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Depdc5 knockout rat: A novel model of mTORopathy

Elise Marsan a,b,c,d,1, Saeko Ishida a,b,c,d,1, Adrien Schramm a,b,c,d, Sarah Weckhuysen a,b,c,d, Giuseppe Muraca b,c,d, Sarah Leca b,c,d, Ning Liang e,f,g, Caroline Treins e,f,g, Mario Pende e,f,g, Delphine Rousset a,b,c,d, Michel Le Van Quyen a,b,c,d, Tomoji Mashimo h, Takehito Kaneko i, Takashi Yamamoto j, Tetsushi Sakuma j, Séverine Mahon a,b,c,d, Richard Miles a,b,c,d, Eric Leguern a,b,c,d,k, Stéphane Baulac a,b,c,d, Stéphanie Baulac a,b,c,d,k

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ABSTRACT

DEP-domain containing 5 (DEPDC5), encoding a repressor of the mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway, has recently emerged as a major gene mutated in familial focal epilepsies and focal cortical dysplasia. Here we established a global knockout rat using TALEN technology to investigate in vivo the impact of Depdc5-deficiency. Homozygous Depdc5−/− embryos died from embryonic day 14.5 due to a global growth delay. Constitutive mTORC1 hyperactivation was evidenced in the brains and in cultured fibroblasts of Depdc5−/− embryos, as reflected by enhanced phosphorylation of its downstream effectors S6K1 and rpS6. Consistently, prenatal treatment with mTORC1 inhibitor rapamycin rescued the phenotype of Depdc5−/− embryos. Heterozygous Depdc5+/− rats developed normally and exhibited no spontaneous electroclinical seizures, but had altered cortical neuron excitability and firing patterns. Depdc5+/− rats displayed cortical cytomegalic dysmorphic neurons and balloon-like cells strongly expressing phosphorylated rpS6, indicative of mTORC1 upregulation, and not observed after prenatal rapamycin treatment. These neuropathological abnormalities are reminiscent of the hallmark brain pathology of human focal cortical dysplasia. Altogether, Depdc5 knockout rats exhibit multiple features of rodent models of mTORopathies, and thus, stand as a relevant model to study their underlying pathogenic mechanisms.

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1. Introduction

DEP-domain containing 5 (DEPDC5) is the most common known cause of familial focal epilepsies (nearly 70 unrelated cases described so far). Germline heterozygous inactivating DEPDC5 mutations are reported in a broad spectrum of focal epileptic syndromes including familial focal epilepsy with variable foci (FFVE; OMIM 604364), autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE; OMIM 600513), familial temporal lobe epilepsy (FTLE; OMIM 600512) and rare Rolandic epilepsies (OMIM 245570) (Dibbens et al., 2013; Ishida et al., 2013; Lal et al., 2014; Martin et al., 2014; Picard et al., 2014). Patients present focal seizures with variable age at onset, typically during childhood. Brain MRI is usually normal, although within families, some individuals may have a focal cortical dysplasia (FCD; OMIM 607341) (Baulac et al., 2015; Scerrri et al., 2015; Scheffer et al., 2014), which is the most common cause of severe, drug-resistant childhood epilepsy. FCD is a malformation of cortical development characterized by cortical dyslamination and the presence of dysmorphic neurons (FCD type Ila) and balloon cells (FCD type IIb) (Blumcke et al., 2011). Recently,
germline DEPDC5 mutations have also been reported in sporadic patients with FCD or hemimegalencephaly (D’Gama et al., 2015).

DEPDC5 does not encode a membrane ion channel, unlike most other epilepsy genes. Instead, DEPDC5 acts in vitro as a GTPase-activating protein for RagA/B, and forms part, together with NPR2 and NPR3, of the GATOR1 complex to inhibit the mechanistic target of rapamycin complex 1 (mTORC1) pathway (Bar-Peled et al., 2013; Panchaud et al., 2013). Recently, mutations in NPR2 and NPR3 have also been reported in familial focal epilepsies associated with FCD (Ricos et al., 2016; Sim et al., 2016). The mTORC1 pathway is a major and ubiquitous signaling cascade that regulates multiple cellular processes including cell growth, proliferation and protein synthesis (Laplante and Sabatini, 2012). It is dysregulated in several neurological disorders associated with neurodevelopmental cortical malformations and intractable seizures (Lim and Crino, 2013), including FCD, hemimegalencephaly and tuberous sclerosis complex (TSC; OMIM 191100 and 613254) due to mutations in TSC1 and TSC2 genes, encoding inhibitors of the mTORC1 pathway. Numerous rodent models of TSC have been engineered and recapitulate part of the neuropathological features of the disease (Bateup et al., 2013; Meikle et al., 2007). Knockout mice of Depdc5 partners, Npr2 and Npr3 have also been recently generated, but have not been explored at the neurological level (Dutchak et al., 2015; Kowalczyk et al., 2012).

To date, the in vivo function of Depdc5 in mammals and its role in epilepsy and cortical malformations remain unknown. Here, we generated a global knockout rat of Depdc5 to model its loss-of-function and investigate the resulting consequences in the brain.

