Supplemental Information

Supplemental Experimental Procedures

Generation of knockout mice. Mouse genomic DNA harboring the Emi2 locus was retrieved from the BAC clone RP23-297G4 (ResGen, PKB986) and introduced into the pBlight-TK vector. Using recombineering technique, loxP recombination sites and a neomycin-selection cassette were introduced flanking the third exon of the mouse Emi2 genomic locus (Lee et al., 2001). The resulting targeting vector (PKB1034) was linearized by NotI digestion and electroporated into ES cells. Following positive and negative selection with Geneticin and ganciclovir, respectively, genomic DNA from surviving ES cell colonies was used to screen for homologous recombination by Southern hybridization (Fig. 1B). Correctly targeted ES cell clones (3041, 3044, 3051) were identified and used for the generation of the Emi2 conditional knockout mouse strain. The $Emi2^{flox}$ allele was generated by crossing Emi2 conditional knockout mice with β-actin–Flpe transgenic mice (Rodriguez et al., 2000) [strain name: B6.Cg-Tg(ACTFLPe) 9205Dym/J; stock no.: 005703.; The Jackson Laboratory] to remove the neomycin cassette. $Emi2^{flox}$ mice were then crossed with β-actin–Cre transgenic mice [strain name: FVB/N-Tg(ACTB-cre)2Mrt/J; stock no.: 003376; The Jackson Laboratory] (Lewandoski et al., 1997) to obtain $Emi2^{+/null}$ mice that were further intercrossed to obtain $Emi2^{null/null}$ knockout mice. $Cdk1^{AF}$ mice were generated as a conditional knockin strain as has been described previously (Adhikari et al., 2016). Conditional knockin mice were maintained as heterozygous $Cdk1^{+/LSL\text{-}T14AY15F}$ mice (hereafter referred to as $Cdk1^{+/SAF}$). Upon crossing to β-actin–Cre transgenic mice (Lewandoski et al., 1997), heterozygous knockin mice ($Cdk1^{+/T14AY15F}$, hereafter referred to as $Cdk1^{+/AF}$) displayed early embryonic lethality and died at E3.5 (data not shown). Heterozygous knockin mice crossed to other Cre strains such as Stra8-Cre or ROSA26-CreERT2 are viable and are referred to as $Cdk1^{+/AF}$ Stra8-Cre or $Cdk1^{+/AF}$ ROSA26-CreERT2. Rosa26-CreERT2, Stra8-Cre, Mvh-Cre mice were obtained from Jos Jonkers/Anton Berns, David Page and Kui Liu, respectively.

Generation of antibodies. Rabbit polyclonal antibodies against Emi2 were raised using a mix of two peptides mapping to amino acid residues 234-252 (CGANVTTSVTPVSSLIA KIK) and 585-596 (HKKRGLCSRLAC) of mouse Emi2 protein, using an established protocol (Berthet et al., 2003). To improve specificity of the antibody, protein extracts from Emi2 knockout testes were run on polyacrylamide gels and transferred to PVDF membranes. Sera collected post-immunizations were first incubated overnight at 4°C with these membranes and then affinity purified using peptide HKKRGLCSRLAC that does not occur in the knockout sequence because of premature truncation. The specificity of the antibodies was further tested by Western blotting using protein extracts from various mouse tissues. A band of expected size (~72 kDa) was detected in wild-type testis, but not in Emi2KO testis and other somatic tissues (Figure S6). A similar procedure was used to raise antibodies against mouse cyclin A1, where we used the peptide CRQSSKSGVALPPVGQG mapping to amino acid residues 3-18. Diluted serum was used for Western blotting against cyclin A1.

Chromosome spreads and immunofluorescence analysis of mouse testis. Testes were excised from male mice euthanized at ages P21-P23 and placed in PBS. The tunica albuginea was removed and seminiferous tubules were gently teased apart with blunt forceps. The seminiferous tubules were placed in a hypotonic extraction buffer (30 mM Tris pH 8.2, 50

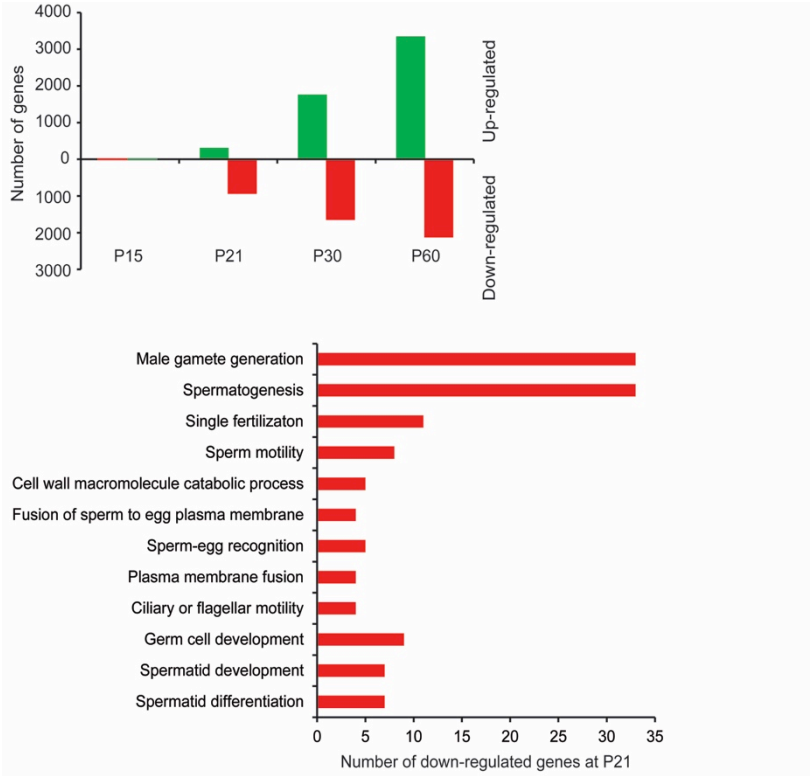
mM Sucrose, 17 mM Trisodium Citrate Dihydrate, 5 mM EDTA) and incubated for one hour with gentle rotation at room temperature. Small portions of the seminiferous tubules were placed in 100 mM Sucrose pH 8.2 droplets (around 60 μl) and pipetted repeatedly (around 50 times) using a cut 200 μl tip until the solution turned turbid. Tubular remnants were removed from the suspension. A polylysine coated slide was dipped in freshly prepared fixative (1% PFA, 30 mM Sodium Tetraborate pH 9.2, 0.15% TritonX-100) and the seminiferous tubule suspension was placed at the edge of the slide and allowed to drip along the length of the slide. Slides thus prepared were placed in a humidified chamber at 4°C for 6-12 hours, and stored at -20°C until further use.

