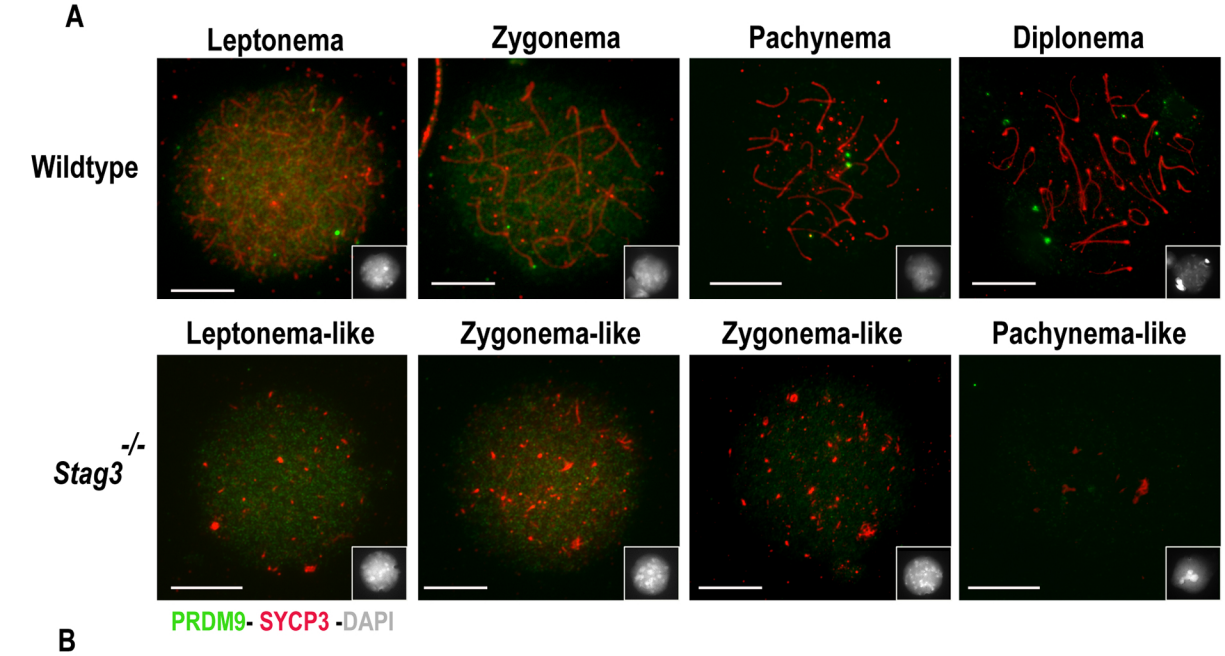
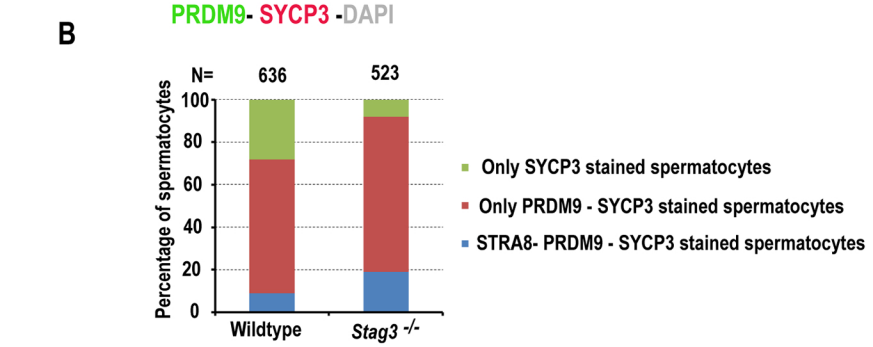
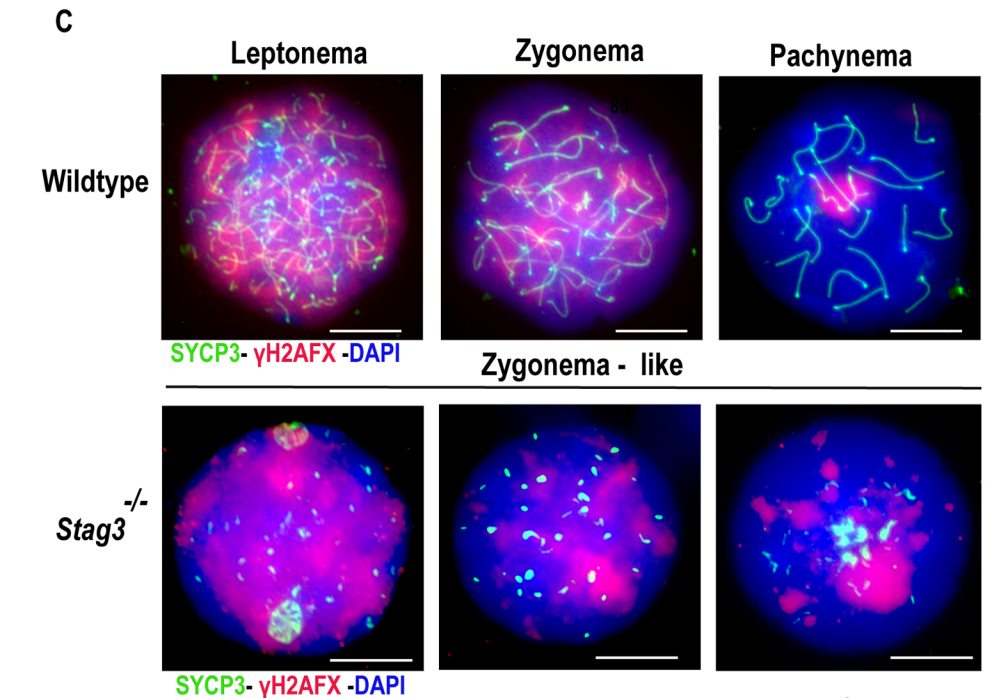

A
Leptonema
Zygonema
Pachynema
Diplonema
Wildtype
Leptonema-like
Zygonema-like
Zygonema-like
Pachynema-like
Stag3 -/-
PRDM9- SYCP3 -DAPI


B
N=
636
523
Percentage of spermatocytes
100
80
60
40
20
0
Wildtype
Stag3 -/-
Only SYCP3 stained spermatocytes
Only PRDM9 - SYCP3 stained spermatocytes
STRA8- PRDM9 - SYCP3 stained spermatocytes


