to MECP2, which either in excess or abatement, results in RTT-like impairments (Chao and Zoghbi, 2012). The absence of FXYD1 in otherwise normal mice results in impaired novel location recognition behavior as it occurs in MECP2^{1lox}-deficient mice (Stearns et al., 2007). Complete loss of *Fxyd1* expression does, however, rescue the impairment in novel location behavior of *Mecp2*-deficient mice, suggesting that the impact of Fxyd1 gene dosage on different functions varies according to the brain region and function involved.

Although MECP2 deficiency causes a devastating disease, it appears that this condition is reversible (Guy et al., 2007; Robinson et al., 2012). However, achieving a correct *MECP2* dosage remains a significant obstacle to the use of MECP2 as a target in drug-mediated therapeutic intervention (Chao and Zoghbi, 2012). An alternative approach is the regulation of MECP2 targets, as observed when manipulating BDNF and IGF1 level in mouse models (Chang et al., 2006; Tropea et al., 2009). Adding to these results, we show here that manipulating *Fxyd1* expression rescues a HC-dependent cognitive function. Because FXYD1 is a protein localized to the cell membrane, it should be readily accessed by small molecules designed to modify its function, and thus provide an additional tool for therapeutic intervention in RTT.

4. Material and Methods

4.1. Animals

Heterozygous female mice carrying a truncated MECP2 protein (*Mecp2*^{308}; JAX: strain B6.129S-*Mecp2*tm1Hzo/J; *Mecp2*^{308}) were bred to homozygous male mice lacking the *Fxyd1* gene (Jia et al., 2005). They were genotyped using PCR protocols previously described (Jia et al., 2005; Shahbazian et al., 2002). The F1 offspring resulting from these crosses was backcrossed for over five generations onto the C57BL6/J background. The F1 female mice were wild-type (WT) or heterozygous (HT) for the *Mecp2* mutations, and heterozygous for the *Fxyd1* deletion. The F1 male mice were WT or homozygous for the *Mecp2*^{308} mutation) and HT for the *Fxyd1* deletion. By crossing F1 mice, six possible genotypes were generated (see below) and the males were used for behavioral testing when they were 6–9 months of age. The genotypes studied were: (1) *Mecp2* WT/*Fxyd1* WT, (2) *Mecp2*^{308}/*Fxyd1* WT, (3), *Mecp2*^{308}/*Fxyd1* HT (4) *Mecp2*^{308}/*Fxyd1* KO, (5) *Mecp2* WT/*Fxyd1* HT, and (6) *Mecp2* WT/*Fxyd1* KO. The animals were housed under a 12:12 h light/dark cycle (lights on at 0700) and given free access to food and water. All experiments were conducted in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee of OHSU.

4.2. RNA extraction and real-time PCR

Four and 6 month-old *Mecp2*^{308} mutant mice were euthanized by CO_2 inhalation followed by decapitation. The FC, CB and HC were rapidly dissected and snap frozen on dry ice. Samples were kept at −85°C until RNA extraction.

Total RNA was extracted with the RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. To remove DNA contamination, RNA samples were treated with the DNA-free DNase I kit (Ambion, Austin, TX) according to the manufacturer's protocol. RNA concentrations were determined by spectrophotometric trace (Nanodrop, ThermoScientific, Wilmingtom, DE). Four hundred ng of total RNA were reverse transcribed (RT) using the Omni RT Kit (Qiagen, Valencia, CA) in the presence of random hexamer primers (Invitrogen, Carlsbad, CA), as suggested by the manufacturer.

