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SLX2 interacting with BLOS2 is differentially expressed during mouse oocyte meiotic maturation

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Abbreviations: SC, synaptonemal complex; TI, telophase; LEs, lateral elements; TF, transverse filaments; CEs, central element; COC, cumulus-oocyte complexes; GV, germinal vesicle; GVBD, germinal vesicle breakdown; pro MI, prometaphase I ;MI, metaphase I; MII, metaphase II; MO, morpholino; AI, anaphase I; MTs, microtubules; dpc, day post-coitus; dpp, post-partum ; DSBs,double-strand breaks; CoIP, co-immunoprecipitation; γH2AX, phosphorylated histone variant H2AX; SLX2, SYCP3-like X-linked 2; Xlr, X-linked lymphocyte regulated; SYCP1, synaptonemal complex protein 1; SYCP3, synaptonemal complex protein 3; SYCE2, synaptonemal complex central element protein 2

Gametogenesis is a complex biological process of producing cells for sexual reproduction. Xlr super family members containing a conserved COR1 domain play essential roles in gametogenesis. In the present study, we identified that Slx2, a novel member of Xlr super family, is specifically expressed in the meiotic oocytes, which is demonstrated by western blotting and immunohistochemistry studies. In the first meiotic prophase, SLX2 is unevenly distributed in the nuclei of oocytes, during which phase SLX2 is partly co-localized with SYCP3 in synaptonemal complex and γH2AX in the nucleus of oocytes. Interestingly, the localization of SLX2 was found to be switched into the cytoplasm of oocytes after prometaphase I during oocyte maturation. Furthermore, yeast two-hybrid and coimmunoprecipitation studies demonstrated that SLX2 interacts with BLOS2, which is a novel centrosome-associated protein, and co-localized with γ-Tubulin, which is a protein marker of chromosome segregation in meiosis. These results indicated that SLX2 might get involved in chromosomes segregation during meiosis by interaction with BLOS2. In conclusion, SLX2 might be a novel gametogenesis-related protein that could play multiple roles in regulation of meiotic processes including synaptonemal complex assembly and chromosome segregation.

Introduction

Gametogenesis is a process of gamete formation, which includes meiotic division and mitotic division. Meiosis is the process by which diploid germ cells from both sexes of sexually reproducing organisms produce haploid gametes, such that pairs of gametes from each sex fuse during fertilization to give diploid offspring. The hallmark of meiosis is the reduction of DNA content by half through 2 cell divisions of germ cells whose DNA have been duplicated once just before meiosis starts.[1] In the first division, homologous chromosomes pair, exchange DNA, and then separate from each other, while in the second, sister chromatids of each of 2 combinations of homologous chromosomes separate from each other. Synaptonemal complex (SC) and chromosomes separate may play a universal role in gametogenesis.

The SC is a complex protein structure that assembles homologous chromosomes during the meiotic prophase to ensure continuous and stable association along the homologous chromosomes. SC is composed of lateral elements (LEs), transverse filaments (TF), and central element (CEs).[2] Many important proteins that participated in the assembly of SC have been isolated and characterized.[3] SYCP1 (synaptonemal complex protein 1) SYCE1 and SYCE2, are the components of central element of SC. In mice lacking SYCE2 protein, the sex chromosomes are unaligned, not forming a sex body.[4] SYCP3 (synaptonemal complex protein 3) is a member of Xlr (X-linked lymphocyte regulated) superfamily,

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whose members contain a conserved COR1 domain.[5] SYCP3 is essential for both formation and maintenance of the AEs and LEs of SC.[6] SYCP3 is required for male fertility, and the Sycp3$^{-/-}$ male germ cells die around the zygotene stage of meiosis.[7] Thus SLX2 proteins, which share homologies with SYCP3 may be also related to the mammalian meiosis. To date, SLX2 have been found to be highly expressed in the testis and may be involved in homologous recombination, double-strand break repair, and sex chromosome inactivation during the meiosis.[8,9] However, the expression profile and potential functions of SLX2 have not been clearly investigated in oocyte during the meiosis.

