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XLID CUL4B mutants are defective in promoting TSC2 degradation and positively regulating mTOR signaling in neocortical neurons

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ABSTRACT

Truncating or missense mutation of cullin 4B (CUL4B) is one of the most prevalent causes underlying X-linked intellectual disability (XLID). CUL4B-RING E3 ubiquitin ligase promotes ubiquitination and degradation of various proteins. Consistent with previous studies, overexpression of wild-type CUL4B in 293 cells enhanced ubiquitylation and degradation of TSC2 or cyclin E. The present study shows that XLID mutant (R388X), (R572C) or (V745A) CULB failed to promote ubiquitination and degradation of TSC2 or cyclin E. Adenoviruses-mediated expression of wild-type CUL4B decreased protein level of TSC2 or cyclin E in cultured neocortical neurons of frontal lobe. Furthermore, shRNA-mediated CUL4B knockdown caused an upregulation of TSC2 or cyclin E. XLID mutant (R388X), (R572C) or (V745A) CUL4B did not downregulate protein expression of TSC2 or cyclin E in neocortical neurons. By promoting TSC2 degradation, CUL4B could positively regulate mTOR activity in neocortical neurons of frontal cortex. Consistent with this hypothesis, CUL4B knockdown-induced upregulation of TSC2 in neocortical neurons resulted in a decreased protein level of active phospho-mTOR^{Ser2448} and a reduced expression of active phospho-p70S6K^{Thr389} and phospho-4E-BP1$^{Thr37/46}$, two main substrates of mTOR-mediated phosphorylation. Wild-type CUL4B also increased protein level of active phospho-mTOR^{Ser2448}, phospho-p70S6K^{Thr389} or phospho-4E-BP1$^{Thr37/46}$. XLID CUL4B mutants did not affect protein level of active phospho-mTOR^{Ser2448}, phospho-p70S6K^{Thr389} or phospho-4E-BP1$^{Thr37/46}$. Our results suggest that XLID CUL4B mutants are defective in promoting TSC2 degradation and positively regulating mTOR signaling in neocortical neurons.

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1. Introduction

Intellectual disability (ID) is found in ~1%–2% of the human population and is more prevalent in males than in females [1–3]. The etiology of intellectual disability (ID) is heterogeneous, and the most prevalent cause is the mutations at various loci of the X chromosome [1–4]. Up to now, mutations of 102 genes of the X chromosome have been shown to be associated with X-linked intellectual disability (XLID) [5]. Based on the presence or absence of other symptoms, XLID can be divided into syndromic and nonsyndromic forms [1–4]. Previous molecular genetic studies demonstrated that truncating or missense mutations of cullin 4B (CUL4B) gene located on the Xq24 locus were observed in male ID patients with other CNS syndromes, including relative macrocephaly, unprovoked aggressive outbursts and intention tremor [6–10]. CUL4B mutation is one of the most frequently mutated genes underlying XLID [6–10]. XLID truncating or missense mutations of CUL4B are believed to cause the impaired CUL4B function in the brain and resulting intellectual disability [6,7]. However, further studies are required to investigate the functional consequence of XLID mutations on CUL4B-mediated physiological effects and molecular pathogenic mechanisms underlying XLID mutant CUL4B-induced intellectual disability in the brain.

Over 1000 distinct E3 ligases, which belong to either the HECT family or the RING family, are found in eukaryotic cells [11,12]. Cullin 4B is a member of family of seven mammalian cullins (cullin 1, 2, 3, 4A, 4B, 5 and 7), which bind to a small RING protein ROC1 or ROC2 (for RING of cullins) and form the largest class of E3 ligases, the cullin-RING E3 ligases [11–13]. In contrast to RING E3 ligases that bind substrates directly, the cullin-RING E3 ligase complex does not interact with substrate directly. Instead, cullin-mediated ubiquitination requires substrate recruiting receptors that are typically joined to cullin-RING E3 ligase complex by a linker protein [11–13]. The C-terminal domain of CUL4B binds to ROC1 or ROC2, which recruits and allosterically activates an E2 enzyme that transfers ubiquitin to the substrate, and assembles into a CUL4B-RING E3 ubiquitin ligase (CRL4B). N-terminal domain of CUL4B interacts with DDB1 (damaged DNA binding protein 1), which acts as the linker protein and links CRL4B to a family of DWD (DDB1-binding WD40) proteins that function as substrate receptors

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and recruit different substrates to CUL4B-ROC-E2 catalytic core. CUL4B-RING E3 ubiquitin ligase has been shown to cause ubiquitylation and subsequent 26S proteasome-mediate degradation of several proteins, which are implicated in DNA replication, cell cycle control, chromatin formation, DNA damage repair, signal transduction and development [12].

In mammalian cells, Ser/Thr protein kinase mTOR (mammalian target of rapamycin) functions as the essential component of two distinct multi-protein signaling complexes called mTORC1 and mTORC2 [14]. The rapamycin-sensitive mTORC1 regulates various intracellular processes, including translation, autophagy, cell cycle and microtubule dynamics [14,15]. Kinase activity of mTOR is controlled by various stimuli such as trophic factors, hormones, neurotransmitters, amino acids, cell energy status and cellular stress [14]. Activation of receptor tyrosine kinases or neurotransmitter receptors leads to the activation of phosphatidyl-inosital-3 kinase (PI3K), and activated PI3K promotes the formation of phosphatidylinositol-3,4,5-trisphospate (PIP3). An increased level of PIP3 causes the recruitment of phosphoinositide-dependent kinase 1 (PDK1) and AKT/protein kinase B (PKB) to the cell membrane and subsequent phosphorylation of AKT by PDK1. Active phospho-AKT activates mTORC1 by phosphorylating a GTPase activating protein TSC2/tuberin, which forms the tuberous sclerosis complex (TSC) with TSC1/hamartin [16]. TSC1/TSC2 complex negatively regulates mTORC1 signaling by inhibiting the Rheb (Ras homolog enriched in brain) GTPase, a positive regulator of mTORC1. AKT phosphorylation of TSC2 inhibits activity of TSC1/TSC2 complex, which allows GTP-bound Rheb to accumulate and activate the mTOR of mTORC1. Then, active and phospho-mTOR of mTORC1 acts primarily by phosphorylating 4E-BP (eukaryotic translation initiation factor 4E binding protein) and p70 ribosomal S6 protein kinase (p70S6K), which are two main regulators of mRNA translation and ribosome biogenesis [14,15]. In the brain, mTOR signaling regulates survival, differentiation and development of neurons [14]. Proper mTOR activity is required for axon guidance, dendrite development, dendritic spine morphogenesis and synaptogenesis [14,17]. The mTOR signaling cascade also mediates long-term synaptic plasticity including late long-term potentiation (LTP) or mGluR-dependent long-term depression (LTD), which underlies processes of learning and memory formation [18–21]. Long-lasting synaptic plasticity and memory require new protein synthesis [18,19]. Previous studies reported that late LTP- or mGluR-LTD-inducing stimulation induced mTOR-mediated phosphorylation of downstream targets, 4E-BP and p70S6K, and subsequent activation of translation machinery in the dendrites [18–20,22,23]. Therefore, dysregulation of mTOR signaling activity is believed to be one of molecular pathogenic mechanisms underlying intellectual disability [21].

