Meiotic Sex Chromosome Inactivation Is Disrupted in Sterile Hybrid Male House Mice

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ABSTRACT In male mammals, the X and Y chromosomes are transcriptionally silenced in primary spermatocytes by meiotic sex chromosome inactivation (MSCI) and remain repressed for the duration of spermatogenesis. Here, we test the longstanding hypothesis that disrupted MSCI might contribute to the preferential sterility of heterogametic hybrid males. We studied a cross between wild-derived inbred strains of *Mus musculus musculus* and *M. m. domesticus* in which sterility is asymmetric: F_1 males with a *M. m. musculus* mother are sterile or nearly so while F_1 males with a *M. m. domesticus* mother are normal. In previous work, we discovered widespread overexpression of X-linked genes in the testes of sterile but not fertile F_1 males. Here, we ask whether this overexpression is specifically a result of disrupted MSCI. To do this, we isolated cells from different stages of spermatogenesis and measured the expression of several genes using quantitative PCR. We found that X overexpression in sterile F_1 primary spermatocytes is coincident with the onset of MSCI and persists in postmeiotic spermatids. Using a series of recombinant X genotypes, we then asked whether X overexpression in hybrids is controlled by *cis*-acting loci across the X chromosome. We found that it is not. Instead, one large interval in the proximal portion of the *M. m. musculus* X chromosome is associated with both overexpression and the severity of sterility phenotypes in hybrids. These results demonstrate a strong association between X-linked hybrid male sterility and disruption of MSCI and suggest that *trans*-acting loci on the X are important for the transcriptional regulation of the X chromosome during spermatogenesis.

FORTY years ago, Lifschytz and Lindsley (1972) proposed the provocative hypothesis that the X chromosome is inactivated during male meiosis and that disruption of this process could lead to sterility. The idea that failure of meiotic sex chromosome inactivation (MSCI) might cause sterility is significant in speciation genetics since it provides a possible explanation for the widespread observation that in crosses between species, sterility typically appears first in the heterogametic sex (Haldane 1922; Forejt 1996; Presgraves 2008).

Whether MSCI takes place in *Drosophila* has been debated for several decades (Kremer *et al.* 1986; McKee and Handel 1993; Hense *et al.* 2007; Vibranovski *et al.* 2009). Current evidence suggests that expression on the X chromosome is suppressed relative to the autosomes during spermatogenesis but that this suppression precedes meiosis and thus does not reflect MSCI (Meiklejohn *et al.* 2011; but see Vibranovski *et al.* 2012). Likewise, the sex chromosomes are not differentially silenced in chicken oocytes (Guioli *et al.* 2012). Therefore, failed MSCI cannot explain hybrid sterility in all female-heterogametic taxa. In therian mammals, however, MSCI is well established (Solari 1974; McKee and Handel 1993; Namekawa *et al.* 2007). Although no MSCI-essential loci have been found on the sex chromosomes, many of the autosomal genes that regulate this process have been identified, and the cellular progression of MSCI is well defined in the lab mouse (*e.g.*, Turner *et al.* 2004; Ichijima *et al.* 2011).

In reproductively normal male mice, both sex chromosomes are transcriptionally active during mitosis and early prophase I of meiosis (leptotene–zygotene). At the start of pachytene, the X and Y undergo global chromatin remodeling and transcriptional silencing of all protein-coding genes, and are sequestered from the autosomes in a heterochromatin domain known as the sex body (reviewed in Handel 2004; Turner 2007). The sex body is disassembled at the end of prophase I but the sex chromosomes remain

condensed and transcriptionally repressed through the two meiotic divisions (Namekawa *et al.* 2006; for exceptions on the Y see Vernet *et al.* 2011, 2012). Importantly, MSCI is essential for male fertility. Cells with defective MSCI are eliminated by late pachytene (Turner *et al.* 2006; Royo *et al.* 2010). Thus, global failure of MSCI causes meiotic arrest and complete sterility. Whether failed MSCI is a cause of hybrid male sterility is an open question.