For immunofluorescence analysis, slides with chromosome spreads were washed 3 times with PBS, followed by incubation for one hour with blocking buffer (PBS with 0.1% Tween-20 and 10% donkey serum) in a humidified chamber at room temperature. Chromosome spreads were then incubated overnight with primary antibodies diluted in blocking buffer in a humidified chamber at 4°C. Slides were washed three times in PBST (PBS with 0.1% Tween-20) and incubated with appropriate Alexa Fluor-conjugated secondary antibodies diluted in blocking buffer in a humidified chamber for one hour at room temperature. Following three washes in PBST, chromosome spreads were counterstained with DAPI and images were taken using Zeiss Axioimager Z1 epifluorescence microscope.

Antibodies. For immunofluorescence analysis, the following antibodies were used: SYCP3 – Santa Cruz Biotechnology #SC-20845, phospho-histone H3 – Cell Signaling #9701, γH2AX – Upstate #07-164, SYCP1 – Abcam #15090-100, SP56 – QED Bioscience #55101, Emi2 (described above). For Western blotting, the following antibodies were used: Emi2 and cyclin A1 (described above), cyclin B1 – Cell Signaling #4135, Cdk1 and Cdk2 [as described in (Berthet et al., 2003)], HSP90 – BD Biosciences #610418, Actin – Santa Cruz Biotechnology #SC-1616, GAPDH – Cell Signaling #2118.

Microarray analysis. For microarray analysis, testis RNA was isolated using Life Technologies' PureLink RNA mini kit. cRNA was prepared with Illumina(r) TotalPrep RNA kit according to the manufacturer's protocol. The triplicate expression data microarrays were normalized using quantile normalization with limma and beadarray in R using the reference database Illumina MouseRef-8 V2. Only probes with an absolute fold change >1.5-fold and a Q-value <0.01 were considered as significantly differentially expressed. Microarray data were submitted to the GEO repository and can be viewed using accession number GSE77309.

Immunofluorescence analysis of human testes. Human testicular tissue was obtained from a prostate cancer patient who underwent bilateral orchidectomy as part of his cancer treatment and a patient who underwent Testicular Sperm Extraction (TESE) for Intracytoplasmic Sperm Injection at the Academic Medical Center, Amsterdam, The Netherlands. National Ethical approval was obtained and patients gave informed consent to use their tissues for research purposes. Tissues were fixed in modified Methacarn (89% Methanol and 11% glacial acetic acid) and paraffin-embedded tissues were sectioned and processed for anti-Emi2 antibody immunostaining. Both patients had normal intact spermatogenesis based on testis histology. The images shown in Figure 6H are representative images obtained using tissue from the TESE patient.



Top genes downregulated at P21

Gene symbol	Gene name
Mycbpap	MYCBP associated protein
Adam24	a disintegrin and metallopeptidase domain 24 (testase 1)
Acsbg2	acyl-CoA synthetase bubblegum family member 2
Capza3	capping protein (actin filament) muscle Z-line, alpha 3
Catsper1	cation channel, sperm associated 1
Catsper2	cation channel, sperm associated 2
Catsper3	cation channel, sperm associated 3
Catsper4	cation channel, sperm associated 4
Cep57	centrosomal protein 57
Csda	cold shock domain protein A
Ccna1	cyclin A1
Ehmt2	euchromatic histone lysine N-methyltransferase 2
Ggn	gametogenetin
Ggnbp2	gametogenetin binding protein 2
Gpx4	glutathione peroxidase 4
Hils1	histone H1-like protein in spermatids 1
Klhl10	kelch-like 10 (Drosophila)
Odf1	outer dense fiber of sperm tails 1
Odf2	outer dense fiber of sperm tails 2; similar to outer dense fiber of sperm tails 2
Odf3	outer dense fiber of sperm tails 3
Acrbp	proacrosin binding protein
Prm1	protamine 1
Prss21	protease, serine, 21
Spag4l	sperm associated antigen 4-like
Spata16	spermatogenesis associated 16
Spata19	spermatogenesis associated 19
Spata20	spermatogenesis associated 20
Tssk2	testis-specific serine kinase 2
Tssk4	testis-specific serine kinase 4
Txndc2	thioredoxin domain containing 2 (spermatozoa)
Txndc8	thioredoxin domain containing 8
Tnp1	transition protein 1
Zfp296	zinc finger protein 296

Fig. S1. Related to Fig. 3. Gene expression analysis of Emi2 knockout testes. Microarray analysis of Emi2 knockout testes at ages P15, P21, P30, and P60 (n=2 per genotype/age) revealed zero differentially regulated genes at P15. Genes downregulated from P21 onwards included those involved in spermatogenesis as well as sperm function.

