Figure S1

A

```
D3Z423|RNF212B_Mm    -MDWFHCNQCFRKD--GAHFFVTSCGHIFCKKCMT---LEKCAVCGNLCKHLALSDNLKP
F6TQD1|RNF212_Mm     MASWVFCNRCFQSPHRKSSFSLTSCGHVYCHSCLLKGTKNECVICQAPCQTVLLSKHTNS
                       .*..**:**:.    : * :*****::*:.*:     ::*.:*   *: : **.: :

D3Z423|RNF212B_Mm    QEKKFFKSPVETALQCFSHISQVWCFQKKQTDLLIAFYKDRITKLEAAVKEAQEMAASQN
F6TQD1|RNF212_Mm     NIQTFFLGIDGLCKKYSQETSQISEFQEKHRRRLVAFYQEKISQLEESLRKSVLQ----I
                     : :.** .    . :  .. **:  **:*:   *:***:::*::** :::::

D3Z423|RNF212B_Mm    KELSALRKE-NGELKKILDILKGSPSCYYGSRLTTPRPVGITSPSQSVAPRPSSHHSSQV
F6TQD1|RNF212_Mm     KQLQSMRSSQQPAFNKIKNSVSTKPNGYLFLPPNSSLPDRIESMDIDLTPPARKPEMSAG

D3Z423|RNF212B_Mm    VSRSSSMESIPYTVAGMGHVEQ-GSRGLHVRSTPGDSYTETPSPASTHSLSYRPSS----
F6TQD1|RNF212_Mm     PSRISVI--SPPQDGRMGSVTCRGPQ--HLSLTPSHASMTKASRVPPLQMPYKELSPPPA
                      ** * :   *   .  ** *  * :  *:  **.:   . * .   .: *:  *

D3Z423|R2NF12B_Mm    ------ASSGQGVSFRPFFSGDSGHT----RVLTPNNSGRRESRTTPESLPGFQLPVLQT
F6TQD1|RNF212_Mm     SQLSSRATQGPSPSVSSSWTGPPRQPISISGLLQRQCAGSAS----P---RGMDTEKMSP
                           *:.* . *.   ::*   :      :*  : :*  .    *    *::   :.

D3Z423|RNF212B_Mm    FYQQRQMGL-ARKDGWNISR--
F6TQD1|RNF212_Mm     FLPSTPTNLRSVASPWHACVHR
                     *  .   .* :  . *: .
```

B

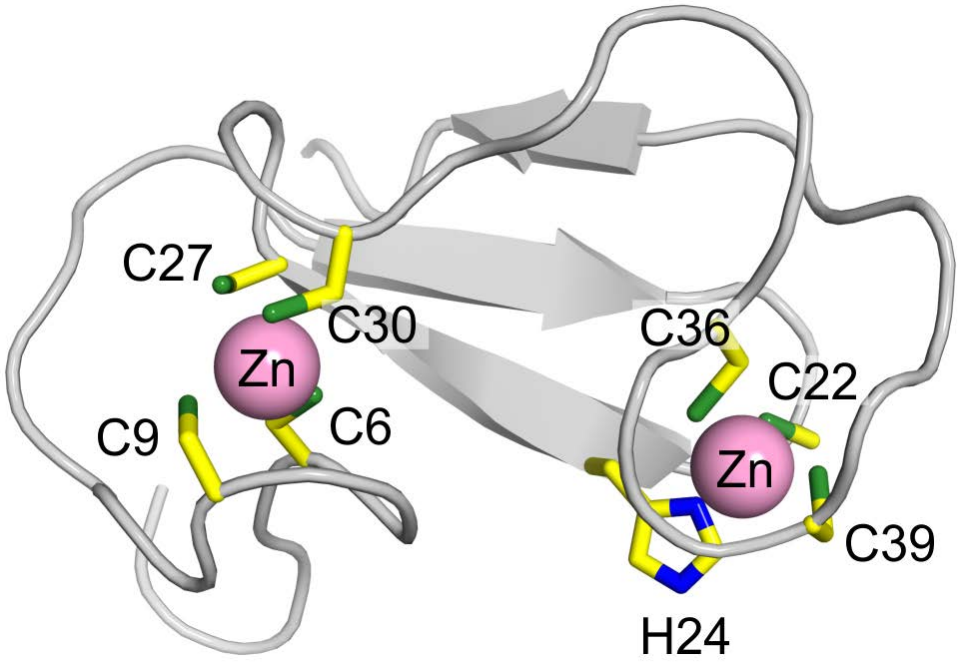


Figure S1. (A) Clustal alignment of mouse RNF212B and RNF212. The cysteine residues involved int the catalytic domain are coloured in yellow and the histidine in green. **(B)** Ribbon representation of the structure of the Zinc Finger domain of 212B predicted by AlphaFold (AlphaFold Protein Structure Database entry D3Z423). The side chains of the residues that form the Zn-binding sites are shown as sticks. The Zn2+ were model by local superimposition of the residues that coordinate the Zn atoms in the crystal structure of the RING domain of Trim69 (PDB 6YXE) (PubMed: 33021497). The figure was created with Pymol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

Figure S2

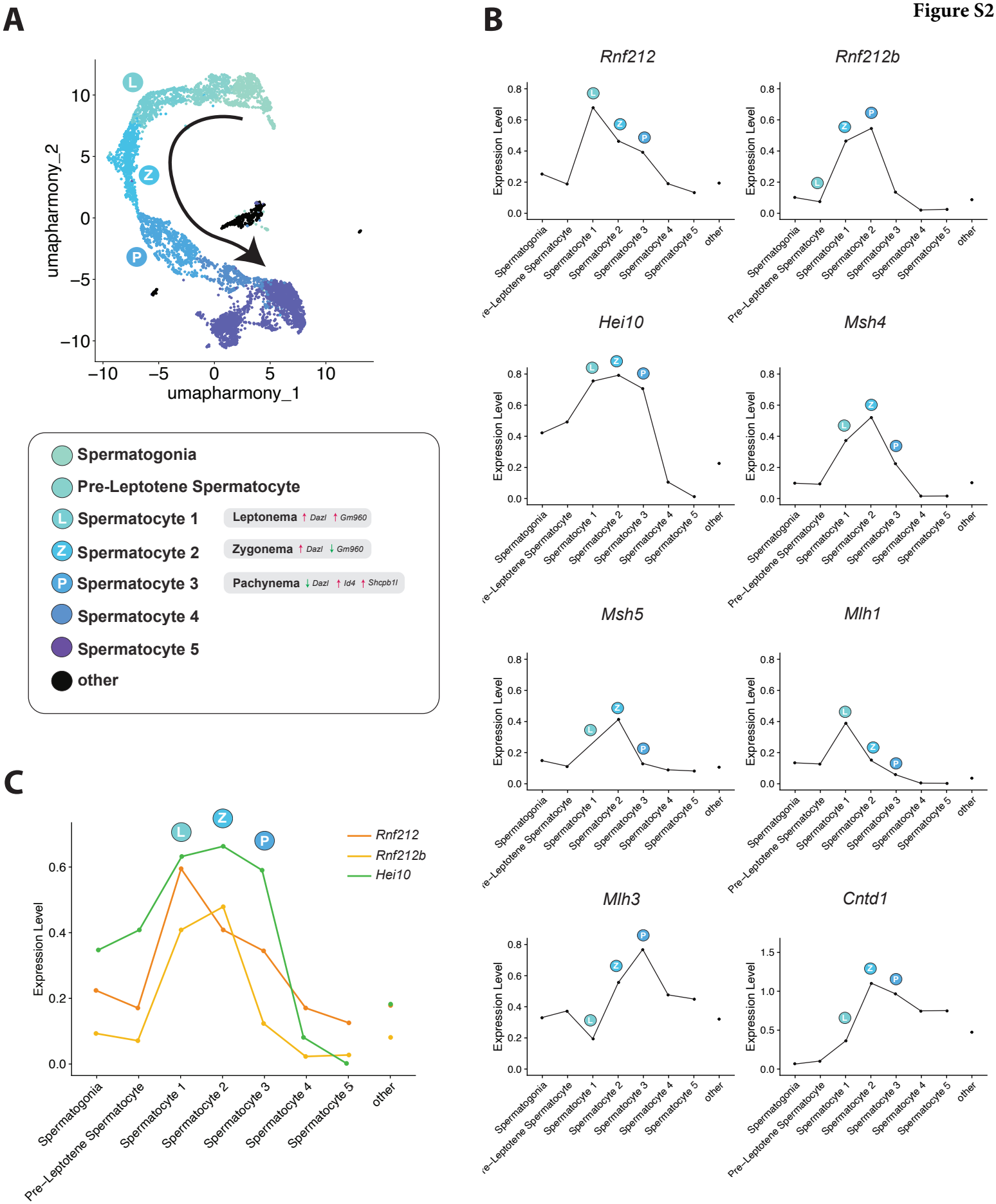

A
B
C
umapharmony_1
umapharmony_2
Spermatogonia
Pre-Leptotene Spermatocyte
Spermatocyte 1
Spermatocyte 2
Spermatocyte 3
Spermatocyte 4
Spermatocyte 5
other
Leptonema ↑ Dazl ↑ Gm960
Zygonema ↑ Dazl ↓ Gm960
Pachynema ↓ Dazl ↑ Id4 ↑ Shcpb1l
Rnf212
Rnf212b
Hei10
Msh4
Msh5
Mlh1
Mlh3
Cntd1
Expression Level

Figure S2. (A) Single cell RNA sequencing was performed on three 10-week-old male DBA/2J mice with analysis restricted to spermatogonia (n = 1030) and spermatocytes (n = 4512), resulting in 8 distinct clusters shown via unified manifold approximation and projection (UMAP) visualization. Gene markers from Ascenção et al., (2024) were used to define each cluster. Cell type annotations follow expected pseudotime trajectory prediction (arrow). **(B)** Average expression level of key crossover genes throughout all 8 annotated clusters. Expression was determined by pseudo-bulking cells from each library to ascertain an average count value. **(C)** Rnf212 (orange), Rnf212b (yellow), and Hei10 (green) were comparatively plotted to highlight differential developmental timings throughout meiotic prophase I (Leptonema = L, Zygonema = Z, Pachynema = P).

Ascenção, C., Sims, J. R., Dziubek, A., Comstock, W., Fogarty, E. A., Badar, J., Freire, R., Grimson, A., Weiss, R. S., Cohen, P. E., & Smolka, M. B. (2024). A TOPBP1 allele causing male infertility uncouples XY silencing dynamics from sex body formation. ELife, 12, RP90887. https://doi.org/10.7554/eLife.90887

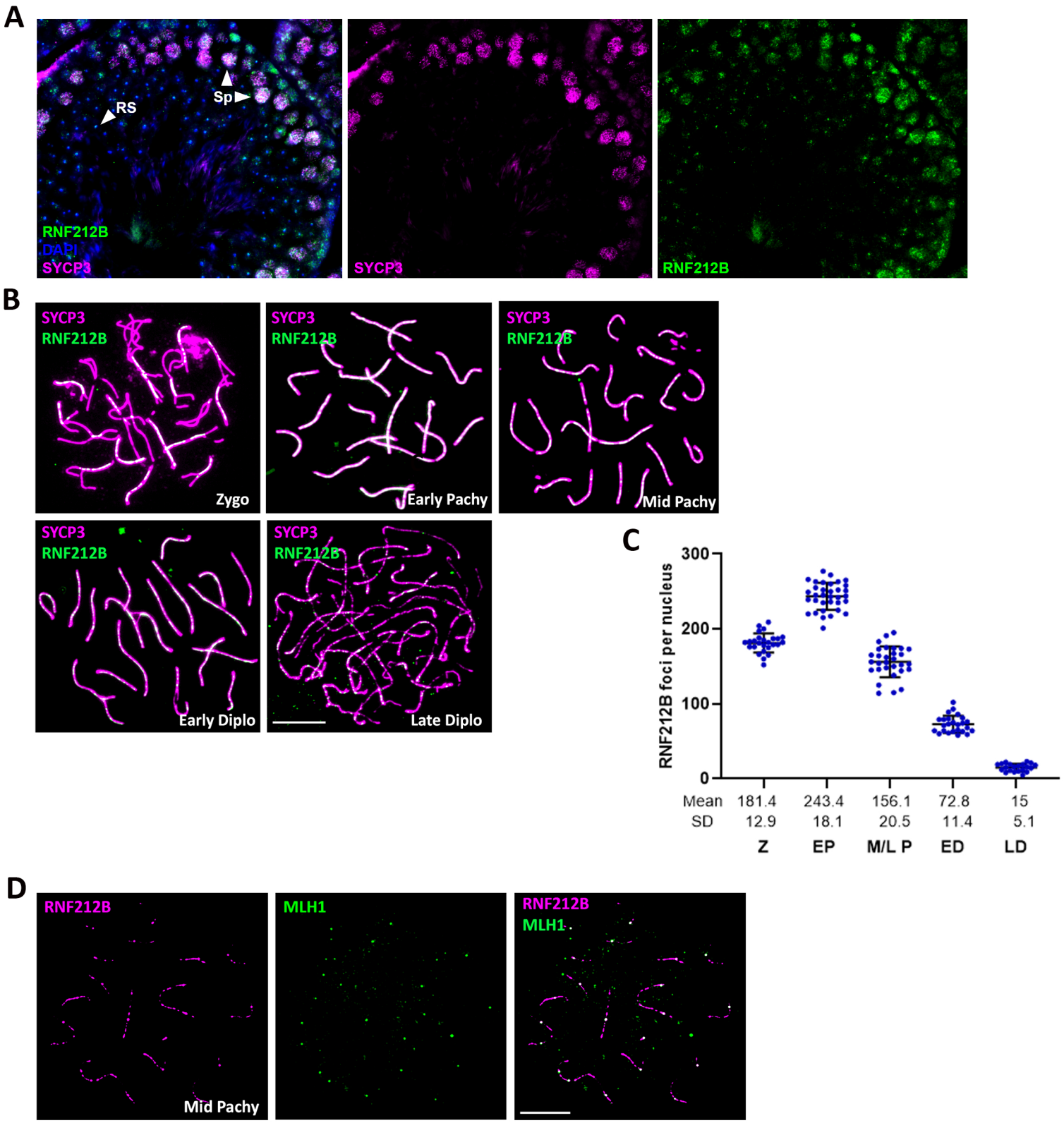


Figure S3. Dynamics of RNF212B foci and colocalization with MLH1 in oocytes. (A) Co-labeling of RNF212B (green), SYCP3(magenta), and DAPI (blue) on formalin-fixed testis sections of adult C57BL/6J mice. RS-round spermatid cells and Sp- spermatocytes. Spermatocytes were characterized as SYCP3 positive cells. **(B)** Double Immunolabelling of RNF212B and SYCP3 from zygotene to diplotene oocytes. RNF212B unloading from the axes is delayed in comparison with spermatocytes, with RNF212B foci persisting at the remaining synapsed LEs in early and late diplotene spermatocytes. **(C)** Plot quantitation of RNF212B foci in oocytes showing higher number than in spermatocytes (see Figure 1C). **(D)**. MLH1 colocalizes with the brighter foci of RNF212B in mid-pachytene oocytes. Bar in panels 10 µm. All experiments have been carried out in at least 2 mice and 15 cells per mouse.