2. Materials and methods

2.1. Animals

Depdc5<sup>mkl<sup>+/+</sup></sup> and Depdc5<sup>mkl<sup>-/-</sup></sup> rats were generated and deposited at the National Bio Resource Project Rat in Japan (www.anim.med.kyoto-u.ac.jp/nbr). Animal care and experimental procedures were approved by the French ministry of research (authorization number 75-1319, project number 03539.02). All efforts were made to minimize the number of animals needed and their suffering.

2.2. TALEN-mediated genome editing in rats

A pair of TALENs targeting exon 2 of rat Depdc5 (Ensembl: ENSRNOT00000085788) was designed and constructed using a two-step assembly method with a Platinum Gateway kit previously reported (Sakuma et al., 2013). The target sites were located immediately downstream of the ATG start codon. TAL Effector Nucleotide Targeter 2.0 (https://tale-nt.cac.cornell.edu/) did not predict off-target site. We selected the Fisher 344 (F344) rat strain since we previously proved that it is a pertinent model for genetically-determined epilepsies, as exemplified by the Kenai (Ishida et al., 2012), Scnta (Mashimo et al., 2010) or Lgl1 (Baulac et al., 2012) mutant rats. TALENs were microinjected into fertilized eggs of F344, and then transferred into the oviducts of pseudopregnant Wistar female rats, as previously described (Mashimo et al., 2013). Genomic DNA of founder rats was extracted from tail biopsies. TALEN target site was amplified using the following primers: forward 5′-AGCAGACATCTCGCTTGT-3′ and reverse 5′-TCTGGCCCA CTCAATTGAC-3′. Successful germline transmission was confirmed by DNA sequencing of first backcross generation rats. These rats were backcrossed five times with wildtype F344 rats. Genotypes were assessed by the analysis of the PCR products from tail DNA with a high-resolution electrophoresis caliper system. A 267 bp band was detected for wildtype, a 279 bp band for Depdc5<sup>mkl<sup>+/+</sup></sup> mutant and a 251 bp band for Depdc5<sup>mkl<sup>-/-</sup></sup> mutant.

2.3. Assessment of embryo vitality

Pregnant rats were injected with lethal dose of pentobarbital sodium (50 mg/kg, i.p.). Embryos were collected by cesarean-section and kept on a warming pad to maintain their body temperature at 37 °C. To assess vitality of embryos aged E12.5 to E16.5, heartbeat was directly observed under a binocular microscope. For E21.5 embryos, cardiac activity was assessed by electromyogram (Neurosoft).

2.4. Rapamycin injection

Rapamycin (LC laboratories) was dissolved at 20 mg/ml in 100% ethanol and stored at −20 °C. Before use, it was diluted to 1 mg/ml in 0.25% Tween 80 and 0.25% polyethylene glycol 300 in PBS. A single dose of 1 mg/kg rapamycin was intraperitoneally injected to pregnant Depdc5<sup>-/-</sup> dams at E13.5 as previously reported (Ma et al., 2014).

2.5. Rat embryonic fibroblast culture

Rat embryonic fibroblasts (REFs) were collected at E13.5 from Depdc5<sup>-/-</sup> interbreeding and cultured in standard medium: Dulbecco’s Modified Eagle’s Medium (DMEM) (Life Technologies) containing 10% fetal bovine serum and 1% penicillin/streptomycin. Rapamycin (20 nM dissolved in 100% ethanol) was added to Earle’s Balanced Salt Solution (EBSS) during the whole starvation period until the harvesting of REFs.

2.6. Cytofluorometric rat embryonic fibroblast size analysis

1 × 10⁶ REFs (passage 6 to 8) were seeded in 100 mm diameter dishes, allowed to proliferate overnight, and then cultured in DMEM without serum for 48 h and starved in EBSS for 3 h. REFs were harvested by gentle trypsinization, washed twice in PBS and fixed in 80% ethanol in PBS. After overnight fixation at −20 °C, fixed cells were washed in PBS, resuspended in DNA staining solution containing 0.20 μg/ml propidium iodide, 100 μg/ml RNaseA and 20 mM EDTA in PBS, and incubated for 30 min at 37 °C. REF soma sizes were analyzed by flow cytometry: the mean cytofluorometric forward scatter (FSC) of minimum 800 stained cells was determined by gating on propidium iodide fluorescence using BD Biosciences FACSCalibur with CellQuest software as previously described (Acosta-Jaquez et al., 2009).

2.7. Western blots

Whole heads of E12.5 embryos were flash-frozen in liquid nitrogen, homogenized with lysing matrix D (MP Biomedicals) in cell lysis buffer (Cell Signaling) containing phosphatase inhibitors and protease inhibitors (Roche). REFs (passage 5) were nutrient-starved with EBSS for 7 h, washed with PBS and then lysed in the same lysis buffer. Similar amount of protein was loaded in each lane in 3–8% Tris–acetate gel for Depdc5 and 10% Bis-Tris–acetate gel (NuPAGE) for phosphorylated S6K1 and rpS6. Western blots were done using the following primary antibodies: anti-DEPDC5 (Cell Signaling, #H6302, 1/100), anti-actin (Sigma Aldrich, #A2066, 1/1000), anti-phosphorylated S6K1 (Thr389) (Cell Signaling, #9205, 1/250) and anti-phosphorylated rpS6 (Ser240/244) (Cell Signaling, #5364, 1/4000). Signal quantification was performed using MultiGauge software (Fuji Film).

