Supplementary information

Supplementary Methods, Fig 1-4 are included.

Supplementary Methods

Production of antibodies

Polyclonal antibodies against mouse Rad21L(a.a.175-487), mRad21(a.a.181-500), mRec8(a.a.194-561), mCENP-C(a.a.1-403), mSA3(a.a.1031-1241), mScp1(a.a.1-160) and mSMC1α(a.a.821-1015) were produced by inserting cDNA fragments in-frame with pET28c (Novagen, Madison, WI) in *E. coli* strain BL21-CodonPlus(DE3). All the His-tagged recombinant proteins were solubilized in a denaturing buffer (6 M HCl-Guanidine, 20 mM Tris-HCl [pH 7.5]) from the inclusion body and purified by Ni-NTA (QIAGEN) under denaturing conditions. The recombinant proteins were used to immunize ICR mice or rabbits. The antibodies were affinity-purified from the immunized serum with immobilized peptides on CNBr-activated sepharose (GE healthcare). The affinity-purified antibodies against Rad21L and Rad21 were further purified by adsorption to Rad21 and Rad21L-conjugated sepharose respectively to remove cross-reactive IgG.

Antibodies and reagents

The following antibodies were used: rabbit polyclonal antibodies against Rad21L, mRad21, mRec8, mCENP-C, SMC1 (Abcam), SMC3 (Abcam), SCP3 (Abcam), CENP-U/MLF1 phosphoT78 (Abcam); mouse polyclonal antibodies against Rad21L, mRad21, mRec8, mSA3, mSMC1α, mScp1, α-tubulin DM1A (Sigma), mScp3 (Kawashima et al, 2010); goat polyclonal antibodies against SA2 (Abcam); and human polyclonal antibody against ACA (MBL).

Immunostaining of chromosome spreads from spermatocytes and fetal oocytes

Chromosome spreads were prepared from spermatocytes and fetal oocytes as described (Peters et al, 1997). For fetal oocytes immunostaining, ovaries from E13.5-19.5 embryos or postnatal day2 newborn mice were removed and treated with collagenase and trypsin. Sex type of each fetal embryo was determined by PCR using primer sets as follows;

Sry-F: 5’-CAGCCCTACAGCCACATGAT-3’ ;

Sry-R: 5’-GAGTACAGGTGTGCAGCTCTA-3’ ;

Rapsyn-F: 5’-AGGACTGGGTGGCTTCCAACTCCCAGACAC-3’ ;

Rapsyn-R: 5’-AGCTTCTCATTGCTGCGCGCCAGGTTCAGG-3’ .

For immunofluorescence staining of meiocytes at zygotene/pachyten transition, chromosomes were spread by cytospin. Immunofluorescence staining was performed using rabbit anti-Rad21L (1:500), rabbit anti-mRad21 (1:500), rabbit anti-mRec8 (1:500), rabbit anti-mScp3 (1:500), rabbit anti-mCENP-C (1:1000), rabbit anti-CENP-U phospho-T78 (1:500), mouse anti-Rad21L (1:1000), mouse anti-mRad21 (1:1000), mouse anti-mRec8 (1:1000), mouse anti-mScp3 (1:1000), mouse anti-mScp1 (1:1000) and human polyclonal ACA (1:100) antibodies. DNA was counterstained with 3 μg/ml Hoechst 33342. Images were captured with Deltavision and processed with DeltaVision SoftWorx software (Applied Precision). Line scan plots were obtained using ImageJ program.

Immunostaining of Metaphase I chromosomes from GV oocytes

Oocytes were fixed 2 h and 6 h after release from GV stage by withdrawing IBMX from M16 medium (Sigma) supplemented with 10% FBS. Images were captured with an FV1000 confocal laser scanning microscope and processed with FLUOVIEW software (Olympus).

