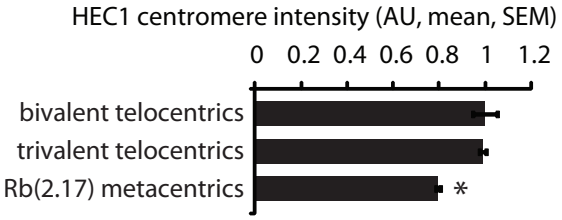
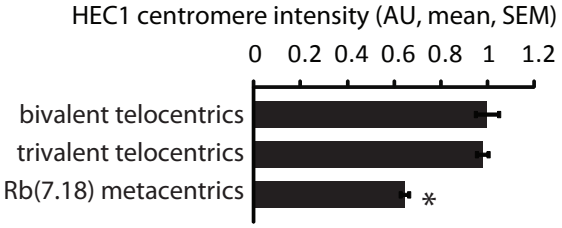
Figure S1

A

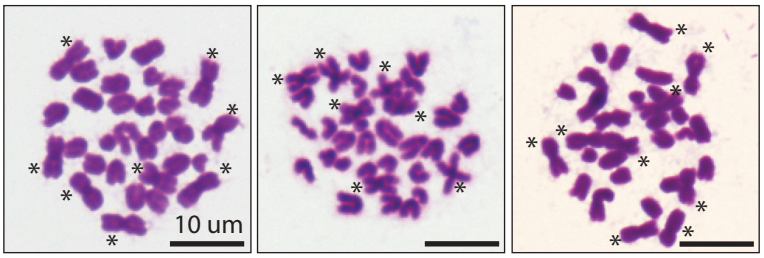

HEC1 centromere intensity (AU, mean, SEM)
0 0.2 0.4 0.6 0.8 1 1.2
bivalent telocentrics
trivalent telocentrics
Rb(2.17) metacentrics
*

B

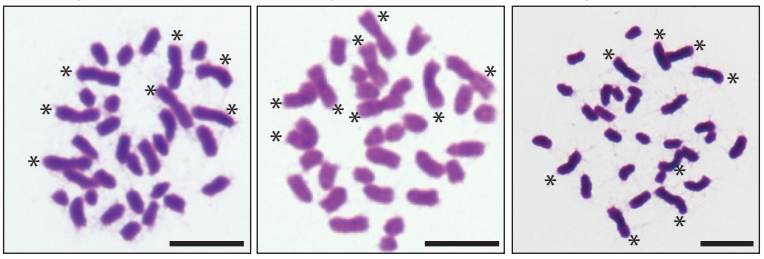

HEC1 centromere intensity (AU, mean, SEM)
0 0.2 0.4 0.6 0.8 1 1.2
bivalent telocentrics
trivalent telocentrics
Rb(7.18) metacentrics
*

Supplementary Figure 2

A Barcelona locality
(La Granada), EBAR



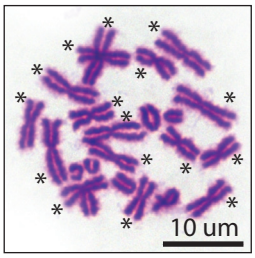
male 1, 2n=32 male 2, 2n=33 male 3, 2n=31



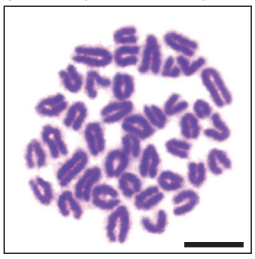
female 1, 2n=33 female 2, 2n=32 female 3, 2n=32

			Rb fusion between chromosomes no.					
EBAR	**sex**	**2n**	**(3.8)**	**(4.14)**	**(5.15)**	**(6.10)**	**(9.11)**	**(12.13)**
130925/1	M1	32	-	HOM	HOM	-	HOM	HOM
130925/2	M2	33	-	HOM	HOM	-	HET	HOM
130925/3	M3	31	-	HOM	HOM	HET	HOM	HOM
130926/1	F1	33	HET	HOM	HOM	HET ?	HET	-
130926/2	F2	32	HET	HET	HOM	-	HOM	HOM
130926/3	F3	32	-	HET	HOM	HET	HOM	HOM