Figure S4

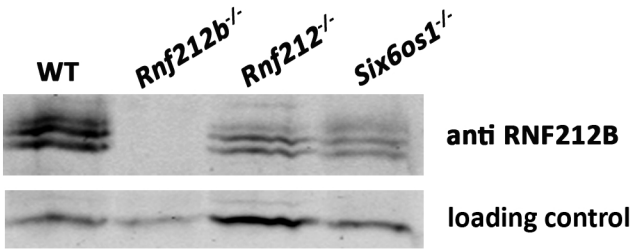
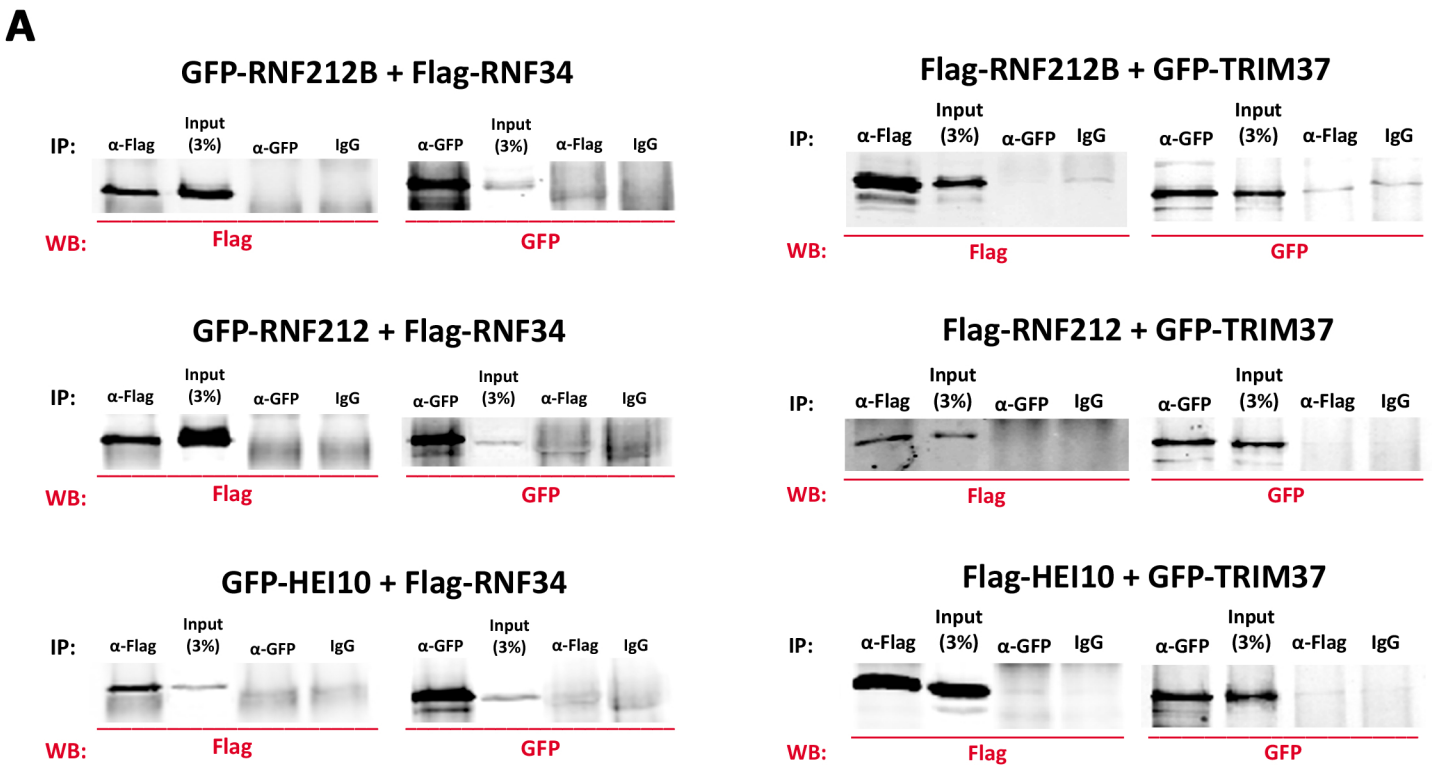


Figure S4. Western blot analysis of RNF212B expression in WT, Rnf212b-/- , Rnf212-/- and Six6os1-/- testis tissue extracts.

Figure S5



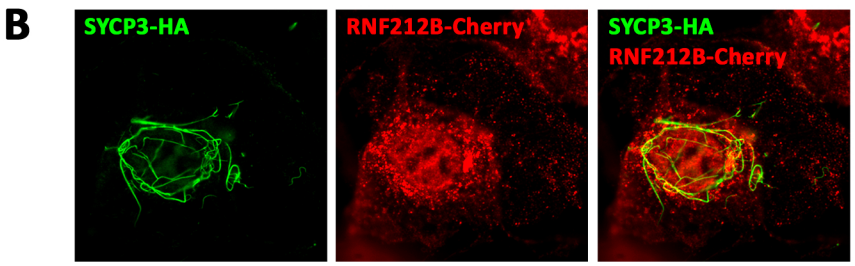


Figure S5. **(A)** Co- immunoprecipitation of RNF212B, RNF212 and HEI10 with RNF34 and TRIM37. HEK293T cells were co-transfected with plasmids encoding RNF212, RNF212B, HEI10, RNF34 and TRIM37 tagged with FLAG or GFP. Protein complexes were immunoprecipitated overnight with an antibody anti-FLAG, anti-GFP or IgGs (as a negative control) and analyzed by immunoblotting. Negative interaction of RNF212B RNF212 and HEI10 with RNF34 and TRIM37 is shown. **(B)** COS7 cells immunofluorescence shows no co-localization of transfected RNF212B with SYCP3. COS7 cells were transfected to express RNF212B (fused to Cherry) along with SYCP3 (fused with HA). Scale bars: 10 µm.

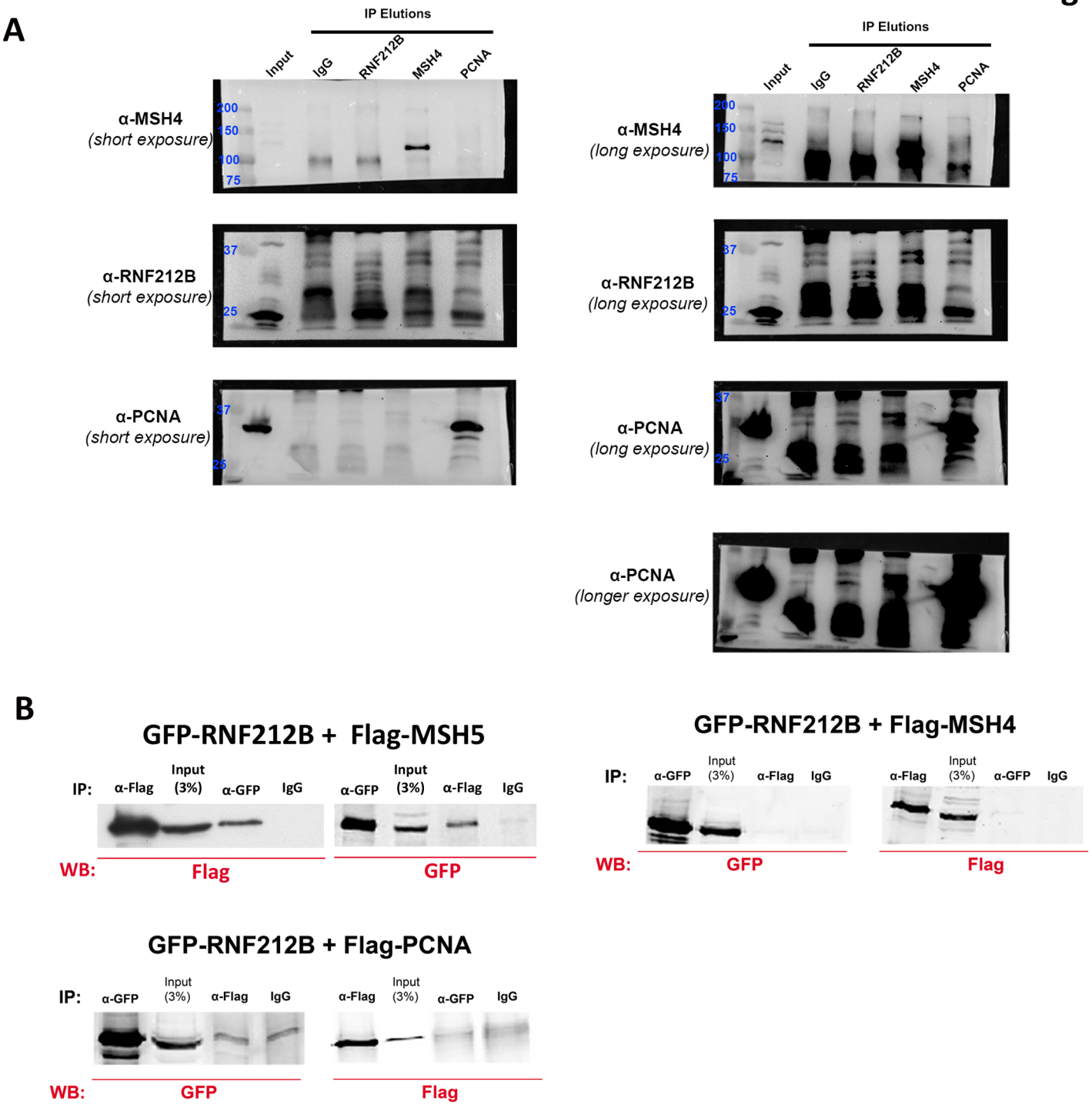


Figure S6. (A) Entire blots corresponding to Figure 3D. **(B)** Co- immunoprecipitation of RNF212B, with MSH5, MSH4 and PCNA. HEK293T cells were co-transfected with plasmids encoding RNF212B, MSH5, MSH4 and PCNA tagged with FLAG or GFP. Protein complexes were immunoprecipitated overnight with an antibody anti-FLAG, anti-GFP or IgGs (as a negative control) and analyzed by immunoblotting. Positive interaction of RNF212B with MSH5 (left upper panel) and negative interaction of RNF212B with MSH4 (right upper panel) and with PCNA (left lower panel) are shown.

Figure S7

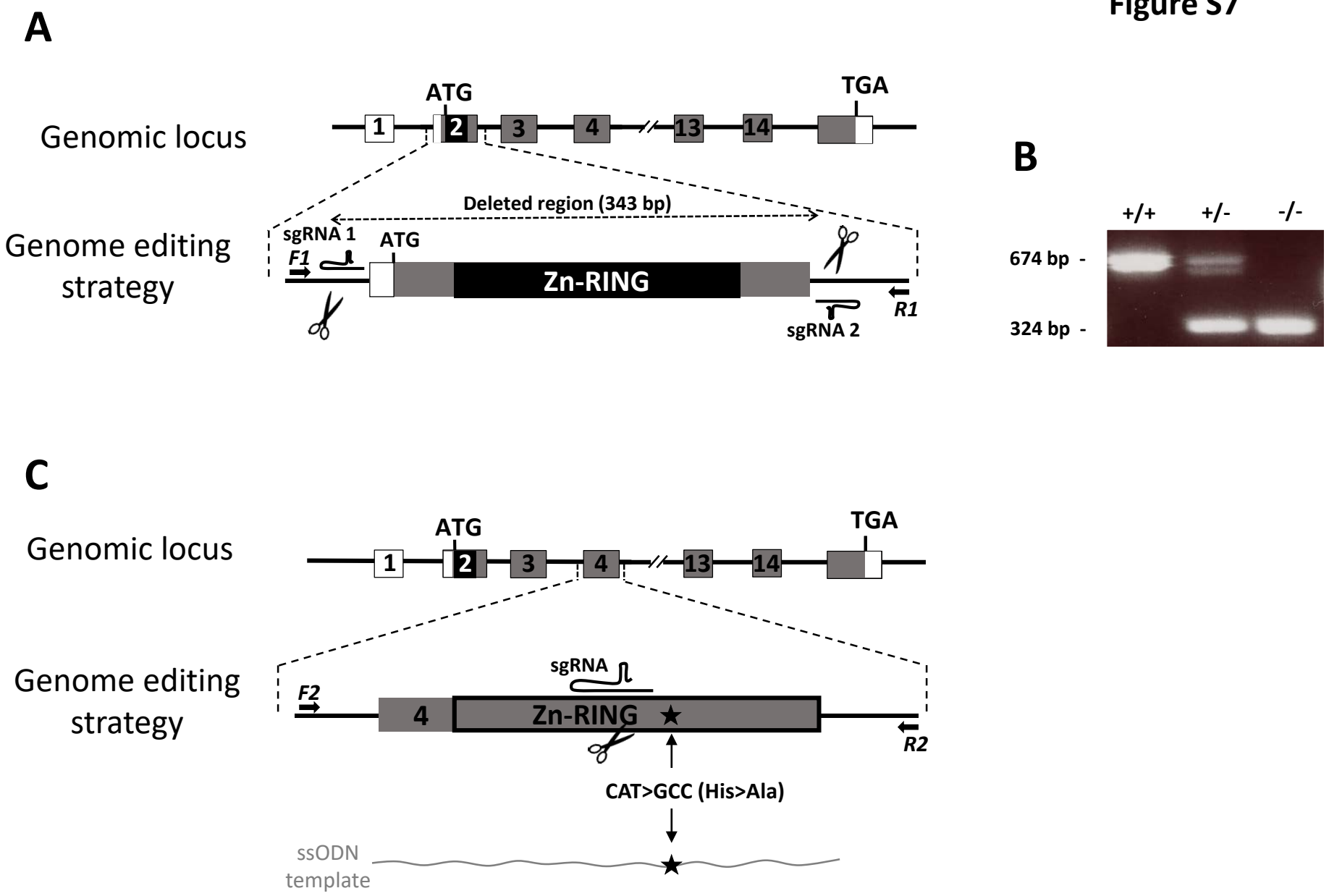
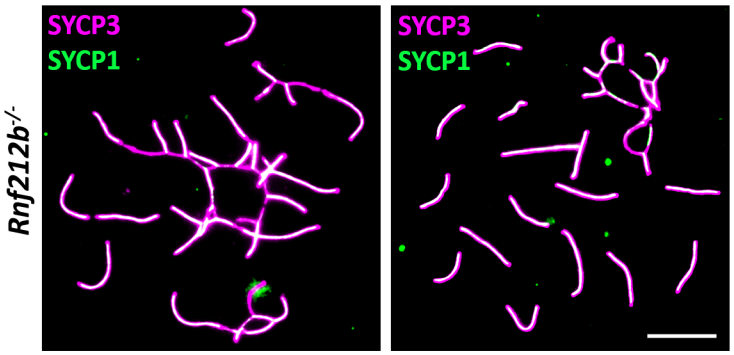

