The E3 Ubiquitin Ligase Protein Associated with Myc (Pam) Regulates Mammalian/Mechanistic Target of Rapamycin Complex 1 (mTORC1) Signaling in Vivo through N- and C-terminal Domains

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Background: Pam and its homologs act as key regulators of axon guidance and outgrowth and synapse development. Pam regulates mTORC1 signaling in vivo, and attenuated mTORC1 signaling may partly explain the axonal defects in Phr1-deficient mice. mTORC1-dependent and -independent mechanisms contribute to neurodevelopmental defects in Phr1 deficiency. Understanding how Pam regulates synapse development will have direct relevance for diverse aspects of neural development.

Results: Pam regulates mTORC1 signaling in vivo, and attenuated mTORC1 signaling may partly explain the axonal defects in Phr1-deficient mice. mTORC1-dependent and -independent mechanisms contribute to neurodevelopmental defects in Phr1 deficiency.

Significance: mTORC1-dependent and -independent protein synthesis, and aberrant mTORC1 signaling is reported in many neurological disorders (4–6). The TSC proteins act as a central hub relaying signals from diverse cellular pathways to control mTORC1 activity (2, 3). mTORC1 signaling plays an important role in neurodevelopment by modulating both general and neuronal activity-dependent protein synthesis. More importantly, long-lasting forms of synaptic plasticity and memory are dependent on new protein synthesis, and aberrant mTORC1 signaling is reported in many neurological disorders (4–6).

Tuberous sclerosis complex (TSC),4 in which slow growing, benign hamartomas form in multiple organs, is caused by mutations in tumor suppressor genes encoding hamartin (TSC1) and tuberin (TSC2), respectively. TSC-associated CNS defects, including seizures and mental retardation, and features of autism spectrum disorder are present in 30–60% of individuals with TSC (1). The TSC1-TSC2 protein complex functions as a critical negative regulator of mammalian/mechanistic target of rapamycin (mTOR) complex 1 (mTORC1)-mediated signaling. The TSC proteins act as a central hub relaying signals from diverse cellular pathways to control mTORC1 activity (2, 3).

Pam and its homologs (the PHR protein family) are large E3 ubiquitin ligases that function to regulate synapse formation and growth in mammals, zebras, Drosophila, and Caenorhabditis elegans. Phr1-deficient mouse models (Phr1ΔN,9 and Phr1Magellan8,9), with deletions in the N-terminal putative guanine exchange factor region and the C-terminal ubiquitin ligase region, respectively exhibit axon guidance/outgrowth defects and striking defects of major axon tracts in the CNS. Our earlier studies identified Pam to be associated with tuberous sclerosis complex (TSC) proteins, ubiquitinating TSC2 and regulating mTORC1-dependent and -independent protein synthesis.

Here, we examine the potential involvement of the TSC/mTOR complex in vivo signaling pathway in Phr1-deficient mouse models. We observed attenuation of mTORC1 signaling in the brains of both Phr1ΔN,9 and Phr1Magellan mouse models. Our results establish that Pam regulates mTORC1 signaling in vivo through two distinct domains. To further address whether Pam regulates mTORC1 through two functionally independent domains, we undertook heterozygous mutant crossing between Phr1ΔN,9 and Phr1Magellan mice to generate a compound heterozygous model to determine whether these two domains can complement each other. mTORC1 signaling was not attenuated in the brains of double mutants (Phr1ΔN,9/Mag), confirming that Pam displays dual regulation of the mTORC1 pathway through two functional domains. Our results also suggest that although dysregulation of mTORC1 signaling may be responsible for the corpus callosum defects, other neurodevelopmental defects observed with Phr1 deficiency are independent of mTORC1 signaling. The ubiquitin ligase complex containing Pam-Fbxo45 likely targets additional synaptic and axonal proteins, which may explain the overlapping neurodevelopmental defects observed in Phr1 and Fbxo45 deficiency.
Regulation of mTORC1 Signaling by Pam

