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Research paper

Non-canonical C-terminal variant of MeCP2 R344W exhibits enhanced degradation rate

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ABSTRACT

Rett Syndrome (RTT) is a neurodevelopmental disorder caused by pathogenic variants in the *MECP2* gene. While the majority of RTT-causing variants are clustered in the methyl-CpG binding domain and NCoR/SMRT interaction domain, we report a female patient with a functionally uncharacterized *MECP2* variant in the C-terminal domain, c.1030C>T (R344W). We functionally characterized MECP2-R344W in terms of protein stability, NCoR/SMRT complex interaction, and protein nuclear localization in vitro. MECP2-R344W cells showed an increased protein degradation rate without significant change in NCoR/SMRT complex interaction and nuclear localization pattern, suggesting that enhanced MECP2 degradation is sufficient to cause a Rett Syndrome-like phenotype. This study highlights the pathogenicity of the C-terminal domain in Rett Syndrome, and demonstrates the potential of targeting MECP2 protein stability as a therapeutic approach.

1. Introduction

Rett Syndrome (RTT) is a severe neurological disorder caused by pathogenic variants in the X-linked *MECP2* gene which encodes methyl-CpG-binding protein 2 (MECP2). MECP2 plays crucial roles throughout neurodevelopment including neuronal maturation and plasticity (Mellios et al., 2018). Classic RTT is a progressive neurodevelopmental disorder primarily affecting females, which is characterized by apparently normal psychomotor development during the first six to eighteen months of life, followed by a short period of developmental stagnation, then rapid regression in language and motor skills, followed by long-term stability. During the phase of rapid regression, repetitive and stereotypic hand movements replace purposeful hand use. Additional findings include fits of screaming and inconsolable crying, autistic features, panic-like attacks, bruxism, episodic apnea and/or hyperpnea, gait ataxia and apraxia, tremors, seizures, and acquired microcephaly (Chahrour and Zoghbi, 2007; Neul et al., 2010). Atypical RTT is thought to be milder, incomplete, and have a protracted clinical course. Regression occurs later (age one to three years) and is not as severe as that in classic Rett Syndrome, as hand use may be preserved and stereotypic hand movements may be minimal or atypical. RTT affects approximately 1 in 10,000 female births, 90–95% of which is caused by loss-of-function variants in *MECP2* (Ip et al., 2018; Lyst and Bird, 2015; Yusufzai and Wolffe., 2000).

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MECP2 is identified as a nuclear protein that preferentially binds to DNA with methylated CpG and is believed to be primarily involved in transcriptional repression (Lamonica et al., 2017; Lyst et al., 2013). The majority of missense pathogenic variants are clustered within the two functional domains of MECP2, the methyl-CpG binding domain (MBD) and the NCoR/SMRT interaction domain (NID) located within the transcriptional repressive domain (TRD). Among the hundreds MECP2 variants that cause RTT, eight of them (R106W, R133C, T158M, R168 *, R255 *, R270 *, R294 * and R306C; NM_004992.4) account for more than 60% of the reported cases (Lyst and Bird, 2015; Neul et al., 2008). There are also several small insertion or deletion variants in the carboxyl-terminal domain (CTD) of MECP2 that lead to C-terminal truncations, making up 5–14% of cases (Bebbington et al., 2010; Neul et al., 2008).

Although the CTD is not recognized as a functional domain of MECP2, increasing evidence shows that CTD variants are implicated in RTT (Bienvenu et al., 2000; Fendri-Kriaa et al., 2012; Guy et al., 2018; Moncla et al., 2002). A C-terminal deletion study reported a drastic reduction in protein levels despite normal MECP2 function or recruitment (Guy et al., 2018), proposing that the CTD is important in terms of MECP2 protein stability. Similar speculation has also been deduced from results showing faster degradation in truncated MECP2 proteins than full-length MECP2 variants (Yusufzai and Wolffe., 2000).

Here, we report a female patient with a functionally uncharacterized *MECP2* variant in the CTD, encoding the amino acid substitution R344W. The patient is a 20-year-old female who presents RTT-like phenotype, including a period of regression, deficits in fine motor skills, toe walking, and stereotypical hand flapping. Though she only met parts of the main criteria of RTT, she also demonstrated supportive symptoms such as impaired sleep pattern, diminished response to pain, hypotonia, scoliosis, inappropriate screaming or laughing episodes, and thus was diagnosed as atypical RTT.

The CTD variant, p.(R344W), was first reported by Laccone and co-workers in a male patient with RTT-like Syndrome (Laccone et al., 2002). Fendri-Kriaa described another patient with RTT that carried a p.R344G variant, affecting the same amino acid residue but with a glycine substitution (Fendri-Kriaa et al., 2012). Although the change of R344W in MECP2 had been predicted to be likely disease-causing, without functional studies of this variant, it remains to be a variant of uncertain significance (VUS).