Mouse oocyte maturation is a precisely orchestrated multi-stage process that occurs from fetal development.[10,11] One key event in meiotic maturation is germinal vesicle breakdown (GVBD), which marks the onset of meiosis I and the beginning of oocyte maturation. After GVBD, microtubules assemble to chromosomes during the prometaphase I (pro MI) stage, and then chromosomes migrate to the central plate of the bipolar spindle and remain in a well-aligned state during the MI stage.[12,13] Subsequently, anaphase I (AI) ensues, followed by extrusion of the first polar body and the spindle migrates to the cortex. Then, the oocyte emits the first polar body, followed by the formation of the metaphase II (MII) spindle located beneath the plasma membrane.[14-16] The formation of meiotic spindles is important for proper meiotic cell cycle progression and chromosome segregation in oocyte. Accurate chromosome segregation (during mitosis and meiosis) is one of the most fundamental molecular and cellular processes that sister chromosomes or homologous chromosomes attach to microtubules emanating from spindle. Microtubules (MTs) are the main constituents of spindles and involved in key processes such as mitosis, maintenance of cell shape, and intracellular trafficking. γ-Tubulin is a specialized member of the tubulin family and has been shown to play an essential role in MTs nucleation at the centrosome of animal cells.[17] γ-Tublin also play an important role in MTs nucleation from the MTs organizing centers.[18] BLOS2, a centrosomal protein belonging to the BLOC-1 complex, co-localizes and interacts with γ-Tublin, a major component of centrosome.[19,20] Interestingly,BLOS2 was found to be highly expressed in testis and localized in centrosomes and myosin V globular tail domains in vesicle-like structures.[21] These indicated that BLOS2 may be associated with centrosome and MTs aggregation and chromosome segregation in germ cells.

Here we showed that SLX2 is differentially expressed during mouse oocyte meiotic maturation. SLX2 is unevenly distributed in the nucleus of oocytes in the prophase I, at which stage SLX2 is partly co-localized with SYCP3 and γH2AX in the SC. From pro-MI to MII, SLX2, which shares a similar distribution pattern as γ-Tublin, is distributed in the cytoplasm of oocytes. Moreover, SLX2 interacts with BLOS2 through yeast two-hybrid screening and Co-IP. These data suggested that SLX2 could be involved in SC assembly and chromosome segregation during the gametogenesis.

Results

The expression profile of Slx2 mRNAs and protein in murine oocytes

To investigate Slx2 expression pattern in oocytes, multiple developmental stages of oocyte were collected. Results showed that Slx2 mRNAs and protein are detected in the testis and ovaries of 1 day postpartum (dpp) as detected by RT-PCR and western blotting assays (**Fig. 1**). Interestingly, Slx2 mRNAs are only detected in the 1 dpp ovaries, but not detected in the other stages after birth, including adult ovaries (**Fig. 1A**). Slx2 mRNA was detected in 14.5 d post-coitus (dpc) ovary (probably more exactly 13.5 dpc as indicated by the protein expression results), but no other developmental stages as detected by Q-PCR (**Fig. 1B and C**). SLX2 protein was detected as early as on 13.5 dpc (**Fig. 1D**), when meiosis of female germ cells is initiated, and sustained a steady level until the first day after birth and became undetectable afterwards.

SLX2 protein was localized in the meiotic oocytes at prophase I

To further examine the distribution of SLX2 in different ovaries at developmental stages, immunostaining was performed on ovary sections (**Fig. 2A**) and chromosome spreads (**Fig. 2B**). SLX2 was present in the nuclei of oocytes on 15.5 dpc ovary sections. After 2 d in vitro culture of 15.5 dpc ovaries, the nuclear level of SLX2 in oocytes seemed to decrease, while the cytoplasmic level seemed to increase. This phenomenon became more apparent at 7 d post-culture. SLX2 was also observed in the nuclei of the oocytes on the 1 dpp ovary sections. Immunohistochemistry results showed that Slx2 protein is present in the meiotic oocyte.