We previously identified protein associated with Myc (Pam) as a TSC2 interactor and demonstrated a genetic interaction between the homologs of these proteins in Drosophila (7). Human Pam belongs to an evolutionally conserved family of large proteins (PHR family), including Phr1 (for Pam/Highwire/RPM-1) in mouse, Highwire (HIW) in Drosophila, RPM-1 (regulator of presynaptic morphology 1) in Caenorhabditis elegans, and Esrom in zebrafish, and all family members encode >80 exons averaging about 4000 amino acids. These proteins contain multiple conserved motifs, which include regulator of chromosome condensation (RCC) homology domains (RHD-1 and RHD-2) with inferred guanine exchange factor (GEF) activity, a Myc-binding domain, a B-box zinc finger, and a C-terminal RING zinc finger (RZF) domain, which is a hallmark motif for E3 ubiquitin (Ub) ligase activity (8–10). Several genetic studies demonstrated that Pam homologs regulate presynaptic growth and development (11–15). Members of the PHR family have been suggested to control a variety of signaling pathways, including JNK/p38 MAPK signaling in Drosophila and C. elegans (16–18), bone morphogenetic protein signaling in Drosophila (19), and cAMP signaling in mammalian cells (20). In addition, RPM-1 positively regulates a Rab GTPase pathway to promote vesicular trafficking via late endosomes, thereby regulating synapse formation and axon formation (21). Results obtained from both Drosophila and C. elegans suggest that the highly conserved RZF domain is critical for the E3 Ub ligase activity of Pam homologs, particularly for the regulation of synapse development (16, 17, 22). Our previous work demonstrated that Pam interacts with specific E2 enzymes and is capable of ubiquitinating TSC2; furthermore, suppression of Pam in primary dissociated neurons results in stabilization of TSC2 and down-regulation of mTORC1 signaling (23). Because Ub and ubiquitination enzymes have emerged as key regulators of synapse development, function, and plasticity (24, 25), our findings suggested that Pam, as an E3 Ub ligase and a regulator of TSC/mTOR signaling, could play an essential role in neuronal activity-dependent protein synthesis and synapse development and function in mammalian neurons.

Phr1 mouse models generated in recent years demonstrate the importance of Phr1 in axon guidance and outgrowth and synapse development. In an N-ethyl-N-nitrosourea-induced genetic screen for morphological defects in mouse embryonic motor neurons, mutants harboring a premature stop codon in exon 63 of Phr1 were isolated and named Magellan (referred to here as Phr1Mag). The Phr1 gene contains ∼86 exons, and a premature stop codon in exon 63 of the Phr1Mag allele results in deletion of the C-terminal region of Pam containing the E3 Ub ligase domain. However, a truncated N-terminal segment of the protein encompassing the RHD region (putative GEF) is stably expressed in these mutants (26). In the genetically engineered model of Phr1 described by Bloom et al. (27), exons 8 and 9, encoding 70 amino acids adjacent to the highly conserved RHD, are deleted in-frame either conditionally in selected regions of the brain or constitutively in all organs. In this Phr1Δ8,9 model, the C-terminal E3 Ub ligase motif is intact. These mouse models are valuable in understanding the functions of N- and C-terminal motifs, the RHD (putative GEF function) and the RZF (E3 Ub ligase activity), respectively.

