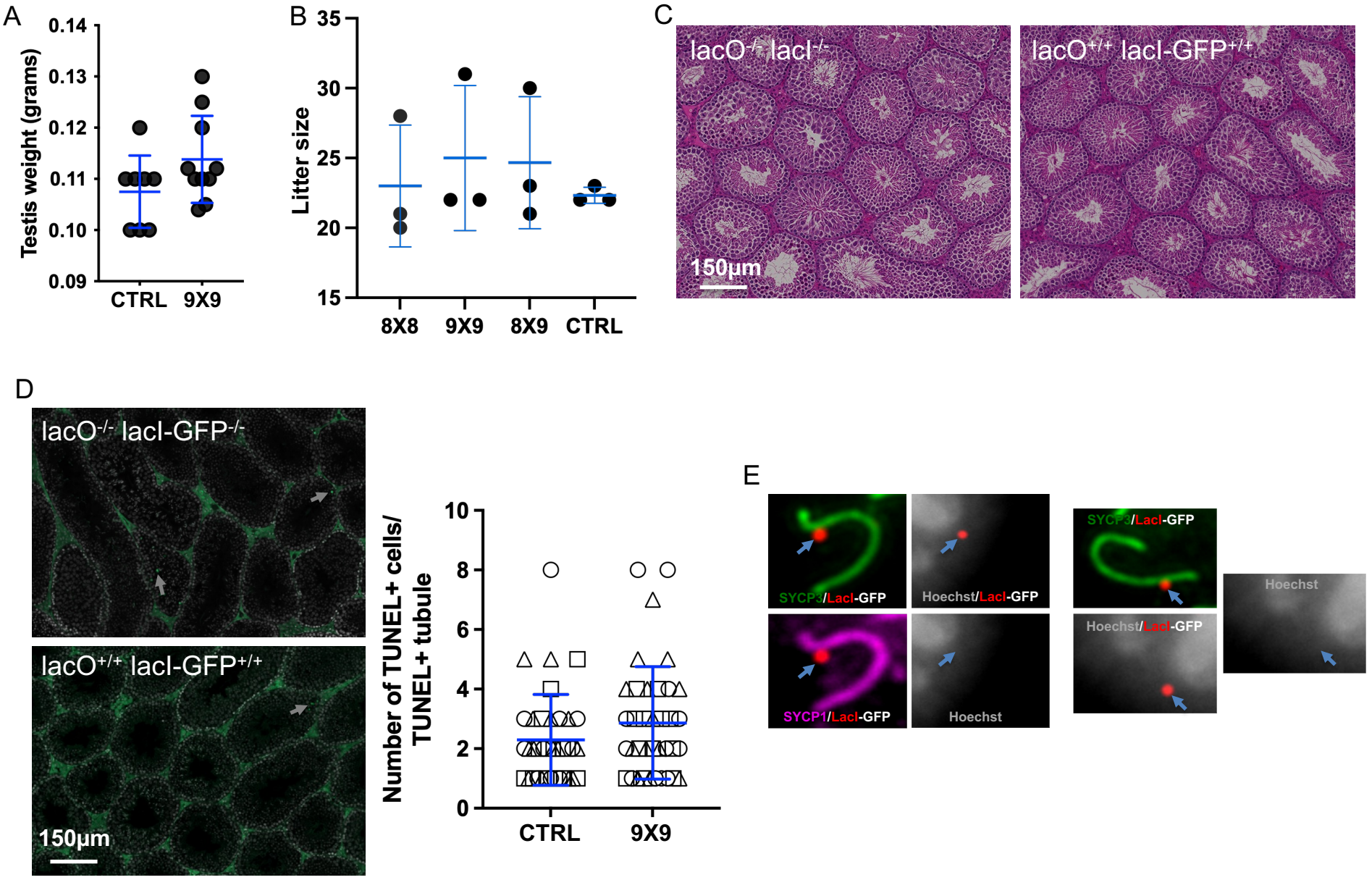
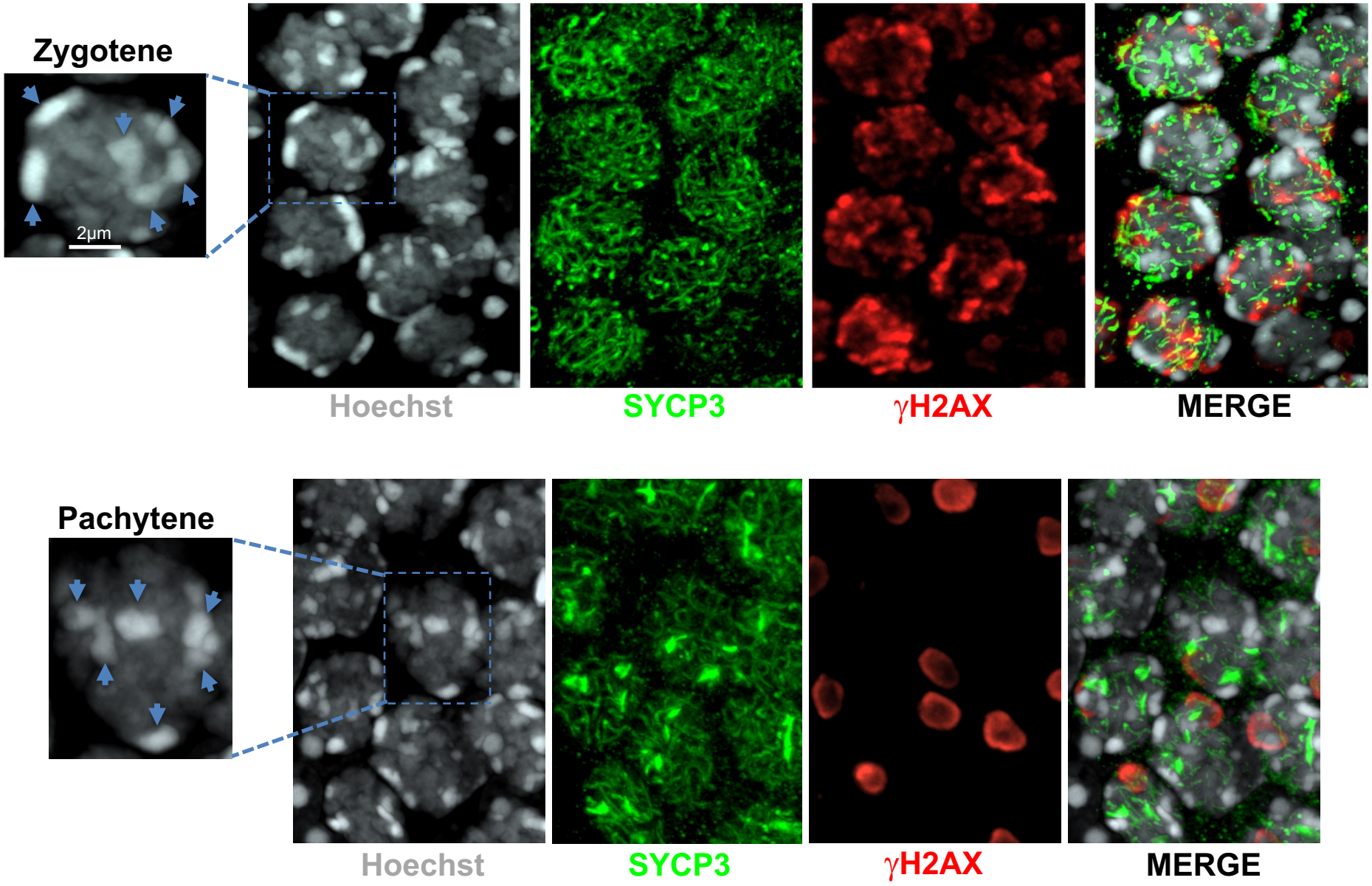