To confirm that SLX2 was indeed localized to SC, chromosome spreads of oocytes at the zygotene and pachytene stages were immunostained against SLX2 and SYCP3. Chromosome spreads at different stages of meiosis of female germ cells were prepared, and SLX2 expression was examined in comparison with SYCP3 (**Fig. 2B**), an essential component of the SC. In oocytes at prophase I, SLX2 is unevenly distributed in the whole nuclei from leptotene to zygotene stage, while it is concentrated to the SC at the pathytene stage. To confirm that SLX2 was expressed in the meiotic oocytes at prophase I, double staining of SLX2and γ-H2AX was performed. The results showed that SLX2 is co-localized in the nuclei of oocytes at zygotene stages with γ-H2AX, a biomarker for DNA double-strand breaks (**Fig. 3A**). The confocal laser scanning results showed that SLX2 protein is highly restricted to the areas containing DAPI bright satellite sequences (**Fig. 3B**). The results suggest that SLX2 might be involved in the SC assembly and DNA recombination in oocyte meiotic maturation by cooperating with SYCP3 and γ-H2AX.

Physical interaction between SLX2 and BLOS2

In order to define the biochemical function of SLX2, yeast two-hybrid screenings were performed to identify its potential binding partners. BLOS2, a centrosomal protein belonging to the BLOC-1 complex, was identified as a binding partner of SLX2

through yeast two-hybrid screening. The interaction was further confirmed by their co-immunoprecipitation (CoIP) from the cell lysate of HEK293T cells that were transfected with FLAG-SLX2 and BLOS2-GFP plasmid constructs (**Fig. 4A and B**).

Subcellular localization of SLX2 during mouse oocyte meiotic maturation

The subcellular localization of SLX2 at different stages of meiotic maturation was examined by immunofluorescent staining to further examine the expression of SLX2. No evident signal of SLX2 was detected in the GV stage. During the pro MI, SLX2 was localized in the nuclei of the oocytes. Subsequently, the protein of SLX2 was localized in the cytoplasm of oocytes during oocyte maturation. To further investigate the roles of SLX2 in mouse oocyte meiosis, double staining of SLX2 and γ-Tublin was performed. Increasingly, SLX2 and γ-Tublin were co-localized to the spindle at MI, telophase (TI), and MII stages (**Fig. 5**) as revealed by immunohistochemistry techniques. In addition, BLOS2, a centrosomal protein belonging to the BLOC-1 complex co-localizes and interacts with γ-Tublin as described in previous studies.[19] These results indicated that SLX2 might play potential roles in mouse oocyte spindle organization/stability by forming a scaffold with BLOS2 and γ-Tublin.

Discussions

Synaptonemal complex (SC) formation is a key step of meiosis. SYCP3 protein participated in the assembly of SC which is a zipper-like structure composed of LEs, TF, and CEs.[3] The sequence of SLX2 shares high homologies with SYCP3, an essential component of SC. In spermatogenesis, SLX2 have been found to be highly expressed in the testis and may be involved in homologous recombination, double-strand break repair, and sex chromosomes inactivation during the meiosis.[9] In the present study, we found that SLX2 is unevenly distributed in the nucleus of oocytes in meiosis I, which is partly co-localized with SYCP3 in SC. The distribution of SLX2 in meiotic spermatocytes and oocytes implies that Slx2 should play an important role in the process of meiosis. In addition, distribution of SLX2 is similar to γ-H2AX, and the 2 proteins were co-localized in the meiotic oocytes at stages of pro MI to MII. γ-H2AX is phosphorylated at the sites of DSBs, which, in turn, recruits DNA repair factors to participate in DSBs repair after homogenous recombinant.[22] It is possible that γ-H2AX may recruit SLX2 during the double-strand repair and homologous recombination in meiosis. Also, SLX2 and γ-H2AX might be related to the sex chromosomes inactivation during male meiosis. Given the spatial distribution of SLX2 in meiotic oocytes, we hypothesize that SLX2 acting as a novel meiosis-associated protein should play important roles in regulation of meiotic processes.

