RESEARCH ARTICLE

RHAMM deficiency disrupts folliculogenesis resulting in female hypo fertility

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ABSTRACT

The postnatal mammalian ovary contains the primary follicles, each comprising an immature oocyte surrounded by a layer of somatic granulosa cells. Oocytes reach meiotic and developmental competence via folliculogenesis. During this process, the granulosa cells proliferate massively around the oocyte, form an extensive extracellular matrix (ECM) and differentiate into cumulus cells. As the ECM component hyaluronic acid (HA) is thought to form the backbone of the oocyte-granulosa cell complex, we deleted the relevant domain of the Receptor for HA Mediated Motility (RHAMM) gene in the mouse. This resulted in folliculogenesis defects and female hypofertility, although HA-induced signalling was not affected. We report that wild-type RHAMM localises at the mitotic spindle of granulosa cells, surrounding the oocyte. Deletion of the RHAMM C-terminus in vivo abolishes its spindle association, resulting in impaired spindle orientation in the dividing granulosa cells, folliculogenesis defects and subsequent female hypofertility. These data reveal the first identified physiological function for RHAMM, during oogenesis, and the importance of this spindle-associated function for female fertility.

KEY WORDS: Gametogenesis, Folliculogenesis, Hypo fertility, Spindle, RHAMM, Oriented mitosis, Centrosome

INTRODUCTION

The postnatal mammalian ovary contains primary oocytes, each enclosed in a single layer of somatic granulosa cells, forming the so-called primordial ovarian follicles (Pepling and Spradling, 2001). These immature oocytes, which are arrested in prophase of Meiosis I (Eppig, 1993), acquire meiotic and developmental competence during folliculogenesis, i.e. the transition from the small primordial follicle to the large multilayered pre-ovulatory follicle. During this process, the oocyte progressively grows while the granulosa cells proliferate and differentiate (reviewed in Li and Albertini, 2013).

Folliculogenesis requires oocyte-granulosa cell interaction, which is stabilised by extracellular matrix (ECM) components and, in its last two stages, depends also on hormones. Following activation, the primordial follicles undergo four successive stages of development, defined by oocyte growth and by the number and type of cells surrounding the oocyte (Hoage and Cameron, 1976; reviewed by Pepling, 2006): accompanied by massive expansion of the granulosa cell layer, the primordial follicles give rise to (i) primary follicles which in turn progress to (ii) secondary follicles. Stimulated by gonadotropins (FSH and LH), further proliferation of the granulosa cells gives rise to the (iii) multi-layered antral follicle containing antral cavities (Messinis et al., 2010). In the final stage of folliculogenesis, the granulosa cells differentiate into mural and cumulus cells (the latter surrounding the oocyte), a single large antral cavity is formed and the oocyte reaches its final growth in the so-called (iv) preovulatory follicle, ready for ovulation. The fully-grown oocyte is tightly enclosed into several layers of thousands of cumulus cells, held together by gap junctions and stabilized by extensive ECM. This is called the cumulus-oocyte complex (reviewed by Russell and Salustri, 2006). The cumulus-oocyte association is retained through ovulation, whereupon the follicle wall ruptures releasing the complex.

Granulosa cell proliferation and multilayered cell complex formation around the maturing oocyte and its ECM components, are essential features of female germ cell maturation. Distinct changes in the morphology of the proliferating granulosa cells and the precise spatial orchestration of their mitotic divisions, give rise to the multi-layered follicle (Da Silva-Buttkus et al., 2008). However, only few of the stimulating and inhibitory regulatory molecules, which obviously accompany this complex process, are known (e.g. Fan et al., 2008; Fan et al., 2009; reviewed by Sánchez and Smits, 2012).

A significant portion of the ECM of the follicles consists of hyaluronan (or hyaluronic acid, HA). Both oocytes and cumulus cells produce HA during folliculogenesis (Salustri et al., 1992; Ueno et al., 2009). There is yet no functional evidence that HA is essential for folliculogenesis, although the co-expression and the time courses of hyaluronan synthases and hyaluronan binding surface proteins suggest such a role (Ohla et al., 1999; Schoenfelder and Einspanier, 2003). HA could simply serve as a structural component or it could induce a signalling cascade. For both functions, cells must possess one or several hyaluronan-binding receptors. Two major surface receptors for HA have been reported, CD44 and RHAMM (Hardwick et al., 1992; Hofmann et al., 1998a; Lesley et al., 1992; Yoneda et al., 1990); the latter’s
HA receptor function has also been disputed (Hofmann et al., 1998b; Jiang et al., 2013).

For both receptors, links to extracellular signalling have been reported. However the physiological and developmental functions of RHAMM are unknown. In cell models, RHAMM has been reported to mediate HA-induced ERK1/2 pathway activation and cell migration (Hardwick et al., 1992, Hamilton et al., 2007). Apart from these putative extracellular activities, RHAMM functions in centrosomal and acrosomal spindle assembly (Maxwell et al., 2003; Groen et al., 2004) as well as in spindle orientation (Dunsch et al., 2012) have been documented in cell extracts and cultured cells.

