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Short Communication

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Farnesylation-defective Rheb Increases Axonal Length Independently of mTORC1 Activity in Embryonic Primary Neurons

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Rheb (Ras homolog enriched in the brain) is a small GTPase protein that plays an important role in cell signaling for development of the neocortex through modulation of mTORC1 (mammalian-target-of-rapamycin-complex-1) activity. mTORC1 is known to control various biological processes including axonal growth in forming complexes at the lysosomal membrane compartment. As such, anchoring of Rheb on the lysosomal membrane via the farnesylation of Rheb at its cysteine residue (C180) is required for its promotion of mTOR activity. To test the significance of Rheb farnesylation, we overexpressed a farnesylation mutant form of Rheb, Rheb C180S, in primary rat hippocampal neurons and also in mouse embryonic neurons using *in utero* electroporation. Interestingly, we found that Rheb C180S maintained promotional effect of axonal elongation similar to the wild-type Rheb in both test systems. On the other hand, Rheb C180S failed to exhibit the multiple axon-promoting effect which is found in wild-type Rheb. The levels of phospho-4EBP1, a downstream target of mTORC1, were surprisingly increased in Rheb C180S transfected neurons, despite the levels of phosphorylated mTOR being significantly decreased compared to control vector transfectants. A specific mTORC1 inhibitor, rapamycin, also could not completely abolish axon elongation characteristics of Rheb C180S in transfected cells. Our data suggests that Rheb in a non-membrane compartment can promote the axonal elongation via phosphorylation of 4EBP1 and through an mTORC1-independent pathway.

Key words: Axons, mTOR protein, Rheb protein, Protein farnesylation

INTRODUCTION

In the developing brain, axonal polarization and elongation are the earliest steps in neuronal differentiation [1] and the process of neuronal polarity establishment, having been studied in primary cultures of cortical and hippocampus neurons [2]. The very first steps in the process of neuronal polarization have been defined as releasing of four to five lamellipodia and small protrusion veils for the developing neuron and one of neurites elongating rapidly to become the cell's axon while other minor neurites become

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dendrites [3, 4]. In the process of axonal polarization/elongation, mTOR (mammalian target of rapamycin) has been reported as a key regulator of axogenesis. mTOR is a serine/threonine kinase and a central regulator of various processes in mammalian cells via its major two downstream targets of EBP1 (eIF-4E binding protein 1), which initiates eukaryotic translation, and S6K (70kDa ribosomal S6 protein kinase), which promotes cell growth. Rheb (Ras homolog enriched in brain) is a small GTPase Ras family member, and it plays an important role in the PI3K/AKT/TSC/mTORC1 signaling pathway to activate mTOR [5]. In neuronal growth, mTOR signaling has been shown to promote cell growth and axon formation [6-11] and inhibition of mTORC1 by its specific inhibitor, rapamycin, blocks axon formation. Conversely, activation of Rheb activity enhances axonal growth and multiple axon formation [12]. The key mechanism for mTORC1 apparently involves activation of local translation of proteins, required for axonal growth and axonal outgrowth in response to external cues such as Ephrins [13]. mTOR-dependent local translation products related to axon specification, CRMP2 (collapsing response mediator protein 2) and tau, are also mediated by Rheb activation. Rheb-mTORC1 activation of S6K also increases in axon formation, and constitutively active S6K also leads to formation of multiple axons [17]. Activation of Rheb also promotes intrinsic sensory axon regrowth in dorsal root ganglia (DRG) models of nerve injury [14].

Because mTOR signaling complex forms at the lysosomal membrane, it is believed that the anchoring of these complex components at the membrane is important. Accordingly, Rheb shares the 'CAAX motif' sequence identity with Ras, shown to be required for membrane localization of each GTPase via farnesylation. As such, this motif is a target for farnesyltransferase inhibitors that can block Rheb and Ras processing and function [18]. With a farnesyl-transferase inhibition via farnesylthiosalicylic acid, Rheb was dispersed to the cytosol and its function was blocked. It also prevented S6K activation induced by a constitutively active mutant of mTOR, indicating that farnesylation of Rheb is critical for the mTOR activation [19].

Recent research has shown that Rheb also activates pathways other than mTOR signaling. For instance, constitutively active mutant of Rheb (Rheb S16H) also induced GSK3β, which can contribute to axonal growth by induction of MAP1 expression [16]. Thus, the importance of Rheb farnesylation in the neuronal growth is not yet completely understood. Here, we investigated the effect of mutation of the CAAX motif in Rheb as required for farnesylation of Rheb on axonal morphology changes in the primary hippocampal neuron cultures. The farnesylation-block of Rheb was achieved via C180S mutation of the molecule. We show that overexpression of Rheb C180S mutant also induces elongation of axonal length but without formation of multiple axons as seen with the overexpression of wild-type Rheb. Increased phosphorylation of 4EBP1 is also seen with overexpressed Rheb C180S and seen in an mTOR independent fashion. Therefore, farnesylation-defective Rheb points to an mTOR1 independent pathway in axogenesis and a non-canonical role for Rheb.