Employing these models, we show here that mTORC1 signaling is compromised in the brains of both Phr1Mag and Phr1Δ8,9 models; however, the TSC1-TSC2 complex is stabilized only in Phr1Mag mice and not in Phr1Δ8,9 mice. In addition, we generated a compound heterozygous model (Phr1Δ8,9/Mag) that demonstrates complementation of each domain with respect to mTORC1 signaling. Collectively, our results provide both in vitro and in vivo evidence that Pam/Phr1 regulates mTORC1 signaling through two functionally independent domains. We also provide in vivo evidence that thinning of the corpus callosum reported in Phr1-deficient mice may be due to down-regulation of mTORC1 signaling.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—All antibodies were purchased from Cell Signaling Technology, with the exception of anti-α-tubulin and anti-FLAG antibodies (Sigma), anti-HA antibody (Covance), anti-Myc antibody (clone 9E10, Developmental Studies Hybridoma Bank, University of Iowa), anti-actin and anti-GAPDH antibodies (Santa Cruz Biotechnology), anti-Rheb (Ras homology enriched in brain) and anti-MyCBP2 (ab86078) antibodies (Abcam), anti-dual-leucine zipper kinase (DLK) antibody (Dr. Lawrence Holman, University of Michigan Medical School), anti-Fbxo45 antibody (Dr. Hideyuki Okano, Keio University, Tokyo, Japan), and anti-TSC1 antibody (28). Affinity-purified PF3, an anti-Pam/Phr1 rabbit polyclonal antibody, was generated in our laboratory against a maltose-binding protein-Pam fusion protein (amino acids 1–150). The Rheb shRNA clone was purchased from Open Biosystems. Stimulation experiments were performed using insulin (Sigma). Rapamycin was purchased from Calbiochem. The Myc-Rheb overexpression plasmid was a kind gift from Dr. Kun-Liang Guan (University of California, San Diego, CA), and Myc-tagged PamF1 and PamF3 plasmids were described previously (29). Lipofectamine 2000, Lipofectamine, and PLUS reagent were purchased from Invitrogen.

Phr1 Mouse Models and Genotyping—Phr1Mag stock mice were obtained from Dr. Samuel L. Pfaff (Salk Institute for Biological Studies, La Jolla, CA) (26), and conventional Phr1Δ8,9 stock mice have been described previously (27). To generate compound heterozygous Phr1Δ8,9/Mag mice, heterozygous Phr1Δ8,9/+ mice were crossed with heterozygous Phr1Mag/+ mice. Day 18.5 embryos were collected from timed pregnant matings. All genotyping was performed by PCR analysis using genomic DNA isolated from ear punch or tail samples. For Phr1Δ8,9 mutant mice, the presence of the WT allele or mutant allele was verified as described previously (27). Phr1Mag/+ mice were genotyped using PCR methods, followed by restriction enzyme digestion using HpyBI (Fermentas) to separate the WT and mutant alleles as described (26). Genotyping of the compound heterozygous Phr1Δ8,9/Mag mouse model utilized methods for both Phr1Δ8,9 and Phr1Mag mice.

Standard and Real-time RT-PCR—Total RNA was isolated using the RNasy mini kit (Qiagen) according to the manufacturer’s instructions. First-strand cDNA was generated using SuperScriptII reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. RT-PCR for Phr1 was carried out employing two sets of mouse-specific primers: the
5’-Phr1 (exon 3) primer GCTGACATCGAAACTCAGTACA and the 3’-Phr1 (exon 3/4) primer CCCAACCTCAATAATCTTGGCA, as well as the 5’-Phr1 (exon 71) primer TGAAGGACACATGGTTGGA and the 3’-Phr1 (exon 72) primer GATATGGCGTGTTCCCATTC. Real-time RT-PCR was performed in triplicate using the Roche LightCycler® 480 system. Mouse-specific primers were designed using the web-based program Primer3 (Version 4.0). Primers included the 5’-Fbxo45 primer GCTGGAATCTGGTGGA and the 3’-Fbxo45 primer CCCACGCTCAAAGCTAGTAAG. Reactions were performed using iTaq™ SYBR® Green Supermix with ROX (Bio-Rad). Acquired data were analyzed using LightCycler® 480 software. Gene expression levels were normalized to Gapdh. As a control for standard and real-time RT-PCR, mouse-specific Gapdh primers (5’-AGTTCGTTGTGAACGGATTTG and 3’-TGTAGACCATGTAGTTGAG-GTCA) were utilized.