C
Leptonema
Zygonema
Pachynema
Wildtype
SYCP3- γH2AFX -DAPI
Zygonema - like
Stag3 -/-
SYCP3- γH2AFX -DAPI

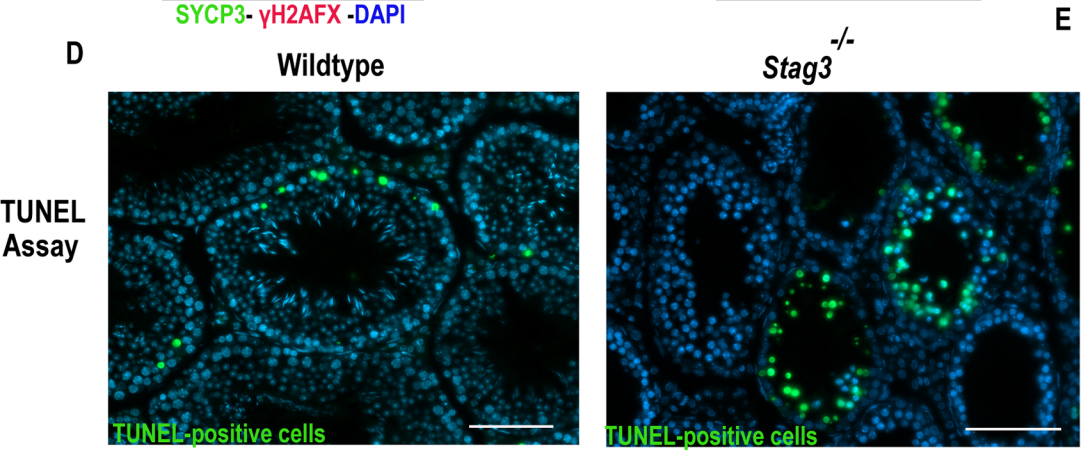

D
Wildtype
Stag3 -/-
TUNEL Assay
TUNEL-positive cells
TUNEL-positive cells
E
Average number of TUNEL positive cells per tubule section
30
25
20
15
10
5
0
***
Wildtype
Stag3 -/-

**Figure S1**. ***Stag3*** **loss leads to abnormal meiotic prophase and meiotic arrest in mouse testis. Related to Figure 1.**

(**A**) Wildtype (WT) and *Stag3*$^{-/-}$ spermatocyte chromatin spreads immunolabeled using antibodies against PRDM9 (green) and SC protein SYCP3 (red). DNA is stained with DAPI (grey), shown in the lower right corner of each image. Spermatocyte meiotic sub-stages are denoted on top of each image panel. Scale bars= 10μm.

(**B**) Stacked bar chart showing frequencies of different spermatocyte morphologies in wildtype (WT) and *Stag3*$^{-/-}$ testes based on the staining patterns of three meiotic proteins: STRA8, SYCP3, and PRDM9, from Figure 1B. In pre-leptotene and leptotene cells STRA8, SYCP3 and PRDM9 co-express (blue bar), zygotene (-like) spermatocytes co-express SYCP3 and PRDM9 (red bar). Pachytene (-like) spermatocytes lack PRDM9 staining and stain only with SYCP3 (green bar).

(**C**) Immunofluorescence staining of spermatocyte chromatin spreads from wildtype (WT) and *Stag3*$^{-/-}$ testes, stained with anti-SYCP3 (green) and γH2AFX (red); DNA is stained with DAPI (blue). Wildtype (WT) spermatocyte prophase I sub-stages are indicated, and examples of *Stag3*$^{-/-}$ chromosome spreads showing different staining patterns are provided. Scale bars= 10μm.

(**D**) Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay on wildtype (WT) and *Stag3*$^{-/-}$ testis sections from 8 week old mice showing apoptotic germ cells (green); DNA is stained with DAPI (blue); scale bars=100μm.

(**E**) Average number of TUNEL-positive cells per testis seminiferous tubule cross-section. The *Stag3*$^{-/-}$ tubules show significantly higher numbers of apoptotic cells than wildtype (B6N) mice ($P < 0.01$ for both comparisons; Mann–Whitney U test).