A
Genomic locus
ATG
TGA
1
2
3
4
13
14
Deleted region (343 bp)
Genome editing strategy
sgRNA 1
F1
ATG
Zn-RING
R1
sgRNA 2
B
+/+
+/-
-/-
674 bp
324 bp
C
Genomic locus
ATG
TGA
1
2
3
4
13
14
Genome editing strategy
sgRNA
F2
4
Zn-RING
R2
CAT>GCC (His>Ala)
ssODN template

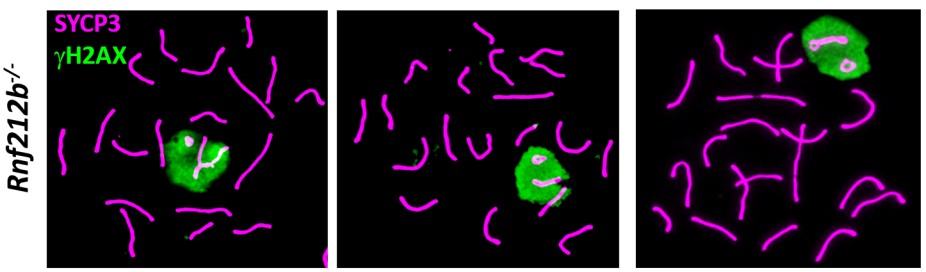
Fig. S7. Genome editing of the mouse *Rnf212b* gene. **(A)** Generation of a null mutant allele of *Rnf212b*. Diagrammatic representation of the mouse *Rnf212b* locus (WT) and the CRISPR/Cas9 genome editing strategy performed, showing two sgRNA flanking exon 4. The edited allele leads to a deletion of the exon 2 encoding the first ATG and the first residues of the Zn-finger domain. Coding (grey/black boxes) and non-coding (white boxes) regions are indicated. ATG, start codon; TGA, stop codon (*). Oligonucleotides employed for the genotyping are indicated (F1 and R1). **(B)** PCR amplification of tail genomic DNA from the three genotypes indicated using primers F1 and R1. **(C)** Generation of the H24A mutant allele of *Rnf212b*. Diagrammatic representation of the mouse *Rnf212b* locus (WT) and the CRISPR/Cas9 genome editing strategy performed, showing the sgRNA located in exon 4 and the ssODN used as template. The edited allele leads to an aminoacidic change (His>Ala) on the Zn-RING of exon 4. Coding (grey boxes) and non-coding (white boxes) regions are indicated. The star symbol represents the His>Ala mutation on exon 4. ATG, start codon; TGA, stop codon (*). Oligonucleotide sequences employed for the amplification of exon 4 are also indicated (F2 and R2).

A



	WT	*Rnf212b*$^{-/-}$	*Rnf212*$^{-/-}$
% Synapsis defects	7.2	25.7	23.9
% Normal	92.8	74.3	76.1

B



	WT	*Rnf212b*$^{-/-}$	*Rnf212*$^{-/-}$	DKO
% XY defects and sex body extended	0	32.2	30.4	33.3

C

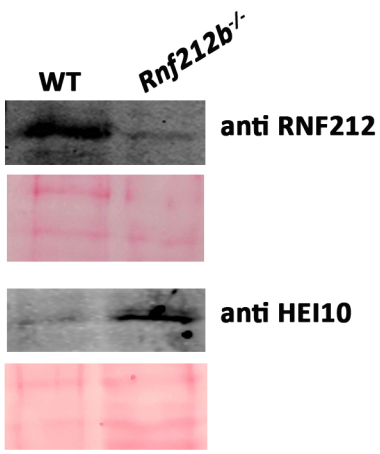


Figure S8. Characterization of *Rnf212b*-deficient mice. (A)Double immunolabeling of SYCP3 and SYCP1 in pachynema oocytes from *Rnf212b-/-* oocytes showing synapsis defects. The lower table show comparative quantitation among the indicated genotypes. **(B)** Double immunolabelling of γH2AX and SYCP3 in *Rnf212b*$^{-/-}$ pachynemas with XY-structural abnormalities showing extended γH2AX signal beyond the Sex Body into autosomal domain (left and center panels) and restricted γH2AX signal at the Sex Body (right panel). The lower table show comparative quantitation of this phenotype among the indicated genotypes. **(C)** Western blot analysis of RNF212 and HEI10 expression in WT and *Rnf212b-/-* testis tissue extracts.

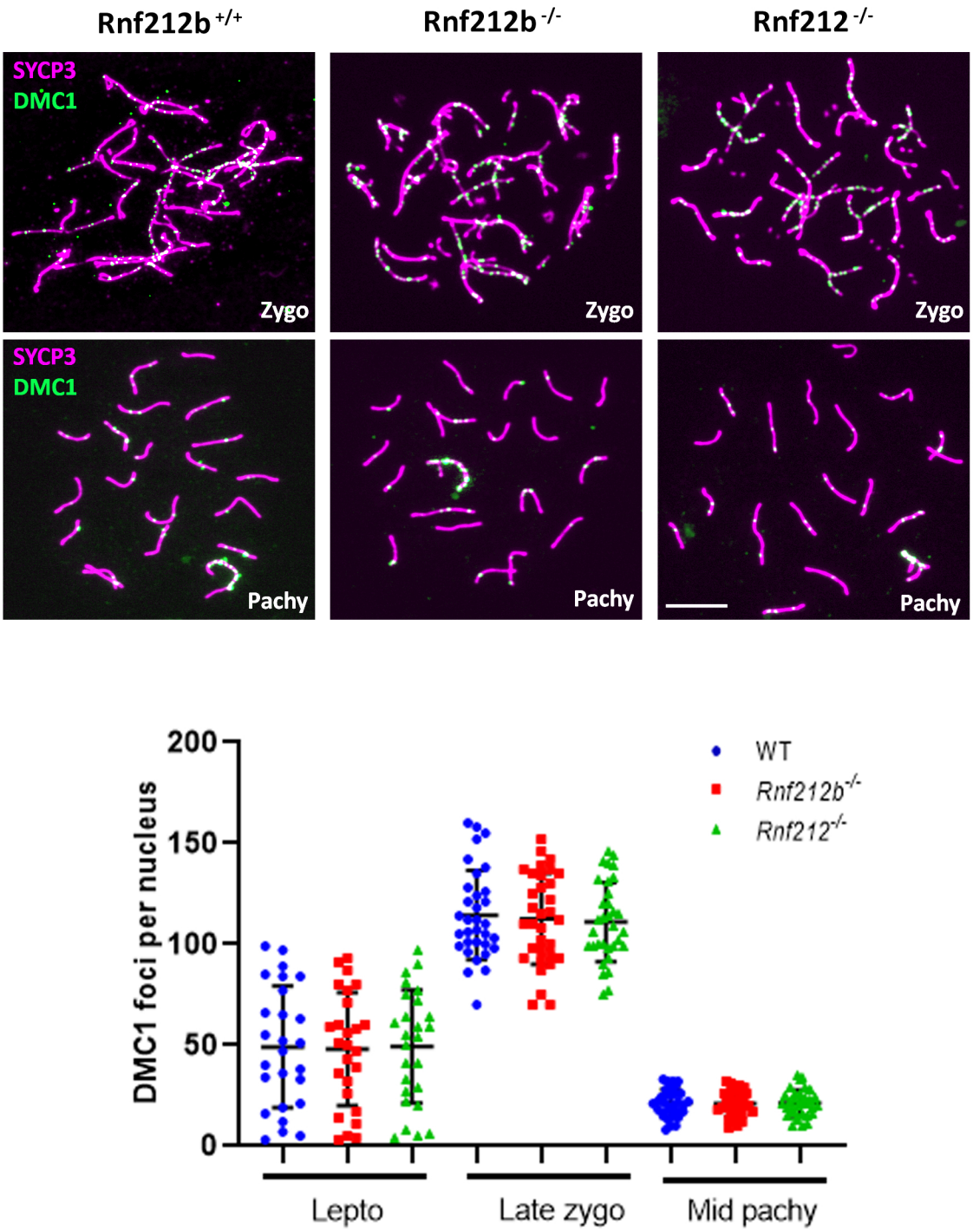


Figure S9. DMC1 recombinase in *Rnf212b*$^{-/-}$, *Rnf212*$^{-/-}$ and wild type spermatocytes. Double immunolabeling of DMC1 and SYCP3 in zygonema and pachynema spermatocytes from the indicated genotypes. Plots on the lower part of the panel represent the quantification of foci for each genotype and stage. Two-tailed Welch's t-test analysis: p= 0.1, no significant differences. Bar in all panels, 10 μm.

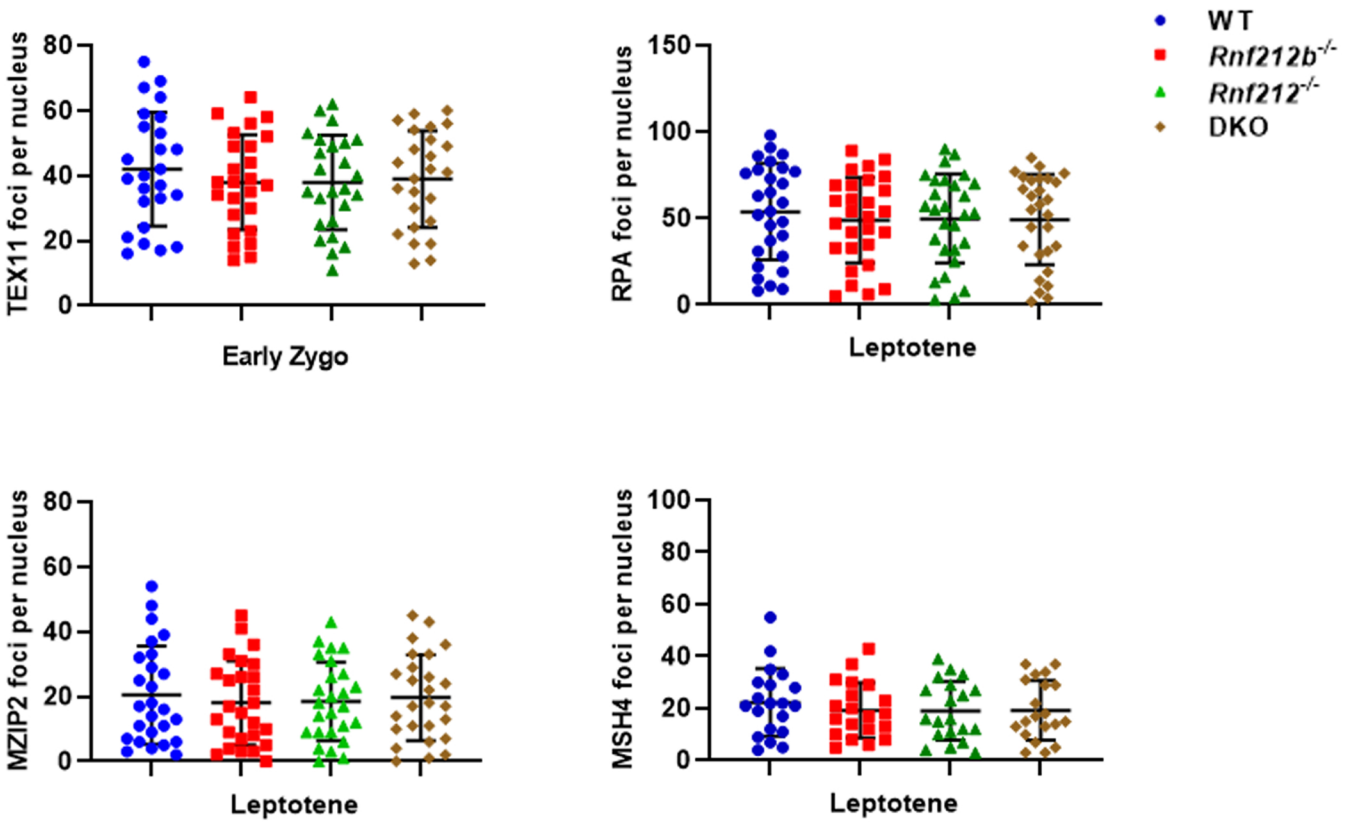


Figure S10. Initial formation of TEX11, MZIP2, MSH4 and RPA foci in wild type, Rnf212b$^{-/-}$, Rnf212$^{-/-}$ and Rnf212b$^{-/-}$ Rnf212$^{-/-}$ (DKO) spermatocytes. Plots represent the quantification of foci for each genotype and stage. Two-tailed Welch's t-test analysis: p= 0.1, no significant differences. All experiments in have been carried out in at least 2 mice and 15 cells per mouse.