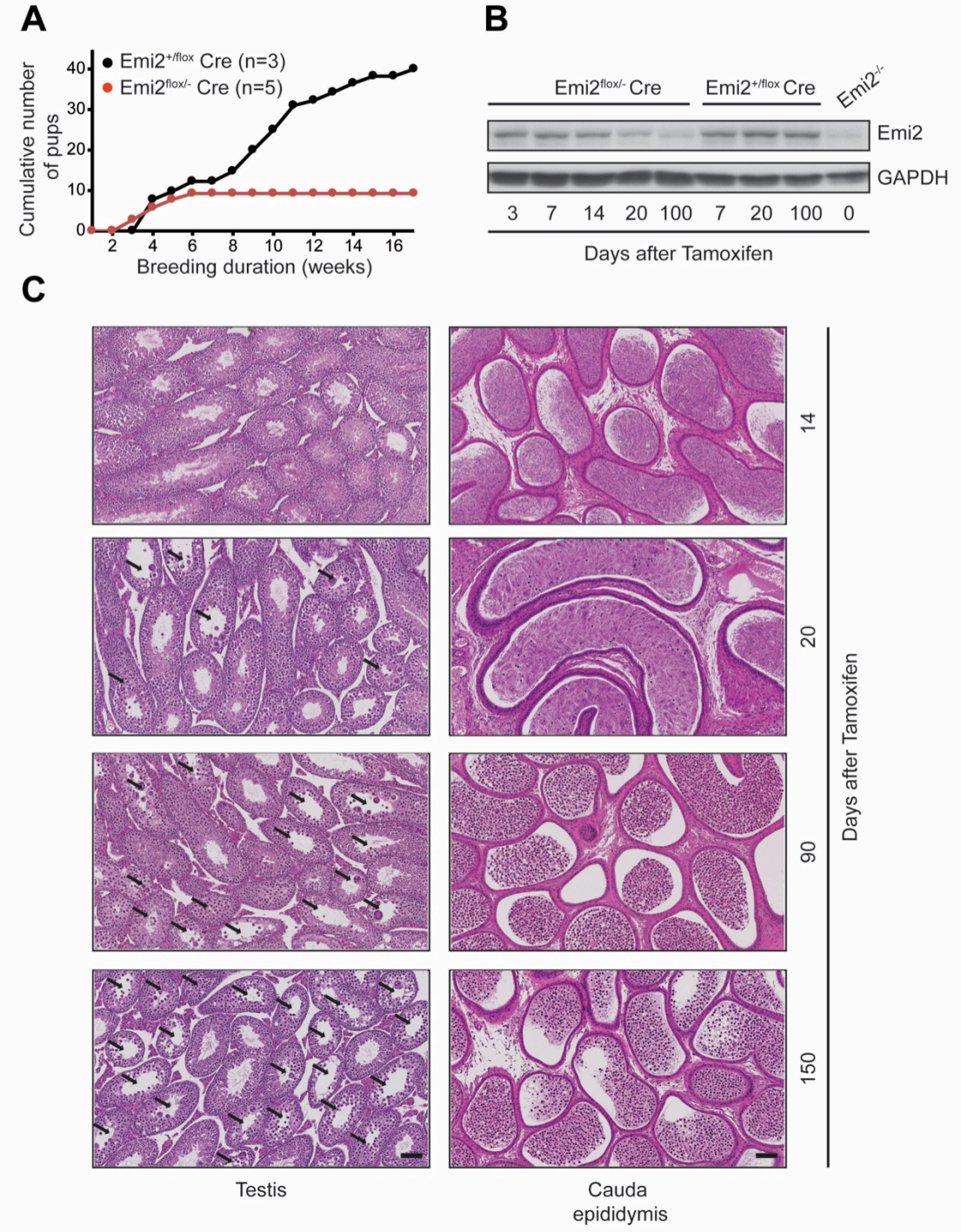


Fig. S2. Related to Fig. 3. Deletion of Emi2 in adult male mice results in sterility. (A) Emi2$^{flox/-}$ ROSA26-CreERT2 adult male mice (n=5) or littermate controls (n=3) were injected with tamoxifen and bred with wild-type females for 12-16 weeks. (A) Graph depicts the average cumulative number of pups born to one male mated with two females over the breeding period. (B-C) Emi2$^{flox/-}$ ROSA26-CreERT2 adult male mice or littermate controls (n=2) were injected with tamoxifen and testes were excised at different time points. Protein extracts of testes excised at different time points following tamoxifen administration were subjected to SDS-PAGE and Western blotting with the indicated antibodies (B). Histopathological analysis of testes excised at different time points following tamoxifen administration revealed progressively increasing number of apoptotic diplotene spermatocytes (arrows ➡) and decreasing number of sperm. Scale bar: 100 μm (C).