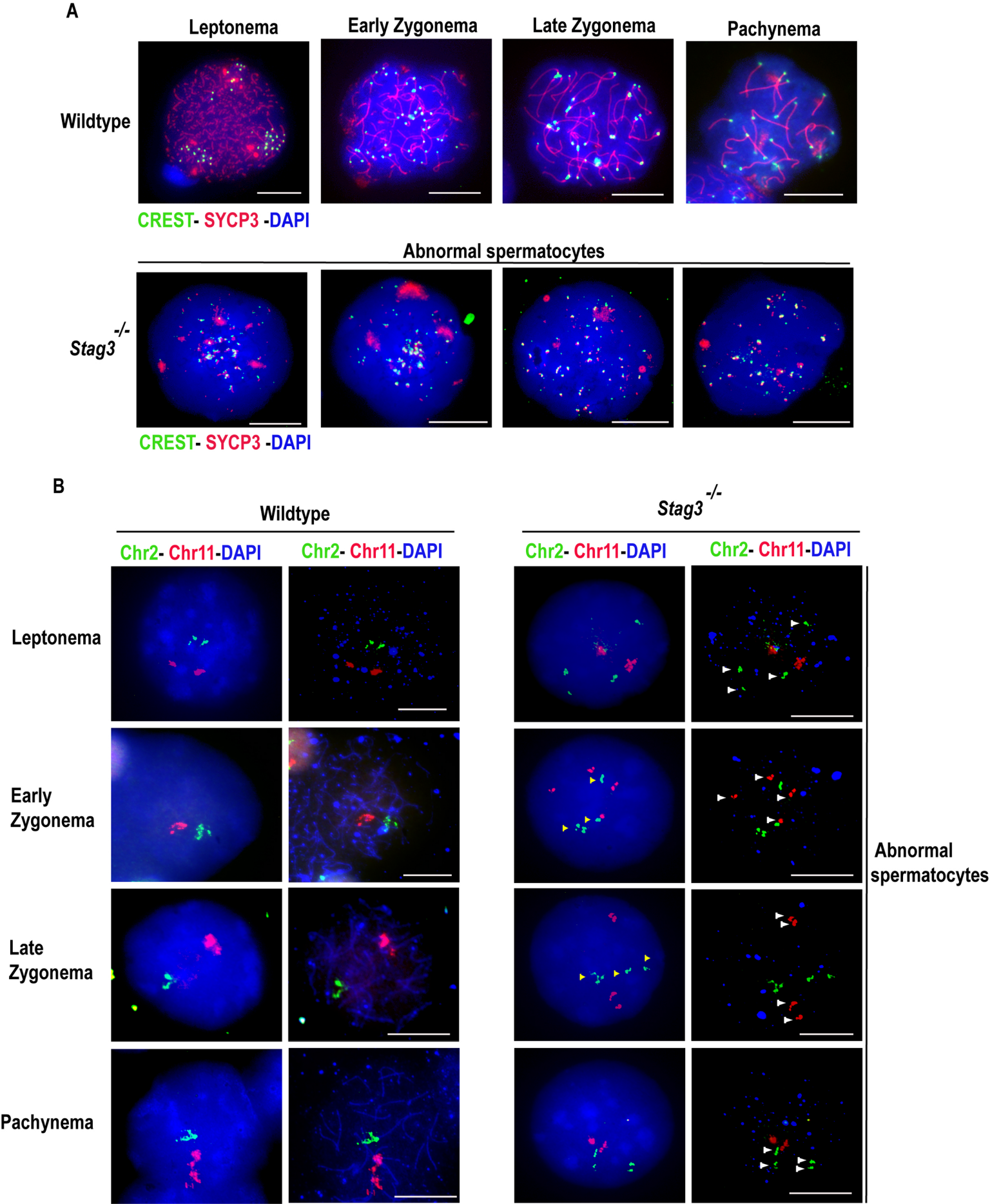

A
Leptonema
Early Zygonema
Late Zygonema
Pachynema
Wildtype
CREST- SYCP3 -DAPI
Abnormal spermatocytes
Stag3 -/-
CREST- SYCP3 -DAPI
B
Wildtype
Stag3 -/-
Chr2- Chr11-DAPI
Chr2- Chr11-DAPI
Chr2- Chr11-DAPI
Chr2- Chr11-DAPI
Leptonema
Early Zygonema
Late Zygonema
Pachynema
Abnormal spermatocytes

**Figure S2. Premature loss of sister chromatid cohesion in *Stag3*$^{-/-}$ spermatocytes. Related to Figure 1.**

(**A**) Meiotic chromatin spreads from wildtype and *Stag3*$^{-/-}$ spermatocytes, immunolabeled with antibodies against the centromere (green, CREST) and SC protein SYCP3 (red); DNA is stained with DAPI (blue). Scale bars=10μm.

(**B**) Wildtype and *Stag3*$^{-/-}$ spermatocyte chromatin spreads stained with DAPI (blue) and hybridized with two 200-kilobase probes against Chrs 2 (green) and 11 (red) distal to the centromere. Note the lack of sister chromatid cohesion in *Stag3*$^{-/-}$ spermatocytes (yellow arrows for 3 distinct FISH signals, white arrows for 4 distinct FISH signals, respectively). Scale bars= 10μm.