The aim of this study is to characterize the functional alterations of a functionally uncharacterized non-canonical variant, R344W, in RTT. This work underlines the importance of missense variants outside canonical MECP2 domains in RTT, and serves as evidence that MECP2 protein stabilization could be a therapeutic potential for RTT patients carrying variants that speed up MECP2 protein degradation.

2. Method

2.1. Constructs

The coding-region of wild type (WT) *MECP2* gene was obtained from the pCMX-Gal4-hMECP2 plasmid (Addgene, #48082), a gift from Dr. Huda Zoghbi. A FLAG tag in the C-terminus of WT MECP2 was first introduced through PCR, and then a R344W mutation (c.1030C>T) was produced by site-directed mutagenesis in the WT MECP2 insert (Fig. 1D). Subsequently, these two inserts were respectively ligated to the plasmid backbone to generate pCMV-MECP2-FLAG plasmids. For immunoprecipitation, we ligated these two inserts to pCMV-Myc plasmid introducing Myc tag in the N-terminus to MECP2-WT and MECP2-R344W. For nuclear localization studies, EGFP-tagged MECP2 plasmids were constructed by ligating MECP2-WT and MECP2-R344W inserts into pEGFP-C1 plasmids (Clontech Laboratories). All plasmids were extracted using Miniprep kit and FavorPrep Plasmid Extraction Midi kit and verified through Sanger sequencing.

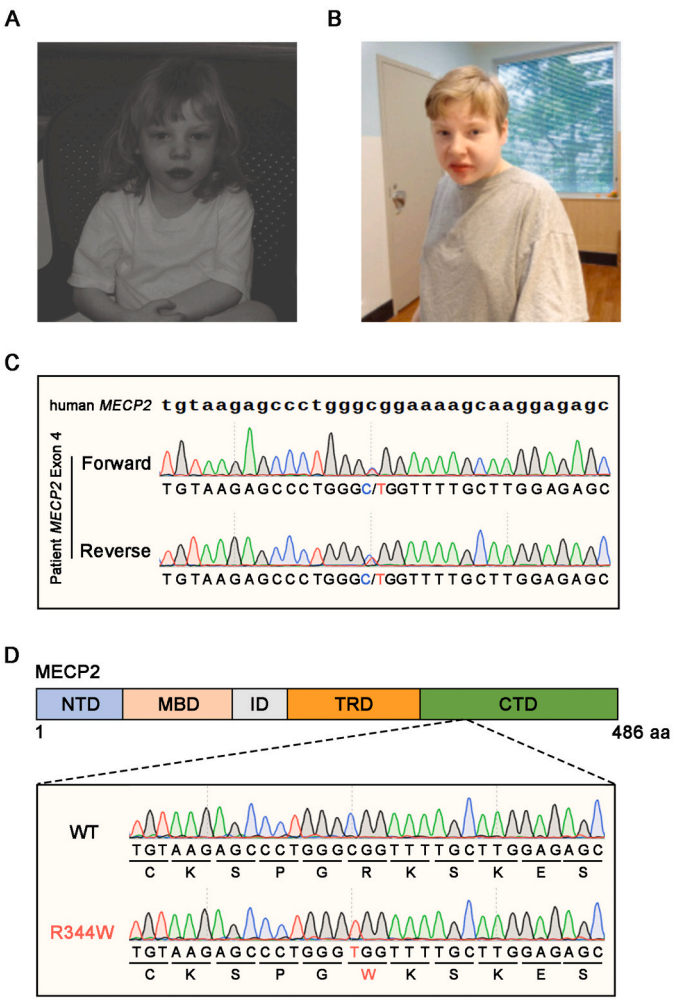

Fig. 1. Characterization of an uncanonical *MECP2* variant. (A). The patient at the age of 3. (B). The patient at the age of 18. Mild dysmorphology, including cupids bow, mild upslanting palpebral fissures, tubular nose, progressed over time. (C). Forward and reverse sequences of *MECP2* exon 4 of the patient. One allele of *MECP2* contains a reported disease-causing variant c.1030C>T in the patient. Both sequences are aligned to the reference genomic sequence of *MECP2* from NCBI GenBank. (D). Construction of MECP2-WT and MECP2-R344W variant plasmids.

2.2. Cell culture

Mouse N2a cells and HEK293T cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C and 5% CO2.

2.3. Antibodies

For immunoblotting, antibodies to FLAG (Sigma-Aldrich, F1804), NCoR (Bethyl, A301–146A), TBLR1 (Abcam, A300–408A), HDAC3 (Sigma, WH0008841M2) and α-tubulin (Santa Cruz, sc-23950) at 1:1000 dilution, and c-Myc (Santa Cruz, sc40) at 1:500 dilution were used for probing. For co-immunoprecipitation, 1μg of antibody to c-Myc was employed for precipitation.

