The small GTPase *Rheb* is required for spermatogenesis but not oogenesis

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Abstract

The process of germ cell development is under the tight control of various signaling pathways, among which the PI3K-Akt-mTOR pathway is of critical importance. Previous studies have demonstrated sex-specific roles for several components of this pathway. In the current study, we aimed to evaluate the role of *Rheb*, a member of the small GTPase superfamily and a critical component for mTORC1 activation, in male and female gametogenesis. The function of *Rheb* in development and the nervous system has been extensively studied, but little is known about its role in the germ line. We have exploited genetic approaches in the mouse to study the role of *Rheb* in the germ line and have identified an essential role in spermatogenesis. Conditional knockout (cKO) of *Rheb* in the male germ line resulted in severe oligoasthenoteratozoospermia and male sterility. More detailed phenotypic analyses uncovered an age-dependent meiotic progression defect combined with subsequent abnormalities in spermiogenesis as evidenced by abnormal sperm morphology. In the female, however, germ-cell specific inactivation of *Rheb* was not associated with any discernible abnormality; these cKO mice were fertile with morphologically unremarkable ovaries, normal primordial follicle formation, and subsequent follicle maturation. The absence of an abnormal ovarian phenotype is striking given previous studies demonstrating a critical role for the mTORC1 pathway in the maintenance of primordial follicle pool. In conclusion, our findings demonstrate an essential role of *Rheb* in diverse aspects of spermatogenesis but suggest the existence of functionally redundant factors that can compensate for *Rheb* deficiency within oocytes.

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Introduction

The PI3K/AKT/mTOR signaling pathway is highly conserved among diverse species and plays important roles in the regulation of cell growth. Among other functions, this pathway serves as a key mediator for cellular responses to both the environment and the intracellular milieu, including growth factors, nutrient availability, oxygen levels, and intracellular energy charge (Laplane & Sabatini 2009). Working through this pathway, these environmental and intracellular signals exert many changes in cellular physiology to maintain homeostasis. Among these, changes in the rate of protein translation and turnover, and cell-cycle progression, are among the best characterized (Laplane & Sabatini 2009).

The cell survival and growth kinase PI3K represents one important node of this pathway. PI3K converts membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol (3,4,5)-trisphosphate (PIP₃). PIP₃ acts as a second messenger by membrane-recruitment and activation of the serine–threonine kinase Akt. Pten, a powerful inhibitor of PI3K signaling, acts to

convert PIP₃ back to PIP₂, thereby suppressing Akt activation. Akt in turn phosphorylates and thereby inhibits Tsc2, and also phosphorylates many additional downstream targets including the Foxo transcription factors, which also play multiple roles in cell growth and proliferation (John *et al.* 2008, Goertz *et al.* 2011, Tzivion *et al.* 2011). The AMP kinase (AMPK) is another important node of this pathway, acting via Tsc2. AMPK is activated by energy stress/low nutrient levels (which result in high intracellular levels of AMP) and also phosphorylates Tsc2. Tsc2 can also be phosphorylated by additional kinases (e.g. Redd1, Erk, and Ikkβ) in response to other environmental cues (Laplane & Sabatini 2009). Tsc2 and its physical partner, Tsc1, are structurally unrelated but form an obligate complex whose principal (and indeed, only well-documented) role is as negative regulator (via *Rheb*) of mTORC1 (one of the two physically and functionally distinct signaling complexes in which mTOR is a key component, the other being mTORC2). The only known function of Tsc1 is to stabilize Tsc2 and prevent its degradation.

Rheb is a small GTPase and member of the Ras family and is ubiquitously expressed in mammals. Tsc2 contains a GAP (GTPase-activating) domain that stimulates the intrinsic GTPase activity of Rheb, thereby promoting the conversion of Rheb-GTP into Rheb-GDP. Whereas Rheb-GTP potentially activates mTORC1, Rheb-GDP is inactive. Rheb directly activates mTORC1 through mechanisms that are not well understood. These findings are based on numerous genetic and biochemical studies performed in vertebrate and vertebrate model systems and appear to be universal. For example, in *Tsc1* or *Tsc2* mutant cells, mTORC1 is constitutively active, potentially stimulating cell growth (Fingar & Blenis 2004, Zou *et al.* 2011).

mTORC1 in turn regulates protein translation largely through two proteins that are key components of the translational control machinery, ribosomal protein S6 (S6) and 4E-binding protein (4EBP), to potentially drive protein translation and cell growth. Phosphorylation of p70S6 kinase (S6K) results in the activation of S6, which allows for the formation of the mRNA translation machinery. mTORC1 also increases protein synthesis by inactivating the translational repressor 4EBP which binds to and inhibits the eukaryotic translation initiation factor 4E (Laplanche & Sabatini 2009).