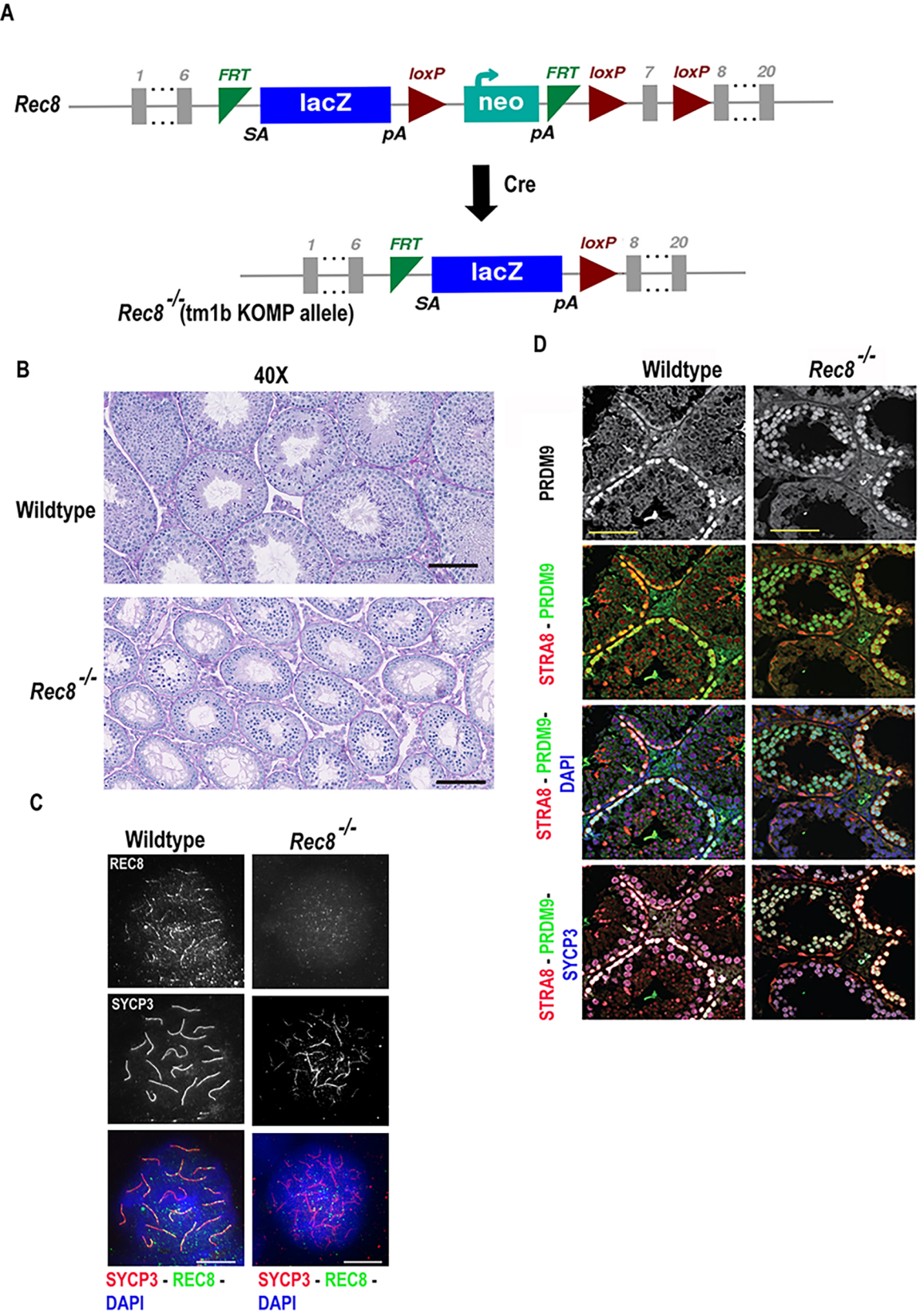

A
Rec8
1
6
FRT
lacZ
SA
pA
loxP
neo
pA
FRT
loxP
7
loxP
8
20
Cre
1
6
FRT
lacZ
SA
loxP
8
20
pA
Rec8 -/- (tm1b KOMP allele)
B
40X
Wildtype
Rec8 -/-
C
Wildtype
Rec8 -/-
REC8
SYCP3
SYCP3 - REC8 - DAPI
SYCP3 - REC8 - DAPI
D
Wildtype
Rec8 -/-
PRDM9
STRA8 - PRDM9
STRA8 - PRDM9- DAPI
STRA8 - PRDM9- SYCP3

**Figure S3**. **Features of the *Rec8* mutant used in this study. Related to Figure 2.**

(**A**) A beta-galactosidase-containing cassette was introduced into the *Rec8* gene in C57BL/6N-derived embryonic stem (ES) cells. The *Rec8* knockout-first reporter allele was generated by a SOX2-Cre-recombinase system to delete exon 7 of the gene. For further details please refer to International Knockout Mouse Consortium (IKMC) website https://www.komp.org/ProductSheet.php?cloneID=873812.

(**B**) PAS-stained testis sections from 8 week old wildtype and *Rec8*$^{-/-}$ males. Note meiotic arrest in *Rec8*$^{-/-}$ testis. Scale bars=100µm.

(**C**) Spermatocyte chromatin spreads labeled with antibodies against SC proteins SYCP3 (grey/red) and cohesin subunit REC8 (grey/green). DNA is stained with DAPI (blue). *Rec8*$^{-/-}$ spermatocytes arrest at a zygotene-like stage, lacking synapsis between homologous chromosomes, and exhibiting synapsis between sister chromatids. Scale bars=10µm.

(**D**) Immunofluorescence staining of testis sections with antibodies against PRDM9 (grey/green), STRA8 (red), SYCP3 (blue). Chromatin was stained with DAPI (blue); scale bars=100µm. Expression of PRDM9 is not obviously affected in *Rec8*$^{-/-}$ testes.