It is worth to note the alteration of SLX2 distribution in the meiotic oocytes. At prophase I, SLX2 protein was first found in the nucleus of oocytes at leptotene stage (**Fig. 2B**). After that, SLX2 protein was highly concentrated into bright dots, which were unevenly distributed in the whole nucleus during zygotene. From leptotene to zygotene, the distribution patter of SLX2 matched well with that of γ-H2AX, which was similar to results found in the meiotic spermatocytes.[9] Interestingly, at pachytene stage, the distribution of SLX2, sharing part co-localization with SYCP3, was remarkably restricted to the SC, during which stage γ-H2AX was not found, because the double-strand breaks had been repaired (**Fig. 2B**). Using the confocal scanning system with high resolution, SLX2 was defined to a specific territory of satellite sequences (**Fig. 3B**), where the chromosome fragments being rich of satellite sequences did not align well. The lack of recruitment of γ-H2AX might contribute to the spatial alteration of SLX2 at pachytene stage. With the accomplishment of the first meiotic prophase, SLX2 decreased to baseline at diplotene. Additionally, when oocytes continue meiotic maturation after the primary arrest, SLX2 reappeared in the cytoplasm, where SLX was co-localized with γ-Tublin from pro MI to MII (**Fig. 5**).

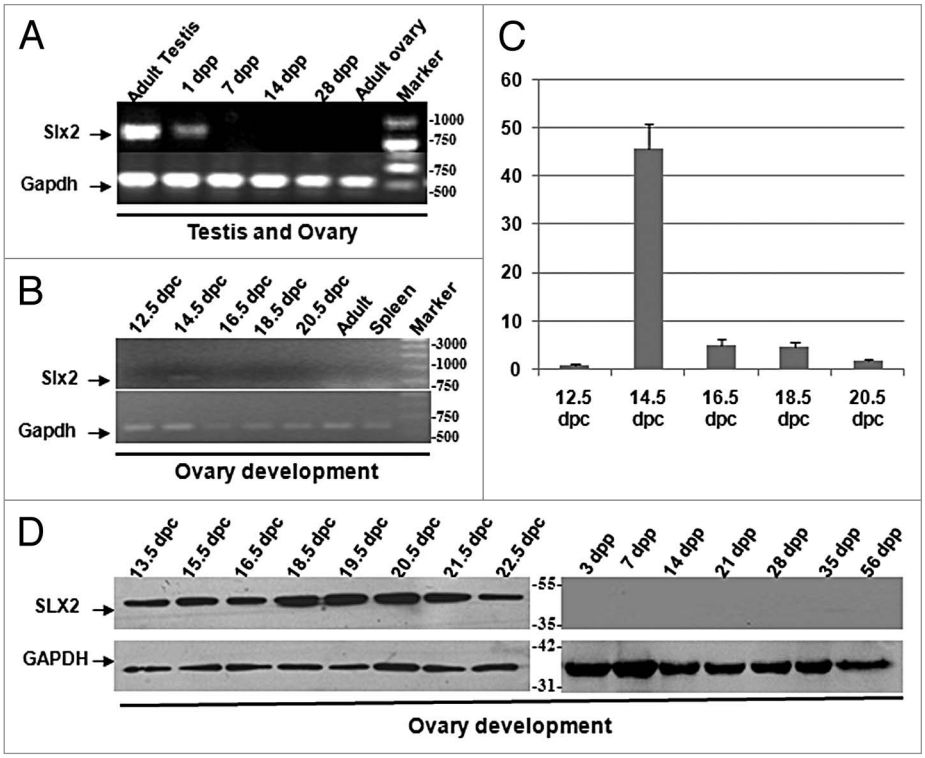

Figure 1. Expression of mouse Slx2 gene detected by RT-PCR, Q-PCR, and western blotting in the testis and oocytes. (**A**) Slx2 gene detected by RT-PCR; Expression levels in the RT-PCR experiments were normalized to those of Gapdh. (**B**) Slx2 gene detected by Q-PCR; Samples were run in triplicate, and error bars represent the standard deviation. (**C**) Slx2 gene detected by Q-PCR with histogram; (**D**) Slx2 protein detected by western blotting.

Contrary to the results in oocytes, SLX2 protein was not found in meiotic spermatocytes after the prophase I.[9] This unique pattern of SLX2 suggests that SLX2 might play more multiple and complicated roles in oocytes than that in spermatocytes.