The abundance of *Fxyd1* mRNA was quantified by real-time PCR, as previously described using primers that target a *Fxyd1* segment contained within the *Fxyd1* mRNA coding region (Deng et al., 2007). All real-time PCR reactions were performed using an ABI Prism 7900HT Real-Time PCR system; threshold cycles (CTs) were detected by SDS 2.2.1 software (Applied Biosystems, Foster City, CA). Relative standard curves were constructed from serial dilutions of one reference sample cDNA (RT of 400 ng total RNA from the CB of a wild-type mouse, serially diluted from 1/10 to 1/500). The CTs from each sample was referred to the relative curve to estimate the corresponding *18s* and *Fxyd1* mRNA content/sample. Thereafter, the *Fxyd1* mRNA content of each sample was normalized for procedural losses using the respective *18S* rRNA values, and expressed in arbitrary unit (AU). The primers and probe used to detect *18S* rRNA were purchased as a kit (TaqMan Ribosomal RNA Control Reagents Kit, Applied Biosystems, Foster City, CA). *Fxyd1* primers and fluorescent probe, chosen within the *Fxyd1* mRNA coding region (NM_019503), were designed using Primer Express 2.0 software (Applied Biosystems). The forward and reverse primers were 5′-TCCATGGCCAGTGCAGAA-3′ (corresponding to nt 46-53 in the coding sequence of *Fxyd1* mRNA) and 5′-ATGAAGAGGATCCCAGCGATA-3′ (complementary to nt 126-146), respectively. The internal fluorescent oligodeoxynucleotide probe sequence 5′-ACGATTACCACACCCTGCGGATCG-3′ (complementary to nt 92-115) was covalently linked to the fluorescent dye, FAM, at the 5′-end, and to the quencher dye, TAMRA, at the 3′-end (Applied Biosystems). Real-time PCR reactions were performed in a total volume of 10 μl, each reaction containing 2 μl of cDNA, 5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems) and 3 μl of primers and probes mix (250 nM of *Fxyd1* and *18S* fluorescent probes, 300 nM of *Fxyd1* primers and 10 nM of *18S* primer. The real-time PCR program used consisted of an initial annealing period of 2 min at 50°C, followed by 10 min of denaturing at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C.

4.3. Antibodies

FXYD1 was detected in western blots using rabbit polyclonal antibodies (Abcam, Cambridge, MA) directed against the segment comprised between amino acid 50 and the C-terminus of human FXYD1; MECP2 was also detected using rabbit polyclonal antibodies (Cell Signaling, Lake Placid, NY), directed against amino acids 465-478 of mouse MECP2. GAPDH was detected using mouse monoclonal antibody 6C5 (Abcam, Cambridge, MA).

4.4. Protein extraction and western blotting

When analyzing RNA and protein content in the same samples (Fig. 1), proteins were extracted from the samples used for RNA extraction as recommended by Morse et al. (Morse et al., 2006). Briefly, after homogenizing the tissue and passing the resulting lysate through a RNA binding column (Rneasy mini kit, Qiagen), flow-through from that initial

passage and the subsequent washes were collected and kept overnight at −20°C. The proteins were precipitated by centrifugation (10 min at 4,000*g*), followed by three washes with 100% ethanol. Protein pellets were air-dried and kept at −85°C until assay. At this time, they were resuspended in Non-Reducing Sample Buffer (NRSB, 62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 1 mM sodium Fluoride, 1 mM sodium orthovanadate, 10 μg/ml leupeptin and pepstatin A, 10 μg/ml aprotinin, and 1 mM PMSF) following a protocol available at http://www.millipore.com/userguides/tech1/mcproto442#2. Proteins were measured with the Pierce 660 nm Protein Assay and the Ionic Detergent Compatibility Reagent (Thermo Fisher Scientific, Rockford, Il). Twenty μg of protein were loaded on a precasted Tris-HEPES Pierce 4–20% protein gel (Thermo Fisher Scientific,) under denaturing conditions (3% β-mercaptoethanol) and transferred to an Immobilon-P membrane (Millipore, Billerica, MA) using a gel transfer device (Idea Scientific Co., Minneapolis, MN) at 100 mAmp/23 V for 2h at 4°C. The membrane was blocked in 5% nonfat milk-TBST (Tris buffer saline with 0.05% Tween 20) and incubated overnight at 4°C with both the FXYD1 and GAPDH antibodies diluted 1:1,000 and 1:20,000, respectively. The next day, the membranes were washed (four changes of TBST every 15 min), before incubating them for 1h at room temperature with both goat anti-rabbit and goat anti-mouse antibodies conjugated to HRP (both from Zymed Laboratories, San Francisco, CA) diluted 1: 25,000 in TBST buffer. After four more washes with TBST buffer, immunoreactivity was visualized using Pierce ECL reagents (Thermo Fisher Scientific, Rockford, IL) and UltraCruz autoradiography Blue films (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was then incubated overnight at 4°C with MECP2 antibodies (1:2,000) followed by 1 h incubation with goat anti-rabbit antibody conjugated to HRP (1:25,000) and the immunoreaction was visualized as described above.