Culture of OA-induced Meta I spermatocytes

Culture of OA-induced Meta I spermatocytes were performed as described (Wiltshire et al, 1995). The isolated spermatocytes were cultured in the presence or absence of 10 μM GW843682X (TOCRIS) for 2 h and then induced to undergo metapahase I by the addition of 5 μM okadaic acid (OA) for 6 h.

RT-PCR

Total RNA was isolated from tissues using Trizol (Invitrogen). cDNA was generated from 0.5 μg of RNA using Superscript III (Invitrogen) followed by PCR amplification using Ex-Taq polymerase (Takara). Sequences of primers used to generate RT-PCR products from cDNA are as follows:

mREC8-1504F: 5-'ctgcccatgctgcctgaacttcctg-3';

mREC8-1732R: 5'-gcttctgttgttccacaagaaggatc-3';

GAPDH-F: 5'-ttcaccaccatggagaaggc-3';

GAPDH-R: 5'-ggcatggactgtggtcatga-3';

Rad21L-1306F: 5'-ggaatgatttctccagctgttgag-3';

Rad21L-1651R: 5'-tcacatcttatagaacattggtccc-3';

mRad21-1745F: 5'-ctatcagtttgcttgagctgtgtcg-3';

mRad21-1908R: 5'-tcagataatatggaaccgtggtcc-3';

mSA3-3537F: 5'-ttcaggctctggcttgggcaagcagc-3';

mSA3-3722R: 5'-cagaaatcctccatgttcagctctg-3';

mSMC1b-3516F: 5'-ttacatcaaggaacagtcaacttgc-3';

mSMC1b-3702R: 5'-ctattgttcattggggttggggttg-3';

mScp2-86F: 5'-tgcgcccacagcctaaagtgtctgc-3';

mScp2-309R: 5'-tattaagtcatccaacttgcggagg-3';

Preparation of testis extracts and immunoprecipitation

To prepare testis extracts, testes were removed from male C57BL/6 mice, detunicated, and then resuspended in extraction buffer (20 mM Tris-HCl [pH 7.5], 200 mM KCl, 0.4 mM EDTA, 0.1% TritonX100, 10% glycerol, 1 mM β-mercaptoethanol) supplemented with Complete Protease Inhibitor (Roche). After homogenization, the cell extracts were filtrated to remove debris. The soluble cytoplasmic/chromatin-unbound fraction was collected after ultra-centrifugation at 100,000*g* for 30 min. The insoluble pellet was washed 2 times with buffer (10 mM Tris-HCl [pH 7.5], 1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10% glycerol) and digested with Micrococcus nuclease (0.008 units/ml) at 4°C for 60 min. The solubilized fractions were removed after centrifugation at 20,000*g* for 10 min at 4°C. The chromatin fractions were extracted from the insoluble pellet by high salt extraction buffer (20 mM HEPES-KOH [pH 7.0], 400 mM KCl, 5 mM $MgCl_2$, 0.1% Tween20, 10% glycerol, 1 mM β-mercaptoethanol) supplemented with Complete Protease Inhibitor. The solubilized chromatin fractions were collected after centrifugation at 100,000*g* for 30 min at 4°C.

For immunoprecipitation of cohesin complexes, 5 μg each of affinity-purified anti-Rad21L, anti-Rec8, anti-Rad21 and control rabbit IgG antibodies were added to the cytoplasmic extracts, and the extracts were incubated for 1 h at 4°C followed by incubation with the ProteinA-Dynabeads (Invitrogen) for 1 h at 4°C with rotation. The bead-bound proteins were eluted with 90 μl of elution buffer (100 mM Glycine-HCl [pH 2.5], 150 mM NaCl), and then neutralized with 10 μl of 1 M Tris-HCl [pH 8.0]. The immunopreciptates were run on the NuPAGE Tris-acetate 3-8% gradient gel using Tris-acetate-SDS running buffer with NuPAGE antioxidant (Invitrogen). Co-immunoprecipitated proteins were probed with anti-Rad21L, anti-Rec8, anti-Rad21, anti-SMC3, anti-SMC1α, anti-SMC1, anti-SA3, anti-SA2, and Tubulin.

