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Ube3a mRNA and protein expression are not decreased in Mecp2^{R168X} mutant mice

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Abstract

Mutations in the transcriptional repressor *methyl CpG binding protein 2 (MeCP2)* are responsible for most cases of Rett Syndrome (RS), a severe neurodevelopmental disorder characterized by developmental regression, minimal speech, seizures, postnatal microcephaly and hand stereotypies. Absence of the maternal copy of ubiquitin protein ligase 3A (*UBE3A*) results in Angelman syndrome, also a severe developmental disorder that shares some clinical features with RS. As MeCP2 regulates gene expression, this has led to the hypothesis that MeCP2 may regulate *UBE3A* expression; however, there are conflicting reports regarding the expression of *Ube3a* in MeCP2 null mutant mice. We have generated a novel MeCP2 mutant knock-in mouse with the mutation R168X, one of the most common mutations in patients with RS. These mice show features similar to RS, including hypoactivity, forelimb stereotypies, breathing irregularities, weight changes, hind limb atrophy, and scoliosis. The male mice experience early death. Analysis of *Ube3a* mRNA and protein levels in the Mecp2^{R168X} male mice showed no significant difference in expression compared to their wildtype littermates.

Keywords

Rett Syndrome; Angelman Syndrome; MeCP2; UBE3A; mouse model

1. INTRODUCTION¹

Rett syndrome (RS) is a severe neurodevelopmental disorder characterized by apparently normal initial development followed by slowing of development and head growth (Hagberg et al., 1983; Hagberg et al., 1985; Hagberg et al., 2002; Rett, 1966). Purposeful hand skills are

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¹Abbreviations: AS – Angelman Syndrome; MeCP2 - methyl CpG binding protein 2; qRT-PCR – quantitative real-time reverse transcriptase polymerase chain reaction; RS – Rett Syndrome; UBE3A - ubiquitin protein ligase 3A; UBE3A-ATS – UBE3A antisense transcript

lost and replaced by characteristic stereotypies. Spoken language is lost. Patients may also develop seizures, breathing irregularities, sleep disturbance, autistic symptoms and scoliosis. Mutations or deletions in *methyl-CpG-binding protein 2 (MECP2)* located at Xq28 are detectable in 96% of patients with RS (Amir et al., 1999; Moretti and Zoghbi, 2006). A small percentage of RS patients with early onset seizures have mutations in *cyclin dependent kinase-like 5 (CDKL5)* (Evans et al., 2005; Scala et al., 2005; Tao et al., 2004). CDKL5 may play a role in phosphorylation and regulation of MeCP2 (Mari et al., 2005).

Because MeCP2 binds to methyl CpG dinucleotides, an early leading hypothesis was that it serves as a global repressor of transcription. However, transcriptional profiling studies have failed to reveal a global de-repression of transcription in the setting of MeCP2 deficiency (Tudor et al., 2002). An alternate hypothesis that MeCP2 instead regulates transcription in a highly specific and selective manner has thus been raised. Strong evidence for this hypothesis is the finding that MeCP2 regulates *BDNF* in a calcium and phosphorylation dependent manner (Chen et al., 2003; Zhou et al., 2006).

Angelman syndrome (AS) is an imprinting disorder caused by a decrease in or loss of function of the maternal copy of *ubiquitin protein ligase E3A (UBE3A)* located at 15q11q13 (Kishino et al., 1997; Magenis et al., 1987). This protein, unlike MeCP2, is not a regulator of gene transcription but is involved in the ubiquitination pathway, which targets specific proteins for degradation. AS patients exhibit profound speech deficits, gait ataxia, seizures, characteristic EEG, postnatal acquired microcephaly, sleep disturbance, and an unusually happy demeanor with propensity to paroxysms of laughter (Angelman, 1965; Clayton-Smith and Laan, 2003; Williams et al., 1995). Several of these features are in common with RS, including the speech deficits, acquired microcephaly, sleep disturbance, and seizures. These similarities suggest that *UBE3A* could be a target for regulation by MeCP2. However, recent studies designed to test this hypothesis yielded conflicting results (Jordan and Francke, 2006; Makedonski et al., 2005; Samaco et al., 2005).

The study by Samaco *et al* looked at two lines of mutant *Mecp2* adult mice, as well as *post mortem* brain tissue from RS patients (Samaco et al., 2005). One mouse line studied, *Mecp2^{tm1.1Jae}*, was constructed with an exon 3 deletion and characterized as functionally *Mecp2* null (Chen et al., 2001). The other mouse line studied was *Mecp2^{tm1.1Bird}*, which is *Mecp2* null (Guy et al., 2001). They reported a significant reduction in expression of both *UBE3A* RNA and protein products. Next, Makedonski *et al* investigated newborns of one of the same mouse lines studied by Samaco *et al* (*Mecp2^{tm1.1Bird}*) as well as *post mortem* brain tissue from RS patients and a lymphoblast cell line from an RS patient (Makedonski et al., 2005). They also reported reductions in *UBE3A* RNA and protein expression. Jordan and Francke then reported studies on both mouse lines, *Mecp2^{tm1.1Jae}* and *Mecp2^{tm1.1Bird}*, at 3 and 21 days of age (Jordan and Francke, 2006). However, in direct contrast to the previous two reports, no significant changes in *Ube3a* RNA or protein expression were detected. This lack of consensus prompted us to investigate *Ube3a* expression in a novel *Mecp2* mutant mouse line designed in our laboratory.

2. RESULTS

***Mecp2^{R168X}* mice show RS features**

We designed a line of *Mecp2* mutant mice, which have the most common mutation associated with RS, R168X, knocked-in the mouse gene (*Mecp2^{R168X}*) (Bienvenu and Chelly, 2006). This sequence change replaces a codon for arginine with a stop codon. Using site directed mutagenesis, we altered the AGA sequence coding for arginine at codon 168 to a TGA coding for stop (Fig 1A). The mutant transcript is transcribed, and is easily detectable by RT-PCR (Fig. 1B). The amplicon was confirmed to be *Mecp2* by sequencing, which also confirmed the

presence of the mutation in the mouse line. The mutant transcript is relatively stable compared to the WT transcript (Fig 1C). The *Mecp2*^{R168X} mice do not express full length wild type *Mecp2* (Fig. 1D). These *Mecp2* mutant mice differ from the mice previously used to study the impact of *Mecp2* on *Ube3a* expression, which had null mutations.