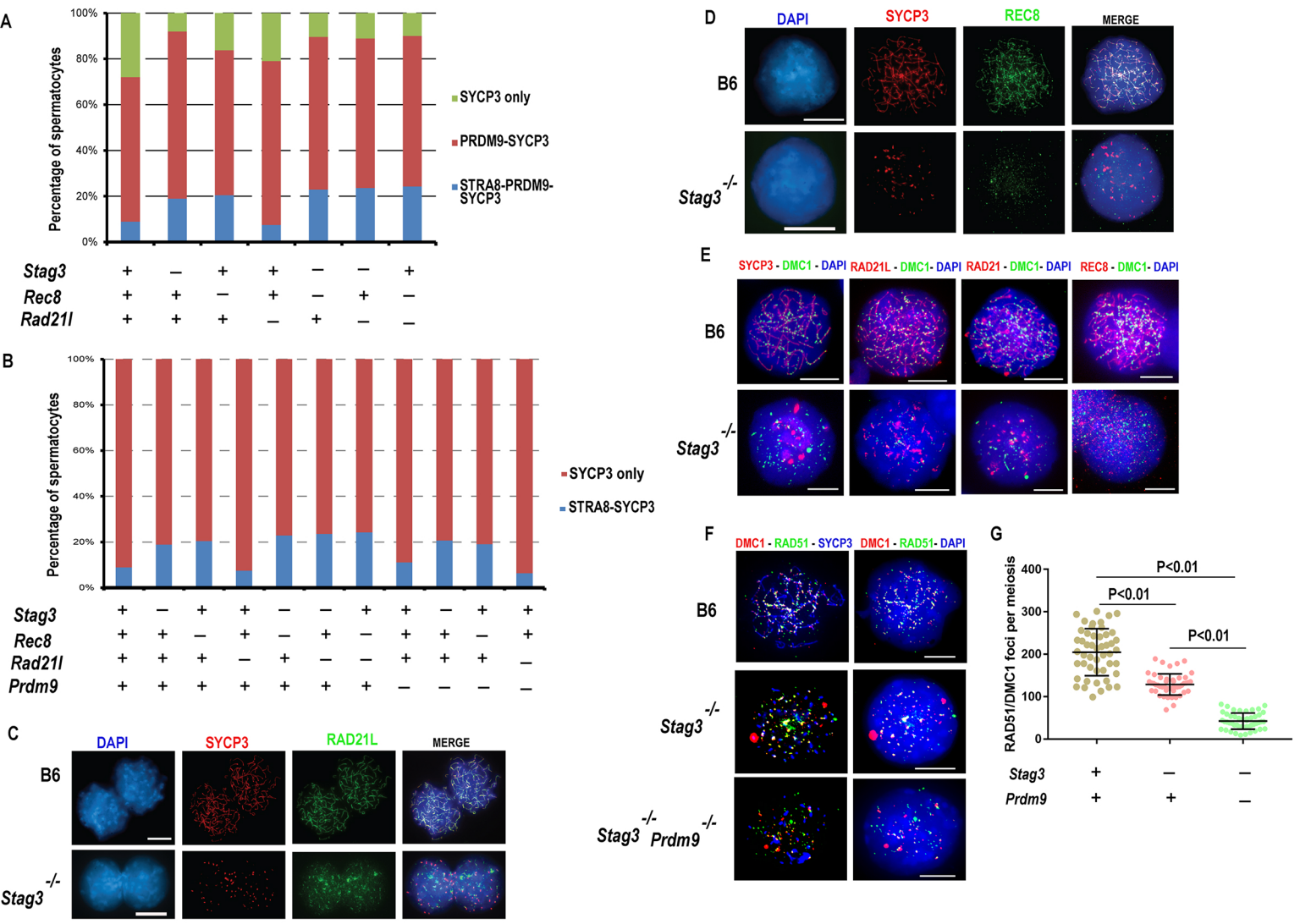

A
100%
80%
60%
40%
20%
0%
Percentage of spermatocytes
SYCP3 only
PRDM9-SYCP3
STRA8-PRDM9-SYCP3
Stag3 + – + + – – +
Rec8 + + – + – + –
Rad21l + + + – + – –
B
100%
80%
60%
40%
20%
0%
Percentage of spermatocytes
SYCP3 only
STRA8-SYCP3
Stag3 + – + + – – + + – + +
Rec8 + + – + – + – + + – +
Rad21l + + + – + – – + + + –
Prdm9 + + + + + + + – – – –
C
DAPI
SYCP3
RAD21L
MERGE
B6
Stag3 -/-
D
DAPI
SYCP3
REC8
MERGE
B6
Stag3 -/-
E
SYCP3 - DMC1 - DAPI
RAD21L - DMC1- DAPI
RAD21 - DMC1- DAPI
REC8 - DMC1- DAPI
B6
Stag3 -/-
F
DMC1 - RAD51 - SYCP3
DMC1 - RAD51- DAPI
B6
Stag3 -/-
Stag3 -/- Prdm9 -/-
G
RAD51/DMC1 foci per meiosis
400
300
200
100
0
P<0.01
P<0.01
P<0.01
Stag3 + – –
Prdm9 + + –

**Figure S4**. **Spermatocyte profile in cohesin- and *Prdm9* deficient single and double mutants. Related to Figure 2.**

(**A**) Stacked bar chart showing frequencies of different spermatocyte morphologies in wildtype (B6) and cohesin mutant testes based on the staining patterns of three meiotic proteins: STRA8, SYCP3, and PRDM9. In pre-leptotene and leptotene cells STRA8, SYCP3 and PRDM9 co-express (blue bar), zygotene (-like) spermatocytes co-express SYCP3 and PRDM9 (red bar). Pachytene (-like) spermatocytes lack PRDM9 staining and stain only with SYCP3 (green bar).

(**B**) Stacked bar chart showing frequencies of different spermatocyte morphologies in wildtype (B6), cohesin and *Prdm9* single and double mutant testes based on the staining patterns of two meiotic proteins: STRA8 ,SYCP3 and DAPI. Pre-leptotene and leptotene spermatocytes are STRA8, SYCP3 (blue bar), while other spermatocytes are only SYCP3 positive (red bar).

(**C**) Sparse chromosome axis-like structures with short linear profiles or punctate foci stained with RAD21L, REC8, and RAD21 in *Stag3*$^{-/-}$ spermatocytes. Immunolabeled wildtype and *Stag3*$^{-/-}$ spermatocytes stained with antibodies against SC protein SYCP3 (red) and cohesin subunit RAD21L (green). DAPI was used for chromatin staining (blue). Scale bars=10μm.

(**D**) Wildtype and *Stag3*$^{-/-}$ spermatocytes stained with antibodies against SC protein SYCP3 (red) and cohesin subunit REC8 (green). DAPI was used to stain DNA (blue). Note the difference in staining pattern between RAD21L and REC8 in *Stag3*$^{-/-}$ spermatocytes. Scale bars=10μm.

(**E**) Immunofluorescence staining of wildtype and *Stag3*$^{-/-}$ spermatocyte chromatin spreads with antibodies against SC and axis protein SYCP3 and cohesin subunits RAD21L, RAD21 and REC8 (red) and DSB repair protein DMC1 (green). Note occasional co-localization between axis proteins and DMC1 signal in *Stag3*$^{-/-}$ spermatocytes. DNA is stained using DAPI (blue). Scale bars=10μm.

(**F**) Immunofluorescence detection of RAD51(green) and DMC1 foci (red) in wildtype, *Stag3*$^{-/-}$ and *Stag3*$^{-/-}$ *Prdm9* $^{-/-}$ spermatocyte chromatin spreads also labeled with antibody against SYCP3 (blue; right panel). Left panel: DNA is stained with and DAPI (blue). Scale bars=10μm. Note co-occurance between RAD51 and DMC1 signal in spermatocytes.

(**G**) Dot-plot showing quantification of Co-localized RAD51/DMC1 foci in wildtype, *Stag3*$^{-/-}$ and *Stag3*$^{-/-}$ *Prdm9* $^{-/-}$ spermatocytes. Error bars=Standard deviation. P-values were calculated using Mann-Whitney U test with Tukey's multiple testing corrections.