2.8. Histology and immunohistochemistry

Whole embryos (E15) were rapidly removed from their chorion and fixed overnight in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Postnatal day (P) P11 and 4 week old rats were injected with a lethal dose of pentobarbital sodium (50 mg/kg, i.p.) and perfused with...
As previously described (Mahon and Charpier, 2012; Paz et al., 2009), software (Hamamatsu Photonics) to analyze digital slides mined from Nissl-stained brain sections using NDP.view2 viewer and hematoxylin were collected. To visualize whole embryo gross anatomy and adult brain cortical architecture, sections were stained with Nissl-staining and hematoxylin–eosin using standard techniques. Cell size was determined from Nissl-stained brain sections using NDP.view2 viewer software (Hamamatsu photonics) to analyze digital slide files. After manual drawing of cell margins, we measured the area from at least 50 large layer IV–V cells. The largest 30 cells per animal were included in the measurements for statistical comparison.

Immunostaining experiments were performed using standard procedure with primary anti-phosphorylated rpS6 Ser240/244 antibody (Cell Signaling, #5364, 1/200) and secondary fluorescent anti-rabbit Alexa 488 antibody (Invitrogen, 1/1000). Nuclei were stained with DAPI. Images were acquired and analyzed through the Nanozoomer software (Hamamatsu photonics). TUNEL assays were performed using In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s instructions and images were acquired using a Zeiss apotome.

2.9. In vivo intracellular electrophysiology

Adult rats were anesthetized with 40 mg/kg sodium pentobarbital and 50 mg/kg ketamine (i.p.). Incision and pressure points were repeatedly infiltrated with 2% lidocaine. In line with behavioral EEG experiments (Supplementary data), a small craniotomy was made above the parietal association cortex, allowing combined intracellular and electrocorticographic (ECoG) recordings. The rats were subsequently maintained in a narcotized and sedated state, monitored with the ECoG and heartbeat rate, by injections of fentanyl (3 μg/kg, i.p.) repeated every 20–30 min as previously described (Polack et al., 2009). Rats were paralyzed with gallamine triehydride (40 mg/2 h, i.m.) and artificially ventilated. Body temperature was maintained at 37 °C with a homeothermic blanket.

ECoG signals were amplified by a differential AC amplifier (Model 1700; A-M Systems), band pass filtered at 1 Hz–1 kHz, and digitized at 3 kHz (Power1401; Cambridge Electronic Design). Intracellular glass micropipettes were filled with 2 M potassium acetate (60–80 MQ). Current-clamp recordings were amplified using an Axoclamp 900A amplifier (Molecular Devices) in bridge mode, filtered at 6 kHz, and digitized at 20 kHz. Recordings were obtained from pyramidal neurons located at depths ranging from 415 to 1525 μm below the cortical surface (Paxinos, 2005). All data were acquired and analyzed using Spike2 software (Spike2 version 7.16; Cambridge Electronic Design).

Neurons were labeled with neurobiotin (Vector Laboratories) added to the intrapipette solution (1%). At the end of experiments, rats were deeply anesthetized with pentobarbital (200 mg/kg, i.p.) and transcardially perfused with 0.3% glutaraldehyde–4% PFA in PB. The morphology of stained neurons was revealed histochemically as described previously (Polack and Charpie, 2006).

To generate firing frequency versus injected current (F–I) relationships, the firing rate was measured in response to 500 ms depolarizing current pulses of increasing intensity. Each current intensity was applied 3–10 times and the corresponding firing responses were averaged. As previously described (Mahon and Charpie, 2012; Paz et al., 2009), we applied linear regressions to F–I curves and determined the threshold current for action potential (AP) generation, extrapolated as the x-intercept of the linear fit, and the neuronal gain, defined as the slope of the F–I curve. The spontaneous firing rate and average membrane potential was calculated from continuous recordings of at least 40 s. Membrane input resistance was calculated as the slope of the linear portion of the voltage–current relationships (500 ms hyperpolarizing pulses of increasing intensity) and the membrane time constant was derived from an exponential decay fit applied to the initial part of the current-induced hyperpolarizations.

Neuronal physiological classes were established as in previous studies (Degeliet al., 2002; Nunez et al., 1993). Intrinsic bursting cells were characterized either by an initial high-frequency (typically >200 Hz) burst of APs followed by a relatively tonic firing or by consecutive bursts of APs. Regular spiking slow adapting neurons exhibited a sustained discharge of APs with a low frequency adaptation along the current step, while regular spiking fast adapting cells responded to high intensity (typically >0.6 nA) current step by an initial sequence of 3–8 APs restricted to the first 50–100 ms of the response followed by a depolarizing plateau with few or no APs.

2.10. Statistical analysis

Statistical analyses were done with GraphPad and SigmaStat software. The results are presented as mean ± SEM. Normality was assessed by Kolmogorov–Smirnov normality test. Comparisons between two groups were performed by unpaired two-tailed Student’s t-test for parametric data and by Mann–Whitney test for non-parametric data. Comparisons between multiple groups were performed by 1-way ANOVA with Tukey’s multiple comparison test as post-hoc analysis for parametric data and by a Kruskal–Wallis test for non-parametric data.