Previous study using cell lines demonstrated that CUL4-DDB1 ubiquitin E3 ligase regulates mTOR activity through promoting the degradation of an inhibitor of mTOR signaling by ubiquitin-dependent proteolysis [24]. Interestingly, TSC2, a negative regulator of mTOR activity, in HEK 293 or U2OS cells has been shown to be recruited by a DWD protein FBW5 to CUL4B-ROC1 E3 ubiquitin ligase, resulting in the subsequent polyubiquitination and degradation of TSC2 [25]. A recent study [26] and our unpublished RT-PCR assays indicated that CUL4B mRNA and protein are highly expressed in various regions of the mouse brain including the cerebral cortex, cerebellum and hippocampus. Therefore, it is possible that CUL4B positively regulates mTOR signaling activity of cerebral cortex, which plays an essential role in controlling long-term synaptic plasticity and learning and memory function, via promoting the removal of TSC2. XLID mutant CUL4B is likely to lose the ability to cause the ubiquitination and degradation of TSC2, leading to the dysregulated mTOR signaling activity in neocortical neurons and resulting intellectual disability. In the present study, our results indicate that XLID truncating or missense mutations impair the ability of CUL4B to promote ubiquitylation and degradation of TSC2 and that XLID CUL4B mutants are defective in positively regulating mTOR activity in neocortical neurons.

2. Materials and methods

2.1. Construction of point mutant or truncated CUL4B

Influenza hemagglutinin epitope (HA, YPYDVPDYA) was added to the C-terminus of human wild-type CUL4B by performing PCR amplification. According to our previous study [27], oligonucleotide-directed mutagenesis using PCR amplification was performed to prepare cDNA encoding XLID mutant (R572C) or (V745A) CUL4B [6]. HA-tagged cDNA of XLID truncated (R388X) CUL4B [6,7] was obtained by performing PCR amplification using the full-length cDNA of CUL4B as the template. DNA mutations of CUL4B were confirmed by performing dideoxy DNA sequencing.

2.2. Stable expression of wild-type or XLID mutant CUL4B in HEK 293 cells

The cDNA of HA-tagged wild-type or XLID mutant CUL4B was subcloned into a mammalian expression vector pcDNA3 (Invitrogen) and transfected to HEK 293 cells using Lipofectamine 2000 (Invitrogen). Two days after the transfection, 293 cells expressing wild-type, (R572C), (V745A) or (R388X) CUL4B were selected by adding 1.5 mg/ml G418 (geneticin sulfate) to culture medium. Positive clones were confirmed by performing Western blot analysis as described below and maintained in the medium containing 0.5 mg/ml G418.

2.3. Preparation of primary cultured neocortical neurons

Cultured neocortical neurons of frontal cortex were prepared as described previously [28]. Briefly, frontal lobe of cerebral cortex was dissected from postnatal 1 day- or 2 days-old mice and incubated with DMEM/F12 medium containing pronase (0.5 mg/ml) and DNase I (0.3 mg/ml) for 50 min at 37 °C. Tissue fragments were then triturated, and dissociated cells were plated onto poly-L-ornithine-coated six-well dishes. Neocortical neurons were cultured in DMEN/F12 medium supplemented with 5% fetal bovine serum and 5% horse serum. From the second day in culture, proliferation of glial cells was prevented by adding 5′-fluoro-2′-deoxyuridine and uridine into the medium.

2.4. Preparation of recombinant adenoviruses

AdEasy Adenoviral Vector System (Stratagene) was used to prepare recombinant adenoviruses containing cDNA of wild-type or XLID mutant CUL4B by homologous recombination [28]. Briefly, cDNA of wild-type, (R572C), (V745A) or (R388X) CUL4B was subcloned into pShuttle-CMV vector provided in the kit. The resulting plasmid was cotransformed into *Escherichia coli*. BJ5183 cells with adenoviral backbone plasmid pAdEasy-1. Recombinant adenoviral plasmid was then transfected into HEK 293 cells. Following the amplification in HEK 293 cells, viral stocks were purified by CsCl gradient ultracentrifugation. Twenty-four hours after plating, cultured neocortical neurons were infected with recombinant adenoviruses.

2.5. Infection of recombinant lentiviruses containing shRNA of CUL4B

Lentiviral vector pLKO.1 containing short hairpin RNA (shRNA) targeting mouse CUL4B (clone ID, TRCN0000012791; target sequence, 5′-CCACGTACCTATACAGAA-3′) was obtained from National RNAi Core Facility at the Academia Sinica and used for preparation of recombinant lentiviruses. One day after plating, cultured neocortical neurons were infected with recombinant lentiviruses containing shRNA of CUL4B.

2.6. Immunoprecipitation of ubiquitinated TSC2, cyclin E or TSC1 in HEK293 cells expressing CUL4B

HEK293 cells stably expressing wild-type or XLID mutant CUL4B were homogenized and solubilized with ice-cold lysis buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM Tris–HCl (pH7.4), 1 mM EDTA, 5 μg/ml pepstain, 5 μg/ml leupetin, 5 μg/ml aprotinin, and 0.3 mM PMSF. Following the centrifugation at 15,000 *g* for 20 min at 4 °C, the supernatant (1 mg protein) was incubated with 50 μl of protein A-agarose suspension for 3 h at 4 °C. After the centrifugation, the supernatant was incubated with 1 μg of monoclonal anti-FK-2 antibody against polyubiquitinated and monoubiquitinated proteins (Enzo Life Sciences) and 50 μl of protein A-agarose suspension overnight at 4 °C. Following the centrifugation at 4 °C, the beads were washed three times with lysis buffer. Subsequently, SDS-PAGE sample buffer was added to the beads, and the sample was heated at 95 °C for 5 min. The supernatant containing the immunocomplex was obtained after the centrifugation and resolved on SDS-polyacrylamide gel. Then, immunoblotting assay with anti-TSC2 polyclonal antibody, monoclonal anti-cyclin E antiserum or polyclonal anti-TSC1 antibody was performed as described below.

2.7. Western blot analysis

Protein samples were prepared by homogenizing cultured neocortical neurons infected with adenoviruses or lentiviruses and HEK 293 cells stably expressing wild-type or mutant CUL4B with SDS sample buffer. Subsequently, protein lysate or the immunocomplex was fractionated on 8 or 10% SDS-polyacrylamide gel and transferred to PVDF membrane. Then, the membrane was incubated at 4 °C overnight with one of the following diluted primary antibodies: (1) Anti-HA monoclonal antiserum (Cell Signalling Technology). (2) Polyclonal anti-CUL4B antibody (Sigma). (3) Anti-mTOR polyclonal antibody (Cell Signaling Technology). (4) Polyclonal anti-phospo-mTOR^{Ser2448} antiserum (Cell Signalling Technology). (5) Anti-p70S6K polyclonal antibody (Cell Signaling Technology). (6) Polyclonal Anti-phospho-p70S6K^{Thr389} antiserum (Cell Signaling Technology). (7) Anti-4E-BP1 polyclonal antibody (Cell Signaling Technology). (8) Polyclonal anti-phospho-4E-BP1$^{Thr37/46}$ antiserum (Cell Signalling Technology). (9) Anti-TSC2 polyclonal antiserum (Santa Cruz). (10) Monoclonal anti-cyclin E antibody (Santa Cruz). (11) Anti-TSC1 polyclonal antiserum (Epitomics). After being washed, the membrane was incubated with horse anti-mouse or donkey anti-rabbit horseradish peroxidase-linked secondary antibody. Then, immunoreactive proteins were visualized by using enhanced chemiluminescence kit (GE Biosciences). To confirm the equal amount of protein samples loaded, membrane was stripped and reblotted with monoclonal anti-actin antibody (Chemicon). Gel bands were quantified with the aid of densitometer (Molecular Dynamics Model 375A) and normalized with reprobed actin signal on the same blot.