Male hemizygotes are more severely affected than the female heterozygotes. The males have a shortened lifespan of 85.8 ± 24.2 days (\pm SD; n=43) (Fig 2A). We observed forelimb stereotypies, hindlimb atrophy, hypoactivity, and breathing irregularities. The males showed great variability in weight as compared to the wildtype littermates that became more accentuated with age (Fig 2B and 2C). While some of the affected males were similar in weight to their unaffected littermates, one subset showed failure to thrive (Fig. 2D) and another showed excessive weight gain (Fig. 2E). By 7 weeks they showed significant hindlimb claspings (Fig. 2F and 2G) and breathing irregularities.

The female heterozygotes showed significant symptoms by approximately 6 months. Lifespan was much longer than that of the males, however, with multiple females surviving more than 1 year. They also showed significant hindlimb claspings and breathing irregularities starting after 6 months of life.

Ube3a and Ube3a-antisense transcript levels are similar in *Mecp2*^{R168X} mice and wild type (WT) mice

We collected cortical brain tissue samples from seven male *Mecp2*^{R168X} mice and seven male WT littermates between the ages of six and seven weeks. In this age range, the male mice are severely affected. Using real-time quantitative PCR (qRT-PCR), *Ube3a* mRNA expression levels showed no significant difference in the affected mice as compared to their WT littermates (Fig. 2A). We also tested expression levels of the *Ube3a* antisense transcript (*Ube3a*-ATS) using the same seven pairs of mice (Fig. 2B). *UBE3A* is imprinted in brain, and so its transcription occurs from only one allele (Rougeulle et al., 1997; Vu and Hoffman, 1997). When *UBE3A* is not transcribed, an alternate transcript, *UBE3A*-antisense (*UBE3A*-ATS), is produced from the antisense strand (Chamberlain and Brannan, 2001; Rougeulle et al., 1998; Runte et al., 2001). Disruption of the maternal *Ube3a* transcript in murine brain correlates with elevated expression of *Ube3a*-ATS (Landers et al., 2005). Expression levels between the same WT and the *Mecp2*^{R168X} groups again showed no significant difference.

Ube3a protein product levels are similar in *Mecp2*^{R168X} mice and WT mice

Cortical brain tissue samples from the same seven male *Mecp2*^{R168X} mice and seven WT littermates were also used to analyze *Ube3a* protein expression. The samples were analyzed by Western blot (Fig. 3A) and quantified by densitometry relative to a reference protein, β -actin (Fig. 3B). We did not detect any significant difference between the affected males and their WT littermates. In fact, both groups had virtually identical expression levels.

3. DISCUSSION

Conceptually, MeCP2 regulation of *UBE3A* is an attractive hypothesis, given the similarities between RS and AS. However, the literature is far from a clear consensus. Two groups using mouse models and human post-mortem brain tissue observed decreases in *UBE3A* RNA and protein in the setting of MeCP2 deficiency (Makedonski et al., 2005; Samaco et al., 2005), while a third group found no change using the same two mouse models as the previous two groups (Jordan and Francke, 2006).

We chose to probe this question using our novel model, which is unique in that it carries a common *Mecp2* mutation found in patients with RS. Study of such mutant models with

common mutations knocked-in can greatly enrich the information gained by knock-out models. These models may more closely mimic actual disease states and may help to provide information on genotype-phenotype correlations within heterogeneous diagnostic categories. It is possible that some *Mecp2* mutations may have dominant negative activity, which could lead to a more complex picture than simple loss of function.

Our studies showed no significant differences between *Ube3a* mRNA and protein levels between the WT and *Mecp2*^{R168X} groups. We chose to study males at the 6 to 7 weeks of age because the males are more severely affected than the females, and this is the most affected age range among the males, just prior to death. We hypothesized that if there were a difference in *Ube3a* expression, it would be best demonstrated in this most severely affected group of mice.

Mecp2 function, however, has recently been demonstrated to be strikingly dynamic (Zhou et al., 2006). *Mecp2* is phosphorylated following neuronal activation, which could then lead to very specific time limited changes in gene expression. One could hypothesize that *Mecp2* might truly impact *Ube3a* expression, but only at discrete and specific times. Mouse model studies, or even post-mortem brain tissue, may have difficulty detecting such time specific effects. Further studies on neurons in culture may better test the hypothesis that *Mecp2* regulates *Ube3a*. Alternatively, the effect could be for a short time at a very specific age or developmental point, including the prenatal period. Our data at this time, however, do not support the hypothesis.

4. EXPERIMENTAL PROCEDURE

Mouse lines

Site directed mutagenesis was performed using the Transformer Site-Directed Mutagenesis Kit (CLONTECH). Sequence alteration was confirmed by sequencing (Massachusetts General Hospital DNA Core Facility). The targeting construct containing a Neomycin resistance (neo) cassette flanked by lox P sites and the mutant sequence was electroporated onto 129SvJ embryonic stem cells. The neo cassette was cloned from pLITMUS-28 (New England Biolabs). Neomycin-resistant colonies were isolated, expanded and characterized by PCR using MeCP2 and Neo cassette specific primers (sequences available upon request). Cells with the correct recombination were injected into C57BL/6 blastocytes and transferred to pseudopregnant females. Chimeric mice were bred with 129S6/SvEv Tac mice. The F₁ mice with germ line transmission of R168X were crossed with Cre Recombinase-expressing mice to remove the neo cassette, yielding *Mecp2*^{R168X} mice. The mice used in the experiments described here were back crossed through a minimum of 10 generations. Care and handling of the mice was conducted in concordance with Institutional Animal Care and Use Committee approved protocols.