A
Testis weight (grams)
0.14
0.13
0.12
0.11
0.10
0.09
CTRL
9X9
B
Litter size
35
30
25
20
15
8X8
9X9
8X9
CTRL
C
lacO-/- lacI-/-
150μm
lacO+/+ lacI-GFP+/+
D
lacO-/- lacI-GFP-/-
lacO+/+ lacI-GFP+/+
150μm
Number of TUNEL+ cells/ TUNEL+ tubule
10
8
6
4
2
0
CTRL
9X9
E
SYCP3/LacI-GFP
Hoechst/LacI-GFP
SYCP1/LacI-GFP
Hoechst
SYCP3/LacI-GFP
Hoechst/LacI-GFP
Hoechst

Supplementary Figure 1. Characterization of lacO and lacO-lacI-GFP transgenic mice. (A) Testis size comparison between control (lacO-lacI-GFP negative) and transgenic lacO-lacI-GFP mice littermates. The graph shows the differences in testes weight. Horizontal bars represent the mean and standard deviation. (B) Breeding results obtained from mice carrying lacO-lacI-GFP insertions in chromosome 8 or 9 and compared to lacO-lacI-GFP negative mice. Horizontal bars represent the mean and standard deviation. 8X8 cross, average ± SD, 23.0 ± 4.4, n = 3 individual crosses, P = 0.99, Tukey's test; 9X9 cross, 25.0 ± 5.2, n = 3, P = 0.86; and 8X9 cross, 24.7 ± 4.7, n = 3, P = 0.89) comparable to those of the control (22.3 ± 0.57, n = 3). More details in Table S1. (C) Histological analysis of testes obtained from lacO-lacI-GFP negative and positive mice. Paraffin-embedded testis sections stained with Hematoxylin & Eosin are shown. (D) Terminal deoxynucleotidyl transferase dUTP nick end nuclear labeling (TUNEL) assay showing no significant changes in the number of apoptotic cells per seminiferous tubules of lacO-lacI-GFP transgenic (9X9) and control mice (CTRL). (E) Two representative images showing magnified meiotic chromosomes from mice with lacO-lacI-GFP inserted into chromosome 9 and immunostained for SYCP3, SYCP1, and lacI (the latter marked with blue arrows). Cells were stained with Hoechst to monitor for changes in chromatin condensation around lacO-lacI-GFP.


Zygotene
2μm
Hoechst
SYCP3
γH2AX
MERGE
Pachytene
Hoechst
SYCP3
γH2AX
MERGE

Supplementary Figure 2. Fluorescently labeled peri-centromeric heterochromatin can be used to visualize chromosome movements. Single-plane fluorescent images from 3D stacks of zygotene and pachytene nuclei in wild-type spermatocytes obtained from whole-mounted and immunostained seminiferous tubules. Hoechst is used to mark pericentromeric heterochromatin (marked by arrows). SYCP3 and γH2AX are used to determine the meiotic stage.

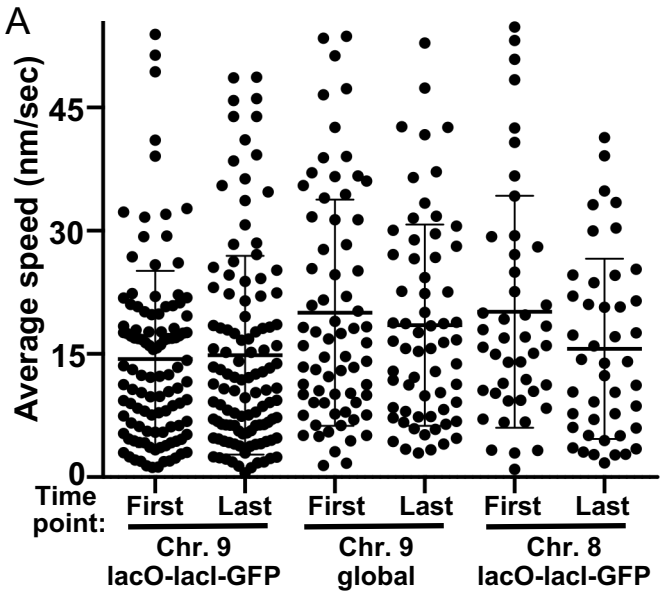
Average Speed

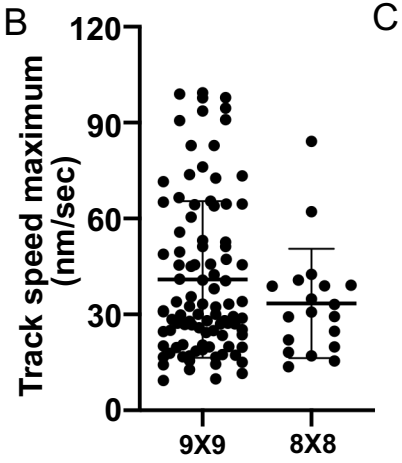
Tukey's multiple comparisons test	Compared values		Summary	Adjusted P Value
Chr. 9 Early – Chr. 9 Mid	7.45 ± 3.21	17.34 ± 8.28	****	<0.0001
Chr. 9 Early – Chr. 9 Late	7.45 ± 3.21	14.94 ± 6.91	**	0.0019
Chr. 9 Early – Chr. 8 Mid	7.45 ± 3.21	18.00 ± 7.23	****	<0.0001
Chr. 9 Early – Early Global	7.45 ± 3.21	7.48 ± 3.48	ns	>0.9999
Chr. 9 Early – Mid Global	7.45 ± 3.21	19.36 ± 6.95	****	<0.0001
Chr. 9 Mid – Chr. 9 Late	17.34 ± 8.28	14.94 ± 6.91	ns	0.5657
Chr. 9 Mid – Chr. 8 Mid	17.34 ± 8.28	18.00 ± 7.23	ns	0.9991
Chr. 9 Mid – Early Global	17.34 ± 8.28	7.48 ± 3.48	****	<0.0001
Chr. 9 Mid – Mid Global	17.34 ± 8.28	19.36 ± 6.95	ns	0.8069
Chr. 9 Late – Chr. 8 Mid	14.94 ± 6.91	18.00 ± 7.23	ns	0.6891
Chr. 9 Late – Early Global	14.94 ± 6.91	7.48 ± 3.48	*	0.0182
Chr. 9 Late – Mid Global	14.94 ± 6.91	19.36 ± 6.95	ns	0.2020
Chr. 8 Mid – Early Global	18.00 ± 7.23	7.48 ± 3.48	***	0.0007
Chr. 8 Mid – Mid Global	18.00 ± 7.23	19.36 ± 6.95	ns	0.9896
Early Global– Mid Global	7.48 ± 3.48	19.36 ± 6.95	****	<0.0001