2.8. Statistical analysis

All results are expressed as the mean ± S. E. value of n experiments. Statistical significance among multiple experimental groups is determined by one-way ANOVA followed by Dunnett's test. Unpaired student's *t*-test (two-tailed) is used to determine the significant difference between two groups of data. A p value < 0.05 is considered significant.

3. Results

3.1. XLID CUL4B mutants fail to promote ubiquitylation and degradation of TSC2 and cyclin E in HEK 293 cells

(R572C), (V745A) or truncated (R388X) CUL4B mutation was found in male XLID patients [6,7]. Therefore, we prepared cDNA encoding mutant (R572C), (V745A) or C-terminal truncated (R388X) human CUL4B. To characterize wild-type or XLID CUL4B using immunoblotting assay, influenza hemagglutinin epitope (HA) was added to the C-terminus of wild-type or mutant CUL4B protein. Then, we investigated the functional consequence of XLID mutation by stably expressing wild-type or XLID mutant CUL4B in HEK 293 cells. Western blotting analysis using anti-HA antiserum showed that similar to HA-tagged wild-type CUL4B, 293 cells stably transfected with the cDNA of HA-tagged (R572C) or (V745A) CUL4B expressed a high level of ~110 kDa-immunoreactive protein (Fig. 1). As expected, molecular weight of HA-tagged truncated (R388X) CUL4B expressed was reduced to ~41 kDa (Fig. 1).

In the present study, we hypothesized that XLID mutations impair the activity of CUL4B-RING E3 ubiquitin ligase and that XLID CUL4B mutants fail to promote the degradation of protein substrates. In accordance with s previous study showing that wild-type CUL4B in HEK 293 cells promoted the degradation of TSC2 [25], our Western blot analysis showed that protein expression of TSC2 was significantly downregulated in HEK 293 cells stably expressing wild-type CUL4B (Fig. 2A). On the other hand, XLMR mutant (R388X), (R572C) or (V745A) CUL4B overexpressed in 293 cells failed to significantly decrease the protein level of TSC2 (Fig. 2A).

In addition to TSC2, wild-type CUL4B-RING E3 ligase has also been shown to enhance the degradation of cyclin E in HEK 293 cells [29,30]. To further confirm our hypothesis that XLID truncating or missense mutations impair the abilityof CUL4B to promote degradation of protein substrates, we also visualized protein level of cyclin E in 293 cells stably expressing wild-type or XLID mutant CUL4B. Immunoblotting assays showed that protein level of cyclin E was significantly decreased in 293 cells expressing wild-type CUL4B (Fig. 2B). In contrast, compared to control HEK 293 cells, protein expression of cyclin E was not altered in 293 cells stably transfected with cDNA of mutant (R388X), (R572C) or (V745A) CUL4B (Fig. 2B).

Overexpression of wild-type CUL4B decreased protein level of TSC2 or cyclin E in HEK293 cells, indicating that wild-type CULB promotes degradation of TSC2 or cyclin E by ubiquitinating TSC2 or cyclin E. In contrast to wild-type CUL4B, stable expression of XLID mutant (R388X), (R572C) or (V745A) CUL4B did not affect the protein level of TSC2 or cyclin E in HEK293 cells, suggesting that XLID mutant CUL4B failed to promote ubiquitylation of TSC2 or cyclin E. To prove this hypothesis, an equal amount of solubilized cellular extract prepared from control HEK293 cells or 293 cells overexpressing wild-type or XLID mutant CUL4B was immunoprecipitated with monoclonal anti-

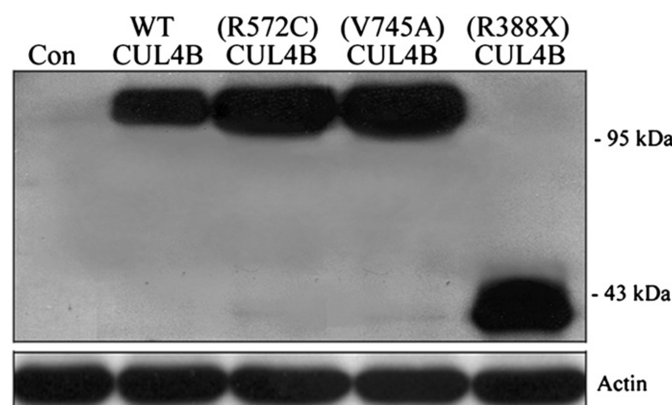

Fig. 1. Stable expression of HA-tagged wild-type or XLID mutant CUL4B in HEK 293 cells. Following the stable transfection of cDNA encoding HA-tagged wild-type or XLID mutant CUL4B, immunoblotting assays using anti-HA antibody indicated that wild-type, (R572C) or (V745A) CUL4B (M.W. = ~110 kDa) was stably expressed in HEK 293 cells. Molecular weight of C-terminal truncated (R388X) CUL4B expressed in 293 cells was reduced to ~41 kDa.