RT-PCR and Real-time qRT-PCR

Mouse brain cortex samples were harvested and immediately placed in RNAlater (Ambion). Total RNA was isolated from cortex tissue using the RNeasy Mini Kit (Qiagen) and reverse transcribed to cDNA using the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics) following manufacturers' protocols. qRT-PCR was then performed on a MiniOpticon MJ Mini Personal Thermal Cycler (Bio-Rad) using the following conditions: 95°C for 3 minutes, followed by 36 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. All samples were processed in triplicate. The primer sequences were as follows: MeCP2 forward (ACA GCG GCG CTC CAT TAT C), MeCP2 reverse (CCC AGT TAC CGT GAA GTC AAA A), *Ube3a* forward (GCG AGC AGC TGC AAA GCA TCT AAT), *Ube3a* reverse (AGC TTG CTC CTT TCT TGG AGG GAT), *Ube3a*-ATS forward (TTG TAT ACA GGA AGC TAA TGG GG) (Landers et al., 2005), *Ube3a*-ATS reverse (CAA AAG TTT ACA AAT

AAA TAA TGT TCC)(Landers et al., 2005), β -actin forward (AGT GTG ACG TTG ACA TCC GTA), and β -actin reverse (GCC AGA GCA GTA ATC TCC TTC T). The fold change in expression was calculated using the standard curve method with β -actin as a reference control gene. All qRT-PCR products were analyzed using gel electrophoresis on 3% agarose, as well as by melting curve analysis. All samples were observed to have a single product with the expected length in gel electrophoresis, and the melting curve analysis showed a single peak.

Western blot analysis

Mouse brain cortex samples were harvested and immediately placed on dry ice, then stored at -80°C until use. The tissue was disrupted by sonication in lysis buffer (Sigma C3228). Protein was quantified with a bicinchoninic assay (Pierce), and $10\mu\text{g}$ was resolved by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Western blots were tested as indicated with a β -actin specific antibody (Abcam ab8227), a Ube3a specific antibody (Abcam ab10488) or a Mecp2 N-terminal specific antibody (Sigma M7443). Secondary antibodies were goat anti-mouse peroxidase conjugate (Sigma A0168) or goat anti-rabbit peroxidase conjugate (Upstate 12–348) as appropriate. All samples were tested at least in duplicate.

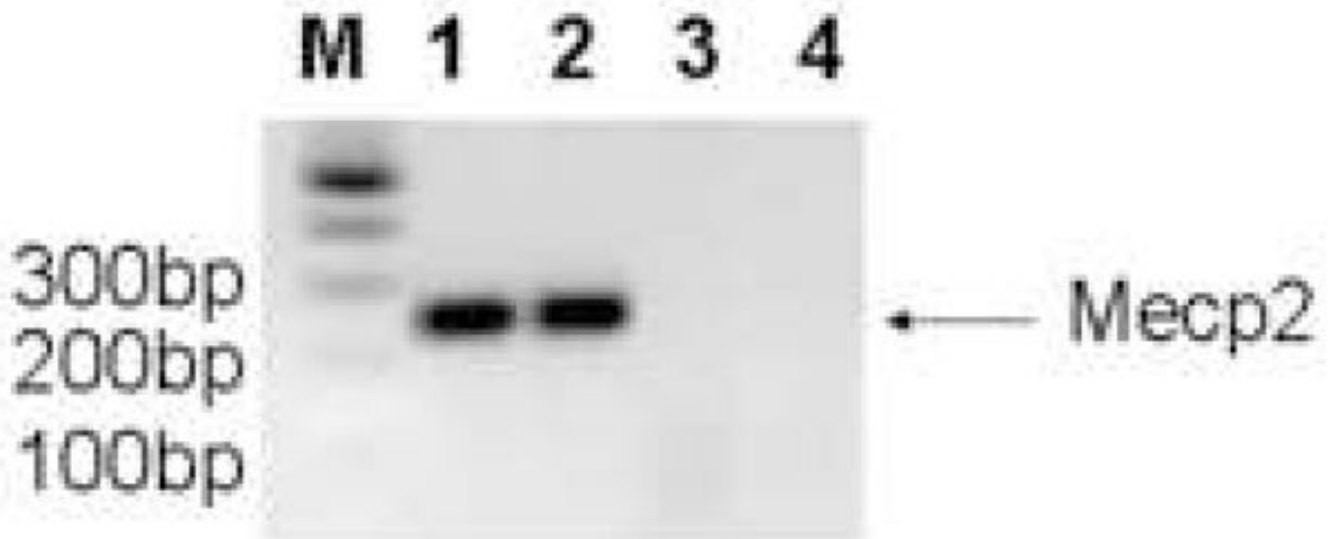
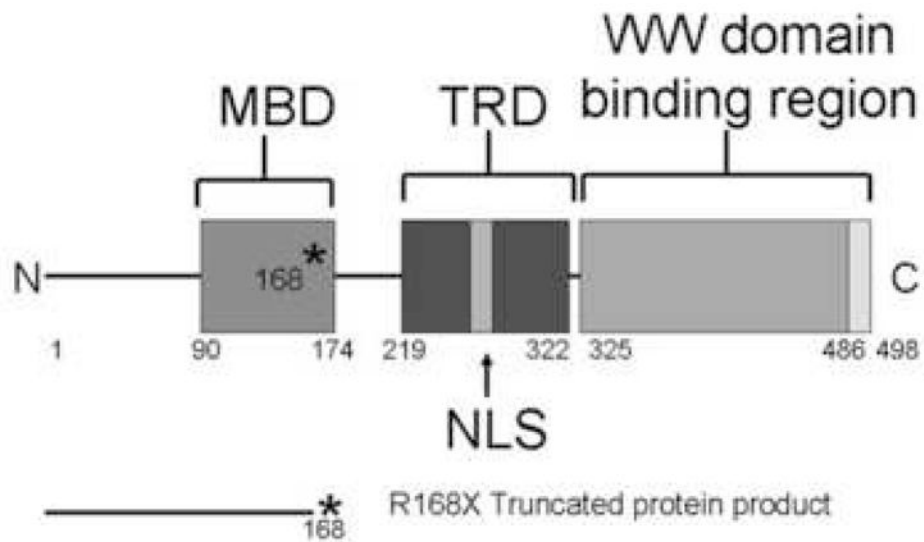
Acknowledgments