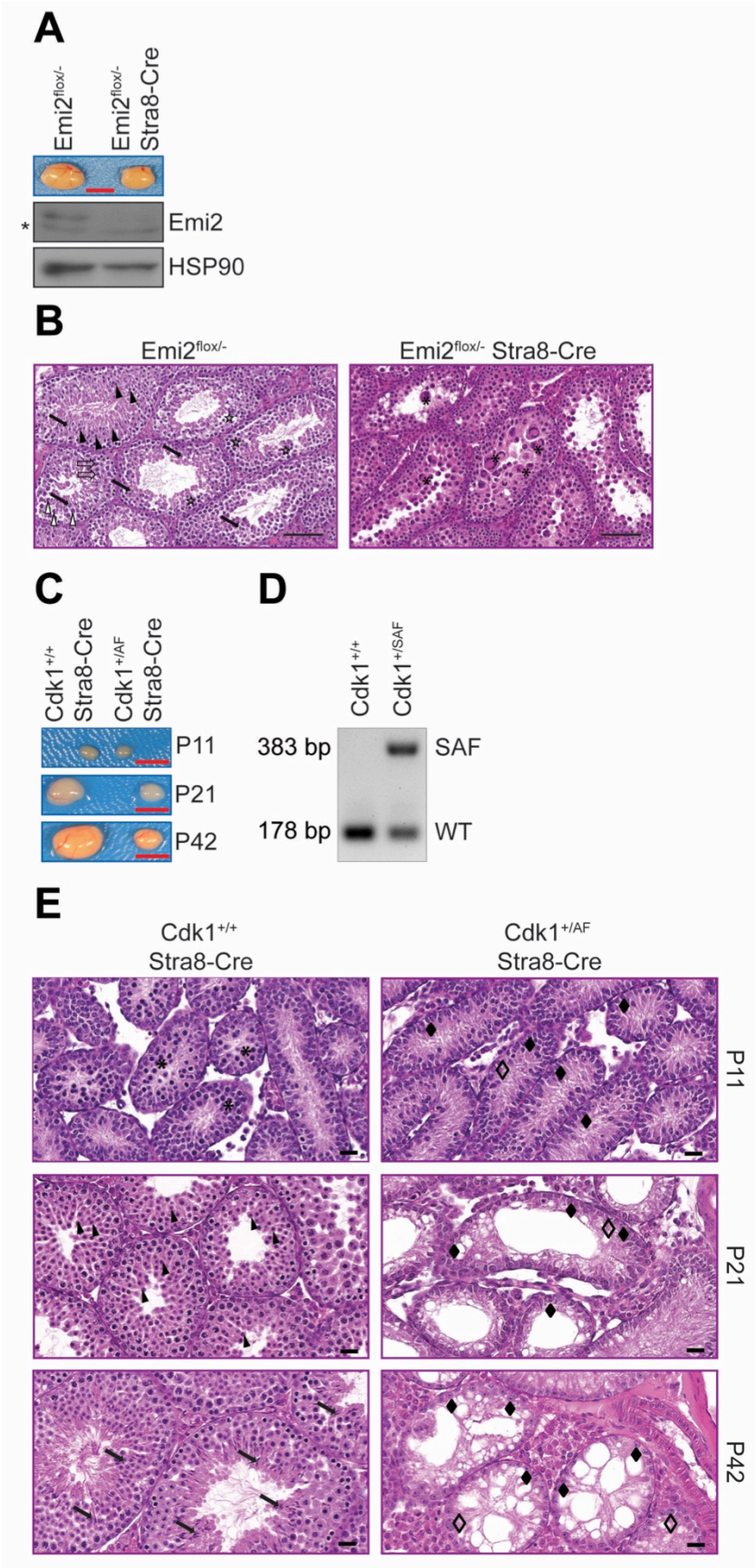


Fig. S3. Related to Fig. 3. Germ cell-specific knockout of Emi2 and activation of Cdk1^{AF}. (A) Testes from Emi2$^{flox/-}$ Stra8-Cre mice are smaller than littermate controls and the absence of Emi2 was confirmed by Western blot analysis. *Non-specific band. Scale bar 5mm. (B) H&E stained sections from testis from Emi2$^{flox/-}$ Stra8-Cre mice display arrest of spermatocytes at pachytene/ diplotene I. Open asterisks indicate normal pachytene spermatocytes, open arrows indicate normal diplotene spermatocytes, open arrow heads indicate meiotic metaphases in spermatocytes, black arrow heads indicate round spermatids, black arrows indicate elongating spermatids, asterisks indicate mono- or multi-nuclear apoptotic pachytene/ diplotene spermatocytes. Scale bar: 100 μm. (C) Testes from Cdk1$^{+/AF}$ Stra8-Cre mice collected at various ages are smaller than littermate controls. Scale bar: 5 mm. (D) Presence of Cdk1^{SAF} locus was confirmed by genotyping of testis. (E) H&E stained sections from testes from Cdk1$^{+/AF}$ Stra8-Cre mice displayed early meiotic arrest, displaying mainly tubules with only Sertoli cells (black diamonds ♦) and only very rare spermatogonia (open diamonds ◊). In the left panel the most advanced germ cell type is indicated: pachytene spermatocytes in P11 (asterisks *), round spermatids in P21 (black arrowheads ▲) and elongated spermatids in P42 (black arrows ➡). Scale bar: 20 μm. Results are representative of 3 mice per genotype.