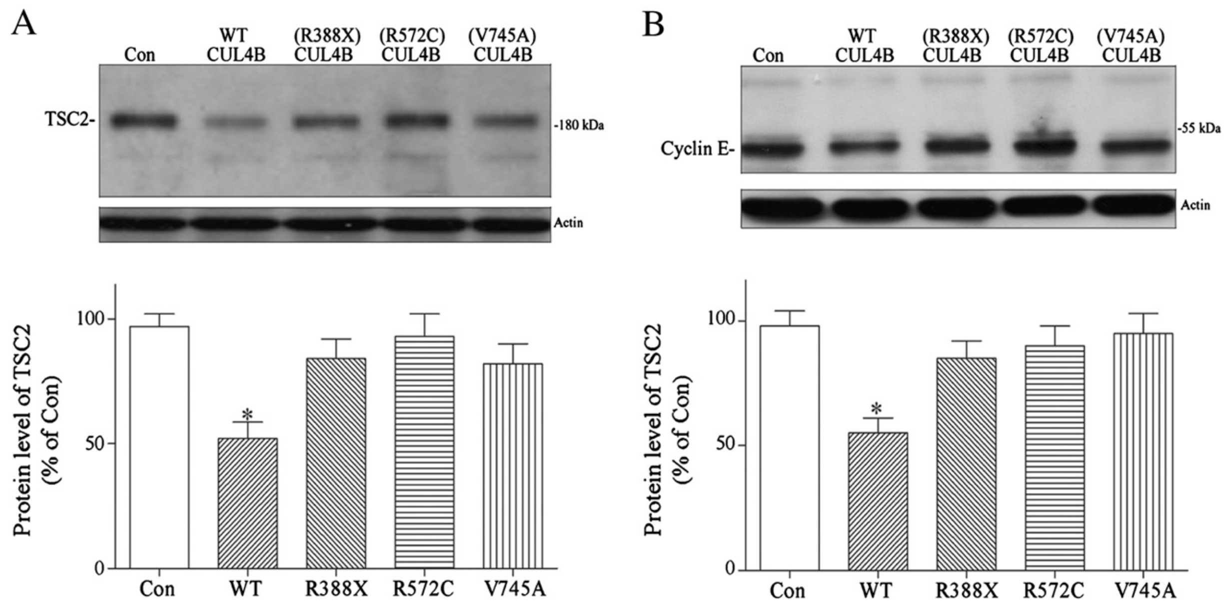

Fig. 2. XLID CUL4B mutants fail to downregulate protein expression of TSC2 or cyclin E in HEK293 cells. (A) Immunoblotting assays showed that compared to control cells, protein level of TSC2 was significantly reduced in HEK293 cells stably expressing wild-type CUL4B. In contrast to wild-type CUL4B, XLID mutant (R388X), (R572C) or (V745A) CUL4B did not significantly decrease TSC2 protein level. (B) Western blot analysis indicated that protein expression of cyclin E was significantly downregulated in 293 cells expressing wild-type CUL4B. Note that protein level of cyclin E was not significantly altered in HEK 293 cells stably expressing XLID mutant (R388X), (R572C) or (V745A) CUL4B. Each bar represents the mean ± S. E. value of 6–7 experiments. *P<0.01 compared to control cells (one-way ANOVA followed by Dunnett's test).

FK-2 antiserum that recognizes ubiquitinated proteins, and then immunoprecipitates were analyzed by Western blot assay with anti-TSC2 antibody. Compared to control 293 cells, stable expression of wild-type CUL4B significantly increased the protein level of high-molecular-mass polyubiquitinated TSC2 (Fig. 3A). In contrast, XLID mutant (R388X), (R572C) or (V745A) CUL4B failed to significantly promote ubiquitylation of TSC2 (Fig. 3A). Immunoprecipitation using anti-FK-2 antibody and subsequent immunoblotting assay of immunocomplex using anti-cyclin E antiserum also demonstrated that compared to control non-transfected 293 cells, overexpression of wild-type CUL4B promoted ubiquitination of cyclin E (Fig. 3B). On the other hand, XLID mutant (R388X), (R572C) or (V745A) CUL4B did not augment ubiquitylation of cyclin E (Fig. 3B). As a control, immunoprecipitation using anti-FK-2 antibody and subsequent Western blot assay using anti-TSC1 antiserum showed that neither wild-type CUL4B nor XLID CUL4B mutants enhanced ubiquitination of TSC1 (Fig. 3C).

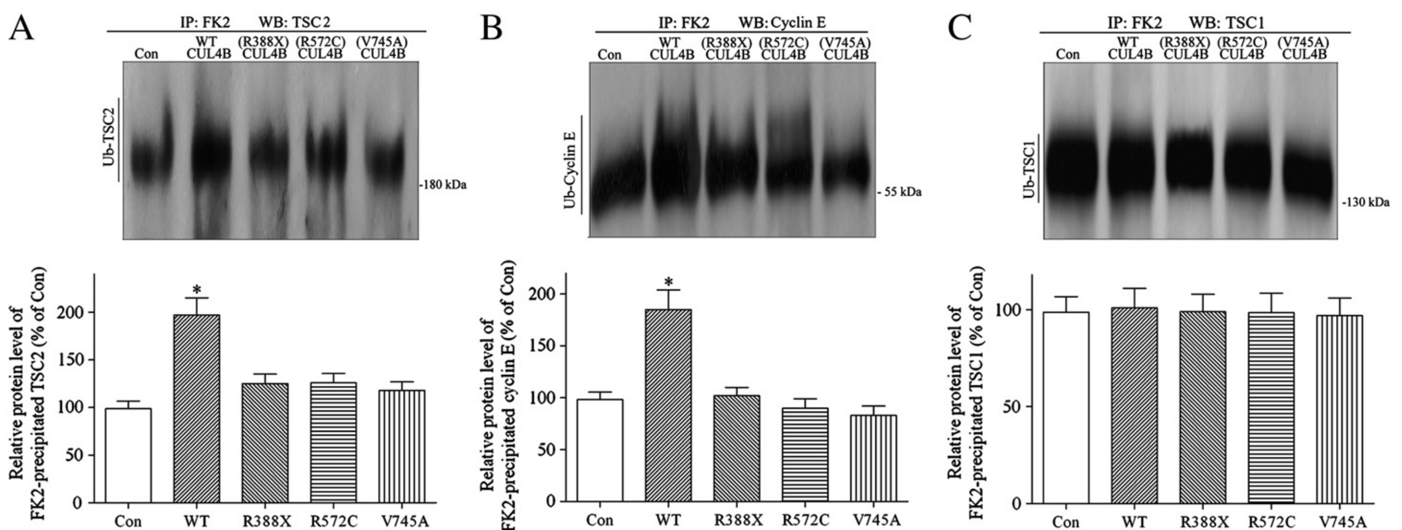

Fig. 3. XLID CUL4B mutants are defective in promoting ubiquitylation of TSC2 or cyclin E. (A) The same amount of solubilized cellular extract prepared from HEK293 cells expressing wild-type or XLID mutant CUL4B was immunoprecipitated with anti-FK-2 antibody, which recognizes ubiquitinated proteins, and visualized by subsequent immunoblotting assay using anti-TSC2 antiserum. Note that ubiquitylation of TSC2 was greatly enhanced in HEK293 cells expressing wild-type CUL4B. XLID mutant (R388X), (R572C) or (V745A) CUL4B did not significantly promote TSC2 polyubiquitination. (B) Solubilized cellular extract was immunoprecipitated by ant-FK-2 antibody and then visualized by Western blot analysis using anti-cyclin E antiserum. Wild-type CUL4B significantly increased ubiquitylation of cyclin E. In contrast, XLID mutant (R388X), (R572C) or (V745A) CUL4B failed to enhance ubiquitination of cyclin E. (C) Immunoprecipitating cellular extract with anti-FK-2 antibody and subsequent immunoblotting assay using anit-TSC1 antiserum showed that wild-type or XLID CUL4B mutants did not promote ubiquitylation of TSC1. Each bar shows the mean ± S. E. value of 5 experiments. *P<0.01 compared to control cells (one-way ANOVA followed by Dunnett's test).