2.4. SDS-PAGE and Western blot

For cycloheximide chase analysis, proteins were separated in 10% SDS-PAGE gel at 120 V for 1.5 h and were subsequently transferred to a nitrocellulose membrane at 90 V for 2 h. For co-immunoprecipitation

experiments, proteins were separated in 7.5% SDS-PAGE gels at 120 V for 1.5 h and were subsequently transferred to a nitrocellulose membrane at 90 V for 2 h. Membranes were blocked with 5% milk in TBST at room temperature (RT) for 1 h, followed by overnight primary antibody incubation with shaking at 4 °C. On the next day, the membranes were rinsed with TBST for 5 min four times and were incubated with secondary antibody for 1 h with shaking at RT. After rinsing with TBST, ECL were used for protein detection and film exposure.

2.5. Cycloheximide chase analysis

HEK293T cells were seeded in a 6-well plate and were transfected with pCMV-MECP2-FLAG plasmids for 12 h using polyethyleneimine (PEI). For degradation analysis, 24 h after transfection, cells were treated with 200 μg/ml of cycloheximide (TOCRIS) in DMSO, and were collected at 0, 3, 6 and 9-hour time points. For rescue analysis, 24 h after transfection, cells were incubated with 200 μg/ml cycloheximide (TOCRIS) in DMSO for 20 min before 40 μM MG132 treatment and were collected after 9 h of incubation. Cells were harvested and lysed in lysis buffer (100 mM Tris pH 8.5, 100 mM NaCl, 1 mM EDTA and 0.5% NP-40) supplemented with protease inhibitor (Abcam), NaF and NaOV for 30 min. Lysates were centrifuged at 14,000 rpm and 4 °C for 15 min, and the supernatant was collected. Proteins were normalized using the Bradford protein assay, and samples were prepared with constant amount of loading buffer and boiled at 95 °C for 10 min. Proteins were resolved via SDS-PAGE, followed by Western blot analysis.

2.6. Co-immunoprecipitation

HEK293T cells were seeded in 100 mm plates and were transfected with pCMV-Myc-MECP2plasmids for 12 h. 24 h after transfection, using a cell scraper, cells were collected in 1X RIPA buffer (DPBS, 150 mM NaCl, 0.1% SDS, 0.5% Na deoxycholate, 1% NP-40) supplemented with protease inhibitors (leupeptin, antipain, aprotnin, trypsin inhibitor, benzamidine, NaOV and PMSF), 250 units/ml benzonase and 1 mM $MgCl_2$, and were lysed for 30 min and vortexed every 10 min to aid lysis. Lysates were centrifuged at 14,000 rpm and 4 °C for 20 min, and the supernatant was collected. Lysates were incubated with 1 μg of Myc antibody with rotation for 2 h at 4 °C, followed by protein A-Sepharose beads (Sigma, P3391) incubation for another 2 h at 4 °C. Bead-bound protein complexes were centrifuged at 200 g and 4 °C for 3 min and washed with 1X RIPA buffer for three times. Pulled-down proteins were eluted with 2X loading buffer and denatured by boiling at 95 °C for 10 min. Proteins were resolved via SDS-PAGE, followed by Western blot analysis.

2.7. Immunofluorescence

N2a cells were seeded in 4-well chamber slides and were transfected with pEGFP-MECP2-C1. 24 h after transfection, cells were washed with PBS and fixed in fixatives (4% formaldehyde, 4% sucrose) for 15 min at room temperature. Fixed cells were briefly washed (PBS), blocked (2.5% BSA and 0.1% Triton X-100 in PBS) for 15 min and incubated with 4′, 6′-diamidino-2-phenylindole (DAPI) (Sigma) in blocking buffer for 30 min at room temperature. After washing with PBS, the chamber was removed, and the slide was mounted with Fluoromount-G (Southern Biotech). The slides were examined using Leica SP8 confocal microscope for EGFP and DAPI.

2.8. Case report

The patient is a female born at 34 weeks gestation to a G1P0 mother. The pregnancy was complicated by reported exposures to substances (possibly alcohol, cigarettes, other illicit substances), however there was an otherwise unremarkable prenatal course with normal prenatal ultrasounds followed by an uncomplicated vaginal delivery. The patient required a 10 day NICU stay for prematurity and jaundice but was then discharged home. Family history is notable for maternal bipolar disorder and substance use disorder, and paternal attention deficit disorder.

The patient met early developmental milestones; notably she rolled over at 4–5 months of age, sat unassisted at 9 months of age, crawled at 9 months of age, and walked at 13 months of age. She said her first words at one year of age, and was speaking in 2–3 word sentences at two years of life. At three years of age, she was going up and down stairs independently, had 3–4 word phrases, and could feed and dress herself (Fig. 1A). Her growth parameters at the age of three were: weight 13 kg (5th percentile), height 93 cm (10th percentile), head circumference 48 cm ($Z = -1.0$).