Indeed, the results from its interacting proteins support the above hypothesis that the functions of SLX2 are multiple. Previous study showed that SLX2 interacts with TIP60 and SYCE2, which possess different functions in meiosis.[9] In this study, yeast two-hybrid and co-immunoprecipitation studies demonstrated SLX2 interacts with BLOS2. BLOS2, a centrosomal protein belonging to the BLOC-1 complex is a major component of centrosome.[19] In addition, BLOS2 was found to be highly expressed in testis and localizes in centrosomes and myosin V globular tail domains in vesicle-like structures.[8] It is possible that BLOS2 may participate in spermatogenesis or meiosis. Furthermore, BLOS2 interacted with γ-Tublin, which is the main constituent of spindles and plays an important role in microtubule nucleation from the microtubule organizing centers.[18,22,23] In this study, SLX2 physically interacted with BLOS2, while SLX2 and γ-Tublin were co-localized in centrosomes during the oocytes maturation. It is possible that SLX2 interacting with BLOS2 along with γ-Tublin mediates spindle organization/stability. Unfortunately, because the original BLOS2 monoclonal antibody is hard to get for us at present, we cannot confirm SLX2, BLOS2, and γ-Tublin are co-localized in the meiotic oocytes.

In conclusion, we have determined the expression and localization of SLX2 in the mice during the oocyte maturation. The present study not only provided strong evidences that mouse SLX2 was involved in spermatogenesis in meiosis, but also revealed the potential roles of SLX2 in meiotic oocyte. Considering the unique expression pattern and interaction with BLOS2, SLX2 could play multiple roles in regulation of meiotic processes including SC assembly, double-strand repair, homologous recombination, and chromosome segregation.

Materials and Methods

Ethics statement, antibody, and chemicals

All animal experiments were approved by the Institute Committee on Animal Care and Use, Chinese Academy of Sciences. Mice were fed in a temperature-controlled room with a regular diet. The animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals in Beijing. Mouse monoclonal anti-γ-Tublin antibody was obtained from Millipore. Mouse polyclonal anti-SYCP3 antibody and γ-H2AX were obtained from Abcam. Mouse monoclonal anti-γ-Tublin antibody was purchased from Sigma-Aldrich. FITC-conjugated anti-rabbit from Jackson Laboratories. Chemicals for the cell culture were obtained from Invitogen and reagents for the animal treatment were purchased from Sigma.

Oocyte collection and culture

The mice were killed using cervical dislocation. Oocytes were collected from ovaries of 8-wk-old female CD-1 mice in M2 medium supplemented without 2.5 μM milrinone under liquid paraffin oil at 37 °C in an atmosphere of 5% CO_2 in air. The germinal vesicle (GV) oocytes were isolated from follicles by sterile needles, and the cumulus cells surrounding the oocytes were

