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Impaired Reelin-Dab1 signaling contributes to neuronal migration deficits of Tuberous Sclerosis Complex

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SUMMARY

Tuberous sclerosis complex (TSC) is associated with neurodevelopmental abnormalities, including defects in neuronal migration. However, the alterations in cell signaling mechanisms critical for migration and final positioning of neurons in TSC remain unclear. Our detailed cellular analyses reveal that reduced Tsc2 in newborn neurons causes abnormalities in leading processes of migrating neurons, accompanied by significantly delayed migration. Importantly, we demonstrate that Reelin-Dab1 signaling is aberrantly regulated in TSC mouse models and in cortical tubers from TSC patients owing to enhanced expression of the E3 ubiquitin ligase, Cul5, a known mediator of pDab1 ubiquitination. Likewise, mTORC1 activation by Rheb overexpression generates similar neuronal and Reelin-Dab1 signaling defects, and directly upregulates Cul5 expression. Inhibition of mTORC1 by rapamycin treatment or by reducing Cul5 largely restores normal leading processes and positioning of migrating neurons. Thus, disrupted Reelin-Dab1 signaling is critically involved in the neuronal migration defects of TSC.

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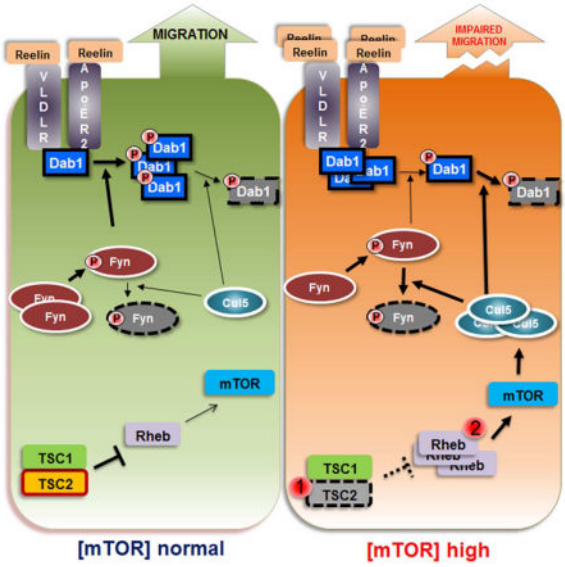
AUTHOR CONTRIBUTION

SK and UM designed experiments and wrote the manuscript. UM carried out most of the experiments, and JP, RP, JC, LH and JM performed part of the phenotypic analyses. LG provided human embryo samples and PB provided tuber samples and related data. PB, SC and MG contributed to data interpretation and manuscript preparation.

Graphical Abstract



Keywords

TSC; cortical tuber; Reelin-Dab1; Cullin 5; neuronal migration; mTOR signaling

INTRODUCTION

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder characterized by brain malformations. Patients often exhibit one or more neurological symptoms, including epilepsy, cognitive disability, and autism (Crino et al., 2006). Cortical tubers, which are consistently found in TSC patients, are directly related to seizures (Feliciano et al., 2012; Prabhakar et al., 2013). TSC results from mutations in *TSC1* (hamartin or TSC1) or *TSC2* (tuberin or TSC2), which encodes GAP (GTPase activating protein) (Sancak et al., 2005). TSC1/2 inhibit the small GTPase Rheb, an activator of mTOR kinase (Kwiatkowski and Manning, 2005). Loss of TSC1 or TSC2 function therefore causes activation of mTOR Complex 1 (mTORC1). Inhibiting mTORC1 with rapamycin corrects many of the pathological features of TSC in mouse models (Carson et al., 2012; Way et al., 2012), including laminar abnormalities, suggesting that aberrant activation of mTORC1 signaling plays a major role in TSC pathophysiology. However, the primary signaling cascade by which activated mTORC1 alters neuronal migration and position is not well understood.

During cortical neurogenesis, newly generated postmitotic neurons initially have a multipolar shape but soon become bipolar with a leading process directed toward the pia and a trailing process directed ventrally (Nadarajah et al., 2003). Early-born neurons migrate to their final position by glia-independent somal translocation: the nucleus and cytoplasm move while the leading process remains fixed. To accomplish the normal inside-out pattern of cortical layering, late-born neurons must navigate long distances and bypass earlier born neurons. The leading process of a late-born neuron follows a course that is guided by glia

until it reaches the marginal zone (MZ). Final positioning then occurs by somal translocation (Nadarajah et al., 2003). Among the many identified signaling pathways that modulate neuronal migration, Reelin-Dab1 signaling is a pivotal and well-defined pathway that regulates migration by translating extracellular cues into cytoskeletal changes (Forster et al., 2010; Frotscher, 2010).

Reelin is a large glycoprotein secreted predominantly by Cajal-Retzius (CR) cells in MZ during cortical neurogenesis. It regulates migration of newborn neurons by promoting neuronal polarity, mediating interactions with glia and stabilizing leading processes for somal translocation (Britto et al., 2011; Dulabon et al., 2000; Franco et al., 2011; Sanada et al., 2004; Sekine et al., 2011). Reelin signals through the VLDLR and ApoER2 receptors to their cytoplasmic adaptor protein Dab1, which is phosphorylated by Src family tyrosine kinases (Arnaud et al., 2003; D'Arcangelo et al., 1999). Phosphorylated Dab1 (pDab1) plays a central role in neuronal migration by activating downstream effectors such as Crk, C3G, Rap1 and PI3K (Ballif et al., 2004; Honda et al., 2011; Jossin and Goffinet, 2007; Park and Curran, 2008; Sekine et al., 2012) and is subject to multiple types of regulation, including ubiquitin E3 ligase Cul5-mediated degradation (Feng et al., 2007).

Our genetic, cellular, and molecular analyses of *Tsc2* conditional knockout (CKO) and knockdown (KD) mice provide evidence that Tsc2 exerts a critical function in cortical laminar organization and MZ maintenance. Remarkably, we have found that the levels of both Reelin and Dab1 are significantly elevated in human cortical tubers and in the forebrain-specific *Tsc2* CKO mice. However, pDab1, a central component of Reelin signaling, is reduced due to aberrantly increased expression of the upstream regulator Cul5. Inhibiting mTORC1 activity restores pDab1 and Cul5 levels to normal. The reduction of Cul5 abrogates migration defects resulting from *Tsc2* KD or Rheb overexpression, providing evidence that Cul5 is a molecular link which mediates crosstalk between mTOR and Reelin-Dab1 signaling pathways in TSC pathogenesis.

RESULTS

Tsc2-deficient neurons have migration defects and invade the marginal zone

To understand the molecular and cellular mechanisms underlying neuropathological features of TSC, we generated a cortical specific CKO of *Tsc2* using Emx1-Cre mice. This CKO expresses Cre in cortical progenitors on embryonic day (E) 9.5 (Gorski et al., 2002), and reduced Tsc2 protein levels were detected as early as E12.5 (Figure S1A). By P0, an increase in cortical thickness was apparent in the *Tsc2* CKO mice (*Tsc2* $^{flox/flox}$; *Emx1-Cre* mice) compared to WT control littermates, similar to other known *Tsc2* or *Tsc1* mutants with cortical deletion. However, in accordance with previous reports of *Tsc1* or *Tsc2* mutants using other Cre drivers such as hGFAP or Synapsin, the increased cortical size of *Tsc2* CKO is not due to a significant increase in the number of neurons (Figure 1D and data not shown). Instead, the increased cortical thickness occurs because the somata of neurons are enlarged. This is demonstrated by comparing the soma size of Cux1+ late born neurons (outlined by N-cadherin, Figure S1B), in the *Tsc2* CKO to WT. This result indicates the critical role of TSC-mTOR in cell growth as opposed to proliferation in generating cortical hyperplasia.

Tsc2 CKO mice exhibited neuronal migration defects, such as a split of hippocampal CA1 and 2 regions into defined layers, diminished MZ and less organized layers of the cerebral cortex (Figure 1A). Similar defects were also observed in *Tsc2* $^{flox/flox}$; *Nestin-Cre* mice and *Tsc2*$^{flox/flox}$; *hGFAP-Cre* mice (data not shown) (Way et al., 2009). The lamination defects in *Tsc2* CKO were further investigated using layer-specific markers, including Cux1 (layers 2–4), FoxP1 (layers 3–5), and FoxP2 (layer 6) at P24. We found that a significant number of Cux1+ upper layer neurons were displaced into the deeper cortex or infiltrated the MZ, although the total number of these cells was largely unchanged (Figure 1B). FoxP1+ neurons also failed to show their normal, tightly restricted distribution, and ectopic FoxP1+ neurons were present in layer 2, and MZ (Figure 1C). We also found fewer and less tightly organized FoxP2+ neurons at P24 (Figure S1C). Overall, absence of Tsc2 was associated with a diffuse distribution of neurons from all layers and excessive upper layer neurons in the MZ.