3.2. XLID CUL4B mutants lose the ability of downregulating protein expression of TSC2 or cyclin E in neocortical neurons of frontal lobe

In the previous sections, our results indicated that XLID CUL4B mutants were defective in promoting the degradation of TSC2 and cyclin E in HEK 293 cells. XLID mutant CUL4B is believed to cause dysregulated neuronal function or development in the frontal lobe of cerebral cortex and resulting intellectual disability [31–34]. In the present study, we hypothesized that CUL4B positively regulates mTOR signaling of frontal cortex by promoting the removal of TSC2, a negative regulator of mTOR activity, and that XLID CUL4B mutants lose the ability to cause the degradation of TSC2, leading to the dysregulated mTOR signaling in neocortical neurons of frontal lobe. Thus, it is essential to show that XLID mutations cause the loss of CULB-mediated downregulation of TSC2 protein expression in neocortical neurons of frontal cortex.

To express wild-type or XLID mutant CUL4B in cultured neocortical neurons of mouse frontal lobe, we prepared recombinant adenoviruses containing the cDNA of HA-tagged wild-type, (R388X), (R572C) or (V745A) CUL4B. Three days after the infection with adenoviruses containing the cDNA of wild-type or XLID mutant CUL4B, Western blot study showed that HA-tagged wild-type, (R572C), (V745A) or (R388X) CUL4B was highly expressed in cultured neocortical neurons of frontal cortex (Fig. 4). Consistent with the results observed in 293 cells, immunoblotting study showed that three days after infecting adenoviruses containing the cDNA of wild-type CUL4B, protein level of TSC2 was greatly reduced in neocortical neurons of frontal lobe (Fig. 5A). In contrast, protein expression of TSC2 was not significantly altered in neocortical neurons expressing XLID mutant (R388X), (R572C) or (V745A) CUL4B (Fig. 5A).

Similar to the finding observed in 293 cells, Western blot assays indicated that overexpression of wild-type CUL4B in neocortical neurons of frontal cortex also decreased protein level of cyclin E (Fig. 5B). On the other hand, three days after the infection of adenoviruses containing cDNA of XLID mutant (R388X), (R572C) or (V745A) CUL4B, a similar protein level of cyclin E was detected in cultured neocortical neurons expressing XLID mutant CUL4B and control non-infected neurons (Fig. 5B).

3.3. Knockdown of CUL4B upregulates protein level of TSC2 or cyclin E in cultured neocortical neurons

If CUL4B promotes the degradation of TSC2 or cyclin E and acts as a physiological regulator of TSC2 or cyclin E protein expression in neocortical neurons of frontal cortex, shRNA-mediated knockdown of CUL4B expression is expected to cause the accumulation of TSC2 or cyclin E in neocortical neurons. Three days after infecting cultured neocortical neurons with lentiviruses containing shRNA targeting CUL4B, about 90% of CUL4B protein expression was suppressed (Figs. 6 and 7). Infection of lentiviruses containing control scrambled shRNA did not affect protein level of CUL4B (data not shown). shRNA-mediated knockdown of CUL4B protein level greatly upregulated protein expression of cyclin E (Fig. 6) or TSC2 (Fig. 7) in cultured neocortical neurons.

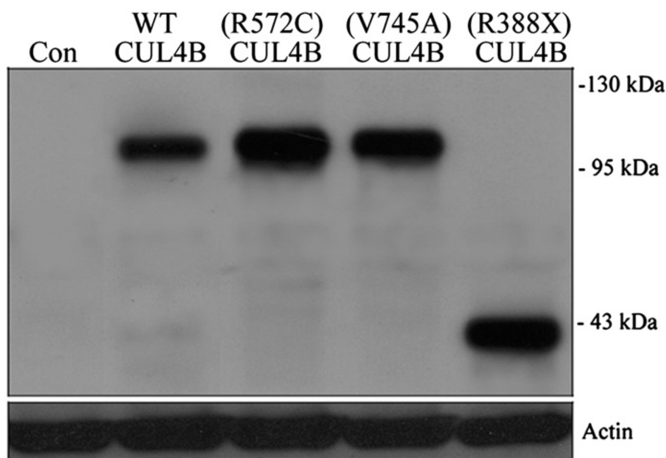

Fig. 4. Recombinant adenoviruses-mediated expression of wild-type or XLID mutant CUL4B in neocortical neurons of frontal lobe. Three days after the infection with recombinant adenoviruses containing the cDNA of HA-tagged wild-type or XLID mutant CUL4B, immunoblotting assays using anti-HA antibody showed that HA-tagged wild-type, (R572C) or (V745A) or C-terminal truncated (R388X)CUL4B was highly expressed in cultured neocortical neurons of frontal cortex.

3.4. XLID CUL4B mutants fail to positively regulate mTOR activity in neocortical neurons

Knockdown of CUL4B-induced upregulation of TSC2 in neocortical neurons of frontal lobe led to a decrease in protein level of active phospho-mTOR^{Ser2448} (Fig. 7) by inhibiting the activity of Rheb GTPase, a positive regulator of mTOR signaling, Then, reduced level of phospho-mTOR^{Ser2448} caused an impaired phosphorylation and activation of p70S6K^{Thr389} and 4E-BP1$^{Thr37/46}$ (Fig. 7), two main effectors of mTORC1 signaling cascade. These results show that wild-type CUL4B positively regulates mTOR activity and is required for normal mTORC1 signaling pathway in neocortical neurons of frontal cortex.

In accordance with our hypothesis that CUL4B acts as a positive regulator of mTOR activity, overexpression of wild-type CUL4B augmented mTORC1 pathway activity of cultured neocortical neurons by significantly upregulating protein expression of active phospho-mTOR^{Ser2448}, phospho-p70S6K^{Thr389} or phospho-4E-BP1$^{Thr37/46}$ (Fig. 8). In contrast, infecting neocortical neurons with adenoviruses containing the cDNA of XLID mutant (R388X), (R572C) or (V745A) CUL4B failed to increase protein level of phospho-mTOR^{Ser2448}, phospho-p70S6K^{Thr389} or phospho-4E-BP1$^{Thr37/46}$ (Fig. 8).

4. Discussion

Molecular genetic studies reported that truncating or missense mutations, including (R388X), (R572C) and (V745A) mutations, of cullin 4B (CUL4B) gene located on the Xq24 locus were observed in male patients with intellectual disability [6,7]. CUL4B mutation is one of the most common mutated genes causing X-linked intellectual disability (XLID) [6–10]. Better understanding of the functional consequence of XLID mutations on CUL4B-mediated physiological effects and possible pathogenic mechanism underlying mutant CUL4B-induced cognitive impairment is essential for the development of therapeutic strategy for mutant CUL4B-induced XLID.