Figure S11

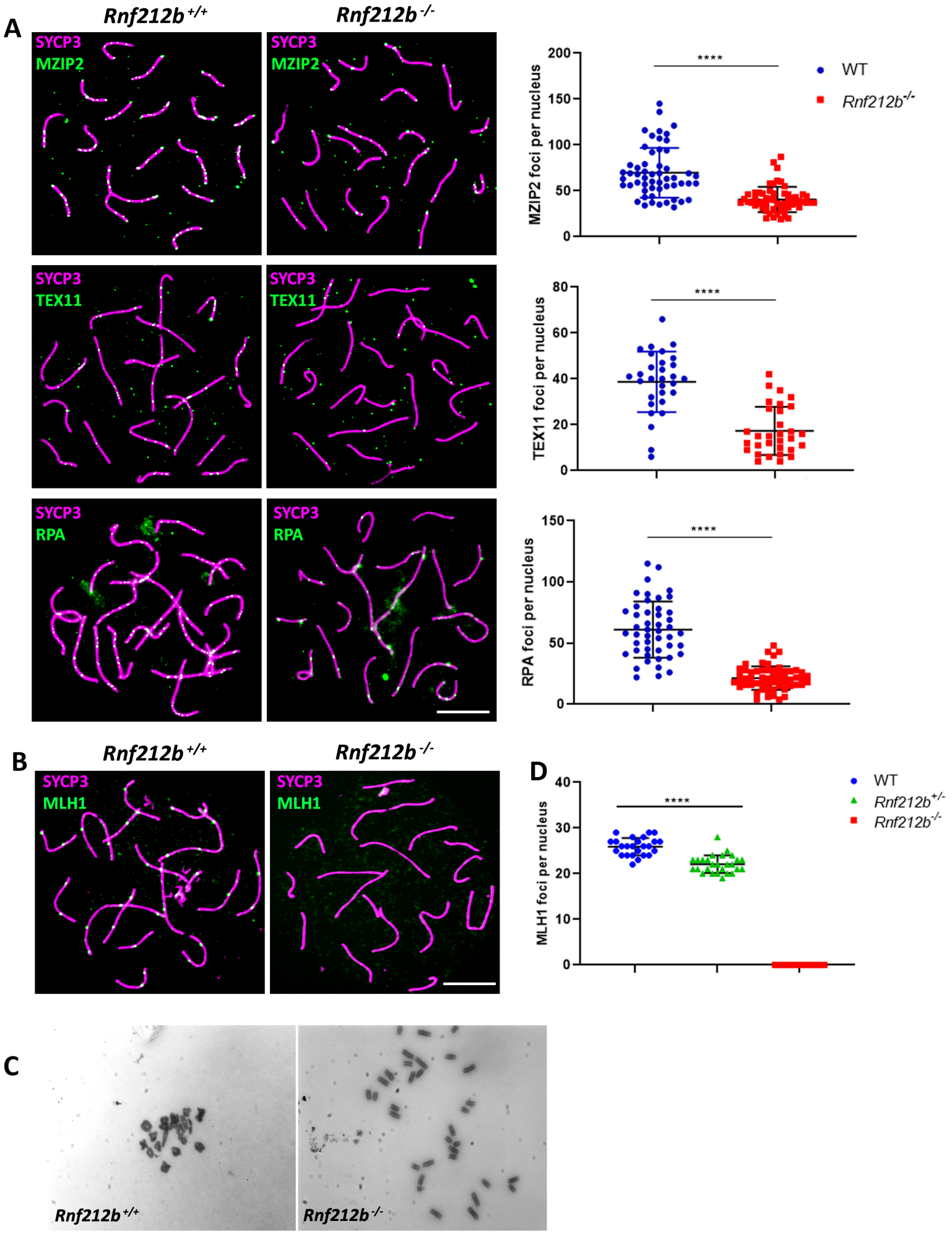
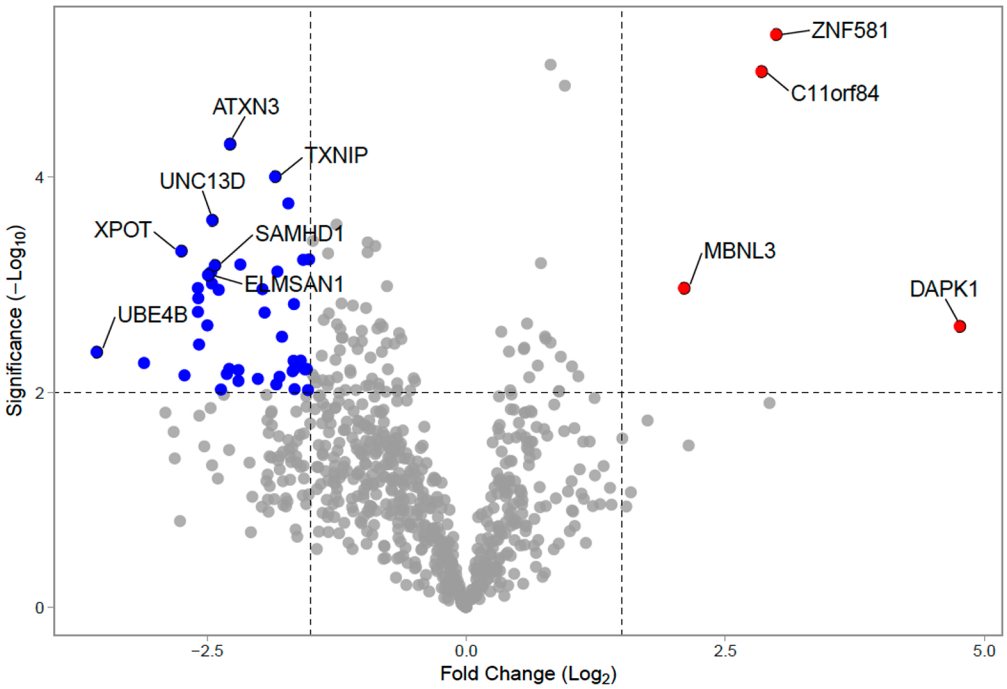

A
Rnf212b +/+
Rnf212b -/-
SYCP3
MZIP2
SYCP3
TEX11
SYCP3
RPA
MZIP2 foci per nucleus
TEX11 foci per nucleus
RPA foci per nucleus
****
WT
Rnf212b -/-
B
Rnf212b +/+
Rnf212b -/-
SYCP3
MLH1
D
MLH1 foci per nucleus
WT
Rnf212b +/-
Rnf212b -/-
C
Rnf212b +/+
Rnf212b -/-

Fig. S11. Localization of proCO factors and Metaphase I cells from *Rnf212b*-deficient oocytes. **(A)** Double immunolabeling of MZIP2, TEX11, RPA with SYCP3 in pachynema oocytes. Number of foci were significantly reduced in *Rnf212b*$^{-/-}$ in comparison with wild type oocyte pachynemas. Plots in the right of the panels represent the quantitation of the values. Two-tailed Welch's t-test analysis: ****p<0.0001. **(B)** Double immunolabeling of MLH1 and SYCP3 in oocyte pachynemas showing the absence on MLH1 foci in *Rnf212b*$^{-/-}$ females in comparison with the presence of at least one MLH1 foci per chromosome in *Rnf212b*$^{+/+}$. (D) Plot representation of the quantitation of MLH1 foci in wild-type (WT), *Rnf212b*$^{+/-}$ and *Rnf212b*$^{-/-}$ pachynemas showing dosage dependent influence. **(C)** Metahnol/acetic metaphase I chromosomes from *Rnf212b*$^{-/-}$ oocytes show most chromosomes as univalents in comparison with all bivalents in the wild-type control oocytes.

A



B

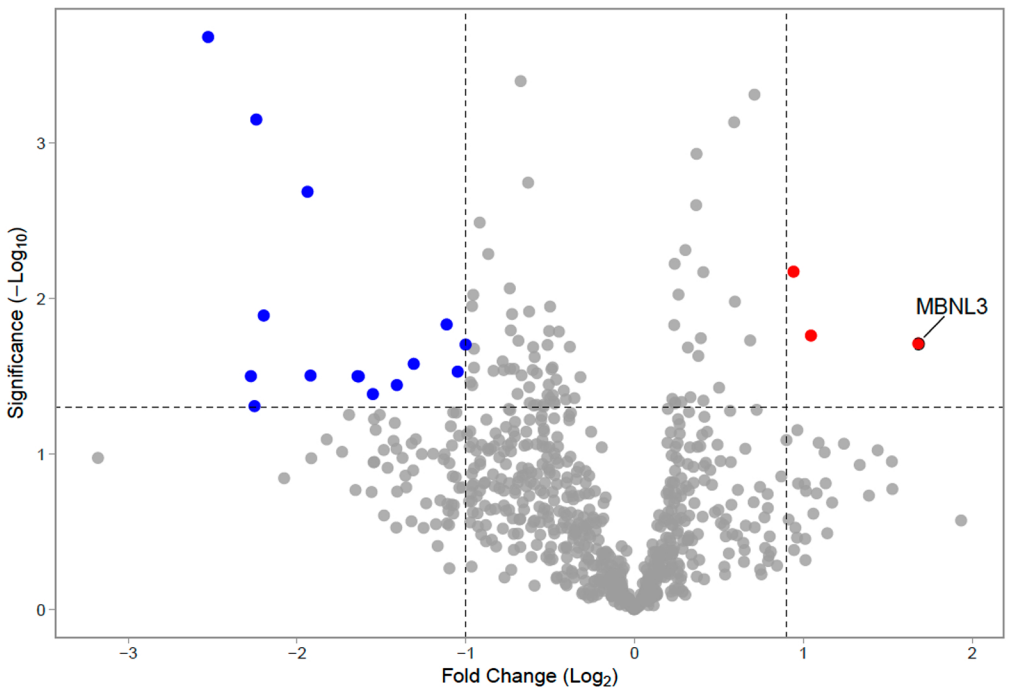
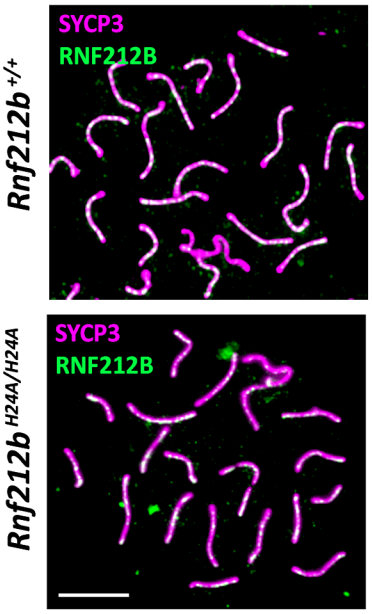


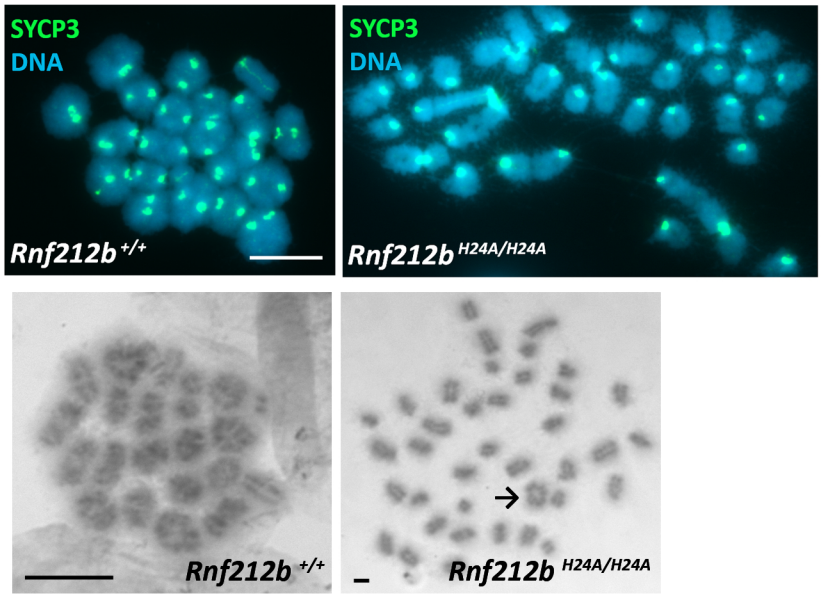
Figure S12. Volcano plot depicting statistical differences in the His-Ubiquitin proteome comparing **(A)** RNF212B-transfected vs empty vector transfected or **(B)** RNF212-transfected vs RNF212B mutant-transfected. Each dot represents a protein.