To explore whether RHAMM is required for gametogenesis, we generated a deletion of the C-terminal putative HA-binding domain as well as the centrosome-targeting sequence of the mouse RHAMM gene (hmmr). The mutant animals are viable, but the RHAMM truncation resulted in female hypofertility. We report here the underlying mechanism, which reveals the first physiological RHAMM function and, in addition, demonstrates the requirement for oriented cell divisions in female gametogenesis.

RESULTS

RHAMM is expressed in the female reproductive organs

RHAMM expression had been detected previously in mRNA extracted from cattle cumulus-oocyte complexes (Schoenefelder and Einspanier, 2003), but its cell specific expression and localization in the reproductive system was unknown. We therefore analyzed RHAMM mRNA expression in the ovary and uterus of wild type mice by radioactive in situ hybridization. RHAMM mRNA was highly expressed along the mitotic epithelium of the uterus, as well as in distinct foci within the ovary that correspond to the ovarian follicles (Fig. 1A). RHAMM expression in the follicles was restricted to the proliferative granulosa cells surrounding the oocyte (Fig. 1B,C). The highest transcript levels were observed in secondary follicles containing highly proliferative granulosa cells (Fig. 1C, arrow) whereas lower hybridization signal intensities were found in follicles with growing antrum (Fig. 1C, arrowhead), characterised by decreased granulosa cell proliferation.

The hmmr<sup>−/−</sup> mouse expresses C-terminus truncated RHAMM, devoid of the centrosome-targeting and HA-binding protein domains

The functions of RHAMM, thus far described in cultured cells, are mediated by its centrosome-targeting and HA-binding domains, which are located in the C-terminus (Fig. 2B) and are responsible for the spindle assembly and cell migration roles of the protein, respectively. To inactivate this region in the mouse, a neomycin cassette with in-frame stop codons was inserted in the mouse genome, replacing a region between exon 10 and 11 of hmmr (Fig. 2A).

RT-PCR analysis of RHAMM mRNA expression, in mouse embryonic fibroblasts (MEF) derived from the resulting mutant animals (hmmr<sup>−/−</sup>), showed that insertion of the neomycin resistance cassette did not prevent mRNA expression of the RHAMM C-terminus (Fig. 2C). However, the premature stop-codon in the neomycin cassette resulted in translation of a truncated protein containing the neomycin-resistance gene product and with the expected molecular weight of 65 kDa, expressed in hmmr<sup>−/−</sup> mutant cells (Fig. 2D). A ~95 kDa protein was detected in wild type samples (hmmr<sup>+/+</sup>), consistent with the predicted molecular weight of the full-length RHAMM protein (Fig. 2D). An antibody raised against a RHAMM N-terminus peptide, thus cross-reacting with both the full-length as well as the C-terminus truncated protein, was used for immunoblotting analysis.

The RHAMM mutant mice are viable

The homozygous hmmr<sup>−/−</sup> mice were viable and normal in appearance. In view of the role of RHAMM in spindle assembly described in vitro, the viability was a surprise. It indicates that the centrosome-targeting function is not essential for mitotic spindle assembly. Also unexpected was that absence of the RHAMM HA-binding motif did not interfere with viability, as HA has been implicated in many cellular processes (Sherman et al., 1994; Toole, 2004). We therefore explored whether HA-mediated signalling is compromised in the hmmr<sup>−/−</sup> background.

The HA-binding domain of RHAMM is dispensable for HA-induced ERK1/2 activation

Because RHAMM was originally identified as mediator of HA-induced ERK1/2 activation (Zhang et al., 1998), we examined whether deletion of the RHAMM HA-binding domain prevents HA-induced ERK1/2 phosphorylation. Wild type and RHAMM mutant MEF were stimulated with high molecular weight HA and the phosphorylated ERK1/2 (p-ERK1/2) protein level was subsequently determined by western blotting. As expected, HA stimulation increased p-ERK1/2 level in hmmr<sup>+/+</sup> MEF. Surprisingly, however, p-ERK1/2 level was also increased in hmmr<sup>−/−</sup> cells (Fig. 2E), indicating that deletion of the RHAMM HA-binding domain is dispensable for HA-induced ERK1/2 phosphorylation. Apparently, HA stimulates ERK1/2 phosphorylation through signalling receptors other than RHAMM.

The hmmr<sup>+/−</sup> female mice display age-dependent hypofertility

Mating of hmmr<sup>+/−</sup> females with wild type hmmr<sup>+/+</sup> male mice revealed an age-dependent hypofertility of the mutant females (Fig. 2G,H). As haplodeficiency of hmmr did not induce fertility defects, hmmr<sup>+/−</sup> mating pairs were used as controls. There was no significant difference between hmmr<sup>+/+</sup> and hmmr<sup>−/−</sup> females in the total number of litter born over a period of 6 months (Fig. 2F), suggesting that the pregnancy of hmmr<sup>+/−</sup> females was normal. However, analysis of the litter size revealed a progressive decrease in the number of offspring/litter of young (8–24 week old) hmmr<sup>+/−</sup> females when compared to controls, which became significant in older animals (>24week old) (Fig. 2G,H). These findings indicate that deletion of the RHAMM C-terminus leads to age-dependent female hypofertility.

