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RHEB1 expression in embryonic and postnatal mouse

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Abstract Ras homolog enriched in brain (RHEB1) is a member within the superfamily of GTP-binding proteins encoded by the RAS oncogenes. RHEB1 is located at the crossroad of several important pathways including the insulin-signaling pathways and thus plays an important role in different physiological processes. To understand better the physiological relevance of RHEB1 protein, the expression pattern of RHEB1 was analyzed in both embryonic (at E3.5–E16.5) and adult (1-month old) mice. RHEB1 immunostaining and X-gal staining were used for wild-type and *Rheb1* gene trap mutant mice, respectively. These independent methods revealed similar RHEB1 expression patterns during both embryonic and postnatal developments. Ubiquitous uniform RHEB1/β-gal and/or RHEB1 expression was seen in preimplantation embryos at E3.5 and postimplantation embryos up to E12.5. Between stages E13.5 and E16.5, RHEB1 expression levels became complex: In particular, strong expression was identified in neural tissues, including the neuroepithelial layer of the mesencephalon, telencephalon, and neural tube of CNS and dorsal root ganglia. In addition, strong expression was seen in certain peripheral tissues including heart, intestine, muscle, and urinary bladder. Postnatal mice have broad spatial RHEB1 expression in different regions of the cerebral cortex, subcortical regions (including hippocampus), olfactory bulb, medulla oblongata, and cerebellum (particularly in Purkinje cells). Significant RHEB1 expression was also viewed in internal organs including the heart, intestine, urinary bladder, and muscle. Moreover, adult animals have complex tissue- and organ-specific RHEB1 expression patterns with different intensities observed throughout postnatal development. Its expression level is in general comparable in CNS and other organs of mouse. Thus, the expression pattern of RHEB1 suggests that it likely plays a ubiquitous role in the development of the early embryo with more tissue-specific roles in later development.

Keywords *Rheb1* gene · Expression pattern · Immunohistochemistry · X-gal staining · Gene trap · Embryos · Adult mice

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Introduction

Ras homolog enriched in brain (RHEB1) is a GTPase that is conserved from yeast to human. 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). Homologues of the *Rheb* gene have been identified in a number of non-mammalian organisms and

mammals including mouse, rat (Yamagata et al. 1994), and human (Gromov et al. 1995; Mizuki et al. 1996). Lower eukaryotes such as yeast or Drosophila have only one gene, whereas mammals have two different *Rheb* genes *Rheb1* (originally abbreviated as *Rheb*) and *Rheb2* (*RhebL1*) (Patel et al. 2003); for review, see (Aspuria and Tamanoi 2004; Heard et al. 2014). Analysis of *Rheb1* mRNA expression profiles showed that *Rheb1* is ubiquitously expressed, whereas *Rheb2* expression is more restricted (Saito et al. 2005). Genetic studies in Drosophila place RHEB1 in the insulin-signaling pathway, downstream of the tuberous sclerosis complex (TSC1/TSC2) and upstream of mTOR/S6K1 (Stocker et al. 2003). RHEB1 protein is a direct target of the tuberous sclerosis complex (TSC1/2), and it relays upstream signals to regulate mTORC1. TSC1/2 inhibits the mTOR/S6K/4EBP1 signaling pathway by stimulating GTP hydrolysis of RHEB1 and its functions between TSC2 and mTOR (Manning and Cantley 2003). Inactivation of TSC1/2 leads to the activation of the RHEB1/mTOR/S6K signaling cascade (Uhlmann et al. 2004). Although the molecular mechanism has not yet been clearly defined, RHEB-GTP activates the target of rapamycin complex 1 (TORC1). TORC1 consists of multiple protein components, including the mammalian target of rapamycin (mTOR) itself and the regulatory-associated protein of mTOR (Raptor), and is a major regulator of cell growth that phosphorylates multiple downstream targets including p70 S6 kinase (S6K) and the eukaryotic initiation factor 4E (eIF4E)-binding proteins 1 and 2 (4E-BP1 and 4E-BP2). RHEB1 activity can be blocked by rapamycin, an inhibitor of mTOR. As a member of TSC1/TSC2/RHEB1/mTOR signaling pathway, RHEB1 participates in the regulation of cell growth, aging, ribosome biogenesis, protein synthesis, actin cytoskeletal organization, autophagy, and metabolism (Heard et al. 2014). The importance of the *Rheb1* gene in tumorigenesis and other pathologies in patients and in mouse has become appreciated in just the last several years. In humans, inactivation of the TSC1–TSC2 complex leads to inappropriate activation of RHEB1 and results in tuberous sclerosis (TS) disease (Tee et al. 2003a, b). TS is an autosomal dominant hamartoma syndrome caused by mutations in tumor suppressor genes *Tsc1/2*. The hallmark of TS is the development of a type of benign tumors called hamartomas found in brain, kidney, lung, and other organs of TSC patients (Mizuguchi and Takashima 2001).

Overexpression and/or amplification of *Rheb1* was detected in most breast, lung, prostate, skin, pancreatic, and melanoma tumor cell lines (Lu et al. 2010; Basso et al. 2005; Zheng et al. 2010). Also, overexpression and/or mutations of *Rheb1* were identified in tumors in different organs of patients including prostate (Chakraborty et al. 2008; Eom et al. 2008; Nardella et al. 2008), breast, head and neck (Lu et al. 2010), uterus, and kidney (Lawrence et al. 2014). Overexpression of RHEB1 in the tissues of experimental mouse models also stimulates tumor growth. For example, tissue-specific RHEB1 expression in basal epidermal keratinocytes of K14-*Rheb1* transgenic mice leads to activation of the mTOR pathway and development of skin tumors (Lu et al. 2010). Moreover, increased level of RHEB1 can produce rapid development of aggressive and drug-resistant lymphomas in experimental mouse models and is highly expressed in some human lymphomas, resulting in activation of downstream mTOR signaling (Mavrakis et al. 2008). Gene targeting of *Rheb1* in mouse leads to embryonic lethality of homozygote embryos around mid-gestation (Goorden et al. 2011; Zou et al. 2011). Embryonic death most likely results from impaired development of the cardiovascular system (Goorden et al. 2011; Tamai et al. 2013). Furthermore, *Rheb1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) are much smaller than control cells and are severely impaired in their ability to proliferate (Goorden et al. 2011). Conditional knockout of *Rheb1* in neural progenitor cells demonstrated that embryonic RHEB1 expression is essential for mTORC1 signaling and myelination in the brain (Zou et al. 2011). Furthermore, conditional *Rheb1* targeting demonstrates that its expression is important for normal functions in T cells (Delgoffe et al. 2011), B cells (Hamada et al. 2009), and male fertility (Baker et al. 2014).

