Spermatogenesis, which involves mitosis and meiosis of male germ cells, is a highly complicated and coordinately ordered process. Cyclin B1 (CCNB1), an important regulator in cell cycle machinery, is proved essential for mouse embryonic development. However, the role of CCNB1 in mammalian spermatogenesis remains unclear. Here we tested the requirement for CCNB1 using conditional knockout mice lacking CCNB1 in male germ cells. We found that ablation of CCNB1 in gonocytes and spermatogonia led to mouse sterile caused by the male germ cells' depletion. Gonocyte and spermatogonia without CCNB1 is unable to proliferate normally and apoptosis increased. Moreover, CCNB1 ablation in spermatogonia may promote their differentiation by downregulating Lin28a and upregulating let-7 miRNA. However, ablation of CCNB1 in premeiotic male germ cells did not have an effect on meiosis of spermatocytes and male fertility, suggesting that CCNB1 may be dispensable for meiosis of spermatocytes. Collectively, these results indicate that CCNB1 is critically required for the proliferation of gonocytes and spermatogonia but may be redundant in meiosis of spermatocytes in mouse spermatogenesis.

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In eukaryotic cells, the onset of M phase is controlled by a common mechanism. Maturation-promoting factor or M-phase-promoting factor (MPF), which is composed by cyclin-dependent kinase 1 (CDK1) and cyclin B, governs M-phase entry in eukaryotic cells.\(^1\)\(^-\)\(^7\) The activation of MPF requires the dephosphorylation of CDK1 and the association of Cyclin B.\(^8\)\(^-\)\(^17\) In amphibian, two B-type cyclins, cyclin B1 (encoded by ccnb1) and cyclin B2 (encoded by ccnb2), have been reported to associate with Cdk1 and promote MPF activation.\(^11\)\(^,\)\(^24\) In frog oocytes, microinjection of ccnb1 or ccnb2 mRNA into immature oocytes could induce germinal vesicle breakdown (GVBD); co-inhibition of ccnb1 and ccnb2 endogenous mRNA translation, but not one of them, with antisense RNAs can inhibit progesterone-induced GVBD.\(^24\) Moreover, in the extracts of active Xenopus eggs, ablation of either ccnb1 or ccnb2 alone was unable to arrest mitosis, but when both cyclin mRNAs were destroyed, the mitosis events were unable to happen.\(^11\) These results suggest that ccnb1 and ccnb2 have redundant roles in the mitosis and meiosis of frog.

However, CCNB1 and CCNB2 were reported to have different localization and expression pattern in mammals,\(^25\)^\(^-\)\(^28\) indicating that they may have distinct roles in mitosis and meiosis of mammalian cells. In human tissue cultured cells, both CCNB1 and CCNB2 are associated with CDK1 and promote CDK1 activation during mitosis; but their localized structure in cell is quite different: CCNB1 to microtubules, CCNB2 primarily to Golgi apparatus.\(^25\)\(^,\)\(^26\) In mouse testis, Ccnb2 mRNAs were found primarily in mitotically dividing spermatocytes, whereas Ccnb1 transcripts were most abundant in the postmeiotic germ cells.\(^27\)\(^,\)\(^28\)

In addition, Ccnb1-null mice die in utero, whereas Ccnb2-null mice is viable and fertile,\(^29\) suggesting that CCNB1 is critically required for mouse embryogenesis, whereas CCNB2 is largely redundant in mouse embryogenesis and productivity. However, whether CCNB1 and CCNB2 have distinct roles in mitosis and meiosis of mammalian cells remain unclear.

Mouse spermatogenesis, which involves mitosis of spermatogonia and meiosis of spermatocytes, is a powerful in vivo system to study the regulation of mitosis and meiosis in mammals. In the present study, we generated conditional knockout mice lacking CCNB1 in different stages of male germ cells to shedding light on the function of CCNB1 in mitosis and meiosis of male germ cells. We found that CCNB1 was critically required for the proliferation of gonocytes and spermatogonia. These cells lacking CCNB1 were unable to proliferate normally and apoptosis increased. We also found that ablation of CCNB1 in spermatogonia might promote their differentiation by downregulating Lin28a and upregulating let-7 miRNAs. However, deletion of Ccnb1 in postnatal, premeiotic male germ cells did not have an effect on spermatocyte meiosis and male fertility, suggesting that CCNB1 may be redundant in meiosis of spermatocytes.