Several studies have implicated some of these key components of the PI3K/AKT/mTOR pathway in spermatogenesis and oogenesis. *Foxo1* is specifically expressed in undifferentiated spermatogonia, and the three Foxos together regulate multiple steps of spermatogenesis including stem cell maintenance and differentiation (Goertz *et al.* 2011, Tarnawa *et al.* 2013). *Pten* is also required for spermatogenesis; its inactivation with the germ line-specific *Vasa-Cre* (*Tg(Ddx4-cre)1Dcas*; Gallardo *et al.* 2007a) results in severe defects in spermatogonial maintenance and differentiation, at least in part through *Foxo1* (Goertz *et al.* 2011). Other studies have implicated mTORC1 as a regulator of spermatogenesis and spermatogonial stem cell maintenance (Hobbs *et al.* 2010).

In the ovary, *Foxo3* is specifically expressed in primordial oocytes, where it serves to potentially restrain their activation. In *Foxo3*-null mice, primordial follicles develop normally, but undergo global activation immediately after they are formed, resulting in a syndrome of deregulated follicle growth and premature female infertility (Castrillon *et al.* 2003, Hosaka *et al.* 2004, John *et al.* 2007, 2008). Strikingly, an identical ovarian phenotype occurs following conditional germ line inactivation of three other PI3K/AKT/mTOR pathway components: *Pten*, *Tsc1*, and *Tsc2*, underscoring the importance of this pathway in the maintenance of the female germ line (John *et al.* 2008, Reddy *et al.* 2008, Adhikari *et al.* 2009, 2010). *Pten* inactivation results in global primordial follicle activation through Akt and consequent phosphorylation and cytoplasmic sequestration/inactivation of *Foxo3* (John *et al.* 2008). *Tsc1* and *Tsc2* inactivation in

oocytes results in global primordial activation associated with mTOR pathway hyperactivity and increased phosphorylation of S6K and 4EBP within the oocytes (Adhikari *et al.* 2009, 2010). These recent mouse models have laid the groundwork for uncovering the regulation of primordial follicle maintenance, but further investigations are required to identify how these canonical PI3K branch components (*Pten*, *Akt*, and *Foxos*) interact with canonical mTOR branch components (*Tsc1/Tsc2*) (Sullivan & Castrillon 2011). To further explore these questions and the role of mTOR signaling in gametogenesis, we studied the impact of *Rheb* inactivation in both the male and female germ line.

Materials and methods

Mouse strains, breeding, and tissue processing and expression analyses

This study was approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee. Generation of both the *Rheb*-floxed and *Vasa-Cre* alleles and genotyping protocols were previously described (Gallardo *et al.* 2007a, Zou *et al.* 2011). *Vasa-Cre* males were crossed with *Rheb^{fl/fl}* females. *Vasa-Cre; Rheb^{fl/+}* male offspring were again crossed to *Rheb^{fl/fl}* females to produce *Vasa-Cre; Rheb^{fl/-}* experimental mice. Matched *Vasa-Cre* negative siblings were used as controls. All tissues were processed immediately after procurement. The tissues were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin, and cut into 5- μ m sections for immunohistochemistry (IHC) or hematoxylin and eosin (H&E) staining. Tissue sections from experimental and control samples were placed on the same slide to ensure identical processing. Expression analyses by digital Northern were conducted as previously described (Gallardo *et al.* 2007b).