In summary, RHEB1 is important for the normal functions of a wide array of different cells. The study of its expression in different tissues and organs will provide an opportunity to better understand its role in normal development and perhaps help to discover novel functions and features.

Initially, the expression of *Rheb1 and Rheb2* genes in human has been studied at the level of mRNA by Northern blot analysis (Gromov et al. 1995) and (Saito et al. 2005), respectively. In mouse, expression pattern of the genes has been studied mostly at the level of mRNA by in situ hybridization with particular focus on the central nervous system (CNS) (Yamagata et al. 1994; Magdaleno et al. 2006), (Allen Developing Mouse Brain Atlas). It is known that expression level of mRNA does not always reflect protein level (Kozak 2007). The discrepancy between mRNA and protein levels can arise for a number of reasons, ranging from stage- or tissue-specific differences in the transcription, stability, or translation of mRNAs, to various steps in translation, or even to posttranslational events affecting protein stability and/or ubiquitination. With this potential complexity in mind, we studied the expression of the RHEB1 protein in wide spectrum of tissues during embryonic and postnatal development in wild-type mice. Moreover, to provide an independent approach to report the expression pattern, we produced and used a mutant *Rheb1* gene trap (referred to below as *Rheb1*$^{\Delta/+}$) mouse.

The promoter trap modification of the gene trap method is the tool to target genes that are active in ES cells (Friedrich and Soriano 1991; Skarnes et al. 1992; Wilson et al. 1995; Fedorov 2004). The promoterless pT1βgeo gene trap vector (Wilson et al. 1995) containing a splice acceptor in front of the β-gal reporter gene and expression of the β-gal gene is controlled by the promoter of the "trapped gene." Hence, the endogenous promoter of the trapped gene drives the expression of the reporter gene and thereby reflects the expression pattern of trapped gene. Thousands of genes (including *Rheb1*) were trapped in ES cells by EUCOMM to generate mutant mice; for review, see (Friedel and Soriano 2010).

Materials and methods

Experimental animals

An ES clone harboring a gene trap vector pT1βgeo in intron 1 of *Rheb1* from EUCOMM was used to generate $Rheb1^{\Delta/+}$ mutant mice. These ES cells (129/Sv background) were injected into (B6D2F1)xB6 blastocysts as described elsewhere (Fedorov et al. 1997). After germ line transmission (GLT), the *Rheb1* targeted allele was transferred into a C57Bl/6N background (Charles River Lab; more than 10 backcrosses). Mutant mice were raised under SPF conditions. All mouse experiments 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 Friedrich-Schiller-University (Jena, Germany).

PCR genotyping

Preparation of tissues from embryonic and adult mice and PCR were done as previously described (Tian et al. 2011). The 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 b.p. To genotype wild-type *Rheb1* and $Rheb1^{\Delta}$ allele, the 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 $Rheb1^{\Delta}$ 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.

Determination of sequence of trapped gene

Rapid amplification of cDNA ends (RACE) method was used by EUCOMM to reveal the fused transcript of the first exon of the *Rheb1* and βgeo cassette and was confirmed by our laboratory. RNA was isolated from brain tissue of 1-month $Rheb1^{\Delta/+}$ mouse using TRIZOL Reagent (Invitrogen Life Technologies, cat #15596018) according to the manufacturer's instructions. RNA was reverse-transcribed using QuantiTect Reverse Transcription Kit (QIAGEN, cat #205311). The resulting cDNA was directly sequenced with Rheb–βGalF primer (Supplementary Table S2).

Quantitative RT-PCR

RNA from the tissues of wild-type mice was prepared and reverse-transcribed as described above and treated with DNase. Equal amounts of input RNA were used for all QRT-PCRs. QRT-PCR was performed using SYBR Green I reagent. Primers for Ex1-Ex5 of *Rheb1* (Supplementary Table S2) and transcript-specific primers for β-actin as an endogenous control were used to evaluate the level *Rheb1* mRNA. The 2-ddCT relative quantification method was used to calculate fold difference in transcript levels between samples. 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).

Embryo generation

Staged embryos were produced by mating between wild type mice or between wild-type females and *Rheb1* GT males. The blastocysts were isolated at E3.5 from uterus, and zona pellucida was removed by incubating with 0.5 % pronase during 2-min incubation at RT. After they were subjected for X-gal staining or for in vitro culture 3 days with 5 % CO_2 for 24 h at 37 °C in DMEM containing 15 % fetal bovine serum without leukemia inhibiting factor. The E9.5–E16.5 embryos were removed from uteri and dissected free of extraembryonic membranes in ice-cold phosphate-buffered saline (PBS) and stained with X-gal (below). Embryo staging and organ identification were performed according to Kaufman (1995).

X-gal staining

Whole-mount specimens and cryostat-sectioned tissue samples were stained as previously described (Fedorov et al. 2001). For whole-mount X-gal staining, $Rheb1^{\Delta/+}$ and wild-type embryos at E9.5–E13.5 were fixed for 15–90 min (depending on embryonic stage) in 4 % paraformaldehyde

at 4 °C. After fixation, embryos were washed three times for 5 min in PBS at RT and stained at 37 °C for 3–12 h (depending on stage) in a solution containing 0.04 % X-gal, 0.01 % sodium deoxycholate, 0.02 % Nonidet P-40, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$. After staining, the specimens were embedded in paraffin, and 10-μm sections were cut and mounted with coverslips. For X-gal staining, E16.5 embryos and tissue samples from adult *Rheb1*$^{\Delta/+}$ mice were rinsed in PBS and freeze-mounted in OCT medium (Sakura, Japan). Frozen 25-μm sections were cut on a cryostat and mounted on Super Frost Plus slides. Slides were stained with X-gal solution, counterstained with eosin, and mounted with coverslips.

