The RNA-binding protein Mex3b regulates the spatial organization of the Rap1 pathway

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INTRODUCTION

RNA-binding proteins (RBPs) are central effectors in the control of co- and post-transcriptional mechanisms that contribute to a diverse array of cellular events (Hogan et al., 2008). Recent large scale analyses have further revealed the role of RBPs and their cognate target RNAs in the assembly of multimolecular complexes at specific cellular sites and in the morphological organization of cells (de Hoog et al., 2004; Lécuyer et al., 2007). However, although mRNA interactome studies have provided evidence that RBPs constitute a very large family comprising >1100 proteins in human (Balza et al., 2012; Castello et al., 2012; Ray et al., 2013), their functions in vivo remain to be determined. Finally, mice with a gene trap insertion in the Mex3c locus display postnatal growth retardation, a skeletal phenotype linked to the impaired translation of the mRNA encoding the insulin-like growth factor 1 in bone-forming cells (Jiao et al., 2012b).

Since their initial description, several reports have sustained the idea that mammalian MEX-3 proteins play several roles in the control of RNA metabolism (Pereira et al., 2013a). MEX3A and MEX3B proteins localize in P-bodies and stress granules, two structures involved in the storage and turnover of mRNAs (Buchet-Poyau et al., 2007; Courchet et al., 2008). MEX3A controls the polarity and stemness of intestinal epithelial cells through the TINO control, respectively, the stability of the transcripts coding for the HLA-A2 MHC class I molecule and the anti-apoptotic protein BCL2 (Cano et al., 2012; Donnini et al., 2004). Interestingly, MEX3C acts as a suppressor of chromosomal instability (Burrell et al., 2012), and, in addition, exhibits a transforming activity when overexpressed in gastric epithelial cells (Jiao et al., 2012). Furthermore, MEX3C and an isoform of MEX3D called Mex3c have been found to enhance mouse energy expenditure, probably through the action of Mex3c in a subpopulation of hypothalamic neurons controlling energy metabolism (Jiao et al., 2012a). This Mex3c mutation has also been found to enhance mouse energy expenditure, probably through the action of Mex3c in a subpopulation of hypothalamic neurons controlling energy metabolism (Jiao et al., 2012a).

To gain further insight into the function of mammalian MEX-3 homologs, we disrupted the Mex3b gene in mouse. We now report that null mice are subfertile owing to a dysfunction of somatic cells in the gonads. In males, the lack of Mex3b impaired the phagocytic properties of Sertoli cells and led to a disorganization of the junctional complexes created between adjacent Sertoli cells that form the blood-testis barrier (BTB). Investigation of the underlying mechanism revealed an unexpected function of Mex3b in the

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regulation of the spatial activation of Rap1 (Rap1a – Mouse Genome Informatics), a small GTPase protein implicated in the control of phagocytosis and cell adhesion, through the stimulation and the recruitment of the Rap1 GTPase-activating protein (Rap1GAP) at the inner face of the plasma membrane.

RESULTS
Histological structure of the gonads is disorganized in Mex3b null mice
To explore Mex3b functions in vivo, we generated mice carrying a conditional Mex3b allele with two loxP DNA sequences framing exon 2, which encodes 463 amino acids out of the 576 amino acids of the Mex3b protein (Fig. 1A). Heterozygous mice carrying one copy of the null allele were generated through crosses with Nestin-Cre transgenic strains that led to the excision of the floxed allele during gametogenesis (Betz et al., 1996). The intercross of Mex3b<sup>+/−</sup> mice and the genotyping of the resulting offspring confirmed the generation of Mex3b null mice (Fig. 1B). Subsequent molecular analyses showed the absence of the Mex3b mRNA (supplementary material Fig. S1A) and protein in both mouse embryonic fibroblasts (MEFs) and testis extracts prepared from Mex3b<sup>−/−</sup> animals (Fig. 1C; supplementary material Fig. S1B). Mex3b null mice were born at expected Mendelian ratio, but 30% of these mice died on the first day after birth (supplementary material Fig. S1C). However, no gross abnormalities were observed upon macroscopic examination. The animals surviving to adulthood were smaller and displayed reduced body weight, a statural and weight deficit that was maintained throughout their lives. Upon breeding of the Mex3b<sup>+/−</sup> heterozygous mice, we observed that the number of pups per month and per female was significantly reduced (0.66 for the breeding of male and female Mex3b<sup>+/−</sup> mice compared with 0.8 for the breeding of wild-type mice) (Table 1). This decrease was even more pronounced when null male or females were crossed to the wild-type mice (0.39 and 0.35, respectively) or when Mex3b<sup>−/−</sup> animals were intercrossed (0.33). This effect could not be ascribed to a sexual behavior phenotype of Mex3b<sup>+/−</sup> or Mex3b<sup>−/−</sup> animals, because vaginal plugs were daily observed in females after pairing.