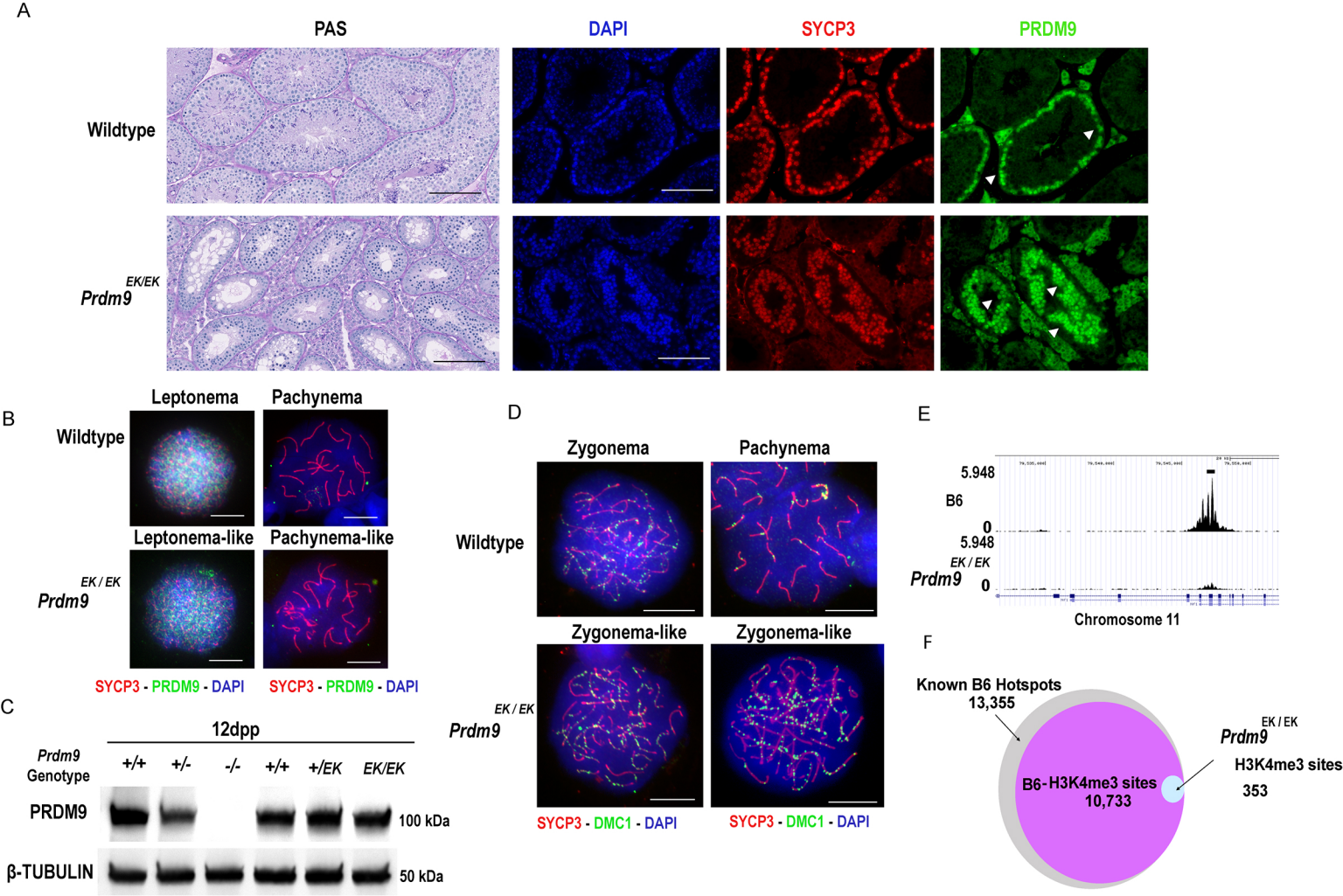

A
PAS
DAPI
SYCP3
PRDM9
Wildtype
Prdm9 EK/EK
B
Leptonema
Pachynema
Wildtype
Leptonema-like
Pachynema-like
Prdm9 EK / EK
SYCP3 - PRDM9 - DAPI
SYCP3 - PRDM9 - DAPI
C
12dpp
Prdm9 Genotype
+/+
+/-
-/-
+/+
+/EK
EK/EK
PRDM9
100 kDa
β-TUBULIN
50 kDa
D
Zygonema
Pachynema
Wildtype
Zygonema-like
Zygonema-like
Prdm9 EK / EK
SYCP3 - DMC1 - DAPI
SYCP3 - DMC1 - DAPI
E
5.948
B6
0
5.948
EK / EK
Prdm9
0
Chromosome 11
F
Known B6 Hotspots
13,355
B6-H3K4me3 sites
10,733
Prdm9 EK / EK
H3K4me3 sites
353

**Figure S5. Meiotic arrest and failure of methyltransferase function in *Prdm9*$^{EK / EK}$ spermatocytes. Related to Figure 2 and Methods S1.**

(**A**) Left panel: Wildtype and *Prdm9*$^{EK / EK}$ testis sections from adult mice stained with PAS. The *Prdm9*$^{EK / EK}$ germ cells exhibit arrest at an abnormal late-zygotene-early-pachytene-like stage. Right 3 panels: Immunofluorescence staining of wildtype and *Prdm9*$^{EK / EK}$ testis sections with antibodies against SC protein SYCP3 (red) and PRDM9 (green); DNA is stained with DAPI (blue). The high expression of PRDM9 in *Prdm9*$^{EK/EK}$ spermatocytes suggests that the expression of PRDM9 is not affected by the E365K substitution mutation. Scale bars=100µm.

(**B**) Wildtype and *Prdm9*$^{EK / EK}$ spermatocyte chromatin spreads immunolabeled using antibodies against PRDM9 (green) and SC protein SYCP3 (red). DNA is stained with DAPI (blue). Spermatocyte meiotic sub-stages are denoted on top of each image panel. Scale bars= 10µm.

(**C**) Immunoblot showing expression of PRDM9 and β-TUBULIN (loading control) in juvenile (12 d postpartum) testes from wildtype ("+/+"), *Prdm9*$^{+ / EK}$ ("+ / EK"), *Prdm9*$^{EK / EK}$ ("EK / EK"), and *Prdm9*$^{- / -}$ ("-/-") males. 50 µg of protein extract was loaded per lane.

(**D**) Wildtype and *Prdm9*$^{EK / EK}$ spermatocytes immunolabeled with anti-SYCP3 (red) and anti-DMC1 antibodies; DNA is stained with DAPI (blue); scale bars=10µm. Almost all *Prdm9*$^{EK / EK}$ spermatocytes fail to repair meiotic DSBs by the pachytene-like stage.

(**E**) Coverage profile of H3K4me3 from a strongly trimethylated *Prdm9*$^{Dom2}$ hotspot on chromosome 11 in B6J (control) and *Prdm9*$^{EK / EK}$ spermatocyte chromatin, revealing failure of methytransferase activity of the mutated PRDM9.

(**F**) Venn diagram showing the number of recombination hotspots with a detectable H3K4me3 peak in wildtype (B6J) and *Prdm9*$^{EK / EK}$ spermatocytes.