Immunohistochemistry

Staged wild-type embryos were fixed overnight in Bouin's solution (4 % paraformaldehyde, 0.15 % picric acid, pH 7.4) at 4 °C followed by paraffin embedding and cut on a microtome 10-μm sections. The sections were then blocked for endogenous peroxidase activity using 3 % hydrogen peroxide for 10 min prior to staining. Adult mice were anesthetized and transcardially perfused with physiological saline (0.9 % NaCl + heparin) followed by fixation in Bouin's solution. The brain was then dissected, placed in fixative for 1 h at RT, and subsequently transferred to 20 % sucrose in PBS for 24 h at 4 °C and freeze-mounted in OCT medium (Sakura, Japan). The frozen brain was sectioned on a cryostat, and 25-μm sections were blocked for endogenous peroxidase activity using 0.3 % hydrogen peroxide in methanol for 30 min prior to staining. The sections of embryonic and adult tissues were blocked for 1 h in 5 % normal goat serum (NGS) in TBST at RT and then were incubated overnight in rabbit polyclonal anti-RHEB1 primary antibody (dilution 1:400; Cell Signaling, # 4935) at 4 °C. Paraffin-embedded 10-μm-thick sections of the internal organs were deparaffined, rehydrated, and microwaved 6 min in 10 mM sodium citrate buffer pH 5.5. Subsequently, the slides were incubated 6 min in peroxidase blocking solution (3 % H_2O_2 in PBS). After antigen retrieval, slides were rinsed in PBS, incubated in 20 % sucrose in PBS at +4 °C for 30 min, washed in PBS, and placed in blocking buffer (2.5 % goat serum in PBS), and then were incubated overnight in rabbit polyclonal anti-RHEB primary antibody (dilution 1:400; Cell Signaling, # 4935) at 4 °C. After incubation with primary antibody, the sections were incubated in biotinylated goat anti-rabbit secondary antibody (dilution 1:500) (Molecular Probes, OR, USA, cat # R-21459) for 30 min at RT in 1.5 % NGS in TBST. Antigen–antibody complexes were detected with the immunoperoxidase system (Vectastain Elite ABC Kit, # PK-6101, Vector Laboratories, Inc). Sections were then counterstained using Mayer's hematoxylin, mounted on slides, and imaged under a Leica DM2000 microscope using LAS V3.7 software. For fluorescent immunohistochemistry, the brains were isolated from the wild-type embryos at E15.5 and placed into 4 % paraformaldehyde made in PBS overnight at 4 °C. Brains were then embedded in 2 % agarose, and 50-μm vibratome sections were collected and used for immunofluorescence staining. Before staining, the sections were blocked for 1 h in 0.1 % cold water fish-skin gelatin/1 % BSA/0.5 % TritonX-100/0.1 M Tris-buffered saline (all from Sigma). After blocking, primary antibodies were added (RHEB1, 1:400; Cell Signaling and Nestin, 1:1000; BD Biosciences) overnight at 4 °C on a rotating shaker and then washed 3× in 10 min each wash in 1× PBS. Alexa Fluor fluorescent secondary antibodies (Life Technologies # R37117) were applied to the sections at 1:1000 in the same blocking solution as listed above with 5 % NGS. Sections were place on a rotating shaker for 1 h protected from light before being washed again 3× in 1× PBS as described above. Sections were then mounted on slides and imaged using a Nikon confocal microscope. Identification of tissues of brain and internal organs of adult mice was performed using Atlas of mouse brain (Paxinos 2008) and histological Atlas of mouse tissues (ACVP 2012).

Results

Identification of gene trap vector integration site

The promoterless pT1βgeo gene trap vector containing a splice acceptor in front of the β-gal reporter gene was used to target the *Rheb1* locus (Fig. 1a, b). The precise integration site of the vector into intron 1 of *Rheb1* was identified using PCR and sequencing analysis. Fifteen forward primers (F24–F38) were designed to cover the sequence of intron 1 of *Rheb1* (Fig. 1c; Supplementary Table S1). DNA of ES clone was subjected to PCR using the forward primers in combination with reverse primer R4 corresponding to En-2 intron region of pT1βgeo. PCR with primers F24 and R4 resulted in PCR product of 650 bp. Sequencing of this fragment confirmed the vector integrated 610 bp downstream of exon 1. The integration site is located at NCBIM37:5:24309691 and NCBIM37:5:24309815 of the mouse genome. Based on these results, the new pair of primers was designed and used for PCR genotyping of *Rheb1*$^{\Delta/+}$ mice in addition to pair of LacZ primers (Fig. 1f, Table S1). Fusion of the first exon of *Rheb1* with the βgeo cassette was originally identified by EUCOMM and confirmed in our laboratory (Fig. 1d, e; Supplementary Table S1).

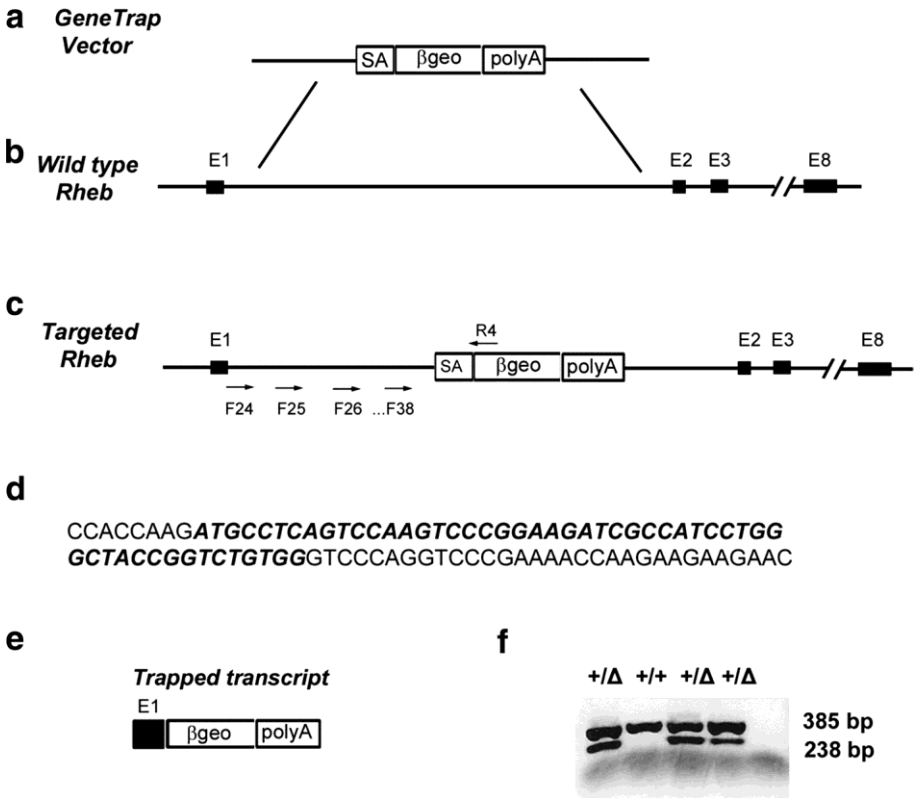

Fig. 1 Gene trap strategy used to disrupt *Rheb1* locus. **a** pT1βgeo gene trap vector does not contain a promoter but contains a splice acceptor in front of the β-geo cassette. **b** Exon–intron structure of wild-type *Rheb1*. **c** Scheme of identification of integration site of pT1βgeo into intron 1 using a set of forward primers F24–F38 and reverse primer R4. **d** Partial sequence of rapid amplification of cDNA ends (RACE) product (5′ → 3′ orientation) of *Rheb1* gene trap ES clone after integration of pT1βgeo. Sequence of exon 1 is shown in *bold italic font*, whereas β-gal is shown in regular font. **e** Scheme of mRNA transcript of trapped allele of *Rheb1*. **f** PCR genotyping of founders harboring gene trap vector. PCR product (238 bp) of trapped allele is indicated by Δ