Cell Culture, Transfection, Transduction, and Western Blotting—HEK293T cells were maintained in high glucose DMEM (Invitrogen) containing 10% fetal bovine serum (Sigma). For overexpression of HA-S6 kinase (S6K), Myc-Rheb, Myc-PamF1, and Myc-PamF3 constructs, HEK293T cells were transfected using Lipofectamine 2000 as recommended by the manufacturer. For Rheb RNAi experiments, 1 day prior to transfection, lentivirus expressing either Rheb shRNA or a non-targeting scrambled control was transduced into HEK293T cells, followed by transfection of HA-S6K alone or with Myc-PamF1 using Lipofectamine and PLUS reagent according to the manufacturer’s recommendations. For serum-deprived conditions, 18–20 h after transfection, cells were serum-deprived for 24 h, followed by immunoblot analysis. For insulin-stimulated conditions, following 24 h of serum deprivation, cells were stimulated for 30 min using a final dilution of 1 μL/ml insulin. Amino acid depletion experiments and rapamycin treatment were performed as described (30). All lysates from mouse tissues or cultured cells were prepared in either 0.5% Nonidet P-40 lysis buffer or standard radioimmuno precipitation assay buffer containing 1X Halt™ phosphatase inhibitor mixture (Thermo Scientific) and 1X Complete protease inhibitor mixture (Roche Applied Science). For S6K immunoprecipitation, protein lysates (500 μg) were incubated with 2–3 μg of primary antibody for 15 h at 4°C, mixed with protein A-Sepharose, and incubated for 3 h. Immunoprecipitates or protein lysates were separated by 4–15% gradient SDS-PAGE (Bio-Rad) and detected with primary antibodies as indicated. For quantification of immunoblotting data, bands were scanned, and intensities were calculated by densitometry (Bio-Rad). Western blotting for endogenous Pam/Phr1 was carried out on whole brain lysates from Phr1Mag and Phr1Δ8,9 mice (embryonic day E17.5) using NuPAGE 3–8% Tris acetate gels (Invitrogen) as described previously (26). Statistical analysis of scanned images was determined using Student’s two-tailed t test.

Histology—Whole mount brains from E18.5 mice were processed by either coronal cryosectioning methods as described previously (27) with minor modifications or by fixation in Bouin’s solution (Sigma), followed by coronal paraffin sectioning. Briefly, for cryosectioning, whole brains were fixed in 4% paraformaldehyde, cryoprotected by serially transferring to 15 and 30% sucrose solutions, sectioned at 20-μm thickness, and stained with a solution of 0.1% aqueous cresyl violet acetate (Sigma). For paraffin sectioning, whole embryos were fixed for 24 h and then washed with 70% ethanol, followed by 6-μm step sectioning and hematoxylin and eosin staining. Paraffin sectioning procedures, including embedding, step sectioning, staining, and analysis, were carried out by the Rodent Histology Core at the Dana Farber/Harvard Cancer Center (Boston, MA).

RESULTS

Compromised mTORC1 Signaling in Phr1 Mutant Mice—We reported previously that suppression of Pam by RNAi in dissociated neurons results in increased TSC2 levels and down-regulation of mTORC1 signaling, supporting the hypothesis that Pam may act as an E3 Ub ligase for TSC2 (23). To test the in vivo role of Pam in TSC/mTOR signaling, we examined two mouse models of Pam, Phr1Mag, in which the C-terminal RZF domain with the E3 Ub ligase activity is deleted (26), and Phr1Δ8,9, with an intact C-terminal RZF motif (27) (Fig. 1A). We observed a reduction of phosphorylation of downstream mTORC1 targets, including S6K (S6K phosphorylated at Thr-389) and S6 (S6 phosphorylated at Ser-240/Ser-244 and Ser-235/Ser-236), in brain lysates of both homozygous mutant models. To our surprise, the down-regulation of phospho-S6K and phospho-S6 was more significant in Phr1Δ8,9 mice, in which the RZF domain is intact, compared with Phr1Mag mice, with the deleted RZF domain (Fig. 1, B and C). The levels of S6K and S6 phosphorylation in brain lysates from both heterozygous mouse models were indistinguishable from those from WT mice. We did not observe any change in phosphorylation of 4E-BP1, another downstream target of mTORC1 signaling in either model, suggesting a differential regulation of this effector protein. We also did not see any change in phosphorylation of Akt and ERK. The compromised mTORC1 signaling was not seen in other embryonic tissues from Phr1Mag and Phr1Δ8,9 mouse models, suggesting that Pam regulation of mTORC1 signaling may be restricted to the brain (data not shown).

