RHEB1 insufficiency in aged male mice is associated with stress-induced seizures

Qi Tian · Pavel Gromov · Joachim H. Clement · Yingming Wang ·
Marc Riemann · Falk Weih · Xiao-Xin Sun · Mu-Shui Dai ·
Lev M. Fedorov

Received: 12 April 2017 / Accepted: 24 August 2017 / Published online: 10 September 2017
© American Aging Association 2017

Abstract The mechanistic target of rapamycin (mTOR), a protein kinase, is a central regulator of mammalian metabolism and physiology. Protein mTOR complex 1 (mTORC1) functions as a major sensor for the nutrient, energy, and redox state of a cell and is activated by ras homolog enriched in brain (RHEB1), a GTP-binding protein. Increased activation of mTORC1 pathway has been associated with developmental abnormalities, certain form of epilepsy (tuberous sclerosis), and cancer. Clinically, those mTOR-related disorders are treated with the mTOR inhibitor rapamycin and its rapalogs. Because the effects of chronic interference with mTOR signaling in the aged brain are yet unknown, we used a genetic strategy to interfere with mTORC1 signaling

selectively by introducing mutations of *Rheb1* into the mouse. We created conventional knockout (*Rheb1*^{+/-}) and gene trap (*Rheb1*^{Δ/+}) mutant mouse lines. *Rheb1*-insufficient mice with different combinations of mutant alleles were monitored over a time span of 2 years. The mice did not show any behavioral/neurological changes during the first 18 months of age. However, after aging (> 18 months of age), both the *Rheb1*^{+/-} and *Rheb1*^{Δ +/-} hybrid males developed rare stress-induced seizures, whereas *Rheb1*^{+/-} and *Rheb1*^{Δ +/-} females and *Rheb1*^{Δ/+} and *Rheb1*^{Δ/Δ} mice of both genders did not show any abnormality. Our findings suggest that chronic intervention with mTORC1 signaling in the aged brain might be associated with major adverse events.

Falk Weih deceased

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11357-017-9997-3>) contains supplementary material, which is available to authorized users.

Q. Tian · Y. Wang · L. M. Fedorov (✉)
OHSU Transgenic Mouse Models Shared Resource, Knight
Cancer Institute, Oregon Health and Science University, 3181 SW
Sam Jackson Park Road, Portland, OR 97239, USA
e-mail: fedorovl@ohsu.edu

P. Gromov
Genome Integrity Unit, Danish Cancer Society Research Center,
Copenhagen, Denmark

J. H. Clement
Department of Hematology and Oncology, Jena University
Hospital, 07747 Jena, Germany

M. Riemann · F. Weih
Leibniz Institute on Aging—Fritz Lipmann Institute,
07745 Jena, Germany

X.-X. Sun · M.-S. Dai
Department of Molecular and Medical Genetics, Oregon Health
and Science University, Portland, OR, USA

L. M. Fedorov
Institut fuer Versuchstierkunde, Friedrich-Schiller-University,
Erlanger Allee 101, 07740 Jena, Germany

Keywords Rheb1 · mTORC1 · Gene targeting · Mice · mTOR inhibitor · Rapamycin

Introduction

RHEB1 belongs to a unique family within the Ras superfamily of GTPases (Urano et al. 2000; Li et al. 2004). *Rheb1* was initially identified as a gene whose expression is rapidly induced upon synaptic activity in the rat hippocampus (Yamagata et al. 1994). Homologs of the *Rheb1* gene have been identified in a number of non-mammalian organisms and in mammals including mouse, rat (Ibid), and human (Gromov et al. 1995; Mizuki et al. 1996); for review, see (Aspuria and Tamanoi 2004; Heard et al. 2014). The RHEB1 protein is a direct target of the tuberous sclerosis complex (TSC1/2), and it transmits upstream-signals to regulate mTORC1. TSC1/2 inhibits the mTORC1/S6K/4EBP1 signaling pathway by stimulating GTP hydrolysis of RHEB1 and its functions between TSC1/2 and mTORC1 (Manning and Cantley 2003). Inactivation of TSC1/2 leads to the activation of the RHEB1/mTORC1 signaling cascade that is accomplished by phosphorylation of its downstream targets that include p70 S6 kinase (S6 K) and the eukaryotic initiation factor 4E (eIF4E)-binding proteins 1 and 2 (4E-BP1 and 4E-BP2) (Uhlmann et al. 2004; Terauchi et al. 2010). RHEB1 together with other members of the TSC1/TSC2/RHEB1/mTORC1 pathway plays an important role in the regulation of cell growth and proliferation, aging, ribosome biogenesis, protein synthesis, actin-cytoskeletal organization, autophagy, and metabolism (Heard et al. 2014). Abnormal function of TSC1/TSC2/RHEB1/mTORC1 signaling results in different pathologies. In humans, mutations in tumor suppressor genes *Tsc1/2* and corresponding inactivation of the TSC1/2 complex leads to inappropriate activation of RHEB1, manifested as tuberous sclerosis complex (TSC) disease (Tee et al. 2003b, 2003a). The hallmark of TSC is the development of a type of benign tumors called hamartomas found in brain, kidney, lung, and other organs of TS patients (Mizuguchi and Takashima 2001). The development of hamartoma in brain causes serious neurological manifestations including epilepsy, autism, and mental retardation, affecting 80–90% of the children with TS. To model TSC specifically in the brain, the neuron specific for *Tsc1* (Meikle et al. 2007) and an astroglia-specific knock out for *Tsc1* (Uhlmann

et al. 2002; Zeng et al. 2008) and *Tsc2* (Zeng et al. 2011) genes were generated in mouse. Inactivation of any of these genes leads to hyperactivation of the RHEB1/mTORC1 pathway, causing severe astrocyte proliferation, neuronal disorganization, and corresponding megalencephaly and seizure development. To generate another TSC mouse transgenic model, human *Rheb1* was cloned under regulation of the promoter of the *nestin* gene, selectively expressing RHEB1 in neuronal stem cells of embryonic and adult brains. Several founders overexpressing RHEB1 in brain had hyperplasia of brain's tissues and they died at the age of 3–4 weeks (Tian, Fedorov, unpublished results). Thus, increased activity of RHEB1/mTORC1 leads to tumor development in different organs of human and other species. On the other hand, the RHEB1 deficiency/insufficiency results in different developmental abnormalities. Gene targeting of *Rheb1* in mice leads to embryonic lethality of homozygous embryos around mid-gestation from impaired development of the cardiovascular system (Goorden et al. 2011; Tamai et al. 2013). Moreover, RHEB1 is vital for adult animals, since tamoxifen-inducible *Rheb1* knock-out in adult animals results in death of homozygous *Rheb1* null mice 18 days after initiating *Rheb1* deletion. A conditional knockout of *Rheb1* in neural progenitor cells that resulted in a RHEB1 expression reduction by > 90% after 2–3 weeks of postnatal development leads to brain growth retardation (50% of weight of w.t. controls) and prominent hypomyelination in all regions of the brain (Zou et al. 2011). Thus, RHEB1 deficiency as well as serious insufficiency and corresponded reduction of the pathway activity downstream of RHEB1 results in embryonic death and severe morphological malformations of postnatal mice. However, it is almost not known how moderate RHEB1/mTORC1 insufficiency can affect the development and functionality of the brain. In particular, it is interesting because treatment of TSC and other disorders resulting from mTORC1 hyperactivity by the TORC1 inhibitor everolimus (rapalog of rapamycin) already results in significant success including reduction of seizure frequency as well as behavioral improvement and tumor regression (French et al. 2016; Franz et al. 2016; Franz 2011; Krueger et al. 2010). However, to support normal or at least improved health conditions of such patients, it might be necessary to have long-term or lifelong application of mTOR inhibitors. Furthermore, the clinical data indicate that the everolimus treatment results in different side effects including pneumonitis,

hyperglycemia, and immunosuppression etc., (for review, Mendus et al. 2015). Clinical manifestations that occur after mTORC1 inhibitor treatment can potentially be divided into two groups. One group can occur as side effects resulting from a toxicity of mTOR inhibitors. Other manifestations can occur as a result of long-term down-regulation of the mTOR pathway per se. Down-regulation of the mTOR pathway without mTOR inhibitors specifically by reduction of RHEB1 expression via gene targeting gives the possibility of better understanding the nature of adverse events related to mTOR inhibition. This is also interesting because RHEB1 is strongly expressed in different regions of the brain responsible for cognition, learning, long short memory, and movement etc. (Tian et al. 2016) and its down-regulation can potentially affect these brain functions.