Sperm analysis and breeding assays

A complete sperm analysis was performed to quantify the output of the testis. Epididymal sperm density was obtained by counting the number of hematoxylin-stained sperm heads in three 20 $\times$ fields for each epididymis. The area in the seminiferous tubule was calculated using the ImageJ (Bethesda, MD, USA) Software package (Schneider *et al.* 2012). Data were graphed as total sperm per square pixel. Epididymal sperm concentration was used as a secondary measure of total sperm output. One epididymis was dissected and placed into 1 ml of PBS. Approximately five cuts were made in the epididymis followed by 30 min incubation at 37 °C to release the sperm from the tissue. Sperm concentration was then measured using a fixed cover slip counting chamber (Cell-Vu, DRM-600). Motility was also noted, but experimental samples contained too few sperm for percentages to be accurately calculated. To study sperm morphology, a small drop of the sperm suspension was placed on a glass microscope slide and spread evenly across the slide with a cover slip. The sperm smear was allowed to dry and was then subjected to Diff-Quik staining (Fisher Scientific, Pittsburgh, PA, USA; 65044C). Previously published criteria were used for the determination of normal sperm

morphology (Wyrobek & Bruce 1975). Breeding assays were performed as previously described (Gallardo *et al.* 2008). Three-week-old experimental and sibling control males were placed with two 6-week-old FVB females and observed for 20 weeks. Females were placed with one FVB male and observed for 18 weeks.

Immunohistochemistry and quantitative analyses of morphology in tissue sections

Slides were deparaffinized in xylene, hydrated in an ethanol series, subjected to antigen retrieval by boiling in 10 mM sodium citrate (pH 6.0) for 15 min, and cooled at room temperature (RT) for 20 min. After peroxidase blocking (3% hydrogen peroxide in water) for 30 min, slides were washed in water and then blocked in BSA (1% in 1 × PBS) for 15 min. The slides were then incubated with primary antibody overnight at 4 °C, subjected to a TBST (1 M Tris-HCl, 5 M NaCl, and 1 × Tween-20) wash series, incubated with secondary antibody for 30 min at RT (ImmPRESS; Vector Laboratories (Burlington, CA, USA)), and subjected to a second TBST wash series. Signal was detected using a DAB liquid chromogen substrate kit (DAKO, Carpinteria, CA, USA). Slides were then counterstained with hematoxylin, rinsed in water, and air-dried.

Antibodies and titers used included Foxo1 (rabbit monoclonal; Cell Signaling Technology (Danvers, MA, USA), #2880; 1:500), Crem1 (rabbit polyclonal; Santa Cruz Biotechnology, #SC-440; 1:1000), mTOR (rabbit monoclonal; Cell Signaling Technology, #2983; 1:1000), p-mTOR (rabbit monoclonal; Cell Signaling Technology, #5536; 1:1000), p-4EBP (rabbit monoclonal; Cell Signaling Technology, #9455; 1:1000), p-S6K (rabbit monoclonal; Cell Signaling Technology, #9234; 1:1000), p-S6 (rabbit monoclonal; Cell Signaling Technology, #4857; 1:1000), pH2AX (rabbit monoclonal; Cell Signaling Technology, #9718; 1:200 000), Ki67 (rabbit monoclonal; Thermo Scientific (Waltham, MA, USA), #RM-9106-FO; 1:500), germ cell nuclear antigen (1:200 IHC) (courtesy of George Enders, University of Kansas). Cell counts were performed to quantify the four principal cellular stages of sperm development (spermatogonia, spermatocytes, round spermatids, and elongated spermatids). Counts for undifferentiated spermatogonia and round spermatids were facilitated by immunostaining these cell types with Foxo1 and Crem1 respectively, as described in the text. Spermatocytes and elongated spermatids were identified and counted based on their distinct morphology in routinely stained histological sections. For each cell type, a minimum of 20 tubules (staged from IV to VII) were counted per mouse.

Figure 1 RNA expression analysis of *Rheb* and *RhebL1*. Graphs show relative expression levels of single probe set for indicated gene across multiple samples; error bars represent s.e.m. Samples are (left to right) *Foxo3*^{+/+} PD1, 3, 7, and 14 ovaries (black), *Foxo3*^{-/-} PD1, 3, 7, 14 ovaries (red), adult ovary, laser-capture microdissected (LCM) primary and secondary oocytes, LCM somatic cells (primary plus secondary granulosa cells and surrounding stroma), eggs, cumulus cells, ES cells, normal adult testis, SI/Sld (germ cell-depleted) adult testis, *Foxo3*^{+/+} E11 embryos, *Foxo3*^{-/-} E11 embryos, adrenal gland, placenta, uterus, bone marrow, spleen, thymus, brain, eye, skeletal muscle, heart, intestine, kidney, liver, and lung. (A) *Ddx4* (*Vasa*) as representative control (germ cell-specific gene in both males and females). (B) *Rheb*. (C) *RhebL1* (probe set #1). (D) *RhebL1* (probe set #2).