When only proteins were analyzed in tissues collected after finishing the behavioral testing (Fig. 3), the proteins were extracted in 400 μl of freshly prepared RIPA lysis buffer (10 mM Tris, pH 7.4, 0.1 % SDS, 0.5% Deoxicholic acid, 1% Triton X-100, NaCl 150 mM, 80 uM aprotinin, 2 uM Leupeptin, 1.5 uM Pepstatin and 1 mM PMSF). The protein content was measured as described above and the proteins were loaded (2 μg/well) onto a precasted 14% Tris-glycine gel (Invitrogen). After transfer to Immobilon-P membranes, the membranes were processed as outlined above for FXYD1, GAPDH, and MECP2 detection. The immunoreactions were visualized using West Dura reagents (Thermo Fisher).

Densitometric analysis was performed using AlphaEaseFC software (Alpha Innotech, Santa Clara, CA), and the values obtained were normalized for procedural losses using GAPDH as the reference.

4.5 Body Weight

Litters were weaned at 21 days of age and male mice were weighed every week until they were 20 weeks of age.

4.6. Behavioral tests

Mice of the six genotypes described in section 4.1 (6–9 months of age) were tested. During week 1, mice were tested for exploratory behavior and measures of anxiety-related behavior in the open field (day 1) and elevated zero maze (day 2), and for sensorimotor function using the rotorod, wire hang, and inclined screen tests (days 3–5). During week 2, we assessed learning and memory function using the novel location and novel object recognition tests (days 8–11).

4.6.1. Open Field—To assess exploratory behavior and measures of anxiety-related behavior, open field activity was assessed individually for 10 min in brightly lit (light

intensity: 1,000 lux) enclosures (16 inch × 16 inch square) equipped with a 16 × 16 array of infrared photocells for measuring horizontal movements and computer-quantified automatically (Kinder Scientific, Poway CA). The total distance moved during the test measured activity levels and the proportion of time spent in the center of the enclosure was used as a measure of anxiety-related behavior. After each assessment of open field activity, the equipment was cleaned with 5% acetic acid to remove residual odors.

4.6.2. Elevated Zero Maze—Measures of anxiety-related behavior were also assessed using the elevated zero maze (1,000 lux). The custom built elevated zero maze consisted of two enclosed areas (safe environment) and two open areas (anxiety-provoking environment), identical in length (35.5 cm; Kinder Scientific, Poway, CA). Mice were placed in the closed part of the maze and allowed free access for 10 min (2 × 5 min bins). A video tracking system (Noldus Information Technology, Sterling, VA) set at 6 samples/second was used to calculate the distance moved, and percent time spent in the open areas of the maze.

4.6.3 Novel Location and Novel Object Recognition—Object recognition was used to assess HC-dependent novel location recognition and HC-independent/FC-dependent novel object recognition. To assess object recognition, mice were individually habituated for three consecutive days to a 16 × 16 inch open-field with clear plexiglass walls (Kinder Scientific, Poway, CA) for 5 min. On the fourth day, the mice were first given three 10 min trials with three plastic objects in different corners of the open field. In subsequent trials, the familiar objects were exchanged with replicas. For the fourth 10 min trial, one of the familiar objects was moved from one corner of the field to another to evaluate HC-dependent novel location recognition. For the fifth 10 min trail, a familiar object was replaced by a novel object to assess HC-independent novel object recognition. There was a 3 min interval between each trial. During this time, the mice were placed back in their home cage and the open field and the objects were cleaned with 5% acetic acid to remove potential odors. The total time spent exploring all objects was compared between trials to assess the familiarization of each mouse with the objects. The difference between the percent time spent exploring the object in the novel location (trial 4) and the percent time spent exploring the same object in its original location (trial 3) as compared to 0 was calculated to assess novel location recognition. The percent time spent exploring the novel object during trial 5 was calculated to assess novel object recognition.

4.7. Sensorimotor function

4.7.1 Rotorod—To assess sensorimotor function, the mice were first tested on the rotorod (Rotamex-5, Columbus Instruments, Columbus, OH). Mice were placed on an elevated rod (3 cm × 9.5 cm spindle 44.5 cm elevated) initially rotating at 5 rpm. The speed of the rotating rod was increased by 1 rpm every 3 sec to a maximum of 24 rpm. Each trial ended when a fall was recorded by photo beams aligned with each individual mouse or if a mouse did not fall from the rod within 300 sec. Mice received 3 trials, 30 min apart, for 3 consecutive days.