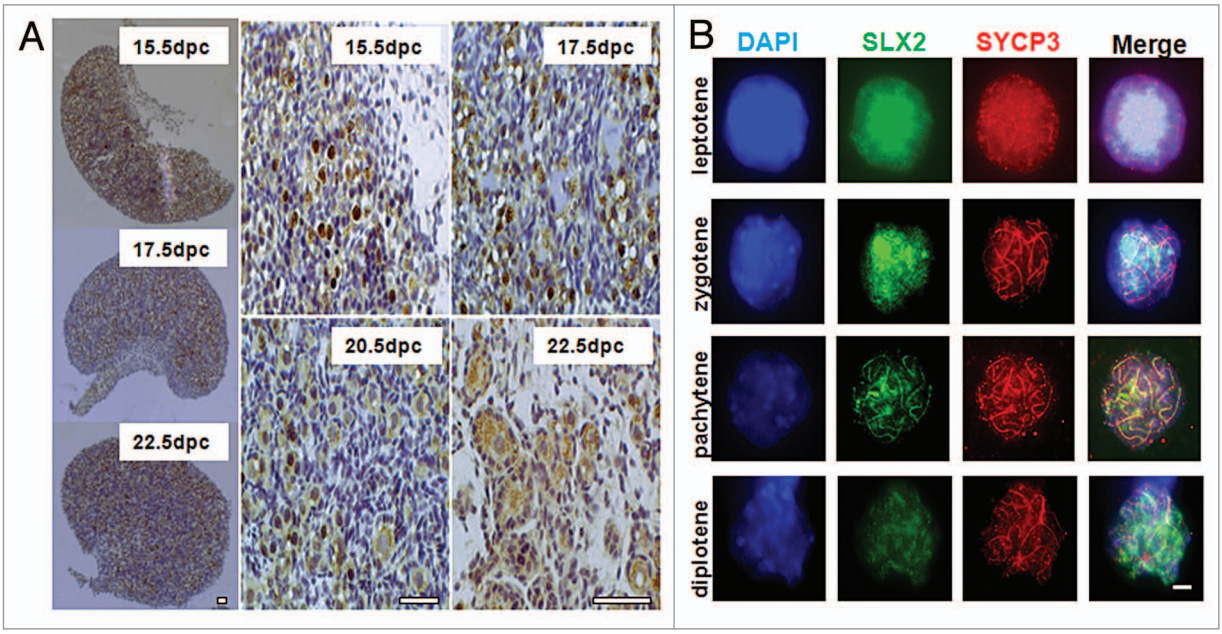

Figure 2. SLX2 expression in oocyte and chromsome spreading detected by imunostaining. (**A**). Ovary sections. 15.5 dpc ovaries were culured for different times to derive the 17.5 dpc and 22.5 dpc ones. SLX2 signal (brown) was developed by using the DAB substrate and nucleus signal (purple) was the hematoxylin staining. Low magnification pictures and high magnification pictures. Scale red bar, 100 μm. (**B**) SLX2 and SYCP3 co-localized in the chromosome spread. Chromosome spreads of oocytes at zygotene and pachytene stages were prepared. SLX2 was stained green. SYCP3 (red) was used as a marker for identifying germ cells at different stages of the prophase of the first division of meiosis. DNA was revealed by the blue DAPI staining. Scale white bar, 10 μm.

removed. Milrinone was used to keep oocytes at the GV stage in M2 medium. Then the oocytes were washed thoroughly with M2 medium and cultured in M2 medium supplemented with 2.5 μM milrinone. After culture, oocytes were used for drug treatment, RT-PCR, western blot and immunohistochemistry, and immunocytochemistry.

RNA isolation and RT-PCR

Analysis of Slx2 gene expression was measured by real-time quantitative PCR. Total RNAs from mouse oocytes at different stages of development were extracted for RT-PCR analysis. Total RNA was extracted from at least 200 oocytes using Trizol reagent (Invitrogen, 15596018), and cDNA was synthesized using the prime Script™ 1st strand cDNA Synthesis Kit (TAKARA, D6110A) according to the manufacturer's protocol. Total RNAs were used as templates for reverse transcription, which followed the manufacturer's protocol and has been described previously.[24] Real-time quantification of mRNA levels was done using SYBR premix Ex TaqTM II kit (TAKARA, DRR081A) according to the manufacturer's protocol. Matchmaker library construction and screening kit and were purchased from Clontech (BD Biosciences). The Slx2 primer pairs are 5′-TTTCCCCGTG GAAGAAGGGA-3′ and 5′-AAAAGAGCGT AGACTCCTCA-3′, which were used to detect the Slx2 mRNA expression. The products of RT-PCR and PCR were analyzed by electrophoresis and stained with ethidium bromide. Relative amounts of cDNA were normalized to Gapdh.

Generation of antibodies and immunofluorescence

The protocol was basically the same as described in our previous studies.[9] Rabbit polyclonal anti-SLX2 antibodies were raised against amino acid residues 1–120 of SLX2. Ovaires were fixed in 10% formaldehyde for paraffin embedding, and 5 μm sections were affixed to the slides. Paraffin sections then were heated at 60 °C for 2 h. The sections were rehydrated in a series of graded ethanol after deparaffinization. Then sections were boiled in citric acid 10 mM (pH 6.0) at 100 °C for 10 min and incubated in 3% hydrogen peroxide (H_2O_2) for 10 min. The tissues were blocked by BDT (3% BSA, 10% normal donkey serum in TBS) and incubated with anti-SLX2 (1:100) for overnight at 4 °C. Pre-immune rabbit serum used as a negative control. After rinsing thoroughly with TBS, sections were incubated with the secondary biotinylated antibody for 15 min at room temperature. And the sections had a reaction with 3,3′-diaminobenzidine tetrahydrochloride (DAB) or analyzed by confocal microscopy. The spreading chromosomes of primary oocytes were performed. The slides were stained using anti-SLX2 (1:100), anti-SYCP3 (1:200, Abcam), anti-γH2AX (1:200, Upstate), and anti-γ-Tublin (1:300). Secondary antibody was the FITC-conjugated or TRITC-conjugated anti-rabbit (1:500) from Jackson Laboratories.