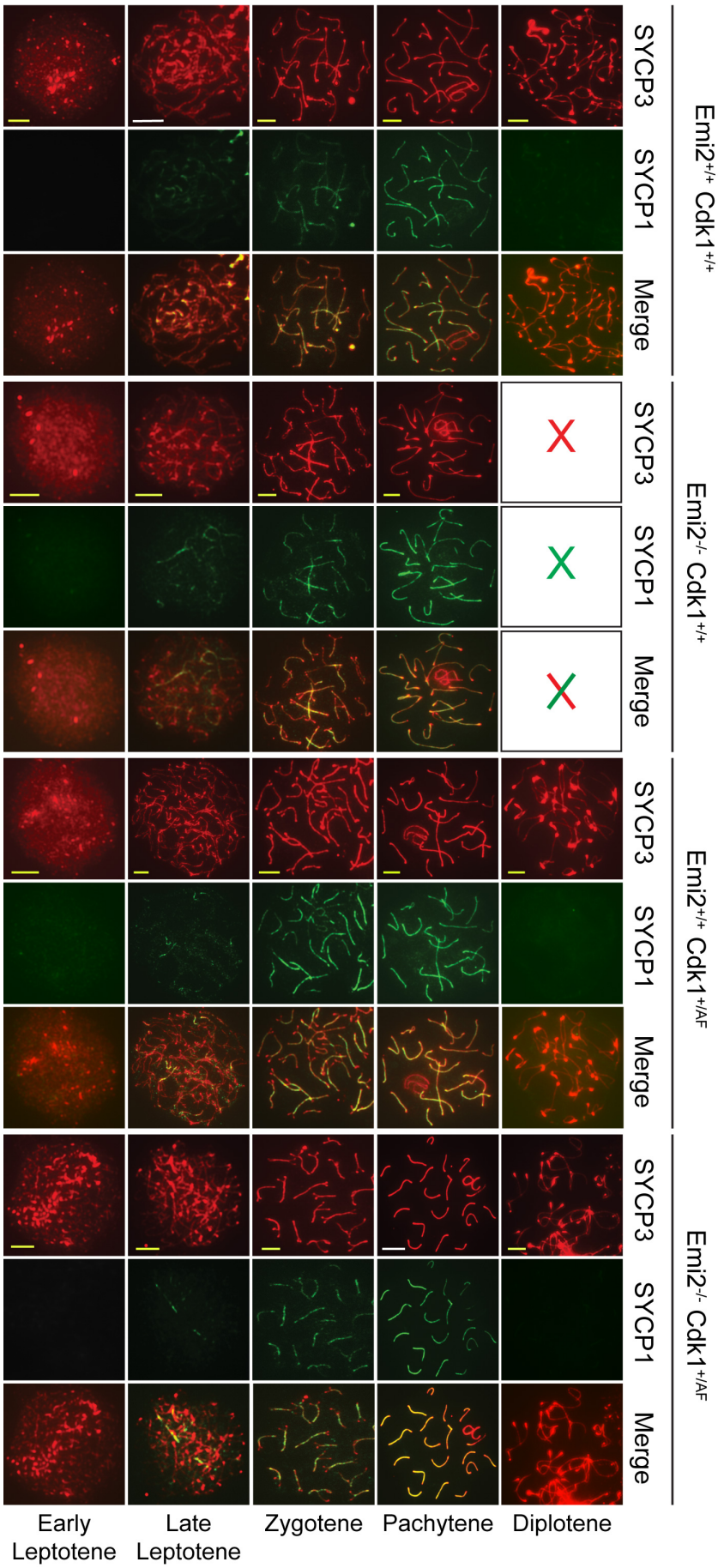


Fig. S4. Related to Fig. 4. Pachytene synapsis is normal in the presence of elevated Cdk1 activity. Testes from $Emi2^{+/+}Cdk1^{+/+}$, $Emi2^{+/+}Cdk1^{+/AF}$, $Emi2^{-/-}Cdk1^{+/+}$, and $Emi2^{-/-}Cdk1^{+/AF}$ mice around 3 weeks of age were used for preparing chromosome spreads that were immuno-stained with the indicated antibodies. For those mice expressing ROSA26-CreERT2, tamoxifen was intraperitoneally injected and testes were excised after 48 hours. Scale bar: yellow 5 μm; white 10 μm. Results are representative of at least three independent experiments. We observed normal progression through prophase I for $Cdk1^{AF}$ ROSA26-CreERT2 (+TAM) spermatocytes; but also observed several aberrant diplotene structures with strong phospho-histone H3 staining and dis-continuous SYCP3 staining, suggesting defects in relocalization of SYCP3 that is expect upon completion of diplotene. Constitutive activation of Cdk1 resulted in early spermatogonial arrest (as evidenced by presence of tubules with only Sertoli cells), accumulation of DSBs during prophase I, and aberrant SYCP3 relocalization.