However, it was around this time when the patient's caregivers noticed challenges in social communication; she did not respond well to other children and was often "in her own world". Though she had developed phrases, she often would repeat phrases during inappropriate times. She often avoided eye contact, and was felt to be unaffectionate. She engaged in restricted and unusual play, often lining up her toys, and had a preference for gazing at light fixtures. She began having many aggressive behavioral outbursts and tantrums, which would sometimes last for 20 min and included self-injurious behavior such as head banging. She was noted to have some hand flapping stereotypies. She underwent evaluation through Developmental Pediatrics, and was thus diagnosed with autism spectrum disorder, and global developmental delay at the age of four. Additional symptoms that were noted by the patient's care providers at age four included toe walking requiring bracing and physical therapy, hypotonia, poor sleep with frequent waking, and insensitivity to pain. She attended early intervention preschool programming and transitioned to public school for kindergarten, where she required a full Individualized Educational Plan (IEP) for educational support, which was in place until she graduated from high school. At the age of 10 years old, the patient was noted to have continued trouble with communication; she had not gained any complex language skills since the age of three, remaining with simple words and phrases only. Her behavior problems had worsened, where she had frequent episodes of self-injurious behavior and dangerous elopement incidents. Atypical antipsychotic medications were added to her regimen to attempt to manage these behaviors. At the time of this writing, the patient is now 20 years old. She continues to have difficult sleep patterns, sometimes staying up all night for 4–5 nights in a row. She can ambulate independently, but occasionally has dyspraxia. She requires oral medication for constipation management. She has mild kyphosis. She is described as overall happy, and does have occasional inappropriate laughing spells.

A screening electroencephalogram (EEG) was done at the time of autism diagnosis (at the age of four) and was found to be normal. Notably, the patient did not demonstrate any seizure symptoms throughout her developmental course. A magnetic resonance imaging (MRI) was completed at the age of four, which demonstrated mild abnormalities, namely multifocal hyperintense signal abnormalities on T2 and FLAIR sequence in the periventricular region and centrum semiovale bilaterally, areas of FLAIR hyperintensity identified in the posterior periventricular white matter, right periventricular white matter along the body of the lateral ventricle, right frontal subcortical white matter and high right centrum semiovale, and T2 prolongation and FLAIR signal within the superior cerebellar white matter. Additionally, it was noted that the temporal lobe myelination was incomplete particularly in the medially and anteriorly multifocal areas of T2 and FLAIR prolongation within the periventricular white matter and centrum semiovale. The findings were felt to represent gliosis from prior insult (prematurity) versus incomplete myelination.

Genetic testing was sent at the age of four, and was notable for a normal karyotype and normal Fragile X repeat expansions. *MECP2* sequencing was sent (Baylor Diagnostic Laboratory) and returned with a VUS in *MECP2*, c.1030C>T (R344W) (Fig. 1C). Parental segregation was pursued, and this variant was not found in the mother, while paternal

testing was not available. A formal diagnosis of Rett Syndrome was not made due to the uncertainty of the variant, and the patient not meeting all clinical criteria for Classic RTT. Genetics was reconsulted when the patient was 18 years of age to reconsider the *MECP2* finding (Fig. 1B). At this time additional genetic testing was sent in an attempt to identify an alternative genetic molecular diagnosis. A microarray returned normal. A broad exome-based 2500 gene commercial panel (Autism ID Xpanded Panel, GeneDx) was sent and did not identify any pathogenic single nucleotide variants or copy number variants. At that time, the patient was then diagnosed with atypical Rett Syndrome with a known variant in *MECP2*, no alternative diagnosis, and clinical criteria (regression/stagnation in language and fine motor skills, partial loss of spoken language, occasional dyspraxic gait, and mild hand stereotypes, with supportive criteria of impaired sleep pattern, abnormal muscle tone, kyphosis, inappropriate laughing, and diminished response to pain). Consideration was given to MRI findings meeting exclusion criteria, however the RTT exclusion criteria notes that brain injury must cause neurological problems. With normal development noted in the first two years of life, these MRI findings would be unlikely to explain regression and stagnation of language skills.