To study the effect of a prolonged moderated reduction in the RHEB1/mTORC1 pathway, we targeted *Rheb1* in mice by two different methods. Generation of our *Rheb1* Gene Trap (*Rheb1*^{Δ/+}) mice were described recently (Tian et al. 2016). By using RHEB1 immunostaining and X-gal staining, we showed that RHEB1 protein expresses in different regions of the brain including the cerebral cortex, subcortical regions (counting hippocampus), cerebellum (particularly in Purkinje cells), and other organs during embryonic and postnatal development. A RHEB1 activation of mTOR is dependent on farnesylation (Hanker et al. 2010). Therefore, in a second model, we inactivated the *Rheb1* gene in embryonic stem cells (ES) cells by targeting the last exon of *Rheb1* containing the carboxyl terminus (so-called CSVM motif) that is responsible for the farnesylation and activation of the RHEB1 protein. These RHEB1-insufficient mouse lines were used to study how long-term reduction of RHEB1/mTORC1 signaling can induce behavioral changes and/or neurological disorders.

Results

Generation and phenotype of *Rheb1*-deficient/insufficient mice

The *Rheb1* gene was targeted in ES cells via two strategies. The scheme of the *Rheb1* by conventional gene targeting is presented on Fig. 1a–d. After homologous recombination of targeting vector with the *Rheb1* locus *Rheb1*^{+/-}, ES cells were used to generate chimeric mice and finally *Rheb1*^{+/-} mice. *Rheb1*^{+/-} embryos and adult

animals displayed no obvious anatomical defects. Adult animals were fertile and did not demonstrate reduced survival due to haplo-insufficiency. However, no viable homozygous mutant animals were observed among 312 offspring produced by mating of heterozygous mice, while 101 wild-type and 211 heterozygous mice were born at the expected ratio 2:1. To determine the time of lethality of the *Rheb1*^{-/-} embryos, we analyzed the E8.5–E10.5 embryos derived from an F2 mating. The analysis of the embryos showed that the *Rheb1*^{-/-} embryos (12 from 45 observed) exhibited lethality and/or growth retardation at E8.5–E11.5 (Fig. 1e). Living growth-retarded and dead *Rheb1* null embryos together accounted for approximately 25% of conceptuses, providing evidence that *Rheb1* deletion had no effect on implantation. Histological sections of *Rheb1*^{-/-} embryos commonly showed cardiovascular defects similar to those described earlier (Goorden et al. 2011; Tamai et al. 2013) (data not shown). Generation of *Rheb1*^{Δ/+} (*Rheb1* GT) gene trap mice and the expression pattern of the β -gal reporter gene in their tissues was described recently (Tian et al. 2016). The mice have correct integration of the gene trap vector into intron 1 of *Rheb1*, and expression of chimeric RHEB1 (Exon1)- β -Gal protein is regulated by the promoter of endogenous *Rheb1*. The *Rheb1* GT animals were subjected to the same analysis as *Rheb1* KO mice. To our surprise, the *Rheb1*^{Δ/Δ} embryos and adult mice were normal although both alleles of *Rheb1* were targeted. Further, we bred *Rheb1*^{Δ/+} and *Rheb1*^{+/-} mice to study how combinations of different *Rheb1* mutant alleles can influence on the mouse phenotype. In contrast to *Rheb1*^{-/-} embryos, the hybrid *Rheb1*^{Δ/-} embryos developed normally. Moreover, *Rheb1*^{Δ/-} hybrid mice were born according to Mendelian distribution; they looked normal and were fertile, suggesting an incomplete inactivation of the *Rheb1*^Δ allele.

Gene expression in tissues of *Rheb1*-insufficient mice

Expression of the *Rheb1* gene in mutant ES cells and mice were studied on the mRNA and protein level. qRT-PCR of *Rheb1* mRNA in ES cells using the pair of primers related to Ex 2 and Ex 5, which were not affected by targeting, showed similar levels in *Rheb1*^{+/+}, *Rheb1*^{+/-}, and *Rheb1*^{Δ/+} ES cells (Fig. 2a). It appears that *Rheb1*⁻-targeted allele transcribes truncated mRNA *Rheb1* transcript in *Rheb1*^{+/-} ES cells, but this transcript apparently decays because no truncated RHEB1 protein was later identified by Western blotting. In contrast, we found an

Fig. 1 Targeting of the *Rheb1* gene. **a** The eight exons of *Rheb1* coding the carboxyl-terminal motif CSVM were deleted after homologous recombination. **b** Southern blot analysis after digestion of ES cell DNA using BamHI results in 11.4 and 7.3 kb WT and KO fragments, respectively. Position of the external probe is indicated. **c** PCR genotyping of ES cells and *Rheb1* KO mice; 400 and 200 b.p. wild-type and KO fragments, correspondingly. **d** PCR genotyping of the embryos at E9.5 after mating of *Rheb1*^{+/-} mice. **e** Whole-mount lateral views of wild-type (left) and abnormal *Rheb1*^{-/-} (right) embryos at E11.5

approximately twofold reduction of *Rheb1* mRNA in *Rheb1*^{+/-} ES cells when a pair of primers was used with at least one of primer binding to exon 8 (Fig. 2b). qRT-PCR with both pairs of primers showed that *Rheb1*^{Δ/+} ES cells have *Rheb1* mRNA levels similar with *Rheb1*^{+/+} ES cells in spite of one *Rheb1* allele was “trapped”. Analysis of *Rheb1* mRNA levels in mouse embryos at E 9.5 with a pair of primers covering unaltered exon 1 region showed that embryos express around fourfold more compared to equivalent ES cells (Fig. 2c). Similarly to ES cells, a decrease of *Rheb1* mRNA was also identified in *Rheb1*^{+/-} embryos when the used primers were related to deleted exon 8 (Fig. 2d). No *Rheb1* mRNA was detected in *Rheb1*^{-/-} embryos. This confirmed that homozygous *Rheb1* disruption lead to complete inactivation of the gene. The level of RHEB1 protein was tested in MEFs of different genotypes by Western blotting (Fig. 3a, b). The amount of RHEB1 was decreased (approximately twofold) in lysates of *Rheb1*^{+/-} MEFs. Some (non-significant) decrease was also identified in *Rheb1*^{Δ/Δ} or *Rheb1*^{Δ/+} MEFs compared with wild-type MEFs (Fig. 3c, d). Thus, analysis of the expression of mRNA and protein

in *Rheb1* mutant embryos showed that *Rheb1* was knocked out by conventional gene targeting but it was not inactivated completely by the gene trap approach. *Rheb1* is highly expressed in the brain of embryos and young mice (Tian et al. 2016). It was interesting to learn how its expression in the brain of wild type and *Rheb1* mutant mice changes with age. The protein level of RHEB1 in the brain of wild-type mice slightly decreased with age between 1 month and 2 years, but it was not statistically significant (Fig. 3e, f). The *Rheb1*^{+/-} and *Rheb1*^{Δ/-} mice showed double RHEB1 reduction in the brain compared with wild-type mice of the same age. Similarly to wild-type mice, small age-related reduction (non-significant) of RHEB1 protein levels were identified in total brain lysates of the *Rheb1*^{+/-} and *Rheb1*^{Δ/-} mutant mice.