Employing standard and real-time RT-PCR, we observed no significant difference in expression of RNA in the brains of both models compared with WT mice (Fig. 2, A and B, and data not shown), which is in agreement with the original reports on these models (26, 27). Our N-terminal anti-Pam antibody PF3 detected a stable truncated Pam protein in homozygous Phr1Mag mouse brain lysates (Fig. 2A, right panel), which is consistent with the earlier study (26). However, PF3 was unable to detect a stable protein product in homozygous Phr1Δ8,9 mouse brain lysates with an in-frame deletion of exons 8 and 9 (Fig. 2B, middle panel), which could be due to masking of the epitope of the antibody employed. Using anti-MYCBP2 antibody (ab86078, Abcam), which was raised against Pam amino acids 2425–2475, we observed expression of Phr1 at a significantly reduced level in Phr1Δ8,9 mutant mouse brain compared with WT littermate brain (Fig. 2B, right panel). Protein expression of Phr1 in the Phr1Δ8,9 model has not been reported earlier. Given these observations, we cannot rule out the possibility that this extremely large protein is unstable under our experimental conditions.
Increased Levels of TSC1/2 in Phr1Mag Mouse Brain but Not in Phr1<sup>Δex8,9</sup> Mouse Brain—We showed previously that Pam binds to the TSC1-TSC2 complex and that the RZF motif in Pam is responsible for ubiquitinating TSC2 (7, 23). Based on these results, in the absence of the Ub ligase activity, the TSC2 protein level is expected to increase in Phr1Mag mouse brain lacking the RZF motif (Ub ligase domain). We therefore examined the protein levels of TSC1 and TSC2 in brain lysates of Phr1Mag and Phr1<sup>/H9004<sup>8,9</sup> mutants compared with the wild-type littermate controls; however, the levels of TSC1 and TSC2 proteins did not change in Phr1<sup>/H9004<sup>8,9</sup> mutants compared with the respective control littermates (Fig. 3). Quantitative RT-PCR analysis of Tsc1 and Tsc2 revealed no change in the RNA levels in Phr1Mag mutants (data not shown), consistent with Pam acting as an E3 Ub ligase to regulate stability of TSC proteins. Our results also suggest that the down-regulation of mTORC1 observed in Phr1<sup>/H9004<sup>8,9</sup> mice is not mediated through the TSC proteins. Genetic studies have established the dual-leucine zipper kinase DLK1/Wallenda as the E3 Ub ligase target in both C. elegans (RPM-1) and Drosophila (HIW) (16–18).

Exogenous Expression of Either N- or C-terminal Segments of Pam Activates mTORC1 Signaling—Our data document that either in-frame disruption of two exons at the N terminus or deletion of the C terminus of Phr1<sup>Mag</sup> is associated with down-regulation of mTORC1 signaling. We therefore tested the possibility that overexpression of N- and C-terminal segments of Pam will result in activation of mTORC1 signaling. To determine whether Pam overexpression could constitutively activate mTORC1 signaling, Myc-tagged Pam N-terminal segment F1 (Myc-PamF1) containing the RHD (amino acids 1–1669) or Myc-tagged Pam C-terminal segment F3 (Myc-PamF3) with the RZF domain (amino acids 3448–4640) (Fig. 4A) was coexpressed with the HA-S6K reporter in HEK293T cells, serum-deprived overnight, and examined for S6K activation. Both the N- and C-terminal segments of Pam activated S6K phosphorylation in a dose-dependent manner as detected by phosphory-
Regulation of mTORC1 Signaling by Pam