Figure S13

A



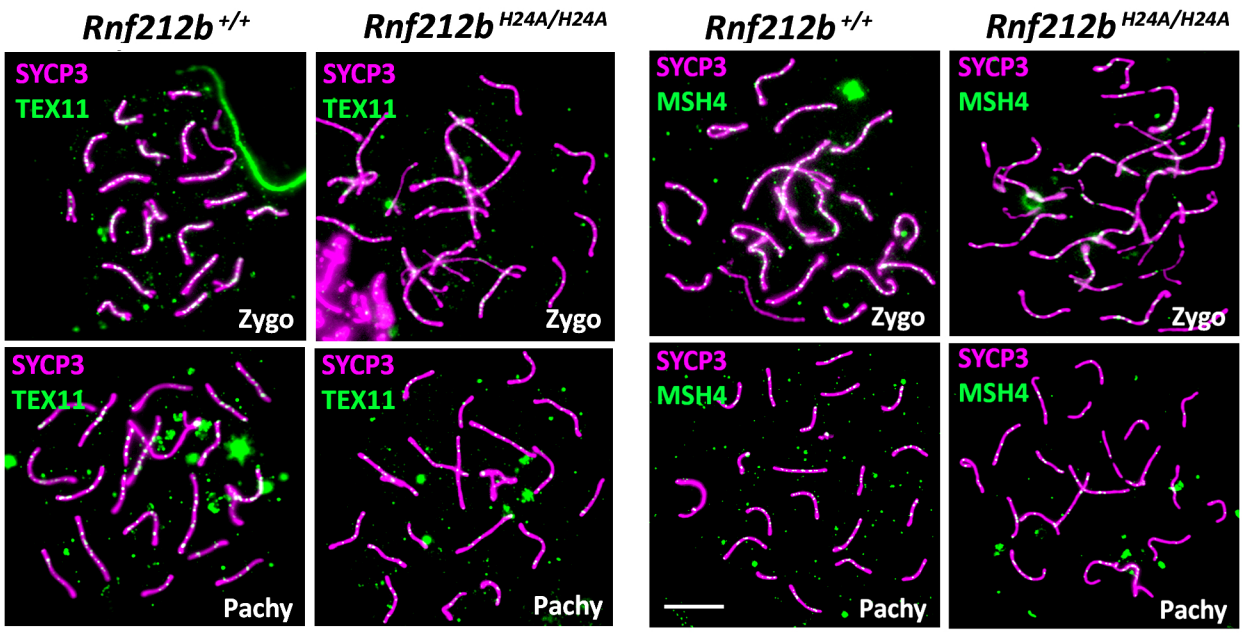
C

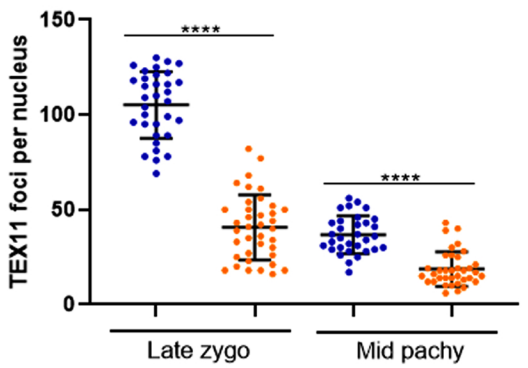


B

	WT	Rnf212b$^{H24A/H24A}$
% X-autosomes fusions	0	8.7
% X Y ring chromosomes	0	11.2
% XY autosynapsis	0	14.9
% Unpaired and X or Y autosynapsis	0	9.9
% Unpaired only	5.3	1.8
% Normal pachytenes	94.7	53.5

D





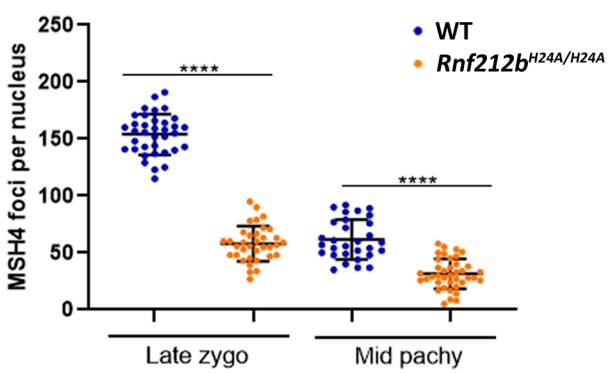


Figure S13. *Rnf2b*$^{H24A/H24A}$ mutants phenocopy Rnf212b$^{-/-}$ mutant mice.
(A) Double immunolabeling of RNF212B and SYCP3 showing foci onto the synapsed LEs of *Rnf2b*$^{H24A/H24A}$ early pachynema similar to the wild type control. **(B)** Numerical quantitation of aberrant configurations in the XY bivalent in Rnf2b$^{H24A/H24A}$ and wild-type spermatocytes (WT). **(C)** Metaphase I cells from chromosome spreads stained with SYCP3 and DAPI (upper panel) and from Methanol/Acetic chromosome preparations stained with Giemsa (lower panel) show the presence of univalents (*Rnf2b*$^{H24A/H24A}$) and a few bivalents in comparison with all bivalents (wild type). **(D)** Immunolocalization of proCO factors TEX11, and MSH4 are decreased in *Rnf2b*$^{H24A/H24A}$spermatocytes at late zygonema and mid-pachynema. Quantitation of the results are shown in plots (right panel). Welch's t-test analysis: ****$p<0.0001$. Bar in all panels, 10 μm.

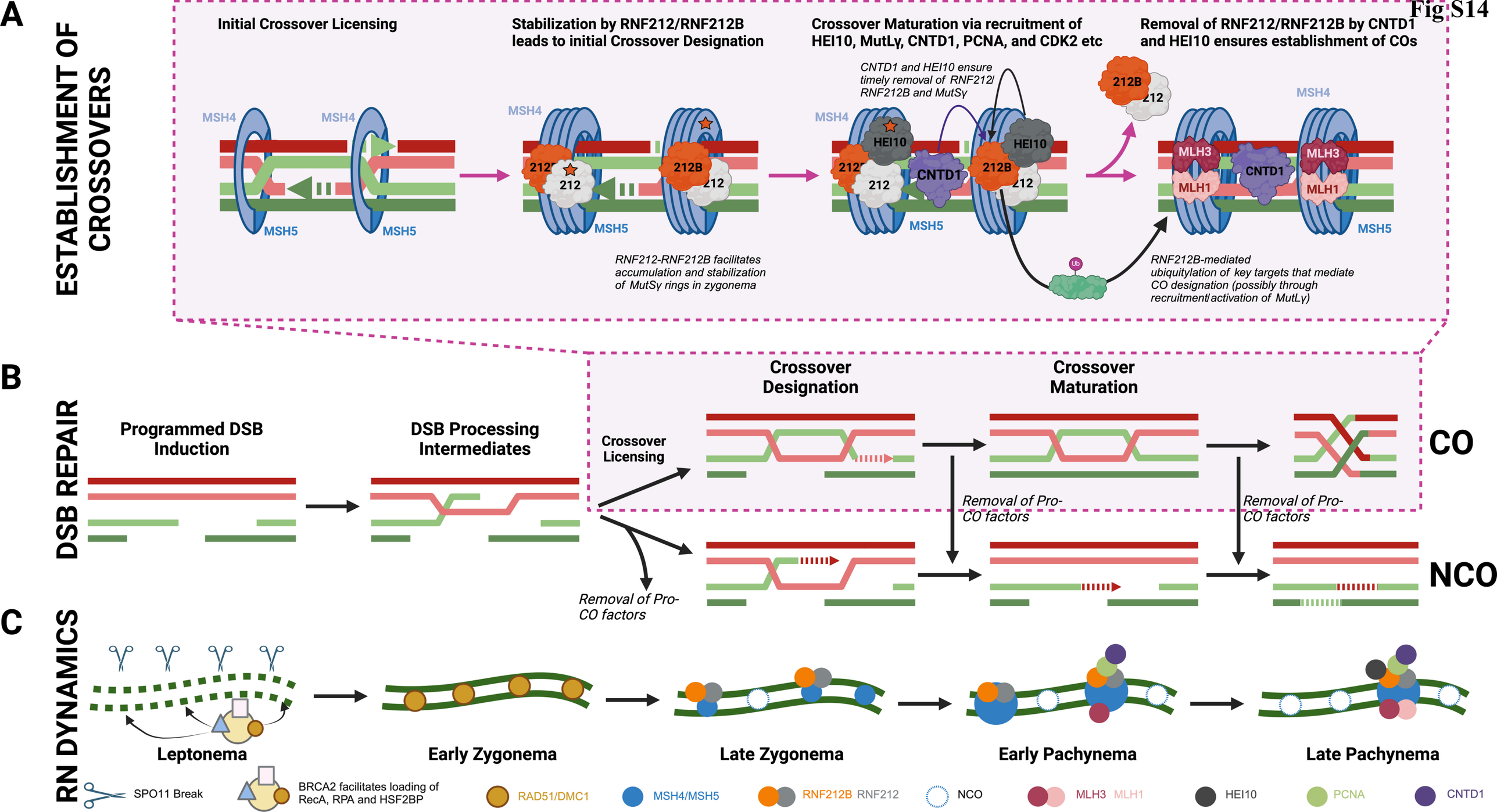

A
ESTABLISHMENT OF CROSSOVERS
Initial Crossover Licensing
Stabilization by RNF212/RNF212B leads to initial Crossover Designation
Crossover Maturation via recruitment of HEI10, MutLγ, CNTD1, PCNA, and CDK2 etc
Removal of RNF212/RNF212B by CNTD1 and HEI10 ensures establishment of COs
MSH4
MSH5
212
212B
HEI10
CNTD1
MLH3
MLH1
Ub
CNTD1 and HEI10 ensure timely removal of RNF212/ RNF212B and MutSγ
RNF212-RNF212B facilitates accumulation and stabilization of MutSγ rings in zygonema
RNF212B-mediated ubiquitylation of key targets that mediate CO designation (possibly through recruitment/activation of MutLγ)
B
DSB REPAIR
Programmed DSB Induction
DSB Processing Intermediates
Crossover Designation
Crossover Maturation
Crossover Licensing
CO
NCO
Removal of Pro-CO factors
Removal of Pro-CO factors
Removal of Pro-CO factors
C
RN DYNAMICS
Leptonema
Early Zygonema
Late Zygonema
Early Pachynema
Late Pachynema
SPO11 Break
BRCA2 facilitates loading of RecA, RPA and HSF2BP
RAD51/DMC1
MSH4/MSH5
RNF212B RNF212
NCO
MLH3 MLH1
HEI10
PCNA
CNTD1

Figure S14. A model to explain the involvement of RNF212B (and RNF212) in driving crossover decisions in mammalian meiosis. The middle panel ("**B. DSB REPAIR**") shows a cartoon of DSB repair progression and is roughly aligned with the corresponding sub-stages of prophase I in the bottom panel ("**C. RN DYNAMICS**"), the staging for which is defined by the status of the synaptonemal complex. The top panel ("**A. ESTABLISHMENT OF CROSSOVERS**") shows the molecular players involved in the processes of Crossover licensing, Designation, and Maturation, but focused on the roles and the genetic/biochemical interactions of RNF212 and RNF212B. In mouse, CO decision making is a dynamic process that begins in zygonema with the loading of the MutSγ complex (MSH4/MSH5) at only a subset of RecA-processed DSB sites (depicted by RAD51/DMC1 loading). Accumulation of MutSγ clamps is facilitated by RNF212/RNF212B, achieving a minimum threshold for CO designation and further maturation in early zygonema, coincident with the successive loading of first CNTD1 and then HEI10. RNF212, RNF212B, and HEI10 function in a co-ordinated fashion to regulate accumulation, stability, and turnover of CO mediators (such as MutLγ) and Pro-CO factors (such as CDK2, not shown, and others). Some or all of these may be SUMO or Ub targets of these RNF proteins. One key target of RNF212B action, for example, could be PCNA, whose action in somatic cells, and localization in meiotic prophase I, could suggest an important action in activating the MutLγ complex. However, other possible targets of RNF212B could also mediate these processes that result in CO designation. Importantly, at each stage of this process, the deselection and/or failure to load appropriate complexes (including RNF212 and RNF212B) results in the switch towards an NCO fate, as depicted in the "RN DYNAMICS" panel, resulting in a fixed and highly regulated frequency and distribution of COs across the genome. Note: only key protein players are included in this model for simplicity; orange stars indicate RNF212/RNF212B interactors identified in the current work. Figure created with the assistance of biorender.com.

Supporting material and methods

Single Cell RNA-sequencing

Three 10-week-old DBA/2J male mice from Jackson Laboratories (#000671; Bar Harbor, ME) were used as a pooled sample to generate a single cell suspension of testis cells as previously described (1) The resulting cell suspension was then split into thirds and used for various enrichment techniques: FACS (to enrich for spermatocytes), MACS (to enrich for spermatogonia), and unenriched (which will have mostly post-meiotic cells). One third served as our unenriched library and was submitted immediately to the Cornell DNA Sequencing Core Facility for further processing. Another third was subjected to magnetic-activated cell sorting (MACS) using the CD117 MicroBeads (Miltenyi Biotech #130-097-146) to enrich for cKIT+ spermatogonia following the manufacturers protocol. Sorted cells were re-suspended in 1 mL 0.04% BSA in 1X dPBS then submitted to the Cornell DNA Sequencing Core Facility for further processing. The final third was subjected to fluorescent activated cell sorting (FACS) to enrich for pachytene spermatocytes, as described previously (2). We sorted on a Sony MA900 FACs machine, tuned to emit at 488 nm with a 100µM nozzle. Sorted cells were submitted to the Cornell DNA Sequencing Core Facility for further processing. FACS was performed at the Cornell Flow Cytometry Facility.

Cells were processed on the 10X Genomics Chromium System aiming to capture 5000-7000 cells per sample as described previously (1)using the 10X Chromium Single Cell 3' RNA-seq v3 kit (10X genomics, PN-1000075; Pleasanton, CA) following the manufacturer's protocol. The sequencing data was aligned to the mouse reference genome (mm10) using the cellranger (v3.1.0) pipeline to generate count matrices (10x Genomics).

Preprocessing of the three individual samples follows the general workflow outlined by (3). The entire analysis was completed using R (v4.2.1). Briefly, the default SoupX (v1.4.5) workflow was used to remove ambient RNA signal. Individual samples were then preprocessed using standard Seurat (v4.4.0) workflow. Low quality cells with fewer than 500 features, fewer than 1000 transcripts, or more than 30% of unique transcripts derived from mitochondrial genes were removed. Next, we used DoubletFinder (v2.0) to identify and remove putative doublets in each dataset, individually. Estimated doublet rates were computed by fitting the total number of cells after quality filtering to a linear regression of the expected doublet rate published in the 10x Chromium handbook. Estimated homotypic doublet rates were also computed using the modelHomotyic function. Following pre-processing and quality filtering, datasets were merged and batch-correction was performed using Harmony (github.com/immunogenomics/harmony) (v1.0).

Generation of CRISPR/Cas9-Edited Mice.