Behavior of *Rheb1* mutant mice

Mice of both *Rheb1* mutant lines were monitored for more than 2 years. Young females and males separated by gender after weaning were maintained as three to four

Fig. 2 Comparison of *Rheb1* mRNA expression by qRT-PCR in *Rheb1*-insufficient ES cells and embryos. **a** *Rheb1* mRNA expression in ES cells using a pair of primers covering the Ex 2-Ex 5 region of *Rheb1* mRNA. **b** *Rheb1* mRNA expression in ES cells using a pair of primers covering the Ex7-Ex 8 region of *Rheb1* mRNA. **c** *Rheb1* mRNA expression in ES cells and 9.5-days-old embryos using a pair

of primers covering Exon 1. No significant change of mRNA levels in *Rheb1*^{+/-} embryos; however, a decrease is visible in *Rheb1*^{Δ/-} embryos. **d** *Rheb1* mRNA expression in ES cells and 9.5-day-old embryos using a pair of primers covering exon 8. The number of dishes with ES cells or embryos of each genotype is indicated. *Statistical difference versus wild type control ($p < 0.05$)

litter-mates per cage for 3–4 months. To identify possible influence of the mutations of *Rheb1* on behavior, the mutant *Rheb1*^{+/-} and *Rheb1*^{Δ/-} males and females were subjected to a social interaction (SI) test. A 5-min SI test was performed with *Rheb1*^{+/-} and *Rheb1*^{Δ/-} test mice and unfamiliar, age-matched stimulus B6N mice of the opposite sex. SIs between *Rheb1*^{+/-} and *Rheb1*^{Δ/-} test males and stimulus B6N females were not different from *Rheb1*^{+/-} and *Rheb1*^{Δ/-} test females and stimulus B6N males. Furthermore, there were no obvious differences in the behavior of *Rheb1* mutant mice and the behavior of control mice when both test and stimulus animals were wild-type B6N mice (data not shown). After SI testing, the mice were kept up to 1 year as breeding pairs with different combinations of *Rheb1*-mutant alleles. No obvious behavioral changes were detected when the mice were cared for in the breeding cages.

Neurological abnormality of *Rheb1* mutant mice

Breeding pairs were separated at the age of 1 year, and several females and males of every genotype were

singly housed in cages for an additional year. The mice of either sex with different combinations of *Rheb1* wild-type and/or mutant alleles showed no neurological disorders up to the age of 18 months. However, upon opening cages, some *Rheb1* mutant mice at around the age of around 18 months showed abnormal reactions that appeared to resemble stress-induced seizures. We found that only *Rheb1*^{+/-} and *Rheb1*^{Δ/-} males had the seizure-like neurological abnormality (Table 1). Moderate seizures were induced by cage opening and/or transfer of mouse into new cages and reached stage 1–2 according the Racine scale (Fig. 4a, b). Since reactions of mice with these two manipulations did not show significant differences, they were combined and labeled as the reactions on cage opening. Even though all *Rheb1*^{+/-} and *Rheb1*^{Δ/-} males repeatedly manifested the convulsions during a period of observation, seizures were not detected consistently every week. The frequency of convulsion events upon opening the cage was 0.59 ± 0.16 and 0.66 ± 0.50 mouse/week for *Rheb1*^{+/-} and *Rheb1*^{Δ/-} males, respectively. If a mouse did not manifest convulsions, it was given a score of 0. Mean

Fig. 3 Analysis of RHEB1 protein expression in tissues of *Rheb1*-insufficient mice. **a** RHEB1 expression in MEFs produced from 13.5-day-old *Rheb1* KO embryos. **c** RHEB1 expression in MEFs produced from *Rheb1*-insufficient embryos with combinations of different mutant *Rheb1* alleles. **e** RHEB1 expression in the brain of *Rheb1*-insufficient mice of different age. **b, d, f** The quantification of RHEB1 expression by ImageJ. The number of the dishes with MEFs or mice of each genotype is indicated. *Statistical difference versus wild-type control ($p < 0.05$)

Table 1 Stress-induced seizures in 18–20-month-old *Rheb1* mutant mice

Genotype, sex	Number of mice (seizure prevalence)	Frequency of seizure events reacting to cage opening	Seizure severity reacting to cage opening	Frequency of seizure events reacting to the open space	Seizure severity reacting to the open space
<i>Rheb1</i> ^{+/+} , ♂	5 (0)	0	0	0	0
<i>Rheb1</i> ^{+/+} , ♀	5 (0)	0	0	0	0
<i>Rheb1</i> ^{+/-} , ♂	4 (4)	0.59 ± 0.16	1.00 ± 0.86	0.84 ± 0.50	2.63 ± 1.36
<i>Rheb1</i> ^{+/-} , ♀	4 (0)	0	0	0	0
<i>Rheb1</i> ^{+Δ} , ♂	4 (0)	0	0	0	0
<i>Rheb1</i> ^{+Δ} , ♀	4 (0)	0	0	0	0
<i>Rheb1</i> ^{ΔΔ} , ♂	4 (0)	0	0	0	0
<i>Rheb1</i> ^{ΔΔ} , ♀	4 (0)	0	0	0	0
<i>Rheb1</i> ^{Δ/-} , ♂	4 (4)	0.66 ± 0.5	0.88 ± 0.75	0.92 ± 0.70	2.94 ± 1.19
<i>Rheb1</i> ^{Δ/-} , ♀	4 (0)	0	0	0	0

Mice were regularly observed weekly during 8 weeks. The seizure manifestations were recorded upon opening the cage and after being placed in an open space. Frequency of seizure events were counted as number of weeks when an individual mouse manifested seizures divided on 8 weeks of observation, and then the mean and standard deviation was calculated for each group of four mice of the same sex and genotype. Seizure severity scores on the Racine scale were averaged for each individual mouse manifested over the 8 weeks, and then the mean and standard deviation was calculated for four mice with the same sex and genotype. The seizures were identified in *Rheb1*^{+/-} and *Rheb1*^{Δ/-} males only. All *Rheb1*^{+/-} and *Rheb1*^{Δ/-} males manifested seizures at some point; however, they were not detected every week. Monitoring of the main mouse group ended at 24 months. Three female mice (two *Rheb1*^{+/-} and one *Rheb1*^{Δ/-}) were monitored for two additional months; no seizures were observed.

Fig. 4 Stress-induced seizures in *Rheb1* $^{\Delta/-}$ mutant males at age 20 months evaluated according to the Racine scale. **a–d** neurological phenotype of *Rheb1* $^{\Delta/-}$ mutant males. **a** Mouse reaction (stage 1) on the opening of cage. **b** Mouse reaction (stage 2) on transfer into new cage. **c** Reaction on transfer in a novel environment (open space). Both reactions (**c**, **b**) characterized by aberrant tail position (resembling Straub tail). **d** Severe reaction of *Rheb1* mutant male in a novel environment characterized by generalized clonic convulsions; the mouse fell onto its side (stage 4). **e**, **f** Normal exploratory behavior of *Rheb1* $^{\Delta/+}$ mutant male and a wild-type female in the novel environment (open space) correspondingly

convulsion stages were scored as 1.00 ± 0.86 and 0.88 ± 0.75 for *Rheb1* $^{+/+}$ and *Rheb1* $^{\Delta/-}$ males, respectively. The transfer of animals to a novel environment (a clean open platform) resulted in more dramatic effects. The frequency of convulsion events upon transfer to the open space reached 0.84 ± 0.50 and 0.92 ± 0.70 mouse/week for *Rheb1* $^{+/+}$ and *Rheb1* $^{\Delta/-}$ males, respectively. Moreover, the mice manifested even more severe seizures. Behavior was often characterized by aberrant tail position (Fig. 4c), resembling Straub tail (Bilbey et al. 1960) that occurs as a sensitive biological reaction to morphine. Furthermore, seizures could sometimes reach stage 4, which is characterized by clonic convulsions (Fig. 4d). Convulsion achieved 2.63 ± 1.36 and 2.94 ± 1.19 for *Rheb1* $^{+/+}$ and *Rheb1* $^{\Delta/-}$ males, respectively. These evoked seizures of *Rheb1*-insufficient males lasted an average of 35 ± 13 s and thereafter, the mice recovered; none died. Males and females of other *Rheb1* mutant genotypes, including *Rheb1* $^{+/+}$ and *Rheb1* $^{\Delta/-}$ females, demonstrated normal behavior without any neurological abnormalities (Fig. 4e, f).