RHEB expression in mouse embryos

Both X-gal histochemistry and RHEB1 immunohistochemistry (IHC) stainings were used to describe RHEB1 expression pattern in mouse embryos beginning from embryonic day 3.5 (E3.5) through E16.5. After integration of the pT1βgeo vector, the β-gal reporter gene is controlled by the promoter of *Rheb1* and thereby reflects the pattern of expression of the trapped *Rheb1* locus. Initially, β-gal expression was tested in ES cells, and positive X-gal staining was identified (Fig. 2a). Subsequently, activity was tested on embryos of different developmental stages after mating of wild-type females with $Rheb1^{\Delta/+}$ males. Eight of seventeen E3.5 embryos were X-gal positive, whereas all 14 wild-type embryos (after breeding wild-type female and male) were negative (Fig. 2b). We did not perform PCR genotyping of embryos at this stage, but the 47 % of β-gal positive embryos closely reflects the expected 50 % Mendelian distribution, confirming that green–blue embryos were indeed $Rheb1^{\Delta/+}$ mutants. Similar results were produced in embryos at E6.5 cultured in vitro: 3/7- and 0/6 X-gal-positive embryos from wild-type mutant and wild-type crosses, respectively (Fig. 2c, d). Later, the coincidence of positive X-gal staining (Figs. 2e–h, 3) with genotype and Mendelian distribution of $Rheb1^{\Delta/+}$ embryos was confirmed by their PCR genotyping at E9.5, E12.5, and E16.5. Whole-mount staining of embryos at E9.5 and E12.5 revealed a ubiquitous expression pattern (Fig. 2e–h) that is confirmed on histological sections (Fig. 2i, j). Thus, X-gal staining showed the uniform ubiquitous RHEB1 expression pattern on the studied stages (E3.5–E12.5 embryos). We noted a particularly intense expression of RHEB1 in the developing CNS, specifically in the neuroepithelial layer of the midbrain, forebrain and neural tube, and dorsal root ganglia. To gain data about *Rheb1* expression on the second half of gestation, the study was extended on histological sections of E13.5 wild-type embryos by immunohistochemistry (IHC) with an anti-RHEB1 antibody. In spite of early-stage embryos showing uniform ubiquitous RHEB1 expression in CNS and other organs (Fig. 2b–i), the expression in the embryos in the second half of gestation became more complex. Thus, the E13.5 embryos have still ubiquitous expression; however, the intensity of staining varied in different tissues (Fig. 2k). Differential expression is more visible on E16.5 $Rheb1^{\Delta/+}$ embryos after X-gal staining. (Figure 3a; Supplementary Fig. S1b).The $Rheb1^{\Delta/+}$ embryos at E16.5 also revealed RHEB1/β-gal expression in different regions of brain. A significant level of expression was found in cerebral hemispheres, lens of the eye, spinal cord, and spinal ganglia (Fig. 3a). If intensity of staining of different parts of CNS looks similar, then the intensity of staining of other organs varied among them. The greatest level of expression was found in heart (Fig. 3a, b), all regions of intestine (Fig. 3a, d), and urinary bladder (Fig. 3a, c).