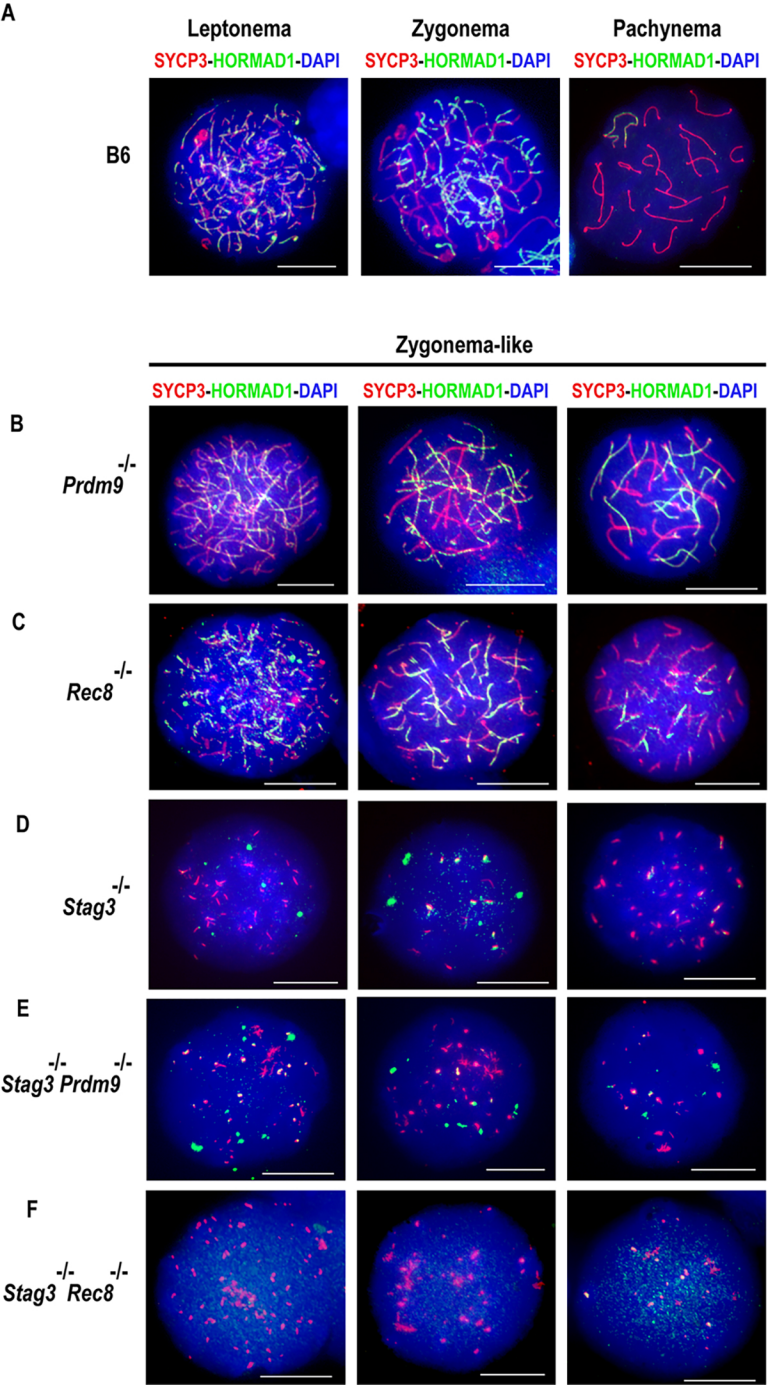

A
Leptonema
Zygonema
Pachynema
SYCP3-HORMAD1-DAPI
SYCP3-HORMAD1-DAPI
SYCP3-HORMAD1-DAPI
B6
Zygonema-like
SYCP3-HORMAD1-DAPI
SYCP3-HORMAD1-DAPI
SYCP3-HORMAD1-DAPI
B
Prdm9 -/-
C
Rec8 -/-
D
Stag3 -/-
E
Stag3 -/- Prdm9 -/-
F
Stag3 -/- Rec8 -/-

**Figure S6**. **Cohesin proteins are essential for HORMAD1 localization on the chromosome axis. Related to Figure 5.**

Immunofluorescence staining of wildtype and different mutant spermatocyte chromatin spreads using antibodies against SC protein SYCP3 (red) and axis protein HORMAD1 (green). DNA is stained with DAPI (blue). For each strain, three representative patterns of staining are provided to better capture the full phenotype.

(**A** & **B**) Immunolabeled chromatin spreads from B6N (wildtype) and *Prdm9*$^{-/-}$ spermatocytes with HORMAD1 and SYCP3. Note co-localization of HORMAD1 with SYCP3 in wildtype and *Prdm9*$^{-/-}$ spermatocytes on unsynapsed axes. Scale bars: 10µm.

(**C**) Immunostained meiotic chromatin spreads from *Rec8*$^{-/-}$ spermatocytes. Note the intermittent staining of HORMAD1 signal on SYCP3 stained axis. Scale bars: 10µm.

(**D**, **E** & **F**) Immunofluorescence staining of *Stag3*$^{-/-}$, *Stag3*$^{-/-}$*Prdm9*$^{-/-}$ and *Stag3*$^{-/-}$ *Rec8*$^{-/-}$ spermatocyte chromatin spreads. Both SYCP3 and HORMAD1 fail to form a linear axis-like profiles, but occasionally co-localize. Scale bars: 10µm.