3. Results

3.1. Generation of a global Depdc5 knockout rat

DEPDC5-related epilepsies are likely to result from a loss-of-function since the majority of mutations leads to a premature stop codon, targeted by nonsense-mediated mRNA decay (Ishida et al., 2013; Picard et al., 2014). We used transcription activator-like effector nuclease (TALEN)–mediated genome editing to generate a global Depdc5 knockout rat and to investigate the effects of Depdc5-deficiency in vivo. TALEN mRNAs targeting exon 2 of rat Depdc5 were microinjected into fertilized eggs of Fisher 344 (F344) rats, to yield two founder mutant rats named Depdc5em1kyo (c.40_44delins17/p.Gly15*) and Depdc5em2kyo (c.39_55delinsT/p.Lys13fs*8) (Fig. 1A). Successful gene targeting was confirmed by Western blot analysis of whole head lysates from living embryos, with an anti-Depdc5 antibody. Depdc5 protein was absent in homozygous Depdc5em1kyo and Depdc5em2kyo rats, and expression was reduced in heterozygous littermates (Fig. 1B). The homozygous knockout Depdc5em1kyo and Depdc5em2kyo rat strains were therefore indifferentially referred to as Depdc5+/−, and the heterozygous as Depdc5+/−.

3.2. Embryonic lethality and growth delay in Depdc5+/− rats

While Depdc5+/− rats interbred, Depdc5−/− pups were never observed at birth. Deletion of both alleles resulted in in utero lethality, as reported in knockout rodents of other genes of the mTORC1 pathway such as Tsc1 (Kobayashi et al., 2001) and Tsc2 (Onda et al., 1999; Rennebeck et al., 1998). We assessed the timing of the embryonic lethality by collecting embryos from Depdc5−/− interbred rats at gestation dates between E12.5 and E21.5 (Table 1). All Depdc5−/−/− embryos were alive until E13.5. However, at E14.5, E15.5 and E16.5, only 50% (7/14), 40% (2/5) and 21% (3/14) of living Depdc5−/− embryos were observed. Depdc5−/− embryos at E13.5–E16.5 were smaller than Depdc5+/− and Depdc5+/+ littermates (Fig. 1C). With less developed limbs and optic vesicles, we conclude that global growth delay precedes embryonic lethality observed from E14.5.

Measurements of whole-mount sections from living E14.5 embryos confirmed that Depdc5−/− embryos are smaller (n = 7; 6.9 ± 0.2 mm) than Depdc5+/− embryos (n = 7; 7.9 ± 0.2 mm) (P < 0.01; Mann–Whitney test) (Fig. 1D). TUNEL assays revealed the presence of apoptotic cells in the liver of E14.5 Depdc5−/− embryos (Fig. 1E),
suggesting important impairment of this organ, which may be the cause of premature death. This result reveals that Depdc5 plays an important role in liver development, and recalls the liver defects observed in knockout mice of Depdc5 partners, Nprl2 and Nprl3 (Dutchak et al., 2015; Kowalczyk et al., 2012) acting in the GATOR1 complex. The cerebral neocortical structures of embryonic Depdc5−/− rats were also

Fig. 1. Depdc5−/− rats die embryonically and present growth delay. (A) DNA sequences of wild-type and TALEN-mutated Depdc5 sequences. TALE: transcription activator-like effector, FokI: restriction endonuclease FokI. TALE binding sequences are shown in blue. Deleted bases are framed, and inserted bases are indicated by the red letters. Depdc5em1kyo strain carries a deletion of 5 bp and an insertion of 17 bp, and Depdc5em2kyo a deletion of 17 bp and an insertion of 1 bp at the targeted site. (B) Western blots of head lysates (60 μg of protein each) from living embryonic day 12.5 (E12.5) embryos derived from Depdc5em1kyo and Depdc5em2kyo strains with Depdc5 and actin antibodies. The Depdc5 specific band is indicated by an arrow, the upper band is nonspecific. (C) Representative picture of embryos from three litters at E13.5, E14.5 and E16.5. Picture of E16.5 embryo is composed of two images. Scale bar: 2 mm. (D) Representative Nissl-stained whole body sagittal sections of living E14.5 (Depdc5+/+, n = 7; Depdc5−/−, n = 7) embryos. Scale bar: 1 mm. (E) Representative images of TUNEL staining on fetal liver from living E14.5 (Depdc5+/+, n = 3; Depdc5−/−, n = 3) embryos. Scale bar: 100 μm. (F) Higher magnification view of brain regions on Nissl-stained sections. From left to right panel: neocortex, lateral ventricle and ganglionic eminence. Histogram indicates quantification of neocortical thickness (indicated by black arrows), lateral ventricular and ganglionic eminence surfaces (dashed-lines). Scale bar: 50 μm, 500 μm and 250 μm respectively. Number of embryos is indicated in brackets. Error bars represent mean ± SEM. n.s., not significant; *P < 0.05; **P < 0.01 (Mann–Whitney test).
affected, in line with the role of DEPDC5 in malformations of the cortical development in humans. Analysis of Nissl-stained whole-embryo sections revealed the neocortex was thinner and the surfaces of both lateral ventricles and ganglionic eminences were smaller in Depdc5<sup>−/−</sup> embryos than in their Depdc5<sup>+/+</sup> littermates (Fig. 1F).