Because MEX-3 contributes to the maintenance of the C. elegans germline, we decided to investigate further the effects of its deletion on the histology of mouse gonads. Examination of secondary follicle in ovaries of 6-month-old Mex3b<sup>−/−</sup> female mice revealed a gross disorganization of the granulosa layers with apparent cellular piknosis (supplementary material Fig. S1D). In Mex3b<sup>−/−</sup> males, the analysis of testes cross-sections of animals ranging from 3 to 6 months of age showed that the architecture of the seminiferous epithelium was altered significantly (Fig. 1E). At 6 months, the lumen within a third of the seminiferous tubules was obstructed (Fig. 1E), a phenotype that did not worsen with aging up to 18 months. We predicted that this obstructive phenotype would decrease the effective sperm count in Mex3b null males. Indeed, when we quantified the number of sperm cells flushed from the caudal epididymis of Mex3b<sup>−/−</sup> males versus wild-type males, we found a two- to threefold reduction in the sperm count compared with that of the wild type at 40 days and at 3 months (Fig. 1D).

However, under microscopic observation we observed neither overt abnormalities of spermatozoan morphology, nor sperm cell motility...
defects. We confirmed that Mex3b is expressed in Sertoli cells as well as in pachytene spermatocyte and round spermatids; although the level of Mex3b mRNA was very low in pachytene spermatocytes (supplementary material Fig. S1E). The three other Mex3 genes are also expressed in total testis and have a pattern of expression similar to that of Mex3b (supplementary material Fig. S1E). Importantly, we did not observe any compensatory increase in the expression of the three other mex-3 homologs in Mex3b−/− tes testes and in Mex3b−/− Sertoli cells (supplementary material Fig. S1F). Thus, these data indicate that the knockout of Mex3b adversely affects the histological architecture of the gonads in females and males and further suggest that the lack of Mex3b perturbs the functions of gonadal somatic cells, i.e. granulosa and Sertoli cells, resulting in the observed subfertility.

**The loss of Mex3b specifically affects Sertoli cells**

Taking these findings into consideration, we chose to focus our study on the role of Mex3b in spermatogenesis. The seminiferous epithelium is composed of two cell types: the germ and Sertoli cells. Somatic Sertoli cells are large polarized cells that extend from the basement membrane to the lumen of the tubules. These cells act as a stem cell niche and a nurturing microenvironment for the germ cells during their differentiation from diploid spermatogonia to haploid spermatooza (Russell and Peterson, 1985). About 30 germ cells at different stages of their maturation interact with each Sertoli cell. Specific types of junctions between adjacent Sertoli cells form the BTB, which constitutes an immune-privileged site protecting postmeiotic germ cells (Cheng and Mrk, 2012). Finally, Sertoli cells clear apoptotic germ cells and residual bodies that are derived from the excess cytoplasmic content and organelles shed by spermatids during their differentiation.

To investigate whether the phenotype observed in the seminiferous tubules resulted from a perturbation of the Sertoli or of the germ cell functions, cross-section of tubules were immunostained with an anti-

**Table 1. Fertility assessment of Mex3b knockout mice**

<table>
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<tr>
<th>Crosses</th>
<th>Fertility*</th>
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<tr>
<td>Male +/- x female +/- (n=5)</td>
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</tr>
<tr>
<td>Male +/- x female +/- (n=9)</td>
<td>0.66</td>
</tr>
<tr>
<td>Male +/- x female +/- (n=6)</td>
<td>0.39</td>
</tr>
<tr>
<td>Male +/- x female +/- (n=7)</td>
<td>0.35</td>
</tr>
<tr>
<td>Male +/- x female +/- (n=6)</td>
<td>0.33</td>
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</tbody>
</table>

*The average number of pups per female per month during the period of fertility.

We then investigated whether the disruption of Mex3b impacted on the differentiation of Sertoli cells. For that purpose, we determined by quantitative RT-PCR the level of the mRNA coding for clusterin, the major protein synthesized by differentiated Sertoli cells that is deposited on sperm membranes (Plotton et al., 2005). As shown in Fig. 2C, there was no significant difference of the clusterin/vimentin mRNA ratio between wild-type and Mex3b−/− Sertoli cells. We also quantified transferrin, inhibin and lactate dehydrogenase (Ldha) mRNA. Transferrin is an iron transporter implicated in the regulation of residual bodies phagocytosis by Sertoli cells (Yefimova et al., 2008); inhibin B is produced in the testis, mainly by Sertoli cells and its expression positively correlates with Sertoli cell function and spermatogenesis (O’Connor and de Kretser, 2004); finally, lactate dehydrogenase A (LDHA) is expressed by differentiated Sertoli cells and the lactate produced from pyruvate by LDHA is exported and used by germ cells as an energy metabolite (Boussoar and Benahmed, 2004). As indicated in Fig. 2C, we observed solely a weak but significant increase of Ldha transcript in the Mex3b−/− Sertoli cells. Taken together, these data indicate that the lack of Mex3b results in a significant increase in the number of Sertoli cells, and alters the germ cell/Sertoli cell ratio, but without affecting the apparent differentiation of Sertoli cells.

To ascertain that the testis phenotype was due to an intrinsic defect specific to Sertoli cells, we generated mice with a disruption of Mex3b targeted to this cell type. For that purpose, we crossed Mex3bFlox/Flox mice with transgenic mice expressing the Cre recombinase under the transcriptional control of the anti-Müllerian hormone (Amh) gene promoter (Lécureuil et al., 2002). In male testis, AMH is uniquely expressed in Sertoli cells and not in germ cells. Previous studies using this AMH::Cre transgenic strain have shown that the Cre activity is detectable in Sertoli cells from mouse embryonic day 15 to adulthood (Lécureuil et al., 2002). Breeding of Mex3b floxed males expressing the Cre recombinase under the control of the Amh promoter with Mex3b floxed females leads to the same reduction of fertility than that observed upon breeding of Mex3b null male mice with wild-type females (Table 2). These data indicate that the targeted invalidation of Mex3b to Sertoli cells recapitulates the male subfertility phenotype observed with the total Mex3b knockout.