Furthermore, since RHEB1 is highly expressed in the cerebellum, we suggested that its double and long-term

reduction can potentially lead to ataxia. However, we did not observe any visible symptoms of ataxia such as loss of equilibrium, body sway, tremor, or other manifestations.

Discussion

To investigate a potential effect of prolonged reduction of RHEB1 on the behavioral and on the functionality of the brain, we targeted the *Rheb1* gene by two different methods. The mice produced by conventional targeting of exon 8 of *Rheb1* have a similar phenotype, including double reduction of RHEB1 in *Rheb1* $^{+/+}$ mice and embryonic lethality of *Rheb1* $^{-/-}$ embryos, as other *Rheb1* knockout mice described previously (Goorden et al. 2011). In our second model, we targeted *Rheb1* by gene trap method (Tian et al. 2016). We showed that a pT1 β geo gene trap vector inserted within intron 1 of *Rheb1* and B-GAL accurately reflected the promotor activity of the “trapped” gene. Initially, it was suggested that the *Rheb1*- β geo allele must lead to the expression of a highly truncated fusion protein encoding only 17 amino acids of RHEB1 fused to β -GAL. However, to

our surprise, after the correct integration of the gene trap vector, the MEFs and brain tissues of the *Rheb1*^{Δ/+} or *Rheb1*^{Δ/Δ} mice expressed normal level of full-length RHEB1. This indicated that the “trapped” allele transcribed a fusion transcript and the primary *Rheb1* mRNA processed and translated into a full-length RHEB1 protein. A similar phenomenon was earlier described where a gene trap insertion lead to a hypomorphic allele that expressed β-GAL reporter and transcribed full-length mRNA of the targeted gene *Vrk1* (Wiebe et al. 2010). Moreover, a complete compensation of full-length *Map-4* mRNA transcription for the gene trap insertion was described; homozygote mice had expression of B-GAL reporter and normal level of full-length *Map-4* mRNA (Voss et al. 1998). It seems that splicing of the gene trap is incomplete and the transcription machinery ignores the En-2 splice acceptor and SV40 polyadenylation signal of the pT1βgeo vector and compensates somehow the synthesis of normal amount of full-length RHEB1. Therefore, *Rheb1*^{Δ/Δ}, *Rheb1*^{Δ/+}, and wild-type mice have the similar levels of expression of RHEB1 protein, whereas *Rheb1*^{Δ/-} have twofold reduced expression like *Rheb1*^{+/-} mice. Comparison of the amount of RHEB1 protein in total brain lysates of young versus old mice showed a slight (non-significant) age-related reduction of RHEB1 protein as in wild type as well as in RHEB1-insufficient mice. We did not compare RHEB1 expression levels in different brain regions; it is therefore not known whether the RHEB1 decrease is specific to certain brain regions or instead reflects a uniform decrease throughout the brain. It is possible that RHEB1 reduction in some region(s) of the brain below some critical level can initiate rare stress-induced seizures.

It is well known that a reduced function of TSC1 and TSC2 proteins in patients leads to hyperactivation of the RHEB1/mTOR pathway in the brain and results in severe morphological changes and serious neurological manifestations including autism and mental retardation in humans (Caban et al. 2017; Jeong and Wong 2016). By analogy with patients, the mice with targeted *Tsc1* or *Tsc2* showed hyperactivation of the mTOR pathway, have serious behavioral, neurological abnormality and early post-natal mortality (Zeng et al. 2008, 2011; Abs et al. 2013; Meikle et al. 2007; Goorden et al. 2007). On the other hand, it is unknown how a long-term RHEB1 insufficiency and related reduced activity of the mTORC1 pathway influences neuronal functions, cognition, and behavior. Our *Rheb1*^{+/-} and *Rheb1*^{Δ/-}

mutant mice had an approximately twofold reduction of RHEB1 in total lysates of the brain and its level did not change significantly with age. Since RHEB1 is highly expressed in the brain regions responsible for different cognitive functions such as learning, long-term and short-term memory, and social behavior etc., we suggested that such RHEB1 insufficiency during aging would lead to some neurological abnormality and/or visible changes in behavior. So, these mice were subjected to long-term monitoring. To exclude an unpredicted effect of insertion of the gene trap vector on the behavioral phenotype, the *Rheb1*^{Δ/+} and *Rheb1*^{Δ/Δ} mice were also subjected to monitoring although they expressed normal amounts of RHEB1. Routine observations and special social interaction tests did not identify any behavioral differences of any of our *Rheb1*-mutant mice compared to wild-type control animals. This is in agreement with recently published results of the group of Elgersma (Goorden et al. 2015). They used several other behavior tests (Morris water maze, contextual fear conditioning and rotarod) and showed that even 75% reduction of RHEB1 and downstream mTORC1 signaling in the brain did not affect synaptic plasticity and hippocampus-dependent learning and memory of mice. Thus, our results and the data of Elgersma's group demonstrated that long-term reduced RHEB1 expression do not affect a social behavior and cognitive functions of RHEB1-insufficient mice.

Further, since RHEB1 has significant expression in the brain, we monitored the *Rheb1* mutant mice to detect any neurological disorders. In spite of RHEB1 being highly expressed in the cerebellum, its long-term twofold reduction and downregulation of members of mTORC1 pathway correspondingly do not cause any visible symptoms of ataxia. Surprisingly, we found that RHEB1 reduction initiated stress-induced seizures in 1.5–2-year-old mice. Interestingly, only old RHEB1-insufficient males were prone to seizures. It seems that during life span, the brain structure and its functions are affected by RHEB1 insufficiency in cooperation with some additional modulators such as the sex. Manifestation of this neurological disorder exclusively in old males can potentially arise due to many different factors. During development, mTOR drives growth and reproduction, while causing aging and age-related diseases later in life for review (Blagosklonny 2010). The males of different species (including human) show shorter lifespan compared with females (for review, Yashin et al. 2010; Gowaty 2007; Clutton-Brock and

Isvaran 2007). It might be that the seizures of old RHEB1-insufficient males are somehow connected with the mTORC1 pathway and due to a faster process of aging in males versus females. Another potential cause is the fundamental difference in the genome between female and male mice, related to the X and Y sex chromosomes. Other factor(s) might be a difference of the male and female endocrine systems that interacts dynamically with the brain and behavior throughout life (Duarte-Guterman et al. 2015; Sanchis-Segura and Becker 2016). These differences might modulate the activity of mTORC1 or downstream members of the mTORC1 pathway in neuronal cells of old RHEB1-insufficient males.

The mTORC1 inhibitors everolimus and other rapalogs are specifically used against seizures in young and middle-aged TSC and epilepsy patients (Jeong and Wong 2016; Franz et al. 2016). Moreover, everolimus is used in combination with other medications for the treatment of a wide spectrum of diseases (for review, Calimeri and Ferreri 2017; Pelletier et al. 2016; Blair et al. 2017). Several papers reported about the usage of everolimus to treat different diseases in old patients (Donati et al. 2017; Furian et al. 2014; Wei and Lai 2015). However, there are no reports published about long-term treatment of seizure-prone old patients or old experimental animals by mTORC1 inhibitors. RHEB1 insufficiency has a similar effect on the mTORC1 signaling pathway as rapamycin or other rapalogs and should protect against seizures in general. Instead of this positive effect, a long-term RHEB1 insufficiency in our old males initiated stress-induced seizures. In spite of the general success of everolimus application against TSC, some undesirable effects are known. Some TSC patients with medically refractory epilepsy exhibit exacerbated seizure frequency despite continuous treatment with everolimus and that requires that the treatment be discontinued (Krueger et al. 2010; Krueger et al. 2016). Seizures in old RHEB1-insufficient mice suggest that an increase of convulsions in some patients after treatment by mTOR1 inhibitors are not side effects of such inhibitors, rather to the downregulation of RHEB1/mTORC1 signaling below a normal level.