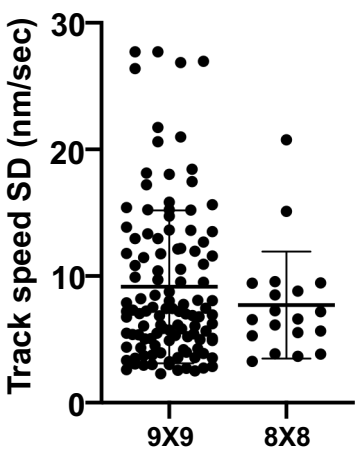
Track displacement length

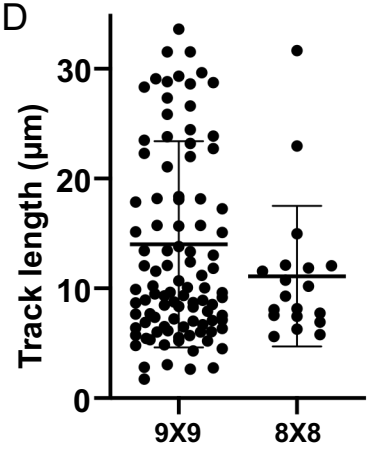
Tukey's multiple comparisons test	Compared values		Summary	Adjusted P Value
Chr. 9 Early – Chr. 9 Mid	2.63 ± 1.66	4.06 ± 1.88	*	0.0235
Chr. 9 Early – Chr. 9 Late	2.63 ± 1.66	4.58 ± 3.30	**	0.0061
Chr. 9 Early – Chr. 8 Mid	2.63 ± 1.66	5.40 ± 1.84	***	0.0002
Chr. 9 Early – Early Global	2.63 ± 1.66	2.41 ± 1.12	ns	0.9995
Chr. 9 Early – Mid Global	2.63 ± 1.66	4.17 ± 1.48	ns	0.0870
Chr. 9 Mid – Chr. 9 Late	4.06 ± 1.88	4.58 ± 3.30	ns	0.8053
Chr. 9 Mid – Chr. 8 Mid	4.06 ± 1.88	5.40 ± 1.84	ns	0.0918
Chr. 9 Mid – Early Global	4.06 ± 1.88	2.41 ± 1.12	ns	0.0564
Chr. 9 Mid – Mid Global	4.06 ± 1.88	4.17 ± 1.48	ns	0.9998
Chr. 9 Late – Chr. 8 Mid	4.58 ± 3.30	5.40 ± 1.84	ns	0.7322
Chr. 9 Late – Early Global	4.58 ± 3.30	2.41 ± 1.12	*	0.0142
Chr. 9 Late – Mid Global	4.58 ± 3.30	4.17 ± 1.48	ns	0.9774
Chr. 8 Mid – Early Global	5.40 ± 1.84	2.41 ± 1.12	***	0.0007
Chr. 8 Mid – Mid Global	5.40 ± 1.84	4.17 ± 1.48	ns	0.3639
Early Global– Mid Global	2.41 ± 1.12	4.17 ± 1.48	ns	0.1067

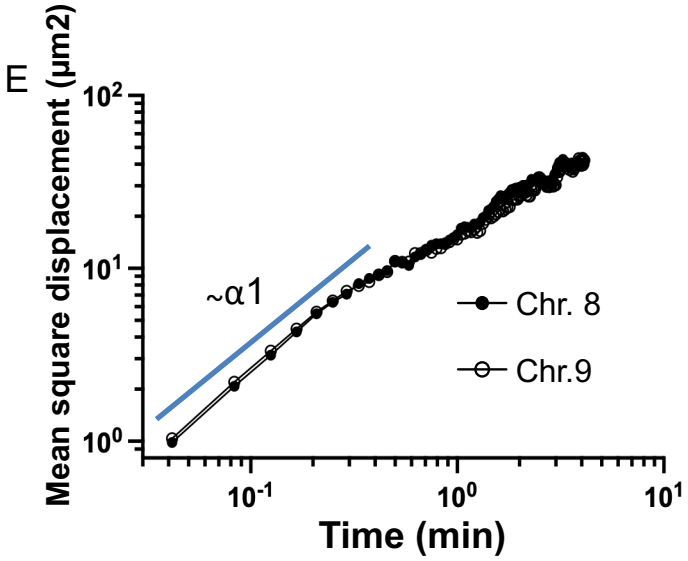
Supplementary Figure 3. lacO-lacI-GFP spot dynamics statistics. Tables containing statistics associated with average speed and track displacement length for chromosomes 9 and 8. Measurements for global (pericentromeric heterochromatin marker) and individual loci (revealed by the lacO-lacI-GFP insertion) motions are considered.