Statistical analysis

Data were graphed and analyzed using GraphPad Prism 5 (La Jolla, CA, USA). Two-tailed Student's *t*-tests were used to evaluate significance and calculate *P* values, with threshold values as described in the results or figure legends. Error bars represent S.E.M. values. A *P* value of <0.05 was considered significant.

Results

Expression of *Rheb* and *Rheb1* in the gonad and the adult germ line

We first characterized the expression of *Rheb* and its closest homolog in the murine genome, *Rheb1* (52% identical and 73% similar at the amino acid level). We took advantage of a previously described microarray-based platform for digital Northern analysis of reproductive tissues and cell types (Gallardo *et al.* 2007b, Contreras *et al.* 2010). The samples subjected to expression profiling included WT and *Foxo3*-null ovaries at postnatal day (PD) 1, 4, 7, and 14 (selected because primordial follicles make up a disproportionately greater fraction of the ovarian mass early in life, leading to good representation of primordial follicle transcripts). In addition, laser-capture microdissected (LCM) oocytes and surrounding granulosa cells from primary to secondary follicles (i.e. early growing follicles) were included given the important role of the mTOR/PI3K pathway in early follicle growth (John *et al.* 2008, Adhikari *et al.* 2009, 2010). Other samples included superovulated eggs, ES cells, normal testis, germ cell-deficient SI/SI(d) testes, embryonic day 11 (E11) embryos, and diverse adult tissues (Fig. 1).

Ddx4 (a.k.a. *Vasa*) was included as a representative germ cell-specific control validating the samples and methodology. *Ddx4* was expressed in all PD1–14 ovarian samples and in LCM oocytes, but was expressed at low (nearly baseline) levels in the adult ovary, consistent with the fact that oocytes make up only a very small percentage of the ovarian mass (Gallardo *et al.* 2007b). Among adult tissues, *Ddx4*, was expressed at high levels only in the testis (only tall peak in the right half of the panel) but nearly absent in germ cell-deficient testes, as expected given *Ddx4*'s germ cell-specific expression. In contrast, *Rheb* was much more broadly expressed with high expression across all tissues, including ovaries and testis, where it appears to be expressed in both the germ line and somatic cells. In contrast, *Rheb1* was expressed at lower apparent levels overall (with lower signal strengths), but like *Rheb* was also very broadly expressed. Although *Rheb* was represented on the array by a single probe, *Rheb1* was represented by two separate probes. Note that the expression peaks from the second probe are virtually superimposable on the first, consistent with faithful readout of gene expression levels across probes. Neither *Rheb* nor *Rheb1* were differentially expressed in *Foxo3*-

null ovaries (red bars), arguing that neither genes is a direct *Foxo3* target. Thus, in summary, *Rheb* is expressed in gonads and the female germ line, making it a plausible candidate for a biological role in the germ line.

Conditional inactivation of *Rheb* in the germ line with *Vasa-Cre*

A floxed *Rheb* allele (*Rheb^f*) was crossed with the germ cell-specific Cre recombinase, *Vasa-Cre*. This strategy allows for prenatal deletion of *Rheb* in both the male and female germ lines, as *Vasa-Cre* is expressed in both male and female germ cells beginning at E15 (Gallardo *et al.* 2007a). Available PCR genotyping protocols permit specific amplification of the WT, floxed, and null (i.e. Cre-recombined) *Rheb* alleles. Confirmation of *Vasa-Cre*-mediated excision of the floxed third exon of the *Rheb* locus was performed through PCR genotyping of both the *Rheb*-floxed and *Rheb*-null alleles in *Vasa-Cre;Rheb^{f/+}* mice. Both the WT and floxed *Rheb* alleles were detected in multiple tissues of *Vasa-Cre;Rheb^{f/+}* mice: however, the floxed allele was clearly less abundant in the testis, consistent with germ cell Cre recombination and *Rheb* inactivation (Fig. 2A). The *Rheb*