### 3.3. Prenatal rapamycin rescues growth delay and embryonic lethality in Depdc5<sup>−/−</sup> rats

Previous in vitro studies have reported that Depdc5 acts as a repressor of the mTORC1 signaling pathway (Fig. 2A) (Bar-Peled et al., 2013; Panchaud et al., 2013). If mTORC1 pathway were involved in the embryonic lethality of Depdc5<sup>−/−</sup> rats, prenatal administration of the mTORC1 inhibitor rapamycin might rescue their phenotype. We injected a single

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E, embryonic day; P, postnatal day; numbers in parentheses refer to percentage of living Depdc5<sup>−/−</sup> embryos with heartbeat detected.

Fig. 2. mTORC1 pathway is upregulated in Depdc5<sup>−/−</sup> embryos. (A) Schema of the Depdc5-mTORC1 signaling pathway. (B) Top panel: Representative picture of living E21.5 embryos treated with rapamycin (Depdc5<sup>+/+</sup>, n = 14; Depdc5<sup>+/−</sup>, n = 23; Depdc5<sup>−/−</sup>, n = 4). Scale bar: 1 cm. Bottom panel: Histogram showing the percentage of living Depdc5<sup>−/−</sup> embryos treated or not with prenatal rapamycin. (C) Top panel: Western blot of brain lysates (30 μg of protein) of E12.5 littermate embryos with phosphorylated rpS6 on Ser240/244 (pS6) and actin antibodies. Bottom panel: Histogram of densitometry analysis of pS6 normalized with actin from two independent Western blots. (D) Top panel: Western blot of REF lysates (15 μg of protein) derived from littermate embryos with phosphorylated S6K1 on Thr389 (pS6K1), phosphorylated rpS6 on Ser240/244 (pS6) and actin antibodies after amino acid starvation, with or without rapamycin. Bottom panel: histogram of densitometry analysis of pS6K1 and pS6 normalized with actin from above Western blot. (E) REF soma sizes, expressed as mean cytofluorometric forward scatter (FSC), in standard or amino acid starvation conditions from two independent cytofluorometric REF sizes analysis. Number of embryos is indicated in brackets. Error bars represent mean ± SEM. a.u., arbitrary units; n.s., not significant; *P < 0.05; **P < 0.01; ***P < 0.001 (1-way ANOVA and Tukey’s multiple comparison post-hoc tests).
The proportion of living Depdc5−/+ embryos increased from 50% to 83% at E14.5 with this treatment. At the projected birth date E21.5, 31% (4/13) of living Depdc5−/+ rats were obtained after prenatal rapamycin treatment (Fig. 2B, bottom). The body length of E21.5 rescued Depdc5−/+ embryos (n = 4; 3.8 ± 0.2 cm) was similar to that of Depdc5+/+ (n = 10; 3.7 ± 0.3 cm) and Depdc5−/− (n = 11; 3.9 ± 0.3 cm) littermate embryos (P > 0.5; Kruskal–Wallis test), indicating that rapamycin also reversed growth delay. While rapamycin enhanced embryonic survival of Depdc5−/+ rats, they showed global edema, and died shortly after birth from impaired maternal–pup interaction. Rapamycin treatment earlier or later than E13.5 did not improve Depdc5−/+ embryo survival (data not shown). The appearance and body length of treated Depdc5+/+ and Depdc5−/+ rats were similar to those of untreated littermates, indicating that rapamycin had no major adverse effects on control rats (Fig. 2B, top). This results provided evidence that mTORC1 pathway is involved in growth delay and embryonic lethality in Depdc5−/− embryos.

To further validate an mTORC1 activity-dependent mechanism, we assessed at the molecular level whether mTORC1 is upregulated in the brains of Depdc5−/− embryos. Thus we monitored by Western blot mTORC1 activation through the phosphorylation state of one of its downstream effector, the ribosomal protein S6 (rpS6, on Ser240/244). rpS6 phosphorylation level was significantly enhanced in brain lysates of E12.5 Depdc5−/+ embryos compared to Depdc5+/− and Depdc5−/− littersates, and tended to be higher in Depdc5+/− than in Depdc5−/+ embryos (Fig. 2C). This indicates constitutive activation of mTORC1 in the brains of Depdc5−/− deficient embryos with a gene dosage effect such that levels of phosphorylated rpS6 are higher when both copies of Depdc5 are deleted.