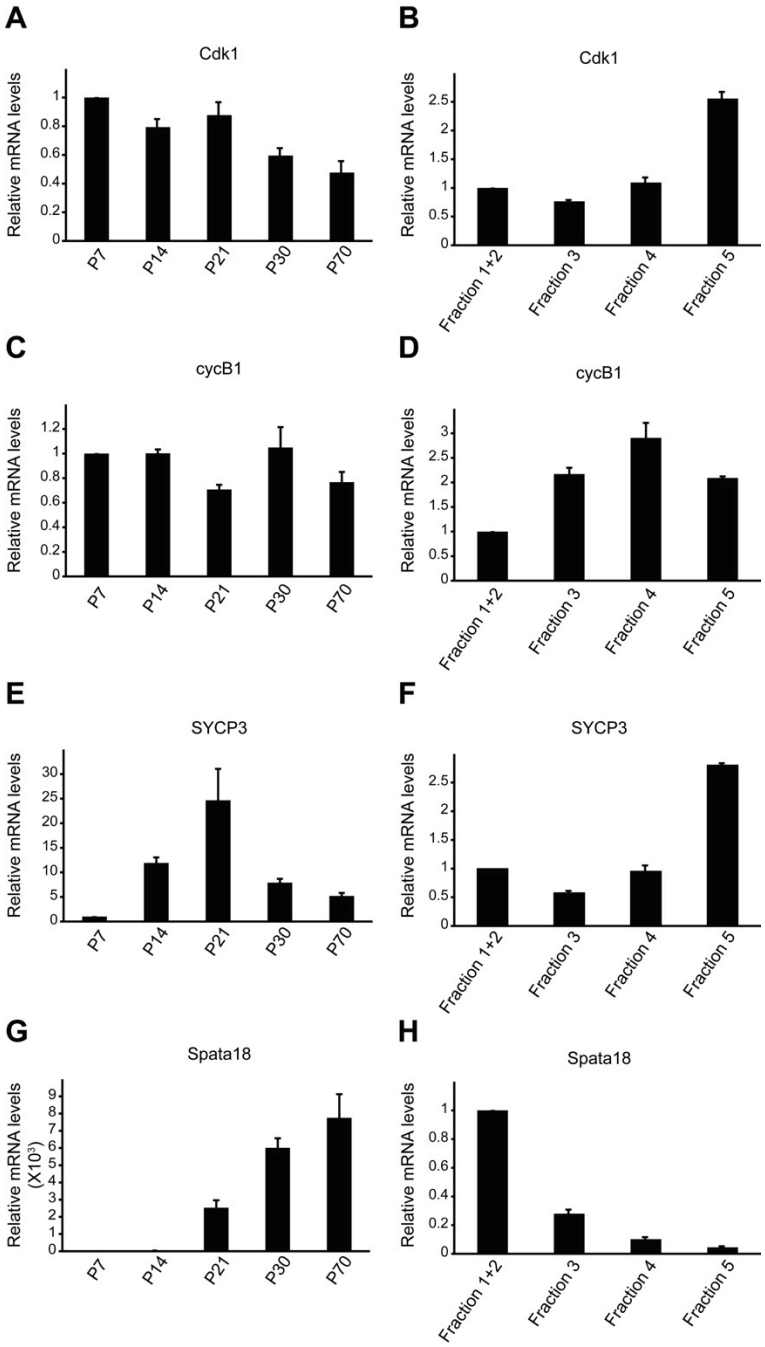


Fig. S5. Related to Fig. 7. mRNA expression analysis. Real-time PCR was performed for various genes using RNA extracted from testes at different ages (A-D) or testicular elutriation fractions (E-H). Expression values were normalized to housekeeping gene Elongation Factor 2 (EF2) and expressed relative to P7 (A-D) or Fraction 1+2 (E-H). Cdk1 mRNA expression decreased with age and was present at highest levels in Fraction 5 (spermatocytes). For cyclin B1, no significant differences were found in expression across various ages and expression was detected at higher levels in fractions 3, 4, and 5 (round spermatids and spermatocytes). Expression profiles for spermatocyte marker SYCP3 and spermatid marker Spata18 were as expected. Highest levels of SYCP3 were detected at ages P14 and P21, and in Fraction 5 (spermatocytes). Spata18 expression increased with age and highest levels were detected in Fraction 1+2 (elongating spermatids). Mean values obtained from testes extracted from three mice per age group are reported in (A-D). For (E-H), results are representative of two independent elutriation experiments, with testes from three mice used per experiment.