MATERIALS AND METHODS

Animals

Experiments in C57BL/6J strain mice (Daehan Bio-Link, Eumseong-gun, South Korea) were conducted in compliance to animal handling guidelines by the Institutional Animal Care and Use Committee (IACUC) of Hallym University (ethical clearance number: Hallym2016-63, South Korea). Similarly, experiments with rats (Orient Bio, Sung-nam si, South Korea) were approved by the IACUC of Korea University (ethical clearance number: KOREA-2016-0195, South Korea). The animals were initially purchased and then housed in individual cages in 12-hour light/dark cycles at 22±2℃, 50±10% humidity with food and water available ad lib. Animal feed was procured commercially (Purina Inc., Sung-nam si, South Korea).

In utero electroporation

In utero electroporation (IUE) was performed as previously described (Saito et al., 2006). Briefly, endotoxin-free DNA plasmid vector preparations at 2 μg/μl were diluted in phosphate buffer saline (PBS) containing 0.01% Fast Green dye (Sigma-Aldrich, St. Louis, MO, USA). The DNA solution was then injected into the lateral ventricles of E15.5 embryos using microinjection with a pulled glass pipette. Before each experiment, the glass pipettes were irradiated with UV light for sterilization. After DNA microinjection, the embryos were electroporated using 5-nm diameter Tweezertrodes (Harvard Bioscience, Holliston, MA, USA). The electroporations were with five 40 V square pulses of 50 ms with 950 ms intervals using a square-wave pulse generator (ECM 830; Harvard Bioscience).

Expression vectors

Oligo primers via PCR were used to produce the mutations for various vectors (Tables 1 and 2). The base vectors that were used included pEGFP-C1 (expressing an N-terminal GFP fusion protein) (Sung Ho Ryu; Postech, South Korea) [19], pCAGIG (co-expressing a bicistronic EGFP) (Connie Cepko; plasmid 11159; Addgene, Watertown, MA, USA) and pmCherry-C1 (expressing an N-terminal mCherry, a mutant fluorescent protein) (Cat. No. 632524; Clontech, Mountain View, CA, USA). pEGFP-C1-Rheb

Table 1. Primer sequences

Insert	Primer
Rheb WT	F- 5'-AATGCCTCAGTCCAAGTC-3' R- 5'-CAGTGTAGTGGCTCGTGC-3'
Rheb C180S	F- 5'-AATGCCTCAGTCCAAGTC-3' R- 5'- CAGTGTAGTGGCTCCTGC -3'.
Rheb D60I C180S	F- 5'-AATGCCTCAGTCCAAGTC-3' R- 5'- CAGTGTAGTGGCTCCTGC -3'.
4EBP1 F113A	F- 5'- ATGTCGGCGGGCAGCAGCT-3' R- 5'- AATTTACAGGTAGAGCCGA -3'.

Table 2. Plasmid list

Experiment	Parental plasmid	Inserts
In utero electroporation	pCAGIG	None
		Rheb WT
		Rheb C180S
In vitro transfection	pEGFP-C1	None
		Rheb WT
		Rheb D60I
		Rheb C180S
		Rheb D60I C180S
	pmCherry-C1	None
		Rheb WT
		Rheb C180S
	pCAGIG	4EBP1 F113A

WT and Rheb D60I constructs (GenBank sequence FQ219039.1) were kindly provided by Dr. Sung Ho Ryu from Postech, South Korea. pCAGGS-4EBP1 F113A construct was from Angelique Bordey (Addgene; plasmid # 81122). The 4EBP1 F113A insert was then subcloned into pCAGIG.

Cryo-sectioning and immunohistochemistry

The *in utero* electroporated brains were fixed in 4% paraformaldehyde at 4℃ overnight, followed by an overnight saturation in 30% sucrose solution in PBS at 4℃. All cortices were subjected for cryo-sectioning (40 μm thickness) with coronal sections, then free-floating onto pre-coated glass (Superfrost-20, Matsunami Glass, Kishiwada, Japan). The sectioned samples were blocked in 3% bovine serum albumin (BSA) in PBS with 0.1% Triton X-100. Immunostaining was carried out according to standard protocols. Briefly, brain sections were blocked for 1 h in 3% BSA PBS and then incubated at 4℃ overnight with primary antibody against GFP (Abcam, Cambridge, UK; 1:1000) and Cux1 (Santa Cruz, Dallas, TX, USA; 1:200) overnight at 4℃. The samples were then washed 4 times for 5 min in PBS. Incubation with the secondary antibody (Alexa fluor conjugated; Life Technologies, Carlsbad, CA, USA) was then performed for 2 h at room temperature. After extensive washing, the tissue sections were mounted in mounting media for microscopy. The fluorescence images were taken using Zeiss LSM710 confocal microscope (Zeiss, Oberkochen, Germany).

Primary cultures

Tissue fragments were dissected out from embryonic day 18 (E18) rat hippocampus samples (Orient Bio, South Korea) and were digested with papain (Worthington Biochemical, Lakewood, NJ, USA) containing L-cysteine (15.76 mg/ml). Dissociated primary rat hippocampal neurons were then transfected by nucleofection (Mirus Bio, Madison, WI, USA) and cultured in Neurobasal medium (Invitrogen, Carlsbad, CA, USA) with B-27 supplement (ThermoFisher, Waltham, MA, USA). The dissected neurons were plated on coverslips coated with Poly-L-lysine (Sigma-Aldrich) at a concentration of 5x105 cells in a 24 well-plate and cultured at 37℃ in humidified, 5% CO2 incubator.