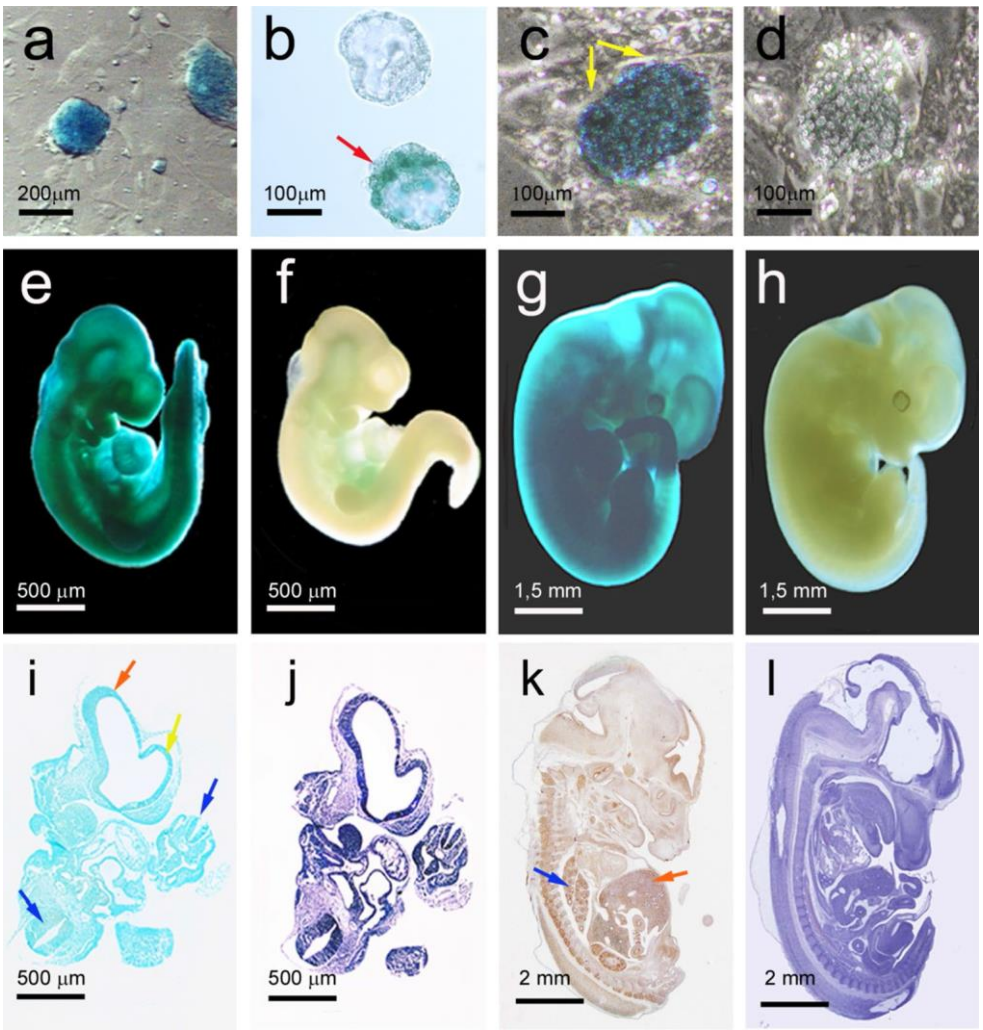

Fig. 2 Detection of RHEB1 expression in wild-type and *Rheb1*$^{\Delta/+}$ mouse embryos at E3.5–E13.5. **a** Colonies of *Rheb1*$^{\Delta/+}$ ES cells expressing β-gal on the wild-type MEF. **b** wild-type (*top*) and *Rheb1*$^{\Delta/+}$ embryos (*bottom*) at E3.5 (blastocysts);. *Red arrow* shows expressing β-gal (*green*) in the inner cell mass (ICM). **c**, **d** In vitro cultured E6.5 wild-type and *Rheb1*$^{\Delta/+}$ embryos, respectively. β-gal activity is greater in the ICM (*yellow arrows*) of *Rheb1*$^{\Delta/+}$ embryo, than in surrounding trophoblast giant cells. β-gal activity is not found in wild-type embryo (*d*). **e**, **f** Lateral view of *Rheb1*$^{\Delta/+}$ and wild-type embryo at E9.5, respectively; X-gal staining. **g**, **h** Lateral view of *Rheb1*$^{\Delta/+}$ and wild-type embryos at E12.5, respectively; X-gal staining. **i** Sagittal section of *Rheb1*$^{\Delta/+}$ embryo at E9.5. Ubiquitous expression (*light blue*) of β-gal including the neuroepithelial layer of midbrain, forebrain, and neural tube (*red*, *yellow*, and *blue arrows*, respectively). **j** Sagittal section of wild-type embryo at E9.5; hematoxylin–eosin staining. **k** Immunohistochemical detection of RHEB1 expression in wild-type E13.5 embryo (sagittal section); *blue* and *red arrows* point lung and liver, respectively. Immunoperoxidase (*brown*) staining with hematoxylin counterstaining (*blue*). **l** The same embryo stained with hematoxylin–eosin

Moderate expression was also observed in stomach, esophagus, kidney (Fig. S1a, b, c), skeletal muscle, follicles of vibrissae, glans penis (Fig. 3a), with low expression in liver, adrenal gland (Supplementary Fig. S1a, b), pancreas, and testes (not shown). Faint RHEB1/β-gal expression) was detected in lung and liver of *Rheb1*$^{\Delta/+}$ embryos at E16.5 (light blue hue against the pink eosin background; Fig. 3a, Supplementary Fig. S1a, b), whereas it is visible well in wild-type embryos at E13.5 after IHC (Fig. 2k). In contrast to *Rheb1*$^{\Delta/+}$ embryos, no β-gal expression was identified in wild-type embryos at any tested stage including E16.5 (Fig. 3e and data not shown). In summary, both X-gal staining and IHC methods showed that RHEB1 protein expression changes with embryonic age from ubiquitous to differential expression patterns throughout development.

To test a possible participation of RHEB1 in regulation of CNS development, we conducted fluorescent immunostaining of the brain of wild-type embryo at E15.5 using anti-RHEB and anti-Nestin antibody. Nestin is an intermediate filament protein selectively expressed in neuronal stem cells (Sunabori et al. 2008). Fluorescent IHC showed co-localization of both proteins in the neuroepithelial layer

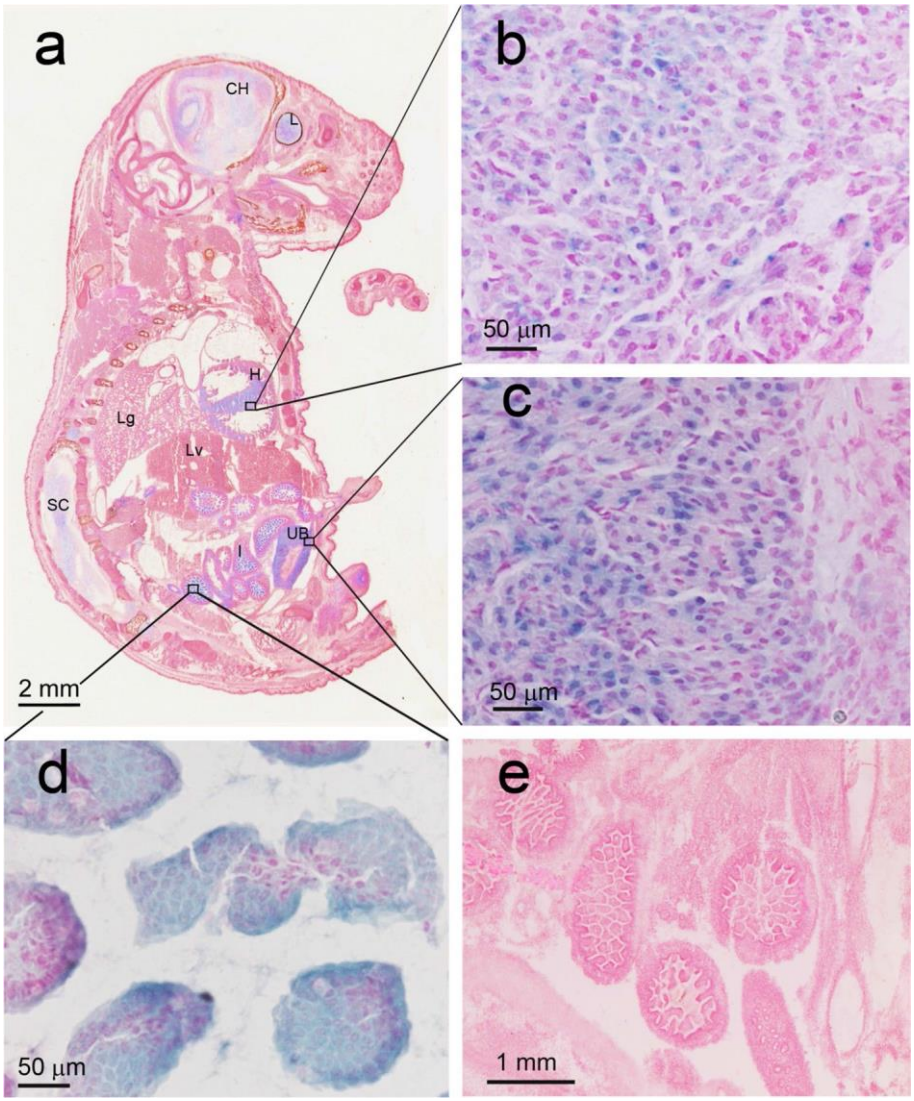

Fig. 3 Identification of RHEB1/β-gal in tissues of *Rheb1*$^{\Delta/+}$ embryos at E16.5 after X-gal staining (*blue*) counterstained with eosin (*red*). **a** Sagittal cryosection, RHEB1/β-gal expression in cerebral hemisphere (CH), lens of eye (L), lung (Lg), liver (Lv), spinal cord (SC), heart (H), intestine (I), urinary bladder (UB). **b** Expression in myocardial cells of right ventricle (cross-sectioned myofibers), **c** in smooth muscle wall of urinary bladder. **d** Epithelial layer of duodenum. **e** Abdominal cavity of wild-type embryo at E16.5 after X-gal staining; sagittal cryosection

of embryonic brain, with projections into the cortex (Supplementary Fig. S2). RHEB1 expression in neuronal stem cells strongly supports suggestion about its participation in regulation of development of CNS.