Previous studies using HEK 293 cell line showed that TSC2 and cyclin E are protein substrates of CUL4B–RING E3 ligase-mediated ubiquitylation and degradation [25,29,30]. We hypothesized that XLID CUL4B mutants are defective in mediating ubiquitylation and degradation of protein substrates in the brain, which results in intellectual disability [6,7]. Our study showed that stable expression of wild-type CUL4B significantly decreased protein level of TSC2 or cyclin E in 293 cells. In contrast, XLMR mutant (R388X), (R572C) or (V745A) CUL4B stably expressed in HEK 293 cells did not downregulate the protein expression of TSC2 or cyclin E. In the present study, immunoprecipitation using anti-FK-2 antibody, which recognizes polyubiquitinated proteins, combined with subsequent Western blot analysis of immunocomplex using anti-TSC2 or anti-cyclin E antiserum indicated that overexpression of wild-type CUL4B augmented ubiquitylation of TSC2 or cyclin E in 293 cells. On the contrary, XLID mutant (R388X), (R572C) or (V745A) CUL4B failed to promote ubiquitination of TSC2 or cyclin E. Our results provide the evidence that XLID mutations cause the loss of CUL4B function and that XLID mutant (R388X), (R572C) or (V745A) CUL4B is defective in promoting ubiquitylation and degradation of TSC2 or cyclin E. XLID mutant (R388X) CUL4B lacks the C-terminal domain, and the location of XLID (R572C) or (V745A) mutation is close to the

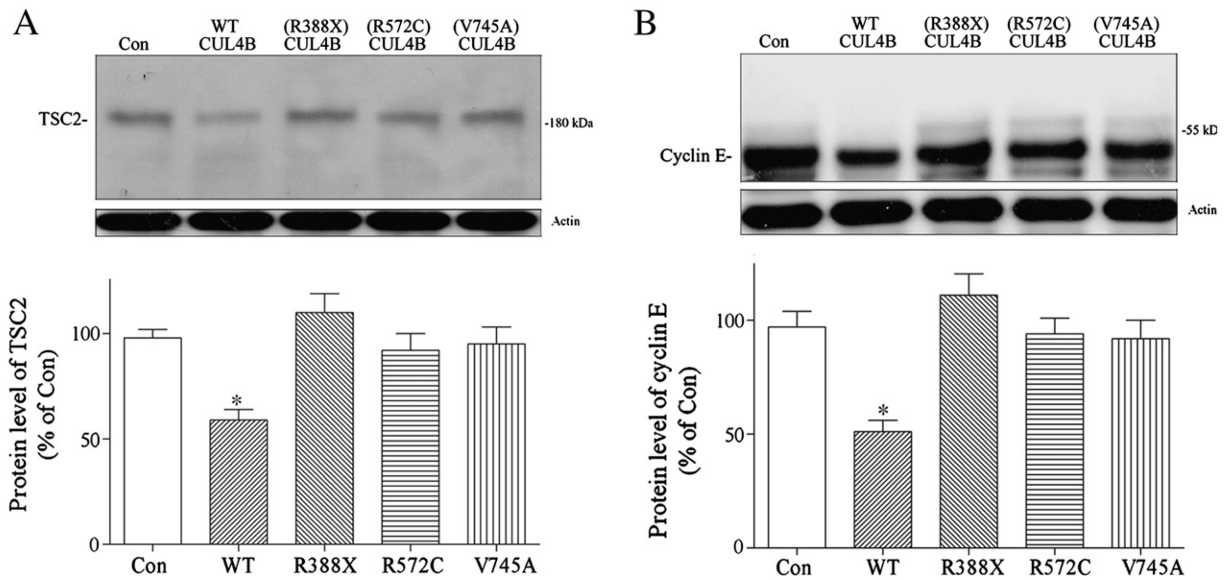

Fig. 5. XLID CUL4B mutants fail to downregulate protein expression of TSC2 or cyclin E in neocortical neurons of frontal lobe. (A) Three days after infecting cultured neocortical neurons with adenoviruses containing cDNA of wild-type or XLID mutant CUL4B, Western blot analysis demonstrated that compared to control non-infected neurons, protein expression of TSC2 was significantly downregulated in neocortical neurons expressing wild-type CUL4B. In contrast, XLID mutant (R388X), (R572C) or (V745A) CUL4B did not significantly alter protein level of TSC2. (B) Immunoblotting assays showed that protein level of cyclin E was significantly decreased in cultured neocortical neurons expressing wild-type CUL4B. On the other hand, XLID mutant (R388X), (R572C) or (V745A) CUL4B failed to affect protein expression of cyclin E. Each bar represents the mean ± S. E. value of 6–7 experiments. *P<0.01 compared to control non-infected neocortical neurons (one-way ANOVA followed by Dunnett's test).

C-terminal domain of CUL4B. It is possible that mutant (R388X), (R572C) or (V745A) CUL4B mutation impairs the binding between the C-terminal domain of CUL4B and RING protein ROC1 or ROC2, which prevents the formation of functional CUL4B-RING E3 ubiquitin ligase.

The mTOR signaling pathway plays an important role in regulating dendritic arborization during the neuronal development [14,17]. Activation of mTOR signaling pathway is functionally linked with local protein synthesis within synapses, leading to the production of proteins required for the formation and maturation of new spines [14,15]. Two phenomena of synaptic plasticity in glutamatergic transmission, late long-term potentiation (LTP) or mGluR-dependent long-term depression (mGluR-LTD), are believed to be cellular substrates for learning and memory [18–21]. Proper translational control via mTOR signaling cascade is required for normal expression of late-LTP and mGluR-LTD [18–20,22,23]. Thus, dysregulation of mTOR signaling activity in the cerebral cortex, which causes an impaired late-LTP or mGluR-LTD and an aberrant dendrite development or spine morphogenesis, could be one of molecular pathogenic mechanisms underlying intellectual disability [21].

XLID is likely to result from dysregulated neuronal function or development in several regions of brain including frontal lobe [31–34]. Therefore, we investigated wild-type or XLID mutant CUL4B modulation of TSC2 or cyclin E protein expression in the neocortical neurons of frontal cortex. Recombinant adenoviruses-mediated expression of wild-type CUL4B significantly decreased protein level of TSC2 or cyclin E in cultured neocortical neurons of frontal lobe. Furthermore, shRNA-mediated knockdown of CUL4B expression led to a significant upregulation of TSC2 or cyclin E protein level, suggesting that CUL4B promotes the degradation of TSC2 or cyclin E and acts as a physiological regulator of TSC2 or cyclin E protein expression in neocortical neurons of frontal cortex. In contrast to wild-type CUL4B, overexpression of XLID mutant (R388X), (R572C) or (V745A) CUL4B failed to downregulate protein expression of TSC2 or cyclin E in cultured neocortical neurons. Therefore, XLID CUL4B mutants are defective in enhancing the degradation of TSC2 or cyclin E in neocortical neurons of frontal lobe.

TSC2 negatively regulates mTOR activity by inhibiting the Rheb GTPase, a positive regulator of mTORC1 signaling cascade [14,15].