Consistently, histological examination of cross-sections of testis of 1- and 2-month-old offspring revealed obstructed seminiferous tubules at a ratio that was highly similar to that observed in Mex3b null mice (Fig. 2D). However, we observed neither a perinatal lethality nor an effect on postnatal growth of these mice. We further confirmed that the Mex3b mRNA was specifically decreased in the testis and not in other organs (Fig. 2E). Quantification of the number of germ cells and Sertoli cells showed a decrease in their ratio that was in the same order of magnitude as the effect observed in Mex3b null mice (Fig. 2F). Thus, these data establish that the testis phenotype caused by the lack of Mex3b is largely due to a defect specific to Sertoli cells.

**Mex3b function promotes phagocytosis**

The clogged lumen of seminiferous tubules observed in the Mex3b null mice could result from an aberrant accumulation of residual bodies as a consequence of a disruption of Sertoli cell phagocytic function. Accordingly, residual bodies were present in early stages of the cycle of Mex3b null mice and Mex3bFlox/Flox; AMH::Cre mice seminiferous epithelium (stages II-III, V and VI), indicating the persistence of material released from elongated spermatids at previous stages (stages VIII-XI) that were not eliminated by Sertoli cells. To test the hypothesis of a Sertoli cell phagocytic dysfunction in the absence of Mex3b, we immunolabeled 15-lipoxygenase (15-LOX;
also known as Alox15), an enzyme that peroxidizes lipids and is known to concentrate within residual bodies (Fischer et al., 2005). As depicted in Fig. 3A, obstructed tubules present in the Mex3b−/− testes showed a marked staining with the anti-15-LOX antibody compared with the tubules of wild-type animals. The same increase of 15-LOX labeling was observed in the tubules of mice with a disruption of the Mex3b locus targeted to Sertoli cells (supplementary material Fig. S3A). Furthermore, Hematoxylin and Scarlett Eosin stained pink the tissue obstructing the tubules, thus confirming that this material was of cytoplasmic origin (supplementary material Fig. S3B). Thus, we conclude that residual bodies accumulate abnormally in the lumen of the tubules of Mex3b knockout mice.

To investigate whether this phenotype resulted from a phagocytic defect, primary culture of Sertoli cells established from Mex3b−/− and wild-type mice were incubated with fluorescent latex beads and their phagocytic capacity was assessed. As shown in Fig. 3B, Mex3b−/− Sertoli cells showed a reduced ability to engulf beads compared with the wild-type Sertoli cells. This phagocytic impairment was not due to a recognition defect of the latex particle as we observed the same capacity of wild-type and Mex3b−/− Sertoli cells to absorb...
beads when placed at 4°C. To confirm these results, Mex3b was knocked down in the TM4 cell line derived from non-transformed BALB/C mouse Sertoli cells (Mather, 1980). With this RNAi approach, we were able to block Mex3b expression specifically (supplementary material Fig. S4A) without affecting the expression of other Mex3 proteins (supplementary material Fig. S4B). Furthermore, using lentiviruses expressing human MEX3B mRNA, which was insensitive to siRNA targeting mouse Mex3b mRNA, we performed rescue experiments by using the human MEX3B wild-type protein fused to the green fluorescent protein (GFP) (supplementary material Fig. S4C). Similar to what we observed in primary Sertoli cells, the reduction of Mex3b levels in TM4 drastically decreased their capacity to ingest latex beads (Fig. 3C). In addition, phagocytosis was restored upon re-expression of the wild-type MEX3B (Fig. 3C). Finally, we studied the expression of the Scl11a2 iron transporter (also called Nramp2/DMT1), which is located in the phagosome of Sertoli cells (Jabado et al., 2002), and of the class B scavenger receptor type I (SR-BI; Scarb1 – Mouse Genome Informatics), which contributes to the phagocytic process of this cell type (Nakagawa et al., 2004). However, qRT-PCR analysis did not reveal significant variation of the mRNA encoding these two proteins when TM4 cells transfected with scramble siRNA were compared with TM4 cells knocked down for Mex3b (supplementary material Fig. S3C).

The previous data indicate that Mex3b is a regulator of phagocytosis in Sertoli cells and thus raise the idea that this protein may exhibit a similar function in professional phagocytic cells. To address this question, phagocytosis assays using the same conditions defined for Sertoli cells were applied to the primary cultures of macrophages differentiated from bone marrow mononuclear cells and showed a consistent 50% reduction in the number of beads per Mex3b−/− macrophage compared with the wild type (supplementary material Fig. S3D). Collectively, these results demonstrate that Mex3b function is required for phagocytosis in Sertoli cells as well as in macrophages.