RHAMM deficiency does not affect oocyte meiotic maturaton

The oocytes reach meiotic and developmental competence during folliculogenesis (Sánchez and Smitz, 2012). After successful completion of folliculogenesis, oocytes resume meiosis and undergo meiotic divisions to produce the gamete awaiting fertilization. Success of meiotic division relies on the assembly of a functional acentrosomal spindle (Kaláb et al., 2011; Dumont et al., 2007). As RHAMM is critical for acentrosomal spindle assembly in Xenopus oocyte extracts (Groen et al., 2004; Joukov et al., 2006), we tested whether mouse female hypofertility, caused by the deletion of the RHAMM C-terminus, is due to acentrosomal spindle assembly defects, in oocyte meiosis.
RHAMM deficiency impairs ovarian folliculogenese

Analysis of the reproductive system of \textit{hmmr}^{+/+} females revealed no abnormalities in the cervix and uterus, despite the high level of RHAMM mRNA expression in the epithelium of the uterus in wild type animals. Since the number of oocytes recovered from the ovaries of \textit{hmmr}^{+/+} mice was approximately a third of those recovered from wild type ones, we asked whether defective oogenesis is the cause of the age-dependent hypofertility of the \textit{hmmr}^{m/m} females.

In order to determine whether the \textit{hmmr}^{m/m} ovaries have a diminished reservoir of oocytes, the ovaries of mice at post-natal day 7 (PND7) were analyzed (Fig. 3). At this time point, primordial follicles’ formation has been completed and the total number of oocytes available for reproduction is fixed (Pepling and Spradling, 2001; Pepling, 2006). The primordial follicles in \textit{hmmr}^{m/m} ovaries appear morphologically normal (Fig. 3D–F). Their quantification (Fig. 3G) indicated a potentially reduced reservoir of oocytes in \textit{hmmr}^{m/m} mutants but no statistically significant difference in the number of these follicles between mutant and wild-type animals.

In ovaries of 10-week-old \textit{hmmr}^{m/m} mice, the number of primary follicles was decreased 5-fold as compared to the controls (Fig. 4G) indicating a defect in the transition from primordial to primary follicles. Moreover, in \textit{hmmr}^{+/+} ovaries, follicles of various maturation stages were present, indicating ongoing folliculogenese (Fig. 4A). In contrast, mostly degenerative (“atretic”) follicles, with increased interstitial tissue, can be seen in a representative section of an \textit{hmmr}^{m/m} ovary (Fig. 4D). Quantification of the ovarian follicles in adult animals, using (one in every five) 4 \( \mu \)m semi-serial sections of complete ovaries (Canning et al., 2003; Tilly, 2003) from 10- and 25-week-old females, confirmed these observations (Fig. 4H). The number of both immature (primary and secondary) and mature follicles (antral and preovulatory) was very significantly decreased in \textit{hmmr}^{m/m} ovaries while decreasing (up to 100-fold) in \textit{hmmr}^{m/m} ones (Fig. 4H).

In conclusion, the \textit{hmmr}^{m/m} adult female mice exhibit a very significant reduction of ovarian follicles, indicating that the RHAMM C-terminus deletion impairs folliculogenese, eventually reducing the pool of fully mature oocytes available for fertilization.

The RHAMM C-terminus localises the protein at the mitotic spindle of granulosa cells

In cultured cells, RHAMM associates with the mitotic spindle (Assmann et al., 1999; Maxwell et al., 2003; Groen et al., 2004; Maxwell et al., 2011) via its C-terminal centrosome-targeting domain (Maxwell et al., 2003). Consistent with these data, RHAMM localised at the mitotic spindle of granulosa cells in wild type ovaries (Fig. 5C), while this localization was completely abolished in \textit{hmmr}^{m/m} granulosa cells (Fig. 5D). Our \textit{in vivo} data, confirmed, therefore, the previous observations in cell culture experiments.

Furthermore, in both wild type and \textit{hmmr}^{m/m} ovaries, the spindle morphology in granulosa cells was normal (Fig. 5A,B), indicating a non-essential role of RHAMM in centrosomal spindle assembly \textit{in vivo}.

RHAMM regulates the oriented mitotic division of granulosa cells

During primary follicle development, granulosa cells have been shown to divide in an oriented manner, with the cell division axis...
perpendicular to the oocyte and basal lamina (Da Silva-Buttkus et al., 2008). This division geometry may promote the formation of the granulosa layers and it has been postulated to be important for folliculogenesis (Da Silva-Buttkus et al., 2008).

During mitosis, the cell division axis is dictated by the positioning of the spindle within the cell, which is perpendicular to the metaphase plate (reviewed by Bornens, 2008). We reasoned that the absence of RHAMM on the spindle of hmmrm/m granulosa cells could perturb spindle positioning and alter cell division geometry.

To test this hypothesis, we analyzed the orientation of granulosa cell division, in 10-week-old mice. Follicles with one or two layers of granulosa cells were selected (Fig. 5E,F) and the long spindle axis of granulosa cells at metaphase or anaphase was used to determine the cell division plane (Fig. 5G, black line). For each granulosa cell, the angle $\theta$ between this plane and the axis determined by the oocyte and granulosa cell centres (Fig. 5G, orange line) was measured (Fig. 5G). In wild type ovaries, as expected, the small $\theta$ (median 11.1°) indicated that granulosa cells orient their spindle axis parallel to the oocyte-granulosa cell axis and perpendicular to the oocyte surface, generating proximal and distal daughter cells.