Western blot analysis

Western blot were performed at standard protocols. Samples from ovaries in vivo or cultured in vitro were electrophoresed on 10% SDS polyacrylamide gels and then transferred to nitrocellulose membranes (Amersham Biosciences AB). The membranes were blocked in TBST (0.5% Tween-20 in TBS) containing 5% nonfat milk powder for 1 h and incubated overnight with a 1:500-diluted anti-SLX2 antibody in TBST, and then washed 3

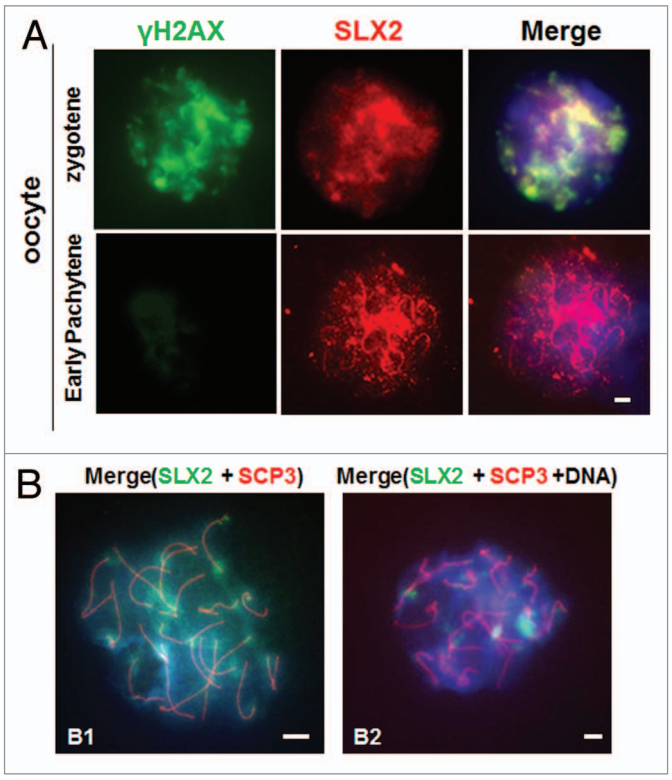

Figure 3. Co-localization of SLX2, SYCP3, and γ-H2AX in the nucleas of oocytes. Chromosome spreads of spermatocytes and oocytes at zygotene and pachytene stages were prepared. (**A**) Expression of SLX2 at the early pachytene stage was weaker than γ-H2AX, and their co-localization could not be detected. SLX2 partially co-localized with γ-H2AX at the zygotene stages. (**B**) Spread chromosomes of primary oocytes stained for SYCP3 and SLX2 (B1). Spread chromosomes of primary oocytes stained for SYCP3, DAPI and SLX2. Images were captured with laser confocal microscope. The scale bars represent 10 μm for all images.

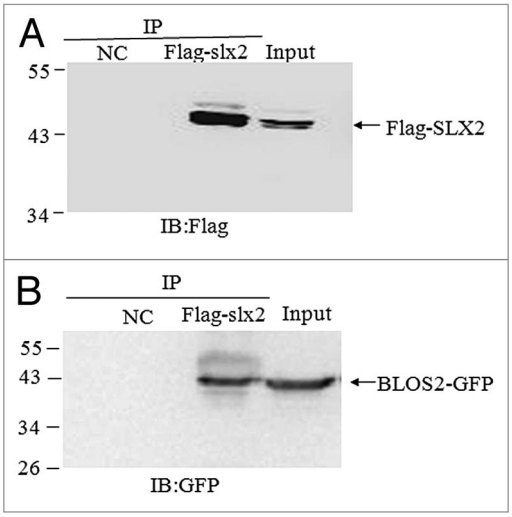

Figure 4. BLOS2 is a binding partner of SLX2. Co-IP using lysate from HEK293T cells that were co-transfected by BLOS2-GFP and FLAG-SLX2. Samples from control and the overexpression group were collected to test by WB.