For immunoprecipitation of mouse CENP-C from the chromatin fraction, 20 μg of affinity-purified anti-mCENP-C and control rabbit IgG antibodies were crosslinked to 200 μl of

protein A-Dynabeads by DMP (Sigma). The antibody-crosslinked beads were added to the solubilized chromatin extracts, and the extracts were incubated for 2 h at 4 °C with rotation. The beads were washed with high salt extraction buffer. The bead-bound proteins were eluted with 90 μl of elution buffer (100 mM Glycine-HCl [pH 2.5], 150 mM NaCl), and then neutralized with 10 μl of 1 M Tris-HCl [pH 8.0]. The immunopreciptates were run on the NuPAGE Bis-Tris 4-12% gradient gel using MOPS-SDS running buffer with NuPAGE antioxidant (Invitrogen).

Identification by mass spectrometry

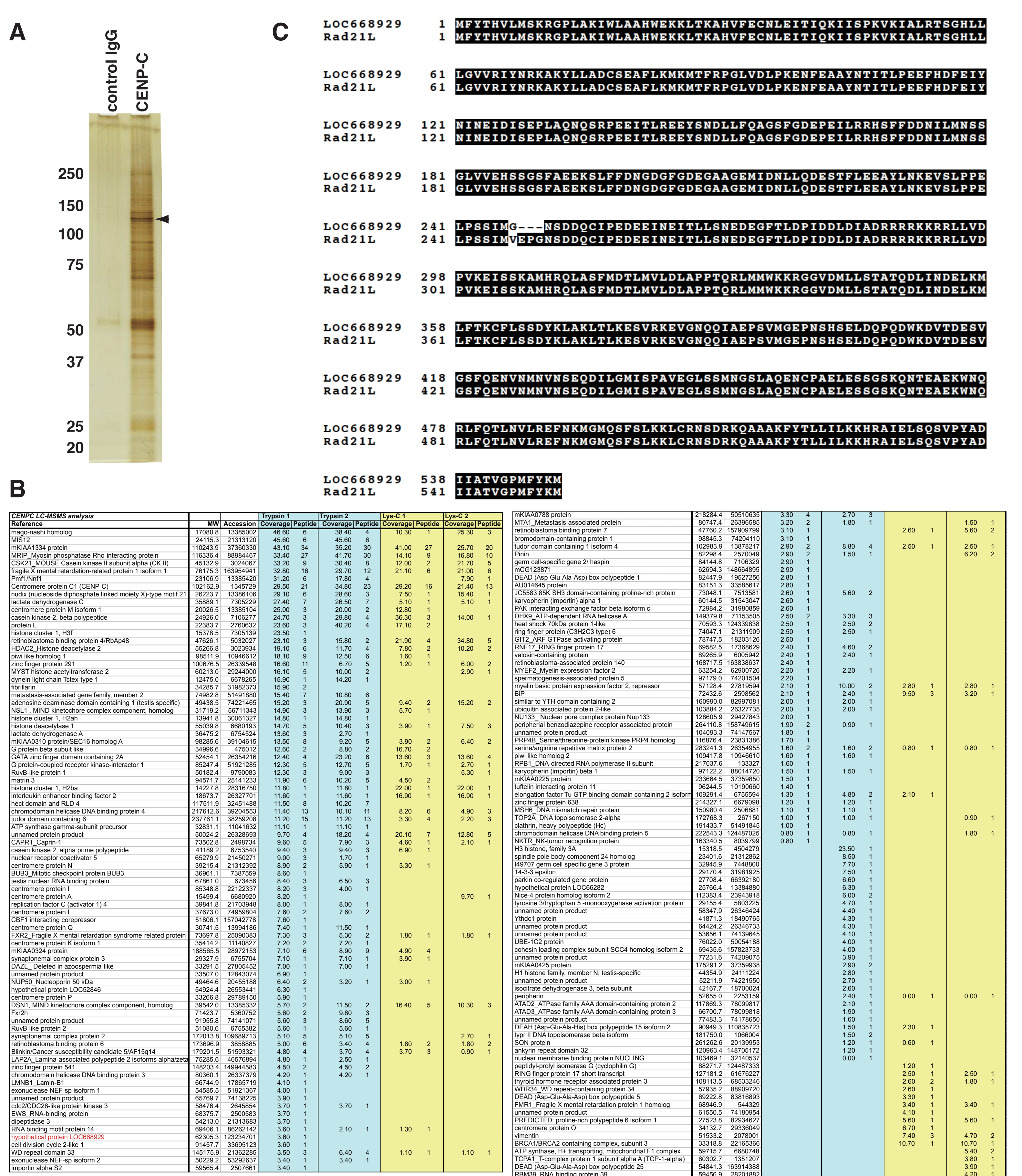
Fractions containing the CENP-C immunoprecipitates were concentrated by precipitation with 10% trichloroacetic acid. The derived precipitates were dissolved in 7 M HCl-Guanidine, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA solution, with 5 mM DTT at 37°C for 30 min, and cysteine SH groups were alkylated with 10 mM iodoacetamide at 37°C for 1 h. After alkylation, the solution was desalted by methanol/chloroform precipitation, and the precipitates were dissolved in 2 M urea, 50 mM Tris-HCl buffer and subjected to trypsin gold (Promega) or Lysil-end peptidase digestion overnight at 37 °C. The resulting mixture of peptides was applied directly to the LC-MS/MS analysis system (Zaplous, AMR, Tokyo, Japan) using Finnigan LTQ mass spectrometry (Thermo Scientific, Waltham, MA) and a reverse phase C18 ESI column (0.2 x 50 mm, LC assist). The protein annotation data were verified in the mouse NCBI sequences using Bioworks software (Ver. 3.3; Thermo Scientific) with quantitation featuring the SEQUEST search algorithm.