RHEB1 expression in the brain of adult mice

To describe RHEB1 expression in postnatal wild-type and *Rheb1*$^{\Delta/+}$mice, both IHC and X-gal stainings were used, respectively. Analysis of the brain of 1-month-old wild-type mice by IHC showed widespread expression of RHEB1 on different cortical regions of brain including, frontal cortex, motor cortex, piriform cortex, and somatosensory cortex (Fig. 4a, b, d, e). Similar expression patterns were visible in the brain of *Rheb1*$^{\Delta/+}$ mice after X-gal staining (Fig. 4c, f). Widespread expression was also identified in different subcortical brain structures, including different nuclei of the thalamus (Fig. 5a, b, e, f), hypothalamus, pons, medulla oblongata, and other regions (data not shown). Strong expression was also identified in hippocampal CA fields (Fig. 5a, b, f). Robust expression was also identified in cerebellum, particularly in Purkinje cells and the cells of granular layers that become easily visible after IHC (Fig. 5c, g) and X-gal staining (Fig. 5d, h). Because of their large size, the Purkinje cells of cerebellum were very useful to observe the intracellular localization of RHEB1 protein. Cytoplasmic and nuclear localization of RHEB1 is visible after IHC as well as X-gal staining (Fig. 5g, h). Hence, we show that *Rheb1* is widely expressed in cerebral cortex and different subcortical structures of adult brain.

RHEB1 expression in other organs of adult mice

In addition to the analysis of the RHEB1 expression in the brain, its expression in several other organs was examined. Because of their robust staining during embryonic

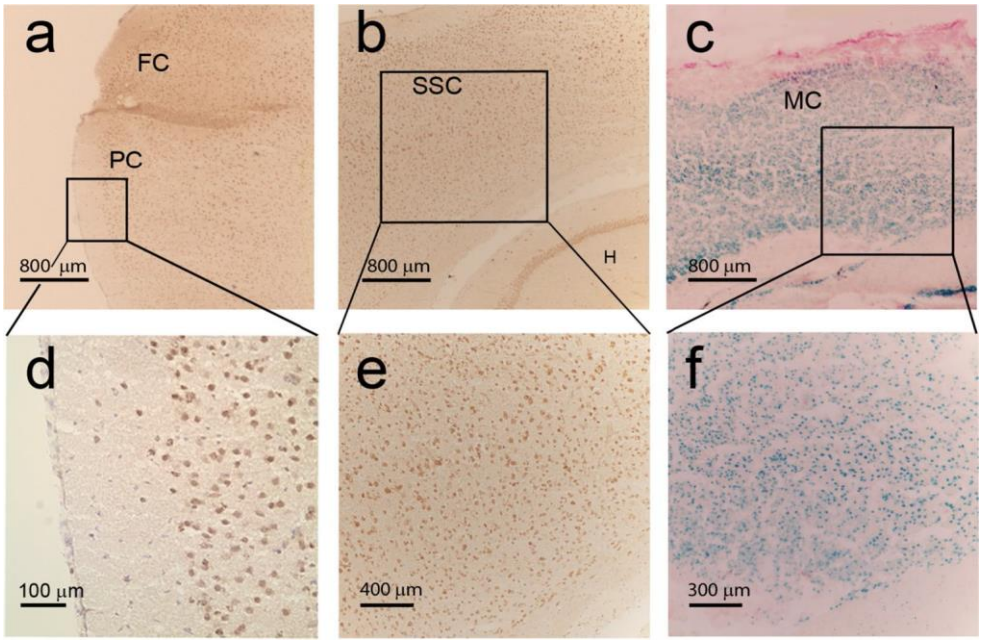

Fig. 4 Detection of RHEB1 expression in the cortex of brain of 1-month-old wild-type and *Rheb1*$^{\Delta/+}$ mice; sagittal sections. **a**, **b**, **d**, **e** RHEB1 expression on different cortical regions of wild-type brain. **c**, **f** RHEB1/β-gal expression in the brain of *Rheb1*$^{\Delta/+}$ mouse. Frontal cortex (FC), motor cortex (MC), piriform cortex (PC), somatosensory cortex (SSC)