In contrast, in the hmmrm/m granulosa cells, the average $\theta$ angle measured was significantly higher, due to a wider distribution of this parameter. This indicated that, in the mutant, spindle orientation was impaired (Fig. 5H). Strikingly, in some cases, spindles were even found positioned perpendicular to the long cell axis, reinforcing the idea that the spindle orientation is determined, in wild type granulosa cells, via the RHAMM activity. The Kolmogorov-Smirnov test showed that the distribution of spindle angles in the mutant cells does not differ from the random distribution ($p=0.6$ for hmmrm/m), in stark contrast with the wild type.
contrast to the wild type in which spindle angles differ very significantly ($p = 1.6 \times 10^{-11}$ for $hmmr^{+/+}$) from the random distribution.

Taken together these data indicate that RHAMM, via its centrosome targeting domain, associates with the spindle of granulosa cells, contributes to its positioning and, in turn, to the geometry of the granulosa cell division. The disoriented positioning of the spindle in $hmmr^{+/+}$ cells most probably contributes to alter the formation of a multilayered and functional follicle.

**DISCUSSION**

**RHAMM expression in the proliferating granulosa cells of the ovarian follicles is consistent with its mitotic upregulation**

We report that RHAMM mRNA is expressed in the adult mouse ovary and localized in ovarian follicles containing highly proliferative granulosa cells, in particular in primary, secondary and pre-antral follicles. In cultured cells, RHAMM mRNA expression is known to be cell cycle regulated, reaching the highest level at G2/M (Sohr and Engeland, 2008). The mRNA labelling observed in uterus and ovarian follicles is therefore consistent with an upregulation of RHAMM expression in highly proliferative tissues.

In its reported (Hardwick et al., 1992; Yang et al., 1993) but also disputed (Hofmann et al., 1998b; Jiang et al., 2013) function as HA receptor, RHAMM would be expected to be expressed in regions of high HA synthesis. In the ovaries, HA is synthesized in the last stage of folliculogenesis, in pre-ovulatory follicles (Zhuo and Kimata, 2001), concomitantly with a surge of expression of
its receptor, CD44 (Ohta et al., 1999; Schoenfelder and Einspanier, 2003; Yokoo et al., 2010). In contrast, RHAMM is expressed at steady state during cattle oocyte maturation (Schoenfelder and Einspanier, 2003) and, in the cell type specific localization reported here, the peak of RHAMM expression occurs in the highly expanding GCs of secondary follicles (Fig. 1). In light of the high proliferative activity of the GCs during folliculogenesis, this expression pattern is consistent with the known upregulation of RHAMM during mitosis (Sohr et al., 2003) and, in the cell type specific localization reported here, the peak of RHAMM expression occurs in the highly expanding GCs of secondary follicles (Fig. 1).

**Activation of ERK1/2 in the hmmr<sup>−/−</sup> genetic background**

The activation of ERK1/2 in granulosa cells is essential for folliculogenesis (Fan et al., 2009). Despite the absence of the HA binding motif of RHAMM in hmmr<sup>−/−</sup> MEFs, HA induced ERK1/2 phosphorylation properly (Fig. 2E). Apparently, HA stimulates ERK1/2 phosphorylation through other signalling receptors (CD44 or LYVE1 which are expressed in the ovary, are candidates) (Prevo et al., 2001) and thus remain unaffected by the RHAMM truncation. Hence absence of ERK1/2 phosphorylation could not be the cause of hypofertility. The slightly elevated HA-independent phosphorylation of ERK1/2 in hmmr<sup>−/−</sup> MEFs (Fig. 2E) has not been observed in the ovaries. Increased ERK1/2 activation is also observed in RHAMM<sup>−/−</sup> ES cells (Jiang et al., 2013), in agreement with our data, but in contrast to a reported defect in ERK1/2 phosphorylation in RHAMM<sup>−/−</sup> MEFs (Tolg et al., 2006). The response to HA in the absence of the HA-binding domain, in the RHAMM C-terminus, raises the question whether RHAMM functions as surface receptor for HA at all. Indeed, this HA-binding domain is unspecific in that it can also bind heparin in vitro (Yang et al., 1994).

We therefore conclude that it is unlikely for the folliculogenesis defect of hmmr<sup>−/−</sup> mice to be due to deficiency in any interaction between HA and RHAMM. Where could the C-terminus of RHAMM be required in oocyte maturation? There are two processes where it could engage in spindle formation: meiosis of the oocytes and mitosis of the proliferating granulosa cells.

**The RHAMM C-terminus is dispensable in oocyte meiotic divisions**

Oocytes gradually acquire developmental and meiotic competence during folliculogenesis, which enable the oocytes to resume meiosis. In meiosis, spindle assembly is essential for the two successive chromosome segregations to give rise to haploid oocytes (Brunet and Maro, 2005; Sánchez and Smitz, 2012). Therefore, rebuilding of the acentriolar microtubule organization center is regarded as one key feature of oocyte competence (Łuksza et al., 2013).