B Greece locality
(Patras) GROL



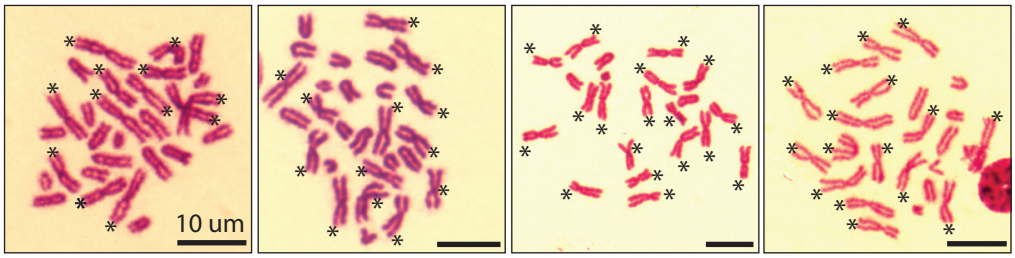
2n=24
(Razata, Faraklada, Nea Artaki)



2n=40

All GROL animals had identical karyotype with homozygous Rb fusions between chromosomes (1.3), (2.5), (4.6), (8.12), (9.16), (10.14), (11.17).

C Madeira locality
(Ponta Delgada) PPOD (Santana) PSAN (Estreito da Calheta) PEDC



2n=28 2n=27 2n=22 2n=24

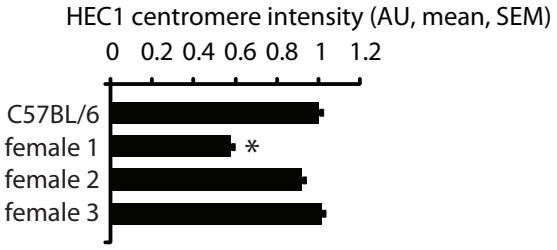
			Rb fusion between chromosomes no.								
PSAN	**sex**	**2n**	**(2.19)**	**(3.8)**	**(4.16)**	**(5.14)**	**(6.7)**	**(9.10)**	**(11.12)**	**(13.17)**	**(15.18)**
130930/2	M1	22	HOM	HOM	HOM	HOM	HOM	HOM	HOM	HOM	HOM
130930/2	M2	22	HOM	HOM	HOM	HOM	HOM	HOM	HOM	HOM	HOM
130930/3	M3	22	HOM	HOM	HOM	HOM	HOM	HOM	HOM	HOM	HOM

			Rb fusion between chromosomes no.							
PEDC	**sex**	**2n**	**(2.4)**	**(3.14)**	**(5.18)**	**(6.7)**	**(8.11)**	**(9.12)**	**(11.16)**	**(13.17)**
130930/2	M4	22	HOM	HOM	HOM	HOM	HOM	HOM	HOM	HOM
130930/2	M5	22	HOM	HOM	HOM	HOM	HOM	HOM	HOM	HOM
130930/3	M6	22	HOM	HOM	HOM	HOM	HOM	HOM	HOM	HOM

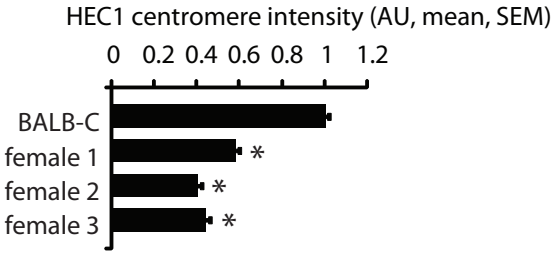
			Rb fusion between chromosomes no.							
PPOD	**sex**	**2n**	**(3.8)**	**(4.5)**	**(9.18)**	**(10.16)**	**(11.12)**	**(14.17)**	**(6.7)**	
130930/2	M7	22	HOM	HOM	HOM	HOM	HOM	HOM	HET	
130930/2	M8	22	HOM	HOM	HOM	HOM	HOM	HOM	HET	
130930/3	M9	22	HOM	HOM	HOM	HOM	HOM	HOM	-	

Figure S3

A MI oocytes, Barcelona - EBAR locality (2n=29-35)



B MI oocytes, Greece locality (2n=40)



C MI oocytes, Greece - GROL locality (2n=24)