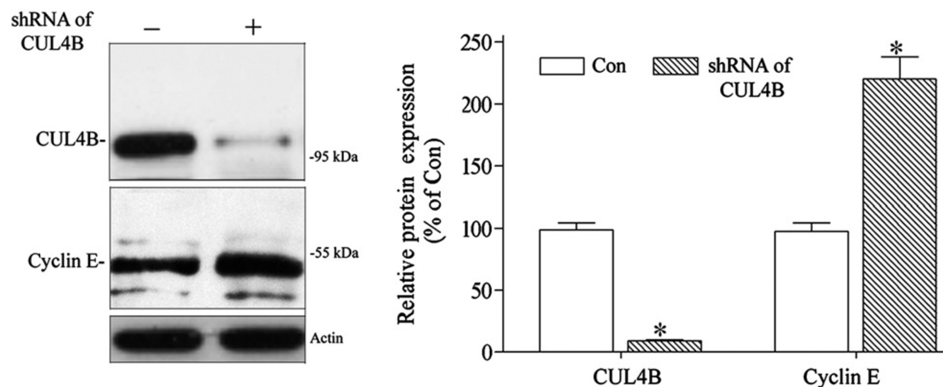

Fig. 6. shRNA-mediated knockdown of CUL4B expression increases protein level of cyclin E in cultured neocortical neurons. Infecting neocortical neurons with lentiviruses containing shRNA targeting CUL4B greatly decreased protein level of CUL4B. Knockdown of CUL4B expression was accompanied by a significant increase in protein level of cyclin E in cultured neocortical neurons of frontal cortex. Each bar shows the mean ± S. E. value of 6 experiments. *P<0.01 compared to control non-infected neurons.

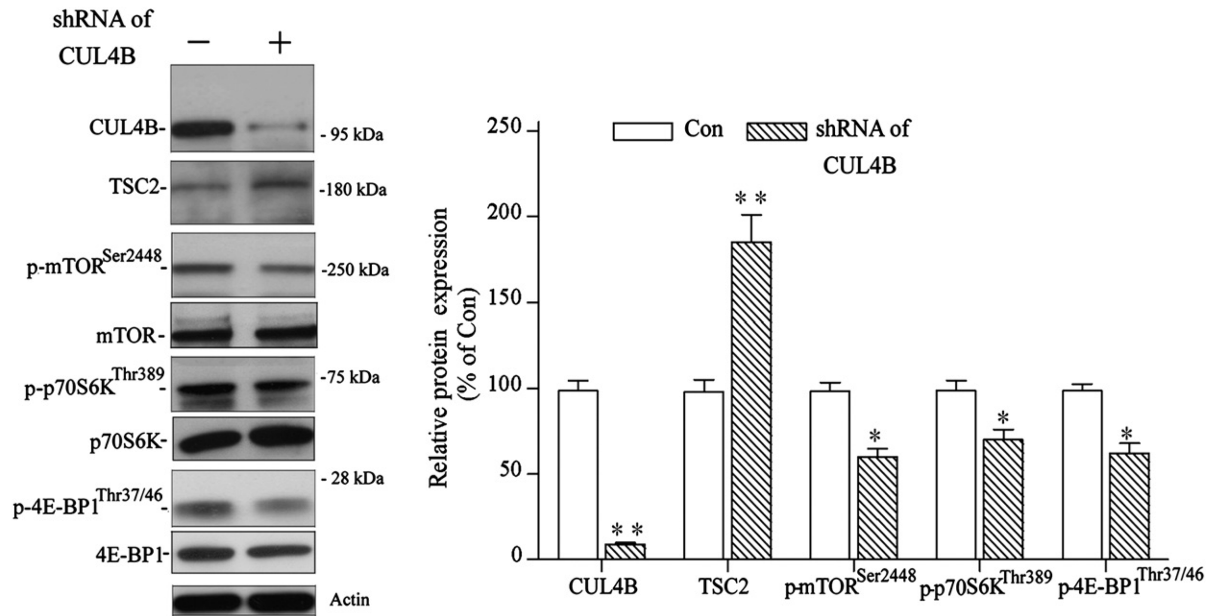

Fig. 7. CUL4B positively regulates the mTOR activity by promoting TSC2 degradation in neocortical neurons of frontal lobe. Three days after infecting cultured neocortical neurons with lentiviruses containing shRNA targeting CUL4B, Western blot analysis indicated a pronounced decrease in CUL4B protein expression, leading to a significant increase in protein level of TSC2. Upregulation of TSC2 expression caused by knockdown of CUL4B resulted in an impairment of mTORC1 signaling pathway, which was indicated by a decreased protein expression of active phospho-mTOR^{Ser2448}, phospho-p70S6K^{Thr389}or phospho-4E-BP1$^{Thr37/46}$ in neocortical neurons of frontal cortex. Each bar represents the mean ± S. E. value of 6 experiments. *P<0.05 compared to control non-infected neocortical neurons. **P<0.01 compared to control non-infected neurons.

The physiological importance of TSC2-mediated negative modulation of mTOR activity was indicated by a previous study showing that heterozygous Tsc2$^{+/-}$ knockout mice displayed the disinhibition of mTOR signaling activity in the brain, which led to a lower threshold for the induction of late long-term potentiation [35]. By causing ubiquitination and promoting degradation of TSC2, CUL4B could play a critical role in regulating the mTOR activity in the neocortical neurons of frontal cortex. Consistent with this hypothesis, knockdown of CUL4B-induced upregulation of TSC2 in neocortical neurons of frontal lobe resulted in a decrease in protein level of active phospho-mTOR^{Ser2448} and a reduced level of active p70S6K^{Thr389} and 4E-BP1$^{Thr37/46}$, two main protein substrates of mTOR-mediated phosphorylation. Overexpression of wild-type CUL4B also significantly enhanced mTORC1 pathway activity of neocortical neurons by increasing protein level of active phospho-mTOR^{Ser2448}, phospho-p70S6K^{Thr389} or phospho-4E-BP1$^{Thr37/46}$. Our results provide the evidence that CUL4B positively regulates mTOR activity and is required for normal mTORC1 signaling pathway in neocortical neurons of frontal cortex. In contrast to wild-type CUL4B, recombinant adenoviruses-mediated expression of XLID mutant (R388X), (R572C) or (V745A) CUL4B did not affect protein level of active phospho-mTOR^{Ser2448}, phospho-p70S6K^{Thr389} or phospho-4E-BP1$^{Thr37/46}$ in cultured neocortical