Immunocytochemistry

Primary neurons were fixed in 4% PFA in 30% sucrose for 15 min at room temperature. After a PBS wash, the cells were blocked for 1 h in 3% BSA in 0.02x TBST and incubated at 4℃ overnight with antibodies against GFP (Abcam; 1:1000), mCherry (Abcam; 1:500), GM130 (Abcam; 1:250), LAMP1 (Abcam; 1:1000), tau-1 (Millipore, Burlington, MA, USA; 1:200), MAP2 (Millipore; 1:2000), p4EBP1 (Thr37/46) (Cell Signaling, Danvers, MA, USA; 1:400) or pmTOR (Ser2448) (Cell Signaling; 1:100). After washing with 1X TBST, the samples were incubated with secondary antibodies (Alexa fluor conjugated; Life Technologies) for 2 h at room temperature and were mounted in mounting media after 3 times wash with 0.5X TBST. All images were observed and captured on a confocal microscope (Leica SP8; Leica, Wetzlar, Germany).

SDS-PAGE and immunoblots

Neuro2A (N2A) cells were procured from JCRB Cell Bank (Osaka, Japan) and cultured at 37℃ in humidified, 5% CO2 incubator. Western blotting involved lysing N2A cells in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM Na3VO4, 5 mM NaF and a protease inhibitor cocktail). Equal amounts of protein were then separated via SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore) and blocked with 5% skimmed milk in 1X TBST. Membranes were probed in primary antibody for overnight at 4℃ with antibodies (all from Cell Signaling) against p4EBP1(Ser65) (1:1000), p4EBP1 (Thr37/46) (1:1000), total 4EBP1 (1:1000), pS6K1 (1:1000) or total S6K1 (1:1000). After washing, the blots were incubated with the appropriate secondary antibody and the protein bands were resolved with Western HRP

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substrate (Luminata Forte; Millipore).

Statistical analysis

Statistical analysis was performed by using GraphPad Prism software (GraphPad, San Diego, CA, USA). The p-values were from one-way ANOVA analysis with a post-hoc Turkey test and data were expressed as mean and SEM.

RESULTS AND DISCUSSION

Farnesylation-inhibition of Rheb leads to axonal outgrowth without the induction of multiple axons

A previous report has indicated that overexpression of wild-type Rheb is associated with an elongated axon [15]. Here, we examined whether a farnesylation-deficient version of Rheb (Rheb C180S) would have any effect on neuronal polarity and axonal length in transfected cells. We cultured primary embryonic day 18 (E18) rat hippocampal neurons after transfection with EGFP co-expressing Rheb WT, Rheb C180S and control vectors and measured length and number of axons of the transfected cells after 3 days *in vitro* (3 *d.i.v.*). We performed immunocytochemistry by tau as an axonal marker and found that there were significant changes in the length of axons by 3 *d.i.v.* (Fig. 1A, arrowhead). Rheb WT-transfection boosted the formation of multiple axons (in 84% of transfected cells) and their length compared with normal control vector transfectants. Interestingly, Rheb C180S transfection also enhanced axonal length (214±9.5 μm) in primary hippocampal neurons, but with the number of single axons being similar to the control-vector transfected cells (Fig. 1B and 1C). A recent report has also stated the consistent effect of an activated form of Rheb and S6K in enhancing formation of multiple axons [17]. We next examined whether Rheb C180S expression would lead to axon elongation *in vivo* and examined the potential of Rheb C180S in formation of callosal axon projections from the layer II/III cortical mouse neurons. Mouse embryos were *in utero* electroporated at E15.5 either with pCAGIG-Rheb C180S or control pCAGIG vector in one hemisphere. At the postnatal stage 5 (P5), the control pCAGIG-labeled axons were elongated and were found to cross the midline to reach the contralateral cortex. By comparison, Rheb WT and Rheb C180S transfectants also projected their axons through the corpus callosum (Fig. 1D). These findings indicated that Rheb C180S also promoted axonal length, but with the neuronal polarity of vector-control transfectants maintained. We measured GFP intensity for the edge of axon fibers in the Cux1 positive zone (Layer II/III). Both Rheb WT and Rheb C180S transfectants had much longer axons and their axon fibers reached the contralateral layers II/III to a significantly higher degree (14 fold for Rheb WT, 12 fold for Rheb C180S) compared with the control vector transfectants (Fig. 1E). We also checked the cellular localization of the farnesylation-defective version of (Rheb C180S) in the form of GFP fusion proteins terminating in Rheb C-terminal sequences in transfected primary rat hippocampal neurons [19]. Compared with Rheb WT GFP fusion protein, Rheb C180S GFP fusion protein had a more diffuse cytoplasmic localization in the transfected cells (Fig. 1F). According to a previous study using the same Rheb C180S construct, this pattern was indicative of Rheb C180S not being concentrated in the endoplasmic reticulum (ER), cis-Golgi, early endosomes, or late endosomes [19].