3. Results

3.1. MECP2-R344W mutation reduces the protein stability of MECP2

We aim to characterize the functional alterations of a functionally uncharacterized non-canonical variant, R344W, in RTT. R344W is located at the C-terminal domain (CTD) of MECP2. The C-terminus of MECP2 has been implicated in affecting MECP2 protein stability, and it has been suggested that its loss-of-function may be attributed to the increased degradation in truncated MECP2 proteins, which is also observed in various MECP2 mutations (Guy et al., 2018; Lamonica et al., 2017; Lyst et al., 2013; Yusufzai and Wolffe., 2000). To determine whether R344W has resulted in reduced MECP2 protein stability, we employed the cycloheximide (CHX) chase assay to study the degradation kinetics of the protein (Lamonica et al., 2017; Yusufzai and Wolffe., 2000). We cultured MECP2 plasmids-transfected HEK293T cells and treated with CHX, a eukaryotic translational elongation blocker, to inhibit new protein synthesis. Western blot analysis shows that while MECP2-WT and MECP2-R344W levels showed no significant difference after 3 h and 6 h of CHX treatment, the MECP2 levels for MECP2-R344W was reduced by approximately 75% after 9 h, which was significantly lower than that of MECP2-WT (Fig. 2A & B). Results here suggest that MECP2-R344W has reduced protein stability, which supports previous work showing reduced protein level in CTD deletion-frameshift mutants (Guy et al., 2018), and is consistent with our hypothesis that R344W in the CTD may contribute to the RTT phenotype by affecting the protein stability of MECP2.

This hypothesis was further tested by evaluating whether the reduced MECP2-R344W protein levels could be rescued through inhibiting the ubiquitin/proteosome pathway, which has been reported to have rescued the MECP2 levels to the greatest extent using the proteosome inhibitor MG132 (Lamonica et al., 2017). We cultured MECP2-R344W plasmid-transfected HEK293T cells and pre-treated the cells with CHX for 20 min before MG132 treatment. Western blot analysis shows that cells treated with MG132 had MECP2-R344W protein levels approximately 1.5 folds higher than that of cells without MG132 treatment (Fig. 3A & B), suggesting that the MECP2-R344W protein levels can be rescued by inhibiting the ubiquitin/proteosome pathway. Intriguingly, we found that MG132 has no effect on MECP2-WT level (Fig. 3A & B). Taken together, these data support the hypothesis that R344W reduces MECP2 protein stability and increased protein degradation rate via the ubiquitin/proteosome pathway.

3.2. R344W does not interfere MECP2 protein interaction with NCoR/SMRT complex components

The role of the methyl-CpG binding domain (MBD) and NCoR interaction domain (NID) in MECP2 is well-established and has been widely reported in many studies; MBD is important for methylated DNA binding specificity, and NID is crucial for the interaction with NCoR/SMRT co-repressor complexes through TBL1 and TBLR1 (Free et al., 2001; Lyst and Bird, 2015; Yusufzai and Wolffe., 2000). Since R344W is not located in or near the NID region, we do not expect that it will affect the MECP2 interaction with the NCoR/SMRT co-repressors. To determine whether R344W in the CTD had any potential involvement in the MECP2 interaction with the NCoR/SMRT co-repressor complexes, we performed co-immunoprecipitation on extracts from MECP2 plasmids-transfected HEK293T cells to study the MECP2 protein-protein interactions. The result from immunoprecipitation reveals that MECP2-R344W did not affect its interaction with NCoR/SMRT co-repressor complexes (Fig. 4A, B).

3.3. R344W does not interfere with MECP2 nuclear localization

A previous X-ray crystallo-graphic analysis suggested that the specificity of MECP2 to methylated CpG is mediated by the conserved hydrophilic region in the MBD, where water molecules had been proposed to enable the DNA-protein interaction as intermediates in earlier studies (Ho et al., 2008; Mayer-Jung et al., 1998; Schwabe, 1997). A dispersed pattern of MECP2 in the nuclei can be observed in MECP2 with mutated MBD or reduced level of DNA methylation (Guy et al., 2011; Nan et al., 1996), indicating failed localization of the protein. While the role of CTD remains unclear, it has been reported that MECP2 showed comparable binding with DNA even with the entire CTD missing, suggesting that CTD is unlikely to involve in DNA binding (Yusufzai and Wolffe., 2000).