As far as we are aware, no direct evidence is yet published that demonstrates whether prolonged down-regulation RHEB1/mTORC1 signaling is a cause of diseases of the nervous system (NS) in human. Nevertheless, its reduction may potentially be involved in development of cognitive and other NS diseases. For

example, a loss of mTOR activity in peripheral lymphocytes has a positive correlation with the progression of Alzheimer's disease (Paccalin et al. 2006). Furthermore, development of some NS diseases may potentially be connected with reduced activity of the RHEB1/mTORC1 pathway in certain regions of the brain. For instance, it is suggested that prefrontal cortex hypomyelination and cognitive symptoms in schizophrenia (SZ) patients can result from a hypoactivation of the RHEB1/mTOR-P70S6K pathway (see Maas et al. 2017 for review). Moreover, the neurons of substantia nigra of post-mortem brains of Parkinson's disease (PD) patients have increased expression of RTP801 protein, an inhibitor of mTORC1 activity, which is requisite for cell death in cellular PD models (Malagelada et al. 2006). Similarly, mTORC1 activity is reduced in striatal tissues from Huntington's disease (HD) patients, as detected by reduced phosphorylation of ribosomal protein S6 (pS6) (Lee et al. 2015).

In summary, our data provide further support that the morphological structure of the brain and its cognitive/behavioral functions are more sensitive to upregulation of the RHEB1/mTORC1 pathway than its down-regulation. However, unexpected stress-induced seizures in old RHEB1-insufficient males suggest that long-term down-regulation of the RHEB1/mTORC1 pathway can have negative effects.

It is particularly important because the current rapid increase in the proportion of elderly people may lead to a significant increase of the fraction of patients with epilepsy and other neurological disorders. Although many elderly people develop seizures, provoked by acute illness, in many others, the seizures occur without any obvious precipitating cause (Brodie et al. 2009). Therefore, to better understand potential connection of chronic down-regulation of the RHEB1/mTORC1 with the manifestation of seizures and other NS disorders in older human, a quantitative analysis of RHEB1 expression and mTORC1 pathway activity must be performed in different regions of the brain of old *Rheb1* mutant mice in future. Such an investigation is of particular interest because of evidence suggesting that the development of SZ, PD, and HD in patients result from down-regulation of RHEB1/mTORC1 in certain regions of the brain. Furthermore, the electroencephalogram and other electrophysiological approaches and patho-histological examination of old brain in details will help to identify a cause(s) of seizures. Moreover, we hope that such investigations will help to understand

why old *Rheb1* mutant male mice only develop stress-induced seizures.

Materials and methods

Construction of the targeting vector

Since activity of proteins in the RAS family depends on farnesylation of the carboxyl terminus (so-called CAAX motif), we targeted the last (8) exon of *Rheb1* containing the CSVM motif, the functional homolog of CAAX in RAS. To generate the *Rheb1* targeting vector, the pKO Scrambler vector V901 (Lexicon) was used as backbone. For positive selection, a 1.6-kb *AscI* fragment containing the *neomycin* (*neo*) resistance gene (V800 vector, Lexicon) was inserted into the *AscI* site of vector V901. For negative selection, a 2 kb *Rsr* II fragment containing the *thymidine kinase* (*tk*) gene was isolated from V830 vector (Lexicon) and inserted into the *Rsr* II site of V901 vector. Genomic DNA corresponding to the *Rheb1* locus was isolated from a lambda phage library derived from 129/Sv mouse DNA. A 5.8-kb *Bgl* II-*Kpn* I fragment containing exons 4–7 of *Rheb1* (homology 1) was cloned into *Bgl*III-*Kpn*I site. A 2.3-kb *Xho*I-*Xho*I fragment downstream of exon 8 (homology 2) was Klenov-fill ended and inserted into a *Sma*I site of vector V901. The *Not*I-linearized *Rheb1*-targeting vector was electroporated into CJ7 ES cells of genetic background 129/Sv and G418 (geneticin) (200 µg/ml), and GANC (gancyclovir) (2×10^{-6} M) was used for positive and negative selection correspondingly. Targeted clones were identified by Southern blot analysis according to standard protocol (Southern 1975) using a 3' probe on *Bam*HI-cut genomic DNA (Fig. 1a, b). A 32 P-labeled PCR fragment spanning a region of 700 bp downstream of homology 2 region was used as probe for Southern blot hybridization. Integrity of genomic DNA was later confirmed through sequencing of a 550-bp fragment upstream of *Rheb1* targeting vector.

Generation of *Rheb1* haplo-insufficient mice

Chimeric mice were created by injecting *Rheb1*-targeted ES cell clones into C57BL/6J (B6J) blastocysts according to standard protocol (Fedorov et al. 1997). After germline transmission (GLT), the *Rheb1*-targeted allele was transferred to a C57BL/6N (B6N) background (Charles River Lab; more than 10 backcrosses).

Rheb1^{Δ/+} mutant mice were produced early and maintained on B6N genetic background (Tian et al. 2016). All mouse studies were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committees of Oregon Health and Science University (Portland, OR, USA) and University of Jena (Germany). All mice were housed under SPF conditions, handled and sacrificed in accordance with institutional guidelines.

PCR genotyping

Live-born animals were screened for the presence of mutant *Rheb1* in tail DNA using Southern blotting and/or PCR. Preparation of tissues from adult mice and PCR were done using standard methods. The forward (F) primer 5'-CAGCCCACGTAATGAAGTGC-3' and reverse (R) primer 5'-TTGTGCCTCCTGTA GCTCAG-3' produce a 400-bp fragment-covered junction of exon 8 and post-exon 8 region of the wild-type *Rheb1* allele, whereas a combination of N-primer 5'-GCATGCTCCAGACTGCCTTG-3' (PGK-promoter of *neo* gene) with the same reverse (R) primer amplified a 200-bp fragment of the targeted allele of *Rheb1* using the following PCR protocol: 94 °C for 5 min followed by 35 cycles: 1 min at 94 °C, 1 min at 54 °C, and 30 s at 72 °C. Embryonic tissues for PCR were prepared as described earlier (Tian et al. 2011). Genotyping of *Rheb1*^{Δ/+} gene trap mice were done as described earlier (Tian et al. 2016). Presence of the pT1βgeo vector was identified with primers corresponding to the β-galactosidase gene: forward primer 5'-GCG TTG GCA ATT TAA CCG CC-3' and reverse primer 5'-CAG TTT ACC CGC TCT GCT AC-3'. The resulting PCR product was 450 bp. To genotype the wild-type *Rheb1* and *Rheb1*^Δ alleles, the following additional pairs of primers were used. The sense primer *RhebF1235* 5'-CCT AGA CTG GAC CCC TCA CA-3' and antisense primer *RhebR1620* 5'-ACG TGA CAG TCC CCT GTT CC-3' were used to amplify a 385-bp fragment of wild-type allele, whereas a combination of the same sense *RhebF1235* primer and mR4 5'-TGT GGG AAA GCC TTC AAA GGG-3' amplifies a 238-bp fragment of the *Rheb1*^Δ allele. PCR was performed using the following protocol: 95 °C for 5 min followed by 35 cycles: 45 s at 95 °C, 45 s at 58 °C and 1 min at 72 °C followed by 6 min extension at 72 °C.