Farnesylation-defective Rheb also leads to phosphorylation 4EBP1 in regulating axonal specification

To explore the activity of Rheb C180S in relation to the mTORC1 pathway, we transfected mouse neuroblastoma Neuro2A cells with EGFP co-expressing Rheb WT, Rheb C180S or control vectors (Fig. 2A and 2B). The transfection generated differential phosphorylation of mTORC1 effectors (phospho-S6K, pS6K $Ser^{240/244}$; phospho-EBP1, p4EBP1 Ser^{65} and p4EBP1 $Thr^{37/46}$). We observed an increase in pS6K levels with Rheb WT (124±6.7% of control) transfection compared with the control vector transfected cells; in contrast, there was a decrease in the levels of p70S6K with Rheb C180S transfection (70±6.1% of control) (Fig. 2A and 2B), suggesting a partial dominant-negative effect on the activity of p70S6K. Surprisingly, Rheb C180S transfected cells had an increase in their p4EBP1 levels compared with the control-vector transfectants. This was seen with both p4EBP1 Ser^{65} (127±9% of control) and p4EBP1 $Thr^{37/46}$ (137±7.3% of control) antibody staining (Fig. 2A and 2B). This led us to conclude that altering the Rheb farnesylation status of Rheb has a differential effect on the activity of the two mTORC1 pathway substrates, namely S6K and 4EBP1. 4EBP1 is a known regulator of cap-dependent translation and is also reported to be downstream of Rheb-mTOR pathway with phosphorylation of 4EBP1 being specifically localized to the axon [15-17]. To further explore the axonal elongation link between Rheb C180S and 4EBP1, we examined levels of p4EBP1 $Thr^{37/46}$ in Rheb WT, Rheb C180S and control transfected E18 rat hippocampal neurons. At the 3 *d.i.v.* stage for these cells, we observed the intensity of p4EBP1 antibody staining being widely increased in the cell body and the axons for both Rheb WT and Rheb C180S transfectants over the control vector transfected cells (Fig. 2C~E). Interestingly, Rheb C180S transfected cells had even higher levels of p4EBP1 than Rheb WT transfected ones (Fig. 2C~E). To explore the contribution of 4EBP to axonal elongation, we transfected cells with 4EBP1 F113A, a mutant of 4EBP. This mutant resists phosphorylation by mTORC1 as it constitutively binds to eIF4E, thus