**Mex3b controls cell-cell adhesion**

Sertoli cells form the BTB, allowing a physical separation between blood vessels and the seminiferous tubules through strong intercellular adhesion. Therefore, we next examined the consequences of Mex3b ablation on cell-cell interaction between Sertoli cells. For that purpose, we performed an ultrastructural analysis on ultra-thin sections of testes. As depicted in Fig. 4A, Sertoli cells in control testes are well organized and the BTB is lined with endoplasmic reticulum cisternae and clearly delimited. By contrast, the basal region of Mex3b−/− Sertoli cells appears distended and locally interrupted, revealing an expansion of

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**Table 2. Fertility assessment of Sertoli-specific Mex3b-deficient mice**

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<td>Male Flox/Flox × female Flox/Flox (n=6)</td>
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<tr>
<td>Male AMH:Cre; Flox/Flox × female Flox/Flox (n=6)</td>
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<td>Male AMH:Cre; Flox/Flox × female AMH:Cre; Flox/Flox (n=6)</td>
<td>0.31</td>
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*The average number of pups per female per month during the period of fertility.

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**Fig. 3. Phagocytic function of Sertoli cells depends on Mex3b.** (A) Immunodetection of the residual bodies component 15-lipoxygenase (white) on cross-sections of 6-month-old mouse testes of the indicated Mex3b genotypes. Stage IV seminiferous tubes are shown. Nuclei were stained with DAPI. (B) Epifluorescent images showing latex beads (red) phagocytosed by primary Sertoli cells isolated from wild-type and Mex3b−/− mouse testes. Nuclei were stained with DAPI. Histogram indicates the average number of phagocytosed beads per DAPI-stained cell (n=200 cells per condition). (C) Projection of confocal sections showing latex beads (white) phagocytosed by TM4 Sertoli cells depleted for endogenous Mex3b by siRNA and re-expressing the human MEX3B protein fused to GFP. Cells were counterstained with phalloidin-TRITC and z-sections of confocal acquisition are depicted to show bead incorporation inside the cells. Histogram indicates the average numbers of engulfed fluorescent beads per phalloidin-TRITC-stained cell (n=200 cells per condition). Error bars represent s.e.m.
intercellular spaces between adjacent cells. The inset in Fig. 4A shows focal disruption of the BTB, thus confirming that the barrier integrity was severely compromised in Mex3b−/− seminiferous tubules.

Then, we examined the expression of N-cadherin (N-Cad; Cdh2 – Mouse Genome Informatics) and connexin 43 (Cx43; Gja1 – Mouse Genome Informatics), two molecules that contribute to the formation of the BTB and are constituent proteins of adherens and gap junctions, respectively (Li et al., 2010; Newton et al., 1993). Immunofluorescence staining of the wild-type mouse seminiferous epithelium with antibodies recognizing these two proteins identified a belt-like structure near the basal lamina that corresponds to the BTB (Fig. 4B). By contrast, the labeling of N-Cad and Cx43 was weaker and more diffuse in the Mex3b knockout mice seminiferous tubules (Fig. 4B).

Abnormal permeability of the BTB may result in the release of germ cells in the bloodstream that leads to the mounting of an autoimmune response and the production of anti-sperm antibodies. Therefore, we measured the level of anti-sperm antibodies in the serum of mice with a genetic disruption of Mex3b in Sertoli cells. As shown in Fig. 4C, anti-sperm antibodies were detected in the serum of mice deficient for Mex3b in Sertoli cells at a level that was higher than the limit of the test positivity, in contrast to observations in the wild-type mouse. Consistently, a biotin marker injected under the testicular tunica albuginea of Mex3b knockout mice was able to diffuse in the lumen of seminiferous tubules in a reproducible manner, whereas it was blocked by the intact BTB of wild-type mice (Fig. 4D). These data confirm that the tightness of the BTB was weakened in Mex3b-deficient seminiferous tubules.

Finally, to reinforce these data, we studied N-Cad and Cx43 expression in TM4 cells knocked down for Mex3b. Staining of these two intercellular junction components decorating the surface of TM4 cells inhibited by siRNA and re-expressing the human MEX3B protein. N-cadherin and connexin 43 (red) were immunostained and counterstained with DAPI.

Fig. 4. Mex3b controls adhesion properties of Sertoli cells. (A) Electron microscopy images of intercellular junctions in testes sections from wild-type and Mex3b-deficient mice. Arrows indicate cellular junctions constituting the BTB between two adjacent Sertoli cells (Ser1 and Ser2). Insets show magnification of the cellular junction region. Scale bars in insets: 60 nm. (B) Confocal microscope images of wild-type and Mex3b-deficient mice testis sections immunostained for N-cadherin and connexin 43 (red) (6-month-old mice). DAPI was used to stain nuclei and white boxes indicate the area magnified in the inserts. (C) Detection of anti-sperm cell antibodies in serum of wild-type and Mex3b−/−; AMH::Cre-deficient mice. Red line indicates the limit of ELISA test’s significance according to the manufacturer (threshold of test positivity). (D) Epifluorescence images of sections from adult wild-type and Mex3b-deficient mice testis, previously injected with a biotin tracer that was allowed to diffuse in the seminiferous tubes of living mice for 30 min. (E) Confocal microscope images of TM4 cells with endogenous Mex3b inhibited by siRNA and re-expressing the human MEX3B protein. N-cadherin and connexin 43 (red) were immunostained and counterstained with DAPI.
Mex3b interacts with Rap1GAP

While exploring possible mechanisms accounting for the role of Mex3b in phagocytosis and in intercellular adhesion, our attention was drawn to a large-scale yeast two-hybrid screen that found that the GTPase-activating protein Rap1GAP binds to MEX3B (Stelzl et al., 2005). Rap1 is a small GTP-binding protein that cycles between an active, GTP-bound, and an inactive, GDP-bound state (Caron, 2003; Gloerich and Bos, 2011). Interestingly, Rap1 proteins...
are key regulators of phagocytosis and cell-cell adhesion. Rap1GAP, the putative MEX3B interactor, belongs to a group of related molecules which increase the intrinsic GTPase activity of Rap1, thereby antagonizing the function of Rap1 guanine nucleotide exchange factors (Rap1-GEFs) that load GTP on Rap1 (Polakis et al., 1991; Rubinfeld et al., 1991).