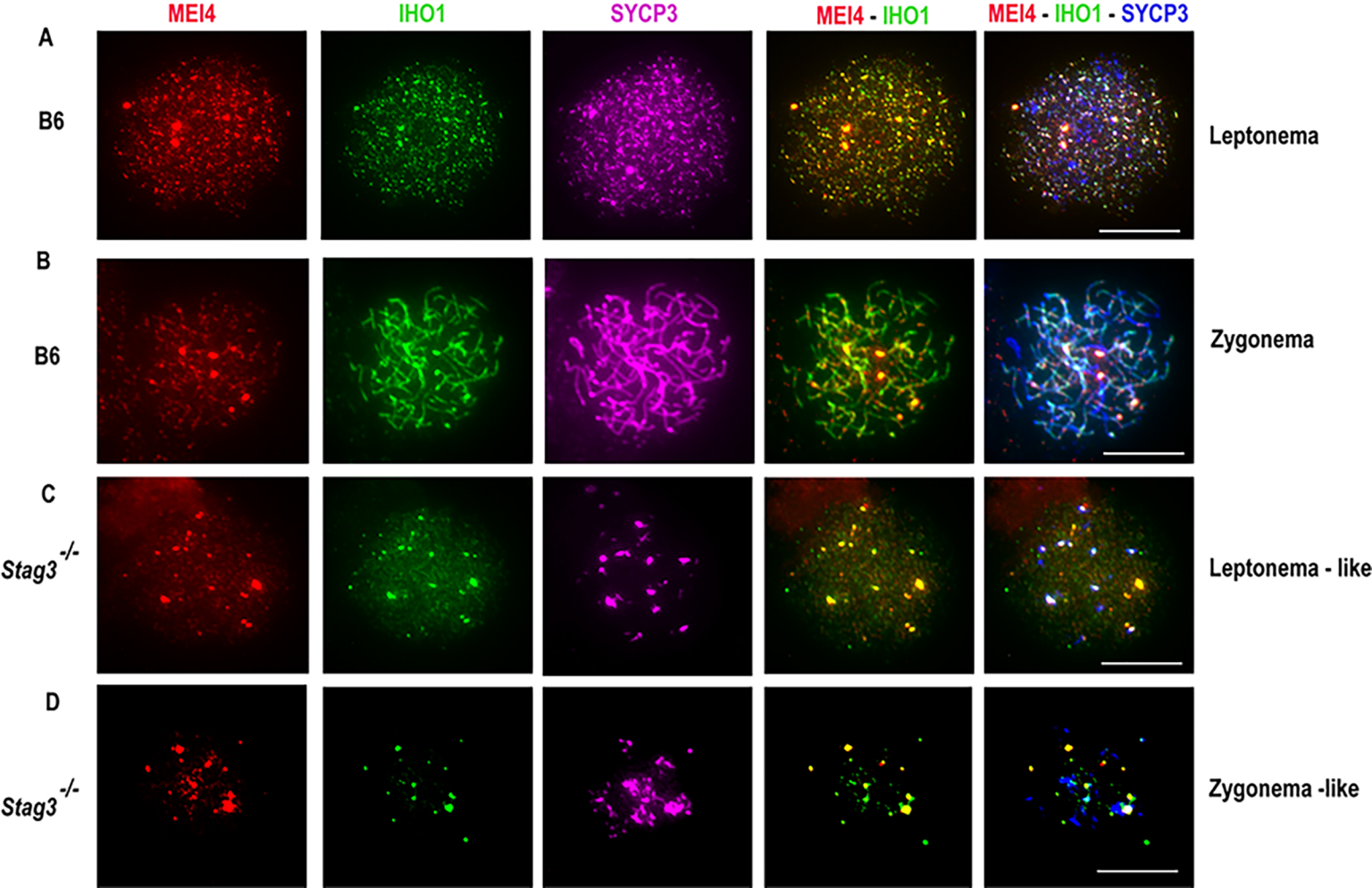

MEI4
IHO1
SYCP3
MEI4 - IHO1
MEI4 - IHO1 - SYCP3
A
B6
Leptonema
B
B6
Zygonema
C
Stag3 -/-
Leptonema - like
D
Stag3 -/-
Zygonema -like

**Figure S7. IHO1and MEI4 co-localize in the absence of a meiotic cohesin axis. Related to Figure 5.**

(A & B) B6 leptotene and zygotene spermatocyte chromatin spreads immunolabeled with antibodies against pre-DSB protein MEI4 (red), axis protein IHO1 (green), and SC protein SYCP3 (purple or blue). Note co-localization of IHO1 and MEI4. Scale bars: 10μm.

(C & D) Stag3-/- leptotene and zygotene (-like) spermatocyte chromatin spreads stained with antibodies against pre-DSB protein MEI4 (red), axis protein IHO1 (green) and SC protein SYCP3 (purple or blue). Scale bars: 10μm. Note that IHO1 and MEI4 co-localize in Stag3-/- spermatocytes in spite of the absence of a meiotic cohesin axis.

| | PRDM9 | STAG3 | RAD21L | REC8 | RAD21 | SMC3 | H3 |
|---|---|---|---|---|---|---|---|
| Input | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| IgG | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| PRDM9-IP | 3.08 | 0.23 | 0.00 | 0.20 | 0.00 | 0.74 | 0.30 |
| PRDM9-IP + DNase | 1.64 | 0.26 | 0.00 | 0.18 | 0.00 | 0.75 | 0.00 |

**Table S1. PRDM9 interacts with cohesin complex subunits in mouse spermatocytes. Related to Figure 1.**

Quantification of protein bands from Figure 1E. All values were normalized to input. Histone H3 IP was used to detect efficiency of DNase treatment.

| | PRDM9 | STAG3 | RAD21L | REC8 | RAD21 | SMC3 | H3 |
|---|---|---|---|---|---|---|---|
| Input | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| IgG | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 |
| SMC3-IP | 0.45 | 2.01 | 9.74 | 0.54 | 1.75 | 1.27 | 0.14 |
| SMC3-IP + DNase | 0.43 | 1.18 | 6.52 | 0.28 | 1.78 | 1.02 | 0.01 |

**Table S2. SMC3 interacts with PRDM9 and cohesin complex subunits in mouse spermatocytes. Related to Figure 1.**

Quantification of protein bands from Figure 1F. All values were normalized to input. Histone H3 IP was used to detect efficiency of DNase treatment.

| Genotyping Primers | Primer Sequence | Comments |
|---|---|---|
| *Stag3* Mt F | CGG TCG  CTA CCA TTA CCA GT | |
| *Stag3* Wt F | TCT CAT CTC TAC AAT GCT TGC AC | |
| *Stag3* Common | AGA AGA GTA GCC CAA GAG AAG G | |
| *Rad21L* Wt F | TCT CAT GCT TCG TGC TTG AC | |
| *Rad21L* Wt R | ATG GCC ACT AAT GGG TTC AG | |
| *Rad21L* Mt R | TTC GTT CTT GGC CAT TCT CT | |
| *Rad21L* Mt F | CGG TCG CTA CCA TTA CCA GT | |
| *Prdm9*_hayashi Common | AGG AAT CTT CCT TCC TTG CTG TCG | |
| *Prdm9*_hayashi Wt R | ATT TCC CTG TAT CTT CTT CAG GAC T | |
| *Prdm9*_hayashi Mt R | CGC CAT TCA GGC TGC GCA ACT GTT | |
| *Prdm9*_EK_ F | ACT GAA GTA GAG CTT TGA AAC TGG G | Sequencing based genotyping |
| *Prdm9*_EK_R | TGG TCC ATT TCT TGC TTC ACC | Sequencing based genotyping |
| *Rec8* Wt F | CAC TCA CAC TGG ATG TGG TAA TG | |
| *Rec8* Wt R | TGT TTG TAT GCA CCC TTG GA | |
| *Rec8* Mt F | TCC TGG GAT TAC TGA TGT CCA | |
| *Rec8* Mt R | CGG TCG CTA CCA TTA CCA GT | |
| *Spo11* Mt F | GCC AGA GGC CAC TTG TGT AG | |
| *Spo11* Wt F | TCA GGA CAG GGC ATA GCA GT | |
| *Spo11* Common | CTG CTC AGG GAG GAG AAC AC | |

**Table S3. Primers required for genotyping different mouse mutants. Related to STAR Methods.**

In the table, “Wt” refers to wildtype and “Mt” refers to mutant-specific genotyping primers.