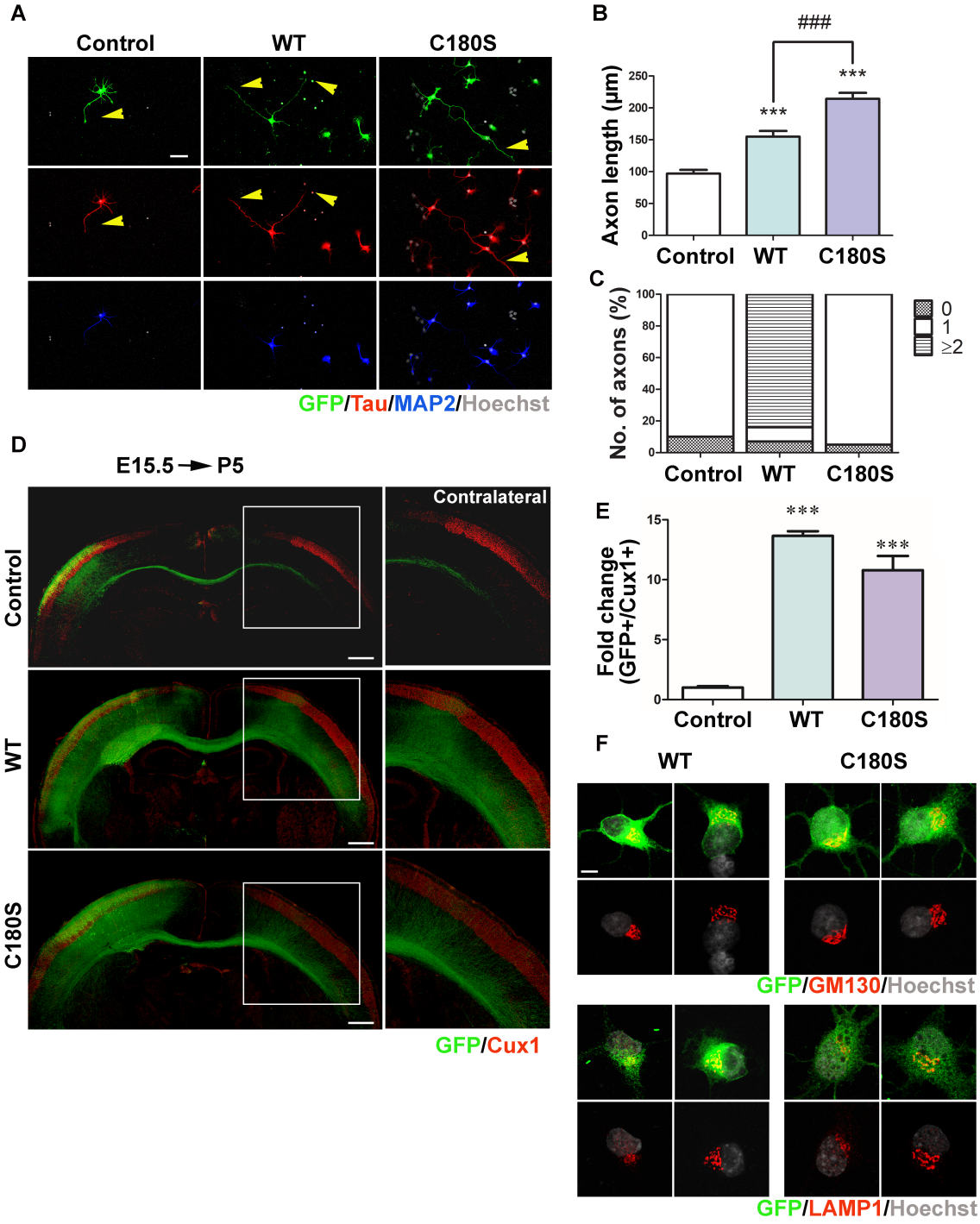

Fig. 1. Rheb C180S expression induced axonal elongation. (A) Primary rat hippocampal neurons were transfected with plasmids for EGFP fusion proteins of Rheb WT, Rheb C180S or control vector constructs at E18 and stained and photographed on 3 *d.i.v.* for GFP (*green*), tau (*red*, axonal marker) and MAP2 (*blue*, dendrite marker) along with a Hoechst staining (*white*, nucleus). Arrowheads indicate terminal ends of the axon. The scale bar is 50 μm. (B) The length of GFP-positive axons (mean±S.E.; *and $^{\#}$, *p<0.05 vs. control, ***p<0.0001, $^{\#}$p<0.05 vs. Rheb WT, $^{\#\#\#}$p<0.0001 with one-way ANOVA and Tukey *post hoc* test, compared with control; n>35). (C) The percentage of neurons without an axon (0), with a single axon (1), and with multiple axons (≥2). (D) Confocal images of coronal sections of postnatal 5 days (P5) mice transfected by *in utero* electroporation with pCAGIG-Control, pCAGIG-Rheb WT, and pCAGIG-Rheb C180S and at E15.5 and immunostained for GFP (*green*) and Cux1 (*red*). The scale bar is 500 μm. (E) Distribution of GFP positive cells in contralateral Layer II (mean±S.E.; *, *p<0.05 vs. control with one-way ANOVA and Tukey *post hoc* test, compared with control; n>5 animals). (F) Primary rat hippocampal neurons were transfected with plasmids for EGFP fusion proteins of Rheb WT, Rheb C180S constructs at E18 and stained and photographed on 3 *d.i.v.* for GFP (green), GM130 (red, Cis-Golgi marker), LAMP1 (red, Lysosome marker) along with a Hoechst staining (white, nucleus). The scale bar is 5 μm.

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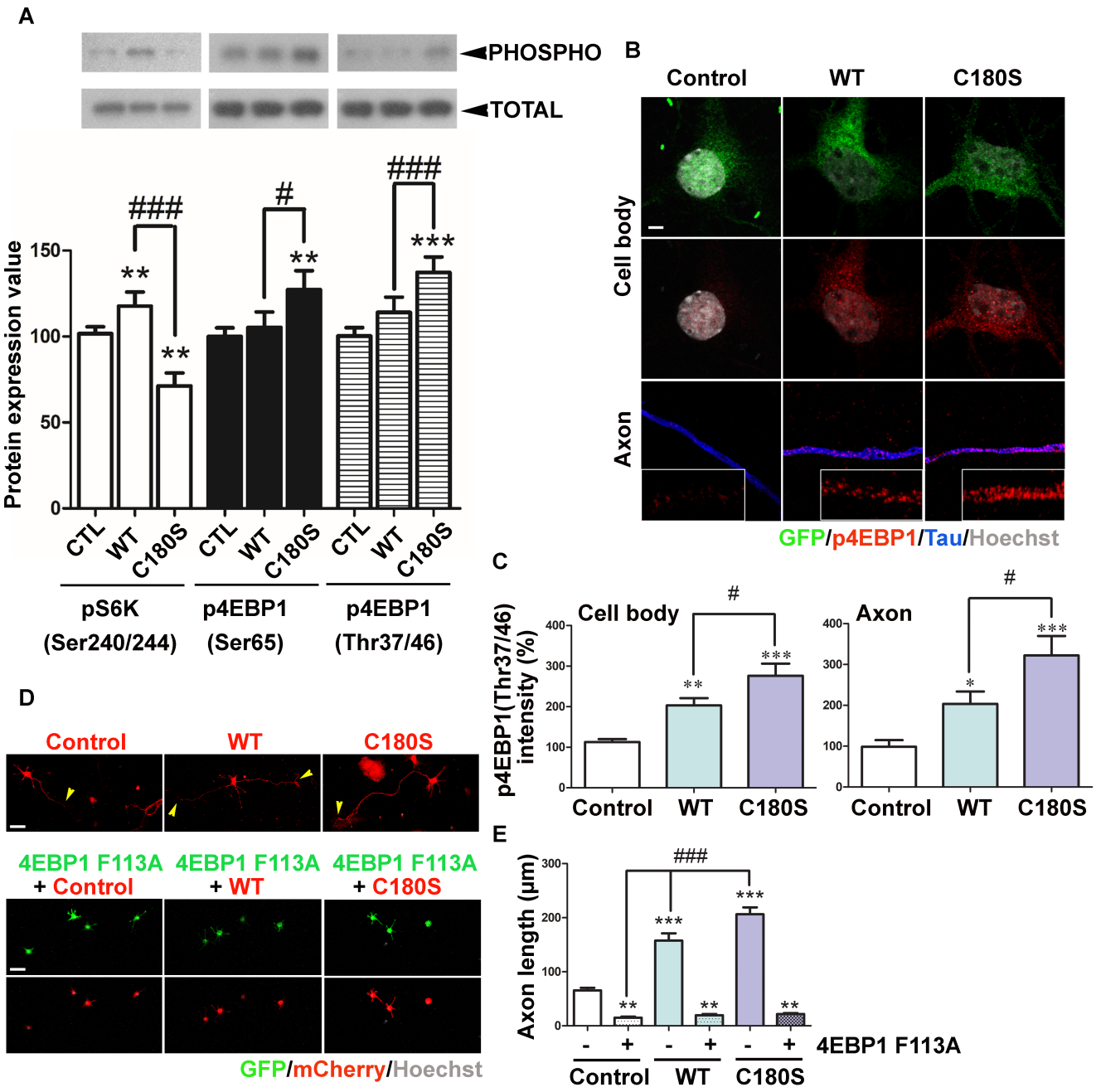