While some sex-linked genes are expressed in haploid spermatids (Hendriksen *et al.* 1995; Namekawa *et al.* 2006; Mueller *et al.* 2008; Reynard *et al.* 2009), most remain repressed for the duration of spermatogenesis (Namekawa *et al.* 2006; Turner *et al.* 2006), a state termed postmeiotic sex chromatin repression (PSCR). Male lab mice with defective PSCR are subfertile or sterile due to reduced sperm count and morphologically abnormal sperm (Roest *et al.* 1996; Cocquet *et al.* 2009; Achame *et al.* 2010). Although different genes are implicated in chromosome-wide repression in primary spermatocytes (*e.g.*, *H2ax*, Fernandez-Capetillo *et al.* 2003; *Brca1*, Turner *et al.* 2004; *MDC1*, Ichijima *et al.* 2011) *vs.* postmeiotic spermatids (*e.g.*, *Hr6b*, Achame *et al.* 2010; *Sly*, Coquet *et al.* 2009), it is thought that PSCR is a downstream consequence of MSCI at the epigenetic level (Turner *et al.* 2006).

We previously studied gene expression in sterile male hybrids between the house mouse subspecies, *Mus musculus musculus* and *M. m. domesticus* (Good *et al.* 2010). In the cross between wild-derived inbred strains *musculus*[PWK] and *domesticus*[LEWES], hybrid male sterility is asymmetric and strongly X linked: both F_1 and late backcross males with a *musculus*[PWK] X chromosome are sterile or nearly so whereas males with a *domesticus*[LEWES] X are normal (Good *et al.* 2008a,b). Strikingly, a large number of genes on the *musculus*[PWK] X chromosome are overexpressed in the testes of sterile F_1 males (Good *et al.* 2010). However, because whole testes comprise germ cells from all stages of spermatogenesis, together with somatic cells, we could not determine whether overexpression is specific to germ cells in which the X is normally silenced or repressed (Good *et al.* 2010).

Here, we test the hypothesis that the timing of X chromosome overexpression in sterile hybrid male house mice is consistent with disrupted MSCI. We used fluorescence-activated cell sorting (FACS) to isolate discrete germ cell populations from reciprocal F_1 males and quantified the expression of X-linked genes using real-time reverse-transcription PCR (qRT–PCR). If disrupted MSCI explains overexpression of the *musculus*[PWK] X in sterile F_1 males then X-linked genes should be overexpressed in meiotic cells subject to MSCI, whereas expression should be normal in cells in which the X is transcriptionally active. We found strong support for this prediction. Therefore, we then sought to define the genetic architecture of disrupted MSCI on the *musculus*[PWK] X. We hypothesized that disrupted MSCI in hybrid males results from local mismatch between loci in the *domesticus*[LEWES] autosomal genome and *cis*-acting sequences

distributed along the *musculus*[PWK] X. To test this, we selected genes along the length of the X that were previously shown to be overexpressed in sterile F_1 testes (Good *et al.* 2010) and measured whole-testis expression in F_1 males with recombinant X chromosomes (X introgression F_1s). If MSCI is disrupted in *cis*, only *musculus*[PWK] alleles should be overexpressed. Contrary to this prediction, we found that both *musculus*[PWK] and *domesticus*[LEWES] alleles were overexpressed in some introgression lines, identifying a region of the X chromosome likely to contain *trans*-acting loci that are important for the regulation of gene expression on the X chromosome during spermatogenesis.

Materials and Methods

Animals

The wild-derived inbred strains used in this study were purchased from the Jackson Laboratory (http://www.jax.org) and were maintained at the University of Arizona Central Animal Facility. Pure F_1 males were produced from reciprocal crosses between PWK/PhJ (*musculus*[PWK]) and LEWES/EiJ (*domesticus*[LEWES]). To generate F_1 males with recombinant X chromosome genotypes we crossed homozygous *musculus*[PWK] or *domesticus*[LEWES] X introgression line females to pure heterosubspecific males. Sex chromosome genotypes for the seven X introgression F_1s are shown in Figure 2A; see Good *et al.* (2008b) and Campbell *et al.* (2012) for details. Intrasubspecific controls were produced by crossing *musculus* strains PWK/PhJ and CZECHII/EiJ (*musculus*[CZECHII]), and *domesticus* strains LEWES/EiJ and WSB/EiJ (*domesticus*[WSB]). These intrasubspecific crosses were generated to avoid confounding expression differences within and between subspecies with differences between inbred and heterozygous genotypes (*i.e.*, to remove the effects of inbreeding depression).