Rodent and human oocytes lack centrioles (Szollosi et al., 1972; Hertig and Adams, 1967). Hence acentriolar - and as a consequence anastral - spindles assemble during the meiotic divisions that conclude oogenesis. This is achieved by the chromatin-centered RanGTP gradient nucleating the mitotic spindle microtubules (Brunet and Maro, 2005; Schatten and...
localization at the meiotic spindles of analysis demonstrated correct spindle formation and TPX2 our data suggests that it might do so. Immunofluorescence binding RHAMM N-terminus can also associate with TPX2 and focusing activity (Joukov et al., 2006). Whether the microtubule-targeting domain, might be sufficient for this spindle pole assembly in vitro. In Xenopus egg extracts, XRHAMM is essential for Ran-dependent anastral spindle assembly and it is required for spindle pole focusing via TPX2 (Groen et al., 2004). The C-terminus of XRHAMM, which encodes the centrosome targeting domain, might be sufficient for this spindle pole focusing activity (Joukov et al., 2006). Whether the microtubule-binding RHAMM N-terminus can also associate with TPX2 and function in anastal spindle focusing remains unknown. However our data suggests that it might do so. Immunofluorescence analysis demonstrated correct spindle formation and TPX2 localization at the meiotic spindles of hhmm<sup>+/m</sup> oocytes. Unfortunately analysis of RHAMM localization was inconclusive, likely due to incompatibility of the RHAMM antibody with the oocyte fixation protocol. Given that these spindles are acentrosomal, were RHAMM to localize at the mouse meiotic spindle, the microtubule-targeting domain at the N-terminus of the protein (which is intact in the hhmm<sup>+/m</sup> mouse) might suffice for this localization and for spindle pole focusing.

The RHAMM-dependent spindle orientation in granulosa cells is required for folliculogenesis

In cell extracts and in cultured cells, two RHAMM mitotic functions have been described: maintenance of spindle integrity (Groen et al., 2004; Joukov et al., 2006; Maxwell et al., 2005) and spindle orientation (Dunsch et al., 2012).

Quantification of immature follicles in hhmm<sup>+/m</sup> ovaries at PND7 demonstrate no statistically significant differences to control hhmm<sup>+/+</sup> ovaries, albeit a potentially reduced reservoir of these follicles. What could this signify? Abnormal spindle assembly can eventually lead to chromosomal instability (Ganem et al., 2009). One can hypothesize that aberrant spindle assembly, caused by the RHAMM truncation, could drive chromosomal instability in oogonia, during embryonic hhmm<sup>+/m</sup> mouse development. Such defective oocytes are thought to be eliminated, via programmed germ cell death, in the first two post-natal days (Ghafari et al., 2007; Lobascio et al., 2007; Pepling, 2006), thereby leading to decreased number of primordial follicles by PND7.

However, previous studies indicate that RHAMM depletion is detrimental for acentrosomal spindle integrity (Groen et al., 2004; Joukov et al., 2006), but not for centrosomal spindle formation. RHAMM disruption by siRNA (Neumann et al., 2010; Dunsch et al., 2012) or a blocking antibody (Maxwell et al., 2003) in mammalian cells containing centrosomes does not prevent the majority of these cells to progress through mitosis, despite delays. Furthermore, no defects in spindle architecture of hhmm<sup>+/m</sup> granulosa cells were observed here and our quantification of in vitro spindle defects induced by the RHAMM C-terminus deletion (H.L., A.W. and A.P., unpublished) show them to be mild and to occur at low frequency. As such, they are unlikely to be the single contributor to the very significant folliculogenesis defects of the hhmm<sup>+/m</sup> mouse.

The second mitotic function of RHAMM, demonstrated in cultured cells, is orientation of the mitotic spindle (Dunsch et al., 2012). In human cells, this function is mediated by a central part of the RHAMM coiled coil domain, which interacts with CHICA and, via CHICA, with the molecular motor dynein to orient the mitotic spindle (Dunsch et al., 2012). The corresponding mouse RHAMM domain is not expressed in hhmm<sup>+/m</sup> (Fig. 2).

Oriented divisions, in diverse systems, can be regulated either by cell shape or by cortical cues, both of which activate dynein-dependent spindle orientation mechanisms (O’Connell and Wang, 2000; Fink et al., 2011; Kiyomitsu and Cheeseman, 2012; Xiong et al., 2014). Granulosa cells can be classified to columnar and cuboidal, according to their shape. The long axis of columnar cells is positioned perpendicular to the oocyte surface (Da Silva-Buttkus et al., 2008). This postulates that, in these cells, shape constrains can dictate spindle positioning, via dynein subcortical localization and subsequent dynein-dependent spindle anchoring. In contrast, cuboidal granulosa cells, which exhibit a five-fold higher proliferation and are located mostly adjacent to the oocyte during early follicle development divisions (Da Silva-Buttkus et al., 2008), cannot rely on shape cues for correct dynein localization and spindle orientation. This postulates the existence of a proximal-distal cortical cue in granulosa cells, enabling correct orientation of the spindle. In either case, as spindle-associated RHAMM is critical for dynein-dependent spindle orientation (Dunsch et al., 2012), the RHAMM truncation would impair spindle orientation in granulosa cells – independent of their shape. Indeed, our data confirms this function in vivo, demonstrating that spindle-associated RHAMM is required for spindle and division plane orientation in these cells.