Figure 2 Genotype confirmation of germ cell *Rheb* conditional knockout. (A) An ethidium bromide-stained 1% agarose gel shows the presence of the *Rheb*-floxed allele (850 bp) in tail DNA from a *Rheb* homozygous-floxed mouse as well as all other tissues of a *Vasa-Cre;Rheb^{f/+}* (heterozygous) male. The floxed (f) band is decreased in the *Vasa-Cre;Rheb^{f/+}* testis consistent with *Vasa-Cre*-mediated recombination in germ cells. The wt (+) band (650 bp) is observed in tissues from this heterozygous male mouse, as expected. (B) Cre-mediated excision was further confirmed by the presence of the *Rheb*-null allele (650 bp) in a *Vasa-Cre;Rheb^{f/+}* testis as well as a *Rheb^{f/-}* control testis. The *Rheb*-null allele was not detected in other tissues (e.g. skin, lung, and spleen). NS, non-specific PCR product (~750 bp).

Figure 3 Conditional *Rheb* inactivation results in a progressive defect of spermatogenesis. (A) Intact testes. (Right) *Vasa-Cre; Rheb^{f/f}* and (left) *Rheb^{f/f}* sibling control. Ruler markings, 1 mm. (B) Testis weights in mice of different ages. Red, *Vasa-Cre; Rheb^{f/f}* and blue, *Rheb^{f/f}* sibling control. * $P < 0.05$ and ** $P < 0.005$, by unpaired *t*-test (2 months, $n = 2$; 3 months, $n = 3$; 4 months, $n = 1$ (no statistical test performed); and 5 months, $n = 3$) (C) H&E stained tissue sections from testes of 2- to 5-month-old *Rheb* cKO and sibling control mice. Representative fields illustrate an abnormal organization of seminiferous tubules at the 2-month time point and the age-dependent defect in meiotic progression resulting in the progressive loss of round and elongating spermatids in the 5-month-old testis. Arrowheads indicate large multinucleated germ cells. Bar = 50 μ m.

null allele was detected in the testis of the *Vasa-Cre; Rheb^{f/f}* mouse but not in other tissues (Fig. 2B) again consistent with germ cell-specific Cre activity. Germ line transmission of the *Rheb*-null allele was also confirmed by genotyping progeny from *Vasa-Cre; Rheb^{f/f}* females crossed with WT males; all progeny carried the null allele ($n = 18$), confirming efficient, biallelic ablation of *Rheb* within oocytes.

Gross and histological analyses of gonads from male and female *Rheb* conditional knockout mice

By gross examination, adult *Rheb* conditional knockout (cKO; *Vasa-Cre; Rheb^{f/f}*) testes were much smaller than those of *Vasa-Cre*-negative (*Rheb^{f/f}*) sibling controls (Fig. 3A). A progressive atrophy of the testes was observed in experimental males. *Vasa-Cre; Rheb^{f/f}* testis weights underwent a further twofold decrease between 3 and 5 months of age (Fig. 3B). Testis sections from 3-month-old *Vasa-Cre; Rheb^{f/f}* males exhibited hypospermatogenesis with all stages of sperm present. However, in aged mice the seminiferous tubules were

more severely affected by the loss of *Rheb*. At 5 months of age, there was severely decreased spermatogenesis and while spermatocytes were present, very few round or elongated spermatids were observed (Fig. 3C). In contrast, *Vasa-Cre; Rheb^{f/f}* ovaries were grossly unremarkable (Fig. 4A). Histological analysis of ovary sections showed the presence of growing follicles of all stages (Fig. 4B) and a healthy pool of quiescent primordial follicles (Fig. 4C).

Rheb cKO males have decreased sperm counts and abnormal sperm morphology

A complete sperm workup was performed to identify defects in total sperm count, morphology, and motility. Epididymides from both *Vasa-Cre; Rheb^{f/f}* and *Rheb^{f/f}* controls were examined histologically for the presence of mature spermatozoa (Fig. 5A). Quantification of sperm head density clearly showed a progressive age-dependent decrease in sperm production (Fig. 5B). This decrease was also quantified through epididymal sperm concentration counts (Fig. 5C). By 4 months of age, the

Figure 4 Ovaries of *Rheb* cKO mice are grossly normal and contain growing follicles of all stages, as well as morphologically normal primordial follicles. (A) Intact ovaries of *Rheb* cKO (*Vasa-Cre; Rheb*^{f/f}) females were of similar size and shape relative to sibling (*Rheb*^{f/f}) controls. Bar = 1 mm. (B) H&E stained tissue sections from *Rheb* cKO and sibling control ovaries revealed growing follicles of all stages. Bar = 250 μ m. (C) Higher magnification shows growing primary and secondary follicles. Insets: morphologically normal, quiescent primordial follicles. Scale bar = 50 μ m.