Embryo generation

Staged embryos were produced by mating between wild-type, *Rheb1*^{+/-}, and or *Rheb1*^{Δ/+} (*Rheb1* GT) mice. Embryo isolation and organ identification were performed according to Kaufmann (2003). The histopathological evaluation of mice including both gross and microscopic examination was performed as described recently (Tian et al. 2016).

The embryos at E13.5 were used for the preparation of mouse embryonic fibroblasts (MEFs). Individual embryos were isolated, trypsinized, passaged into 50-mm-diameter dish, and cultured in DMEM containing 10% fetal bovine serum. After formation of a confluent monolayer, MEFs from each dish were genotyped and 1.5×10^6 of cells were used for Western blotting.

Quantitative RT-PCR

RNA from the tissues of wild-type mice was prepared and reverse-transcribed as described above and treated with DNase. 'ing SYBR Green I reagent and pairs of primers related to Ex2-Ex5, Ex7-Ex8, and pairs of primers for single Ex1 or Ex8 of *Rheb1* (Supplemental Table 1). Transcript-specific primers for β -actin as an endogenous control were used to evaluate the *Rheb1* mRNA level. The 2-ddCT relative quantification method was used to calculate fold difference in transcript levels between samples (Livak and Schmittgen 2001). Amplification efficiencies for the primer pairs were verified to be equivalent over a range of template concentrations. qRT-PCR was performed using an Applied Biosystems 7300 Real-Time PCR thermocycler (Applied Biosystems, Foster City, CA).

Western blot analysis

MEF cells and brain tissues from euthanized mice were frozen in liquid nitrogen. Tissue lysates were prepared as described elsewhere (Fedorov et al. 2006). Proteins were detected by anti-RHEB1 mab (Abnova; no. H00006009-M01) diluted 1:500 and anti β -ACTIN mab (Cell Signaling, no. 8H10D10) diluted 1:5000. Secondary goat anti-mouse antibody conjugated to HRP (Bio-Rad, no. 1721011) was diluted at 1:10,000. The signal was detected using a chemiluminescence kit (PerkinElmer, Wellesley, MA). The intensity of the immunoblot bands was quantified by densitometric

scanning with ImageJ analysis software (NIH) and normalized to β -actin (<https://imagej.nih.gov/ij/>).

SI tests

To test a possible behavior changes of the *Rheb1* mutant mice 3–4 months old, *Rheb1*^{+/-} and *Rheb1*^{Δ +/-} males and females were divided in four groups (three mice/group) according to the genotype and sex. Behavior of *Rheb1* mutant mice was evaluated as the interaction between individual male and female by social interaction test as described elsewhere (Panksepp et al. 2007). Two approaches to testing the interaction were used: (a) a *Rheb1* mutant male test mouse was placed with a normal B6N female stimulus mouse and (b) a *Rheb1* mutant female test mouse was placed with a normal B6N male stimulus mouse. Interactions between normal individual B6N males and females were used as appropriate controls. Each *Rheb1* mutant mouse (designated the “test” mouse) was isolated for 24 h within a clean cage that contained fresh cellulose pellet bedding without nesting material. After 24 h, the B6N mouse designated the “stimulus” mouse was added to the cage of the test mouse and the amount of social investigation that test mouse directed towards the stimulus mouse during a 5-min period was monitored. The variables of social investigation recorded included sniffing or snout contact with the head/neck/mouth area (1), direct contact with the anogenital area (2), and social pursuit within one body-length as the stimulus mouse moved continuously throughout the cage (3), and social grooming (4). All behaviors were videotaped (Sony, DCR-VX2100, Japan) and stored on a Dell Pentium IV PC for additional analysis. All behaviors were scored for composite duration and analyzed by software (ButtonBox v.5.0, Behavioral Research Solutions, Madison, WI, USA).

Analysis of induced seizures

To identify possible neurological abnormalities after genetic disruption of *Rheb1*, the mice were observed for two full years. After initially identifying seizures, the mice were regularly monitored to analyze and quantify the convulsions once per week for 8 weeks. The cages were opened and the reactions of the individual mice were recorded. Next, each mouse was gently picked up by the tail and transferred to the surface of an open platform (open space). Severity of seizures was

quantified using a modified Racine scale (Harai et al. 2012; Racine 1972), (0 = no response, 1 = mouth and facial jerks, 2 = convulsive waves axially through the body and/or Straub tail, 3 = forelimb clonus, 4 = rearing, falling down, hindlimb and forelimb clonus; 5 = generalized convulsions with a tonic extension episode and status epilepticus). Frequencies of seizure events and their severity were recorded and summarized separately for the opening of the cage and for the relocation of mice to the open space. During the period of regular observation, handling of mice and scoring of seizures were done by the same investigators. To validate and confirm a seizure phenotype, the convulsions were recorded on photo or video camera.

Statistical analysis

Statistical analysis was performed by Student *t* test using a *P* value of < 0.05 as statistically significant.

Acknowledgements We are saddened to report that, during the writing of this manuscript, our colleague and co-author Dr. Falk Weih passed away. We gratefully acknowledge Alex Derkatch, Galina Fedorova, and James Smart for the expert technical assistance. We also thank Harald Schubert for providing the laboratory space and T. Gridley for the CJ7 ES cells. We acknowledge the DNA core facility at OHSU for the sequencing analysis. We acknowledge Gareth Lahvis and Stephanie Krasnow for their excellent advices. John Crabbe and Pamela Metten are thanked for the helpful discussions and comments on the manuscript. The work was partly supported by Medical Research Foundation (Portland, Oregon), grant no. 9003823, the Office of the Vice President for Research at OHSU, and an NBL-3 grant from Bundesministerium für Bildung und Forschung, Germany.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