Based on the ability of Pam to increase \[^{35}S\]GTP\_H9253 binding to Rheb as well as the release of tritiated \[^3H\]-GDP from Rheb, it was suggested that Pam may act as a GEF for Rheb through its RHD (32). We asked whether Rheb is required for the ability of the PamF1 fragment containing the RHD to activate mTORC1 by employing RNAi-mediated knockdown of Rheb, followed by exogenous expression of PamF1 in HEK293T cells. The activation of S6K by PamF1 under serum-deprived conditions was partially blocked by Rheb RNAi, suggesting that Rheb may be partly responsible for the activation of S6K by PamF1 (Fig. 4D). A decrease in S6K activation by Rheb RNAi in insulin-stimulated cells served as a positive functional readout for Rheb RNAi in these cells (Fig. 3E). As expected, activation of S6K by PamF1 and PamF3 was blocked by rapamycin as well as by amino acid deprivation (data not shown).

Functional Complementation between Phr1Mag and Phr1\[^{Δ8,9}\]Mice with Respect to mTORC1 Signaling—To further validate that Pam regulates mTORC1 signaling in vivo through two functionally independent domains, we generated a compound heterozygous model by crossing heterozygous Phr1Mag mice (Phr1Mag\(/+\)/-H11002) with heterozygous Phr1\[^{Δ8,9}\]/H9004 mice (Phr1\[^{Δ8,9}\]/H9004/+/+H11001) (Fig. 5A). The compound heterozygous Phr1\[^{Δ8,9}/Δ8,9\]Mag mice, similar to the homozygous Phr1\[^{Mag}\] and Phr1\[^{Δ8,9}\] mutants, displayed neonatal lethality, indicating that one WT copy of Phr1 is required for survival. However, mTORC1 signaling (as reflected by phosphorylation of S6K at Thr-389 and of S6 at Ser-240/Ser-244) was not compromised in brain samples obtained from at least 11 compound heterozygous mice compared with either WT or heterozygous littermates.

Genotyping
 Regulation of mTORC1 Signaling by Pam

**FIGURE 4.** Overexpression of either N- or C-terminal fragments of Pam induces 56K phosphorylation in a dose-dependent manner. A, schematic of full-length (FL) Pam, the N-terminal fragment (amino acids [aa] 1–1669) containing RHD-1 and RHD-2 (PamF1), and the C-terminal fragment (amino acids 3448–4640) containing the RZF domain of Pam (PamF3). MBD, Myc-binding domain. B and C, overexpression of the indicated amounts (micrograms) of Myc-PamF1 and Myc-PamF3 (C) along with an HA-S6K reporter in HEK293T cells resulted in an increased level of phospho-S6K (pS6K) compared with controls (pcDNA3 vector, HA-S6K alone) under serum-deprived conditions. Overexpression of Rhabd served as a positive control. D, in serum-deprived HEK293T cells overexpressing Myc-PamF1 along with an HA-S6K reporter (third and fourth lanes), transduction of Rhabd shRNA (Rhabd-KD) resulted in inhibition of phospho-S6K (fourth lane) compared with scrambled (Scr) control shRNA (third lane). The first and second lanes served as controls. E, as expected, transduction of Rhabd shRNA in HEK293T cells resulted in decreased S6K phosphorylation upon insulin stimulation compared with scrambled control shRNA. Immunoblotting using anti-Myc and anti-HA antibodies served as a control. The results are representative of three independent experiments.