Vasa-Cre; Rheb^{f/f} males exhibited severe oligoasthenoteratozoospermia. The predominant morphological defect observed was amorphous head shapes (Fig. 5D).

Rheb is required for male, but not female, fertility

To quantify the effect of *Rheb* cKO on fertility, breeding assays were set up for *Vasa-Cre; Rheb*^{f/f} males and females and *Rheb*^{f/f} sibling controls. The *Rheb* cKO female mice were fertile; their fecundity was indistinguishable from controls and they continued to have litters of normal size up to 18 weeks of age (Fig. 6A and B). The male *Rheb* cKO mice, however, did not produce any offspring over a period of 20 weeks (Fig. 6C and D). This complete male infertility is expected given the oligoasthenoteratozoospermia observed above.

Histomorphometric analyses of gametogenesis expose a defect in meiotic progression

As an initial characterization of the disrupted seminiferous tubules, and to identify potential developmental blocks in spermatogenesis, cell counts were carried out to quantify the numbers of spermatogonia, spermatocytes, round spermatids, and elongated spermatids (Fig. 7A). *Foxo1* expression was used as a convenient marker of undifferentiated spermatogonia (Goertz *et al.* 2011, Tarnawa *et al.* 2013). Immunostaining and counts revealed normal numbers of undifferentiated spermatogonia in *Vasa-Cre; Rheb*^{f/f} testes at all time points (Fig. 7C). Spermatocytes were counted based on their

Figure 5 Total sperm production is drastically decreased and remaining sperm are malformed. (A) Histology of epididymides from control and experimental mice. Bar = 100 μ m. (B) The decrease in epididymal sperm was quantified by sperm head density counts ($n=1$ for all time points and genotypes). (C) Total sperm counts were performed after releasing the sperm from the epididymis. *** $P<0.0001$. Red, *Vasa-Cre; Rheb*^{f/f}, $n=4$ and blue, *Rheb*^{f/f}, $n=5$ sibling controls. (D) The few sperm obtained from the epididymis of *Rheb* cKO mice were all immotile and exhibited abnormal morphology. Bar = 10 μ m; all panels at same magnification.

Figure 6 Serial breeding assays reveal that *Rheb* cKO males are sterile while *Rheb* cKO females are fertile with normal fecundity to 18 weeks of age. (A) Aggregate (i.e. all animals of the same genotype averaged) and (B) individual (i.e. every line represents a single animal) representations of total progeny per female. (C) Aggregate and (D) individual representations of total progeny per male.

large size and distinctive cellular morphology. A significant accumulation of spermatocytes was observed at 2 months but numbers reverted to normal at 3 and 4 month time points. By 5 months of age, a significant decrease in spermatocyte numbers was observed (Fig. 7D). Quantifications of round and elongated spermatids were performed through Crem1 immunostaining and cellular morphology respectively

(Delmas *et al.* 1993; Fig. 7B). Decreases in round and elongated spermatid counts were observed at early time points (Fig. 7C and D). Thus in summary, *Rheb* cKO males exhibited a striking age-dependent decrease in spermatid production and a more modest decrease in spermatocytes, while maintaining normal numbers of undifferentiated spermatogonia. These results point to abnormalities in meiotic progression.

Spermatogonia continue to proliferate and initiate meiosis

In light of the progressive decrease in spermatocytes in the *Vasa-Cre; Rheb^{f/f}* mice, proliferation, and meiotic entry were further evaluated by immunostaining for Ki67 and phosphorylated H2AX (pH2AX) respectively. Ki67 is a marker of actively cycling cells, while H2AX is phosphorylated in response to double-stranded DNA breaks (Hamer *et al.* 2002, Akbay *et al.* 2013). pH2AX nuclear foci serve as a marker of meiotic recombination in response to the formation of chiasma between sister chromatids in leptotene spermatocytes. However, detailed counts of cells positive for Ki67 and pH2AX foci revealed no significant differences between *Vasa-Cre; Rheb^{f/f}* and control males at all time points studied (Fig. 8). Therefore, spermatogonial proliferation and meiotic entry *per se* appears largely unaffected in *Rheb* cKO males, again consistent with a severe defect in meiotic progression.