This distinct orientation of the granulosa cell division plane is likely to facilitate the establishment of orderly concentric layers of granulosa cells and thus contribute to functional communication between granulosa cells and oocyte in the follicle. Such bi-directional communication is crucial for successful follicle development (Wigglesworth et al., 2013; Gilchrist et al., 2004); its impairment would be consistent with the very significant decline in number of immature and mature follicles observed in hhmm<sup>+/m</sup> ovaries.

In summary, we report the first identified physiological function for RHAMM, during oogenesis, and the importance of this function for female fertility. Our data indicate that RHAMM is a critical factor for folliculogenesis and that this spindle-associated protein is required for spindle positioning in granulosa cells, which surround the oocyte during its growth phase. Deletion of the RHAMM centrosome-targeting domain in vivo abolishes its spindle association, resulting in impaired spatial orientation of dividing granulosa cells, folliculogenesis defects and subsequent female hypofertility.

MATERIALS AND METHODS

Construction of the targeting vector and generation of the hhmm<sup>+/m</sup> mouse

The hhmm<sup>+/m</sup> mouse was generated by homologous recombination in ES cells as described previously (Reichardt et al., 1998). To delete the C-terminus (aa 318–794) of the RHAMM protein (accession number NP_0385800), a targeting vector was constructed, containing the promotorless neomycin (neo)–resistance gene, as selection marker, between exon 10 and exon 11 of the full-length hhmm. The in-frame stop codon in the neomycin cassette resulted in premature translation stop, which gave rise to a fusion protein (predicted MW 65 kDa) comprising the RHAMM N-terminus (317 aa) and the neomycin-resistance gene product neomycin phosphotransferase II (264 aa). This targeting vector was introduced into mouse embryonic stem (ES) cells. Clones of transformed, neomycin resistant ES cells were screened for the insertion of the correct modification in the hhmm gene locus. A clone with a correct insertion was chosen and injected into blastocysts to generate chimeric mice. These mice were crossed with C57BL/6 to allow germline transmission of the hhmm mutant gene.

Mouse colony maintenance and genotyping

The colony was maintained by breeding heterozygous animals. Backcrossing to the parental C57BL/6J strain using F1 hybrids, routinely every 10th generation, was employed to avoid production of
inbred lines. Animals were provided with standard laboratory chow and tap water *ad libitum* and kept in accordance with local regulations (TLLV Thüringen, Erfurt, Germany) at constant temperature (22 °C) and light cycle (12-h light, 12-h dark). Animals were sacrificed by CO₂ inhalation. Tissues designated for western blotting analysis were snap frozen on dry ice and stored at −80 °C. For *in situ* hybridization analysis, tissues were frozen in isopentane cooled on dry ice, cut into 20 μm cryo-sections on a cryostat (Leica, Wetzlar, Germany), thaw-mounted on super frost slides and stored at −80 °C until further processing.

Genomic DNA was obtained from tail biopsies according to standard protocols and genotyping was performed by PCR (primers: ex11rev: 5′-TGCAGACGACGACAGTTC-3′, ex10fw: 5′-AGCAAGGATAGAGAAAGGGGTG-3′, neo44rev: 5′-TGATCGACAGACGGCCTT-3′) with an annealing temperature of 63 °C, in order to discriminate between wild type *hmnr*+/− and mutated *hmnr*mut alleles with expected sizes of 681 bp and 503 bp respectively.

**Fertility assays**

Mating of 8- to 12-week-old mice, during 6 months, was used in the quantification of average number of litters. During this period, 25 litters and 115 offspring were born to five RHAMM mutant females versus 28 litters and 199 offspring to five controls.

Mating within two age cohorts, comprising 8- to 24-week-olds or mice older than 24 weeks, was used in the quantification of litter size. The offspring number for a minimum of 25 litters per genotype and age group was quantified, in total 137 litters and 831 offspring were used in the analysis.

**Culture and immortalization of mouse embryonic fibroblasts (MEFs)**

Heterozygous female mice, mated with heterozygous males, were sacrificed at 14.5 days of gestation. The embryos were removed under aseptic conditions, the heads and livers were discarded and the tails were sacrificed at 14.5 days of gestation. Heterozygous female mice, mated with heterozygous males, were mated and immortalization of mouse embryonic fibroblasts (MEFs) was quantified, in total 137 litters and 831 offspring were used in the quantification of average number of litters. During this period, 25 litters and 199 offspring to five controls.

**RT-PCR**

Total RNA was extracted from MEFs and reverse transcribed into cDNA using the High Fidelity cDNA Synthesis kit (Roche). Amplification of the indicated regions of RHAMM was performed with the respective primer pairs listed below. GAPDH was used as control.