4.7.2 Inclined Screen—To determine potential impairments in balance, mice were tested in the inclined screen test. Mice were placed on a 182-cm-long inclined screen (36° incline), 128.5 cm from the top and 53.5 cm from the bottom of the screen, and were allowed to explore for 3 min. Mice have a natural propensity to climb upwards. The total distance moved and the mean velocity of movement were recorded using a Noldus Instruments Ethovision video tracking system. The event recorder of the Noldus software was used to determine the interval of time between the beginning of the test and the first misstep (latency).

4.7.3 Wire Hang—*T*o evaluate muscle strength, mice were lifted up by their tails and slowly placed on a horizontal cotton wire (1 mm in diameter) allowing them to use both fore- and hindpaws to grab the wire. The placing procedure takes 1–2 s and does not require pre-training or habituation. The wire was mounted 60 cm above a horizontal surface. Once the mice grasped the wire, it was a little bent out of the horizontal plane and slightly V shaped. Two sequential trials were given, and the length of time that the mice held onto the wire (latency) was recorded.

4.8. Statistical Analysis

All statistical analyses were performed using SPSS software (SPSS Inc, Chicago, Il), SigmaStat (Systat Software Inc., San Jose, CA) or GraphPad Prism software (GraphPad Software, LaJolla, CA). Genotype differences were analyzed using one or two-way ANOVA followed by post-hoc tests when appropriate. In cases that only two genotypes were compared, the results were analyzed using the Student's *t* test. To analyze the object recognition preference data, one-tailed t-tests were used as *a priori* the % time spent exploring the object in a novel location or the novel object is anticipated to have a higher mean than exploring the object in the familiar location or the familiar objects, respectively. Data are expressed as means ± SEM. $p < 0.05$ was considered significant for all tests.

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Highlights

- *Fxyd1* expression increased in frontal cortex of Mecp2^{308} mutant male mice
- *Fxyd1* mRNA levels increased in hippocampus of *Mecp2^{308}* mutant male mice
- *Mecp2^{308}* mutant mice have impaired hippocampus-dependent novel location recognition
- This impairment is rescued by deletion of the *Fxyd1* gene

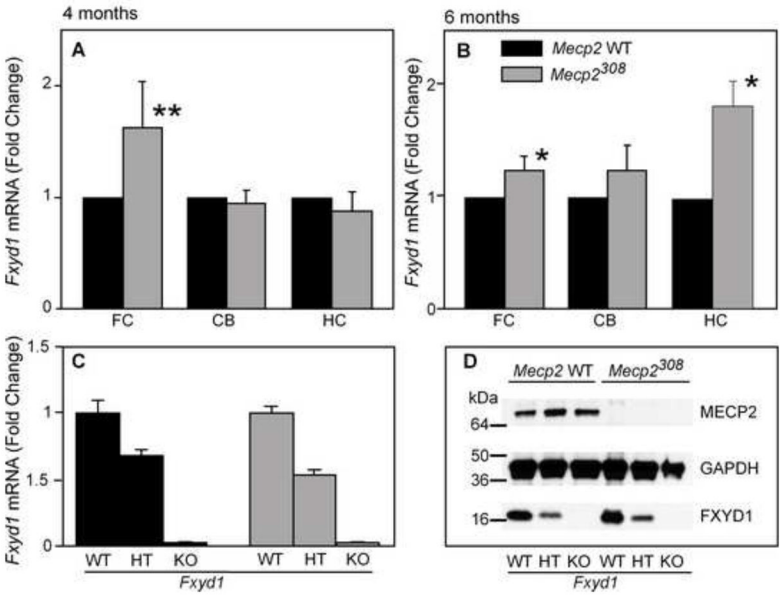

Figure 1. *Fxyd1* mRNA abundance increases in selected brain regions of *Mecp2*^{308} mice