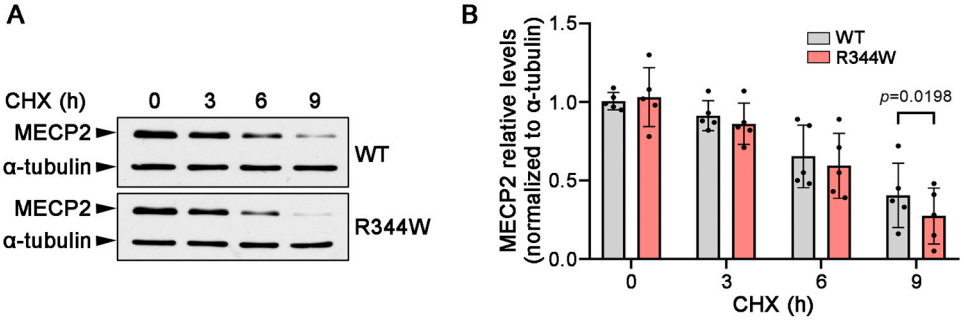

Fig. 2. MECP2-R344W mutation decrease the stability of MECP2 protein. (A) and (B). Decreased MECP2-R344W protein levels following 9 h of CHX treatment. (A.) Western blot analysis of MECP2 protein levels in MECP2 transfected HEK293T cells following 0, 3, 6, 9 h CHX treatment. (B). Grey bars: MECP2-WT protein levels. Red bars: MECP2-R344W protein levels. Values are normalized to α-tubulin of each timepoint. Two-way ANOVA was performed. $P = 0.0198$. All error bars represent the mean ± SEM (n = 5).

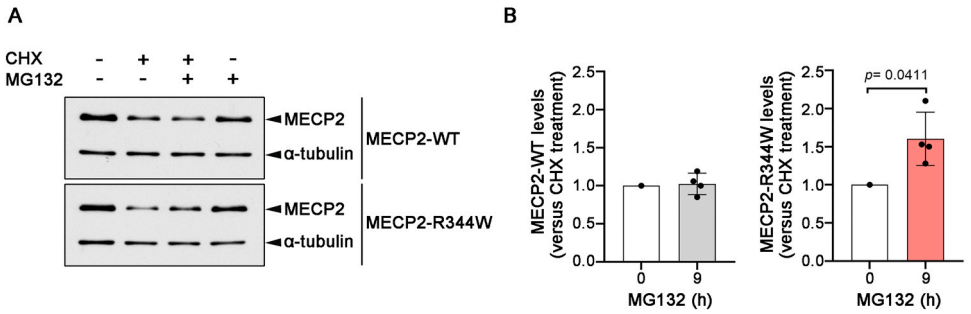

Fig. 3. Blocking proteosome pathway can restore MECP2-R344W protein level. (A) and (B). MG132 treatment rescued FLAG-tagged MECP2-R344W protein degradation. (A). Western blot analysis of MECP2 protein levels of FLAG-tagged MECP2-WT and MECP2-R344W following 9 h CHX treatment with or without MG132. (B). Values are represented as the fold change relative to cells treated with 9 h CHX treatment but without MG132. MECP2-R344W protein level was restored 50% by MG132 compared to untreated group while no rescue effect was observed in MECP2-WT protein levels with 9 h treatment of MG132. Paired t-test was performed. $P = 0.0411$. All error bars represent the mean ± SEM (n = 4).