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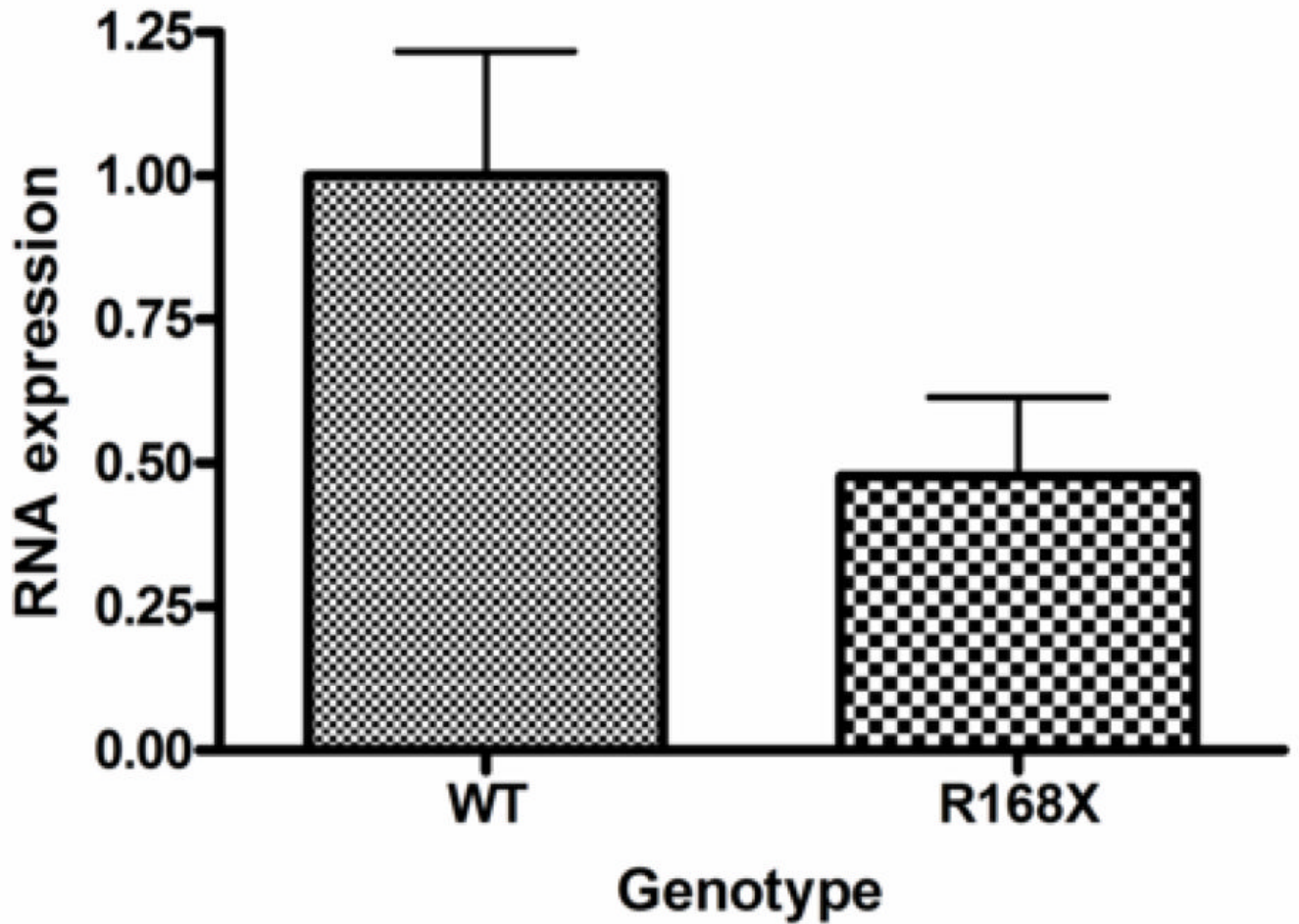
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Relative expression of Mecp2 RNA