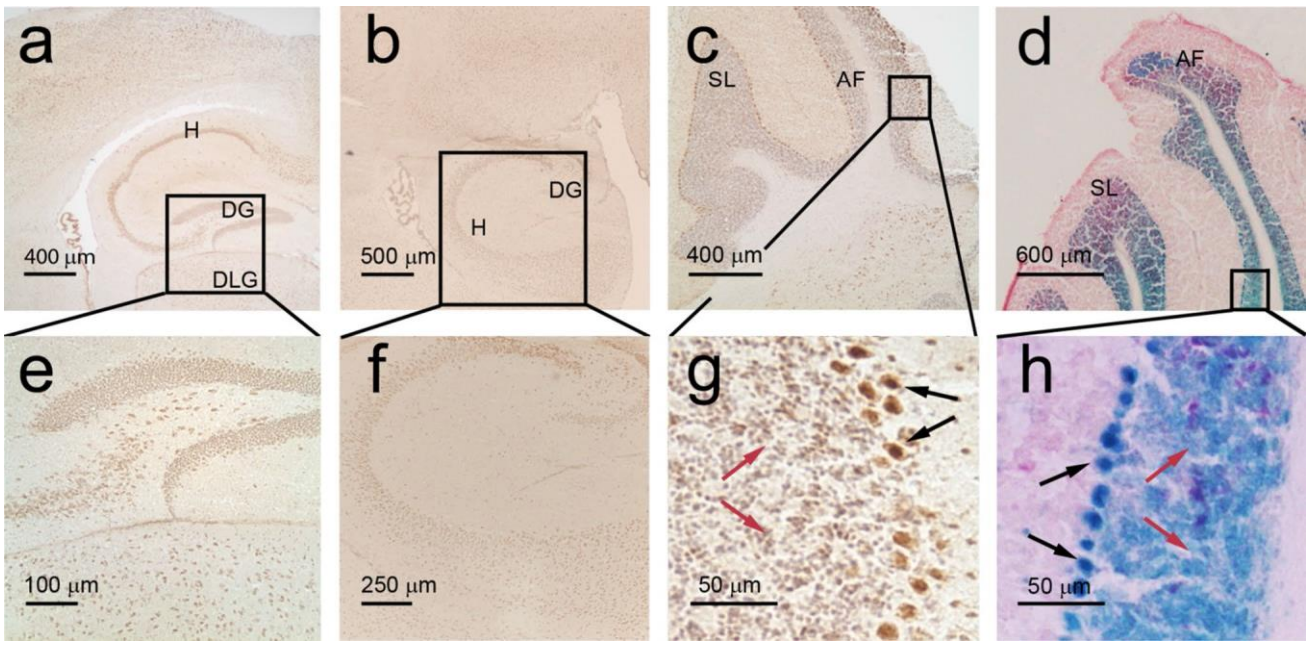

Fig. 5 Detection of RHEB1 expression in the different regions of the brain of 1-month-old wild-type and *Rheb1*$^{\Delta/+}$ mice. **a**, **b**, **e**, **f** RHEB1 expression in different regions on sagittal section of wild-type brain: dentate gyrus (DG), dorsal lateral geniculate nucleus (DLG) of the thalamus, hippocampus (H). **c**, **g** and **d**, **h** RHEB1 expression in cerebellum, of wild-type and *Rheb1*$^{\Delta/+}$ mice, respectively; simple lobule (SL) and ansiform lobule (AF) of cerebellum. **g**, **h** Strong expression of RHEB1 or RHEB1/β-gal in the nuclei of Purkinje cells (*black arrows*) and in the cells of the granular layer (*red arrows*) is observed

development (Fig. 3), we analyzed RHEB1 expression in heart, intestine, urinary bladder, and muscle of adult animals. As shown in Fig. 6a–h, RHEB1 expression remains as intense as in the E16.5 embryo. To compare approximately the level of *Rheb1* transcription in CNS and different organs of adult mice, the level of *Rheb1* mRNA was evaluated by QRT-PCR

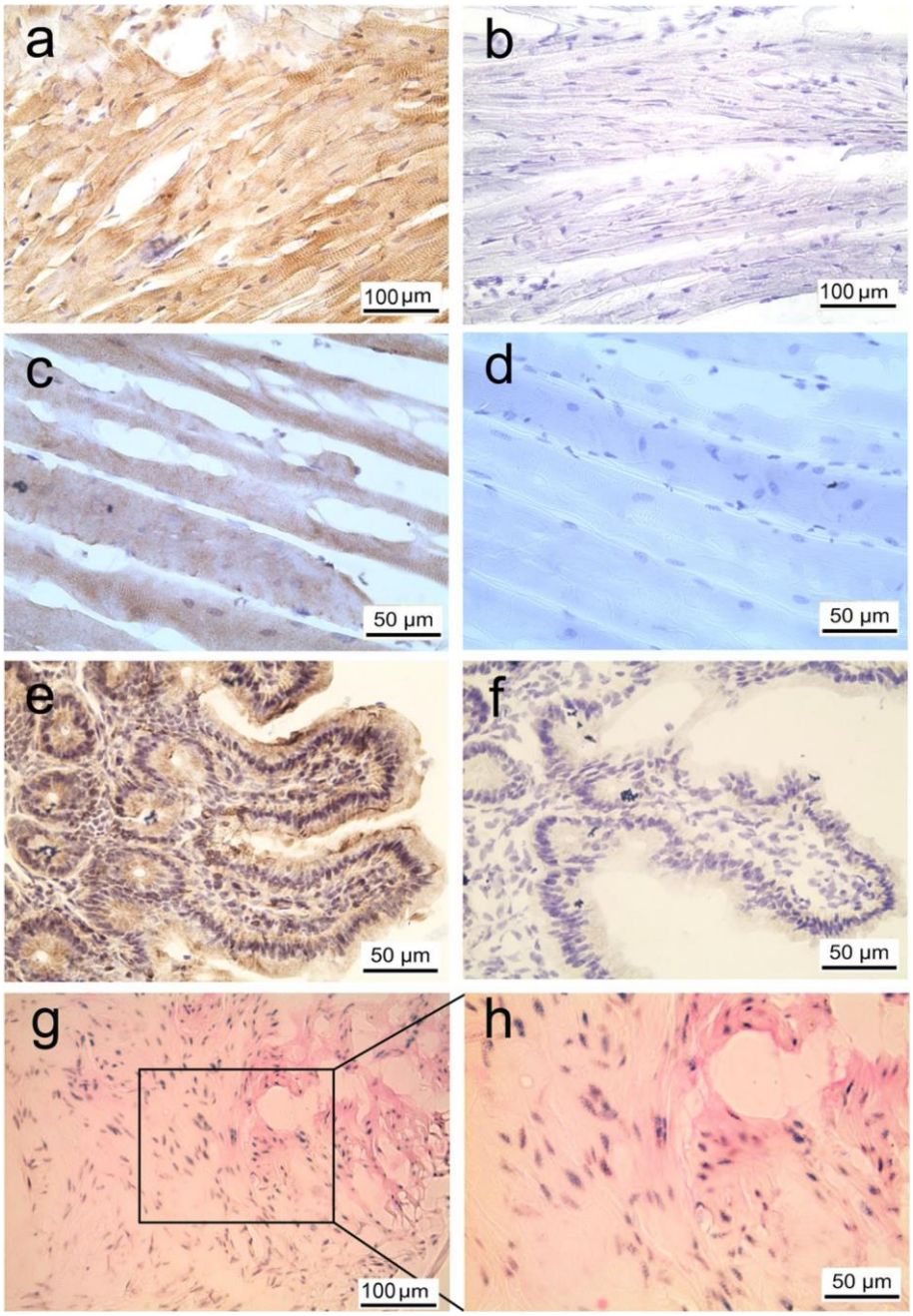

Fig. 6 Immunohistochemical detection of RHEB1 expression on cryosections of the tissues of 4-week-old wild-type and *Rheb1*$^{\Delta/+}$ mice. **a** RHEB1 expression in longitudinally oriented cardiomyofibers of heart, **c** in the myocytes of striated muscle, **e** in epithelium cells of villi of ileum (intestine). **b**, **d**, **f** Corresponding no primary antibody control. Immunoperoxidase (*brown*) staining; in addition, sections were counterstained with hematoxylin (blue). **g**, **h** RHEB1/β-gal expression in smooth muscle of urinary bladder of *Rheb1*$^{\Delta/+}$ mouse after X-gal staining

in several regions of brain and internal organs (kidney and heart) of WT mouse (Fig. S3). Similar high expression was identified in temporal cortex of the brain and heart, whereas other tissue/organs have twofold to threefold less expression. Thus, adult animals have broad spatial expression patterns with different intensity throughout postnatal development.