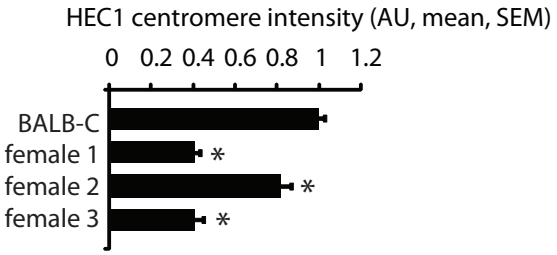
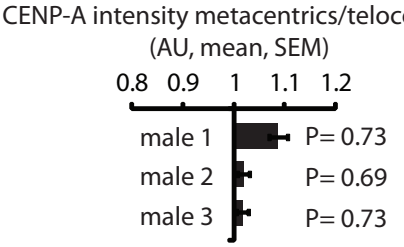
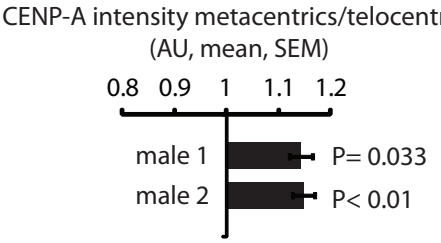


Figure S4

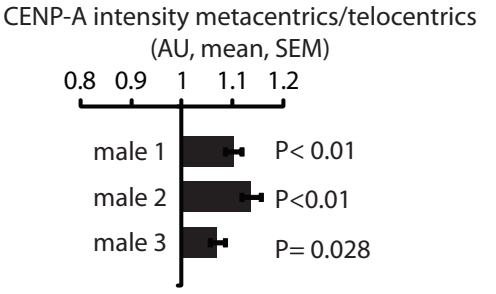
A Barcelona - EBAR (2n=29-35)



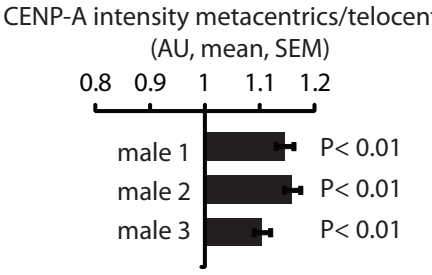
B Greece - GROL (2n=24)



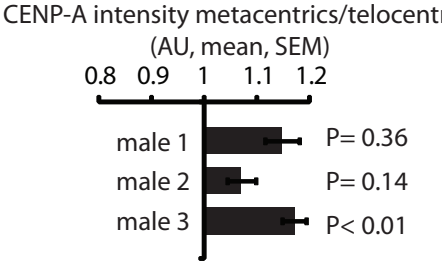
C Madeira - PEDC (2n=24)



Madeira - PPOD (2n=27-28)



Madeira - PSAN (2n=22)



Supplemental Figure Legends

Figure S1. Centromere HEC1 quantification of Rb(2.17) and Rb(7.18) metacentrics. Related to Figure 1. MI oocytes from the Rb(2.17) x CF-1 cross (A, n=70) or the Rb(7.18) x CF-1 cross (B, n=50) were fixed and stained for DNA and HEC1. HEC1 staining was quantified at centromeres from the metacentric and the homologous telocentrics in the trivalent, and from other telocentrics. Asterisks indicate that metacentrics are statistically different from telocentrics ($P<0.05$); AU: arbitrary units.

Figure S2. Karyotypes of mice from natural metacentric populations. Related to Figure 4. Bone marrow cells were collected from mice from the indicated localities, and karyotypes were obtained by Giemsa staining. Representative karyotypes for each animal (EBAR, A) or locality (GROL,B ;PPOD, PSAN, PEDC, C) are shown with the number of chromosomes indicated. Black asterisks mark metacentrics. The tables show the homozygous (HOM) or heterozygous (HET) constitution for a given metacentric for each individual (EBAR) or population (PPOD, PSAN, PEDC).

Figure S3. HEC1 quantification in MI oocytes from natural metacentric populations. Related to Figure 4. MI oocytes from animals from the indicated localities, together with laboratory strain C57BL/6 or BALB/c for comparison, were fixed and stained for DNA and HEC1. HEC1 staining was quantified in randomly chosen centromeres. Each bar represents $n\geq140$ centromeres from at least 8 oocytes from a single animal. Asterisks indicate that centromere HEC1 signal is statistically different from lab strains ($P<0.05$), AU: arbitrary units.

Figure S4. CENP-A quantification in primary spermatocytes from natural metacentric populations. Related to Figure 4. Spermatocytes ($n\geq29$) from males from wild metacentric

populations (EBAR, GROL, PEDC, PPOD, PSAN) were fixed and stained for CENP-A, together with synaptonemal complex protein SYCP2. CENP-A staining was quantified for the metacentrics (n≥91) and for the telocentrics (n≥34) and plotted as a ratio. P values indicate whether the ratio is significantly different from one, AU: arbitrary units.