Fig. 2. Rheb C180S expression induces phosphorylation of 4EBP1. (A) Neuro2A (N2A) cells were transfected with EGFP co-expressing Rheb WT, Rheb C180S or control vectors for 48h before assaying for the expression of pS6K (phospho-S6K, $Ser^{240/244}$), p4EBP1 (phospho-4EBP1 Ser^{65}, $Thr^{37/36}$) and total S6K, 4EBP1 via western blot with the percentages of band intensities shown. (B) Primary rat hippocampal neurons were transfected with plasmids for EGFP fusion proteins of Rheb WT, Rheb C180S or control vectors at E18 and stained on 3 *d.i.v.* with GFP (*green*), p4EBP1 ($Thr^{37/46}$) (*red*), tau (*blue*) antibodies and Hoechst (*white*). The scale bar is 2.5 μm. (C) Percentages for the p4EBP1 intensity levels in the cell body and in the axon (mean±S.E.; *and #, *p<0.05 vs. control, ***p<0.0001, #p<0.05 vs. Rheb WT with one-way ANOVA and Tukey *post hoc* test, compared with control; n>35). (D) Primary rat hippocampal neurons were co-transfected with plasmids mCherry co-expressing Rheb C180S, Rheb WT or control vectors at E18 and stained on 3 d.i.v. with GFP (green), mCherry (red) antibodies (upper panel). Primary rat hippocampal neurons were co-transfected with plasmids for EGFP co-expressing 4EBP1 F113A with mCherry co-expressing Rheb C180S, Rheb WT or control vectors at E18 and stained on 3 d.i.v. with GFP (green), mCherry (red) antibodies (lower panel). Arrowheads indicate terminal ends of the axon. The scale bar is 50 μm. (E) The percentages for the axonal length for each group of transfectants (mean±S.E.; *and #, *p<0.05 vs. control, ***p<0.0001, #p<0.05 vs. 4EBP1 F113A with one-way ANOVA and Tukey post hoc test, compared with control; n>35).