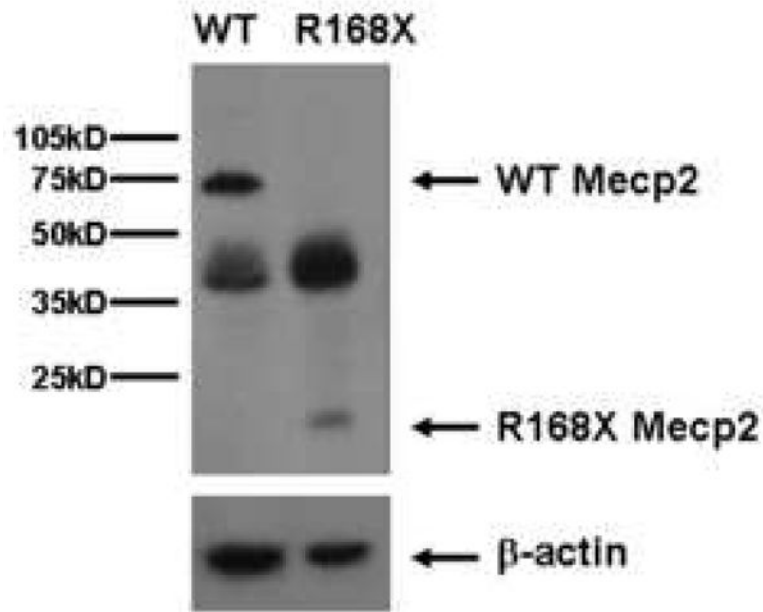
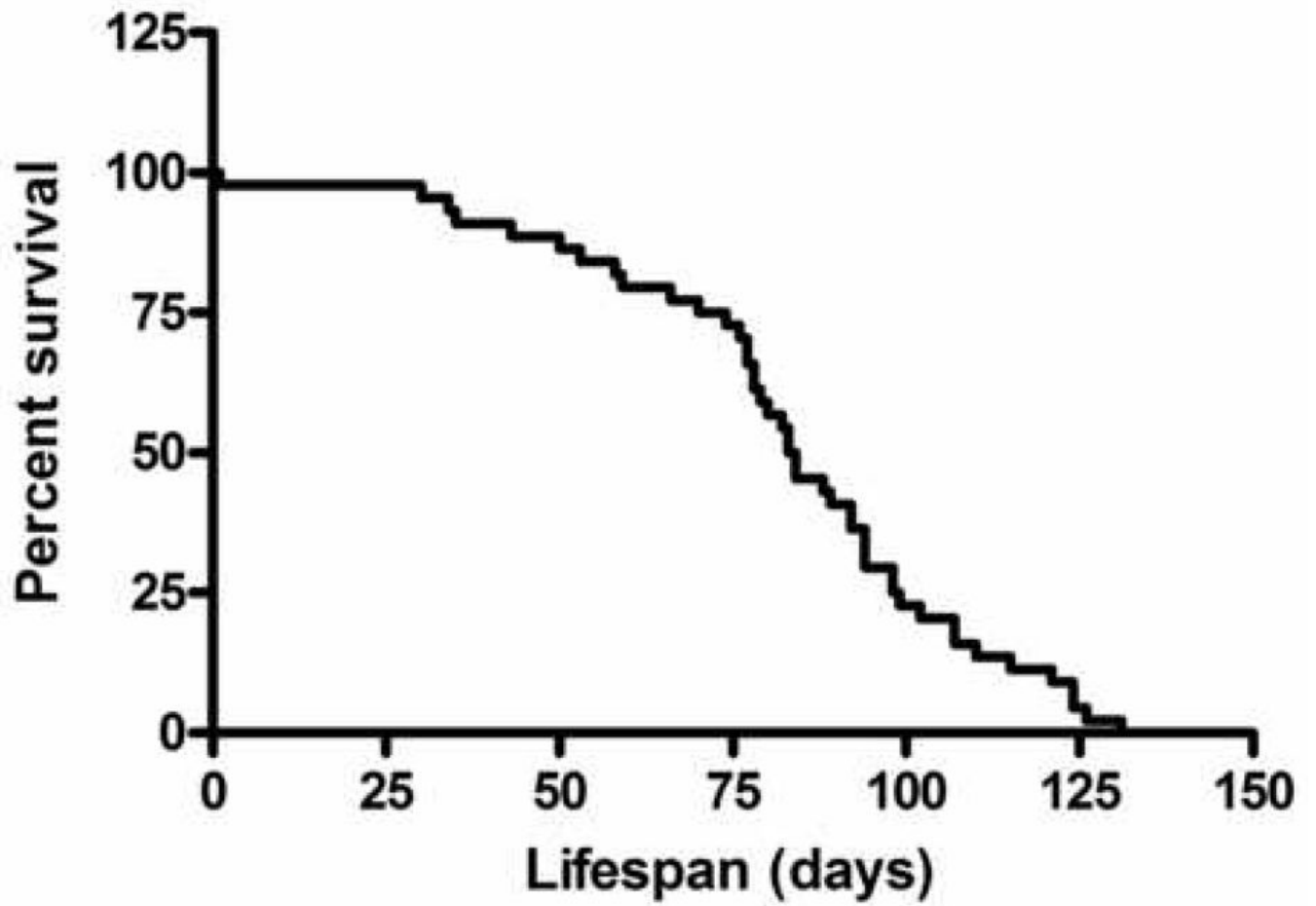