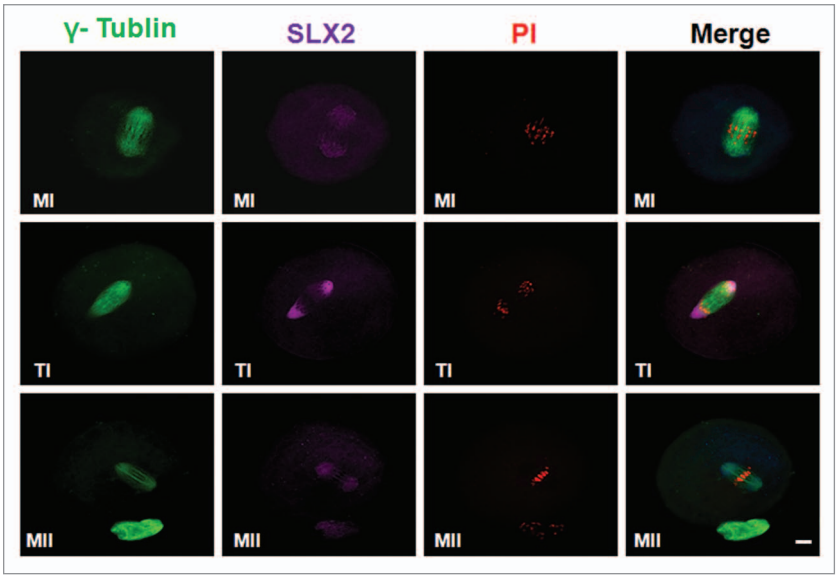

Figure 5. Localization of SLX2 and γ-Tublin during mouse oocyte meiotic maturation from MI to MII. Confocal microcopy showing immunostaining of γ-Tubulin (green), DNA (red), and SLX2 (pink) in oocytes at MI, TI, and MII stage. SLX2 colocalize with γ-Tubulin in spindle polar regions from MI to MII. Fluorescence intensity signals confirming γ-Tubulin (green) and SLX2 (pink) colocalization. Scale bar, 10 μm.

times with TBST. The filters were then incubated for 1 h with alkaline phosphatase (AP)-conjugated anti-mouse IgG (S3721, Promega). The protein expression level was analyzed by Image-Pro plus software 5.1.

Yeast two-hybrid assay

Yeast two-hybrid assay was performed by using the Matchmaker library construction and screening kit (Clontech, Cat. No. K1615-1) with slight modification.[24,25] The bait plasmid was constructed by subcloning mouse SLX2 cDNA fragments encoding the full-length peptide into pGBKT7. The fusion library construction and two-hybrid assay were performed in one step by co-transforming the yeast strain AH109 with ds cDNA, pGADT7-Rec, and pGBKT7. Colonies were picked from SD/-Ade/-His/-Leu/-Trp/X-α-Gal selection plates after 5 d. The absorbance was measured at 405 nm after pelleting the yeast debris. The inserts of selected positive clones were sequenced and identified by searching the NCBI BLAST database.

Co-immunoprecipitation of in vitro translated proteins

Using in vitro transcription and translation system (Lipofectamine 2000, Invitrogen), SLX2 and BLOS2 cDNA fragments were subcloned into pFlag-cmv4 and pEGFP-N1 vector to construct Flag-SLX2- and BLOS2-GFP-tagged plasmids, respectively, and then the 2 tagged plasmid DNA were co-transfected in HEK293T cells. Subsequently, total protein lysates of the cells were incubated with rabbit immunoglobulin (IgG) or anti-GFP antibody for 2 h at 4 °C, followed by incubation with protein A agarose (Santa Cruz) overnight at 4 °C. Agarose beads and captured protein complexes were washed 5 times and suspended in SDS sample buffer for immunoblotting with anti-GFP (1:1000 dilution, Santa Cruz), anti-Flag (1:2000 dilution, Sigma-Aldrich), or anti-SLX2 (1:1000 dilution). pFlag-cmv-4 empty vector and BLOS2-GFP transfected HEK293T cells served as negative controls. Agarose beads and captured protein complexes were washed 5 times and suspended in SDS sample buffer for immunoblotting with anti- Flag or anti-GFP antibody.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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