In order to better understand the basis of the lamination defects, we determined the positions of neurons at P0 using layer-specific markers and *pCAG-GFP* labeling at E15.5 via *in utero* electroporation. Compare to WT, fewer Cux1+ neurons were positioned in the normal upper layers (2–4) and more were distributed in deeper layers of the cortex in *Tsc2* CKO (Figure 1D), suggesting delayed and/or defective migration in *Tsc2* CKO. Layer 5-specific Ctip2+ neurons also spread more broadly in the *Tsc2* CKO than in WT (Figure 1E). Similarly, more migrating neurons labeled at E15.5 and E16.5 by *pCAG-GFP* electroporation were consistently positioned in deeper layers in the *Tsc2* CKO at P0 than in WT (Figure 1F and S1E, respectively). Additional analysis of the migratory behavior by tracking birth-dated postmitotic cells with high BrdU content (injected at E16.5 and analyzed at P1) confirmed the delay in migration of TSC2-deficient late-born neurons (Figure S1F). BrdU+ neurons in the IZ or lower cortical plate overlapped with Cux1 but not with Ctip2, indicating their fate as late-born neurons. To determine whether ectopic neurons at P0 can be correctly positioned at later stages, the distribution of Cux1+ neurons at P7 was examined (Figure S1D). Although a substantial number of ectopic Cux1+ neurons were found in deeper layers, a reduced proportion of neurons was observed in the lower bins, especially bin 6–10, suggesting some catch-up of neuronal migration. Interestingly, the number of Cux1+ neurons in bin 1, which covers mainly MZ, was clearly increased, indicating that progressive increase of late-born neurons in MZ over time. *Tsc2* $^{flox/flox}$; *Nestin-Cre* animals showed similar migration defects and lamination abnormalities (Figure S1G and S1H). Next, we examined the morphology of *Tsc2*-deficient migrating neurons by tracing the processes of GFP labeled neurons whose leading process contacted the MZ in 3D images taken from 100 μm thick sections (Figure 1G). We found significantly increased number of *Tsc2*-deficient neurons display elongated and simplified leading processes compared to WT. Approximately 60 % of *Tsc2*-deficient neurons had less than 3 branches and an average leading process length of 60μm, whereas 36 % of WT neurons had less than 3 branches and the average leading process length was 26μm (Figure 1H and 1I). Although the trailing processes of migrating neurons appeared to be longer in some *Tsc2* CKO neurons, this was not observed consistently (data not shown). Thus, leading process defects are potentially responsible for the delayed migration and abnormal positioning of neurons observed in *Tsc2*-deficient cortices. Possible mechanisms include hindering interactions with radial glia

processes or impairing somal translocation, for which establishment of a stable, adhesive and complex leading process is required (Gil-Sanz et al., 2013).

Reelin-Dab1 signaling is impaired in *Tsc2* CKO

Reelin-Dab1 signaling is critical for maintaining the integrity of the MZ (Kubo et al., 2010), and migration defects in TSC mice models resemble the phenotype of the hypomorphic allele of Dab1, in which infiltration of late-born neurons to MZ, retarded neuronal migration and distinct hippocampal layering defects were reported (Herrick and Cooper, 2002). Therefore, we reasoned that impaired Reelin-Dab1 signaling might be responsible for the migration defects observed in *Tsc2* CKO cortices. In order to test this possibility, the levels of Reelin-Dab1 signaling molecules were examined in the developing cortices at P0 and E16.5, when Tsc2 is largely absent, in conjunction with mTORC1 activation (Figure 2A and S1A). Elevated mTORC1 signaling activity was observed by phosphorylation of S6 (pS6) at Ser 240/244 and found in most cortical plate neurons at P0 (Figure 2B), E16.5, E18.5 and P24, (Figure S2A, S2D and S2B) in the CKOs, while mTORC1 activity was mainly limited to a subset of neurons, including subplate and layer 5 neurons, in WT. Interestingly, immunostaining and Western blot analysis consistently showed that both Reelin and Dab1 proteins were increased in the *Tsc2* CKO compared to WT cortices (Figure 2C–2E, S2C and E). However, RT-PCR analyses showed no change in *Reelin* or *Dab1* at the transcription level (Figure 2F), suggesting abnormal posttranslational regulation in the absence of Tsc2.

Previous studies have established the importance of Dab1 phosphorylation upon activation of Reelin signaling and ubiquitin-mediated down regulation of pDab1 during neuronal migration (Arnaud et al., 2003; Feng et al., 2007). When Reelin signaling is impaired, pDab1 level was concomitantly reduced while total Dab1 protein increases, thus eliciting defective neuronal migration (Arnaud et al., 2003; Bock et al., 2004). In *Tsc2* CKO, the level of pDab1 was significantly reduced at E16.5 and P0 whereas total Dab1 was increased (Figure 2G), consistent with the notion that impaired Reelin-Dab1 signaling might be underlying mechanism in Tsc2-deficient cortex.

To gain further evidence of Reelin-Dab1 signaling defects linked to Tsc2 pathology, Purkinje cell (PC) migration during cerebellar development was examined by eliminating Tsc2 in the PCs using *Nestin Cre* mice. Reelin-Dab1 signaling plays a critical role in PC migration (Gallagher et al., 1998; Perez-Garcia et al., 2004; Yang et al., 2002). Reelin is expressed in postmitotic granule cells, and Reelin receptors and Dab1 are expressed in the PCs, mediating the upward migration of PCs to form the PC plate beneath the EGL layer (Goffinet et al., 1999). Because PCs are a major source of Shh, a mitogen for cerebellar granule cell precursors (Wechsler-Reya and Scott, 1999), PC migration defects in *Reelin* signaling mutants cause cerebellar hypoplasia, which is also consistently found in human patients with mutant *Reelin* (Hong et al., 2000). Our analysis of the $Tsc2^{flox/flox}$; *Nestin Cre*, which deletes Tsc2 in the progenitors of PCs and other cerebellar cells, revealed PC migration defects and a smaller cerebellum with a reduced number of folia (Figure 2H). At P5, when Tsc2 expression is almost completely eliminated in the cerebellum of $Tsc2^{flox/flox}$; *Nestin Cre* mice, Calbindin+ PCs were ectopically localized in the deeper layer of cerebellar plate (arrows). Concomitantly, Dab1 expression in the PCs was clearly increased, especially

in the ectopic PC found in the deeper layer of *Tsc2* $^{flox/flox}$; *Nestin Cre* animals compared to WT littermates (Figure 2I). At E17.5, an increase in Dab1 levels was also observed in the *Tsc2*$^{flox/flox}$; *Nestin Cre* posterior cerebellum (Figure 2I) where PC defects were most prominent, although Reelin levels were not obviously different from the WT (Figure 2I). Because *Tsc2* $^{flox/flox}$; *Nestin Cre* animals cannot survive beyond P5, it is not feasible to investigate defects in granule cells, which migrate downward along Bergmann glia fibers to form the internal granule cell layer during the second postnatal week (Hatten and Mason, 1990; Solecki et al., 2006). To determine whether migration defects are specific to PCs or global, we investigated migration defects in *Tsc2* $^{flox/flox}$; *hGFAP Cre*, in which Cre recombination occurs in the majority of progenitors, but not in the progenitors of early born neurons, including PCs. We found that, although the cerebellum was enlarged and granule cell size appeared to be increased (data not shown), there were no obvious migration/positioning defects of granule neurons, evidence against the idea that abnormal regulation of mTOR signaling causes global migration defects.

Cul5 expression is abnormally regulated in the *Tsc2* CKO

To determine whether the reduction of pDab1 resulted from changes in the level and the activity of Fyn, which directly phosphorylates Dab1, we examined expression of Fyn in the *Tsc2*-deficient cortex. Fyn expression was significantly reduced in the cortical plate as well as in the intermediate zone (IZ) of the *Tsc2* CKO compared to WT (Figure 3A). The total protein and phosphorylated Fyn were also substantially reduced in cortical lysates from the *Tsc2* CKO (Figure 3E–3H). Next we examined the level of total and phosphorylated Src family kinase (SFK) since Src is also known to phosphorylate Dab1 during cortical neuron migration. Western blot analysis of SFK and pSFK suggested that total SFK was slightly increased while pSFK was clearly increased in the *Tsc2* CKO (Figure 3E, 3F and S3C). Because pSFK is widely distributed in the developing cortex, we performed immunohistochemical analysis to determine the cell types with increased pSFK (Figure 3B and S3A). Although pSFK was elevated in both VZ and SVZ of the *Tsc2* CKO, the pSFK level in the cortical plate was much lower than that of WT (Figure 3C). These results suggest that reduction of Fyn and activated SFK in the cortical plate may be responsible for decreased pDab1 in migrating neurons.

Because Cullin 5 (Cul5), a core component of E3 ubiquitin-protein ligase complexes, is involved in the ubiquitin-mediated degradation of pSrc kinase (Laszlo and Cooper, 2009), Fyn (Simo and Cooper, 2013) and pDab1 (Feng et al., 2007), the reduction of pSFK, Fyn and pDab1 in the cortical plate may have resulted from increased expression of E3 ubiquitin ligase Cul5 in *Tsc2*-deficient neurons. To determine the involvement of Cul5 in Tsc2 pathology, we conducted immunostaining and Western blot analyses and found a prominent increase of Cul5 in the *Tsc2* CKO mouse brain (Figure 3D–3F, S3B and S3C). At P0, there was slightly more Cul5 in the cortical plates of WT than in the VZ (Figure 3D). In the *Tsc2* CKOs, however, Cul5 expression was concentrated in the upper portion of the cortical plate and in CR cells, where pS6 expression was enriched. This result provides evidence for abnormal Cul5 up-regulation in *Tsc2*-deficient neurons, including CR cells. RT-PCR and qPCR analyses demonstrated that the elevation of *Cul5* in the *Tsc2* CKO was partly due to transcriptional up-regulation, as *Cul5* transcripts were 5 fold higher than in WT at P0

(Figure 3I and 3J). Together, increased expression of Cul5 may be responsible for pDab1 reduction through excessive degradation as well as inefficient phosphorylation by diminishing pFyn and pSFK resulting in increased total Dab1 is consistently observed in the *Tsc2* CKO.