(**A, B**) *Fxyd1* mRNA levels were significantly increased in the frontal cortex (FC) of 4 and 6 month-old Mecp2^{308} mice when compared to WT littermate controls. At 6 months of age, a significant increase in *Fxyd1* mRNA was also found in the hippocampus (HC) of *Mecp2*^{308} mice. No significant differences were found in the cerebellum (CB) at either 4 or 6 months of age. **p< 0.05, ** p<0.01, t-test *Mecp2*^{308} vs. WT group. (**C**) To ascertain that *Fxyd1* mRNA levels were decreased as expected in mice with one or both *Fxyd1* alleles deleted, *Fxyd1* mRNA levels were measured by real-time PCR in CB samples of mice WT, HT or KO for the *Fxyd1* allele, and having either an intact or a mutated *Mecp2* gene. Regardless of the presence or absence of a mutated *Mecp2* allele, *Fxyd1* HT mice have *Fxyd1* mRNA levels that are half of the *Fxyd1* WT group; *Fxyd1* KOs had undetectable *Fxyd1* mRNA. (**D**) A similar reduction in FXYD1 protein expression was seen by western blot. Bars in **A**–**C** are mean ± SEM. Numbers of animals/genotype; **A**; WT and KO; FC and HC: $n = 6$; CB: $n = 8$. **B**; WT and KO: FC and CB; $n = 9$; HC: $n = 6$. **C**; black bars; WT: $n = 3$; HT: $n = 7$; KO: $n = 2$; white bars; $n = 4$ genotype.

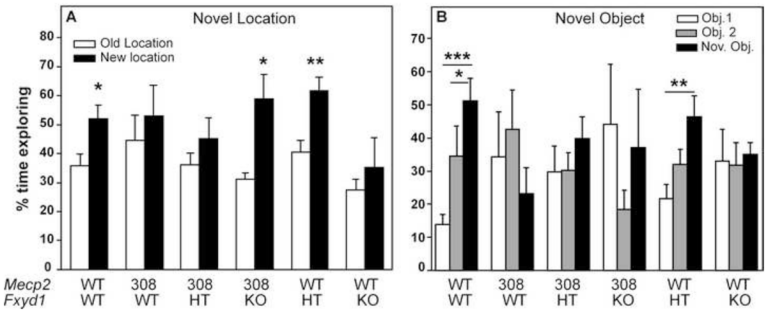

Figure 2. Measures of cognitive function in *Mecp2*^{308} mice with either both *Fxyd1* alleles intact or carrying one or both deleted *Fxyd1* alleles

Male mice of all genotypes were examined for cognitive function in the novel location (**A**) and novel object (**B**) recognition tests. **A**: *Novel location*, *Mecp2*^{308} mice show an impairment in novel location recognition. This impairment was also seen in *Mecp2*^{308}/*Fxyd*1 HT mice, but it was rescued by deleting both *Fxyd1* alleles. Object in old location vs. new location, p=0.016 for *Mecp2* WT/*Fxyd*1 WT; p=0.017 for *Mecp2*^{308}/*Fxyd*1 KO; p=0.005 for *Mecp2* WT/*Fxyd*1 HT (one-tailed test). **B**: *Novel object recognition*, *Mecp2*^{308} mice show impaired novel object (NO) recognition function, and this impairment was not rescued by deleting either one or both *Fxyd1* alleles. Deletion of both *Fxyd1* alleles in *Mecp2* WT mice resulted in an impairment similar to that of *Mecp2*^{308} animals. Effect of genotype on NO recognition: in *Mecp2* WT/*Fxyd*1 WT: F=7.632, p=0.002, p=0.001 for NO vs. Obj. 1, p=0.038 for NO vs. Obj. 2; in *Mecp2* WT/*Fxyd*1 HT: F=5.721, p=0.012, p=0.179 NO vs. Obj. 1, p=0.009 NO vs. Obj. 2 (one way ANOVA). Bars are mean ± SEM. *= p< 0.05, **= p<0.01, ***= p=0.001. Number of animals per genotype; WT/WT: n = 12; *Mecp2*^{308}/WT: n = 6; *Mecp2*^{308}/HT: n = 9; *Mecp2*^{308}/KO: n = 4; WT/HT: n = 7; WT/KO: n = 5.