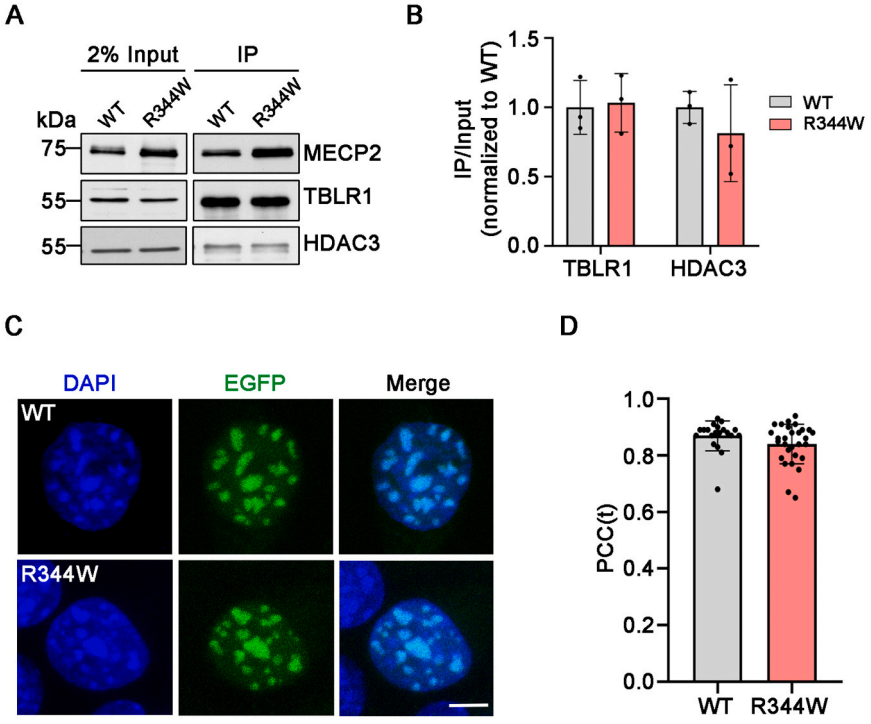

Fig. 4. NCoR/SMRT interaction and nuclear localization isn't interfered by MECP2-R344W mutation. (A) and (B). Undisrupted MECP2-R344W protein interaction with NCoR/SMRT complex components. (A). Co-immunoprecipitation of TBLR1 and HDAC3 from extracts of MECP2-WT and MECP2-R344W transfected HEK293T cells. (B). Values are presented as "Input" divided by "IP" and are shown as the fold change relative to MECP2-WT. Unpaired t-test was performed. All error bars represent the mean ± SEM (n = 3). (C) and (D). Similar MECP2 nuclear localization pattern between MECP2-WT and MECP2-R344W proteins. Mouse neuroblastoma N2a cells that transiently expressed the full-length EGFP-tagged MECP2-WT and MECP2-R344W, representing the co-localization at chromocenters (DAPI; Blue) and the recombinant EGFP-tagged MECP2 protein (Green). Scale bar: 5 μm. (D). Average mean of the Pearson Correlation Coefficient (PCC) values (n = 20 cells for WT and n = 29 for R344W; ± SEM shown). Unpaired t-test was performed.

In this regard, we hypothesize that R344W will have minimal effects on the nuclear localization pattern of MECP2. To examine this, N2a cells were transfected with EGFP-MECP2-WT and -R344W plasmids and were subjected to immunostaining. Clear and visible heterochromatic foci were observed in EGFP-MECP2-WT N2a cells, and a similar nuclear localization pattern was also observed in EGFP-MECP2-R344W N2a cells (Fig. 4C, D). Colocalization analysis using Pearson Correlation Coefficient (PCC) was also performed. High PCC of EGFP and DAPI were observed at the heterochromatic foci (Fig. 4C, D), suggesting that the MECP2 protein was colocalized to DNA, and was not affected by mutation R344W. Consistent with our hypothesis, these results suggest that R344W does not involve in MECP2 nuclear localization.

4. Discussion

While pathogenic variants of *MECP2* in the MBD and NID have been well studied in RTT, little is known about the mutations located outside the functional domains. Studying the mechanisms of different variants in *MECP2* and their function helps facilitate a better understanding of the protein, and may provide insights for therapeutics development. This study reports a female patient with an uncharacterized *MECP2* variant in the C-terminal domain, c.1030C>T (R344W), and characterizes the function of this non-canonical *MECP2* variant in relation to RTT from three aspects, namely protein stability, NCoR/SMRT complex interaction, and protein nuclear localization in vitro. We found that R344W in the CTD of MECP2 increases protein degradation, suggesting

MECP2 deficiency and functional domain impairments due to protein instability is sufficient to lead to RTT-like phenotype, highlighting the pathogenicity of CTD variants in RTT. We also observed no change in protein binding level among MECP2-R344W, TBLR1 and HDAC3 (Fig. 4A, B). Thus, we conclude that the MECP2 recruitment of NCoR/SMRT co-repressor complexes is unlikely to be affected by the R344W variant. However, whether or not R344W variant of MECP2 behaves the same way in neurons as in the non-neuronal model system is still unclear, further examination is required to test in the neuronal context. All in all, these findings provide important evidence to support the notion of targeting MECP2 protein stability as a potential therapeutic approach (Guy et al., 2018; Yusufzai and Wolffe., 2000).

4.1. R344W variant exhibited reduced protein stability

MECP2 protein instability-led MECP2 deficiency has been previously proposed to be an underlying cause of RTT (Guy et al., 2018). Several mutations reported to affect the functional domains of *MECP2*, including T158A, T158M, R133C, R168 * and R255 * , were also found to destabilize the MECP2 protein (Brown et al., 2016; Guy et al., 2018). This raises an interesting question of whether the pathological mechanisms behind these recurring mutations are caused by the conventional functional domains (MBD and NID) alone.

It has been reported that the CTD of MECP2 is involved in maintaining protein stability and might have contributed to the pathogenesis of RTT (Guy et al., 2018; Yusufzai and Wolffe., 2000). Therefore, we hypothesized that R344W, as a CTD variant, may also reduce the protein stability of MECP2. Consistent to previous report (Guy et al., 2018), the reduction in CTD-mutated MECP2 protein level was also observed in our study (Fig. 2). Conversely, our hypothesis is also supported by the partial restoration of MECP2 protein level by blocking the ubiquitin/proteosome degradation pathway via MG132 (Fig. 3).