For developing the *Rnf212b*$^{-/-}$ loss of function mice, sgRNA1 5'-GTAACACTGCTTACCGTTTAC-3' and sgRNA2 5'-GATAATACGGATTCTGTATGT-3' targeting the exon 2 were predicted at

https://eu.idtdna.com/site/order/designtool/index/CRISPR_SEQUENCE. For developing the *Rnf212b*$^{dead/dead}$ model, sgRNA1 5'- CATTTCTTTGTCACCAGCTG -3' on exon 4 was predicted at https://eu.idtdna.com/site/order/designtool/index/CRISPR_SEQUENCE. The designed ssODN (ctacattattcttattctttttctctatctggtccagAATGGATTGGTTTCATTGCAACCAGTGTTTCC GAAAAGATGGGGCCCATTTCTTTGTCACCtcCTGTGGCgccATTTTCTGTAAAA AGTGTATGACTTTGGgtgagtggcttcttttcagatttatcccaaaatgtttaacttagatataatgagtagccatt) contains the mutation (CAT>GCC, that results in the change of His>Ala). The crRNAs, the tracrRNAs and the ssODNs were produced by chemical synthesis by IDT. The crRNA and tracrRNA were annealed to obtain the mature sgRNA. A mixture containing the sgRNAs (20 ng/µl of each annealed sgRNA), 30 ng/µl of recombinant Cas9 protein (IDT) and 10 ng/µl of the ssODN were microinjected into B6/CBA F2 zygotes (hybrids between strains C57BL/6J and CBA/J) at the Transgenic Facility of the University of Salamanca. Edited founders were identified by PCR amplification (Taq polymerase, NZYtech) with primers flanking the edited region. For the *Rnf212b*$^{-/-}$ model: primer F1 5'- GCGAGTTACAGTAAAGGCAAACA -3'; and primer R1 5'- CAGTATGACATTTCCCGACACAA -3'. And for the *Rnf212b*$^{H24A/H24A}$ model: primer F2 5'- CCCACACCCAGTAAACGGTAA -3'; and primer R2 5'- TGCTAAATCAGTTAGTGCCATTCT -3'. PCR products were directly sequenced or subcloned into pBlueScript (Stratagene) followed by Sanger sequencing for a detailed verification of the editing. Selected *Rnf212b* founders, carrying the desired alleles, were crossed with wild-type C57BL/6J mice to eliminate possible unwanted off-targets. Heterozygous *Rnf212b*$^{+/-}$ mice were re-sequenced and crossed to generate the edited homozygotes *Rnf212b*$^{-/-}$. Genotyping was performed by agarose gels analysis of PCR products from genomic DNA extracted from tail biopsies. Hemizygous *Rnf212b*$^{H24A/H24A}$ mice were directly processed for the analysis.

Immunocytology and antibodies

Testes were detunicated and processed for spreading using a conventional "dry-down" technique. Oocytes from fetal ovaries (E16.5-18.5 embryos) were digested with collagenase, incubated in hypotonic buffer, disaggregated and fixed in paraformaldehyde. Antibodies against RNF212B were produced by Proteogenix. Full-length mouse RNF212B (codon optimized for E. coli) was expressed as a RNF212B-7XHis-fusion protein in pET and purified from inclusion bodies using Ni-NTA beads. To generate rabbit polyclonal antibody two rabbits were immunized with the purified protein. Antibodies were then affinity purified against immobilized mouse RNF212-6His fusion protein. The antibodies (titer and specifitiy) against RNF212B were analyzed by protein blot analysis and immunostaining. Meiocytes from males and females were incubated with the following primary antibodies for immunofluorescence: rabbit αRNF212B R2 (1:750, ProteoGenix SAS), mouse αSYCP3 IgG sc-74569 (1:100, Santa Cruz), rabbit α-SYCP3 serum K921 (provided by Dr. José Luis Barbero, Centro de Investigaciones Biológicas, Spain), rabbit αSYCP1 IgG ab15090 (1:200, Abcam), rabbit anti-γH2AX (ser139) IgG #07–164 (1:500, Millipore), mouse αMLH1 51-1327GR (1:20, BD Biosciences), mouse αCDK2 (1:20; Santa Cruz Sc-6248), rat αRPA2 2208S (1:100, Cell Signaling #2208), rabbit αDMC1 (1:500, ProteintechTM), goat αRNF212 (1:200 provide by Dr. Neil Hunter, University of California, Davis), mouse αHEI10 (1:60, Origene Technologies 4H3), rabbit αCNTD1 (1:300 provide by Dr. Attila Toth, Universität Dresden, Germany), rabbit αMSH4(1:50, abcam ab58666; old batch from 2010), guinea pig αSYCE1 (1:100, provide by Dr. Karolinska Institutet, Stockholm, Sweden), mouse αHA (1:500, Cell signaling technology #2367S), Goat αSYCP3 (1:200, R&D Systems #AF3750), rabbit αTEX11 (1:60, provided by Dr. Jeremy Wang, University of Pennsylvania), rabbit αMZIP2 (1:100, provided by Dr. Chao Yu, University of Gothenburg). The secondary antibodies used were goat Alexa 555 α-mouse A-32727, goat Alexa 488 α-mouse A-11001, donkey Alexa 555 α-rabbit A-31572, goat Alexa 488 α-rat A-11006 (1:200, ThermoFisher), goat Alexa 488- Fab α-rabbit 111-547- 003 (1:100, Jackson Immunoresearch). Slides were visualized at room temperature using a microscope (Axioplan2; Carl Zeiss, Inc.) with 63 x objectives with an aperture of 1.4 (Carl Zeiss, Inc.). Images were taken with a digital camera (ORCA-ER; Hamamatsu) and processed with OPENLAB 4.0.3 and Photoshop (Adobe).The secondary antibodies used were goat Alexa 555 α-mouse A-32727, goat Alexa 488 α-mouse A-11001, donkey Alexa

555 α-rabbit A-31572 (1:200, ThermoFisher), goat Alexa 488- Fab α-rabbit 111-547-003 and donkey FITC α-guinea pig 706-095-148 (1:100, Jackson Immunoresearch). Slides were visualized at room temperature using a microscope (Axioplan2; Carl Zeiss, Inc.) with 63 x objectives with an aperture of 1.4 (Carl Zeiss, Inc.). Images were taken with a digital camera (ORCA-ER; Hamamatsu) and processed with OPENLAB 4.0.3 and Photoshop (Adobe). Quantification of fluorescence signals was performed using ImageJ software.

Stimulated emission depletion Microscopy.

Stimulated emission depletion (STED) microscopy (SP8, Leica) was used to generate the super-resolution images. Secondary antibodies for STED imaging were conjugated to Alexa 555 and 488 (Invitrogen). Slides were mounted in Prolong Antifade Gold without DAPI. Fluorescence signals were measured along the 19 autosomal AEs of pachytene cells using the 'Plot profile' tool of ImageJ. Signal intensities were standardized, acquiring values between -1 and + 1, and the overlay profiles of RNF212B and other RNF212 were plotted. Regression analysis for the pair of proteins was performed to determine the correlation between their profiles. The values of the coefficients of determination R2 are shown in the scatter plots.

Histology

For adult male histological analysis, testis were fixed in bouin and were processed into serial paraffin sections and stained with haematoxylin–eosin. For TUNEL assay, testis were fixed in formol 10% and sections were deparaffinized and apoptotic cells were detected with the In Situ Cell Death Detection Kit (Roche) and counterstained with DAPI. Apoptotic cells were pseudocoloured in green. For immunoflouprescence detection of RNF212B slides were boiled in sodium citrate buffer (10mM sodium citrate, 0.05% Tween-20, pH 6.0) for 20min, then incubated with rabbit RNF212B (1:100, this paper) and mouse SYCP3 (1:1000, Abcam) primaries diluted in blocking buffer (1 x PBS, 0.1% Triton, 1% BSA, 3% goat serum) at room temperature for 1 hour. Slides were the washed with 1x PBS + 0.1% Triton. Slides were then incubated with Alexa 647 α-rabbit (1:1000, Jackson Immunoresearch) and Rhodamine α-mouse (1:500, Jackson Immunoresearch). Slide were mounted in DAPI/antifade mix.

Oocyte quantification.

Ovaries from 10-day old mice were fixed in formol 10% overnight, dehydrated, embedded in paraffin, serially sliced into 5 μm thick sections and stained with hematoxylin and eosin. For each mouse, the number of oocytes was counted in every fifth

section of one ovary, and the sum of oocytes from all counted sections was considered the total number of oocytes per ovary. Only those oocytes with a visible nucleus were counted to avoid duplicate count.

Generation of plasmids

Full-length cDNAs encoding RNF212, RNF212B, HEI10, TRIM37, RNF34, MSH2, MSH4, MSH5, MSH6, SYCP1, SYCP3, SIX6OS1, PCNA, PSMA8, TEX12, SYCE1, SYCE2 and SYCE3 were RT-PCR amplified from murine wild-type testis RNA using the following primers: RNF212 ATG_S (CATGGCCAGCTGGGTGTTCTGT), RNF212 STOP_AS (TCGATGAACGCATGCATGCCAG); RNF212B ATG_S (AATGGATTGGTTTC ATTGCAACC), RNF212B STOP_AS (CTATCTGGAAAT GTTCCATCCGTCT); HEI10 ATG_S (CGCCATGTCTTTGTGTGAAGAC), HEI10 STOP_AS (GAGAACTCAGGGATGCAGGAAT); TRIM37 ATG_S (GGCAATGGATGAGCAGAGTGTGG), TRIM37 STOP_AS (GGGACCAGGTAGCTTGTAGCTCGG); RNF34 ATG_S (CCATGAAGGCGGGTGCTACTTC), RNF34 STOP_AS (CCTACTGCAGGGCCCTCAATTTC); MSH2 ATG_S (GCTCTGTGCTCTAGTGGTGTTGGT), MSH2 STOP_AS (CCCAGAACAGCACACAGTAAAGG); MSH4 ATG_S (CATGTGCTGCTTGTTCCTGCGC), MSH4 STOP_AS (CACCGTGTACTGGTCACTCCTC); MSH5 ATG_S (TGTCCTCAGAGTCGCCGAGC), MSH5 STOP_AS (GCGAGGGAGGACTCTCAGAGG); MSH6 ATG_S (CTTCATCATGTCCCGACAAAGC), MSH6 STOP_AS (AAGTCAGCTCAGCTTCCAATGTA); SYCP1 ATG_S (AGGAGGCAGCATGGAGAAGCAAA), SYCP1 STOP_AS (CTCTGTAACCAACATTGGTTTTCTG); SYCP3 ATG_S (GTTTCCTCAGATGCTTCGAGGGT), SYCP3 STOP_AS (TCAGAATAACATGGATTGAAGAGAC) ; SIX6OS1 ATG_S (AGTGTCCAAGATGAATGATAATCTG), SIX6OS1 STOP_AS (GTTCAAAAATAATAACTCAAAAAAAC); PCNA ATG_S (CATGTTTGAGGCACGCCTGAT), PCNA STOP_AS (GCAATGCCTAAGATGCTTCCTC); PSMA8 ATG_S (CGTGATGGCTTCTCGGTATGACAG), PSMA8 STOP_AS (CAGTGACGCTGGCATTAGGTTGAC); TEX12 ATG_S (TCTCCAGAGAATGATGGCAAACC), TEX12 STOP_AS (GGCACATTACTTGTGTAGGGTGT); SYCE1 ATG_S (GAGCAGTATGGCCACCAGACC), SYCE1 STOP_AS (GAGGAGGGTATTAGGTCCTGC); SYCE2 ATG_S (AAAAGACGGAATGGAGCGCCACG), SYCE2 STOP_AS (AGCAGAAGTCAGCATTCACCATC); SYCE3 ATG_S (AGACCAGATGGCTGATTCCGA) and SYCE3 STOP_AS (CAGATGGTAGCTCACTGCTTG).

The full-length cDNAs were cloned into pcDNA3 (EcoRV), pcDNA3 2xFlag (EcoRV), pcDNA3 2xHA (EcoRI), pmCherry-C1 (SmaI) and/or pEGFP- C1 (SmaI) mammalian expression vectors under the CMV promoter. In frame cloning was verified by Sanger sequencing. For the construction of the mutants pcDNA3 2xFlag RNF212B, pcDNA3 2xFlag RNF212 and pcDNA3 2xFlag HEI10 we PCR amplified the mutant construct bearing the two Cys>Ala mutations in the Zn domain (MUT), using the following primers that contain the mutation: RNF212 MUT_F (**GCC**GGGGAGGTGTAC**GCC**CACTCCTGCCTCCTCAAAGGTACAAAGAATG) and RNF212 MUT_R (GCTGGTCAGGCTGAAGGACGA); RNF212B MUT_F

(**GCT**GGCCATATTTTC**GCT**AAAAAGTGTATGACTTTGGAAAAATGTGCT) and RNF212B MUT_R (GCTGGTGACAAAGAAATGGGC); HEI10 MUT_F (**GCT**AATTATCGGAAG**GCT**CGGATCAAGCTCTCTGGTTATGCTTGGGTC) and HEI10 MUT_R (AAGCAGCATGTCTTCACACAAAG).

Immunoprecipitation and western blotting.

HEK293T cells were transiently transfected and whole cell extracts were prepared in a 50mM Tris-HCl pH 7,4, 150mM NaCl, 1mM EDTA, 1% Triton X-100 buffer supplemented with protease inhibitors. Those extracts were cleared with protein G Sepharose beads (GE Healthcare) for 1 h. The corresponding antibodies were incubated with the extracts for 2 h and immunocomplexes were isolated by adsorption to protein G-Sepharose beads o/n. After washing, the proteins were eluted from the beads with 2xSDS gel-loading buffer 100mM Tris-Hcl (pH 7), 4% SDS, 0.2% bromophenol blue, 200mM β-mercaptoethanol and 20% glycerol, and loaded onto reducing polyacrylamide SDS gels. The proteins were detected by western blotting with the indicated antibodies. Immunoprecipitations were performed using mouse αFlag IgG (5µg; F1804, Sigma-Aldrich), mouse αGFP IgG (4 µg; CSB-MA000051M0m, Cusabio), ChromPure mouse IgG (5µg/1mg prot; 015-000-003). Primary antibodies used for western blotting were

rabbit αFlag IgG (1:2000; F7425 Sigma-Aldrich), goat αGFP IgG (1:3000 sc-5385, Santa Cruz), mouse αHA IgG (1:3000, 11 101 R, Biolegend), rabbit αRNF212B R2 (1:3000, ProteoGenix). Secondary horseradish peroxidase-conjugated α-mouse (715-035-150, Jackson ImmunoResearch), α-rabbit (711-035-152, Jackson ImmunoResearch), α-goat (705-035-147, Jackson ImmunoResearch) or α-rat (712-035-150, Jackson ImmunoResearch) antibodies were used at 1:5000 dilution. Antibodies were detected by using Immobilon Western Chemiluminescent HRP Substrate from Millipore.