Reelin-Dab1 signaling is disrupted in cortical tubers

To determine whether Reelin–Dab1 signaling is perturbed in TSC patients, we next examined levels of Reelin-Dab1 signaling proteins in cortical tuber sections. Tubers are focal malformations in the TSC cortex that lack laminar structure. They contain enlarged, dysmorphic neurons and numerous glia cells as well as undifferentiated Nestin+ progenitor-like ("giant") cells that exhibit biallelic loss of *TSC1* or *TSC2* (Crino et al., 1996; Crino et al., 2010). Tubers can be found as early as 19 weeks gestation and likely form as a consequence of abnormal neuronal migration. Additionally, giant cells and dysmorphic neurons retain embryonic cellular and molecular characteristics (Maldonado et al., 2003). Therefore, we considered that tubers might provide a unique system for observing developmental defects, including altered Reelin-Dab1 signaling, at later stages. Accordingly, areas with normal laminar morphology immediately adjacent to tubers failed to show a distinctive immunopositive signal of Reelin, Dab1 or Cul5 (Figure 4A). We never observed abnormal expression of Reelin-Dab1 signaling molecules in the Tsc2 heterozygote cortices at P0, supporting the idea that full deletion of Tsc2 is required for abnormal regulation of Reelin-Dab1 signaling (Figure S3D). Immunostaining of cortical tubers in three cases, which consistently revealed intense Dab1, Reelin and Cul5 expression in the giant cells (Figure 4A and 4B). We also observed elevated Reelin expression in two SEGA (subependymal giant cell astrocytoma) samples and in an additional cortical tuber sample, demonstrating widespread up-regulation of Reelin expression in TSC cases (Figure S4A–S4D). To ensure the specificity of immune detection of Reelin and Dab1 in the cortical tuber sections, we confirmed that, as expected, they were distinctly expressed in the embryonic human brain as a positive control (Figure S4F). Human embryonic cortices at 19 weeks also showed enriched Cul5 expression in the top of the cortical plate, analogous to the mouse cortex at P0. (Figure S4E). Collectively, these findings support the hypothesis that, as in the Tsc2 CKO mice, Reelin-Dab1 signaling is disturbed in human cortical tubers suggesting a potential contribution to TSC pathogenesis.

Prenatal treatment with rapamycin rescued migration and reduced the expression of Dab1 and Cul5

To obtain direct evidence that the Reelin-Dab1 signaling and cellular defects in Tsc2-deficient neurons were the result of the abnormal mTORC1 activation, we treated *Tsc2* CKO mice with rapamycin, a well-established mTORC1 inhibitor. Rapamycin was previously reported to rescue lamination defects in the hippocampus and cortex when administered prenatally to $Tsc2^{flox/flox}$; *hGFAP-Cre* mice (Carson et al., 2012). Consistent with the prediction, the number of neurons with delayed migration was significantly reduced and lamination defects were no longer detectable at P0 when rapamycin was administered during embryogenesis (E13.5-P0) (Figure S5A–S5H). Rapamycin treatment reverted the levels of pDab1 and Dab1 in the *Tsc2* CKO to normal and also restored Fyn expression (Figure 5A–5C). These results suggest that mTORC1 is an upstream regulator of pDab1 and that

neuronal migration caused by abnormal Reelin-Dab1 signaling is mediated by mTOR activity.

We next assessed whether rapamycin treatment reduces Cul5 mRNA and protein expression. Cul5 protein expression in rapamycin treated *Tsc2* CKO cortical sections and extracts was indistinguishable from that of WT littermates. Interestingly, rapamycin effectively reduced expression of Cul5 protein in *Tsc2* CKO but not in WT as compared to vehicle treated animals (Figure 5B and 5C). However, rapamycin treatment significantly reduced *Cul5* mRNA expression in both WT and *Tsc2* CKO mice, although the degree of reduction was more prominent in the *Tsc2* CKO (Figure 5D). Thus, our results with mTORC1 inhibition establish that aberrant regulation of pDab1, Fyn and Cul5 resulted from mTORC1 hyperactivation.

Cul5 reduction rescues the migration defects of *Tsc2* KD neurons

To determine whether Cul5 overexpression is directly responsible for the cellular phenotypes and neuronal migration defects underlying the *Tsc2* CKO, we manipulated Cul5 and Tsc2 expression levels with shRNA. Examination of Cul5 and Tsc2 protein levels in electroporated cortical lysates confirmed the efficacy of *Cul5* and *Tsc2* shRNA (Figure S6A and S6B). We first determined the degree of migration delay caused by *Tsc2* KD. We used *in utero* electroporation to introduce *Tsc2* shRNA (Tsai et al., 2012) or control scrambled shRNA along with *pCAG-GFP* to cortical progenitors at E13.5 and E16.5 and analyzed the position of GFP+ neurons 5 days later (Figure S6C and 6A). Similar to the delayed migration in *Tsc2* CKO shown in Figure 1F, significantly fewer *Tsc2* KD late-born neurons reached the top of the cortex compared to control (Figure 6A). *Tsc2* KD neurons were distributed broadly in the cortical wall and moderately enriched in the deeper layers of the cortex and IZ (Bins 4–7). We also compared the migration defects of *Tsc2* KD neurons with those of neurons overexpressing the 5F Dab1 phosphorylation-defective mutant protein in which five tyrosine (Tyr185, 198, 200, 220 and 232) residues are switched to phenylalanine (Howell et al., 2000). The migration defects were similar to that of Tsc2 CKO (Figure 1F): very few neurons of either type reached the upper layers (Bins 2–3), and most remained in the deeper layers and IZ (Figure 6A and 6B). However, the migration delay was considerably greater when 5F was overexpressed, consistent with the idea that partial reduction, but not complete absence, of pDab1 is likely responsible for the migration defect in *Tsc2* CKO. Because *Tsc2* CKO neurons showed simplified leading processes at P0 (Figure 1G–1I), we traced the leading processes of *Tsc2* shRNA expressing migrating neurons contacting the MZ at P2 (electroporated at E16.5) (Figure 6C). Similar to *Tsc2* CKO, the leading processes of *Tsc2* KD neurons were longer and less branched than those of the control shRNA electroporated neurons (Figure 6D and 6E), supporting the notion that *Tsc2* mutation exerts its effects in a cell autonomous manner.

Next, we attempted to rescue *Tsc2* KD phenotypes by reducing the Cul5 level with *Cul5* shRNA. *Cul5* KD neurons mainly accumulated in the top of the cortical plate and only rarely appeared in deeper layers of cortex (Figure 6A and 6B), consistent with a previous observation (Simo et al., 2010). Importantly, *Cul5* KD almost completely rescued the migration defects of *Tsc2* KD neurons and restored normal migration and distribution

(Figure 6A, 6B and S6C) Furthermore, the leading processes of double shRNA treated neurons recovered the complexity of their branches and their shorter length (~30 μm), comparable to that of control shRNA (~31μm) (Figure 6C–6E). Remarkably, *Cul5* KD also rescued the migration defects of *Tsc2* CKO when *Cul5* shRNA was electroporated at E15.5 and the position of neurons examined at P0 (Figure 6F). Together, our results with Cul5 manipulations suggest that migration delay and leading process defects of *Tsc2*-deficient neurons result from upregulated Cul5 expression.

Rheb overexpression causes neuronal migration delay and impairment of Reelin-Dab1 signaling through upregulation of Cul5 expression

To further establish the role of mTOR signaling in the regulation of Cul5 expression, we activated mTOR by overexpressing Rheb, an mTOR activator and small GTPase enriched in brain. Enriched forced expression of Rheb is achieved using the CAG promoter, which elicits increased pS6 expression in H293 cells (data not shown) and cortical explants (Figure 7B and S7C). Rheb was *in utero* electroporated at E13.5, E14.5 and E16.5, and the positions of neurons were determined five days later (Figure 7A, S7A and S7B). Rheb overexpression under all conditions examined caused a certain degree of neuronal migration delay. Interestingly, the experimental condition of E13.5 electroporation and E18.5 harvest showed the most striking migration delay, similar to *Tsc2* KD, as the majority of neurons remained in the lower portion of the cortical plate and IZ (Figure7A and S6C). This phenotype is almost completely rescued by the *Cul5* KD, supporting the role of abnormal Cul5 upregulation in neuronal migration delay or arrest. To determine changes in Reelin-Dab1 signaling due to Rheb overexpression, Dab1 levels were monitored in protein lysates from electroporated cortical areas at P0 or P2 after inducing Rheb overexpression at E14.5 or E16.5, respectively (Figure 7B and S7C). As expected, Dab1 protein levels were increased upon Rheb overexpression, but reduced by *Cul5* KD despite continued aberrant mTORC1 activation as determined by pS6 expression (Figure 7B). RT-PCR and qPCR analyses demonstrated that forced Rheb expression can increase *Cul5* mRNA in the cortical explant (Figure 7C and 7D). Increased *Cul5* transcription is also achieved through overexpression of Rheb in N2a, a mouse neuroblastoma cell line, providing further evidence that mTOR activation can upregulate Cul5 expression in other cellular contexts (Figure 7E). Interestingly, the expression level of Socs7, an adaptor protein that links phosphoryated substrates to the Cul5 ubiquitin complex, is not significantly changed by either Rheb overexpression or *Tsc2* CKO (Figure 7C–7F). Furthermore, Rheb overexpression did not significantly alter *Dab1* and *Reelin* mRNA expression, supporting the specific transcriptional regulation of *Cul5* by mTOR activation. Together, mTOR activation by downregulated Tsc2 or upregulated Rheb can delay cortical neuronal migration at least partially through impaired Reelin-Dab1 signaling caused by increased Cul5 expression.