Supplemental Experimental Procedures

Oocyte collection and culture

Mouse strains were purchased from the Jackson Laboratory [Rb(6.16)24Lub, stock# 000885; B6Ei.Cg-Rb(7.18)9Lub/J, stock #001615; B6Ei.Cg-Rb(2.17)11Rma/J, stock #001387; C57BL/6J, stock# 000664; BALB/c, stock# 000651; *Mus pahari*/EiJ, stock #002655; CAROLI/EiJ, stock #000926; ZALENDE/EiJ, stock #001392; CAST/EiJ, stock #000928; PWD/PhJ, stock #004660] or Harlan NSA (CF-1, solicitation # DL3171940). Female mice (7-14 wk-old) were hormonally primed with 5U of Pregnant Mare Serum Gonadotropin (PMSG, Calbiochem, cat# 367222) 48 h prior to oocyte collection. Germinal vesicle (GV)-intact oocytes were collected in bicarbonate-free minimal essential medium with polyvinylpyrrolidone and Hepes (MEM-PVP), denuded from cumulus cells, and cultured in Chatot-Ziomek-Bavister (CZB) medium covered with mineral oil (Sigma, cat# M5310) in an humidified atmosphere of 5% CO_2 in air at 37°C. Meiotic resumption was inhibited by addition of 7.5 μM milrinone (Sigma-Aldrich) (*Mus pahari*/EiJ, CAROLI/ EiJ, PWD/PhJ, CAST/EiJ) or 2.5 μM milrinone (all others). Milrinone was subsequently washed out to allow meiotic resumption. Oocytes were checked for nuclear envelope breakdown (GVBD) 1.5 h after the washout, and those that did not enter GVBD were removed from the culture. MI oocytes were fixed 6-7.5 h after GVBD

(depending on mouse species) when the spindle was fully developed and close to anaphase I. All animal experiments were approved by the Institutional Animal Care and Use Committee and were consistent with the National Institutes of Health guidelines.

Sucrose spreading of mouse spermatocyte chromosomes

A modification of a chromosome spreading protocol [S1] was used. Mouse testes were collected from 8-12 wk-old males, and individual seminiferous tubules were transferred to 3 ml of ice cold freshly made hypotonic buffer for 60 min. Small sections of tubules were placed on depression slides (Science Lab, cat # 10-1305-8) in 32 μl of 100 mM sucrose (pH 8.2) and minced with two scalpel blades until most of the tubules were cut and liquid was cloudy. Any large chunks of tubules were removed and another 32 μl of sucrose were added and mixed with the sample, followed by spreading 30 μl of cell suspension on slides dipped into freshly made 1% PFA (pH 9.2, 0.15% Triton X-100 in dH_2O). Slides were then placed directly into a humidified chamber covered with a lid. After 2.5 h the lid was left half-open for an additional 30 min. After drying, slides were washed twice in Photoflo/PBS for 5 min followed by antibody staining.

Oocyte immunocytochemistry

Mouse oocytes were fixed in freshly prepared 2% paraformaldehyde in PBS with 0.1% Triton X-100, pH 7.4, for 25 min at room temperature, placed in blocking solution (PBS containing 0.3% BSA and 0.01% Tween-20) overnight at 4°C, then permeabilized in PBS with 0.3% BSA and 0.1% Triton X-100 for 20 min, and washed in blocking solution for 20 min before primary antibody staining. Human CREST autoimmune serum (1:100 dilution, PerkinElmer) or HEC1 rabbit polyclonal anti-mouse antibody (1:500, a gift from R. Benezra) were used to label centromeres or kinetochores. Rabbit anti-mouse AURKA antibody (1:1000, BETHYL, cat# 800-

338-9579) was used to detect spindle poles. After 1 h incubation with primary antibodies, cells were washed three times for 15 min. Secondary antibodies were Alexa Fluor 594–conjugated goat anti–human or donkey anti-rabbit (1:500, Invitrogen). Cells were washed three times for 15 min in blocking buffer and mounted in Vectashield with bisbenzimide (Hoechst 33342, Sigma-Aldrich) to visualize chromosomes. To improve HEC1 staining, oocytes were placed in ice-cold MEM-PVP medium for 8 min prior to fixation to depolymerize microtubules.