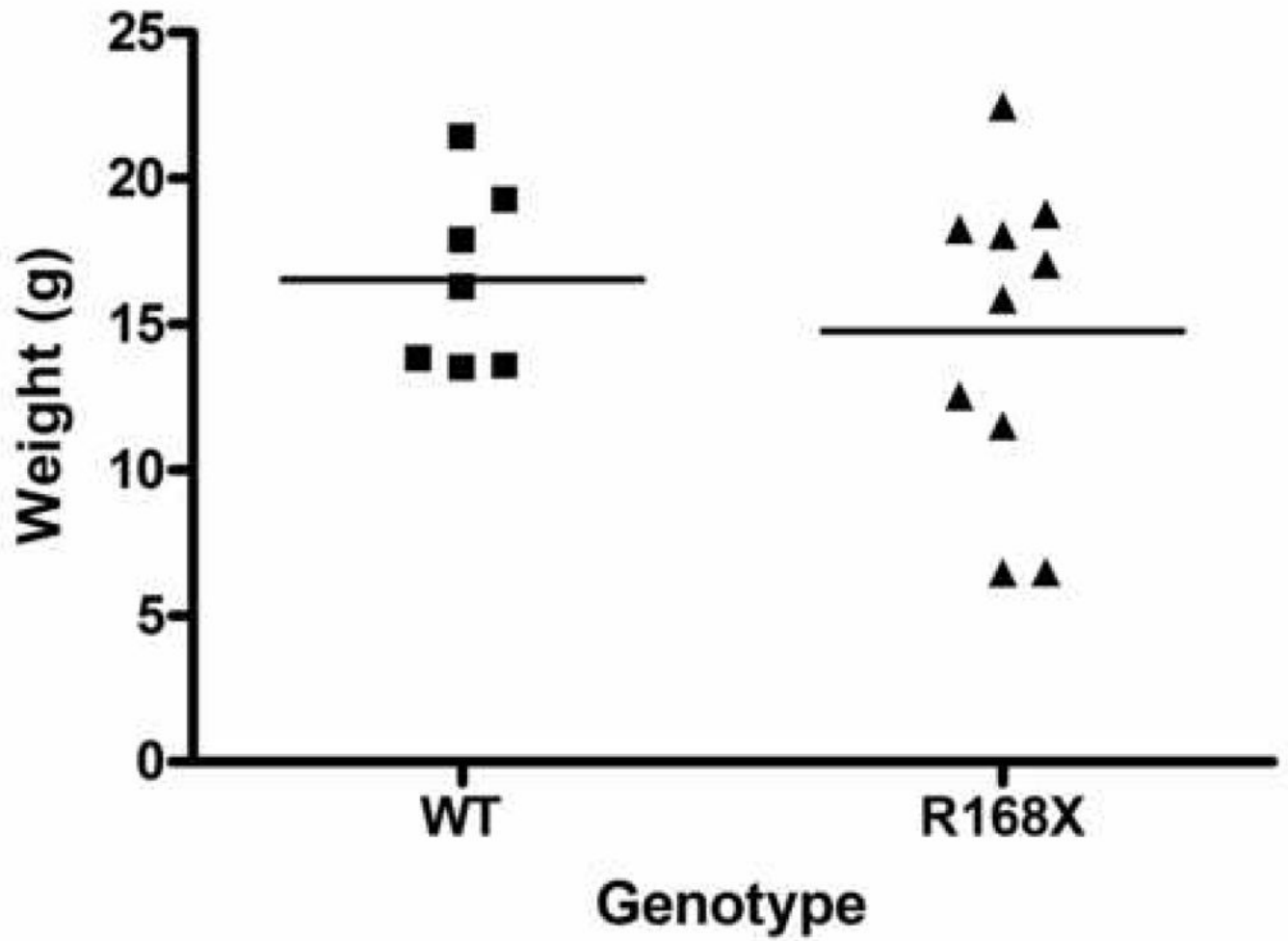
Figure 1. *Mecp2*^{R168X} mutant mice

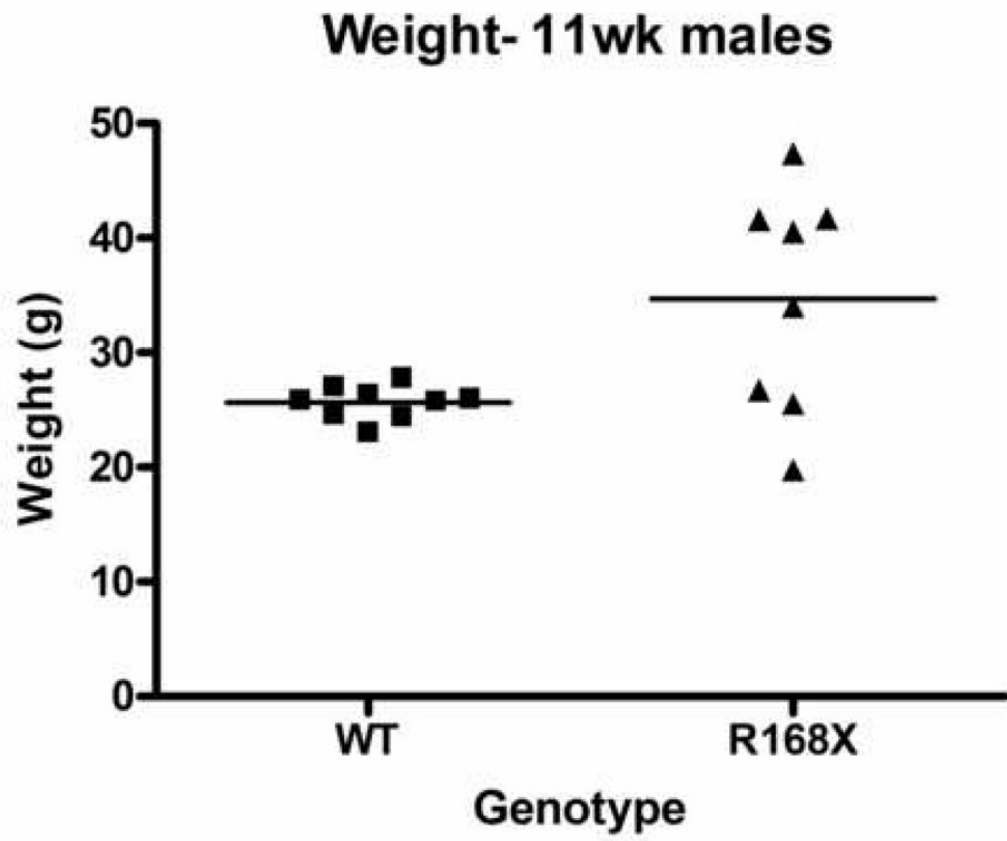
(A) An A to T point mutation was engineered to create a premature stop codon in place of an arginine at codon 168. (B) Confirmation of the mutant transcript by RTPCR. RTPCR was performed with primers flanking the mutation using cDNA reverse transcribed from WT mouse RNA (lane 1), cDNA reverse transcribed from mutant mouse RNA (lane 2), WT mouse RNA (lane 3), and mutant mouse RNA (lane 4). The expected 238bp product was detected in lanes 1 and 2 and confirmed as MeCP2 by sequencing. Larger bands are produced from genomic DNA in lanes 3 and 4 due to the presence of intron 3. (C) The *Mecp2*^{R168X} mutant RNA is relatively stabled compared to the WT transcript. Error bars show standard error of the mean. (D) *Mecp2*^{R168X} mutant mice (right lane) express a small protein which may be a prematurely truncated mutant MeCP2 protein, but do not express the full length WT protein. The WT mice (left lane) express the full length protein.