The activation status of mTOR, S6K, and 4EBP appears to be unaffected in Vasa-Cre; Rheb^{f/f} mice

We then sought to determine the activation status of mTOR pathway components in *Rheb* cKO testes as well as ovaries. Immunohistochemistry was performed for phosphorylated (i.e. activated) mTOR pathway members p-mTOR, p-S6K, p-S6, and p-4EBP using well-validated phosphorylation site-specific antibodies for each of these factors. However, no significant differences were observed in the phosphorylation state of any of these mTOR pathway components in *Rheb* cKO vs control testes, as gauged by staining intensity, when specific spermatogenic stages were compared (Fig. 9).

Discussion

These studies document a male infertility phenotype associated with multiple defects in the meiotic and post-meiotic stages of spermatogenesis in mice with conditional genetic inactivation of *Rheb* in the germ line. Interestingly, given previous studies implicating mTORC1 in stem cell self-renewal, *Rheb* was dispensable for spermatogonial self-renewal and mitotic proliferation (Hobbs *et al.* 2010). However, *Rheb* was necessary for spermiogenesis, as *Rheb* males exhibited

Figure 7 Cell counts reveal meiotic progression defect. (A) Foxo1 and (B) Crem1 immunostains of *Rheb* cKO (*Vasa-Cre; Rheb*^{f/f}) and control testes at 2 and 5 months of age. Bar=50 and 100 μ m respectively. (C) Quantification of Foxo1- and Crem1-positive cell counts in 20 tubules; * P <0.05 and ** P <0.0005 by unpaired *t*-test. (D) Counts of spermatocytes and spermatids in hematoxylin-stained tissue sections. The counts of elongated spermatids closely mirrored the observed decrease in Crem1⁺ round spermatids; * P <0.05 and ** P <0.005 by unpaired *t*-test.

greatly reduced epididymal sperm numbers, and the few sperm present were immotile and exhibited grossly abnormal head morphology. A delay in meiotic progression was also evident at 2 months of age as spermatocyte counts were significantly increased. Immunostains for meiotic initiation-associated double-strand DNA breaks were relatively unaffected, further suggesting that these defects were due to delays or abnormalities in meiotic progression, and not meiotic initiation *per se*. Interestingly, these pleiotropic phenotypes were age-dependent, and in 5-month-old males, the meiotic defect was even more severe, with greatly decreased numbers of post-meiotic spermatids.

A large number of mouse mutants with combined defects in meiosis and spermiogenesis have been described. These mutations affect a wide range of processes, from acrosome and tail formation to misregulation of late spermiogenesis gene expression (Yan 2009). The multiple defects that we have described resemble in several respects the spermatogenesis phenotype

previously described for mice with conditional germ line inactivation of *Dicer*, the miRNA-processing enzyme (Korhonen *et al.* 2011, Romero *et al.* 2011). The *Dicer* cKO is also infertile due to cumulative defects in meiosis and spermiogenesis that result in an oligoasthenoteratozoospermia phenotype. Delayed progression of meiosis was also observed, but the phenotype was manifested during the first wave of spermatogenesis and did not worsen with age (Romero *et al.* 2011). Both *Rheb* and *Dicer* control critical pathways regulating protein expression, albeit through two fundamentally different mechanisms. The biological basis of these *Rheb* and *Dicer* male infertility phenotypes probably resides in this precise regulation of gene expression and translation required to complete the complex transcription-independent processes of meiotic and post-meiotic sperm development. With hundreds of testis-specific genes expressed in spermatocytes and spermatids, the translation of these mRNAs must be perfectly orchestrated to coordinate acrosome biogenesis, tail formation, nuclear

Figure 8 Analyses of cell proliferation and meiotic entry. (A, B, C, D, E, F, G and H) Immunostaining for Ki67 as a marker for cellular proliferation. Bar = 100 μ m. (I, J, K, L, M, N, O and P) Immunostaining for pH2AX as a marker of meiotic entry. Scattered apoptotic germ cells serve as an internal positive control (arrowheads) since apoptotic cells undergo DNA fragmentation and are strongly positive for pH2AX. Bar = 25 μ m.

condensation, and cytoplasm removal. With the loss-of-functional spermatozoa in *Vasa-Cre; Rheb^{f/f}* function in the testis, it is reasonable to hypothesize that regulation of protein translation by mTORC1 is required for spermiogenesis. Although we did not document an obvious defect in spermatogonial function, and the numbers of these cells were unaffected, it is possible that *Rheb* inactivation resulted in some unidentified abnormality (metabolic, transcriptional, etc.) in early stages of spermatogenesis that became manifest at later steps of spermatogenesis.