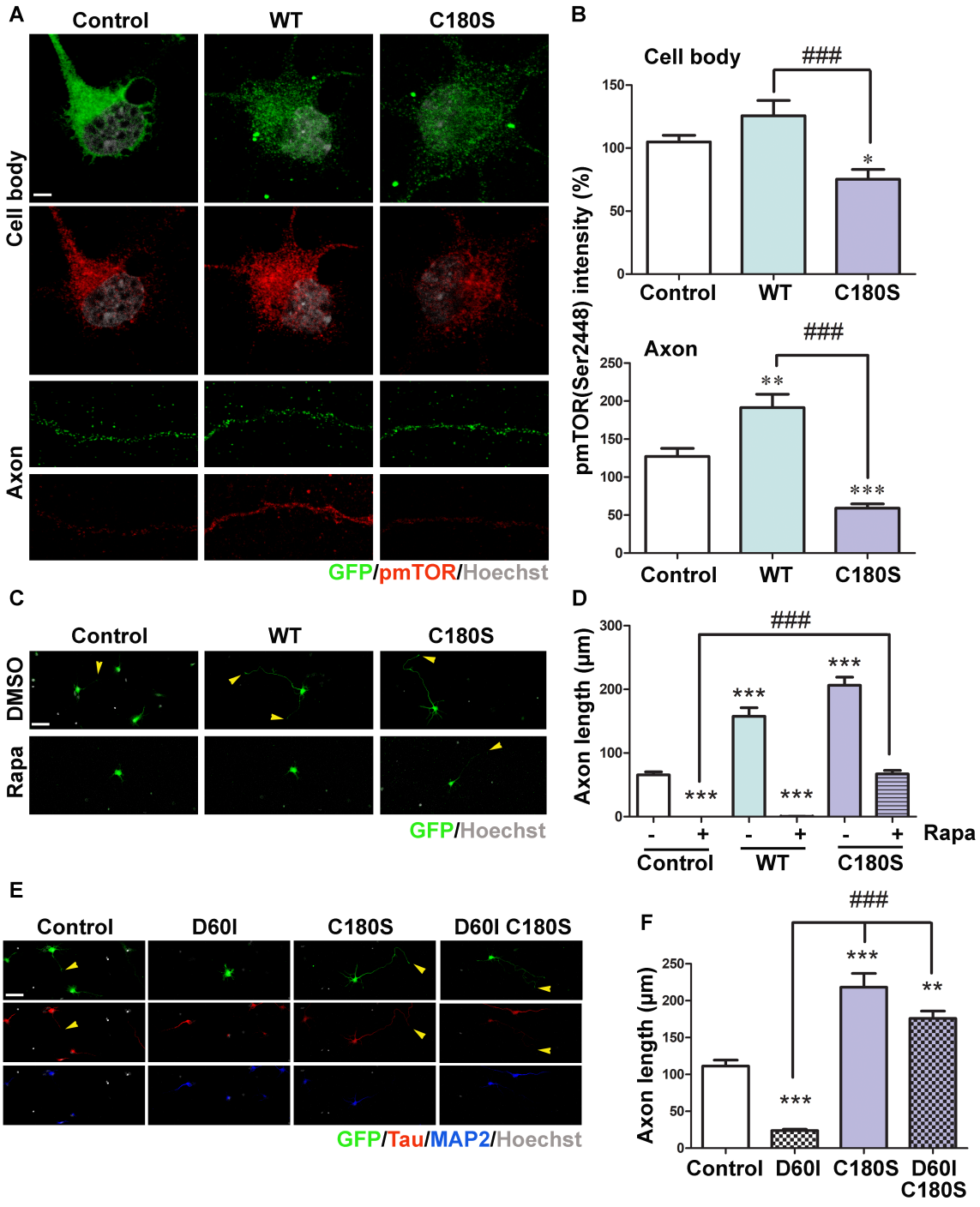

Fig. 3. Rheb C180S expression is not associated with mTORC1. (A) Primary rat hippocampal neurons were transfected with plasmids for EGFP fusion proteins of Rheb WT, Rheb C180S and control vectors at E18 and stained after 3 *d.i.v.* with GFP (*green*), pmTOR (phospho-mTOR^{Ser2448}) (*red*), tau (*blue*) antibodies and Hoechst (*white*). The scale bar is 2.5 μm. (B) The percentages for the pmTOR intensity level in the cell body and the axon (mean±S.E; *and $^{\#}$, *p<0.05 vs. control, ***p<0.0001, $^{\#}$p<0.05 vs. Rheb WT, $^{\#\#\#}$p<0.0001 with one-way ANOVA and Tukey *post hoc* test, compared with control; n>50). (C) DMSO or rapamycin (200 nM) were added to the neurons for 2h after transfected neuron plating and stained after 3 *d.i.v.* with GFP (*green*) antibodies. Arrowheads indicate terminal ends of the axon. The scale bar is 50 μm. (D) The length of GFP-positive axons (mean±S.E.; *and $^{\#}$, *p<0.05 vs. control, $^{\#}$p<0.05 vs. control+rapamycin with one-way ANOVA and Tukey *post hoc* test, compared with control; n>25). (E) Primary rat hippocampal neurons were transfected with plasmids for EGFP fusion proteins of Rheb D60I, Rheb C180S, Rheb D60I+C180S or control vector constructs at E18 and stained and photographed on 3 *d.i.v.* for GFP (*green*), tau (*red*, axonal marker) and MAP2 (*blue*, dendrite marker) along with a Hoechst staining (*white*, nucleus). Arrowheads indicate terminal ends of the axon. The scale bar is 50 μm. (F) The length of GFP-positive axons (mean±S.E.; *and $^{\#}$, *p<0.05 vs. control, ***p<0.0001, $^{\#}$p<0.05 vs. Rheb D60I, $^{\#\#\#}$p<0.0001 with one-way ANOVA and Tukey *post hoc* test, compared with control; n>25).

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inhibiting translation [20, 21]. After transfection with GFP-4EBP1 F113A along with m-Cherry co-expressing Rheb WT, Rheb C180S or control vector in E18 primary hippocampal neurons, axonal lengths were measured for comparison at 3 *d.i.v.* (Fig. 2F~H). We found that for all the transfected cell groups, there was a significant reduction in axon growth when in presence of 4EBP1 F113A (Fig. 2F~H). Taken together, these results indicated that the phosphorylation increase in 4EBP1 by farnesylated-defective version of Rheb was required for the increases seen in formation/elongation of axons.

Increased levels of p4EBP1 due to farnesylation defective Rheb is not dependent on mTORC1 activity

Considering that Rheb WT and C180S induced differential mTOR downstream phosphorylation (Fig. 2A and 2B), we wondered whether increases in p4EBP1 levels due to Rheb C180S were indeed mTORC1 dependent. To verify this in our test cells, we investigated whether Rheb C180S also promoted the phosphorylation of mTOR (pmTOR Ser^{2448}), which is a direct indicator of mTORC1 activity [22]. After transfection in E18 rat primary hippocampal neurons, induction of pmTOR Ser^{2448} intensity was detected in Rheb WT transfectants more than the control vector transfected cells. By contrast, Rheb C180S transfected cells had decreased levels of pmTOR Ser^{2448} in both the axon and the cell body when compared with the control-vector cells (Fig. 3A and 3B), suggesting that Rheb C180S did not enhance mTORC1 activity. Accordingly, axon formation in Rheb WT and control vector transfected cells was completely abolished with rapamycin (200nM), an inhibitor for mTORC1, treated cells. But interestingly, Rheb C180S-transfected neurons maintained their axons at lengths similar for rapamycin-untreated cells (Fig. 3C and 3D). We next examined whether Rheb C180S leading to axon elongation depended on Rheb GTPase activity. We cultured primary embryonic day 18 (E18) rat hippocampal neurons after transfection with EGFP co-expressing GTPase defective Rheb (Rheb D60I), Rheb C180S, Rheb D60I C180S (double mutant) and control vectors. The axon lengths were measured after 3 *d.i.v* for the transfected cells. We performed immunocytochemistry by tau as an axonal marker. Rheb D60I-transfectants had stunted axonal growth (23.8±1.8 μm) compared with the control vector transfectants (111.3±7.8 μm). Surprisingly, the Rheb double mutant (Rheb D60I C180S) transfected neurons had their axon lengths (175.9±9.5 μm) being similar to Rheb C180S transfectants (218.2±17.7 μm) (Fig. 3E and 3F). Taken together, these results indicated that Rheb C180S mediated neurite outgrowth through the increased phosphorylation of 4EBP1, but at least partially independent of mTORC1 activity and Rheb GTPase activity.