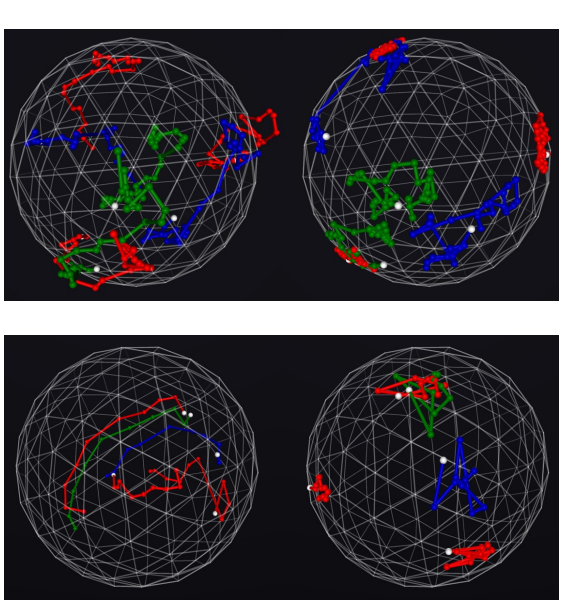




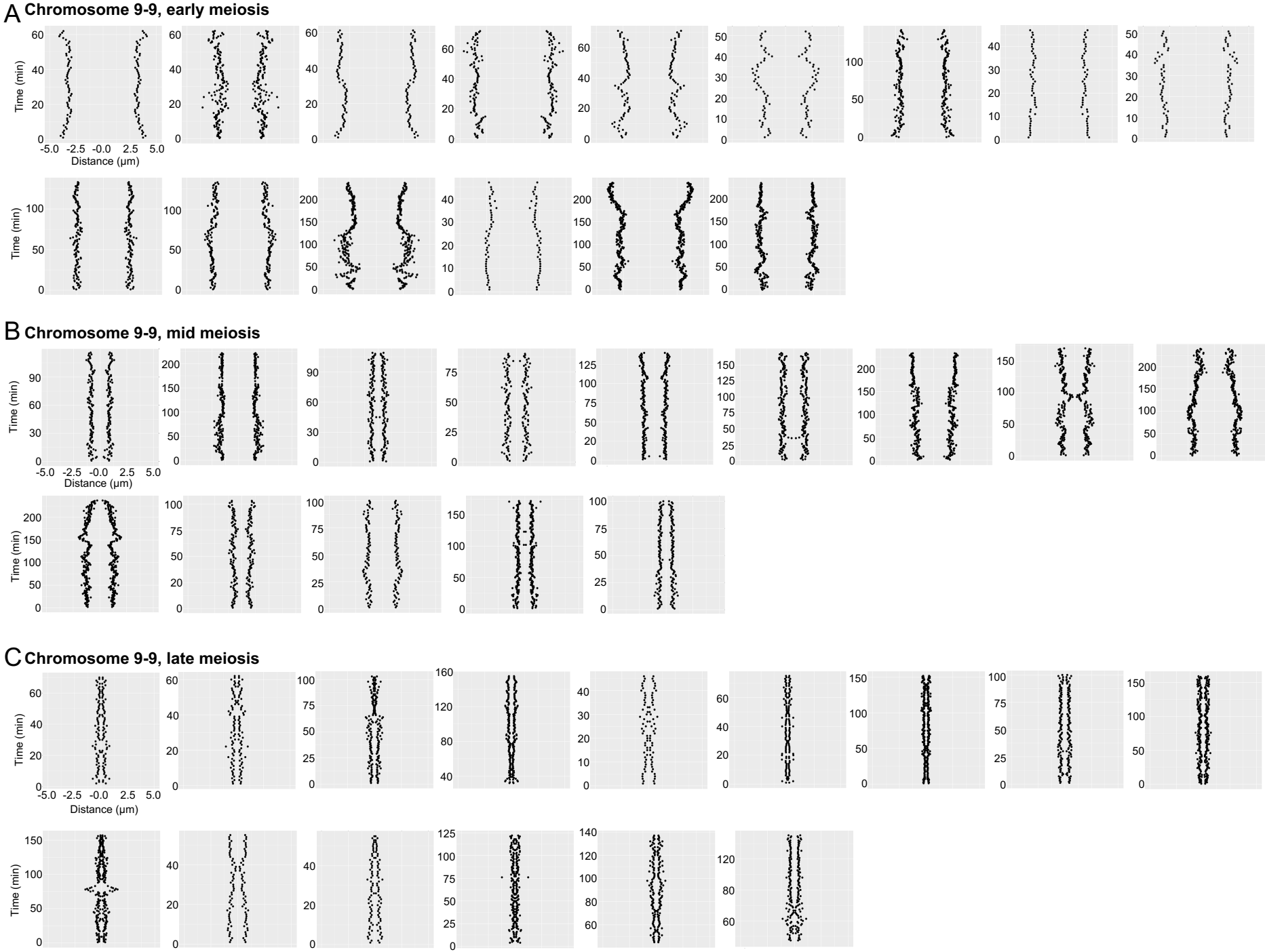


F

	Variable 1	*Variable 2*
Mean	0.2409	0.2181
Variance	0.0387	0.0252
Observations	37	50
Hypothesized Mean Difference	0	
df	68	
t Stat	0.5772	
P(T<=t) one-tail	0.2828	
t Critical one-tail	1.6675	
P(T<=t) two-tail	0.5656	
t Critical two-tail	1.9954	

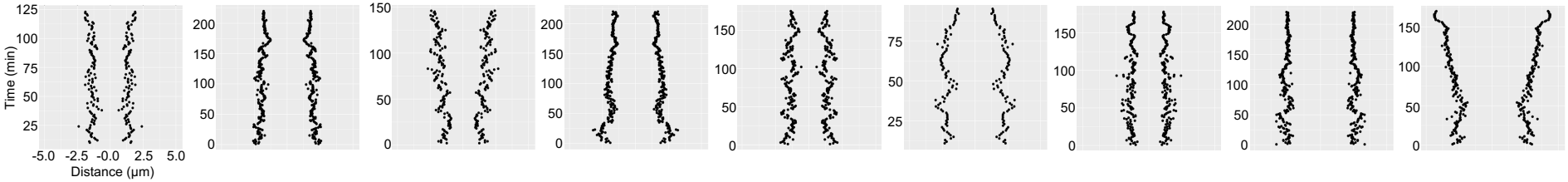


Supplementary Figure 4. Comparative analysis of chromosomes 9 and 8 RPM movements. (A) Average speeds at the first and last time points of a 10-minute RPM measurement period. Each dot represents a measurement obtained from one cell. Chromosome 9 lacO–lacI–GFP spot first versus last time point: 14.4 ± 10.7 vs. 14.8 ± 12.11 (P = 0.999); chromosome 9 Hoechst blobs (global): 21.2 ± 15.2 vs. 19.9 ± 14.4 (P = 0.998); chromosome 8 lacO-lacI-GFP spot: 20.1 ± 14.1 vs. 15.6 ± 10.9 (P = 0.4913). No statistically significant differences were observed between the first and last time points for any measurement. Track speed maximum (B), track speed standard deviation (SD) (C), track length (D), and mean square displacement (E) were compared as parameters of chromosome movements for lacO-lacI-GFP insertions in chromosomes 9 (9X9) and 8 (8X8) in mid meiotic cells. Mean square displacement and the alpha factor were calculated as described in Methods. Values at each time point in the curve correspond to the average of several measurements from cells at mid meiotic stage. (B) track speed maximum, mean ± SD, chr. 9 mid meiosis, 40.92 ± 24.5 nm/sec, number of cells, n = 125, 5 biological replicates. Chr.8 mid meiosis, 33.42 ± 17.1 nm/sec, n = 20, 3 biological replicates. P = 0.21. (C) Track speed standard deviation, chr. 9, 9.15 ± 6.04 nm/sec. Chr.8, 7.71 ± 4.23 nm/sec. P = 0.32. (D) Track length, chr. 9, 14.01 ± 9.39 nm/sec. Chr.8, 11.11 ± 6.41 nm/sec. P = 0.20. (F) Descriptive statistics and P value obtained when comparing RPM characteristics in spermatocytes carrying lacO-lacI-GFP spots that mark both chromosomes 8 and 9 within the same nucleus. Variable 1 and variable 2 correspond to data obtained from 9X9 and 8X8, respectively. (G) Examples of nuclei showing raw versus subtracted nuclear rotation datasets. The red spots are the registration spots, the blue are "extra" Hoechst spots, and the green correspond to lacO-lacI-GFP spots. The sphere represents the measured sphere onto which spots are projected in the algorithm's final step. The upper and lower panels show examples of nuclei from spermatocytes in which chromosomes 9 or 8 are marked by lacO-lacI-GFP insertions, respectively.

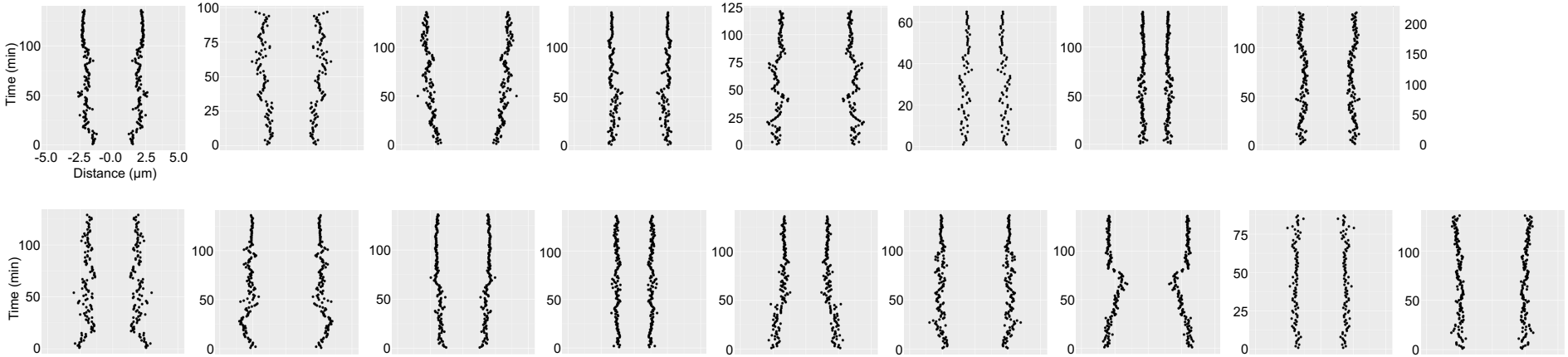

A Chromosome 9-9, early meiosis
B Chromosome 9-9, mid meiosis
C Chromosome 9-9, late meiosis
Time (min)
Distance (μm)

Supplementary Figure 5. Homologous chromosome pairing (chromosome 9X9) across meiotic stages. (A) Examples of Inter-homolog distances in early meiotic cells showing wide separation. (B) Mid meiotic cells exhibit intermediate distances consistent with an intermediate state of pairing. (C) Late meiotic cells show inter-homolog distances corresponding to synapsis.