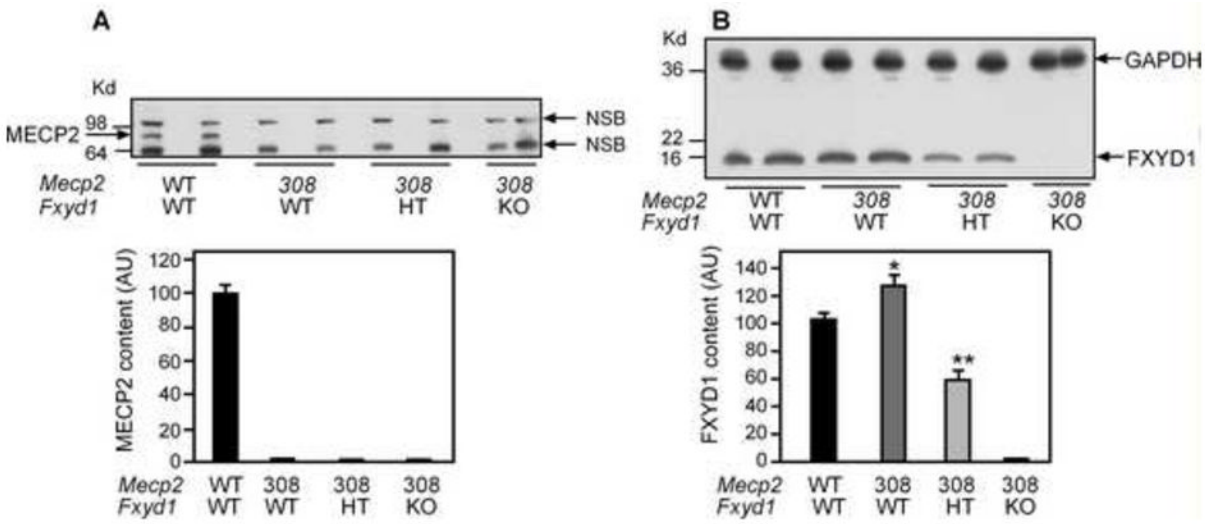

Figure 3. The content of FXYD1 and MECP2 in the FC of mice subjected to behavioral testing as assessed by western blotting

A, *Upper panel:* Representative blots (one from three blot containing proteins derived from 1–2 animals/group) showing that MECP2 is detected in *Mecp2* WT animals, but not in *Mecp2*^{308} mice, regardless of the *Fxyd1* genotype. NSB = non-specific bands present in all samples regardless of *Mecp2* genotype. *Lower panel:* Densitometric analysis depicting the lack of detectable MECP2 in *Mecp2*^{308} mice. **B,** *Upper panel:* Representative blot (one from three blots) showing genotype-dependent changes in FXYD1 content. *Lower panel:* Densitometric analysis of the changes in FXYD1 content detected in the FC of mice of four different genotypes. WT/WT = WT for both *Mecp2* and *Fxyd1; Mecp2*^{308}/WT = *Mecp2*^{308}/*Fxyd1* WT*; Mecp2*^{308}/HT= *Mecp2*^{308} lacking one *Fxyd1* allele; *Mecp2*^{308}/KO = *Mecp2*^{308} lacking both *Fxyd1* alleles. Vertical bars are SEM. *= p< 0.05, **= p<0.01 vs. WT controls (Student t test). Number of animals/genotype from left to right; **A** and **B**: *n* = 6, 4, 6, 5.

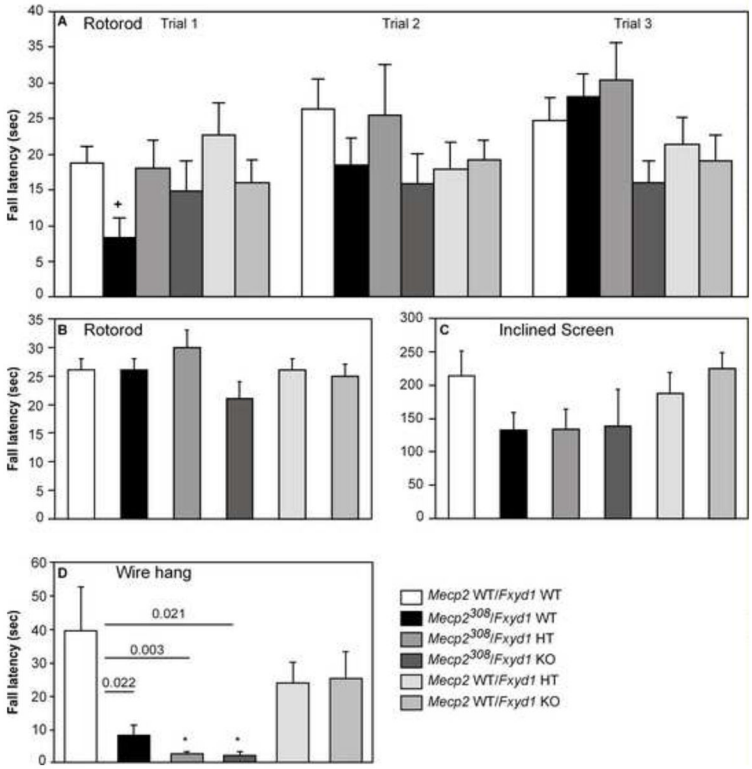

Figure 4. Deletion of one or two Fxyd1 alleles does not rescue sensorimotor impairments of *Mecp2*^{308} mice