Lifespan of Mecp2R168X Males



Weight - 4wk old males







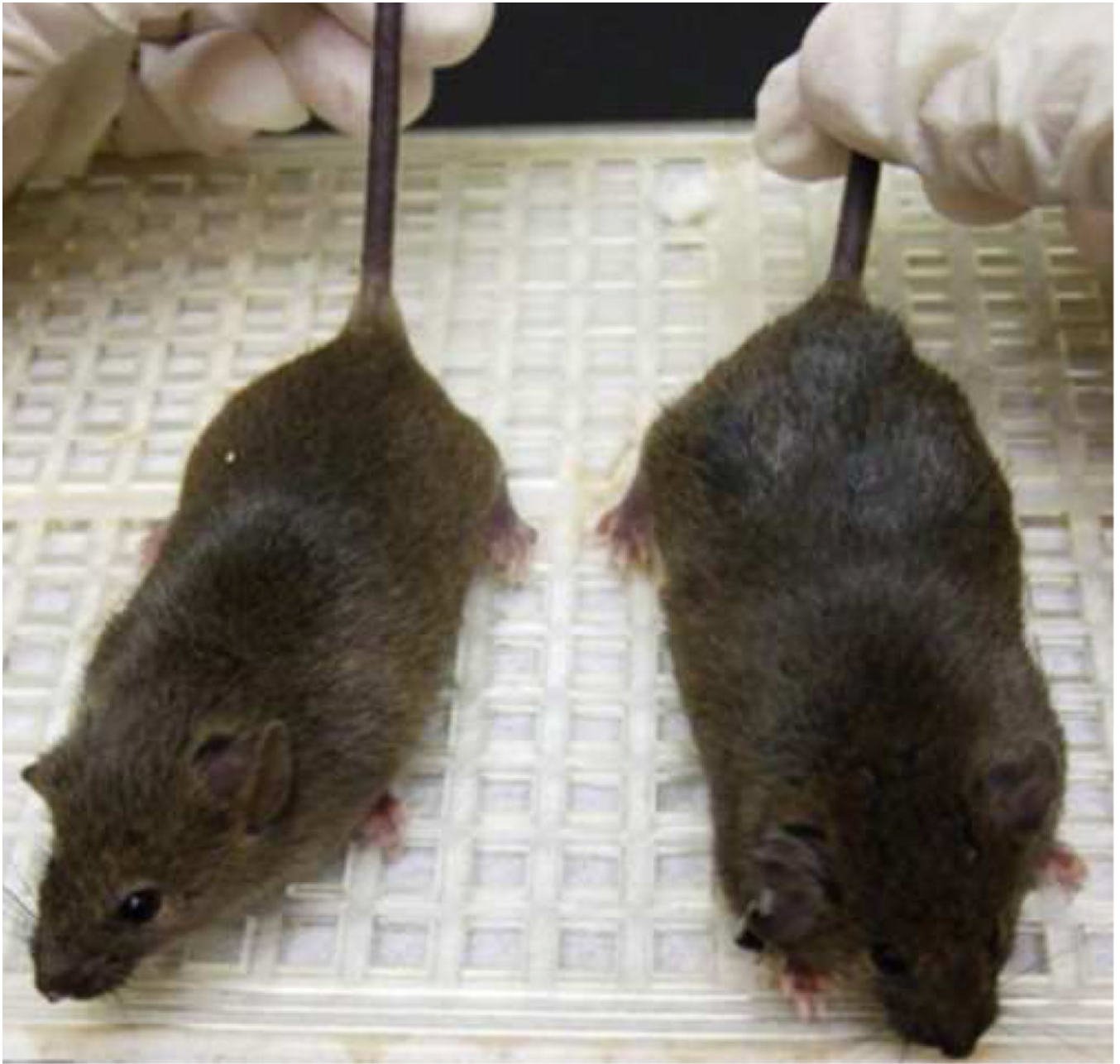






Figure 2. Characterization of Mecp2^{R168X} mutant mice

(A) Lifespan of the Mecp2^{R168X} mutant mice is shortened, with a median lifespan of 83.5 days (n=45). (B) At 4 weeks of age, greater variance in the weight distribution of the affected males was apparent, with numerous low weight mice. (C) In the mice surviving to 11 weeks, weight variance was also apparent, but many more mice were obese. (D) At 3 weeks, an affected hemizygous Mecp2^{R168X} male (mouse on the right) had difficulty gaining weight while his WT littermate thrived. (E) At 7 weeks, an affected hemizygous Mecp2^{R168X} male (mouse on the right) showed greater weight gain his WT littermate. (F) 7 week affected male showing hindlimb clamping. (G) 7 week old WT male littermate showing normal function of hindlimbs.