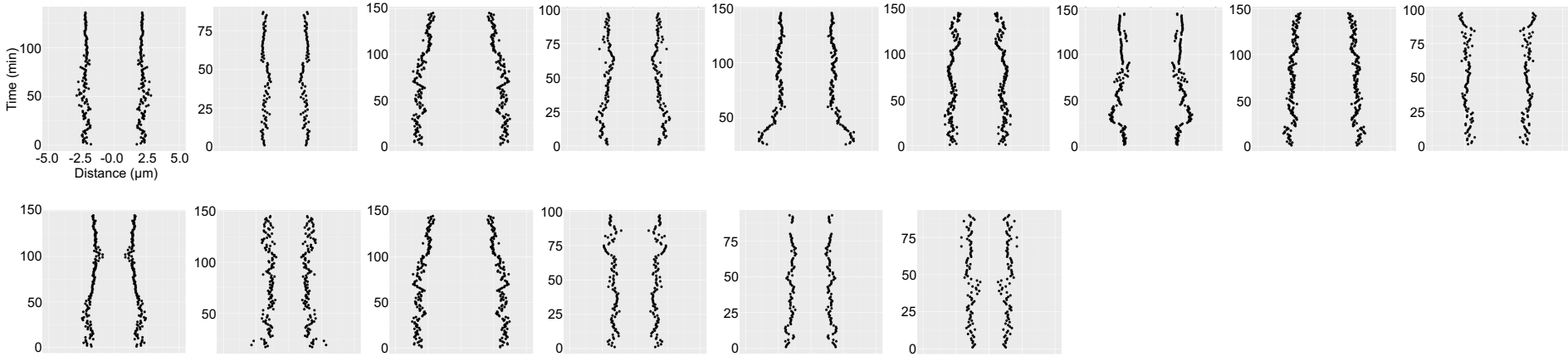
Chromosome 8-9, early meiosis



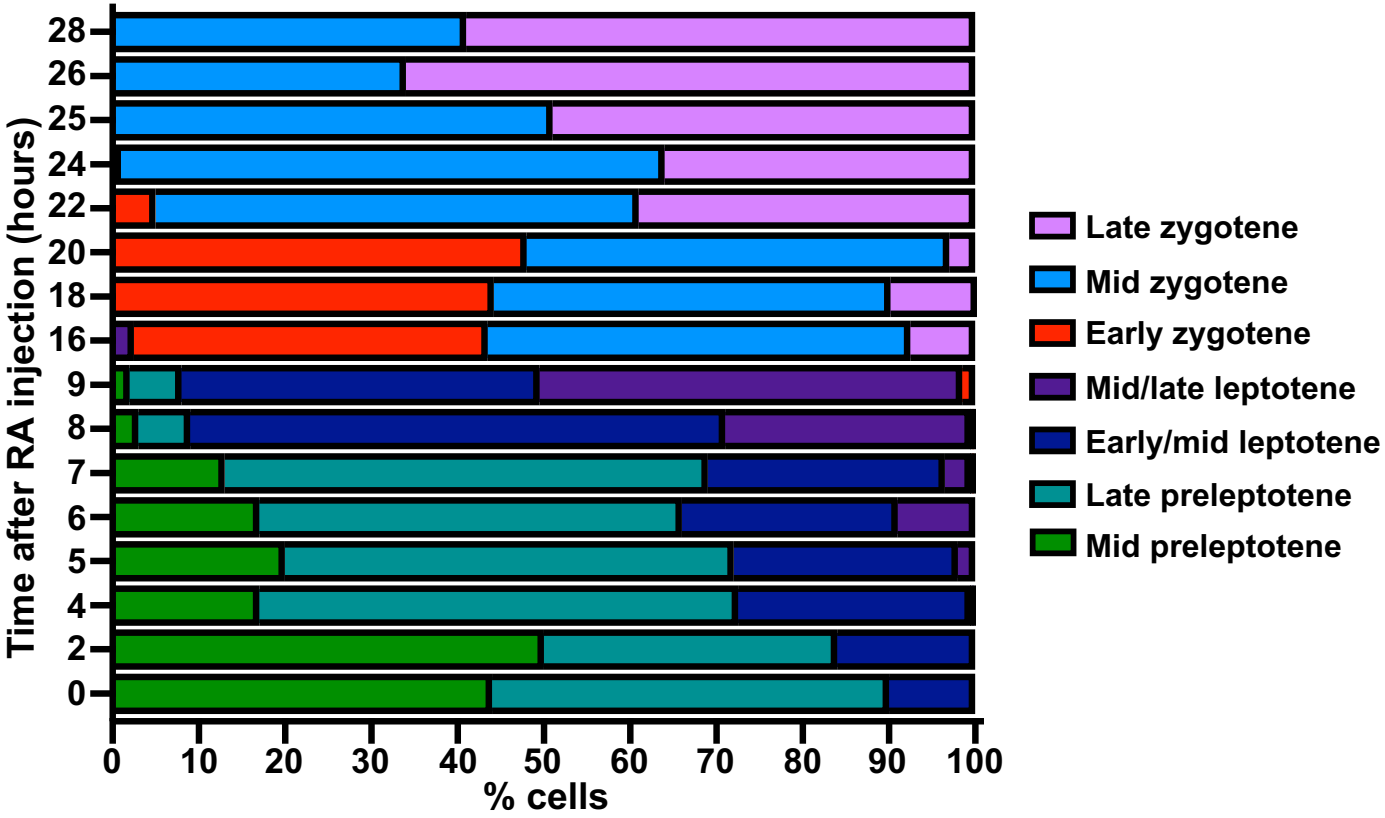
Chromosome 8-9, mid meiosis



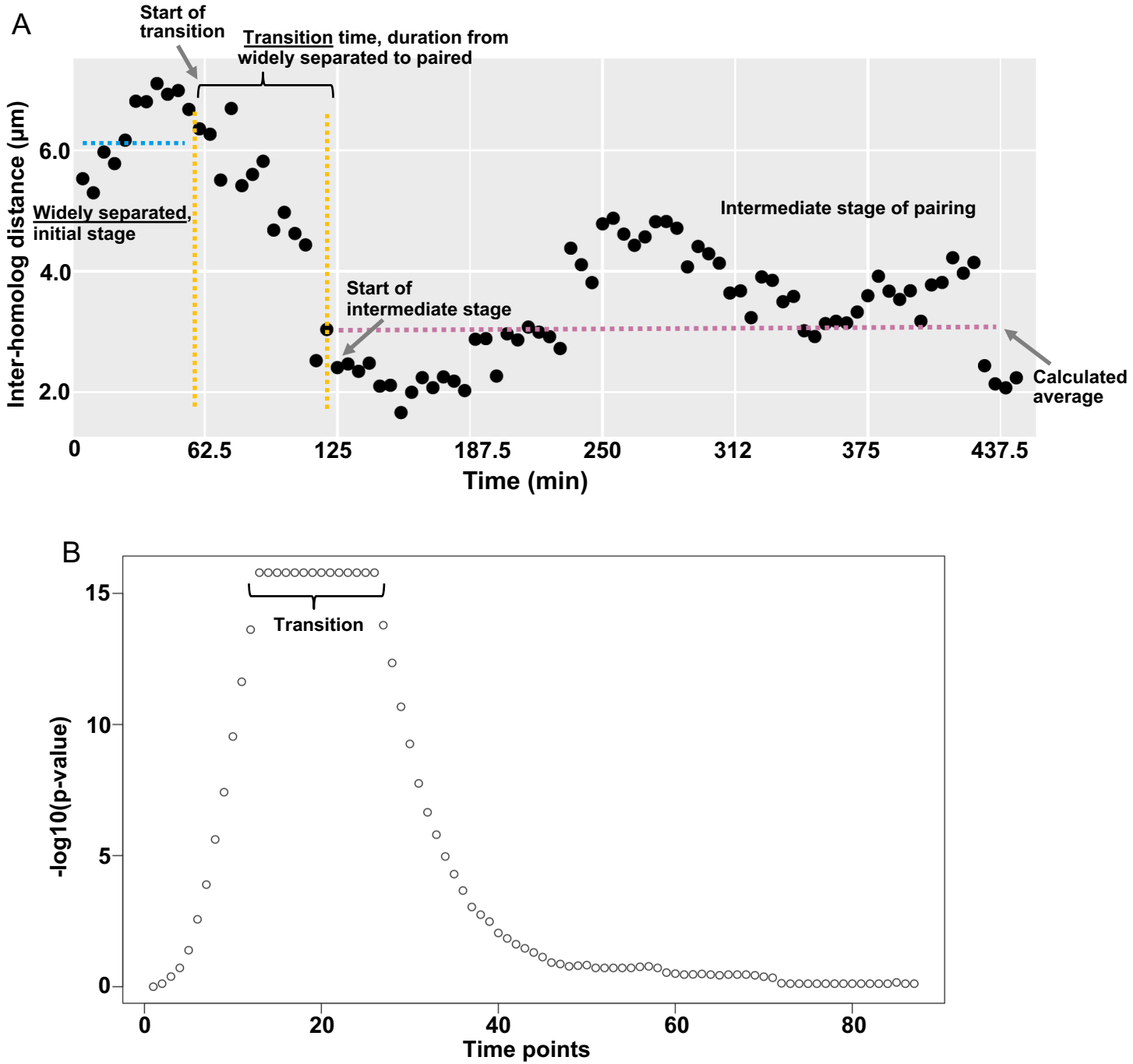
Chromosome 8-9, late meiosis



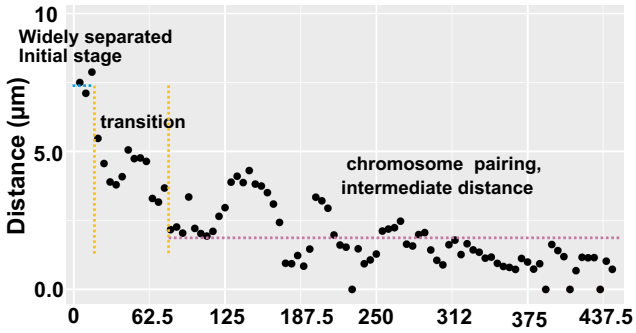
Supplementary Figure 6. Distances between heterologous chromosomes across meiotic stages. Early, mid, and late meiotic cells show a persistent wide separation between the loci on chromosomes 8 and 9.

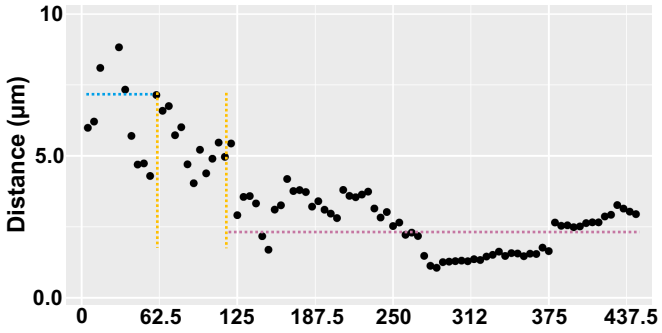

Time after RA injection (hours)
28
26
25
24
22
20
18
16
9
8
7
6
5
4
2
0
0
10
20
30
40
50
60
70
80
90
100
% cells
Late zygotene
Mid zygotene
Early zygotene
Mid/late leptotene
Early/mid leptotene
Late preleptotene
Mid preleptotene

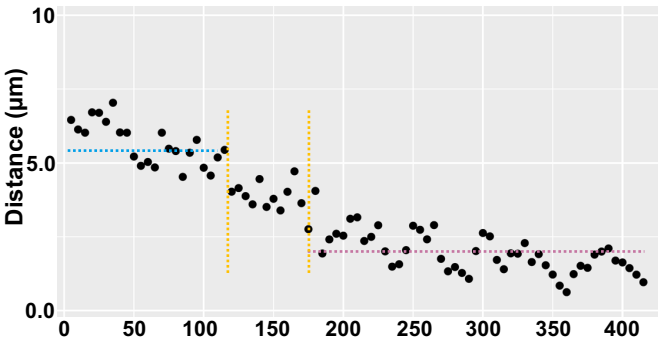
Supplementary Figure 7. Meiotic progression in synchronized mouse spermatocytes. Time-course quantification of meiotic cell populations at various times post-retinoic acid injection, showing synchronized entry into meiosis and progression through prophase I stages. 0 = 6 days and 12 hours after retinoic acid injection.

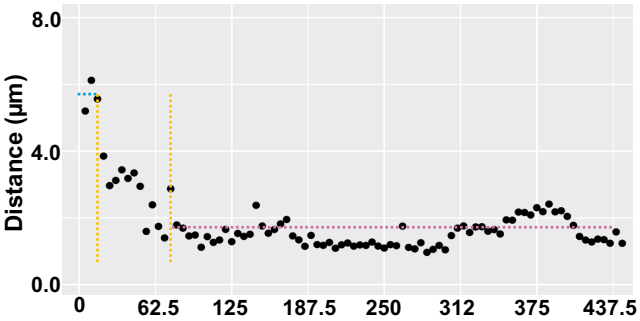