and immunoblotting data from a representative Phr1Δ8,9/Mag litter are shown in Fig. 5 (B and C). These results indicate that mTORC1 down-regulation is not responsible for lethality at birth. Importantly, in Phr1Δ8,9/Mag mice, an intact N terminus on the Phr1Δ8,9 allele interacts with the TSC1-TSC2 complex and regulates mTORC1 signaling in vivo. Homozygous Phr1Δ8,9 mutant mice exhibit CNS morphology defects, including corpus callosum defects, loss of internal capsule axon fiber tracts, and loss of the anterior commissure, among many other observed defects (27). The CNS morphology defects have not been described previously for homozygous Phr1Δ8,9 mice (26); therefore, we examined E18.5 brain sections of these mutant mice by histology and observed a number of defects, including loss of internal capsule axon fiber tracts, absence of anterior commissure, and a narrower corpus callosum (Fig. 6, A and B, and data not shown). We then raised the question of whether any of these defects could be rescued in the compound heterozygous Phr1Δ8,9/Mag mice, in which mTORC1 signaling is not compromised. Interestingly, we observed that the thinner corpus callosum seen in homozygous Phr1Δ8,9 and Phr1ΔMag mice was consistently rescued in eight of eight (four litters) compound heterozygous Phr1Δ8,9/Mag mice and was indistinguishable from the WT littermates (Fig. 6, C–E). However, the defects in axon fiber tracts of the internal capsule and anterior commissure persisted in the compound heterozygous mice (Fig. 6, F–H; and data not shown), strongly suggesting that these defects are mTORC1-independent.

**DISCUSSION**

Pam is a large multidomain protein with a putative GEF homology domain at the N terminus, a Myc-interacting domain in the middle, and a C-terminal RZF domain typical of E3 Ub ligase (9, 10). Our earlier studies showed that the C-terminal domain of Pam interacts with the TSC1-TSC2 complex and regulates mTORC1 signaling by ubiquitinating TSC2 (7, 23). In this study, we have demonstrated that both the N- and C-terminal domains of Pam independently regulate mTORC1 signaling in vitro and in vivo. Two independent mutant mouse models of Pam, Phr1Δ8,9, with an intact C terminus and Phr1ΔMag with a deletion of the C-terminal RZF domain but a stably expressed N-terminal domain, show down-regulation of mTORC1 as observed by decreased phosphorylation of downstream targets S6K and S6. However, phosphorylation of another mTOR target, 4E-BP1, was not affected, consistent with other studies in which differential regulation of mTORC1 downstream targets was reported (36, 37). In agreement with the loss of either the N or C terminus of Pam resulting in down-regulation of mTORC1, overexpression of either the N or C terminus of Pam activates mTORC1 signaling.
Regulation of mTORC1 Signaling by Pam

RHDs have been shown to function as GEFs and stimulate the nucleotide release of small GTPases such as Arf, Rab, and Ran (38). It was recently demonstrated that sphingosine-1-phosphate-induced activation of mTORC1 is mediated by Pam and that Pam may act as a GEF for Rheb (32). Our results show that Rheb may be partly responsible for activation of mTORC1 mediated by the N terminus of Pam; however, it cannot be ruled out that other yet to be defined mechanisms could also play a role in this regulation. Further studies are necessary to understand whether Pam binds to Rheb directly or indirectly as well as whether Pam acts as a bona fide Rheb GEF in vivo. The large size of the Pam protein (~500 kDa) and the inherent difficulties in reliably assaying Rheb GDP/GTP ratios make these studies quite challenging.

It is interesting that the down-regulation of mTORC1 signaling is restricted to brain in Phr1 mouse models, which may be explained by dosage-sensitive effects of Pam loss in the brain. Alternatively, we raised the possibility that HERC1, a 532-kDa E3 Ub ligase with striking structural similarity to Pam and identified as a TSC2 interactor (39), plays a similar role as Pam and compensates for the loss of Pam in tissues other than brain. Interestingly, HERC1 has a C-terminal HECT domain, a hallmark of E3 Ub ligases, as well as an RHD at the N terminus and has been shown to ubiquitinate TSC2 in vitro (39). Therefore, it is tempting to speculate that, similar to Pam, HERC1 could exhibit two distinct modes of regulation on TSC/mTORC1 signaling and that these two large structurally similar proteins could function in a tissue/context-specific manner.