Statistics.

In order to compare counts between genotypes, we used the Welch´s t-test (unequal variances t-test), which was appropriate as the count data were not highly skewed (i.e., were reasonably approximated by a normal distribution) and in most cases showed unequal variance. We applied a two-sided test in all the cases. Asterisks denote statistical significance: *p-value <0.05, **p-value <0.01, ***p-value<0.001 and ****p-value<0.0001.

Ethics statement.

Mice were housed in a temperature-controlled facility (specific pathogen free, spf) using individually ventilated cages, standard diet and a 12 h light/dark cycle, according to EU laws at the "Servicio de Experimentación Animal, SEA". Mouse protocols have been approved by the Ethics Committee for Animal Experimentation of the University of Salamanca (USAL). We made every effort to minimize suffering and to improve animal welfare. Blinded experiments were applied when possible. No randomization methods were applied since the animals were not divided in groups/treatments. The minimum size used for each analysis was two animals/genotype.

Hela transfection and His-Pull downs

HELA cells stably expressing either 10xHis-ubiquitin or 10xHis-SUMO were seeded at 10% confluency in five 15 cm plates with DMEM supplemented with 10% FBS and 100 U/mL penicillin 100 mg/mL streptomycin. Next day, cells were transfected with a transfection mixture of 150 mM NaCl and 60 mg PEI containing 12 mg of FLAG-RNF212b plasmid (Empty vector, WT or MUT). 24 h post-transfection, culture medium was replaced. 48 h post-transfection, cells were scraped in ice-cold PBS and an input sample was taken for immunoblotting analysis. The rest of the sample was lysed in 10 mL of Guanidinium lysis buffer (6M guanidine-HCl, 0.1M Sodium Phosphate, 10mM TRIS, pH 7.8) for subsequent His-Pull down experiments that were performed as previously described (4)). Lysates were homogenized by two rounds of sonication at 80% amplitude during 5 s using a tip sonicator (Q125 Sonicator, QSonica, Newtown, USA). Subsequently, protein concentration was determined by BiCinchoninic Acid (BCA) Protein Assay Reagent (Thermo Scientific) to equalize the total protein amount among

samples. After equalization, cell lysates were supplemented with 5 mM β-mercaptoethanol and 50 mM Imidazole pH 8.0. Dry nickel-nitrilotriacetic acid-agarose (Ni-NTA) beads (QIAGEN) were equilibrated with Guanidinium buffer also supplemented with 5 mM β-mercaptoethanol and 50 mM Imidazole pH 8.0. 60 mL of equilibrated Ni-NTA beads were added to the cell lysates and incubated overnight at 4°C under rotation.

After lysate-bead incubation, Ni-NTA beads were spun down and transferred with Wash Buffer 1 (6 M Guanidine-HCl, 0.1M Sodium Phosphate, 10 mM Tris, 10 mM Imidazole, 5 mM β-mercaptoethanol, 0.2 % Triton X-100, pH 7.8) to an Eppendorf LoBind tube (Eppendorf). Beads were washed with Wash buffer 2 (8 M Urea, 0.1M Sodium Phosphate, 10 mM Tris, 10 mM imidazole, 5mM β-mercaptoethanol, pH 8), then with Wash buffer 3 (8 M urea, 0.1M Sodium Phosphate, 10 mM TRIS, 10 mM imidazole, 5 mM β-mercaptoethanol, pH 6.3) and ultimately, beads were washed twice with Wash buffer 4 (8 M urea, 0.1M Sodium Phosphate, 10 mM TRIS, 5 mM β-mercaptoethanol, pH 6.3). When washing with wash buffer 3 and 4, beads were allowed to equilibrate with the buffer for 15 min under rotation. Finally, proteins were eluted from the beads twice with 20 mL of Elution buffer (7 M urea, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris pH 7.0 and 500 mM imidazole pH 7.0).

Immunoprecipitation from HeLa cell extracts

For electrophoresis and immunoblotting, samples were separated on Novex 4-12% gradient gels (Thermo Fisher Scientific) using NuPAGE® MOPS SDS running buffer (50mM MOPS, 50mM Tris-base, 0.1% SDS, 1mM EDTA pH 7.7) and transferred onto Amersham Protran Premium 0.45 NC Nitrocellulose blotting membranes (GE Healthcare) using a Bolt Mini-Gel system (Thermo Fisher Scientific), which was used for both the gel electrophoresis and the protein transfer to the membrane according to vendor instructions. Membranes were stained with Ponceau-S (Sigma Aldrich) to determine total amount of protein loaded. Next, membranes were blocked with blocking solution (8% Elk milk, 0.1% Tween-20 in PBS) for 1h prior to primary antibody incubation. After 1 h incubation with the secondary antibody, chemiluminescence reaction was initiated with Western Bright Quantum Western blotting detection kit (Advansta – Isogen) for subsequent visualization. Antibodies: α-FLAG mouse monoclonal (Sigma F3165-1MG), α-GFP rabbit (Novus Biologicals NB600-308), α-SUMO2/3 mouse monoclonal (8A2

clone, in-house produced), α-Ubiquitin mouse monoclonal (Santa Cruz sc-8017), α-MBLN3 Rabbit polyclonal (ThermoFisher).

Trypsin digestion of His-Ubiquitin and His-SUMO2/3 purified conjugates.

Eluted proteins were supplemented with ABC to 50 mM. Subsequently, samples were reduced with 1 mM dithiothreitol (DTT) for 30 min and alkylated with 5 mM chloroacetamide (CAA) for 30 min. After an additional reduction with 5 mM DTT for 30 min at RT, conjugates were diluted with 50 mM ABC and digested with 500 ng of sequencing grade modified trypsin (Promega) o/n in dark.

Mass Spectrometry sample preparation

Digested peptides were acidified by adding 2% TriFlourAcetic (TFA) acid. Subsequently, peptides were desalted and concentrated on triple-disc C18 Stage-tips as previously described (68). Stage-tips were in-house assembled using 200 µL micro pipet tips and C18 matrix. Stage-tips were activated by passing through 100µL of methanol. Subsequently 100 µL of Buffer B (80% acetonitrile, 0.1% formic acid), 100 µL of Buffer A (0.1% formic acid), the acidified peptide sample, and two times 100 µL Buffer A were passed through the Stage-tip. Elution was performed in 50 µL of 32,5% acetonitrile, 0.1% formic acid. Samples were vacuum dried using a SpeedVac RC10.10 (Jouan, France) and stored at -20°C. Prior to mass spectrometry analysis, samples were reconstituted in 10 µL 0.1% formic acid and transferred to autoload vials.

LC-MS/MS analysis

All the experiments were analyzed by on-line nanoHPLC MS/MS with a system consisting of an EASY-nLC 1200 system (Thermo) coupled to an Orbitrap Fusion Lumos Trybrid mass spectrometer (Thermo, Bremen, Germany). Samples were measured in a 2-column setup, injected into an in-house packed precolumn and eluted via a homemade analytical nano-HPLC column (50 cm × 75 µm; Reprosil-Pur C18-AQ 1.9 µm (Dr. Maisch, Ammerbuch, Germany) heated in a column oven set to 50°C (Sonation,

Biberach, Germany) The gradient was run from 2% to 36% solvent B. (80% acetonitrile, 0.1% formic acid) in 120 min followed by column re-requilibration. The mass spectrometer was operated in data-dependent MS/MS mode for a cycle time of 3 seconds, with a HCD collision energy of 32. In the master scan (MS1) the resolution was 120,000, the scan range 300-1600, at an AGC target of 400,000 with a maximum fill time of 50 ms. For MS2 the scan range mode was set to automated, and the MS2 scan resolution was 30,000 at an normalized AGC target of 100% with a maximum fill time of 60 ms. Quadrupole isolation width of 1.2 Da. A lock mass correction on the background ion m/z=445.12 was used. Precursors were dynamically excluded after n=1 with an exclusion duration of 45 s, and with a precursor range of 10 ppm. Charge states 2-5 were included

Mass Spectrometry data analysis

All raw data was analyzed using MaxQuant (version 1.6.14) as previously described (69) We performed the search against an in silico digested UniProt reference proteome for Homo sapiens including canonical and isoform sequences (5th July 2021). Database searches were performed according to standard settings with the following modifications: Digestion with Trypsin/P was used, allowing 4 missed cleavages. Oxidation (M), Acetyl (Protein N-term), Phospho (S,T), QQTGG (K) (for SUMOylation sites) and GlyGly (K) (for ubiquitination sites) were allowed as variable modifications with a maximum number of 3. Carbamidomethyl (C) was disabled for SATTs analysis as a fixed modification. Label-Free Quantification was enabled, not allowing Fast LFQ. All peptides were used for protein quantification. Output from MaxQuant Data were exported and processed in MS Excel for further filtering, processing of the data, and visualization. For the statistical analysis, output from the analysis in MaxQuant was further processed in the Perseus computational platform version 1.6.14 (70).

Preparation of germ cell lysates for immunoprecipitation

Germ cell suspension was prepared from 12 week old C57BL6/J males. After dissecting and detunicating the testes, tubules were added to a large drop of PBS, minced for one minute with razor blades, then transferred to a 15mL conical tube in ~2mL of PBS. Slurry

was then vigorously pipetted with a plastic dropper pipette for one minute, the tube was filled with PBS, mixed, then tubules allowed to settle for 3 minutes at room temperature. Supernatant was then filtered through a 70um cell strainer. For Figure 3D, five mice were used and cells were pooled on ice after straining. Cells were then pelleted at 600xg for 5min at 4C, the cell pellet gently resuspended by flicking, then resuspended in lysis buffer (50mM Tris-HCl pH 8.0, 500mm NaCl, 1% Triton, 1mM EDTA, 1 Pierce Protease Inhibitor Mini Tablet (EDTA-free), 0.1mg/mL PMSF) slowly to a final volume of ~3mL. Cells were then sonicated at 23% amplitude, in 12second cycles, 0.4sec on / 0.2sec off, for two cycles with 30sec recovery on ice between. Lysate was cleared by centrifuging at 17,900xg for 20min at 4C. Lysate was collected and protein content was measured using Pierce BCA kit per manufacturers instructions.

Immunoprecipitation and Western blotting from mouse germ cell extracts

For the germ cell suspension IP, testis from 5 mice were used to generate the lysate. 2.95mg protein lysate was used for each IP. Input gel sample corresponds to ~5% of the total input for each IP. For whole testes lysate IP, frozen tubules from 3 mice were used to generate lysate. 3.4mg of protein lysate was used for each IP, and the input gel sample corresponds to ~3% of total input for each IP. For each IP, 10ug of each antibody: rabbit αMSH4 (Abclonal A8556), rabbit αRNF212B R2 (ProteoGenix), rabbit αPCNA (Proteintech 10205-2-AP), normal rabbit IgG (Cell Signaling 2729S) was added to protein lysate then incubated with rocking overnight at 4C. Input gel samples were taken from pooled lysate before dividing into separate IP reactions. Beads from 50uL Protein A Dynabead slurry were equilibrated in lysis buffer (50mM Tris-HCl pH 8.0, 500mm NaCl, 1% Triton, 1mM EDTA, 1 Pierce Protease Inhibitor Mini Tablet (EDTA-free), 0.1mg/mL PMSF) then added to antibody/lysate reaction and incubated at room temperature for 2 hours. Supernatant was removed and beads were washed 3 times with 500uL lysis buffer by slowly pipetting. Bound protein was eluted by resuspending beads in elution buffer (100mM Tris-HCl pH 8.0, 1% SDS, 10mM DTT) and incubating in a 65C water bath for 15mins. Supernatant was then removed from beads, 4X Laemmli sample buffer was added and samples were boiled at 95C for 5-10minutes then either stored at -20C till use or used immediately.

One half of each IP gel sample (elutions and input) was run on an 10% acrylamide SDS-PAGE gel for downstream Western blotting. After SDS-PAGE electrophoresis, protein was transferred to a PVDF membrane using a BioRad Trans-Blot Turbo transfer system after PVDF was equilibrated in methanol then 1X Towbin buffer. Membrane was blocked for 10min at RT with BioRad EveryBlot Blocking Buffer. Primary antibodies used for western blotting were: rabbit αRNF212B R2 (1:2000, ProteoGenix), rabbit αMSH4 (1:500, Abclonal A8556), rabbit αPCNA (1:5000, Proteintech 10205-2-AP). Membranes were washed 3x in TBST, then incubated with 1:5000 anti-rabbit HRP secondary (Invitrogen) for 1 hour at RT, then washed once more 3x in TBST. Pierce ECL was added to membranes then blots were imaged with a BioRad ChemiDoc XRS+ with various exposure times.

SUMOylome MA analysis from mouse samples

We performed the sumoylome on an n=5 analysis of the samples. To maximize the yield, we pooled 8 testes per replicate for wildtype (labeled as "C" in the data), 8 testes per replicate *for Rnf212b* KO (labeled "RKO"), and 5 testes per replicate for *Hei10* KO (labeled "HKO"). Wildtype controls were 17-19 dpp to avoid spermatocytes as *Rnf212b* and *Hei10* lack. Homogenization of testes and subsequent purification of peptides modified by endogenous SUMO2/3 was performed essentially as described previously (50). Frozen testes were stored at −80 °C until further processing, and kept on dry ice until seconds before homogenization. Four 1.4 mm and two 2.8 mm zirconium beads were added per 2 mL homogenizer tube (Precellys), in addition to 10 µL of 0.5 M freshly dissolved chloroacetamide (CAA). All tubes containing beads, testes, and SUMO protease inhibitors, were kept on dry ice (-80 °C) at all times. Only immediately prior to homogenization through bead-milling, 1 mL of room temperature Lysis Buffer (6 M guanidine-HCl, 50 mM TRIS, pH 8.5) was added to all tubes. Bead-milling was performed using a Precellys 24 homogenizer system, at 5,500 rpm for 20 s, with testes already homogenized within several seconds. After the first grinding cycle, tubes were briefly centrifuged at 500g, and subjected to another round of grinding. Following bead-milling, lysates were centrifuged at 500g for 30 seconds and transferred to 15 mL tubes containing one additional volume of Lysis Buffer, in order to separate homogenate from

the zirconium beads. Lysates were subjected to a 10 s pulse of sonication at 30 W, after which they were supplemented with 5 mM CAA and 5 mM TCEP, and incubated at 37 °C for 30 minutes. Next, lysates were centrifuged for 30 min at 3,000g in a swing-out centrifuge with delayed deceleration, carefully decanted into new tubes, and subsequently passed through 0.45 μm disc filters in order to clarify them.