3.4 Upregulation of mTORC1 in Depdc5−/− cultured fibroblasts after amino acid starvation

mTORC1 pathway is sensitive to amino acid starvation. Since in vitro data from mammalian cell lines suggests this sensitivity is abolished by Depdc5 knockdown (Bar-Peled et al., 2013), we examined the effect of Depdc5-deficiency on primary rat embryonic fibroblasts (REFs). REFs derived from Depdc5−/− interbreeding let us compare mTORC1 activity in standard or amino acid-deprived culture media. mTORC1 activity was monitored by the phosphorylation of two downstream effectors: p70S6 kinase 1 (S6K1, on Thr389) and the S6K1 substrate rpS6 (on Ser240/Ser244) as previously reported (Dutchak et al., 2015; Efeyan et al., 2014; Liang et al., 2014). In standard medium, Western blots of REF lysates indicated that phosphorylation levels of S6K1 and rpS6 were similar across genotypes (data not shown). However, after amino acid starvation, phosphorylation states of both S6K1 and rpS6 were consistently increased in REF lysates derived from Depdc5−/− embryos compared to those from Depdc5+/+ and Depdc5−/+ embryos (Fig. 2D). Rapamycin treatment of REFs derived from Depdc5−/− embryos abolished S6K1 and rpS6 phosphorylation, confirming that Depdc5 negatively regulates mTORC1 activity. We also compared mTORC1 activity in REFS derived from Depdc5−/− embryos to REFS derived from Depdc5+/+ embryos to assess the effect of the lack of one copy of Depdc5 on the pathway activity. Semi-quantification of Western blot suggested a tendency to increased phosphorylation states of S6K1 and rpS6 in REFS derived from Depdc5−/− compared to Depdc5+/+, but did not reach significant levels (Fig. 2D). This result is in line with the data obtained above in brain lysates (Fig. 2C), showing robust mTORC1 upregulation in Depdc5−/− embryos, and more subtle effects in Depdc5+/− embryos. A similar upregulation was obtained in REFS derived from a knockout mouse for Nprl2 (Dutchak et al., 2015), one of the two Depdc5 partners in the GATOR1 complex.

Acting via S6K1 and rpS6, mTORC1 is a pivotal regulator of cell size. We therefore assessed the impact of an mTORC1 upregulation on the soma size of REFS derived from Depdc5−/+ and Depdc5−/− embryos, in standard or amino acid starvation condition using flow cytometry analysis. In standard medium, REFS soma sizes did not differ significantly between genotypes. After amino acid starvation, both Depdc5−/+ and Depdc5−/− derived REFS were reduced in size while Depdc5+/− derived REFS remained unchanged (Fig. 2E). These data indicate that Depdc5 is necessary to control the cell growth by repressing mTORC1 pathway during amino acid starvation.

3.5 Brain cortical structural abnormalities in Depdc5−/+ rats

Heterozygous Depdc5+/− rats displayed no differences in gross anatomy, fertility and rates of weight gain compared to control littersmates (Supplementary data, Fig. 1A). Some inactivating deletions in DEPDC5 may be associated with ovarian cancers and glioblastomas (Cancer Genome Atlas: http://cancergenome.nih.gov/). Besides, TSC patients develop tumors associated with an increased mTORC1 activity (Onda et al., 1999). We therefore asked whether Depdc5-deficiency promotes tumor development. No evidence for tumors was apparent in Depdc5+/− rats whole body at ages greater than 12 months (n = 8). Furthermore, we detected no differences between Depdc5+/− and Depdc5−/+ littermate rats survival (up to 18 months).

We next assessed possible defects in brain cortical architecture of Depdc5−/+ rats, since heterozygous DEPDC5 mutations may cause cortical malformation such as FCD type II in humans (Baulac et al., 2015; D’Cama et al., 2015; Scerri et al., 2015) with an increased cortical thickness, blurred gray/white matter demarcation and cortical dyslamination as well as dysmorphic neurons in FCD type Ila, and balloon cells in FCD type Iib (Blümcke et al., 2011). We found that the brain-to-body weight ratios of Depdc5+/− and Depdc5−/+ rats were similar (Supplementary data, Fig. 1B). Nissl-staining of coronal brain sections of Depdc5+/− rats revealed no major defects in the structural brain organization at 4 weeks (Fig. 3A) and 23 weeks (data not shown). Cerebral cortical thickness did not differ between Depdc5+/− (n = 6, 1.22 ± 0.01 mm) and Depdc5−/+ (n = 6, 1.21 ± 0.01 mm) rats (P > 0.05; Mann–Whitney test), neither in gray/white matter boundaries (Fig. 3A). In contrast, the delimitations between the six cortical layers were less distinct in Depdc5+/− rats, particularly between layers I–II (ectopic neurons were observed in layer I) and V–VI (Fig. 3B). These aberrantly positioned cells suggest that neuronal migration was perturbed because of Depdc5 haploinsufficiency. Furthermore, Nissl and hematoxylin & eosin staining revealed the presence of enlarged cells throughout the cortex of Depdc5−/+ rats, especially in layers IV–V (approximately 15 cells/mm²). These cells consisted of both balloon-like cells resembling those seen in FCD type Ib, with no Nissl substance and an eosinophilic cytoplasm (Fig. 3C, top), and of cytomegalic dysmorphic neurons, with an aggregated Nissl substance displaced towards the cell membrane, as seen in FCD type Ila and Ib (Fig. 3C, middle). Balloon-like cells and cytomegalic dysmorphic neurons were never observed in Depdc5+/− littersmates. To confirm a difference in neuronal cell size, we measured the soma size of pyramidal neurons in cortical layers IV–V from Nissl-stained sections. The soma size of the cytomegalic dysmorphic neurons present in cortical layers IV–V of Depdc5+/− rats were significantly greater than the largest pyramidal cell in Depdc5−/+ rats from same layers, with an average area size increase of ~30% (Fig. 3C, bottom).

3.6 Effects of rapamycin treatment on cytomegalic dysmorphic neurons

Are these cytomegalic dysmorphic neurons linked to mTORC1 upregulation? We pursued this question by immunostaining for phosphorylated rpS6 (on Ser240/244) in brain sections from 4-week-old Depdc5−/+ and Depdc5−/− rats. A basal level of rpS6 phosphorylation was detected in normal-appearing (non-cytomegalic) pyramidal neurons from Depdc5+/+ and Depdc5−/+ rats (Fig. 3D, left). In contrast, phosphorylated rpS6 staining of the majority of cytomegalic
dysmorphic neurons in layers IV–V was more intense than nearby normal-appearing cells (Fig. 3D, right). Altogether these results suggest that increased cell size is caused by hyperactivation of the mTORC1 pathway.