- **hmnr** exons 1–5: fw: 5′-GACCCCTTCGGTGTGTCCTCAC-3′; rev: 5′-GCCCTTATGTCGTTGGCCTGG-3′
- **hmnr** exons 7–12: fw: 5′-GGTCAACAGGAAGCATGGACG-3′; rev: 5′- CTACAGCCGACCGCTTGA-3′
- **hmnr** exons 18–21: fw: 5′-AAGGCAACCCCAACTGTACGT-3′; rev: 5′-CCCTGTGGTGTTGCTGTCCT-3′

**Histological analysis, ovarian follicle quantification, orientation of granulosa cell division**

Mouse ovaries and uterus were fixed with 4% paraformaldehyde for 16–24 h and embedded in paraffin. 4 μm thick tissue sections were stained with hematoxylin and eosin according to standard protocol. The sections were analyzed in an Olympus BX41 light microscope and images were acquired using the Cell® software (Olympus).

For ovarian follicle quantification, 4 μm thick serial sections of the whole ovary were prepared and one of every five sections was stained with hematoxylin and eosin. The sections were examined at high magnification and the follicles were counted and the total number of follicles in the sections examined were plotted (see Figs 3 and 4) (Canning et al., 2003; Tilly, 2003).

For the morphological classification of the follicles, the criteria of Pedersen and Peters (Pedersen and Peters, 1968) were applied. Briefly: follicles type 1,2 and 3 were classified as primordial; type 4 and 5 were classified as primary; type 6 as secondary; type 7 as antral and type 8 as pre-ovulatory.

For analysis of the orientation of granulosa cell division, ovary sections, prepared as described above, were scanned with 40× objective in a VS110 virtual microscope (Olympus). From the scanned images, follicles with one or two layers of granulosa cells were selected and the cell division axis of granulosa cells at metaphase or anaphase, was determined: A line dissecting the center of the oocyte, the centre of the mitotic granulosa cell and the basal lamina was defined as the oocyte-basal membrane axis (Fig. 5G, orange line). For cells at anaphase, a line along the metaphase plane was drawn (Fig. 5G, red line) and a second line perpendicular to the metaphase plate (Fig. 5G, black line) was used to define the spindle axis. The angle between oocyte-basal membrane axis and spindle axis was determined using ImageJ (NIH). The follicles of 4 wild type mice ovaries (n=41 mitotic cells) and 8 mutant mice ovaries (n=31 mitotic cells) were thus analyzed.

**Mouse oocyte collection and in vitro maturation**

Oocyte *in vitro* maturation assays were performed as previously described (Brunet et al., 2008). Briefly, oocytes were collected from ovaries of 10- or 26-week-old *hmnr*+/− and *hmnr*mut mice and placed in M2 medium pre-warmed to 37 °C and supplemented with 4 mg/ml BSA and 1 mM milrinone. For video microscopy of oocyte meiotic maturation, oocytes were transferred to a Ludin Chamber containing M2 medium with 4 mg/ml BSA. Time-lapse images were acquired using a Photometrics CCD camera (CoolSnap HQ2) mounted on a Leica HC PL APO 20×0.7 NA objective enclosed in a thermostatic chamber (Life Imaging Service). Images were taken every 15 min for 18–20 h at 20× magnification. Metamorph 7.0 (Universal Imaging) and ImageJ (NIH) software were used for image analysis.

**In situ hybridization**

*In situ* hybridization was performed as described previously (Trajkovic-Arsic et al., 2010). Fresh-frozen sections from wild type ovaries and uterus were hybridized with S35-labeled riboprobes corresponding to nucleotides 1–540 of mouse RHAMM mRNA (accession number NM_013552). Following post-hybridization, the sections were covered with 56°C acetic acid and washed at 60°C and 4°C. Blocking and washes were performed with blocking buffers indicated (immunolabelling, western blotting) were conjugated (1/5000) (Jackson Immunoresearch Laboratories). The following antibodies were used: rabbit polyclonal anti-RHAMM (Rhodius) (Accession number: NP_038580) was used to immunize rabbits; the resulting anti-RHAMM polyclonal antibody was affinity-purified from rabbit serum. A synthetic peptide corresponding to aa1-241 of RHAMM (accession number: NM_013552). Following post-hybridization, the sections were covered with photo-emulsion (NTB, Kodak) and stored at 4°C. Autoradiograms were analyzed and images were acquired under dark-field illumination. Control hybridization experiments employing a sense probe did not reveal any specific hybridization signal.

**Antibodies**

A synthetic peptide corresponding to aal-241 of RHAMM (accession number: NP.038580) was used to immunize rabbits; the resulting anti-RHAMM polyclonal antibody was affinity-purified from rabbit serum. The primary antibodies, used in immunofluorescence (IF) and western blotting (WB) experiments, are shown in Table 1. Immunofluorescence-stained Alexa-conjugated goat or HRP-conjugated donkey secondary antibodies were used in the dilutions indicated: Alexa-fluor-488-conjugated (1/300) or -594-conjugated (1/400) (Molecular Probes, Invitrogen); HRP-conjugated (1/5000) (Jackson Immunoresearch Laboratories). The blocking buffers indicated (immunolabelling, western blotting) were used as primary and secondary antibody diluents.