**Results**

Generation of conditional knockout mice lacking CCNB1 in male germ cells. In order to test the requirement for CCNB1 function in male germ cell mitosis and meiosis, we generated two strains of mice, one specifically lacking CCNB1 in all male germ cells and the other lacking CCNB1...
in postnatal, premeiotic male germ cells. An ES cell line (Clone No. EPD0357_2_A11) from EUCOMM with Ccnb1 gene targeting was used for microinjection to generate the mouse model. To achieve the Ccnb1 gene targeting, an L1L2_Bact_P cassette was inserted at position 100782436 of Chromosome 13 between exons 4 and 5. The cassette, which is flanked by two FRT sites, is composed of lacZ sequence, the first loxP site, neomycin under the control of the human beta-actin promoter and SV40 poly A. The cassette end with the second loxP site and the third loxP site is inserted downstream of the targeted exon 9 at position 100779501. A ‘conditional ready’ (floxed) allele can be created by flp recombinase expression, leaving exons from 5 to 9 flanked by loxP sites (Figure 1a). We then crossed mice homozygous for the floxed Ccnb1 allele (Ccnb1f/f) with Mvh-Cre or Stra8-Cre transgenic mice, which express Cre recombinase under the Mvh promoter and Stra8 promoter, respectively (Figures 1b and c). The mRNA and protein level of Ccnb1 were tested in the adult mice testes by QRT-PCR and western blotting, respectively. Both protein (Figures 1d and e) and mRNA (Figures 1f and g) of Ccnb1 in Mvh-cKO and Stra8-cKO mouse testes were significantly reduced compared with Ccnb1f/f (Control) mice, suggesting that Ccnb1 was efficiently deleted in male germ cells.
Ablation of CCNB1 in gonocytes and spermatogonia led to sterility of male mice caused by germ cells’ depletion.

To inactivate the CCNB1 in male mouse germ cells at the early stage of germ cell development, we ablated CCNB1 in early stage of male germ cells using Mvh-Cre transgenic mice in which Cre recombinase was expressed in male germ cells at embryonic day 15 (E15). Adult Mvh-cKO male mice were overtly normal, but their testes were smaller than the control littermates (Figure 2a) and testis weight of the adult Mvh-cKO mice was strikingly reduced compared with the control littermates (Control: 122.2 ± 5.963 mg; n = 12; Mvh-cKO: 17.9 ± 0.899 mg; n = 12) (Figure 2b). Fertility testing showed that the Mvh-cKO male mice were completely sterile (Figure 2c). Histological analysis indicated that seminiferous tubules of Mvh-cKO mice were small and contained no germ cells (Figure 2d). The epididymis of Mvh-cKO mice were also smaller than the control littermates (Figure 2a) and no spermatozoa were observed (Figure 2d). To explore at which stage the germ cells disappeared in the Mvh-cKO mouse testis, we collected the day postnatal 1 (1 dpn), 3, 7 and 15 dpn control and Mvh-cKO mouse testes and examined the existence of germ cells by staining the germ cells with specific marker TRA98 and Sertoli cell marker SOX9. The results showed that quantity of germ cells in 1 dpn control and Mvh-cKO mouse testes had no difference, but germ cells notably reduced in 3 dpn Mvh-cKO mouse testis (Figure 2e). At 7 dpn, germ cells could proliferate normally in control mouse testes but not in Mvh-cKO mouse testes (Figure 2e). At 15 dpn, almost no germ cells existed in the seminiferous tubules of Mvh-cKO mice (Figure 2e). This phenotype may be...
caused by excessive apoptosis because there are no more spermatogonia. Germ cells were unable to detect at the 18 dpn Mvh-cKO mouse testis (Supplementary Figure S1A). We then investigated the expression of germline-specific genes (Dazl, Oct4, Figla and Mvh) in 7 and 120 dpn mouse testes using RT-PCR. We found that the expression of germline-specific genes significantly reduced in 7 dpn Mvh-cKO mice testis and were unable to detect in 120 dpn Mvh-cKO mice testis (Supplementary Figure S1B). Collectively, these results indicate that deletion of Ccnb1 in early-stage male germ cells (gonocytes and spermatogonia) results in male sterile due to germ cells’ depletion before the first wave of spermatogenesis.