To evaluate if rapamycin could prevent the emergence of cytomegalic and dysmorphic neurons, pregnant *Depdc5*<sup>+/−</sup> dams were treated with rapamycin as described above. We focused the analysis on littermate rats aged P11. At P11, cytomegalic dysmorphic neurons were present in *Depdc5*<sup>+/−</sup> (Fig. 3E, left), but not in *Depdc5*<sup>+/+</sup> littermates. Standard histological analysis of cortical sections from the rapamycin-treated *Depdc5*<sup>+/−</sup> rats showed rapamycin rescued cortical abnormalities (Fig. 3E, middle). Quantitative measurements revealed a significant reduction in cell size and Nissl aggregates after rapamycin treatment in *Depdc5*<sup>+/−</sup> neurons (reduced from ~30% when compared with untreated *Depdc5*<sup>+/−</sup> neurons) (Fig. 3E, right). Thus, *Depdc5* haploinsufficiency results in enhanced cell size and dysmophy of neurons that are preventable by rapamycin treatment, demonstrating an mTORC1-dependent mechanism.

3.7. Changes in pyramidal cell electrophysiology in *Depdc5*<sup>+/−</sup> rats

We searched for possible spontaneous epileptic behavior in *Depdc5*<sup>+/−</sup> rats, since heterozygous *DEPDC5* mutations cause seizures in Human. A video-based approach revealed no clonic or convulsive seizure-like behavior in young (P10 to P28), juvenile (5 to 11 week-old) or adult (12 to 20 week-old) rats (*Depdc5*<sup>+/−</sup>, *n* = 18; *Depdc5*<sup>+/+</sup>, *n* = 10). We did not detect neither seizure-like events nor interictal activity by electroencephalogram in young, juvenile and adult rats (*Depdc5*<sup>+/−</sup>, *n* = 12; *Depdc5*<sup>+/+</sup>, *n* = 9) (Supplementary data, Fig. 2).

In line with this result, we note that all studies on heterozygous global knockout rodents of *Tsc1* and *Tsc2* also reported the absence of spontaneous seizures (Lasarge and Danzer, 2014), to the exception of one global *Tsc1*<sup>+/−</sup> mouse model that displays ictal events during a short postnatal window (~P19) (Lozovaya et al., 2014).

We asked whether *Depdc5*-deficiency could alter the intrinsic properties and excitability of single neurons using in vivo simultaneous electrocorticographic (ECoG) and intracellular recordings of cortical
neurons ($n = 15$ cells from $9$ Depdc5$^{+/+}$ rats, and $13$ cells from $8$ Depdc5$^{−/−}$ rats, with typical pyramidal morphology). Spontaneously, neurons from Depdc5$^{−/−}$ rats were significantly more hyperpolarized and had a lower firing rate ($9$ out of $13$ neurons were silent) than Depdc5$^{+/+}$ rat’s neurons (Fig. 4A and B). This discrepancy was not associated with changes in passive properties since the membrane input resistance and time constant were similar between Depdc5$^{−/−}$ and Depdc5$^{+/+}$ neurons (Supplementary data, Fig. 3). Furthermore, action potential parameters, including voltage threshold, amplitude, maximum rate-of-rise and total width, were not significantly different ($P > 0.05$ for each parameter, Student’s t-test and Mann–Whitney tests) between genotypes.

We next investigated the firing patterns of Depdc5$^{+/+}$ neurons in response to current pulse injections. We recorded three classes of pyramidal neurons with distinct firing patterns: intrinsic bursting (IB), regular spiking slow adapting (RS-SA) and regular spiking fast adapting (RS-FA) (Fig. 4C, left). Pyramidal cells of Depdc5$^{+/+}$ rats displayed a typical ratio for these different firing profiles: IB $13\%$, RS-SA $66\%$ and RS-FA $20\%$ (Degenetais et al., 2002; Nunez et al., 1993). In contrast, the relative proportion of RS-FA neurons was considerably higher in Depdc5$^{−/−}$ rats: IB $15\%$, RS-SA $23\%$ and RS-FA $61\%$ (Fig. 4C, right). These divergences were in line with differences in transfer functions, measured as the mean firing frequency induced by depolarizing current pulses of increasing intensity. The minimal current to induce firing in RS-FA neuron was much higher and the slope of the transfer function was lower than that for RS-SA neurons (Fig. 4D, left). With increased numbers of RS-FA neurons, Depdc5$^{−/−}$ neurons were on average significantly less responsive to excitatory inputs (Fig. 4D, right). We note however that cytomegalic neurons observed in Depdc5$^{−/−}$ rat brains, which were not recorded here, may have pro-epileptic properties, as suggested in humans (Abdijaddid et al., 2015). Thus, Depdc5-deficiency impacts both the overall intrinsic properties and excitability of cortical pyramidal neurons.