A
Start of transition
Transition time, duration from widely separated to paired
Widely separated, initial stage
Intermediate stage of pairing
Start of intermediate stage
Calculated average
Inter-homolog distance (µm)
6.0
4.0
2.0
0
62.5
125
187.5
250
312
375
437.5
Time (min)
B
Transition
-log10(p-value)
15
10
5
0
0
20
40
60
80
Time points

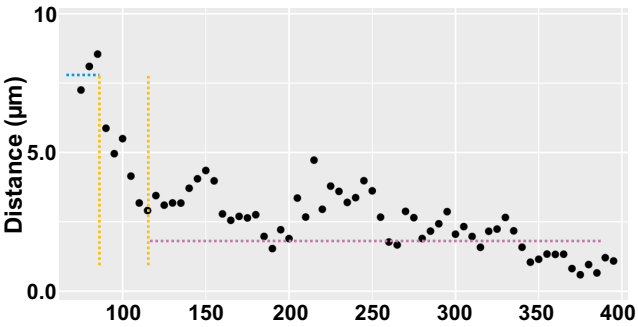
Supplementary Figure 8. Examples of homologous chromosome pairing and stage transition. (A) Representative kymograph showing relatively stable stages of chromosome interactions and transition from widely separated to an intermediate pairing distance. (B) Example of software detection of pairing stages and detection of transitions (Chow method) between pairing stages, supporting a rapid and discrete pairing mechanism. This method detects whether a significant "structural break" or shift occurs in the relationship between variables. The plot displays p-values at -log10(p-value) versus time. Because some p-values were p=0 (and log(0) is undefined), we shifted all p-values by the smallest non-zero p-value for each data set.