Discussion

We provide evidence that impaired Reelin-Dab1 signaling plays an important role in the pathogenesis of aberrant neuronal migration and positioning in TSC. Although we found that Reelin-Dab1 signaling is impaired in *Tsc2* CKO mice, the *Tsc2*-deficient brain did not exhibit the typical *Reelin-Dab1* mutant phenotype of inverted cortical layers and cerebellar

hypoplasia (Howell et al., 1997; Rice et al., 1998; Sheldon et al., 1997). It is therefore likely that *Tsc2*-deficiency causes a reduction, but not complete loss, of Reelin-Dab1 signaling. This interpretation is consistent with the results of several previous reports. First, a hypomorphic allele of *Dab1* mutant showed disturbances of specific aspects of migration, such as infiltration of MZ by late-born cortical neurons and split of the CA1 region into two well-defined layers (Herrick and Cooper, 2002), a phenotype remarkably similar to that of the *Tsc2* CKO mice. Second, mice null for a single type of high-affinity Reelin receptor, Vldlr, showed excessive pyramidal neurons in the MZ but not splitting deficits, whereas mice double null for both high-affinity Reelin receptors, Vldlr and ApoER2, showed the same phenotype as *Reelin* or *Dab1* mutants (Beffert et al., 2006). Third, ectopic Reelin expression driven by the nestin promoter in *Reelin* mutant mice restored preplate splitting but not neuronal positioning (Magdaleno et al., 2002). This last finding provides further support for the notion that the strength and location of Reelin-Dab1 signaling causes the differential phenotypic outcomes largely in an activity-dependent manner. Thus, it appears that delayed neuronal migration and infiltration of MZ are sensitive pathologic indicators of ineffective Reelin-Dab1 signaling.

Abnormal neuronal migration is often found in pediatric neurological disorders with intractable epilepsy and intellectual disability (Guerrini and Parrini, 2010; Verrotti et al., 2010). Our studies have established the molecular link between two critical signaling pathways that are essential for neuronal migration and increase our understanding of the cellular and molecular mechanisms responsible for the abnormal neuronal migration fundamental to TSC pathogenesis. Several lines of evidence support a direct pathogenic role of migration defects in the cortical malformations found in TSC patients. First, lamination defects have been reported in *Tsc1* and *2* mutant mouse models (Carson et al., 2012; Way et al., 2009). Second, *Tsc1* deletion from cortical progenitor cells causes migration defects that lead to white matter heterotopias, and *Tsc1* deletion from SVZ stem cells causes migration defects that lead to subependymal nodules (Feliciano et al., 2012; Feliciano et al., 2011; Zhou et al., 2011). Third, our results clearly demonstrated ectopically positioned PCs and reduced size of the cerebellum at P5 in the *Tsc2* CKO. Patients with the *RELN* (homolog of Reelin) mutation suffer from epilepsy and severe brain malformations, including lissencephaly and cerebellar hypoplasia (Chang et al., 2007; Hong et al., 2000; Ross et al., 2001) Furthermore, mutations of Reelin-Dab1 signaling components or of Rbx2, which encodes subunit of CUL5 ligase complex, cause PC migration defects (Simo and Cooper, 2013). Interestingly, Tsc2 loss does not disrupt migration of cerebellar granule neurons along radial glia processes, supporting the specificity of mTOR pathway-dependent neuronal migration defects due to abnormal Reelin-Dab1 signaling.

Our results demonstrated abnormally increased expression of Reelin in both human patients and mouse models, which may also contribute to the retarded migration and dispersed laminar structure observed. Reelin exerts its effects on neuronal migration and dendritic process development by activating mTOR through upregulation of AKT and PI3K (Jossin and Goffinet, 2007). This suggests that mTOR activation is required for neuronal migration and that during neuronal migration mTOR may participate in a feedback mechanism to regulate Cul5 expression and finely adjust the pDab1 level. A recent study also revealed a

connection between an upstream regulator of mTOR signaling components, the Stk25-Lkb1-stradα complex, and Reelin-Dab1 signaling (Matsuki et al., 2010). Stk25, an adaptor protein that links the polarity proteins Lkb1 and Stradα to Golgi protein GM130, can modulate Reelin-Dab1 signaling antagonistically during the establishment of neuronal polarity and deployment of the Golgi complex in dendrites of hippocampal neurons (Matsuki et al., 2010). Lkb1 is an upstream inhibitory regulator of mTOR that acts by promoting AMP kinase (AMPK)-dependent Tsc2 phosphorylation and is essential to generate neuronal polarity (Barnes et al., 2007; Corradetti et al., 2004; Shaw et al., 2004). AMPK activation in cortical neuronal culture under conditions of metabolic stress reduces mTORC1 signaling through phosphorylation of Tsc2 (Williams et al., 2011). Thus, further studies on the interaction of Reelin-Dab1 and mTOR may find more emerging points directing a common mechanism responsible for neuropathological outcomes of mTORopathies, a group of disorders resulting from mTOR-mediated neurodevelopmental defects (Crino, 2011).

Our results demonstrating that rapamycin restores Reelin-Dab1 signaling activity in the *Tsc2* CKO and that reducing Cul5 rescues migration defects of *Tsc2* KD neurons have important therapeutic potential for neuronal migration disorders due to perturbations of the Reelin-Dab1 and mTOR signaling pathways. This evidence is consistent with the idea that impairments in Reelin-Dab1 signaling may contribute to the neuropathology and neurological manifestations in Pretzel syndrome and PTEN (Phosphatase Tensin homolog)-related diseases such as Cowden syndrome (Nelen et al., 1999) and with the expectation that at least some of these abnormalities may respond to rapamycin. Pretzel syndrome is associated with deletion of the *Stradα* gene (Parker et al., 2013). Knockdown of *Stradα* in developing mouse cortex by *in utero* electroporation produces neuronal migration delay, which is analogous to *Tsc2*-deficiency, and mTORC1 activation is observed in fibroblast cell lines derived from patients and in the cortex of individuals with this disease (Parker et al., 2013). It is therefore extremely interesting that treatment with rapamycin prevents seizures in infants with this syndrome. Intriguingly, the neuron-specific *PTEN* mutant mouse, which models PTEN-related diseases, also shows increased Dab1 protein level and lamination defects (Kazdoba et al., 2012). Because abnormal regulation of mTOR and Reelin-Dab1 signaling is implicated in autism and disorders such as schizophrenia (Beffert et al., 2006; Iafrati et al., 2014), the effects of perturbations of the critical interplay between these signaling pathways may extend beyond neuronal migration and include alterations of dendrite elaboration, spine development and synaptic plasticity. Whether any of these alterations will also respond to rapamycin or other interventions targeting mTOR or Reelin-Dab1 signaling is speculative, but the approach may eventually provide an effective therapy for a subset of neurodevelopmental disorders.

In summary, our study demonstrates abnormal regulation of Reelin-Dab1 signaling activity in the *Tsc2* CKO cortex and describes previously unappreciated molecular pathogenic mechanisms that contribute to aberrant neuronal migration. Furthermore, abnormal expression patterns of Reelin-Dab1 signaling genes, including the putative factor Cul5 and regulatory proteins like SFK (including Fyn), are found in both *Tsc2* CKO mice and human patients. These similarities provide additional support for our hypothesis that disordered regulation of Reelin-Dab activity is the principal cause of Tsc2 neuropathology.

MATERIALS AND METHODS

IRB

Human patient samples were obtained and treated according to the guidelines of the Institutional Review Board of Temple University School of Medicine. Human patients carry *TSC2* mutations and tubers samples were isolated from frontal cortex.

Mice

All the animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committees of Temple University School of Medicine. *Tsc2*$^{flox/flox}$ mice (Hernandez et al., 2007) were bred to Emx1-Cre (Jackson Lab #005628) or Nestin-Cre (Jackson Lab #003771) mice to generate conditional *Tsc2* knockout mice.

Co-immunoprecipitation

Cell lysates from PBS- or rapamycin- treated cerebral cortex were incubated overnight with phosphotyrosine antibody and protein G-sepharose beads (GE Healthcare). Beads were washed extensively with lysis buffer and denatured in SDS sample buffer; samples were examined by Western blot with Dab1 antibody.

RNA isolation and reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from cerebral cortex using TRIzol® reagent (Invitrogen), and reverse transcription reactions were performed with total RNA; sequence information for primers is in the Supplemental method.

Cul5 shRNA and Rheb plasmid generation

Cul5 shRNA construct was generated using the shRNA sequence (Feng et al., 2007) that is effective in cortical neurons (“GCTGCAGACTGAATTAGTAG”) and cloned in *pLentiLox3.7* lentivirus vector that includes a dual promoter and *eGFP* (Rubinson et al., 2003). The *Rheb-GFP* was PCR amplified with the primer set ‘CCTCAGTCCAAGTCCCG’ (forward) and ‘TCACATCACCGAGCACGAAGA’ (reverse) using *pBk-cmv-mGFP* plasmid. For full-length *Rheb* expression vector, PCR product was further cloned into the pCAG vector.

Rapamycin treatment

Rapamycin was initially dissolved in sterile DMSO to a final concentration of 10 mM. Prior to use, this stock was diluted with PBS and injected intraperitoneally at 0.3 mg/kg daily to pregnant females from E13.5.

In utero electroporation

GFP expression plasmid (1 μg/μl) and/or shRNA plasmid and scrambled shRNA (2μg/μl) were electroporated into embryos as previously described (Olson et al., 2006).