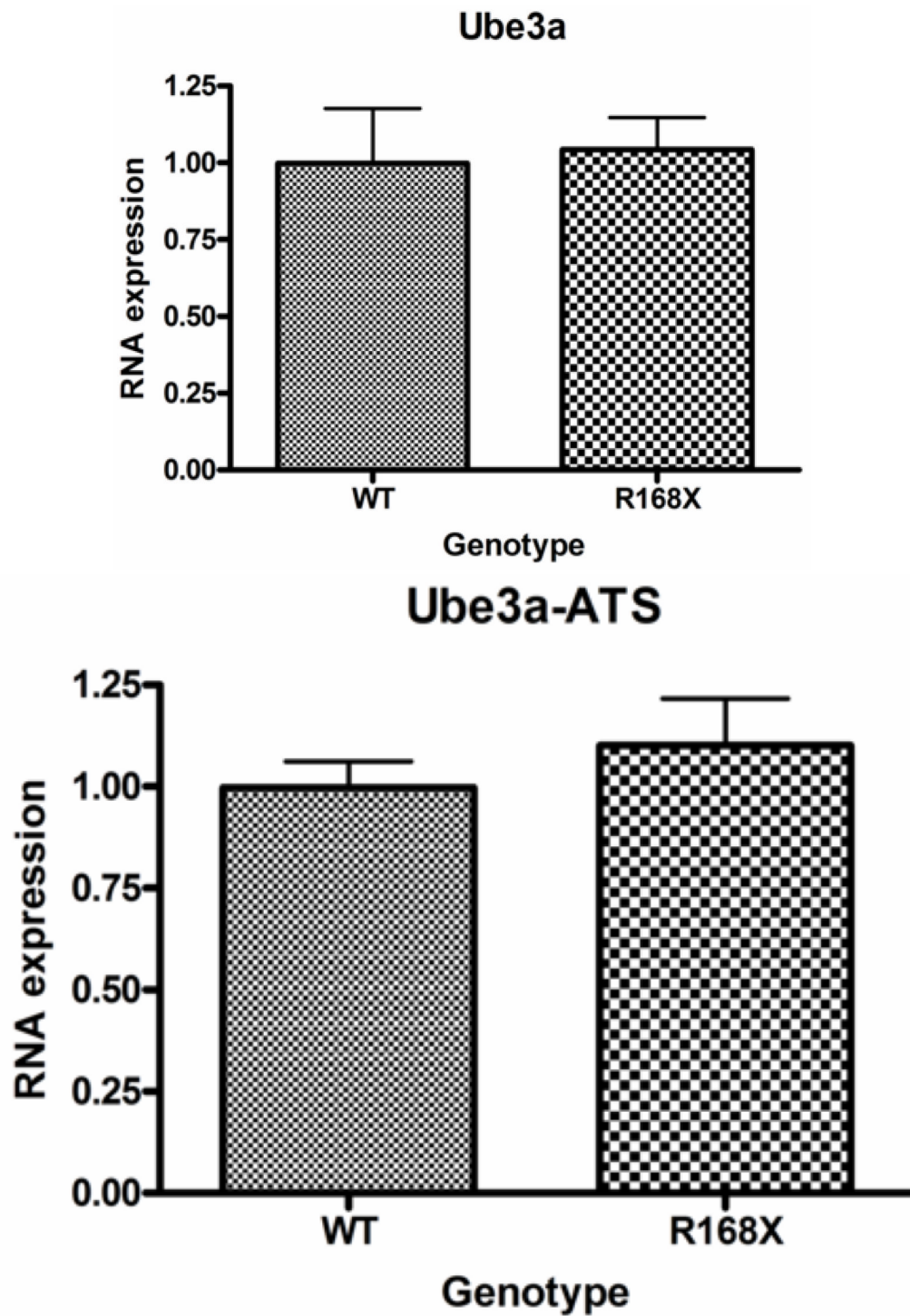


Figure 3. Ube3a and Ube3a-ATS mRNA expression
Male hemizygous *Mecp2*^{R168X} mice no significant difference in Ube3a (A) or Ube3a-ATS (B) mRNA expression in cortical tissue as compared to their WT littermates when analyzed by real time qRT-PCR. Error bars show standard error of the mean.

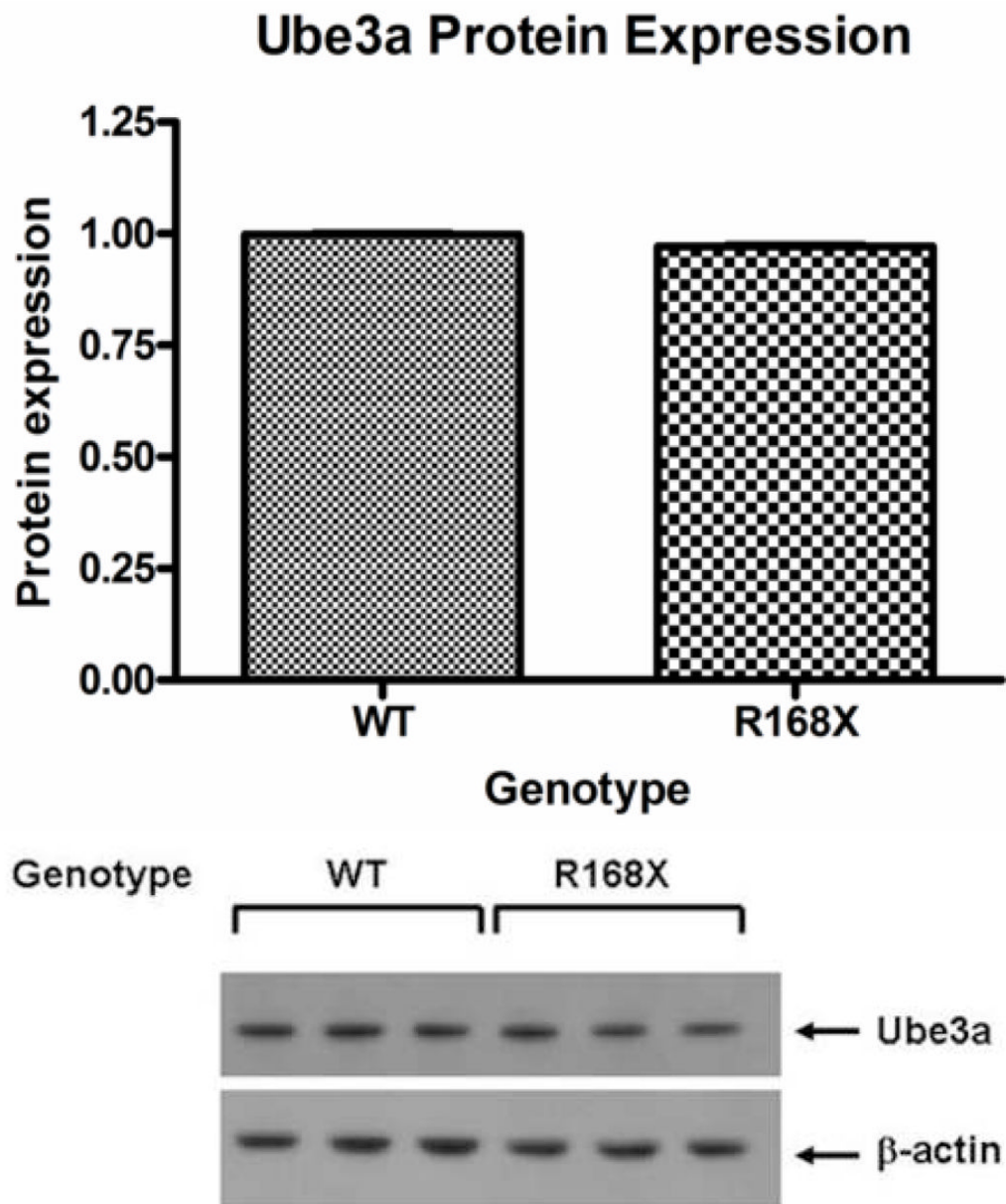


Figure 4. Ube3a protein expression
 (A) Male hemizygous *Mecp2*^{R168X} mice showed no change in Ube3a protein expression in cortical tissue as compared to their WT littermates when analyzed by Western blot. Error bars show standard error of the mean. (B) Western blot showing representative samples from 3 pairs of mice.