References

- Abs E, Goorden SM, Schreiber J, Overwater IE, Hoogeveen-Westerveld M, Bruinsma CF, Aganovic E, Borgesius NZ, Nellist M, Elgersma Y (2013) TORC1-dependent epilepsy caused by acute biallelic Tsc1 deletion in adult mice. *Ann Neurol* 74(4):569–579. <https://doi.org/10.1002/ana.23943>
- Aspuria PJ, Tamanoi F (2004) The Rheb family of GTP-binding proteins. *Cell Signal* 16(10):1105–1112. <https://doi.org/10.1016/j.cellsig.2004.03.019>
- Bilbey DL, Salem H, Grossman MH (1960) The anatomical basis of the straub phenomenon. *Br J Pharmacol Chemother* 15(4):540–543
- Blagosklonny MV (2010) Revisiting the antagonistic pleiotropy theory of aging: TOR-driven program and quasi-program. *Cell Cycle* 9(16):3151–3156. <https://doi.org/10.4161/cc.9.16.13120>
- Blair J, Barry R, Moore DJ, Denniston AK (2017) A comprehensive review of mTOR-inhibiting pharmacotherapy for the treatment of non-infectious uveitis. *Curr Pharm Des* 23(20):3005–3014. doi:<https://doi.org/10.2174/1381612823666170111125550>
- Brodie MJ, Elder AT, Kwan P (2009) Epilepsy in later life. *Lancet Neurol* 8(11):1019–1030. [https://doi.org/10.1016/S1474-4422\(09\)70240-6](https://doi.org/10.1016/S1474-4422(09)70240-6)
- Caban C, Khan N, Hasbani DM, Crino PB (2017) Genetics of tuberous sclerosis complex: implications for clinical practice. *Appl Clin Genet* 10:1–8. <https://doi.org/10.2147/TACG.S90262>
- Calimeri T, Ferreri AJ (2017) m-TOR inhibitors and their potential role in haematological malignancies. *Br J Haematol*. <https://doi.org/10.1111/bjh.14529>
- Clutton-Brock TH, Isvaran K (2007) Sex differences in ageing in natural populations of vertebrates. *Proc Biol Sci* 274(1629):3097–3104. <https://doi.org/10.1098/rspb.2007.1138>
- Donati M, Paolino G, Muscardin L, Panetta C, Donati P (2017) Resolution of benign and malignant sebaceous neoplasms, in a renal transplant patient treated with Everolimus. *Exp Clin Transplant* 15(1):100–102. <https://doi.org/10.6002/ect.2014.0208>
- Duarte-Guterman P, Yagi S, Chow C, Galea LA (2015) Hippocampal learning, memory, and neurogenesis: effects of sex and estrogens across the lifespan in adults. *Horm Behav* 74:37–52. <https://doi.org/10.1016/j.yhbeh.2015.05.024>
- Fedorov LM, Haegel-Kronenberger H, Hirschhain J (1997) A comparison of the germline potential of differently aged ES cell lines and their transfected descendants. *Transgenic Res* 6(3):223–231
- Fedorov LM, Schmittwolf C, Amann K, Thomas WH, Muller AM, Schubert H, Domen J, Kneitz B (2006) Renal failure causes early death of bcl-2 deficient mice. *Mech Ageing Dev* 127(7):600–609. <https://doi.org/10.1016/j.mad.2006.02.009>
- Franz DN (2011) Everolimus: an mTOR inhibitor for the treatment of tuberous sclerosis. *Expert Rev Anticancer Ther* 11(8):1181–1192. <https://doi.org/10.1586/era.11.93>
- Franz DN, Belousova E, Sparagana S, Bebin EM, Frost MD, Kuperman R, Witt O, Kohrman MH, Flamini JR, Wu JY, Curatolo P, de Vries PJ, Berkowitz N, Niolat J, Jozwiak S (2016) Long-term use of everolimus in patients with tuberous sclerosis complex: final results from the EXIST-1 study. *PLoS One* 11(6):e0158476. <https://doi.org/10.1371/journal.pone.0158476>
- French JA, Lawson JA, Yapici Z, Ikeda H, Polster T, Nabbout R, Curatolo P, de Vries PJ, Dlugos DJ, Berkowitz N, Voi M, Peyrard S, Pelov D, Franz DN (2016) Adjunctive everolimus therapy for treatment-resistant focal-onset seizures associated with tuberous sclerosis (EXIST-3): a phase 3, randomised, double-blind, placebo-controlled study. *Lancet* 388(10056):2153–2163. [https://doi.org/10.1016/S0140-6736\(16\)31419-2](https://doi.org/10.1016/S0140-6736(16)31419-2)
- Furian L, Silvestre C, Vallesse L, Baldan N, Donato P, Bonfante L, Cavallin F, Marchini F, Rigotti P (2014) Everolimus associated

- with low-dose calcineurin inhibitors, an option in kidney transplant recipients of very old donors. *Transplant Proc* 46(10): 3390–3395. <https://doi.org/10.1016/j.transproceed.2014.08.044>
- Goorden SM, van Woerden GM, van der Weerd L, Cheadle JP, Elgersma Y (2007) Cognitive deficits in Tsc1+/- mice in the absence of cerebral lesions and seizures. *Ann Neurol* 62(6): 648–655. <https://doi.org/10.1002/ana.21317>
- Goorden SM, Hoogeveen-Westerveld M, Cheng C, van Woerden GM, Mozaffari M, Post L, Duckers HJ, Nellist M, Elgersma Y (2011) Rheb is essential for murine development. *Mol Cell Biol* 31(8):1672–1678. <https://doi.org/10.1128/MCB.00985-10>
- Goorden SM, Abs E, Bruinsma CF, Riemsdijk FW, van Woerden GM, Elgersma Y (2015) Intact neuronal function in Rheb1 mutant mice: implications for TORC1-based treatments. *Hum Mol Genet* 24(12):3390–3398. <https://doi.org/10.1093/hmg/ddv087>
- Gowaty PA (2007) Sex differences in ageing in natural populations of mammals and birds. *J Biosci* 32(7):1221–1222
- Gromov PS, Madsen P, Tomerup N, Celis JE (1995) A novel approach for expression cloning of small GTPases: identification, tissue distribution and chromosome mapping of the human homolog of rheb. *FEBS Lett* 377(2):221–226
- Hanker AB, Mitin N, Wilder RS, Henske EP, Tamanoi F, Cox AD, Der CJ (2010) Differential requirement of CAAAX-mediated posttranslational processing for Rheb localization and signaling. *Oncogene* 29(3):380–391. <https://doi.org/10.1038/onc.2009.336>
- Harai T, Inoue R, Fujita Y, Tanaka A, Horio M, Hashimoto K, Hongou K, Miyawaki T, Mori H (2012) Decreased susceptibility to seizures induced by pentylentetrazole in serine racemase knockout mice. *Epilepsy Res* 102(3):180–187. <https://doi.org/10.1016/j.epilepsyres.2012.06.001>
- Heard JJ, Fong V, Bathaie SZ, Tamanoi F (2014) Recent progress in the study of the Rheb family GTPases. *Cell Signal* 26(9): 1950–1957. <https://doi.org/10.1016/j.cellsig.2014.05.011>
- Jeong A, Wong M (2016) mTOR inhibitors in children: current indications and future directions in neurology. *Curr Neurol Neurosci Rep* 16(12):102. <https://doi.org/10.1007/s11910-016-0708-8>
- Kaufmann MH (2003) *The Atlas of Mouse Development*. Elsevier Academic Press, London
- Krueger DA, Care MM, Holland K, Agricola K, Tudor C, Mangeshkar P, Wilson KA, Byars A, Sahmoud T, Franz DN (2010) Everolimus for subependymal giant-cell astrocytomas in tuberous sclerosis. *N Engl J Med* 363(19):1801–1811. <https://doi.org/10.1056/NEJMoa1001671>
- Krueger DA, Wilfong AA, Mays M, Talley CM, Agricola K, Tudor C, Capal J, Holland-Bouley K, Franz DN (2016) Long-term treatment of epilepsy with everolimus in tuberous sclerosis. *Neurology* 87(23):2408–2415. <https://doi.org/10.1212/WNL.0000000000003400>
- Lee JH, Tecedor L, Chen YH, Montey AM, Sowada MJ, Thompson LM, Davidson BL (2015) Reinstating aberrant mTORC1 activity in Huntington's disease mice improves disease phenotypes. *Neuron* 85(2):303–315. <https://doi.org/10.1016/j.neuron.2014.12.019>
- Li Y, Inoki K, Guan KL (2004) Biochemical and functional characterizations of small GTPase Rheb and TSC2 GAP activity. *Mol Cell Biol* 24(18):7965–7975. <https://doi.org/10.1128/MCB.24.18.7965-7975.2004>
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods* 25(4):402–408. <https://doi.org/10.1006/meth.2001.1262>
- Maas DA, Vallès A, Martens GJM (2017) Oxidative stress, prefrontal cortex hypomyelination and cognitive symptoms in schizophrenia. *Transl Psychiatry* 7:e1171. <https://doi.org/10.1038/tp.2017.138> published online 18 July 2017
- Malagelada C, Ryu EJ, Biswas SC, Jackson-Lewis V, Greene LA (2006) RTP801 is elevated in Parkinson brain substantia nigral neurons and mediates death in cellular models of Parkinson's disease by a mechanism involving mammalian target of rapamycin inactivation. *J Neurosci Off J Soc Neurosci* 26(39):9996–10005. <https://doi.org/10.1523/JNEUROSCI.3292-06.2006>
- Manning BD, Cantley LC (2003) Rheb fills a GAP between TSC and TOR. *Trends Biochem Sci* 28(11):573–576. <https://doi.org/10.1016/j.tibs.2003.09.003>
- Meikle L, Talos DM, Onda H, Pollizzi K, Rotenberg A, Sahin M, Jensen FE, Kwiatkowski DJ (2007) A mouse model of tuberous sclerosis: neuronal loss of Tsc1 causes dysplastic and ectopic neurons, reduced myelination, seizure activity, and limited survival. *J Neurosci Off J Soc Neurosci* 27(21): 5546–5558. <https://doi.org/10.1523/JNEUROSCI.5540-06.2007>
- Mendus D, Rankin-Gee EK, Mustapha M, Porter BE (2015) Increased sensitivity to kindling in mice lacking TSP1. *Neuroscience* 305:302–308. <https://doi.org/10.1016/j.neuroscience.2015.07.075>
- Mizuguchi M, Takashima S (2001) Neuropathology of tuberous sclerosis. *Brain Dev* 23(7):508–515
- Mizuki N, Kimura M, Ohno S, Miyata S, Sato M, Ando H, Ishihara M, Goto K, Watanabe S, Yamazaki M, Ono A, Taguchi S, Okumura K, Nogami M, Taguchi T, Ando A, Inoko H (1996) Isolation of cDNA and genomic clones of a human Ras-related GTP-binding protein gene and its chromosomal localization to the long arm of chromosome 7, 7q36. *Genomics* 34(1):114–118
- Paccalin M, Pain-Barc S, Pluchon C, Paul C, Besson MN, Carret-Rebillat AS, Rioux-Bilan A, Gil R, Hugon J (2006) Activated mTOR and PKR kinases in lymphocytes correlate with memory and cognitive decline in Alzheimer's disease. *Dement Geriatr Cogn Disord* 22(4):320–326. <https://doi.org/10.1159/000095562>
- Panksepp JB, Jochman KA, Kim JU, Koy JJ, Wilson ED, Chen Q, Wilson CR, Lahvis GP (2007) Affiliative behavior, ultrasonic communication and social reward are influenced by genetic variation in adolescent mice. *PLoS One* 2(4):e351. <https://doi.org/10.1371/journal.pone.0000351>
- Pelletier C, Majethia U, Forsythe A (2016) Comparative effectiveness research in renal cell carcinoma: Lenvatinib with everolimus as a potential new treatment option. *J Clin Oncol* 34(7 suppl):27. https://doi.org/10.1200/jco.2016.34.7_suppl.27
- Racine RJ (1972) Modification of seizure activity by electrical stimulation. I. After-discharge threshold. *Electroencephalogr Clin Neurophysiol* 32(3):269–279
- Sanchis-Segura C, Becker JB (2016) Why we should consider sex (and study sex differences) in addiction research. *Addict Biol* 21(5):995–1006. <https://doi.org/10.1111/adb.12382>

- Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 98(3): 503–517
- Tamai T, Yamaguchi O, Hikoso S, Takeda T, Taneike M, Oka T, Oyabu J, Murakawa T, Nakayama H, Uno Y, Horie K, Nishida K, Sonenberg N, Shah AM, Takeda J, Komuro I, Otsu K (2013) Rheb (Ras homologue enriched in brain)-dependent mammalian target of rapamycin complex 1 (mTORC1) activation becomes indispensable for cardiac hypertrophic growth after early postnatal period. *J Biol Chem* 288(14):10176–10187. <https://doi.org/10.1074/jbc.M112.423640>
- Tee AR, Anjum R, Blenis J (2003a) Inactivation of the tuberous sclerosis complex-1 and -2 gene products occurs by phosphoinositide 3-kinase/Akt-dependent and -independent phosphorylation of tuber. *J Biol Chem* 278(39):37288–37296. <https://doi.org/10.1074/jbc.M303257200>
- Tee AR, Manning BD, Roux PP, Cantley LC, Blenis J (2003b) Tuberous sclerosis complex gene products, Tuber and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr Biol* 13(15): 1259–1268
- Terauchi A, Johnson-Venkatesh EM, Toth AB, Javed D, Sutton MA, Umemori H (2010) Distinct FGFs promote differentiation of excitatory and inhibitory synapses. *Nature* 465(7299): 783–787. <https://doi.org/10.1038/nature09041>
- Tian Q, Hanlon Newell AE, Wang Y, Olson SB, Fedorov LM (2011) Complex cytogenetic analysis of early lethality mouse embryos. *Chromosom Res* 19(4):567–574. <https://doi.org/10.1007/s10577-011-9209-4>
- Tian Q, Smart JL, Clement JH, Wang Y, Derkach A, Schubert H, Danilchik MV, Marks DL, Fedorov LM (2016) RHEB1 expression in embryonic and postnatal mouse. *Histochem Cell Biol* 145(5):561–572. <https://doi.org/10.1007/s00418-015-1394-3>
- Uhlmann EJ, Wong M, Baldwin RL, Bajenaru ML, Onda H, Kwiatkowski DJ, Yamada K, Gutmann DH (2002) Astrocyte-specific TSC1 conditional knockout mice exhibit abnormal neuronal organization and seizures. *Ann Neurol* 52(3):285–296. <https://doi.org/10.1002/ana.10283>
- Uhlmann EJ, Li W, Scheidenhelm DK, Gau CL, Tamanoi F, Gutmann DH (2004) Loss of tuberous sclerosis complex 1 (Tsc1) expression results in increased Rheb/S6K pathway signaling important for astrocyte cell size regulation. *Glia* 47(2):180–188. <https://doi.org/10.1002/glia.20036>
- Urano J, Tabancay AP, Yang W, Tamanoi F (2000) The *Saccharomyces cerevisiae* Rheb G-protein is involved in regulating canavanine resistance and arginine uptake. *J Biol Chem* 275(15):11198–11206
- Voss AK, Thomas T, Gruss P (1998) Compensation for a gene trap mutation in the murine microtubule-associated protein 4 locus by alternative polyadenylation and alternative splicing. *Dev Dyn* 212(2):258–266. [https://doi.org/10.1002/\(SICI\)1097-0177\(199806\)212:2<258::AID-AJA10>3.0.CO;2-0](https://doi.org/10.1002/(SICI)1097-0177(199806)212:2<258::AID-AJA10>3.0.CO;2-0)
- Wei KC, Lai PC (2015) Combination of everolimus and tacrolimus: a potentially effective regimen for recalcitrant psoriasis. *Dermatol Ther* 28(1):25–27. <https://doi.org/10.1111/dth.12176>
- Wiebe MS, Nichols RJ, Molitor TP, Lindgren JK, Traktman P (2010) Mice deficient in the serine/threonine protein kinase VRK1 are infertile due to a progressive loss of spermatogenesis. *Biol Reprod* 82(1):182–193. <https://doi.org/10.1095/biolreprod.109.079095>
- Yamagata K, Sanders LK, Kaufmann WE, Yee W, Barnes CA, Nathans D, Worley PF (1994) Rheb, a growth factor- and synaptic activity-regulated gene, encodes a novel Ras-related protein. *J Biol Chem* 269(23):16333–16339
- Yashin AI, Wu D, Arbeev KG, Ukraitseva SV (2010) Joint influence of small-effect genetic variants on human longevity. *Aging (Albany NY)* 2(9):612–620. [10.18632/aging.100191](https://doi.org/10.18632/aging.100191)
- Zeng LH, Xu L, Gutmann DH, Wong M (2008) Rapamycin prevents epilepsy in a mouse model of tuberous sclerosis complex. *Ann Neurol* 63(4):444–453. <https://doi.org/10.1002/ana.21331>
- Zeng LH, Rensing NR, Zhang B, Gutmann DH, Gambello MJ, Wong M (2011) Tsc2 gene inactivation causes a more severe epilepsy phenotype than Tsc1 inactivation in a mouse model of tuberous sclerosis complex. *Hum Mol Genet* 20(3):445–454. <https://doi.org/10.1093/hmg/ddq491>
- Zou J, Zhou L, Du XX, Ji Y, Xu J, Tian J, Jiang W, Zou Y, Yu S, Gan L, Luo M, Yang Q, Cui Y, Yang W, Xia X, Chen M, Zhao X, Shen Y, Chen PY, Worley PF, Xiao B (2011) Rheb1 is required for mTORC1 and myelination in postnatal brain development. *Dev Cell* 20(1):97–108. <https://doi.org/10.1016/j.devcel.2010.11.020>