Peptide preparation for endogenous SUMO-IP

Sample protein concentration was determined using Bradford assay according to manufacturer's instructions. Endoproteinase Lys-C (Wako) was added to samples in a 1:200 enzyme-to-protein ratio (w/w). Digestion was performed overnight, still, and at room temperature. Digested samples were diluted with three volumes of 50 mM ammonium bicarbonate (ABC) to dilute guanidine concentration to 1.5 M, and a second round of overnight digestion was performed by addition of Lys-C in a 1:200 enzyme-to-protein ratio. Digests were acidified by addition of trifluoroacetic acid (TFA) to a final concentration of 0.5%, after which they were centrifuged at 4,250g and at 4°C for 30 min. Clarified digests were carefully decanted into clean 50 mL tubes, after which peptides were purified using C8 Sep-Pak cartridges (Waters) according to the manufacturer's instructions. Sep-Pak cartridges with 500 mg C8 sorbent were used, with one cartridge used per sample. Unrelated small peptides were washed off using 5 mL of 25% ACN in 0.1% TFA. SUMOylated peptides were eluted using 4 mL of 45% ACN in 0.1% TFA. SepPak elutions were collected in 50 mL tubes with small holes punctured into the caps, and then frozen overnight at −80°C. Deep-frozen samples were lyophilized to dryness for 96 h, with the pressure target set at 0.004 mbar and the condenser coil at −90°C.

Crosslinking of SUMO antibody to beads

400 μL of Protein G Agarose beads (Roche) were used to capture 100 μL of SUMO-2/3 antibody (8A2, acquired from Abcam in 2018, ab81371; ~5-10 μg/μL antibody). All washing and handling steps were followed by centrifugation of the beads at 500*g* for 3 min in a swing-out centrifuge with delayed deceleration and careful aspiration of buffers, to minimize loss of beads. Beads were pre-washed 3 times with ice-cold PBS, after which the antibody was added, and the tube was completely filled with ice-cold PBS. Beads and

antibody were incubated at 4°C on a rotating mixer for 2 h, and subsequently washed 3 times with ice-cold PBS. Crosslinking of the antibody to the beads was achieved by addition of 1.2 mL of 0.2 M sodium borate, pH 9.0, which was freshly supplemented with 20 mM dimethyl pimelimidate. Crosslinking was performed for 30 min at room temperature on a rotating mixer, after which the crosslinking step was repeated once. Next, SUMO-IP beads where washed twice with ice-cold PBS, twice with 0.1 M glycine pH 2.8, and three times with ice-cold PBS, after which all beads were pooled in a single 1.5 mL tube and stored until use at 4°C in PBS supplemented with 10 mM sodium azide.

Purification of SUMOylated peptides

Lyophilized peptides were dissolved in 5 mL ice-cold SUMO-IP buffer (50 mM MOPS, 10 mM Na2HPO4, 50 mM NaCl, buffered at pH 7.2). Samples were clarified by centrifugation at 4,250*g* for 30 min at 4°C in a swing-out centrifuge with delayed deceleration. Samples were transferred to new tubes, after which 25 μL SUMO-IP beads were added per sample. Samples were incubated at 4°C for 4 h in a rotating mixer, after which the beads were washed twice with ice-cold SUMO-IP buffer, twice with ice-cold PBS, and twice with ice-cold MQ water. Upon each first wash with a new buffer, beads were transferred to a clean 1.5 mL LoBind tube (Eppendorf). To minimize loss of beads, all centrifugation steps were performed at 500*g* for 3 min at 4°C in a swing-out centrifuge with delayed deceleration. Elution of SUMO peptides from the beads was performed by addition of 50 μL of ice-cold 0.15% TFA, and performed for 30 min while standing still on ice, with gentle mixing every 10 min. The elution of the beads was repeated once, and both elutions were cleared through 0.45 μm spin filters (Millipore) by centrifuging at 12,000*g* for 1 min at 4°C. The two elutions from the same samples were pooled after clarification. Next, samples were pH-neutralized by addition of 10 μL 1 M Na2HPO4, and allowed to warm up to room temperature. Second-stage digestion of SUMOylated peptides was performed with 133 ng of Endoproteinase Asp-N (Roche). Digestion was performed overnight, at 30°C and shaking at 300 rpm.

Peptide preparation for endogenous SUMO-IP

Samples were digested with Endoproteinase Lys-C (Wako) in a 1:200 enzyme-to-protein ratio (w/w). Digested samples (room temperature) were diluted with ammonium bicarbonate (ABC) to dilute guanidine, redigested wit Endoproteinase Lys-C, and after acidification with TFA, were centrifuged. Peptides from the clarified were purified using C8 Sep-Pak cartridges (Waters). SUMOylated peptides were eluted and rozen overnight at −80°C. Deep-frozen samples were lyophilized to dryness for 96 h.

StageTip purification and high-pH fractionation of SUMO-IP samples

Preparation of StageTips and high-pH fractionation of SUMO-IP samples on StageTip, was performed essentially as described previously (50, 71). Quad-layer StageTips were prepared using four punch-outs of C18 material (Sigma-Aldrich, Empore™ SPE Disks, C18, 47 mm). StageTips were equilibrated using 100 µL of methanol, 100 µL of 80% ACN in 200 mM ammonium, and two times 75 µL 50 mM ammonium. Samples were thawed out, and supplemented with 10 µL 200 mM ammonium, just prior to loading them on StageTip. The StageTips were subsequently washed twice with 150 µL 50 mM ammonium, and afterwards eluted as six fractions (F1-6) using 80 µL of 4, 7, 10, 13, 17, and 25% ACN in 50 mM ammonium. All fractions were dried to completion in LoBind tubes, using a SpeedVac for 3 h at 60°C, after which the dried peptides were dissolved using 11 µL of 0.1% formic acid.

MS analysis of sumoylome

All samples were analyzed using an EASY-nLC 1200 system (Thermo) coupled to an Orbitrap Exploris™ 480 mass spectrometer (Thermo). Separation of peptides was performed using 20-cm columns (75 µm internal diameter) packed in-house with ReproSil-Pur 120 C18-AQ 1.9 µm beads (Dr. Maisch). Elution of peptides from the column was achieved using a gradient ranging from buffer A (0.1% formic acid) to buffer B (80% acetonitrile in 0.1% formic acid), at a flow of 250 nL/min. Gradient length was 40 min per sample, including ramp-up and wash-out, with an analytical gradient of 28.5 min. The buffer B ramp for the analytical gradient was as follows: F1: 13-24%, F2: 14-27%, F3-5: 15-30%, F6: 17-32%. The analytical column was heated to 40°C using a column oven, and ionization was achieved using a NanoSpray Flex™ NG (Thermo) with the spray voltage set at 2 kV, an ion transfer tube temperature of 275°C, and an RF funnel level of 50%. Acquisition methodology optimized for endogenous SUMO was used (51). To this end, full scan range was set to 500-1,500 m/z with a precursor selection window of 550-1,500 m/z, MS1 resolution to 120,000, MS1 AGC target to “200” (=2,000,000 charges), and MS1 maximum injection time to “Auto”. Monoisotopic Precursor Selection (MIPS) was enabled in “Peptide” mode, without relaxation of restrictions in case insufficient precursors were observed. Precursor intensity threshold was set to 30,000 charges per second. Precursors with charges 3-6 were selected for fragmentation using an isolation width of 1.3 m/z, and fragmented using higher-energy collision disassociation (HCD) with normalized collision energy of 25. Precursors were excluded from re-

sequencing by setting a dynamic exclusion of 30 s, and with an exclusion mass tolerance of 15 ppm. MS2 resolution was set to 120,000, MS2 AGC target to "200" (=200,000 charges), and MS2 maximum injection time to 666 ms. Data-dependent scans were limited to 2 per duty cycle.

Analysis of SUMOylome MS data

All MS RAW data was analyzed using the freely available MaxQuant software, version 1.5.3.30 (72, 73). All data was processed in a single computational run, with exceptions to default MaxQuant settings specified below. For generation of the theoretical spectral library, the mouse FASTA database was downloaded from Uniprot on the 8th of March, 2022. The mature sequence of SUMO2 was inserted in the database to allow for detection of free SUMO. *In silico* digestion of theoretical peptides was performed with Lys-C, Asp-N, and Glu-N, allowing up to 8 missed cleavages. Variable modifications used were protein N-terminal acetylation, methionine oxidation, peptide N-terminal pyroglutamate, and Lys SUMOylation, with a maximum of 3 modifications per peptide. The SUMO mass remnant was defined as described previously (50); DVFQQQTGG, $H_{60}C_{41}N_{12}O_{15}$, monoisotopic mass 960.4301, neutral loss b7-DVFQQQT, diagnostic mass remnants [b2-DV, b3-DVF, b4-DVFQ, b5-DVFQQ, b6-DVFQQQ, b7-DVFQQQT, b9-DVFQQQTGG, QQ, FQ, FQQ]. Label-free quantification was enabled, with "Fast LFQ" disabled. Maximum peptide mass was set to 6,000 Da. Stringent MaxQuant 1% FDR filtering was applied (default), and additional automatic filtering was ensured by setting the minimum delta score for modified peptides to 20, with a site decoy fraction of 2%. Matching between runs was enabled, with a match time window of 0.5 min and an alignment window of 10 min. For protein quantification, the same variable modifications were included as for the peptide search.

Statistical processing of SUMOylome MS data

To further minimize false-positive discovery, additional manual filtering was performed at the peptide level. All modified peptides were required to have a localization probability of >75%, be supported by diagnostic mass remnants, be absent in the decoy database, and have a delta score of >100 in case SUMO modification was detected on a peptide C-

terminal lysine not preceding an aspartic acid or glutamic acid. Multiply-SUMOylated peptides were accepted with an Andromeda score of >100 and a delta score of >40. SUMO target proteins were derived from the "proteinGroups.txt" file, and all post-filtering SUMO sites were manually mapped. Only proteins containing at least one SUMO site were considered as SUMO target proteins, and other putative SUMO target proteins were discarded. Further statistical handling and quantification of SUMO site and SUMO target protein data was performed using the Perseus software (versions 1.5.5.3 and 1.6.14.0) (5), including $\log_2$-transformation, filtering for presence of sites or proteins in a sufficiently high number of replicates, imputation of missing values (either sampled from a normal distribution below the global detection limit, or using the imputeLCMD package integrated in Perseus version 1.6.14.0), two-tailed two-sample Student's t-testing, and multiple-sample ANOVA testing. Both two-sample and ANOVA testing were performed with permutation-based FDR control, resulting in *p*-values corrected for multiple-hypothesis testing (i.e. *q*-values). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (6) partner repository with the dataset identifier PXD047019 and 34723319.

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Mass-Spectrometry coupled to RNF212B immunoprecipitation from testis extracts.

Protein extracts from detunicated testis were prepared in 50 mM Tris-HCl (pH8), 500 mM NaCl, 1 mM EDTA 1% Triton X100. 200 µg of antibodies RNF212B (three independent replicas from 5 different mice) and IgG from rabbit (negative control) were crosslinked to 100 ul of sepharose beads slurry (GE Healthcare). 20 mg of protein extracts were incubated over night with the sepharose beads. Protein-bound beads were packed into columns and washed in extracting buffer for three times. Proteins were eluted in 100 mM glycine pH3 and analysed by Lc-MS/MS shotgun in LTQ Velos Orbitrap at the Proteomics facility of Centro de Investigación del Cáncer (CSIC/University of Salamanca). A nano-UPLC system (nanoAcquity, Waters Corp., Milford/MA, USA) coupled to a LTQ-Velos-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose/CA, USA) via a nano-electrospray ion source (NanoSpray flex, Proxeon, Thermo) was used for reversed-phase LC-MS/MS analysis. Peptides were dissolved in 2%ACN/0.1% FA and loaded onto a trapping column (nanoACQUITY UPLC 2G-V/M Trap Symmetry 5 µm particle size, 180 µm × 20 mm C18 column, Waters Corp., Milford/MA, USA). Peptides were separated on a nanoACQUITY UPLC BEH 1.7 µm, 130 Å, 75 µm × 250 mm C18 column (Waters Corp., Milford/MA, USA) with a linear gradient from 2% to 35% solvent B (ACN/0.1% FA) at a flow rate of 250 nL/min over 120 minutes. The LTQ-Orbitrap Velos was operated in the positive ion mode applying a data-dependent automatic switch between survey MS scan and tandem

mass spectra (MS/MS) acquisition. Survey scans were acquired in the mass range of m/z 400 to 1600 with 60,000 resolution at m/z 400 with lock mass option enabled for the 445.120025 ion. The 20 most intense peaks having ≥2 charge state and above 500 intensity threshold were selected in the ion trap for fragmentation. Protein identification was done by searching the MS/MS spectra against mouse UNIPROT sequences (UP000000589 release 2023_11_08) with MSFragger search engine (7). Search parameters were as follows: fully tryptic digestion with up to two missed cleavages, 20 ppm and 0.5 Da mass tolerances for precursor and product ions, respectively, oxidation of methionine and acetylation of the protein n-terminus were established as variable modifications and carbamidomethylation of cysteine as fixed modification. The resulting peptide hits were filtered for a maximum 1% FDR using the Percolator algorithm with a q-value cutoff of 0.05. Proteins were filtered for a maximum of 1% FDR using ProteinProphet. Protein identifications were annotated with Mus musculus GO database using PANTHER (8). Results were filtered with GO identifiers: GO:0006281, GO:0006310 and GO:0000795; corresponding to DNA repair, DNA recombination and Synaptonemal complex, respectively. Additionally, results were also filtered by "meio" and "synaptonema". The summatory of total spectral count from each experimental condition was calculated. MS protein intensities and MaxLFQ had been log2 transformed and used to calculate fold change. The table only show those hits with a minimum log2 fold of 0.3.

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