Immunohistochemistry

Paraffin sections (7 μm) were prepared and immunohistochemistry was performed as described (Cho et al., 2012). Primary antibody information can be found in the Supplemental method.

Quantitative analyses

Z-stack images encompassing 100 μm were taken with a confocal microscope (Leica, SP8) every 2 μm. Details of leading process analyses and counting of Cux1- and GFP-expressing cells are described in the Supplemental procedures. We analyzed 3 nonconsecutive sections taken from 3–5 WT- or CKO-electroporated mice. For leading process analyses, we analyzed 30 to 40 neurons from 3 well-electroporated mice for each condition. Student's t-test was applied to assess statistical significance. $P<0.05$ was regarded as statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Arnaud L, Ballif BA, Forster E, Cooper JA. Fyn tyrosine kinase is a critical regulator of disabled-1 during brain development. Current biology : CB. 2003; 13:9–17. [PubMed: 12526739]

Ballif BA, Arnaud L, Arthur WT, Guris D, Imamoto A, Cooper JA. Activation of a Dab1/CrkL/C3G/Rap1 pathway in Reelin-stimulated neurons. Current biology : CB. 2004; 14:606–610. [PubMed: 15062102]

Barnes AP, Lilley BN, Pan YA, Plummer LJ, Powell AW, Raines AN, Sanes JR, Polleux F. LKB1 and SAD kinases define a pathway required for the polarization of cortical neurons. Cell. 2007; 129:549–563. [PubMed: 17482548]

Beffert U, Durudas A, Weeber EJ, Stolt PC, Giehl KM, Sweatt JD, Hammer RE, Herz J. Functional dissection of Reelin signaling by site-directed disruption of Disabled-1 adaptor binding to apolipoprotein E receptor 2: distinct roles in development and synaptic plasticity. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2006; 26:2041–2052. [PubMed: 16481437]

Bock HH, Jossin Y, May P, Bergner O, Herz J. Apolipoprotein E receptors are required for reelin-induced proteasomal degradation of the neuronal adaptor protein Disabled-1. The Journal of biological chemistry. 2004; 279:33471–33479. [PubMed: 15175346]

Britto JM, Tait KJ, Johnston LA, Hammond VE, Kalloniatis M, Tan SS. Altered speeds and trajectories of neurons migrating in the ventricular and subventricular zones of the reeler neocortex. Cerebral cortex. 2011; 21:1018–1027. [PubMed: 20847150]

Carson RP, Van Nielen DL, Winzenburger PA, Ess KC. Neuronal and glia abnormalities in Tsc1-deficient forebrain and partial rescue by rapamycin. Neurobiology of disease. 2012; 45:369–380. [PubMed: 21907282]

Chang BS, Duzcan F, Kim S, Cinbis M, Aggarwal A, Apse KA, Ozdel O, Atmaca M, Zencir S, Bagci H, et al. The role of RELN in lissencephaly and neuropsychiatric disease. American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics. 2007; 144B:58–63.

Cho SH, Kim JY, Simons DL, Song JY, Le JH, Swindell EC, Jamrich M, Wu SM, Kim S. Genetic ablation of Pals1 in retinal progenitor cells models the retinal pathology of Leber congenital amaurosis. Human molecular genetics. 2012; 21:2663–2676. [PubMed: 22398208]

Corradetti MN, Inoki K, Bardeesy N, DePinho RA, Guan KL. Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. Genes & development. 2004; 18:1533–1538. [PubMed: 15231735]

Crino PB. mTOR: A pathogenic signaling pathway in developmental brain malformations. Trends Mol Med. 2011; 17:734–742. [PubMed: 21890410]

Crino PB, Aronica E, Baltuch G, Nathanson KL. Biallelic TSC gene inactivation in tuberous sclerosis complex. Neurology. 2010; 74:1716–1723. [PubMed: 20498439]

Crino PB, Nathanson KL, Henske EP. The tuberous sclerosis complex. N Engl J Med. 2006; 355:1345–1356. [PubMed: 17005952]

Crino PB, Trojanowski JQ, Dichter MA, Eberwine J. Embryonic neuronal markers in tuberous sclerosis: single-cell molecular pathology. Proc Natl Acad Sci U S A. 1996; 93:14152–14157. [PubMed: 8943076]

D'Arcangelo G, Homayouni R, Keshvara L, Rice DS, Sheldon M, Curran T. Reelin is a ligand for lipoprotein receptors. Neuron. 1999; 24:471–479. [PubMed: 10571240]

Dulabon L, Olson EC, Taglienti MG, Eisenhuth S, McGrath B, Walsh CA, Kreidberg JA, Anton ES. Reelin binds alpha3beta1 integrin and inhibits neuronal migration. Neuron. 2000; 27:33–44. [PubMed: 10939329]

Feliciano DM, Quon JL, Su T, Taylor MM, Bordey A. Postnatal neurogenesis generates heterotopias, olfactory micronodules and cortical infiltration following single-cell Tsc1 deletion. Human molecular genetics. 2012; 21:799–810. [PubMed: 22068588]

Feliciano DM, Su T, Lopez J, Platel JC, Bordey A. Single-cell Tsc1 knockout during corticogenesis generates tuber-like lesions and reduces seizure threshold in mice. The Journal of clinical investigation. 2011; 121:1596–1607. [PubMed: 21403402]

Feng L, Allen NS, Simo S, Cooper JA. Cullin 5 regulates Dab1 protein levels and neuron positioning during cortical development. Genes & development. 2007; 21:2717–2730. [PubMed: 17974915]

Forster E, Bock HH, Herz J, Chai X, Frotscher M, Zhao S. Emerging topics in Reelin function. Eur J Neurosci. 2010; 31:1511–1518. [PubMed: 20525064]

Franco SJ, Martinez-Garay I, Gil-Sanz C, Harkins-Perry SR, Muller U. Reelin regulates cadherin function via Dab1/Rap1 to control neuronal migration and lamination in the neocortex. Neuron. 2011; 69:482–497. [PubMed: 21315259]

Frotscher M. Role for Reelin in stabilizing cortical architecture. Trends Neurosci. 2010; 33:407–414. [PubMed: 20598379]

Gallagher E, Howell BW, Soriano P, Cooper JA, Hawkes R. Cerebellar abnormalities in the disabled (mdab1-1) mouse. The Journal of comparative neurology. 1998; 402:238–251. [PubMed: 9845246]

Gil-Sanz C, Franco SJ, Martinez-Garay I, Espinosa A, Harkins-Perry S, Muller U. Cajal-Retzius cells instruct neuronal migration by coincidence signaling between secreted and contact-dependent guidance cues. Neuron. 2013; 79:461–477. [PubMed: 23931996]

Goffinet AM, Bar I, Bernier B, Trujillo C, Raynaud A, Meyer G. Reelin expression during embryonic brain development in lacertilian lizards. The Journal of comparative neurology. 1999; 414:533–550. [PubMed: 10531544]

Gorski JA, Talley T, Qiu M, Puelles L, Rubenstein JL, Jones KR. Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2002; 22:6309–6314. [PubMed: 12151506]

Guerrini R, Parrini E. Neuronal migration disorders. Neurobiology of disease. 2010; 38:154–166. [PubMed: 19245832]

Hatten ME, Mason CA. Mechanisms of glial-guided neuronal migration in vitro and in vivo. Experientia. 1990; 46:907–916. [PubMed: 2209800]

Hernandez O, Way S, McKenna J 3rd, Gambello MJ. Generation of a conditional disruption of the Tsc2 gene. Genesis. 2007; 45:101–106. [PubMed: 17245776]

Herrick TM, Cooper JA. A hypomorphic allele of dab1 reveals regional differences in reelin-Dab1 signaling during brain development. Development. 2002; 129:787–796. [PubMed: 11830577]

Honda T, Kobayashi K, Mikoshiba K, Nakajima K. Regulation of cortical neuron migration by the Reelin signaling pathway. Neurochemical research. 2011; 36:1270–1279. [PubMed: 21253854]

Hong SE, Shugart YY, Huang DT, Shahwan SA, Grant PE, Hourihane JO, Martin ND, Walsh CA. Autosomal recessive lissencephaly with cerebellar hypoplasia is associated with human RELN mutations. Nature genetics. 2000; 26:93–96. [PubMed: 10973257]

Howell BW, Hawkes R, Soriano P, Cooper JA. Neuronal position in the developing brain is regulated by mouse disabled-1. Nature. 1997; 389:733–737. [PubMed: 9338785]

Howell BW, Herrick TM, Hildebrand JD, Zhang Y, Cooper JA. Dab1 tyrosine phosphorylation sites relay positional signals during mouse brain development. Current biology : CB. 2000; 10:877–885. [PubMed: 10959835]

Iafrati J, Orejarena MJ, Lassalle O, Bouamrane L, Gonzalez-Campo C, Chavis P. Reelin, an extracellular matrix protein linked to early onset psychiatric diseases, drives postnatal development of the prefrontal cortex via GluN2B-NMDARs and the mTOR pathway. Molecular psychiatry. 2014; 19:417–426. [PubMed: 23752244]

Jossin Y, Goffinet AM. Reelin signals through phosphatidylinositol 3-kinase and Akt to control cortical development and through mTor to regulate dendritic growth. Molecular and cellular biology. 2007; 27:7113–7124. [PubMed: 17698586]

Kazdoba TM, Sunnen CN, Crowell B, Lee GH, Anderson AE, D'Arcangelo G. Development and characterization of NEX-Pten, a novel forebrain excitatory neuron-specific knockout mouse. Developmental neuroscience. 2012; 34:198–209. [PubMed: 22572802]