We first examined whether endogenous MEX3B and Rap1GAP proteins interacted in cells. Immunoprecipitation of the endogenous human MEX3B from a lysate of human kidney epithelial cells (BOSC cells) followed by a western blot with an anti-Rap1GAP antibody revealed a specific interaction between these two molecules (Fig. 5A). The complex between endogenous Mex3b and Rap1GAP was also detected in mouse Mex3b+/+ testis extracts and, as expected, no Rap1GAP was co-immunoprecipitated with the polyclonal serum recognizing MEX3B when testis extracts were prepared from the Mex3b−/− mice (Fig. 5A). As RBPs may indirectly bind to another protein through RNA bridging, we expressed epitope-tagged Myc-MEX-3B and Rap1GAP-HA and incubated transfected BOSC cell extracts with RNase A after lysis. As shown in Fig. 5B, the complex between Myc-MEX-3B and Rap1GAP-HA did not dissociate upon RNase A treatment, but, on the contrary, the binding between these two proteins was even slightly strengthened under this condition. Thus, our data confirm that MEX3B and Rap1GAP physically interact in an RNA-independent manner.

In order to map the regions of MEX3B involved in Rap1GAP binding, we constructed a series of Myc-tagged MEX3B deletion mutants. Immunoblotting with the anti-HA and anti-Myc antibodies confirmed that Rap1GAP-HA and mutants Myc-MEX-3B were expressed at similar levels after transfection (Fig. 5C). As shown in Fig. 5C, deletion of the first 239 amino acids (MEX-3B 239-569 mutant) did not abolish this interaction whereas truncation of MEX3B after the second KH domain (MEX-3B 1-239) prevented the binding of Rap1GAP-HA. Together, these data show that Rap1GAP interaction surface of MEX3B encompasses the region between the second KH domain and the ring finger motif.

We next wondered whether phagocytic and adhesion defects observed in Mex3b-depleted cells could be linked to the effect on Rap1GAP and the Rap1 pathway. If this hypothesis is true, the silencing of Rap1GAP in TM4 cells should phenocopy the lack of Mex3b. Using this approach, we found that Rap1GAP knockdown resulted in the reduction of both phagocytosis (Fig. 5D) and of N-Cad

Fig. 6. Mex3b regulates Rap1 activity through the recruitment of Rap1GAP at the plasma membrane. (A) Rap1-GTP pulldown from whole testis and purified Sertoli cells lysates assessed by western blot. The ratio of Rap1-GTP/Total Rap1 for each condition is indicated underneath the western blot. (B) Rap1-GTP pulldown from lysates of TM4 cells treated with siRNA targeting mouse Mex3b mRNA and re-expressing human MEX3B-GFP protein. (C) N-cadherin and connexin 43 localization after siRNA and GGTI-298 treatment was visualized by immunofluorescence using confocal microscopy. Cells were counterstained with DAPI. (D) Immunolocalization of Rap1GAP-HA in TM4 cells knocked down for Mex3b expression by siRNA and re-expressing MEX3B-GFP after serum stimulation. (E) Localization of GFP-RBDvalGDS in TM4 cells knocked down for Mex3b expression by siRNA. Cells were exposed to phalloidin-TRITC prior to observation by confocal microscopy. White arrowheads indicate the accumulation of GFP-RBDvalGDS.

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**Table:**

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<th>Condition</th>
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<tr>
<td>Total Rap1</td>
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**Figures:**

(A) Rap1-GTP pulldown from whole testis and purified Sertoli cells lysates assessed by western blot. The ratio of Rap1-GTP/Total Rap1 for each condition is indicated underneath the western blot. (B) Rap1-GTP pulldown from lysates of TM4 cells treated with siRNA targeting mouse Mex3b mRNA and re-expressing human MEX3B-GFP protein. (C) N-cadherin and connexin 43 localization after siRNA and GGTI-298 treatment was visualized by immunofluorescence using confocal microscopy. Cells were counterstained with DAPI. (D) Immunolocalization of Rap1GAP-HA in TM4 cells knocked down for Mex3b expression by siRNA and re-expressing MEX3B-GFP after serum stimulation. (E) Localization of GFP-RBDvalGDS in TM4 cells knocked down for Mex3b expression by siRNA. Cells were exposed to phalloidin-TRITC prior to observation by confocal microscopy. White arrowheads indicate the accumulation of GFP-RBDvalGDS.
and Cx43 at the cell surface, a phenotype that was comparable to what was observed when Mex3b expression was downregulated (Fig. 5E). Furthermore, expression of a truncated version of MEX3B unable to bind RAP1GAP (MEX-3B 1-239) in TM4 cells previously knocked down for Mex3b did not restore membrane localization of N-cadherin and connexin 43 as full-length MEX3B did (Fig. 4E; supplementary material Fig. S5). Altogether, these data are consistent with a positive coupling between MEX3B and Rap1GAP in the regulation of phagocytosis and cell-cell interaction.