Supplementary References

Kawashima SA et al (2010) Phosphorylation of H2A by Bub1 prevents chromosomal instability through localizing shugoshin. *Science* **327**: 172-177

Peters AH et al (1997) A drying-down technique for the spreading of mammalian meiocytes from the male and female germline. *Chromosome Res* **5**: 66-68

Wiltshire T et al (1995) Induced premature G2/M-phase transition in pachytene spermatocytes includes events unique to meiosis. *Dev Biol* **169**: 557-567



Supplementary Fig 1. The hypothetical protein Loc668929 was identified in the co-immunoprecipitates of CENP-C. (A) Immunoprecipitates from the chromatin bound fraction of spermatocytes obtained by anti-mCENP-C antibody or control IgG, were silver stained and subjected to LC-MS/MS analysis. Arrowhead: CENP-C. (B) Proteins detected by LC-MS/MS analysis of two independent digestions either by trypsin or Lysil-end peptidase of mCENP-C co-immunoprecipitates are listed. The number of identified peptides and coverage for each protein are shown. As CENP-C itself is a DNA binding protein, a minor population of SC components and cohesin subunit might be co-precipitated through CENP-C bound small DNA fragments from the chromatin fraction extracted by high salt after MNase digestion. (C) Our cloned cDNA of Rad21L from a mouse testis cDNA library revealed it to have 3 amino acids inserted and one amino acid change at a.a.247-250 compared to the predicted amino acid sequence of the hypothetical protein Loc668929 in the database. This discrepancy is assumed to be due to an incorrect prediction of the intron6-Exon7 junction on the genome sequence of Loc668929. The Rad21L sequence has been submitted to the DDBJ/EMBL/GenBank databases under accession No. AB602048.

```
      Rad21L[Mus musculus]   1 --------------------------------------------------------------------------------