**Immunolabelling**

Paraffin-embedded ovary sections were deparaffinized and rehydrated. For heat-mediated antigen retrieval, the tissue sections were placed in...
citrate buffer (10 mM, pH 6.0) and incubated in a pressure cooker at 125°C for 20 min. The sections were allowed to cool-down for 30 min, washed in PBS, incubated with immunohistochemistry blocking buffer (5% BSA, 5% goat serum, 0.1% triton X-100) for 1 h, followed by primary antibody incubation at 4°C overnight. The samples were washed in PBS (10 min, 3 changes) and incubated with Alexa-conjugated secondary antibodies for 1 h, followed by counter-staining of nuclei with 1 µg/ml DAPI (4′,6-diamidino-2-phenylindole, Sigma) for 3 min. All steps were performed at room temperature, unless otherwise indicated.

Images were acquired on an Axiovert200 microscope equipped with a 12-bit grayscale cooled CCD AxioCamMRm camera (Zeiss). Representative images were brought to a resolution of 300 ppi without re-sampling using Adobe Photoshop (Adobe) and the area of interest was cropped.

ERK1/2 activation assay
MEFs were incubated with serum-free medium for 2 h at 37°C. They were subsequently stimulated for 15 min with the addition of 100 µM high-molecular-weight hyaluronic acid or 10% serum in the medium and processed for immunoblotting.

Preparation of cell protein lysates
Cells were harvested by "scrapping" into ice-cold PBS containing protease and phosphatase inhibitors (10 mM NaV, 50 mM NaF, 50 mM β-mercaptoethanol. 10 mM PMSF 10 µg/ml antipain, 10 µg/ml chymostatin, 1 µg/ml pepstatin A, 2 µg/ml leupeptin, 200 µg/ml aprotinin/AEBSF-HCl, 2 µg/ml aprotinin) and centrifugation (100 g, 5 min, 4°C). The supernatant was discarded and one pellet volume of 2× Laemmli sample buffer (4% w/v SDS, 20% glycerol, 0.2% w/v bromophenol blue, 156 mM β-mercaptoethanol, 100 mM Tris-HCl pH 6.8) was added. Lysate proteins were denatured at 100°C for 5 min, the suspension was sonicated to shear DNA and stored at −80°C.

SDS-PAGE and western blotting
Proteins were separated by SDS-PAGE using 10% acrylamide gel in running buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS). Proteins were transferred onto nitrocellulose membrane (0.2 µm pore size, PROTRAN, Whatman) using a wet transfer system (Mini-Protein, BioRad) at 30 V, overnight in transfer buffer [50 mM Tris, 380 mM glycine, 0.05% (w/v) SDS, 20% (v/v) methanol]. Membranes were stained with Ponceau S solution (0.25% (v/v) Ponceau S (Serva), 40% methanol (Merck), 15% acetic acid (Roth) in H₂O₃ for total protein visualization, briefly washed and then incubated for 30 min in WB blocking buffer (5% non-fat milk, 0.1% Tween-20 in PBS). Membranes were incubated with primary antibody diluted in blocking buffer for 1 h, washed four times with 0.1% Tween-20 in PBS, incubated in secondary, HRP-conjugated, antibody for 45 min and washed as described above. Antibody labelling was visualised by chemiluminescence using the western lightning plus-ECL Kit (Perkin Elmer) and detected on BioMax MR film (Kodak).

Statistical analysis
The fertility assays (Fig. 2) were analyzed by the two tail Student’s t-test, the in vitro oocyte maturation assays (supplementary material Fig. S1) by the Fisher’s Exact test, the granulosa cell spindle angle quantification (Fig. 5) by the Mann-Whitney test. Results are presented as mean±s.d., with error bars denoting the standard deviation. The hypothesis that spindle orientation in granulosa cells is random (Fig. 5) was tested by the Kolmogorov-Smirnov distribution test, applied on the distribution of spindle angles in granulosa cells versus a random distribution, normalising the angle θ (Fig. 5) between 0 and 1 (D = 0.1876, p = 0.6416 for hnm/m; D = 0.5852, p = 1.601×10⁻¹¹ for hnm/m⁻¹).

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Competing interests
The authors have no competing or financial interests to declare.

Author contributions
HL, JM, AW, LF, SB, JH, TK, MHV, HH, KZ, PH, AP designed the experiments and analyzed the data; HL, JM, AW, LF, SB, JH, HH, AP performed the experiments; HL and AP wrote the manuscript; all authors read and commented on the manuscript.

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References

Table 1. Primary antibodies used

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Fig. S1. RHAMM deficiency does not impair oocyte meiotic maturation. (A) Schematic overview of the oocyte meiotic maturation, indicating the different stages and their average duration. GV: germinal vesicle, GVBD: germinal vesicle breakdown, M1: meiosis I, PB1: expulsion of the 1st polar body, M2: meiosis II; blue colour: chromatin, red colour: microtubules. (B–G) Oocyte maturation was analyzed via video microscopy, for oocytes of 10 week-old (B–D) and 26 week-old (E–G) wild type and *hmmrm/m* mutant mice. The number of oocytes at GVBD, M1- or M2-arrested are plotted as cumulative percent. Abbreviations as in A. Despite the reduction in total number of oocytes in *hmmrm/m* mutants, oocyte in vitro maturation assays indicate no defects in oocyte maturation except for a significant delay (p<0.05) in GVBD of the older *hmmrm/m* oocytes (F).