**Mex3b regulates the activity and the spatial localization of Rap1GAP**

Mex3b interaction with Rap1GAP might impact its ability to modulate Rap1 activity. Thus, to determine whether Mex3b could regulate Rap1 activity, we performed a series of pulldown assays to precipitate GTP-bound Rap1 using the Rap1 substrate Ral-GDS (van Triest et al., 2001). A robust and reproducible increase of Rap1-GTP was observed in Mex3b<sup>−/−</sup> testis extracts compared with the Mex3b<sup>+/+</sup> control (Fig. 6A). A similar enhancement of Rap1-GTP was revealed in Mex3b<sup>−/−</sup> primary cultures of Sertoli cells compared with Mex3b<sup>+/+</sup> Sertoli cells (Fig. 6A). We further confirmed these data in TM4 cells after Mex3b silencing. In addition, expression of human MEX3B in TM4 cells treated with siRNA targeting mouse Mex3b reduced the level of Rap1-GTP to that observed in TM4 cells transfected with scrambled siRNA (Fig. 6B) without modification of Rap1GAP RNA or protein levels (supplementary material Fig. S4D,E). Thus, we conclude that Mex3b positively regulates Rap1GAP function and thereby restrains Rap1 activity.

As Mex3b depletion reduces intercellular adhesion and regulates the activity of Rap1 through Rap1GAP, we tested whether the effect of Mex3b on cell-cell contact was dependent on Rap1 activation. We speculated that a mild reduction of Rap1 signaling could rescue the proper localization of N-Cad and Cx43 at the cell surface. To test this idea, Mex3b was silenced in TM4 cells and exposed to the Rap1 inhibitor GGTI-298 (Efuet and Keyomarsi, 2006). Analysis of Rap1-GTP levels by pulldown confirmed the partial inhibition of Rap1 activity after 30 min of exposure to 12.5 μM GGTI (supplementary material Fig. S6A). As shown in Fig. 6C, this treatment led to the redistribution of N-Cad and Cx43 to the cell surface in TM4 cells knocked down for Mex3b. Thus, a sustained activation of Rap1 subsequent to the loss of Mex3b is responsible for the failure of N-Cad and Cx43 to be localized and stabilized at the cell surface.

It has been previously reported that the spatial gradient of Rap1-GTP depends on Rap1GAP activity, which is higher in the periphery than in the central region of the cells (Mochizuki et al., 2001; Ohba et al., 2003). These results prompted us to examine whether Mex3b could regulate Rap1GAP subcellular localization. As previously described (Bucheton-Poutay et al., 2001), exogenously expressed MEX3B-GFP fusion is localized in the nucleus, in the cytoplasm and also decorates the cell cortex (supplementary material Fig. S6B). Accordingly, an epitope-tagged Rap1GAP-HA construct localizes to the cortical plasma membrane of TM4 cells under serum stimulation (Fig. 6D). Strikingly, Mex3b silencing perturbed this cortical localization and led to a more diffuse cytoplasmic pattern. Furthermore, re-expression of MEX3B in Mex3b knocked down cells restored Rap1GAP-HA localization to the plasma membrane (Fig. 6D). These results indicate that Mex3b is required for the proper targeting of Rap1GAP to the inner face of the plasma membrane.

To address this hypothesis directly, we used the fusion protein GFP-RBD<sub>RAP1GDS</sub> which interacts with Rap1-GTP and allows the visualization of active Rap1 through GFP signal (Bivona et al., 2004). Under control conditions, Rap1-GTP was detected in the cytoplasm and was weakly enriched at the plasma membrane of TM4 cells in basal conditions (Fig. 6E). However, upon silencing of Mex3b, we observed a marked increase of the GFP signal with a clear enhancement of the labeling at the plasma membrane, thus reflecting a higher Rap1-GTP concentration at the cell cortex. Furthermore, GGTI treatment reduced the overall GFP signal and clearly diminished the decoration of the inner face of the plasma membrane with the GFP-RBD<sub>RAP1GDS</sub> (supplementary material Fig. S6C). Overall, these results indicate that Mex3b regulates the expression of intercellular adhesion molecules by controlling the level and the localization of Rap1-GTP via its effect on both Rap1GAP activity and transport.

**DISCUSSION**

We report here that Mex3b-deficient mice are subfertile. Investigation of the pathophysiological processes in Mex3b-deficient males showed that residual bodies released by spermatoctyes during their differentiation obstruct a fraction of mouse seminiferous tubules, thus causing a net decrease in the spermatozoa yield. In addition, the BTB is loosened and anti-sperm antibodies are produced. These effects were found to arise from a disruption of phagocytosis and adhesive abilities of Mex3b-deficient Sertoli cells. Exploration of the underlying mechanisms revealed the causative role of a sustained activation of Rap1 at the Sertoli cell cortex that is a consequence of a perturbation of Rap1GAP activity, activity and subcellular localization of which are controlled by Mex3b. Thus, this work unveils a key role for Mex3b in the spatial organization of the Rap1 signaling pathway, which regulates Sertoli cell biological properties.