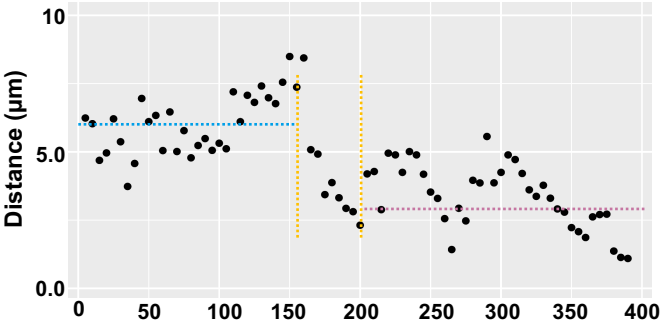

Widely separated
Initial stage
transition
chromosome pairing,
intermediate distance
Distance (µm)
10
5.0
0.0
0
62.5
125
187.5
250
312
375
437.5


Distance (µm)
10
5.0
0.0
0
62.5
125
187.5
250
312
375
437.5

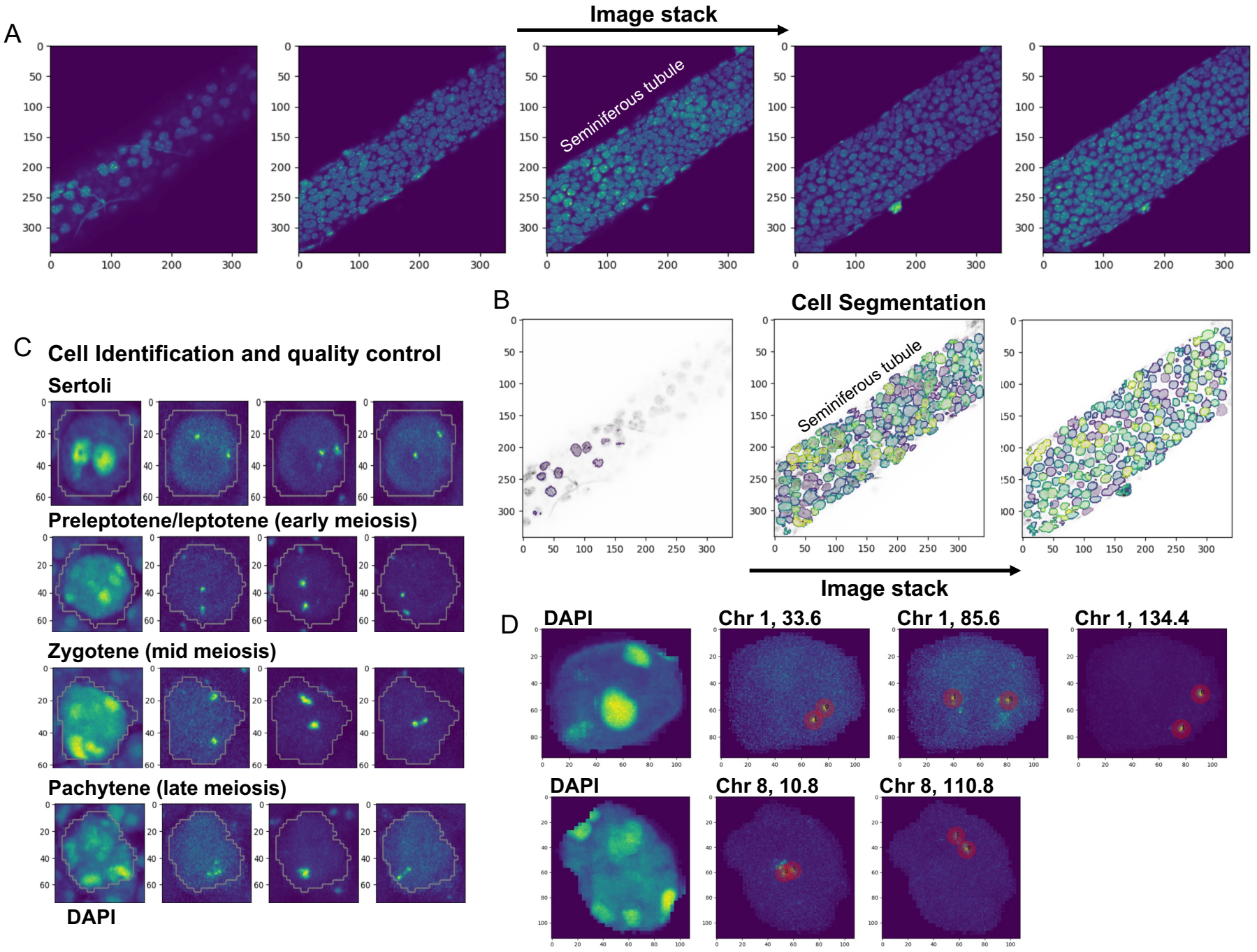

Distance (µm)
10
5.0
0.0
0
50
100
150
200
250
300
350
400


Distance (µm)
8.0
4.0
0.0
0
62.5
125
187.5
250
312
375
437.5

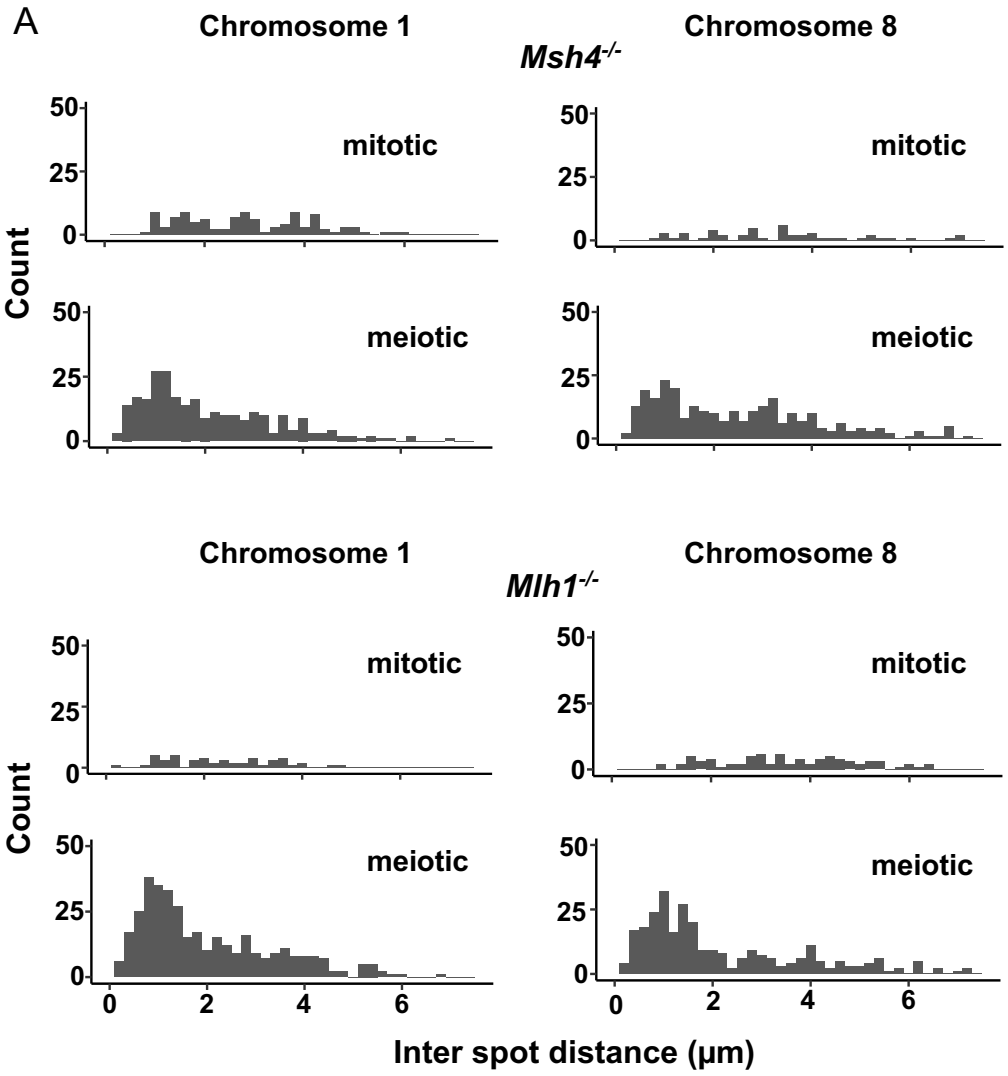

Distance (µm)
10
5.0
0.0
100
150
200
250
300
350
400

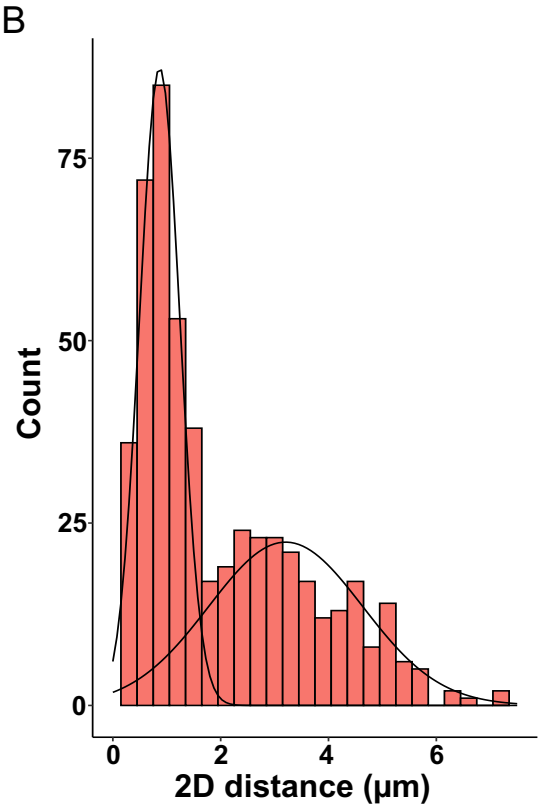

Distance (µm)
10
5.0
0.0
0
50
100
150
200
250
300
350
400

Supplementary Figure 9. Long-term imaging of homolog pairing and transition. Additional kymographs showing stages of chromosome interactions (chromosome 9) and the transition from widely separated to an intermediate pairing distance.


A
Image stack
Seminiferous tubule
B
Cell Segmentation
Seminiferous tubule
Image stack
C
Cell Identification and quality control
Sertoli
Preleptotene/leptotene (early meiosis)
Zygotene (mid meiosis)
Pachytene (late meiosis)
DAPI
D
DAPI
Chr 1, 33.6
Chr 1, 85.6
Chr 1, 134.4
DAPI
Chr 8, 10.8
Chr 8, 110.8

Supplementary Figure 10. Image processing pipeline for meiotic nuclei classification and fluorescent spot segmentation. (A) Representative image of a whole-mounted seminiferous tubule stack used for segmentation. (B) Representative image showing nuclear segmentation for some of the tubule stacks shown in A. (C) Classification of meiotic spermatocytes and mitotic cell types. Cells are identified using the distribution of heterochromatin, nuclear size, and location within a seminiferous tubule. The leftmost column shows DAPI staining, the other three show DNA FISH in three separate channels. (D) Example of fluorescent spot segmentation in two different meiotic nuclei.


A
Chromosome 1
Chromosome 8
Msh4-/-
mitotic
meiotic
Count
Chromosome 1
Chromosome 8
Mlh1-/-
mitotic
meiotic
Count
Inter spot distance (µm)


B
Count
2D distance (µm)

Supplementary Figure 11. Characterization of inter-homolog distance in meiotic prophase cells. (A) The inter-spot distance distribution is similar for chromosomes 1 and 8 in mouse spermatocytes in mid-prophase and mitotic cells. (B) Bimodality in the inter-spot distance distribution in mid meiotic prophase cells.