Kubo K, Honda T, Tomita K, Sekine K, Ishii K, Uto A, Kobayashi K, Tabata H, Nakajima K. Ectopic Reelin induces neuronal aggregation with a normal birthdate-dependent "inside-out" alignment in the developing neocortex. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2010; 30:10953–10966. [PubMed: 20720102]

Kwiatkowski DJ, Manning BD. Tuberous sclerosis: a GAP at the crossroads of multiple signaling pathways. Human molecular genetics. 2005; 14(Spec No. 2):R251–258. [PubMed: 16244323]

Laszlo GS, Cooper JA. Restriction of Src activity by Cullin-5. Current biology : CB. 2009; 19:157–162. [PubMed: 19147357]

Magdaleno S, Keshvara L, Curran T. Rescue of ataxia and preplate splitting by ectopic expression of Reelin in reeler mice. Neuron. 2002; 33:573–586. [PubMed: 11856531]

Maldonado M, Baybis M, Newman D, Kolson DL, Chen W, McKhann G 2nd, Gutmann DH, Crino PB. Expression of ICAM-1, TNF-alpha, NF kappa B, and MAP kinase in tubers of the tuberous sclerosis complex. Neurobiol Dis. 2003; 14:279–290. [PubMed: 14572449]

Matsuki T, Matthews RT, Cooper JA, van der Brug MP, Cookson MR, Hardy JA, Olson EC, Howell BW. Reelin and stk25 have opposing roles in neuronal polarization and dendritic Golgi deployment. Cell. 2010; 143:826–836. [PubMed: 21111240]

Nadarajah B, Alifragis P, Wong RO, Parnavelas JG. Neuronal migration in the developing cerebral cortex: observations based on real-time imaging. Cerebral cortex. 2003; 13:607–611. [PubMed: 12764035]

Nelen MR, Kremer H, Konings IB, Schoute F, van Essen AJ, Koch R, Woods CG, Fryns JP, Hamel B, Hoefsloot LH, et al. Novel PTEN mutations in patients with Cowden disease: absence of clear genotype-phenotype correlations. European journal of human genetics : EJHG. 1999; 7:267–273. [PubMed: 10234502]

Olson A, Sheth N, Lee JS, Hannon G, Sachidanandam R. RNAi Codex: a portal/database for short-hairpin RNA (shRNA) gene-silencing constructs. Nucleic acids research. 2006; 34:D153–157. [PubMed: 16381835]

Park TJ, Curran T. Crk and Crk-like play essential overlapping roles downstream of disabled-1 in the Reelin pathway. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2008; 28:13551–13562. [PubMed: 19074029]

Parker WE, Orlova KA, Parker WH, Birnbaum JF, Krymskaya VP, Goncharov DA, Baybis M, Helfferich J, Okochi K, Strauss KA, et al. Rapamycin prevents seizures after depletion of STRADA in a rare neurodevelopmental disorder. Science translational medicine. 2013; 5:182ra153.

Perez-Garcia CG, Tissir F, Goffinet AM, Meyer G. Reelin receptors in developing laminated brain structures of mouse and human. The European journal of neuroscience. 2004; 20:2827–2832. [PubMed: 15548227]

Prabhakar S, Goto J, Zhang X, Sena-Esteves M, Bronson R, Brockmann J, Gianni D, Wojtkiewicz GR, Chen JW, Stemmer-Rachamimov A, et al. Stochastic model of Tsc1 lesions in mouse brain. PloS one. 2013; 8:e64224. [PubMed: 23696872]

Rice DS, Sheldon M, D'Arcangelo G, Nakajima K, Goldowitz D, Curran T. Disabled-1 acts downstream of Reelin in a signaling pathway that controls laminar organization in the mammalian brain. Development. 1998; 125:3719–3729. [PubMed: 9716537]

Ross ME, Swanson K, Dobyns WB. Lissencephaly with cerebellar hypoplasia (LCH): a heterogeneous group of cortical malformations. Neuropediatrics. 2001; 32:256–263. [PubMed: 11748497]

Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Rooney DL, Zhang M, Ihrig MM, McManus MT, et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nature genetics. 2003; 33:401–406. [PubMed: 12590264]

Sanada K, Gupta A, Tsai LH. Disabled-1-regulated adhesion of migrating neurons to radial glial fiber contributes to neuronal positioning during early corticogenesis. Neuron. 2004; 42:197–211. [PubMed: 15091337]

Sancak O, Nellist M, Goedbloed M, Elfferich P, Wouters C, Maat-Kievit A, Zonnenberg B, Verhoef S, Halley D, van den Ouweland A. Mutational analysis of the TSC1 and TSC2 genes in a diagnostic setting: genotype--phenotype correlations and comparison of diagnostic DNA techniques in Tuberous Sclerosis Complex. European journal of human genetics : EJHG. 2005; 13:731–741. [PubMed: 15798777]

Sekine K, Honda T, Kawauchi T, Kubo K, Nakajima K. The outermost region of the developing cortical plate is crucial for both the switch of the radial migration mode and the Dab1-dependent "inside-out" lamination in the neocortex. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2011; 31:9426–9439. [PubMed: 21697392]

Sekine K, Kawauchi T, Kubo K, Honda T, Herz J, Hattori M, Kinashi T, Nakajima K. Reelin controls neuronal positioning by promoting cell-matrix adhesion via inside-out activation of integrin alpha5beta1. Neuron. 2012; 76:353–369. [PubMed: 23083738]

Shaw RJ, Bardeesy N, Manning BD, Lopez L, Kosmatka M, DePinho RA, Cantley LC. The LKB1 tumor suppressor negatively regulates mTOR signaling. Cancer Cell. 2004; 6:91–99. [PubMed: 15261145]

Sheldon M, Rice DS, D'Arcangelo G, Yoneshima H, Nakajima K, Mikoshiba K, Howell BW, Cooper JA, Goldowitz D, Curran T. Scrambler and yotari disrupt the disabled gene and produce a reeler-like phenotype in mice. Nature. 1997; 389:730–733. [PubMed: 9338784]

Simo S, Cooper JA. Rbx2 regulates neuronal migration through different cullin 5-RING ligase adaptors. Dev Cell. 2013; 27:399–411. [PubMed: 24210661]

Simo S, Jossin Y, Cooper JA. Cullin 5 regulates cortical layering by modulating the speed and duration of Dab1-dependent neuronal migration. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2010; 30:5668–5676. [PubMed: 20410119]

Solecki DJ, Govek EE, Hatten ME. mPar6 alpha controls neuronal migration. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2006; 26:10624–10625. [PubMed: 17050699]

Tsai PT, Hull C, Chu Y, Greene-Colozzi E, Sadowski AR, Leech JM, Steinberg J, Crawley JN, Regehr WG, Sahin M. Autistic-like behaviour and cerebellar dysfunction in Purkinje cell Tsc1 mutant mice. Nature. 2012; 488:647–651. [PubMed: 22763451]

Verrotti A, Spalice A, Ursitti F, Papetti L, Mariani R, Castronovo A, Mastrangelo M, Iannetti P. New trends in neuronal migration disorders. European journal of paediatric neurology : EJPN : official journal of the European Paediatric Neurology Society. 2010; 14:1–12. [PubMed: 19264520]

Way SW, McKenna J 3rd, Mietzsch U, Reith RM, Wu HC, Gambello MJ. Loss of Tsc2 in radial glia models the brain pathology of tuberous sclerosis complex in the mouse. Human molecular genetics. 2009; 18:1252–1265. [PubMed: 19150975]

Way SW, Rozas NS, Wu HC, McKenna J 3rd, Reith RM, Hashmi SS, Dash PK, Gambello MJ. The differential effects of prenatal and/or postnatal rapamycin on neurodevelopmental defects and cognition in a neuroglial mouse model of tuberous sclerosis complex. Human molecular genetics. 2012; 21:3226–3236. [PubMed: 22532572]

Wechsler-Reya RJ, Scott MP. Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. Neuron. 1999; 22:103–114. [PubMed: 10027293]

Williams T, Courchet J, Viollet B, Brenman JE, Polleux F. AMP-activated protein kinase (AMPK) activity is not required for neuronal development but regulates axogenesis during metabolic stress. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108:5849–5854. [PubMed: 21436046]

Yang H, Jensen P, Goldowitz D. The community effect and Purkinje cell migration in the cerebellar cortex: analysis of scrambler chimeric mice. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2002; 22:464–470. [PubMed: 11784791]

Zhou J, Shrikhande G, Xu J, McKay RM, Burns DK, Johnson JE, Parada LF. Tsc1 mutant neural stem/progenitor cells exhibit migration deficits and give rise to subependymal lesions in the lateral ventricle. Genes & development. 2011; 25:1595–1600. [PubMed: 21828270]