Our results indicate that a third of seminiferous tubules was obstructed in Mex3b<sup>−/−</sup> null mice testes and a similar proportion of tubules was affected in males with a Mex3b locus specifically disrupted in Sertoli cells. This incomplete penetrance could be linked to the modifying effects of the genetic makeup of the mice, but we cannot formally exclude a compensatory effect of other Mex3 genes that are expressed in Sertoli cells. We also observed that the multilayers of granulosa cells surrounding the oocyte showed an abnormal histology in Mex3b<sup>−/−</sup> female oocytes. As granulosa cells share the same embryonic origin than Sertoli cells and communicate via gap junctions containing Cx43 (Patek et al., 1991), it is possible that Mex3b regulates granulosa functions and folliculogenesis through the same mechanisms identified in Sertoli cells.

It is well documented that Sertoli cells have a key scavenger function as they eliminate apoptotic germ cells and remove residual bodies released during the cytoplasmic reduction phase of spermiogenesis (Nakanishi and Shiratsuchi, 2004). However, we did not notice a decrease in the clearance of apoptotic germ cells in Mex3b<sup>−/−</sup> null mice. Spermiogenesis occurs at the apical adluminal compartment of the tubules, which contains round elongating/elongated spermatoctyes attached to Sertoli cells via an actin-based adherences junction (AJT) type designated as the apical ectoplasmic specialization (ES) (Lee and Cheng, 2004). Thus, in vivo, the action of Mex3b may be compartmentalized to this region of the Sertoli-germ cell interface to control phagocytic removal of residual bodies, a question that remains to be explored. Although we did not observe an overt reduction of Mex3b<sup>−/−</sup> sperm cell motility, we cannot formally exclude that this Sertoli phagocytic defect may also affect spermiogenesis and consequently decrease the sperm fertilizing fitness.

A net reduction of N-Cad and Cx-43, two junction molecules that contribute to the establishment of the BTB, is observed in the...
semimiferous tubules of Mex3b-deficient mice. Consistently, electron microscopy analysis shows a focal disruption of the BTB in the Mex3b<sup>−/−</sup> testis and experiments using a biotin tracer confirm that the tightness of the BTB is indeed altered. The detection of anti-sperm antibodies in the serum of mice deficient for Mex3b in Sertoli cells convincingly strengthens the notion that the integrity of the BTB is impaired in the absence of Mex3b.

Our conclusion that Mex3b is a regulator of the Rap1 pathway involved in BTB formation is based on the following series of complementary results: (1) endogenous Mex3b binds to Rap1GAP; (2) Mex3b inactivation in mouse prevents Rap1GAP localization at the cell cortex and leads to aberrant Rap1 activation as revealed by biochemical approaches and imaging techniques that identify Rap1-GTP in cells; (3) the membrane localization of N-Cad and Cx43 depends on the ability of Mex3b to bind Rap1GAP and treatment of TM4 cells deficient for Mex3b with the Rap1 inhibitor GGTI-298 rescues the proper cell surface expression of these two cell junction molecules; and finally, (4) Rap1GAP RNAi phenocopies the phagocytosis and cell-cell interactions defects generated by the lack of Mex3b. Thus, these data, together with the observed diminution of the levels of N-Cad and Cx43 transcripts in the absence of Mex3b, raise the idea that this RBP acts at successive steps to regulate the transcriptional stabilization of proper expression of these junction molecules. In conclusion, this study provides insights into the in vivo function of mammalian MEX-3 proteins by ascribing a role to Mex3b in the spatial assembly of the Rap1 pathway, which proves necessary for normal Sertoli cell physiology. This work is congruent with recent large-scale analyses that discovered a general scaffolding role for RBPs in the dynamic organization of localized signaling centers (de Hoog et al., 2004; Lécuyer et al., 2007). As alterations of the integrity of the BTB is a frequent cause of male infertility, it is plausible that mutations affecting either MEX3B or genes coding for components of the Rap1 pathway may account for cases of human spermatogenic defects.

**Materials and methods**

**Mice**

Total and Sertoli cell-specific Mex3b null mice were generated using embryonic stem cell technology as described (Hogan et al., 1994). For details of generation and genotyping, see supplementary materials and methods.

All animal experiments were conducted according to the standard ethical guidelines of the Institut National de la Santé et de la Recherche Médicale (INSERM).

Reproductive ability of mice was assessed by mating 2-month-old mice of the indicated genotype for 9 months. For the counting of sperm cells, cauda epididymis and vasa deferentia were excised. Semen was allowed to exude for 15 min at 37°C and counted using a Malassez grid.

**Cell lines**

MEFs were isolated from E12.5 embryos as previously described. Sertoli cells were isolated from ~10- to 14-day-old mice and cultured as described previously (Weiss et al., 1997). The mouse Tm4Sertoli and human BOSC cell lines were obtained from the American Tissue Culture Collection (ATCC).

**Phagocytosis assay**

Cells were lysed 48 h post-transfection and immunoprecipitation and western blot analyses were performed as described previously (Nony et al., 2003). When indicated, protein extracts were treated with RNase A (0.2 mg/ml; Roche) prior to immunoprecipitation. When performed, Rap1 activation assay was carried out according to the manufacturer’s protocol (Rap1 activation assay kit; Millipore) and quantification of Rap1-GTP/total Rap1 was performed using ImageJ software.