Confocal images were collected as z-stacks at 0.3-µm intervals to visualize all chromosomes (25-30 µm range) using a microscope (DMI4000 B; Leica) equipped with a 63× 1.3 NA glycerol-immersion objective lens, an xy piezo Z stage (Applied Scientific Instrumentation), a spinning disk confocal scanner (Yokogawa Corporation of America), an electron multiplier charge-coupled device camera (ImageEM C9100-13; Hamamatsu Photonics), and an LMM5 laser merge module with 488- and 593-nm diode lasers (Spectral Applied Research) controlled by MetaMorph software (Molecular Devices). For HEC1 quantitation in MI oocytes, regions were drawn manually around individual centromeres (15 per oocyte) using ImageJ, and the mean intensity was calculated at the brightest plane after background subtraction. The average was calculated for each group (CF-1, n=60; C57BL/6J, n= 76; *M. pahari*, n=30; *M. m. domesticus*, n=51; CHPO, n=79; *M. caroli*, n=17; *M. m. castaneus*, n=38; EBAR, n=70; GROL, n=40; Greece 2n=40, n=30) and normalized to the CF-1 strain for comparison. For oocytes from wild mice, HEC1 intensity was normalized to available laboratory strains (C57BL/6J or BALB/c) and then normalized to CF-1 based on ratios calculated from HEC1 staining in these strains. Statistical differences for metacentric (EBAR, GROL) or telocentric (Greece, 2n=40) populations vs. laboratory strains (C57BL/6J and BALB/c) were determined by an unpaired t-test.

For the bivalent positioning assay (Figure 2C-E), GV oocytes from 6-12 wk old CF-1 and CHPO x CF-1 females were collected, matured *in vitro* into MI (7.5-8 h after GVBD), fixed and stained for HEC1, AURKA (or tubulin) and DNA. To preserve the chromosome position within the spindle, no cold treatment was applied. AURKA staining poles (or tubulin) provided a spatial reference for scoring the position of telocentric bivalents (Figure 2C), based on bivalent position in the spindle and orientation with respect to the HEC1-brighter and -dimmer centromeres. To measure the position of CF-1 and CF-1 x CHPO bivalents, the position of each bivalent was calculated (d; in Figure 2D) as the distance from each bivalent, projected onto the pole-pole axis kinetochore, to the spindle midzone (calculate as the midpoint between the two spindle poles). To quantify the differences in centromere HEC1 staining between CHPO metacentrics and CHPO telocentrics (Figure 3B), the HEC1 signal was quantified for most CHPO metacentrics and CHPO telocentrics in each oocyte; the statistical difference between metacentrics and telocentrics was calculated by an unpaired t-test. A representative experiment is shown (Figure 3B), and similar results were obtained in three independent experiments.

Spermatocyte immunocytochemistry

Mouse spermatocytes were spread on glass slides (Fisherfinest, #12-544-3) as described above, incubated for 10 min at room temperature in 0.4% Photoflo (Fisher, cat #1464510)/PBS, followed by 10 min in 0.01% Triton-X100/PBS and 10 min in 10% antibody dilution buffer (ADB)/PBS (ADB: 3g BSA, 10 ml of goat serum, 250 μl of 20% Triton X-100, 10 ml of 10x PBS, into 100ml of dH_2O). Slides were incubated with rabbit monoclonal anti-mouse CENP-A antibody (1:400, clone C51A7, Cell Signaling, cat# 2048) and guinea pig anti-mouse SYCP2 antibody (1:200, a gift from J. Wang) overnight at room temperature in a humidified chamber, washed for 10 min in Photoflo/PBS, Triton-X/PBS and ADB/PBS sequentially, and incubated

for 1.5 h at 37°C with FITC conjugated donkey anti-guinea pig secondary antibody (1:100, Vector Laboratories) and Alexa-594 conjugated donkey anti-rabbit secondary antibody (1:2000, Invitrogen). Slides were then washed three times, 10 min each, with 0.4% Photoflo/PBS and once with 0.4% Photoflo/dH_2O for 10 min and mounted with Vectashield with Hoechst 33342 on a 24x40mm #1.5 cover glass (Thermo Scientific, cat# 152440). From each slide at least 10 primary spermatocytes at the diplotene stage of prophase I were selected based on the distinct SYCP2 staining pattern [S1] and imaged as a z-stack at 0.2-μm intervals to visualize all chromosomes, using the confocal microscope described above. All metacentric and telocentric chromosomes in each cell were identified by the characteristic centromere (CENP-A) position. For CENP-A quantitation the mean intensity at each centromere was calculated from a z-projection. Average CENP-A intensity was calculated after background subtraction for metacentric and telocentric chromosomes. The ratio of metacentric/telocentric was calculated for each cell, then averaged over all animals (3 slides per animal) for each locality. Statistical differences between metacentric and telocentric chromosomes were determined by an unpaired t-test.