     Rad21L1[Homo sapiens]   1 --------------------------------------------------------------------------------
Loc749340[Pan troglodytes]   1 --------------------------------------------------------------------------------
Loc609572[Canis familiaris]  1 MGTLRSLLPAFRVLVQDLEWKLAVAPDSRVPASTAPAAPAWSCAKGARASGAQLLICEMDIISATSSAGSGTQKPYYQLP
     Loc512408[Bos taurus]   1 --------------------------------------------------------------------------------
Loc689823[Rattus norvegicus] 1 --------------------------------------------------------------------------------
  LOC419269[Gallus gallus]   1 --------------------------------------------------------------------------------

      Rad21L[Mus musculus]   1 --------------------------------------------------------------------------------
     Rad21L1[Homo sapiens]   1 --------------------------------------------------------------------------------
Loc749340[Pan troglodytes]   1 --------------------------------------------------------------------------------
Loc609572[Canis familiaris] 81 MDDEWMTVLAFVFEMGREDTQKLRDLEDEETEGWVDSASARLQAEMQVQDQRLLLTLRHLHSVLEELRTESAHWEDARSS
     Loc512408[Bos taurus]   1 --------------------------------------------------------------------------------
Loc689823[Rattus norvegicus] 1 --------------------------------------------------------------------------------
  LOC419269[Gallus gallus]   1 --------------------------------------------------------------------------------

      Rad21L[Mus musculus]   1 -------------------MFYTHVLMSKRGPLAKIWLAAHWEKKLTKAHVFECNLEITIQKIISPKVKIALRTSGHLLL
     Rad21L1[Homo sapiens]   1 -------------------MFYTHVLMSKRGPLAKIWLAAHWEKKLTKAHVFECNLEITIEKILSPKVKIALRTSGHLLL
Loc749340[Pan troglodytes]   1 -------------------MFYTHVLMSKRGPLAKIWLAAHWEKKLTKAHVFECNLEITIEKILSPKVKIALRTSGHLLL