Gonocytes and spermatogonia without Ccnb1 were unable to proliferate normally and apoptosis was increased. To investigate what caused the germ cells depletion in Mvh-cKO male mice, we first examined proliferation of the germ cells in 7 dpn mouse testis. We found that, in the Mvh-cKO mice testis, although germ cells expressed M-phase-specific marker H3pSer10, they were unable to complete the M-phase events, such as chromosomes condensation and nuclear envelop breakdown (Figures 3a and b). We then examined the expression of several apoptosis-related genes in 7 dpn mouse testis by RT-PCR and QRT-PCR and found that the expression of p53, Reprimo and Caspase-3 were notably increased in the Mvh-cKO mouse testis (Supplementary Figure S2, Figures 3c–e). Moreover, immunostaining of p53 showed that some germ cells in 7 dpn Mvh-cKO mouse testes expressed the p53 protein, but no p53-active germ cell was observed in the control littersmates (Figure 3f). In addition, TUNEL assays also showed that the apoptosis signal was remarkably increased in the Mvh-cKO mice testis compared with the control littersmates (Figure 3g). These results suggest that male germ cells depletion in Mvh-cKO mouse testes is probably due to the inhibited proliferation of male germ cells and increased apoptosis.

Ablation of Ccnb1 in postnatal, premeiotic male germ cells does not have an effect on spermatocyte meiosis and male fertility. Because male germ cells were completely disrupted before the first wave of spermatogenesis in Mvh-cKO mice, the function of Ccnb1 in meiosis of spermatocytes remain unclear. To investigate the role of Ccnb1 in meiosis of mammalian male germ cells, we generated the Stra8-cKO mice. In these mice, Ccnb1 floxed allele was deleted by Stra8-Cre in postnatal, premeiotic male germ cells. Testes of adult Stra8-cKO mice were smaller generated the Stra8-cKO mice. In these mice, CCNB1 in meiosis of mammalian male germ cells, we spermatocytes remain unclear. To investigate the role of telo disrupted before the first wave of spermatogenesis in and male fertility.

Ablation of CCNB1 in postnatal, premeiotic male germ cells might promote their differentiation by downregulating the expression of Lin28a and upregulating the expression of let-7 miRNAs. CCNB1 may not have an essential role in spermatogonial germ cells’ differentiation, as Stra8-cKO mice have normal fertility. To investigate the effect of CCNB1 ablation on spermatogonial germ cells’ differentiation, we examined the expression of the gonocytes and undifferentiated spermatogonia-specific genes, Plzf and Lin28a and a differentiating spermatogonia-specific gene c-Kit in 2, 3 and 7 dpn Mvh-cKO and adult Stra8-cKO mouse testis. The expression of c-Kit is significantly reduced in 2 dpn Mvh-cKO mice testis (Figure 5a), but in 3 dpn mouse testis, its expression had returned to normal level (Figure 5b). Moreover, the expression of c-Kit in adult Stra8-cKO mice was strikingly increased (Figure 5d). These observations indicate that ablation of CCNB1 in gonocytes and spermatogonia does not inhibit their differentiation; in contrast, it might promote their differentiation. Interestingly, we observed that, in contrast to 2, 3 and 7 dpn Mvh-cKO mouse testis, the expression of Plzf in adult Stra8-cKO mouse testis was significantly increased (Figures 5a–d). The best interpretation of this phenomenon is that Ccnb1 is not completely depleted in all spermatogonia in Sta8-cKO male mice, as Stra8-Cre just delete Ccnb1 in part of undifferentiated spermatogonia and in all differentiated spermatogonia. We also found that the expression of Gdnf, which can promote self-renewal of undifferentiated spermatogonia, was remarkably increased in the testis of 3 and 7 dpn Mvh-cKO mice and adult Stra8-cKO mice testis (Figures 5b–d). However, even with the higher expression of Gdnf, the expression of Plzf was still notably reduced in 3 and 7 dpn Mvh-cKO mice testis (Figures 3b and c), suggesting that CCNB1 is critically required for the undifferentiated germ cells’ self-renewal. Another interesting phenomenon is that, in contrast to Plzf, the expression of Lin 28a was notably reduced in the adult Stra8-cKO mice testis. In fact, although Plzf and Lin28a were remarkably reduced in 2, 3 and 7 dpn Mvh-cKO mouse testis, the expression of Lin28a reduced more severely than Plzf. These results indicate that LIN28A may be involved in germ cells’ differentiation. LIN28A specifically regulates the maturation of let-7 miRNAs. We then investigated the expression of let-7 miRNAs in the adult Stra8-cKO mouse testes and found that let-7 miRNAs were remarkably increased. Collectively, these results indicate that ablation of CCNB1 might promote the undifferentiated germ cells’
differentiation by downregulating the expression of Lin28a and upregulating the expression of let-7 miRNAs.