Parents genotype:	1st litter	2nd litter	3rd litter	4th litter
8x8				
F Chr.8. LacO^{+}, LacI^{+}	4 females	0 females	3 females	1 female
x	4 males	4 males	1 males	3 males
M Chr.8. LacO^{+}, LacI^{+}	**8**	**4**	**4**	**4**
F Chr.8. LacO^{+}, LacI^{+}	2 females	2 females	2 females	6 females
x	5 males	3 males	3 males	5 males
M Chr.8. LacO^{+}, LacI^{+}	**7**	**5**	**5**	**11**
F Chr.8. LacO^{+}, LacI^{+}	4 females	3 females	2 females	
x	1 male	4 males	2 males	
M Chr.8. LacO^{+}, LacI^{+}	**5**	**7**	**4**	
9x9				
F Chr.9. LacO^{+}, LacI^{+}	2 females	3 females	6 females	4 females
x	6 males	3 males	5 males	3 males
M Chr.9. LacO^{+}, LacI^{+}	**8**	**6**	**11**	**7**
F Chr.9. LacO^{+}, LacI^{+}	3 females	4 females	5 females	3 females
x	2 males	2 males	1 males	2 males
M Chr.9. LacO^{+}, LacI^{+}	**5**	**6**	**6**	**5**
F Chr.9. LacO^{+}, LacI^{+}	1 female	2 females	3 females	
x	3 males	5 males	2 males	
M Chr.9. LacO^{+}, LacI^{+}	**4**	**7**	**5**	
8x9				
F Chr.8. LacO^{+}, LacI^{+}	7 females	4 females	4 females	4 females
x	4 males	1 male	4 males	3 males
M Chr.9. LacO^{+}, LacI^{+}	**11**	**5**	**8**	**7**
F Chr.8. LacO^{+}, LacI^{+}	3 females	4 females	3 females	
x	4 males	4 males	3 males	
M Chr.9. LacO^{+}, LacI^{+}	**7**	**8**	**6**	
F Chr.9. LacO^{+}, LacI^{+}	5 females	3 females	1 female	
x	3 males	1 male	4 males	
M Chr.8. LacO^{+}, LacI^{+}	**8**	**4**	**5**	
CTRL				
F LacO^{-}, LacI^{-}	1 females	2 females	2 females	3 females
x	3 males	7 males	2 males	3 males
M LacO^{-}, LacI^{-}	**4**	**9**	**4**	**6**
F LacO^{-}, LacI^{-}	2 females	3 females	3 females	2 females
x	2 males	5 males	2 males	3 males
M LacO^{-}, LacI^{-}	**4**	**8**	**5**	**5**
F LacO^{-}, LacI^{-}	2 females	3 females	4 females	
x	2 males	3 males	3 males	
M LacO^{-}, LacI^{-}	**4**	**6**	**7**	

Supplementary Table 1. Fertility test.

Antibody	Source	Western blot dilution	Immuno-labeling dilution
α-tubulin	Proteintech, 66031-1-Ig	1:5,000	
LacI	Kerafast, EG1501	1:500	1:100
SYCP3	Pezza Lab.		1:300
SHOC1	Pezza Lab.		1:100
HFM1	Pezza Lab.		1:100

Supplementary Table 2. Antibody list.