Our observation that the down-regulation of mTORC1 seen in both homozygous Phr1 mutants is rescued in compound heterozygous Phr1Δ8,9/Mag mice clearly documents that the N and C termini of Pam can functionally complement each other with respect to mTORC1 regulation. However, similar to the homozygous Phr1Δ8,9 and Phr1Mag mutant mice, the compound heterozygous mutants die at birth, suggesting that mTORC1 down-regulation is not responsible for perinatal lethality and at least one intact wild-type Phr1 allele is required for survival. In addition to defects in sensory and motor neurons, Phr1 knock-out mice exhibit a severe reduction of major axon tracts in the CNS, with narrowing and reduced thickness of the corpus callosum and absence of the anterior commissure (27). We have shown that the narrower corpus callosum seen in homozygous Phr1Δ8,9 and Phr1Mag mutant mice is rescued in compound heterozygous Phr1Δ8,9/Mag mice, supporting the notion that down-regulation of mTORC1 signaling may be responsible for the corpus callosum defects in Phr1 mutant mice. However, other CNS defects persist in the double heterozygous mice, suggesting that they are not mTORC1-dependent. Our results also imply that Pam may regulate mTORC1 signaling independently of Fbxo45, which is consistent with a previous report showing no change in TSC2 protein levels in Fbxo45-deficient mouse brain (34). It is interesting that although Fbxo45 knock-out mice resemble Phr1Δ8,9 mice, with a loss of major axon tracts and the complete absence of the anterior commissure, they differ from Phr1Δ8,9 mice in that
they have no defect in the corpus callosum (34). It is likely that the Pam-Fbxo45 Ub ligase complex could target other proteins/pathways, which, when dysregulated, might result in neural development defects shared between the Phr1\^{+/+} and Fbxo45- deficient mice.

mTORC1-mediated signaling plays an important role in neurodevelopment by modulating both general and neuronal activity-dependent protein synthesis. More importantly, long-lasting forms of synaptic plasticity and memory are dependent on new protein synthesis, and aberrant mTORC1 signaling is reported in many neurological disorders and has emerged as a common biochemical characteristic of many syndromes associated with autism spectrum disorders (6, 40, 41). Recent evidence indicates that mutations in genes such as NLGN3/4, SHANK3, and NRXN1 are associated with altered synaptic function and lead to autism/Asperger syndrome, and the
NLGN/SHANK3/NRXN pathway is also linked to synaptogenesis (4, 42). Pam and its homologs function as regulators of synapse formation and growth, and work from our laboratory has shown Pam to be a TSC/mTORC1 pathway regulator in neurons. Therefore, it is tempting to speculate whether an NLGN/SHANK3/NRXN pathway is also linked to synaptogenesis.

FIGURE 7. Reduced Fbxo45 levels in both Phr1 mutant mouse models. A and B, whole brain lysates and RNA were prepared from E17.5–18.5 littermates and subjected to Western blot and real-time quantitative PCR (qPCR) analyses. Immunoblotting of at least three WT and mutant animal pairs showed diminished levels of Fbxo45 in both Phr1Mag (−/−) (A) and Phr1Δ519 (−/+ −) mice (B) compared with the respective WT (+/+) mice. Representative immunoblots are shown (left panels). Actin and GAPDH served as loading controls. For quantitative PCR analyses of both Phr1 mouse models, expression of Fbxo45 mRNA in embryonic mouse brains from at least three WT and mutant animal pairs is shown (right panels). GAPDH was used for normalization, n.s., not significant. C, immunoblotting of compound heterozygous Phr1Δ519/Δmag mouse brain also demonstrated a decreased level of Fbxo45 compared with WT (+/+) littermates. Experiments represented in A–C were carried out with two independent litters. D, overexpression of HA-Fbxo45 along with an HA-S6K reporter in HEK293T cells did not result in increased phosphorylation of S6K under serum-deprived conditions. Myc-Rheb and the pcDNA3 vector alone served as positive and negative controls, respectively.

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