Discussion

In the present study, we reported the requirement of CCNB1 in spermatogenesis using conditional knockout mice lacking CCNB1 in male germ cells. Ablation of CCNB1 in mouse gonocytes and spermatogonia results in male sterile due to germ cells' depletion, whereas ablation of CCNB1 in postnatal, premeiotic male germ cells does not have an effect on the meiosis of spermatocyte and male fertility. We found that germ cells' depletion in Mvh-cKO mice is probably due to inhibited proliferation and increased apoptosis. We also showed that ablation of CCNB1 in undifferentiated spermatogonia might promote their differentiation by downregulating the expression of Lin28a and upregulating the expression of let-7 miRNAs. These results indicate that CCNB1 is critically required for the proliferation of gonocytes and spermatogonia but may be redundant in spermatocytes. Previous studies showed that CDK1 is essential for mitosis and meiosis in mice. Mammalian somatic cells without CDK1 are unable to complete mitosis while mammalian oocytes or spermatocytes without CDK1 are unable to complete meiosis. In the present study, we show that CCNB1, one regulatory subunit of CDK1, is essential for the mitosis of early-stage male germ cells but may be redundant in meiosis of male germ cells.

The mitosis of male germ cells involves the proliferation of primordial germ cells (PGCs), gonocytes and spermatogonia. The Mvh-Cre has its role beginning from E15, and at 1 dpn recombinase efficiency can reach >95%. Mvh-Cre can delete genes specifically in gonocytes, spermatogonia, spermatocytes and spermatids but is unable to delete genes in PGCs. In this study, the early stage of male germ cells refers to gonocytes and spermatogonia ranging from 1 to 15 dpn.
Ablation of CCNB1 in these cells results in male germ cells depletion caused by inhibited proliferation and increased apoptosis. However, it is unclear whether CCNB1 is required for the proliferation of PGCs. To study the requirement for CCNB1 function in PGCs, an earlier expressed germ cell-specific Cre may be needed.

The first wave of spermatogenesis is different with the adult waves of spermatogenesis. In Mvh-cKO mouse testes, germ cells were completely depleted before the first wave of spermatogenesis. Therefore, it is unclear whether CCNB1 is essential for the spermatogonia in adult male mice. To test the requirement of CCNB1 in spermatogonia in adult male mice, we also deleted CCNB1 in undifferentiated spermatogonia using Ngn3-Cre.49 We found that the male Ccnb1<sup>−/−</sup>; Stra8-Cre<sup>X</sup> Ngn3-Cre (Ngn3-cKO) mice are subfertile; they can generate a little mount of spermatozoa (Supplementary Figure S4E). The testes of Ngn3-cKO mice are smaller than control and almost no spermatozoa exist in epididymis (Supplementary Figures S4A–C). The expression of Ccnb1, Mvh, Stra8, Sycp3 and Prm1 were notably reduced in Ngn3-cKO adult mouse testes (Supplementary Figure S4D), whereas the expression of Plzf, Gfra1, c-kit, p53, Reprimo, Caspase-3 and Gdnf were remarkably increased in Ngn3-cKO adult mouse testes compared with control littermates (Supplementary Figure S4D). The phenotype of Ngn3-cKO mice are more severe than Stra8-cKO mice but less than Mvh-cKO mice. The defects of Mvh-cKO and Ngn3-cKO mice indicate that CCNB1 is critically required for the proliferation of gonocytes and spermatogonia in pubertal mouse testes and for the proliferation of spermatogonia in adult mouse testes.