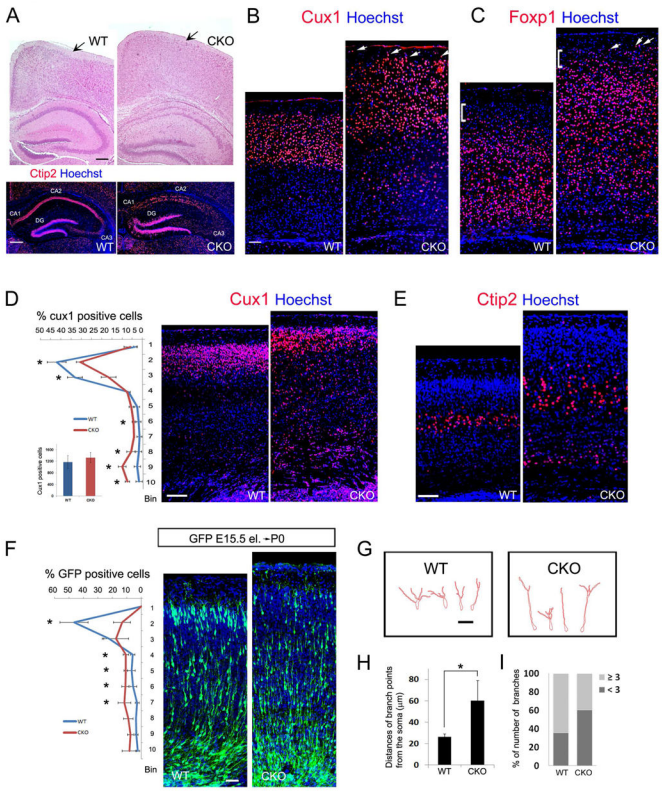

Figure 1. Abnormal neuronal migration causes lamination defects in *Tsc2*-deficient cortex and hippocampus

(A) Histology of WT and *Tsc2* CKO mice at P17 shows reduced thickness of the MZ (arrow), and Ctip2 immunostaining reveals disorganization of hippocampal layers at P24. (B) Cux1+ late-born neurons are excessive in the MZ (arrows) and ectopically localized in the deep cortical layers of *Tsc2* CKO mice at P24 (red bracket). (C) FoxP1+ neurons are widely dispersed and present in layer2 (bracket) and MZ in *Tsc2* CKO at P24 (arrows). (D) Cortex was divided into 10 bins and the percentage of Cux1+ neurons quantified at P0. Graph shows total numbers of Cux1+ cells. (E) Ctip2+ layer 5 neurons show wider distribution in *Tsc2* CKO. (F) Defective migration of *Tsc2* CKO neurons at P0, visualized by *in utero* electroporation with *pCAG-GFP* at E15.5. (G–I) Leading processes of CKO neurons contacting the MZ are longer with fewer branches than WT neurons. Graph depicts percentage with 3–5 or 1–2 branches (40 neurons from 3 WT animals, 40 neurons from 3 CKO animals). *p<0.0001 by Student's t test. Scale bar: 100 μm (A top, B, and C), 200 μm (A bottom), 40 μm (D–F), 20 μm (G). See also Supplemental Figure S1 (related to Figure 1)

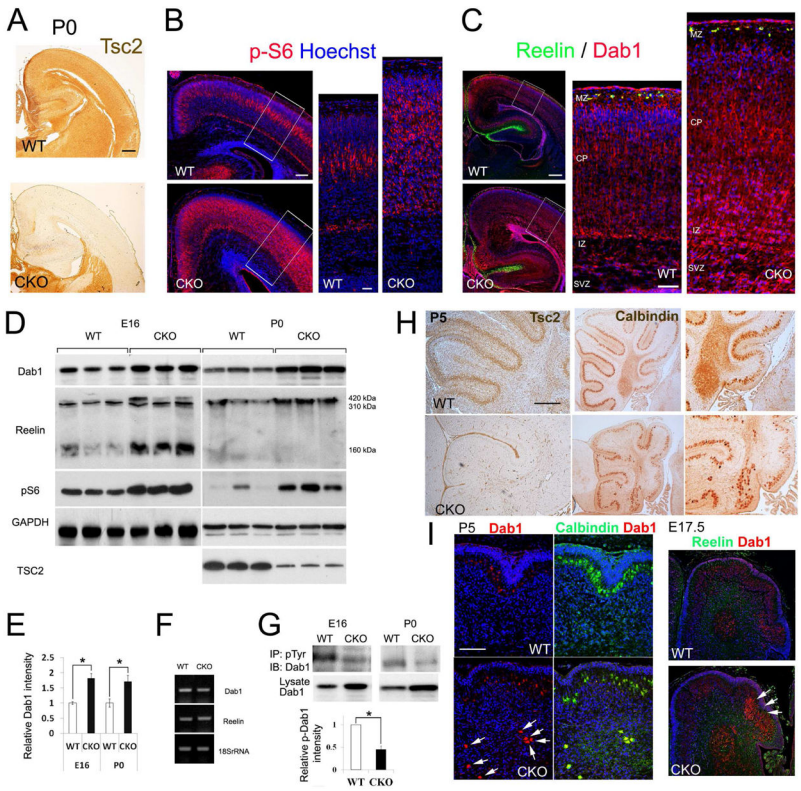

Figure 2. Reelin-Dab1 signal pathway is perturbed in the *Tsc2* CKO

(A–B) Tsc2 immunohistochemistry shows down regulated Tsc2 expression but increased pS6 expression in most regions of *Tsc2* CKO cortex as compared to WT. (C) Dab1 is widely distributed in the cortex at P0 in both WT and *Tsc2* CKO but markedly increased in CKO. (D) Western blots for Reelin and Dab1 from *Tsc2* CKO cortical extracts show highly increased protein levels at both E16.5 and P0, respectively. (E) Dab1 expression relative to GAPDH was quantified by ImageJ. (F) Transcription levels of *Reelin* and *Dab1* are not changed when determined by RT-PCR. (G) Level of pDab1 was decreased in *Tsc2* CKO at E16 and P0. (H) In *Tsc2* $^{flox/flox}$; *Nestin-Cre*, Tsc2 expression is undetectable in cerebellum at P5, whereas intense Tsc2 staining is found in the PC layer of WT. Calbindin+ PCs are positioned aberrantly in the deeper areas, as is most evident in the posterior lobes (enlarged picture). (I) Dab1 expression in PCs is limited to the PC layer in the WT (arrows), whereas intense Dab1 staining is found in ectopic Calbindin+ PCs (arrows) in addition to those in the PC layer in CKO. At E17.5, strong Dab1expression (red) is detected in the posterior PC plates in CKO (arrows) compared to WT. Scale bar: 200 μm (A and C left). 100 μm (B left, H and I), 40 μm (C enlarged), 20 μm (B enlarged). See also Supplemental Figure S2 (related to Figure 2)

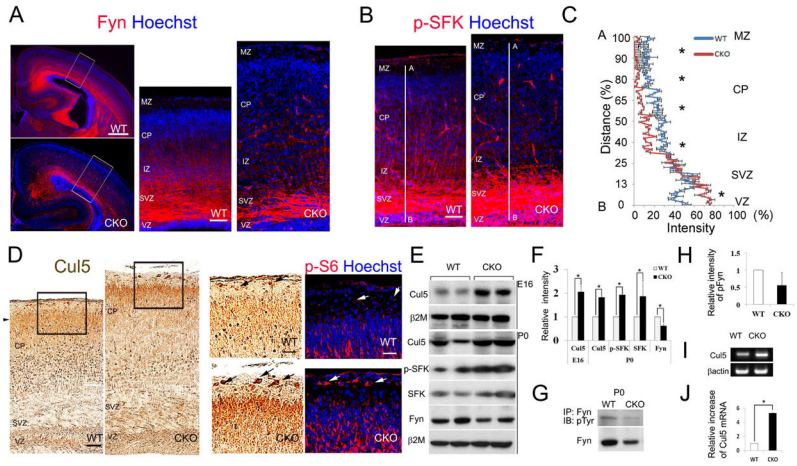

Figure 3. Cul5 expression is abnormally upregulated in the absence of Tsc2

(A) The level of Fyn is reduced in the *Tsc2* CKO. (B) Intense pSFK expression in the VZ and SVZ, but cortical pSFK expression at P0 is lower in the *Tsc2* CKO than WT. (C) Immunostained images imported into ImageJ were deconvolved to further confirm distribution patterns of pSFK expression by measuring fluorescence intensity per pixel across the cortex. (D) Immunostaining of Cul5 in the cortex of WT and *Tsc2* CKO demonstrates focal increase of Cul5 expression in the upper layer of cortical plate in the *Tsc2* CKO as compared to WT. CR cells are visualized by Cul5 and pS6 expression in both WT and *Tsc2* CKO; expression is increased in *Tsc2* CKO. (E and F) Western blot analysis shows more abundant pSFK and Cul5 protein, but reduced Fyn and pFyn expression, in the *Tsc2* CKO compared to WT. (G and H) Level of pFyn is decreased in CKO. (I and J) Higher *Cul5* mRNA level in CKO than in WT, determined by RT-PCR and qPCR. CP: cortical plate, VZ: ventricular zone, SVZ: subventricular zone, IZ: intermediate zone. Scale bar: 200 μm (A left), 40 μm (A right, B and D left), 20 μm (D enlarged). See also See also Supplemental Figure S3 (related to Figure 3)

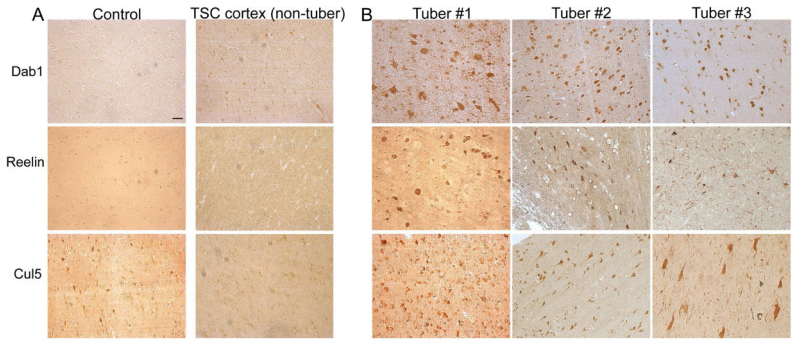

Figure 4. Expression of Reelin-Dab1 signaling proteins is altered in human TSC tubers