Discussion

In prior work, the systematic analysis of *Rheb1* expression was performed in mice at age E9.5 to postnatal day 56 (p. 56), specifically focusing on the level of mRNA in the CNS (Allen Developing Mouse Brain Atlas) and (Magdaleno et al. 2006). In the present study, we examined the expression of RHEB1 protein in a wide spectrum of mouse tissues from both preimplantation (E3.5) and postimplantation embryonic stages as well as from adult mice. Moreover, we used wild-type and *Rheb1*$^{\Delta/+}$ mice that allowed us to use two independent methods for assessing RHEB1 expression. In general, RHEB1 IHC and X-gal staining demonstrated similar expression patterns during both embryonic and postnatal development. We found that RHEB1 is actively expressed in preimplantation embryos at E3.5 and

perhaps earlier. It seems that although RHEB1 is actively expressed in preimplantation stages, its deficiency does not affect preimplantation development but is critical soon after implantation in the period of organogenesis, and *Rheb1* $^{-/-}$ embryos die on mid-gestation (Goorden et al. 2011; Zou et al. 2011). Moreover, we observed that embryos at the age E3.5–E12.5 have uniform ubiquitous RHEB1 expression, whereas in E13.5 and E16.5 embryos and later in adult mice, the expression shifts from uniform ubiquitous to a more complex, tissue-specific pattern. Different level of expression is more prominent on the section of *Rheb1*$^{\Delta/+}$ E16.5 embryos (Fig. 3; Supplementary Fig. S1). Although IHC and X-gal staining showed similar expression patterns for wild-type RHEB1 and targeted RHEB1/β-gal proteins, there were staining differences among some organs like in embryonic lung and liver at E13.5 and E16.5. Our gene trap vector pT1βgeo contains *neo* gene, and its expression might slightly reduce RHEB1/β-gal expression in these organs. Based on these observations, we cannot conclude that RHEB1 expression disappeared completely in some organs/tissues. More likely, it is gradual reduction in the expression in some tissues during embryonic development after E12.5, and therefore, in some cases, we cannot see low level of expression on histological section at later stages. To conclude complete absence of expression would require laser capture of individual cells with quantification via QRT-PCR.

RHEB1 protein expression pattern in the brain of wild-type and *Rheb1*$^{\Delta/+}$ embryos and adult mice was similar to *Rheb1* mRNA expression pattern of wild type published previously (Allen Developing Mouse Brain Atlas) and (Magdaleno et al. 2006). Interestingly, we identified the high level of RHEB1 protein not only in different regions of the brain but also in several internal organs such as heart, intestine, and urinary bladder of embryos and adult animals. The comparison of RHEB1 expression levels in internal organs versus brain by QRT-PCR showed that the levels are comparable in general. The strong RHEB1 synthesis in the internal organs might also infer the functional significance of RHEB1 in these organs, and abnormalities in the regulation of RHEB1 in these tissues can initiate pathological changes including cancer.

The goal of our study was to analyze the overall expression pattern of RHEB1 protein in different tissues of mouse. Although our primary goal was not to focus on the details of intracellular localization of RHEB1, we nevertheless observed RHEB1 localization in some cells. The localization RHEB1 in cytoplasm is visible in the cells of brain and internal organs of wild-type mice after IHC as well as in the cells of *Rheb1*$^{\Delta/+}$ mice after X-gal staining. Analysis of nuclear staining is more complicated. In most tissues, it is difficult to conclude that we detected RHEB1 nuclear localization in general even though it looks like nuclear staining. Some cells are small like the cells of the cerebellar molecular layer. In other cases, it is not possible to discriminate details of X-gal staining between subcellular nuclear compartments or perinuclear regions such as the Golgi apparatus or endoplasmic reticulum. The question will be answered after immunofluorescent staining via confocal microscopy in future studies. However, Purkinje cells of the cerebellum are sufficiently large to permit detection of the intracellular localization of RHEB1 protein. In these cells, the cytoplasmic and nuclear localization of RHEB1 is clearly visible after IHC as well as X-gal staining. To perform intracellular signaling from plasma membrane inside cells, the proteins of Ras family have a C-terminal CAAX (C = cysteine, A = aliphatic, X = terminal amino acid) motif which is responsible for the association with plasma and endo membranes; for review, see (Schmick et al. 2015). In contrast to other proteins of RAS superfamily, endogenous RHEB1 has an atypical CAAX motif (CSVM) (Aspuria and Tamanoi 2004). The identification of RHEB1 in cell compartments demonstrated in general localization to the Golgi apparatus, endoplasmic reticulum (Buerger et al. 2006; Jiang and Vogt 2008; Hanker et al. 2010), vesicular structures (Saito et al. 2005; Buerger et al. 2006; Sancak et al. 2008), and mitochondria (Ma et al. 2008). Our surprising observation of RHEB1 localization in the nuclei of the Purkinje cells of cerebellum is supported by fact that other members of TSC1/2/RHEB1/mTOR signaling pathway including tuberin, an upstream regulator of RHEB1 and mTOR target of RHEB1, are also localized in the cytoplasm and nucleus of cells (Rosner et al. 2007). Moreover, by using multiphoton microscopy, EGFP-RHEB1 localization was recently detected in both cytoplasmic and nuclear regions in living HEK293, HeLa, and SHO transiently transfected cells (Yadav et al. 2013). The level of RHEB1 expression was evaluated as ~40 % within the cell nucleus and ~60 % within the cytoplasm, the Golgi apparatus, and ER together.

We identified nuclear localization of RHEB1 in Purkinje cells by both IHC and X-gal staining.

To enter into the nucleus, the nuclear proteins penetrate the nuclear membrane through the nuclear pore complex (NPC) (Davis 1995) or through nuclear import pathways (Sorokin et al. 2007). Nuclear import depends on interaction between the nuclear localization signal (NLS) of nuclear protein and its import receptor. NLS is a polybasic sequence comprised of multiple lysines (K) or arginines (R). Lin et al. (2014) showed that template PxxK[KR] x[KR]xx (a very common NLS motif) may have many modifications and depended on binding affinity to its import receptor, and it can potentially have different nuclear import activity. Exon 1 of *Rheb1* presents in both wild-type *Rheb1* and mutant *Rheb1/β-gal* gene of our experimental mice. After translation of Exon 1, the N-terminus of both

proteins (wild-type RHEB1 and targeted RHEB1/β-gal) contain **P**QS**K**S**RK** amino acid sequence that is similar to **P**xx**K**[**KR**]x[**KR**]. We suggest that PQSKSRK motif functions as a NLS to import RHEB1 into nuclei. The role and precise localization of RHEB1 within the cell nucleus is unclear and must be studied in more detail in the future. Finally, we extended here our knowledge about RHEB1 expression on the protein level during embryogenesis and postnatal development in the CNS and several internal organs. Our studies will thus contribute to further elucidating the function of RHEB1 in its normal biological context as well as its role in the pathogenesis of different diseases.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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