GDNF, secreted by Sertoli cells, is a factor essential for undifferentiated spermatogonia self-renewal.51 We found that expression of Gdnf was normal in 2 dpn Mvh-cKO mouse testes but significantly increased in 3 and 7 dpn Mvh-cKO and
Figure 5 Deletion of Ccnb1 in germ cells promoted their differentiation and Lin28a/let-7 axis involved in this process. (a) Real-time PCR analysis of Plzf, c-Kit, Lin28a and Gdnf expression in 2 dpn control and Mvh-cKO mice testis. (b) Real-time PCR analysis of Plzf, c-Kit, Lin28a and Gdnf expression in 3 dpn control and Mvh-cKO mice testis. (c) Real-time PCR analysis of Plzf, c-Kit, Lin28a and Gdnf expression in 7 dpn control and Mvh-cKO mice testis. (d) Real-time PCR analysis of Plzf, c-Kit, Lin28a and Gdnf expression in 90 dpn control and Stra8-cKO mice testis. (e) Real-time PCR analysis of let-7 family miRNAs expression in 90 dpn Control and Stra8-cKO mice testis. In panels (a–e), ≥3 samples were used in each group in qPCR.
adult Stra8-cKO and Ngn3-cKO mouse testes compared with control littermates. These results suggest that ablation of CCNB1 in germ cells does not inhibit the secretion of GDNF; in contrast, the expression of GDNF is increased. Consisted with the high expression of Gdnf in Stra8-cKO and Ngn3-cKO mice testis, the expression of Plzf was remarkably increased, indicating that high expression of Gdnf may promote the self-renewal of undifferentiated spermatogonia. However, the higher expression of Gdnf in 3 or 7 dpn Mvh-cKO mouse testes was unable to promote the proliferation of gonocytes or spermatogonia. These observations indicate that CCNB1 is critically required for the proliferation of gonocytes and spermatogonia. Even when high GDNF exists but not CCNB1, the gonocytes and spermatogonia are unable to proliferate normally.

The expression of Plzf and c-Kit were notably increased in adult Stra8-cKO and Ngn3-cKO mouse testis compared with control littermates, whereas in Mvh-cKO mouse testis these two genes were markedly reduced. The best interpretation of this phenomenon is that CCNB1 was not ablated in all spermatogonia in Stra8-cKO and Ngn3-cKO mice, and the spermatogonia with normal level of CCNB1 could proliferate normally. Moreover, high level of GDNF in Stra8-cKO and Ngn3-cKO mouse testes further promote the proliferation of these CCNB1-remaining spermatogonia. In short, CCNB1 was not completely deleted in all spermatogonia in Stra8-cKO and Ngn3-cKO mice, and mice spermatogonial stem cell (SSC) pool still exist, SSCs can self-renew; however, in Mvh-cKO mice, CCNB1 was ablated in all spermatogonia and SSC pool was unable to maintain and finally led to germ cells’ depletion and male sterility (Supplementary Figure S5).

We also showed that germ cells’ depletion in male Mvh-cKO mice is due to inhibited proliferation and increased apoptosis in germ cells. However, ablation of CCNB1 might not inhibit spermatogonial differentiation; in contrast, it may promote spermatogonial differentiation. LIN28A is a marker of undifferentiated spermatogonia and highly expressed in undifferentiated spermatogonia in mice.34,35,37 LIN28A mainly represses the maturation of let-7 miRNAs, whereas the maturation of other miRNAs is largely unaffected by LIN28A.42–44 Because the miRNAs of Lin28a are themselves let-7 targets, this LIN28A/let-7 axis regulates proliferation and differentiation of PGCs and spermatogonial progenitor cyclic expansion.34,36,37 In the present study, we found that, in contrast to higher expression of Plzf, the expression of Lin28a was remarkably reduced in adult Stra8-cKO mouse testis. Moreover, though the expression of Plzf and Lin28a were notably reduced in 2, 3 and 7 dpn Mvh-cKO mouse testis, Lin28a reduced more severely than Plzf. In addition, we also found that the mature miRNA level of let-7 family were notably increased in adult Stra8-cKO mouse testis. These results indicate that high level of c-Kit expression in Stra8-cKO mice testes is probably due to the change of LIN28A/let-7 axis, with low level of Lin28a and high level of let-7 promoting the differentiation of spermatogonia.