4.2. R344W variant showed no observable effect on MECP2 nuclear localization

While MBD remains to be the primary site for methylated CpG recognition, the CTD of MECP2 has been shown to facilitate DNA and chromatin association (Chandler et al., 1999; Guy et al., 2018). Evidence from DNase I footprinting of nucleosome core showed that a C-terminal deletion mutant of MECP2 (MECP2 1–404) failed to protect DNA in the nucleosome and had significantly reduced protection to the linker DNA (Chandler et al., 1999). These results also demonstrated that the specific nucleosome profile of methylated CpGs affects MBD recognition, and that the methylated CpGs exposed in the nucleosome core were less protected by deletion mutant than a full-length MECP2, concluding that the CTD of MECP2 contributes to both the protein affinity for DNA and nuclease protection (Bianciardi et al., 2016; Chandler et al., 1999). These results demonstrated the possible involvement of the CTD in facilitating the MBD in MECP2 methylated DNA binding, suggesting an important role of a non-classical domain on the functional domains. Although how R344W affects the MBD hence methylated DNA binding of MECP2 is beyond the scope of this study, it would be interesting to test the findings and investigate whether these changes are clinically significant in the future.

Nevertheless, it appears unlikely that R344W would affect nuclear localization pattern of MECP2 (Fig. 4C, D). Nuclear localization signal (NLS) is a conserved region that can be found within the TRD (Guy et al., 2011; Piccolo et al., 2019). NLS has been identified as a short motif that interacts with nuclear import factors KPNA3 and KPNA4, and mediates active transport through the nuclear pore complex, but intriguingly, is not found to be necessary for MECP2 nuclear localization (Lyst et al., 2018; Nan et al., 1996). Prior work has demonstrated that MECP2 proteins are abundantly retained within the nucleus and concentrated in heterochromatic foci when NLS is disrupted (Lyst et al., 2018). The same study also demonstrated that disease progression was not affected in vivo with NLS being blocked (Lyst et al., 2018). Conversely, substantial cytoplasmic MECP2 was detected when both MBD and NLS were mutated, suggesting that MBD affinity for DNA plays a role in nuclear localization (Lyst et al., 2018). Furthermore, a study showed that MECP2 with MBD deletions cannot be localized to heterochromatin (Nan et al., 1993; Nan et al., 1996). Together, these results revealed that an intact MBD alone, rather than NLS, is necessary and sufficient for MECP2-DNA affinity, hence specific MECP2 nuclear localization and retention (Lewis et al., 1992; Lyst et al., 2018; Nan et al., 1993; Nan et al., 1996). Therefore, consistent with the current evidence, R344W is unlikely to affect nuclear localization of MECP2.

4.3. Challenges of Rett Syndrome therapies and therapeutic potential

Our findings also suggest the potential of restoring the MECP2 protein level through pharmacological intervention. In fact, RTT is not regarded as a neurodegenerative disorder because neurons do not die and are not damaged permanently in the absence of MECP2 (Guy et al., 2007). The feasibility of rectifying RTT-like phenotype using genomic manipulation has been demonstrated in mouse in vivo studies (Garg et al., 2013; Guy et al., 2007). De novo expression of MECP2 in symptomatic RTT adult mice showed reversible phenotype and restored neuronal functions (Guy et al., 2007). Systemic delivery of exogenous MECP2 into RTT mice also reversed and stabilized RTT-like symptoms, and showed improvements in behavioral, cognitive, and respiratory functions (Garg et al., 2013).

Though gene therapy may be a viable RTT treatment in the future, there are still many challenges ahead. Appropriate mode of delivery and choice of vector are needed to minimize uneven distribution of gene editing materials. While systemic injection of gene editing materials through the tail vein improved symptoms in mice, it has been reported that site-specific intracranial injection developed parkinsonism, likely due to local overexpression of MECP2 at the injection sites (Garg et al., 2013). Dose control is another major problem as overexpression confers the risk of developing symptoms of MECP2 Duplication Syndrome due to X-inactivation in females. X-inactivation in females ensures dosage compensation by silencing either one of the two copies of X-chromosome. Since the *MECP2* gene is located on the X-chromosome, it is important to deliver sufficient MECP2 to restore non-functional cells without affecting cells carrying the functional MECP2 (Sandweiss et al., 2020). Moreover, since the clinical application of gene therapy is still under development, fewer patients may see the benefit of said therapy. On these premises, our findings suggested that MECP2 protein stabilization is a potential therapeutic alternative for treating RTT-like phenotype, with fewer ethical concerns, better dose control, and relatively low cost of production. Further investigation on the therapeutic potential of MECP2 protein stabilization for RTT will be the focus of future studies. While our findings have come to a conclusion that R344W reduces MECP2 protein stability, the underlying mechanism and clinical significance are yet to be elucidated. All in all, these findings support the claim that targeting MECP2 protein stability could be a potential therapeutic direction in the future. Our results offer precision medicine implications for specific patients with functional MECP2 proteins with increased degradation. However, for the patients with dysfunctional MECP2 proteins, enhancing protein stability would not likely have therapeutic value.

Compliance with ethical standards statement

All procedures performed in studies were in accordance with the ethical standards of the institutions of the authors.

Informed consent

Written and informed consent for publication was obtained for case report from legal guardians of the patient.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibneur.2023.09.007.

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