Centromere counting assay in MII eggs

6-12 wk-old Rb(6.16)24Lub x CF-1 females were primed with 5U PMSG and 48 h later with 5U of equine chorionic gonadotropin (eCG, Sigma, cat# C1063-1VL), and 12 h later *in vivo* matured MII eggs were collected. Eggs were treated with hyaluronidase enzyme (3 mg/ml in MEM-PVP medium, Sigma) for 10 min until cumulus cells fell off, then incubated in 100 μM kinesin-5 inhibitor, monastrol, in CZB media for 1.5 h in 5% CO_2 in air at 37°C. Eggs were fixed in 2% PFA, stained for centromeres (CREST) and DNA (Sytox green, 1:5000, Molecular Probes), mounted into Vectashield, and imaged as z-stacks at 0.3-μm intervals using the confocal

microscope described above. Centromeres in MII eggs and in some fraction of polar bodies were counted manually using ImageJ software. In addition, in ~ 50% of eggs the combination of DNA and kinetochore staining was used to confirm the presence of the morphologically distinct Rb fusion metacentric. The centromere counting analysis was performed blind by mixing images from Rb(6.16)24Lub x CF-1 MII eggs with images from telocentric CF-1 MII eggs. After the analysis, the origin of each image was revealed to determine the fraction of false positive results (i.e., CF-1 counted as 38 centromeres). Only 2% of CF-1 MII eggs were miscounted, indicating high reliability of the centromere counting assay. Centromere counts from 168 MII eggs (>10 females, 4 independent experiments) were analyzed with chi-square test ($\chi 2=6.88$, $p=0.0087$) showing that the segregation ratio 60% : 40% was statistically significant (n=101 with 40 centromeres, n=67 with 38 centromeres).

Collection of wild mice

Three males and three females from each natural metacentric population (with the exception of GROL, where only 2 males were trapped) were live-trapped during September and October 2013 in commensal habitats within the known geographic range of a given metacentric population (PSAN, PPOD, PEDC, EBAR, GROL). Mice with all telocentric chromosomes were trapped in June 2013 from three distinct places in Greece: Razata (Cephalonia Island, N 38° 10' 57.7", E 20° 30' 51.0"), Faraklada (N 37° 12' 0.6", E 21° 360' 55.3") and Nea Artaki (Halkida, N 38° 29' 15.7", E 23° 37' 32.4"). GV oocytes were collected, matured *in vitro* for 7 h after GVBD to metaphase I, fixed and stained for HEC1 and DNA. In Greece and Barcelona, 2 - 3 females from standard laboratory mouse strains (C57BL/6J, BALB/c) were subjected to the same oocyte collection, fixation and staining procedure. Spermatocyte chromosome spreads (3 slides per

animal) were stained for CENP-A, SYCP2, and DAPI. Glass slides with stained MI oocytes and spermatocytes from each locality were shipped to Philadelphia for imaging and analysis.

Karyotypes from each animal were obtained from cell suspensions prepared from bone marrow [S2] as follows: bone marrow cells expelled from the mouse femur were subjected to a hypotonic shock in 0.56% (0.075M) KCl for 30 min at 37º C (no mitotic inhibitor was used during the protocol to avoid compromising oocyte/spermatocyte meiosis). A pre-fixation step with methanol and glacial acetic acid (3:1) was performed, followed by three rounds of fixation with ice-cold fixative. Air-dried slides with cell suspensions were prepared and stained with 5% Giemsa to confirm the diploid number of each individual.

Supplemental References:

S1. Morelli, M. A., Werling, U., Edelmann, W., Roberson, M. S., and Cohen, P. E. (2008). Analysis of meiotic prophase I in live mouse spermatocytes. Chromosome Res. *16*, 743–60.

S2. Ford, C. E. (1966). The use of chromosome markers. In Tissue Grafting and Radiation (Academic Press), pp. 197–206.