Stra8-cKO mice have normal fertility, albeit their testis is smaller than control, suggesting that CCNB1 may be redundant in meiosis of spermatocytes. CCNB2, which is also highly expressed in testis, may compensate the loss of CCNB1 in mutant mice. To investigate whether CCNB1 is compensated by CCNB2 in meiosis of spermatocytes, we intend to generate Ccnb2−/−; Ccnb1fl/fl; Stra8-Cre mice. We found that the fertility of male Ccnb2−/−; Ccnb1fl/fl; Stra8-Cre mice is remarkably reduced. When we crossed male Ccnb2−/−; Ccnb1fl/fl; Stra8-Cre with female Ccnb2−/−; Ccnb1fl/fl; Stra8-Cre or Ccnb2−/−; Ccnb1fl/+ mice for 3 months, only one or three pups were born. Until now, the Ccnb2−/−; Ccnb1fl/fl; Stra8-cKO male mice have not been produced. The observations suggest that CCNB2 might compensate the role of CCNB1 in meiosis of spermatocytes in mutant mice.

In summary, CCNB1 is critically required for the spermatogenesis. Ablation of CCNB1 in gonocytes and spermatogonia rendered them unable to proliferate normally and male sterility increased, finally resulting in germ cells’ depletion and male sterility. However, ablation of CCNB1 in premeiotic male germ cells did not have effect on meiosis of spermatocytes and male fertility, suggesting that CCNB1 may be redundant in meiosis of spermatocytes, and other cyclins such as CCNB2 may compensate the role of CCNB1 in meiosis of spermatocytes in mutant mice. We also found that ablation of CCNB1 in spermatogonia may promote their differentiation by down-regulating Lin28a and upregulating let-7 miRNAs. Our study is the first to prove in vivo the essential role of CCNB1 in mammalian spermatogenesis. To better understand and cure some cell cycle abnormal diseases such as cancer, it is important to understand fully the function of CCNB1 in vivo.

Materials and Methods

Animals. Conflotargeting ES cells were obtained from the European Conditional Mouse Mutagenesis Program. Conflot floxed mice were generated with the standard procedure. Genotypes were identified by PCR analysis (F: 5′-CAAGCACTTTACCAGGAACTAT-3′; R: 5′-GTCAAGAACAGCTACTGTTAC-3′). The wild-type allele generated a band at 673 bp, while the floxed allele was at 475 bp and cKO allele could not generate band by using these primers. The mice obtained were from mixed backgrounds of 129 and C57BL6/J. Mvh-Cre (The Jackson Laboratory, Bar Harbor, ME, USA, stock no. 008594) and Stra8-Cre (The Jackson Laboratory, stock no. 006208) mice were used in the present study and were described previously.36,31 All animals were kept in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute of Zoology (IOZ), Chinese Academy of Sciences (CAS), Beijing, China.

qRT-PCR and RT-PCR analysis. Total RNA was isolated from testes using TRIzol (TIANGEN, Beijing, China) according to the manufacturer’s instructions. The Ct values were normalized to the internal control (GAPDH for RNA and U6 for microRNA) and presented as a relative expression level. All primers for qRT-PCR and RT-PCR are described in Supplementary Tables S1 and S2, respectively.

Western blotting analysis. Proteins of testis were separated on 10% SDS-PAGE gels and transferred to PVDF membranes and probed with primary antibodies as follows: CCNB1 (Abcam, Cambridge, UK, ab72, 1 : 500); GDNF (Bioworld, Minnesota, USA, MB001, 1 : 5000) and followed with secondary antibodies conjugated to horseradish peroxidase (ZSGB-BIO, Beijing, China) at a dilution of 1 : 5000 and detected by the ECL System (Pierce, Waltham, MA USA).
Tissue collection and histological examination. Mouse testes and epididymides were collected and weighed, fixed in 4% paraformaldehyde and then embedded in paraffin. Five sections of each testis and epididymis (5 μm, taken 200 μm apart) were stained with hematoxylin–eosin for normal histological analysis. Immunohistochemistry (IHC) staining was performed with the standard procedure, using horseradish peroxidase-conjugated anti-IgG secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA), visualized with 3',3-diaminobenzidine. The slides were counterstained with hematoxylin.

For immunofluorescence (IF) staining, tissue sections were dewaxed and rehydrated and then antigen retrieved in 10 mM sodium citrate buffer. Sections were blocked (5% BSA) and incubated with primary antibodies at 4 °C overnight. Sections were washed and incubated with FITC or TRITC-conjugated secondary antibodies (1: 200; Jackson ImmunoResearch) for 1 h at room temperature and 4 °C. Successful conception was defined by the presence of vaginal envelope breakdown and chromosome condensation in amphibian oocytes. Proc Nat Acad Sci USA 1979; 76: 2799–2802.

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