With regard to the control of protein translation, Rheb and mTORC1 act through canonical pathways including the phosphorylation of 4EBP and S6K. We were unable to document, by *in situ* methods or western blotting, significant alterations in the phosphorylation state of these key mTOR effectors. Several possibilities may be considered. First, by western blotting, the significant somatic cell contamination of whole testes lysates could make it difficult to detect differences in phosphorylation levels (we observed no differences through these methods – unpublished data). We also failed to detect differences in the phosphorylation levels in these factors

(and hence significant alterations in mTORC1 signaling) by potentially more sensitive *in situ* methods, although immunohistochemistry may also be insensitive to relatively modest changes in target epitope levels. It is also possible that unknown feedback loops serve to preserve Tsc/mTOR signaling in the absence of *Rheb*. Although a precise molecular mechanism of action was not determined, the *Rheb* cKO is the first genetic *in vivo* study which implicates *Rheb* in the regulation of spermatogenesis.

Despite the complete sterility of *Rheb* cKO males, cKO females were fertile and exhibited normal fecundity. We observed no alterations in primordial follicle numbers, or follicle maturation, by careful histological analyses. This result is somewhat surprising in light of previous studies clearly demonstrating a central role of mTOR signaling in the regulation of primordial follicle activation. The global follicle activation phenotype previously documented in *Tsc1* or *Tsc2* cKO females was associated with dramatic upregulation of the mTOR signaling pathway (demonstrable with the same immunohistochemical assays performed in our study;

Figure 9 Analysis of mTOR and downstream effectors. Immunostains with phosphorylation site-specific antibodies against canonical mTOR pathway members in both ovaries (left) and testes (right). Bar = 250 and 100 μ m respectively. (A, B, C and D) Total mTOR, (E, F, G and H) p-mTOR, (I, J, K and L) p-S6K, and (M, N, O and P) p-4EBP.

Adhikari *et al.* 2009, 2010), yet conditional inactivation of *Rheb* in oocytes did not have any observable effect on follicle maturation. Although different *Cre* deleter lines were used in these studies (*Vasa-Cre* and *Gdf9-Cre*), this does not appear to provide an adequate explanation, as both *Cre* deleter lines have been extensively utilized and well documented to lead to efficient *Cre*-mediated recombination in primordial oocytes (Castrillon *et al.* 2003, Lan *et al.* 2004, Gallardo *et al.* 2007a, Adhikari *et al.* 2009). If mTORC1 activation played an essential role in the recruitment of primordial follicles, *Rheb* inactivation would be predicted to result in a primordial follicle arrest phenotype in the absence of mTOR signaling secondary to *Rheb* inactivation. Explanations for the lack of phenotype may include the action, within the oocyte, of functionally redundant members of the GTPase superfamily, such as the *Rheb* homolog *Rheb11*. Although global *Rheb11* KO mice were viable and fertile (Zou *et al.* 2011), we have documented *Rheb11* expression in the oocyte (Fig. 1C and D), which could

serve to compensate for the failure of complete *Rheb* inactivation to affect any necessary thresholds of mTOR activation (Tee *et al.* 2005). A *Rheb* germ cell cKO in a *Rheb11*-null background may provide insights into this hypothesis. Alternately, an unknown *Rheb* homolog, or perhaps a more distantly related Ras-like GTPase, may provide this function. Furthermore, cellular functions including the control of apoptosis, differentiation, and the actin cytoskeleton regulation have been proposed to be modulated by non-canonical (i.e. mTORC1-independent) functions of Tsc1/Tsc2 (Neuman & Henske 2011) and it is possible that such mTOR-independent actions of the Tsc complex also contribute to the lack of a *Rheb* ovarian phenotype.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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