Sensorimotor functions were examined with the rotorod (**A**, **B**), inclined screen (**C**) and wire hang (**D**) tests. **A,** Performance during each of 3 trials on the rotorod test. There was an effect of genotype on the latency to fall of the rod when comparing the *Mecp2* WT/*Fxyd1* WT group to *Mecp2*^{308}*/Fxyd1* WT mice (t=2.916, +: p=0.009). However, there was no significant effect of genotype when comparing all genotypes at once (one-way ANOVA, F=1.692, p=0.151). **B**, There was no effect of genotype on rotorod performance when examining the overall performance of each group (average of 9 trials, 3 trials/day on 3 consecutive days, one-way ANOVA, F=1.171, p=0.374). **C**, There was no effect of genotype on the latency to fall in the inclined screen test (one-way ANOVA, F=1.722, p=0.146). **D**, The wire hang test showed an effect of genotype on fall latency (one-way ANOVA, F = 4.442, p = 0.002 followed by post-hoc Newmann-Keuls pairwise comparison tests (p values are indicated on the graph). *Mecp2*^{308} mice fell faster that WT controls and this impairment was not rescued by deletion of either one or both *Fxyd1* alleles. Mice with either one or both deleted *Fxyd1* alleles and normal *Mecp2* alleles behaved like WT controls. Bars are mean ± SEM. Number of animals/genotype from left to right; **A** and **B**: n = 11, 9, 9, 12, 10, and 12; **C** and **D**: n = 8, 11, 12, 8, 12, and 6.

Table 1

Body weight gain (g)

Genotype (n)	Weight (g)			
	4 weeks	10 weeks	13 weeks	20 weeks
Mecp2 WT/*Fxyd1* WT	12.9±1 (5)	25.4±0.8	27.8±1	29.4±1
Mecp2^{308}/*Fxyd1* WT	12.5±0.6 (7)	24±0.6	25.8±.07	28.4±0.6
Mecp2^{308}/*Fxyd1* HT	12.8±0.4 (10)	23.9±0.6	25.8±0.6	28.2±0.7
Mecp2^{308}/*Fxyd1* KO	10.9±0.6 (10)	23.7±0.5	24.5±0.6	28.7±0.9
Mecp2 WT/*Fxyd1* HT	10.6±1 (9)	22.7±0.6	24.5±0.6	27.1±0.5
Mecp2 WT/*Fxyd1* KO	12.1±0.5 (6)	23.3±0.7	25.6±0.6	27.6±0.7

Values are mean ± SEM. (n), Number of animal per group.

Table 2

Anxiety-related behavior in the open field and elevated zero maze

Genotype (n)	Open Field (% Time spent in center)	Elevated zero maze (% time spent in open areas)	Elevated zero maze (total distance moved, cm)
Mecp2 WT/*Fxyd1* WT	9±4 (5)	19.3±5 (11)	1238±98 (11)*
Mecp2^{308}/*Fxyd1* WT	5±1 (9)	19.5±3 (10)	1924±220 (10)
Mecp2^{308}/*Fxyd1* HT	7±1 (10)	19.8±3 (11)	2043±162 (11)
Mecp2^{308}/*Fxyd1* KO	7±2 (9)	37.7±6 (10)*	1881±212 (10)
Mecp2 WT/*Fxyd1* HT	7±1 (9)	22.6±5 (9)	1761±145 (9)
Mecp2 WT/*Fxyd1* KO	7±1 (7)	28.4±4 (10)	1923±224 (10)

Measurement of anxiety-related behavior in the open field test did not change according to genotype. In the elevated zero maze, there was a significant effect of genotype on time spent in open areas ($F = 2.727$, $p=0.029$; $p<0.05$ for *Mecp2*^{308}/*Fxyd1* KO vs. *Mecp2* WT/*Fxyd1* HT, *Mecp2*^{308}/*Fxyd1* WT and *Mecp2*^{308}/*Fxyd1* HT) and the total distance moved ($F = 2.687$, $p=0.03$; $p<0.05$ for *Mecp2* WT/*Fxyd1* WT vs. *Mecp2*^{308}/*Fxyd1* HT). (n), Number of animal per group.