(A) Control human cortex and non-tuber region of TSC patient cortex show very low Reelin, Dab1, and Cul5 expression. (B) Reelin, Dab1 and Cul5 are highly expressed in giant cells (GCs) in the tuber and some is also expressed in astrocytes (smaller cells) in three independent cases of cortical tubers. Scale bar 50 μm. See also Supplemental Figure S4 (related to Figure 4)

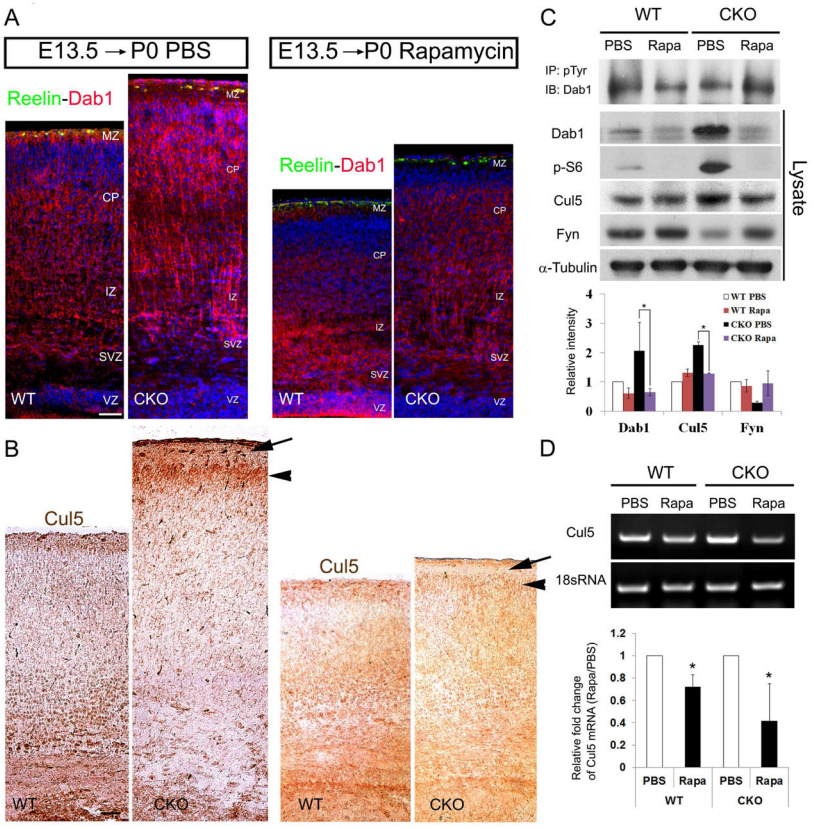

Figure 5. Rescue of Tsc2 mediated migration defects by inhibition of mTOR

(A and B) Rapamycin treatment (E13.5-P0) decreases Dab1 and Cul5 expression in *Tsc2* CKO. (C) Western blot analysis of *Tsc2* CKO and WT at P0 after rapamycin treatment demonstrates the restoration of expression level of Dab1, pDab1 and Cul5. Expression level of Dab1, Cul5 and Fyn relative to GAPDH was quantified by ImageJ. (D) Decreased level of *Cul5* transcripts in both WT and *Tsc2* CKO after rapamycin treatment shown by RT-PCR and quantitative PCR. Scale bar: 40 μm (A and B). See also Supplemental Figure S5 (related to Figure 5)

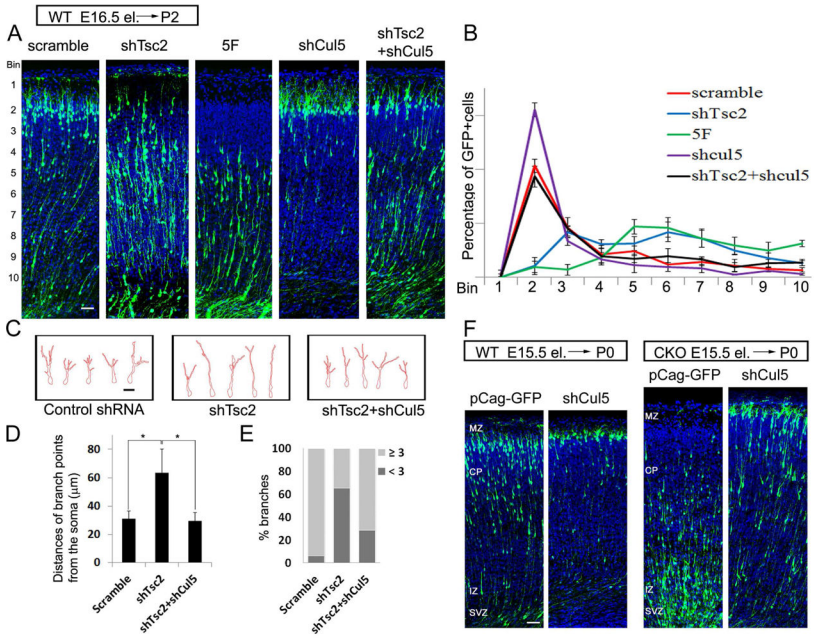

Figure 6. Rescue of migration defects of *Tsc2* KD neurons by reduction of Cul5

(A) *Tsc2* and *Cul5* shRNA constructs, control shRNA (Scrambled) and non-phosphorylatable Dab1 (5F) constructs were electroporated at E16.5. (B) Neuronal migration defects were analyzed at P2 by quantifying the percentage of GFP+ neurons in each bin. (C–E) *Tsc2* KD neurons have elongated and less branched leading processes compared to control shRNA expressing neurons. *Cul5* shRNA rescues the leading process phenotype of *Tsc2* KD neurons (*Tsc2* shRNA N=32 from 3 cortices, *Scrambled* N=31 from 3 cortices, *Cul5; Tsc2* double shRNA, N=31 from 3 cortices, *p<0.0001 by Student's t test). (F) Scrambled shRNA control or *Cul5* shRNA was electroporated to WT or *Tsc2* CKO cortices at E15.5. Delayed migration of *Tsc2* CKO neurons is rescued by *Cul5* shRNA at P0. CP: cortical plate, SVZ: subventricular zone, IZ: intermediate zone. Scale bar: 40 μm (A and E), 20 μm (B). See also Supplemental Figure S6 (related to Figure 6)

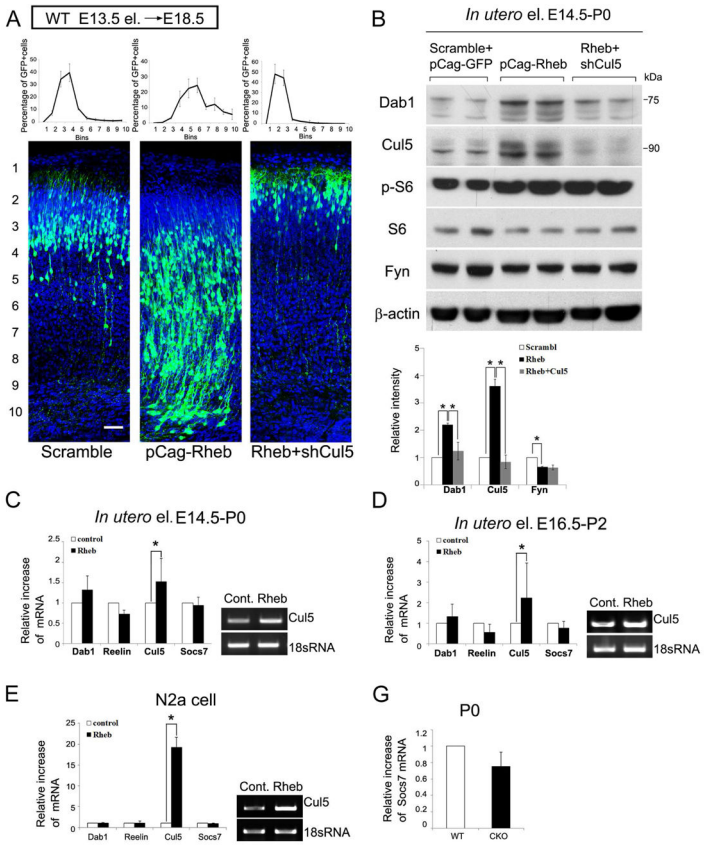

Figure 7. Rheb overexpression impairs neuronal migration and Reelin Dab1 signaling by enhancing Cul5 expression

(A) pCAG-Rheb is electroporated with scrambled shRNA control or along with *Cul5* shRNA at E13, and the position of electroporated neurons analyzed at E18.5 by quantifying the percentage of GFP+ neurons in each bin. (B) Western blot analyses of pCAG-Rheb electroporated cortical areas reveal increased Cul5 and Dab1 protein levels compared to control construct-electroporated areas. The protein level of Dab1 and Cul5 is substantially reduced by concurrent expression of shCul5 and Rheb in spite of unchanged level of pS6. (C–E) Transcription levels of *Dab1*, *Reelin*, *Cul5*, and *Socs7* are compared in Rheb-electroporated vs. control vector-electroporated cortices (C and D) and N2a cells (E) by qPCR and RT-PCR. (F) The level of *Socs7* transcripts in the *Tsc2* CKO is similar to that of WT at P0. Scale bar 40 μm. See also Supplemental Figure S7 (related to Figure 7)