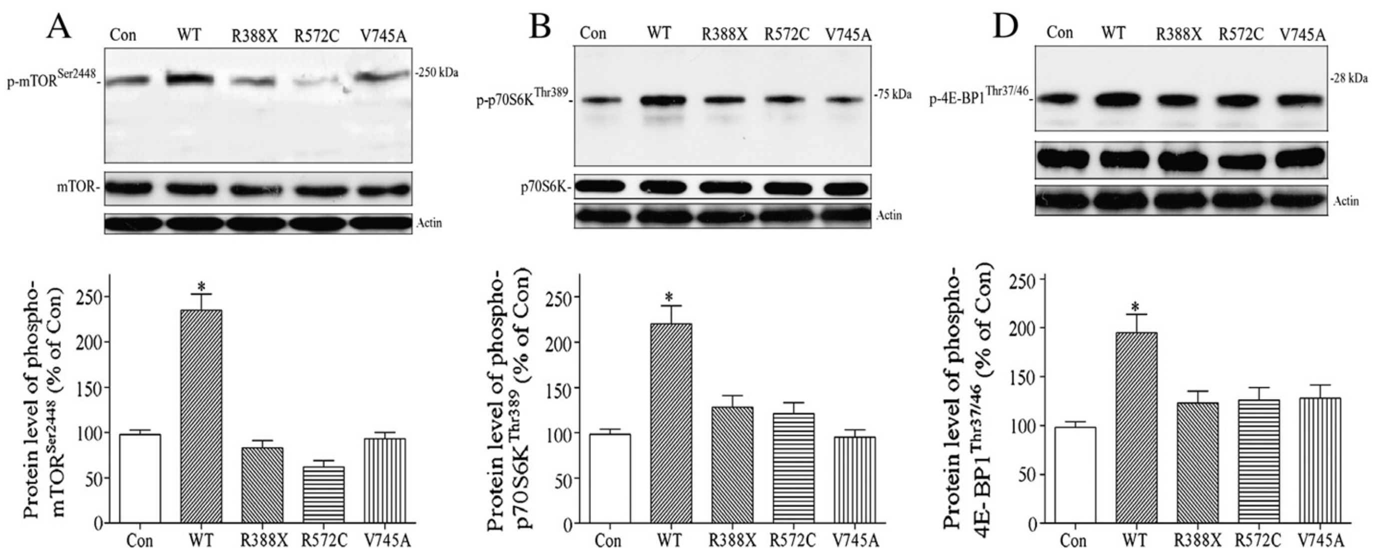

Fig. 8. XLID CUL4B mutants fail to positively regulate mTOR signaling in neocortical neurons of frontal cortex. (A) Infecting neocortical neurons with adenoviruses containing cDNA of wild-type CUL4B promoted the activation and phosphorylation of mTOR^{Ser2448}. Overexpression of XLID mutant (R388X), (R572C) or (V745A) CUL4B failed to enhance the phosphorylation of mTOR^{Ser2448}. (B) Western blot study showed that protein expression of active phospho-p70S6K^{Thr389} was significantly upregulated in neocortical neurons expressing wild-type CUL4B. In contrast, XLID CUL4B mutants did not increase protein level of phospho-p70S6K^{Thr389}. (C) Wild-type CUL4B expressed in neocortical neurons significantly upregulated protein expression of active phospho-4E-BP1$^{Thr37/46}$. XLID mutant (R388X), (R572C) or (V745A) failed to significantly affect protein level of phospho-4E-BP1$^{Thr37/46}$. Each bar shows the mean ± S. E. value of 6 experiments. *P<0.01 compared to control non-infected neocortical neurons (one-way ANOVA followed by Dunnett's test).

neurons. This finding indicates that XLID CUL4B mutants are defective in positively regulating mTOR activity in neocortical neurons of frontal lobe.

Our results suggest that one of CUL4B-mediated physiological functions in neocortical neurons of frontal lobe is to promote TSC2 degradation and enhance mTOR signaling activity. On the contrary, XLID mutant (R388X), (R572C) or (V745A) CUL4B is defective in promoting degradation of TSC2 and augmenting mTOR signaling cascade in neocortical neurons of frontal lobe. Therefore, one of the possible pathogenic mechanisms underlying XLID mutant (R388X), (R572C) or (V745A) CUL4B-induced male patient's cognitive dysfunction may be the impairment of XLID CUL4B mutant-mediated degradation of TSC2 and subsequent accumulation of TSC2, leading to downregulation of mTORC1 pathway activity and impaired neuronal development and long-term synaptic plasticity.

Cyclin E is a component of the core cell cycle machinery and is highly expressed in proliferating cells. A high level of cyclin E is also found in the postmitotic neurons in the nervous system [36–38]. Our study shows that overexpression of wild-type CUL4B in neocortical neurons of frontal cortex decreased protein level of cyclin E. Furthermore, protein expression of cyclin E was upregulated in cultured neocortical neurons infected with shRNA of CUL4B. Consistent with our hypothesis that XLID mutations cause the loss of CUL4 B function, XLID mutant (R388X), (R572C) or (V745A) CUL4B failed to alter protein level of cyclin E in cultured neocortical neurons. Abnormal elevation of pro-apoptotic cyclin E level has been shown to cause apoptotic cell death of cerebellar granule neurons, midbrain dopaminergic neurons and neocortical neurons [35–37]. A recent study also reported that cyclin E forms complexes with cyclin-dependent kinase 5 and regulates synaptic plasticity and memory formation through controlling the number of synapses and dendritic spines [39]. Therefore, wild-type CUL4B is likely to exert a neuroprotective effect on neocortical cells and modulate synaptic and memory function of neocortical neurons of frontal lobe by negatively regulating protein level of cyclin E. In contrast, XLID mutant (R388X), (R572C) or (V745A) CUL4B exhibited an impaired ability to promote the degradation of cyclin E, suggesting that XLID CUL4B mutants lose the ability of protecting neocortical neurons and regulating synaptic plasticity and memory formation in the frontal cortex via downregulating protein expression of cyclin E.

In the present study, our shRNA-mediated knockdown experiments suggest that CUL4B functions as a physiological regulator of TSC2 or cyclin E protein expression in neocortical neurons of frontal cortex. Recently, CUL4B knockout mice were also prepared to study physiological functions of CUL4B and possible pathogenic mechanism of XLID CUL4B mutant-induced cognitive impairment [26,40]. Embryonic lethality with pronounced growth inhibition and increased apoptosis in extra-embryonic tissues were observed in CUL4B knockout mice, indicating that CUL4B plays an essential developmental role in the extra-embryonic tissues [40]. Interestingly, the deficiency of CUL4B in the hippocampus led to the reduced dendritic arborization and impaired spatial memory function of rescued conditional CUL4B knockout mice [26]. This finding raises the possibility that loss of CUL4B causes the accumulation of TSC2 and diminished mTOR signaling activity, resulting in an impaired dendritic development and memory function of the hippocampus [26].

In addition to TSC2 and cyclin E studied in the present study, CUL4B–RING E3 ubiquitin ligase also promotes ubiquitination and degradation of β-cantenin, androgen receptor, estrogen receptor and peroxiredoxin III [41–43]. Wnt/β-cantenin signaling pathway is involved in proper neuronal development and function of the CNS [44]. Androgen and estrogen receptors regulate synaptic function and synaptic plasticity in the brain [45,46]. Peroxiredoxin III could affect neural development through regulating the reactive oxygen species (ROS) level [43,47]. Further study is required to determine whether XLID mutant (R388X), (R572C) or (V745A) CUL4B is also defective in promoting the degradation of β-cantenin, androgen receptor, estrogen receptor or peroxiredoxin III, resulting in impaired neuronal development and synaptic plasticity.

In summary, the present study provides the evidence that wild-type CUL4B positively regulates the mTOR activity and exerts a neuroprotective effect in the neocortical neurons of frontal cortex by promoting degradation of TSC2 and cyclin E, respectively. In contrast to wild-type CUL4B, XLID (R388X), (R572C) and (V745A) CUL4B mutants are defective in downregulating protein expression of TSC2 or cyclin E in the neocortical neurons of frontal lobe. The mTOR signaling cascade is required for neuronal development, long-term synaptic plasticity and cognitive function in the cerebral cortex [14,15,18–21]. Our results suggest that XLID (R388X), (R572C) and (V745A) mutations impair CUL4B-mediated degradation of TSC2 and cause resulting accumulation of TSC2 and inhibition of mTOR activity, leading to the malfunction of frontal cortex and intellectual disability.

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