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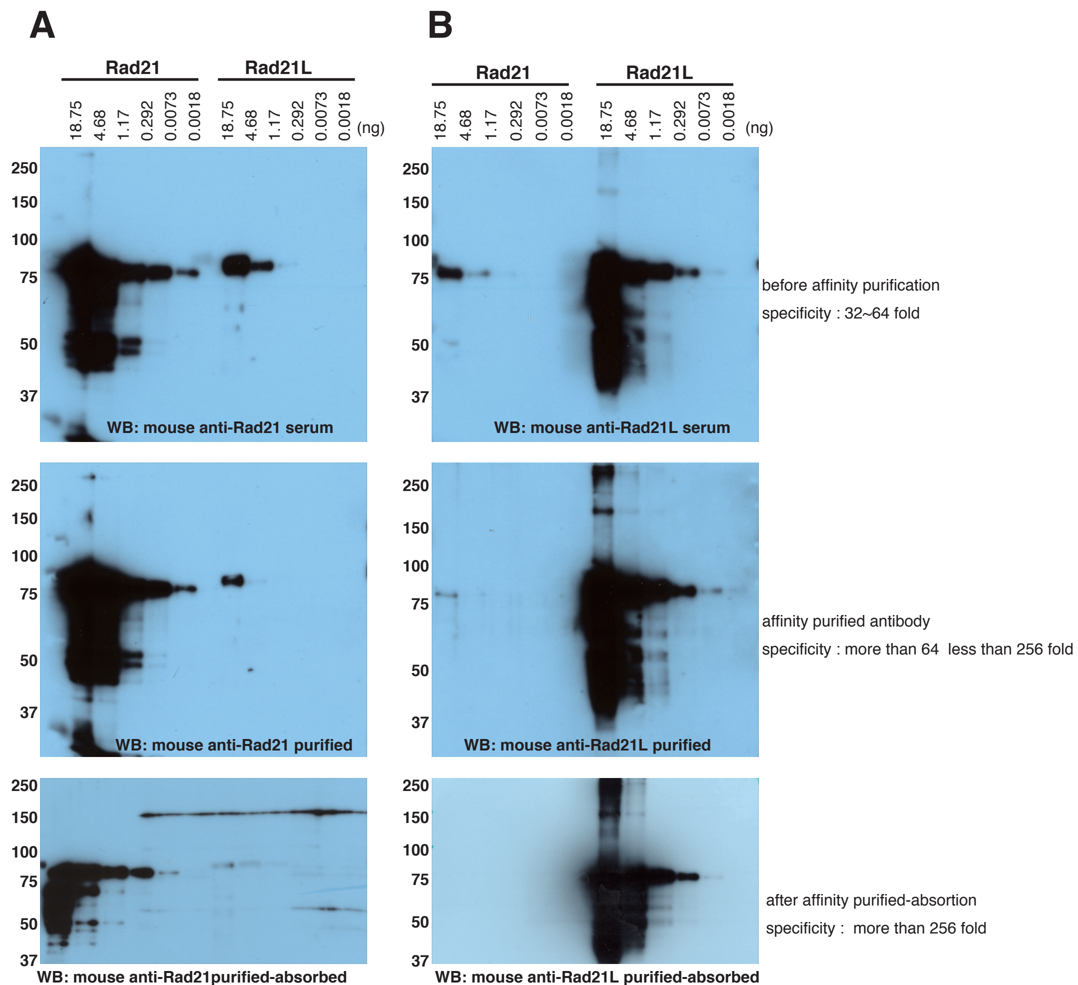
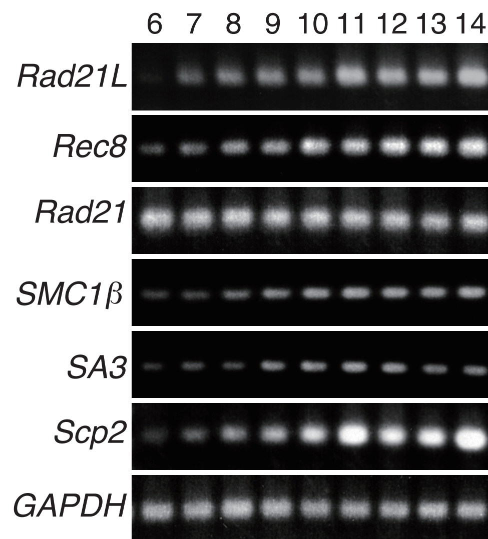
Supplementary Fig 2. Rad21L protein homologs are conserved in vertebrates.
Multiple alignment shows the amino acid sequences of *Mus musculus* Rad21L protein and its homologs including hypothetical proteins in other vertebrate species, *Homo sapiens, Pan troglodytes, Canis familiaris, Bos taurus, Rattus norvegicus, Gallus gallus*.



Supplementary Fig 3. Specificity of anti-Rad21L and anti-Rad21 antibodies.
The antigens Rad21L (a.a.175-487) and Rad21(a.a.181-500) used to generate antibodies show some similarities to each other. To exclude the possibility that the anti-Rad21 and anti-Rad21L antibodies crossreact, the antibody specificities were examined by western blotting against a 4-fold dilution series of recombinant Rad21 and Rad21L proteins (0.0018ng-18.75ng). (A) Western blotting was performed with unpurified anti-Rad21 serum (upper panel), affinity-purified anti-Rad21 antibody (middle panel), and affinity-purified anti-Rad21 antibody, which was further absorbed with the immobilized Rad21L column (lower panel). (B) Western blotting was performed with unpurified anti-Rad21L serum (upper panel), affinity-purified anti-Rad21L antibody (middle panel), and affinity-purified anti-Rad21L antibody, which was further absorbed with the immobilized Rad21 column (lower panel). The titration demonstrates that our anti-Rad21 and anti-Rad21L antibodies show more than 256 fold specificity to Rad21 and Rad21L, respectively.



Supplementary Fig 4. ***Rad21L*** **is expressed concomitantly with meiotic entry.**
The mRNA expression for the indicated genes was analyzed by RT-PCR. The RNA samples were taken from juvenile male testes at 6 to 14 days after birth.