Our results indicate that cytosolic localization of a farnesylation-defective version of Rheb (Rheb C180S) can induce axonal elongation, similar to Rheb WT. This was also independent of its GTPase activity, but still dependent on the phosphorylation of 4EBP1. We found that the wild-type and farnesylation-defective versions of Rheb exert their promotional roles in axonal growth using different downstream cascades as there were differences in the phenotype of the transfected neurons between the two groups. Rheb WT transfection induced multiple axons per cell whereas Rheb C180S only maintained axonal elongation to a specific single axon (Fig. 1). This Rheb mutant also induced callosal axonal elongation in the developing neocortex of mouse embryos (Fig. 1). Previous reports have shown a Rheb WT promoting axonal elongation and disrupting radial migration. This abnormal activation of mTORC1 impairs Reelin-Dab1 signaling, contributing to neuronal migration defects [42, 43]. However, Rheb C180S only promoted callosal axon extension, but radial migration was only marginal (Fig. 1). Collectively, these results indicate that Rheb WT and Rheb C180S share a common activity in promoting axonal elongation, but not for the other phenotypes in the two different model systems.

We reasoned that these were caused by different downstream signaling cascades. A previous report has shown that Rheb WT induces the formation of multiple axons dependent on the activation of the Rheb-mTORC1-S6K axis [17]. However, Rheb C180S overexpression increased neither phosphorylation of S6K nor mTORC1 activation. On the other hand, Rheb C180S strongly promoted 4EBP1 phosphorylation. With respect to 4EBP1, its expression is important in promoting axon specification. Hyperphosphorylation of 4EBP1 is required to disrupt its interaction with the translation initiation factor eIF4E, leading to activation of cap-dependent translation. Previously, two reports have shown that mTOR-insensitive unphosphorylated-4EBP1 and 4EBP1 Δ24, both capable of constitutively binding to eIF4E, also prevented axon specification [15, 17]. We conjecture that Rheb C180S induced p4EBP1 levels, but not through an activation of mTORC1. Rheb C180S transfected neurons had decreased pmTORC1 levels in both the cell body and the axons (Fig. 3). In neurons, rapamycin is known to block both pS6K and p4EBP1 levels and axon specification from suppression of mTORC1 [15]. This was also seen in our experiments for both control and Rheb WT transfectants (Fig. 3D). Rheb C180S-transfected cells maintained their axons even in presence of rapamycin, suggesting that increases in p4EBP1 levels in transfected cells were not dependent on increased mTORC1 activity. This phosphorylation of 4EBP1 may be occurring non-canonically and occurring via an arm of the Rheb pathway that is mTORC1 independent.

We have currently no clear answer of what could be the mecha-

nism for Rheb C180S in promoting axonal elongation in an mTORC1-independent manner, but we may speculate several possible mechanisms. As opposed to mTORC1, farnesylation-block of Rheb may be able to upregulate another member of mTOR, mTORC2. In this pathway, farnesylation-block of Rheb may promote cytoskeletal protein formation downstream of mTORC2 via PKC (protein kinase C), Rho, Rac, and thus promoting axon elongation [29, 30-32]. It has been reported that Rheb regulates dendritic spine morphogenesis in mTORC1-independent ways via an interaction of Rheb with syntenin [40, 41]. Thus, other partner kinases participating in phosphorylation of 4EBP1 may mediate cytosolic Rheb actions. For example, Cdc2 can phosphorylate 4EBP1 [33]. Cdc2 activation is also positively linked to axon regeneration [34]. Another candidate is PDK1 which can modulate the phosphorylation of 4EBP1 [35]. As PDK1 contains a PH (pleckstrin homology) domain, disrupting its PH domain also decreased axon length [36]. Src ($p60^{src}$) also leads to 4EBP1 phosphorylation in a rapamycin-resistant manner [37] and Src kinases have been implicated in guidance and growth of axons as Src promotes stability of the growth cones [38]. In addition, PAK2 is a newly described effector of TSC1/TSC2 complex, upstream of Rheb-mTORC1, and it affects 4EBP1 phosphorylation in an mTORC1-independent fashion [39].

In summary, farnesylation-defective Rheb can promote phosphorylation of 4EBP1 and axonal elongation in an mTORC1 independent manner, suggesting that Rheb may regulate axonal growth while dissociated from the lysosomal membrane during the axonal elongation in developing neurons.

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