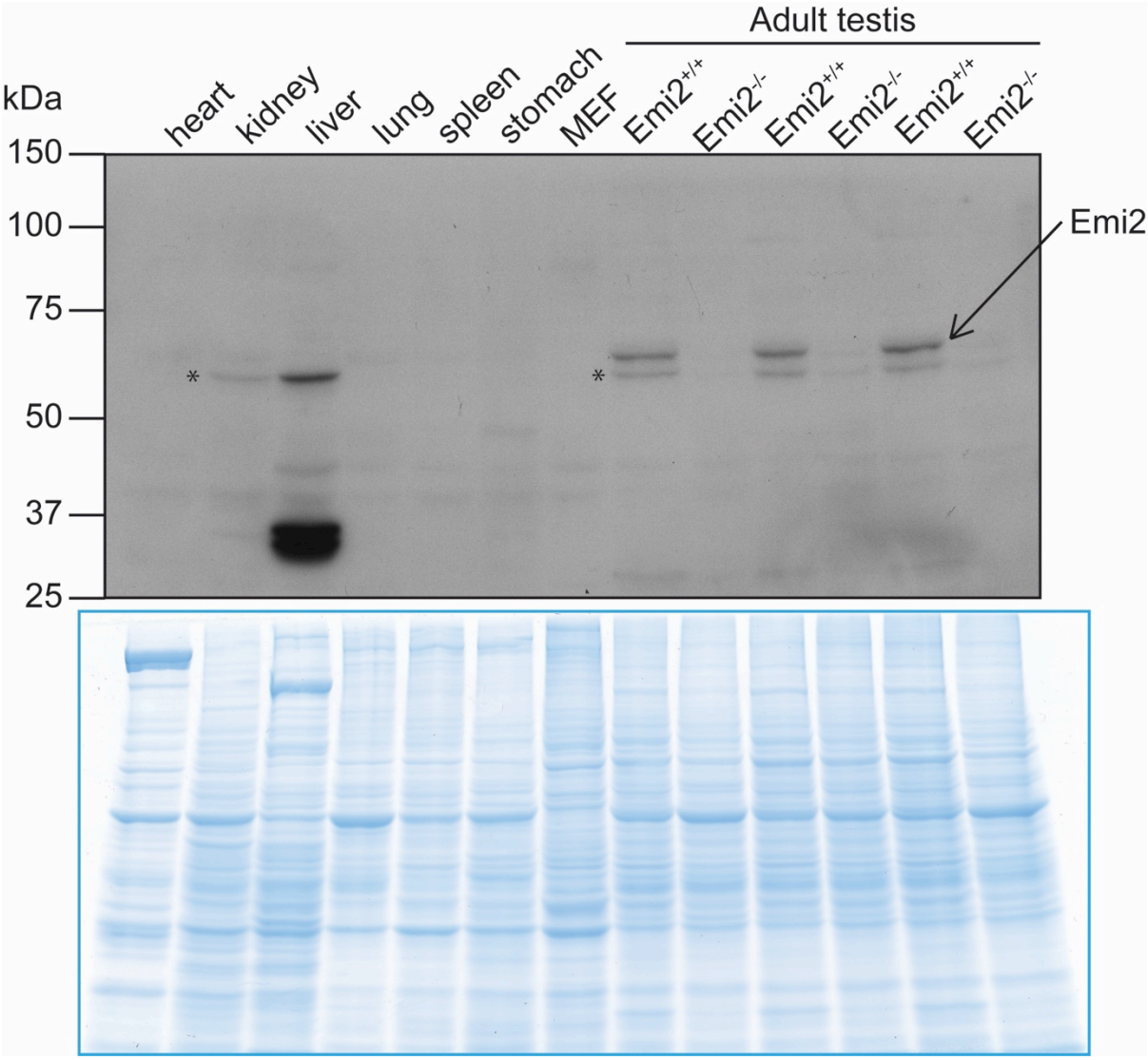


Fig. S6. Related to Fig. 1 and 7. Validation of antibodies generated against Emi2. Protein extracts from various mouse tissues including Emi2KO testes were resolved by SDS-PAGE and subjected to Western blotting with home-made antibodies against Emi2. Specific band of expected size (72kDa) was observed only in wild-type testes. *Non-specific band observed in kidney, liver, and testes (top panel). Coomassie blue-stained gel (bottom panel) served as loading control. Results are representative of two independent experiments.