**Western blot and immunoprecipitation**

Cells were lysed 48 h post-transfection and immunoprecipitation and western blot analyses were performed as described previously (Nony et al., 2003). When indicated, protein extracts were treated with RNase A (0.2 mg/ml; Roche) prior to immunoprecipitation. When performed, Rap1 activation assay was carried out according to the manufacturer’s protocol (Rap1 activation assay kit; Millipore) and quantification of Rap1-GTP/total Rap1 was performed using ImageJ software.

**Antibodies and reagents**

Mouse monoclonal anti-myc (9E10), anti-GFP (Roche Applied Science), anti-HA (16B12, Covance), anti-tubulin (gift from L. Lafanache, Université Joseph Fourier-Grenoble; Institut Albert Bonniot, Grenoble), Mouse monoclonal anti-myc (9E10), anti-GFP (Roche Applied Science), anti-HA (16B12, Covance), anti-tubulin (gift from L. Lafanache, Université Joseph Fourier-Grenoble; Institut Albert Bonniot, Grenoble) antibodies were used in western blots at 1:5000, 1:1000, 1:250,000 and 1:1000, respectively. Rabbit polyclonal anti-Mex3b antibody was used for western blots at 1:750 and recognizes both mouse and human Mex3b. Rap-1 inhibitor GGTI-298 was purchased from Sigma-Aldrich.

**Histological sections, immunofluorescence staining and electron microscopy**

Tissues samples were placed in 4% formaldehyde or Alcohol Bouin’s overnight and embedded in paraffin or directly frozen at −80°C. For details of staining, see supplementary materials and methods.

After the staining, vimmentin-positive cells (Sertoli cells) were counted as well as germ cells with the Hematoxylin counterstain in seminiferous tubules. For stages I-VII, all round spermatids were counted and for stages VII-XII all pachytenic spermatocytes were enumerated based on the established classification of seminiferous tubules stages (Russell and Peterson, 1985) (n=100 seminiferous tubules on six male mice at each age point).
Immunofluorescence analyses on frozen section and cells were performed as described previously (Carette et al., 2010). N-cadherin, connexin 43 and 15-lipoxygenase (gift from P. Sutovsky, University of Missouri, Columbia, USA) antibodies were used at 1:100. Immunolocalization of Rap1GAP-HA and human MEX3B-GFP protein was determined after overnight deprivation of serum and a short activation (10 min).

For electron microscopy, testis pieces were fixed with glutaraldehyde, post-fixed in reduced osmium, dehydrated and embedded in Epon as previously described (Lablack et al., 1998). The sections were analyzed with a JEOL 1200EX electron microscope (Institut des Neurosciences, Grenoble, France).

Detection of anti-sperm cell antibodies

Sperm antibodies were measured in the serum of 3-month-old wild-type and Mex3b<sup>Flox/Flox</sup>, AMH−/Cre deficient mice using the ELISA kit from Cusabio as specified by the manufacturer’s instructions.

Biotin tracer studies

The permeability of the BTB was assessed by using a biotin tracer as described previously (Meng et al., 2005). For details, see supplementary materials and methods.

Cloning and transfection

Myc-Mex-3b and Myc-Mex-3b mutants were cloned in Myc-pCMV-Tag3B vector (Stratagene) as described previously (Buchet-Poyau et al., 2007). pm210/Rap1GAP-HA and GFP-RBDB<sub>3AD</sub> (gift from R. Phillips, University of North Carolina, Chapel Hill, USA) constructs were also used. For the rescue experiments, pLENTI CAG-human MEX3B fused with GFP were designed to infect TM4 cells and ensure that siRNA against mouse Mex3b did not reduce expression of human MEX3B-GFP.

Short interfering RNAs (siRNAs) against the mouse Mex3b and Rap1GAP gene and the non-targeting control siRNA were purchased from Dharmacon. Goedel-GAP was designed to infect TM4 cells and ensure that siRNA against mouse Rap1GAP did not reduce expression of human RAP1GAP.

RNA isolation and RT-PCR analysis

Total RNA was extracted from cell lines or tissues using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Then, RNAs were reverse transcribed to cDNA using the Omniscript Reverse Transcription Kit (Qiagen) and real-time RT-PCR was performed using the MXP-3000 PCR system (Stratagene). RNA of pachytene spermatocytes (PS) or round spermatids (RS) were obtained by centrifugal elutriation. The sequences of the different primers used for RT-PCR are listed in Supplementary Table S1.

Statistical analysis

All experiments were carried out in triplicate or with a significant number of individuals and statistical analyses were performed with the statistical package GraphPad Prism using the unpaired Student’s t-test (confidence intervals 99%). Values are given as mean and standard error of the mean (s.e.m.).

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.L.B., K.B.-P., N.C., J.C. and M.B. conceived and designed the experiments. M.L.B., K.B.-P., C.T. and N.C. performed a large part of the experiments from mouse phenotyping to molecular biology studies. O.D., E.F. and C.A.-R. contributed to biochemistry and imaging experiments. J.-P.R. engineered the lentiviral vectors expressing MEX3 proteins and D.N. produced lentiviral particles. D.S. contributed to the interpretation of the electron microscopic images. S.R. and S.K. discussed regularly the results concerning the fertility phenotype, suggested experiments and shared protocols and expertise. D.B., P.C. and F.G. participated to the generation of the mouse models. P.D. and M.H.P. were involved in the characterization of the tests phenotype, in the primary culture of Sertoli cells and in the phagocytosis assays. M.B., M.L.B. and N.C. wrote the manuscript.

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Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.108514/DC1

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