4. Discussion

Humans that carry heterozygous loss-of-function mutations of DEPDC5 exhibit focal epileptic syndromes with or without developmental brain cortical malformations. We introduced loss-of-function Depdc5 mutations in F344 rats to mimic human haploinsufficiency. This novel knockout animal model shows Depdc5 is required for global growth and embryonic viability. Heterozygous deletion of Depdc5 in rat leads to brain cortical abnormalities with cytomegalic and dysmorphic phosphorylated rpS6-positive neurons due to upregulation of the mTORC1 pathway.

In vitro, Depdc5 represses the activity of the amino-acid sensing branch of the mTORC1 pathway (Bar-Peled et al., 2013; Panchaud et al., 2013). Thus, we assessed in vivo how mTORC1 signaling is affected by Depdc5-deficiency. Deletion of both Depdc5 alleles induced an embryonic lethality, observed from E14.5, preceded by global growth delay, consistent with the ubiquitous expression of Depdc5 in whole body. Rapamycin, a specific mTORC1 inhibitor, when administered prenatally, rescued the growth defect and improved the survival of Depdc5$^{−/−}$ embryos, providing evidence of a direct link between Depdc5 and mTORC1 signaling cascade. We further demonstrated that deletion of both alleles of Depdc5 produces increased phosphorylation of mTORC1 downstream effectors associated with enlargement of soma size of cultured rat embryonic fibroblasts (REFs). Our data also show that REFs derived from Depdc5$^{−/−}$ embryos did not repress mTORC1 activity during amino acid starvation, confirming mTORC1 senses amino acid levels via Depdc5. Altogether, both the rapamycin
rescue and the upregulation of the mTORC1 pathway, demonstrates that Depdc5-deficiency leads to early developmental mTORC1-dependent defects. Similar effects are described in global and condition- al knockout mouse models of mTORopathies including Tsc1 (Anderl et al., 2011; Ma et al., 2014) and Tsc2 (Way et al., 2012). We note, however, that the phenotype of Depdc5-deficient rats is less severe since Tsc1 and Tsc2 rodent models succumb earlier, during mid-gestation (from E9.5).

Lack of a single Depdc5 copy reflects the genotype of patients who are heterozygous for Depdc5 mutations. In rats, such a loss induced al- terations in the neuronal development including aberrant cortical lamin- ation, indicative of neuronal migration defects. Furthermore Depdc5−/− knockouts exhibit a conduction, throughout the cortex of Depdc5−/− rats (from age P11) of cells, resembling cytomegalic dys- morphic neurons and balloon cells in FCD type II patients, which typically exhibit an increased cell soma size of 50–100% (Lim and Crino, 2013). We furthermore demonstrated that these morphological changes were due to mTORC1 pathway hyperactivity, and that prenatal administration of rapamycin was effective in preventing their apparition. Thus this model shares neuropathological hallmarks with sporadic FCD type II patients presenting dysmorphic cortical neurons (Baybis et al., 2004; Miyata et al., 2004). Accordingly, phosphorylated rpS6-positive dysmorphic neurons were also seen in postoperative brain tissue from a FCD type II patient with a DEPDC5 mutation (Scerri et al., 2015). These brain pathological findings also parallel the phenotype in Tsc1 condi- tional knockout mouse with enlarged dysmorphic neurons associated with increased mTORC1 activity (Meikle et al., 2007). Similar to the other Tsc1 and Tsc2 brain-specific knockout rodent models (Goto et al., 2011; Meikle et al., 2008; Tsai et al., 2013), rapamycin treatment was ef- fective in this model and improved the cortical abnormalities. This result confirms an mTORC1-dependent mechanism leading to the features reminiscent of FCD in heterozygous Depdc5+/− rats. However, despite these developmental cortical abnormalities, we did not detect spontaneous seizures in Depdc5+/− rats. In Depdc5+/− F344 rats, as in most other Tsc1−/− and Tsc2+/− global knockout rodent models, gene dosage effects may ensure that deletion of a single copy is not sufficient to trigger seizures. Therefore, we speculate that investiga- tion of epileptic activity will necessitate viable homozygous animals (Abdijadid, S., et al., 2015). Basic mechanisms of epileptogenesis in pediatric cortical dysplasia. CNS Neurosci. Ther. 21, 92–103. Acosta-Jaquez, H.A., et al., 2009. Site-specific mTOR phosphorylation promotes mTOR1-mediated signaling and cell growth. Mol. Cell. Biol. 29, 4308–4324. Anderl, S., et al., 2011. Therapeutic value of prenatal rapamycin treatment in a mouse brain model of tuberous sclerosis complex. Hum. Mol. Genet. 20, 4604–4614. Bar-Peled, L., et al., 2013. A tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1. Science 340, 1100–1106. Bateup, H.S., et al., 2013. Excitatory/inhibitory synaptic imbalance leads to hippocampal hyperexcitability in mouse models of tuberous sclerosis. Neuron 78, 510–522. Baulac, S., et al., 2015. Familial focal epilepsy with focal cortical dysplasia due to DEPDC5 mutations. Ann. Neurol. 77, 675–683. Baulac, S., et al., 2012. A rat model for LGI1-related epilepsies. Hum. Mol. Genet. 21, 3546–3557. Baybis, M., et al., 2004. mTOR cascade activation distinguishes tubers from focal cortical dysplasia. Ann. Neurol. 56, 478–487.