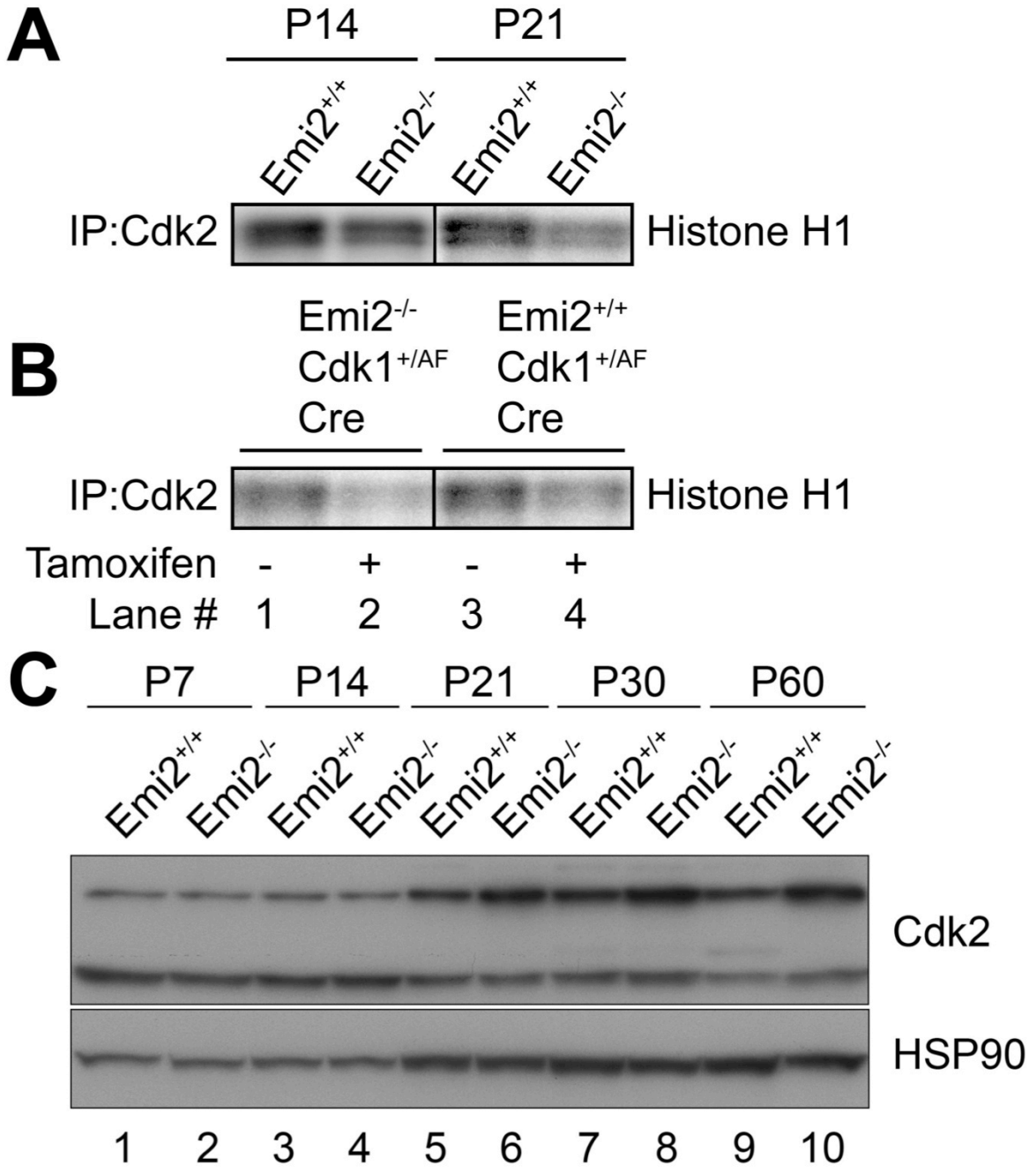


Fig. S7. Related to Fig. 5 and 7. Cdk2-associated activity is decreased in Emi2 knockout testes. Protein extracts from testes isolated from control (Emi2$^{+/+}$ or Emi2$^{+/-}$) or Emi2KO (Emi2$^{-/-}$) mice at P14 and P21 were subjected to immunoprecipitation (IP) with antibodies against Cdk2 and kinase activity was measured using radiolabeled ATP and histone H1 as substrate (A). Same experiment as in (A) but using extracts of Emi2$^{-/-}$Cdk1$^{+/AF}$Cre and Emi2$^{+/+}$Cdk1$^{+/AF}$Cre (B). Results are representative of at least three independent experiments. (C) Protein expression of Cdk2 in testis extracts from different stages as indicated from wild-type and Emi2KO mice. HSP90 serves as loading control. Results are representative of three independent experiments.

Table S1: Related to Fig. 1. Genotyping and genomic primers

Primer name	Primer Sequence (5' to 3')
Genotyping primer P1, PKO2025	CAGAGATTTGCTACATAGAC
Genotyping primer P2, PKO2026	CCTGTCTGGGGCTACGTTGA
Genotyping primer P3, PKO2028	ACACAGAGGAATGCAGCAAA
5' Probe Forward, PKO1253	TCCTACTTTATTCCCAACGGTT
5' Probe Reverse, PKO1254	GCAAACATAGCATGCCATAGAA
3' Probe Forward, PKO1365	CTGATGAAGCTTTAAAGCCTTG
3' Probe Reverse, PKO1366	ACATGGAGCAATAAAACACACA
5'Probe Forward, PKO380	GTGTATAATCACAGAAACAGGC
5'Probe Reverse, PKO381	AGTCCCCGCACCTCGTAAC
3'Probe Forward, PKO382	GGTTTGTTGTAAAGCTGTTGTC
3'Probe Reverse, PKO383	TGGCTGCTGACTACTTCAAT
StopAF forward genotyping primer, PKO2226	GAAAGCAGTGAGGGTTTAATGA
StopAF reverse genotyping primer, PKO2237	CATACAGTCCTCTTCACATCCA
Recombination testing primer Pr1, PKO2226	GAAAGCAGTGAGGGTTTAATGA
Recombination testing primer Pr2, PKO174	TGACTATATTTGGATGTCGAAG
PKO2212	GATACTCAAAGAGTTCAACTTAGAAGA
Sequencing primer, PKO2222	AAGGGGTCCCAGGGCTGTGCCT
5'Probe Forward, PKO380	GTGTATAATCACAGAAACAGGC

Table S2: Related to Fig. 7. Real time PCR primers

Primer name	Primer Sequence (5' to 3')
Emi2 Forward, PKO2144	CTTGTATAGGGCTTGCTTG
Emi2 Forward, PKO2145	TTATACAGTGCTTGGAACG
Emi2 Reverse, PKO2146	GCCCCCTGAGCACTTGCT
Emi2 Reverse, PKO2147	CCTGGCAACCTTCACATA
Cdk1 Forward, PKO2006	ACGAGGTAGTGACGCTGTGG
Cdk1 Reverse, PKO2007	GCCCAGAGCTCTGAAGATCC
cyclin B1 Forward, PKO2863	GCAGAACAGTTGTGTGCCCA
cyclin B1 Reverse, PKO2864	GTCACAAAGGCGAAGTCACC
SYCP3 Forward, PKO4716	AGCCGCTGAGCAAACATCT
SYCP3 Reverse, PKO4717	CTGGAGCCTTTTCATCAGC
Spata18 Forward, PKO4447	GTGCTACACAGGATTTCCAAC
Spata18 Reverse, PKO4448	ACTTTCAGCCAGATCGTCTTG
EF2 Forward, PKO3215	CACTTACCATCCCCCGTCAC
EF2 Reverse, PKO3216	CTTTGGGGTCGCAGCTCTTA

Supplemental References

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