All males were maintained in cages containing a maximum of three same-sex sibs until 50 days, after which they were caged singly for at least 20 days and killed by cervical dislocation. The reciprocal F_1 males used in the targeted cell-type expression experiment ($n = 5$/genotype) were 70–73 days old. The reciprocal F_1 and X introgression F_1 males used in the whole-testis expression experiment were 70 days old ($n = 3$/genotype). Testes were dissected immediately after euthanasia, cut into two sections, and stored in RNA*later* (Ambion) at $-20°$ until processing. Data collection for individual measures of testis mass, sperm count, and sperm abnormality is described in Campbell *et al.* (2012).

Preparation and staining of testicular single cell suspensions for FACS

Single cell suspensions were prepared according to Getun *et al.* (2011) with minor modifications. Briefly, interstitial cells were removed by digesting freshly dissected detunicated testes with 1mg/ml collagenase I (Sigma) in Gey's balanced salt solution (GBSS, Sigma). Tubules were

digested with 1 mg/ml trypsin (Worthington) in GBSS with 1 mg/ml collagenase and germ cells were released by gentle pipetting with a wide orifice transfer pipette for 90 sec/testis. Cells were prestained with 0.16 mg/ml Hoechst 33343 (Invitrogen) and 0.8 mg/ml trypsin in GBSS. Trypsin was inactivated with 0.16 mg/ml fetal calf serum, and final staining was performed by bringing Hoechst concentration to 0.36 mg/ml. Cells were filtered twice through 70-μm nylon mesh, stained with propidium iodide (0.002 mg/ml), and stored on ice protected from light until sorting. All digestion and staining steps were carried out at 33° for 15 min with agitation at 120 rpm; DNase (0.004 mg/ml) was added at every step to eliminate cell clumps. Immediately before processing, cells were spun down for 4 min at $\sim$200 $\times$ g in a clinical centrifuge and resuspended in plain GBSS.

FACS and validation

Cell sorting was performed using a BD FACSAria IIu (BDBiosciences, San Jose, CA). Propidium iodide discriminates dead from viable cells whereas Hoechst differentiates viable cells based on DNA content and chromatin structure (see Bastos *et al.* 2005 for details). Propidium iodide was excited at 488 nm and detected at 610/20 with a 595LP filter. Hoechst was excited at 405 nm, with Hoechst Blue being detected at 450/50 nm and Hoechst Red at 630/30 nm with a 600LP filter. All samples were run with an 85-μm nozzle at <5000 events/sec. Cell populations enriched for spermatogonia and primary spermatocytes from early prophase I (LZ, leptotene/zygotene), mid-pachytene–diplotene primary spermatocytes (PD), secondary spermatocytes (SS), and postmeiotic round spermatids (RS) were collected into RLT buffer (Qiagen) with 1% betamercaptoethanol and stored on ice until processing.

We validated the purity of targeted cell populations in two ways. First, a subset of each cell population was collected into fixative (3:1 methanol:glacial acetic acid), spread on slides, and stained with 5% Giemsa (ENG Scientific). Cells were examined at 100× on a phase-contrast microscope and the number of cells of the targeted type was counted for a minimum of 50 cells/population. Second, five autosomal genes that are enriched in different spermatogenic cell types were assayed in each cell population with qRT–PCR as described below: *Igfr1* (spermatogonia/early prophase I), *Hormad1* (pachytene), *Hist1h1t* (mid-late pachytene), and *Acrv1* and *Protamine 1* (round spermatids).

RNA extraction and cDNA synthesis

RNA from FACS cells was extracted within 24 hr of each sort using the RNeasy Plus kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized the same day using SuperScript III (Invitrogen) with oligo(dT) and $\sim$200 ng template RNA per 50 μl reaction. RNA extraction and cDNA synthesis from whole testis were as for cells, but with $\sim$10 μg template RNA per 40 μl cDNA reaction.

Selection of experimental loci and primer design

We assayed seven X-linked genes in FACS cells. To determine whether X overexpression was restricted to genes associated with particular spermatogenic time points we chose three genes that are normally highly expressed prior to MSCI in mitotic spermatogonia and early prophase I spermatocytes (*Ammecr1*, *Rps6ka6*, *Fmr1*), three postmeiotic genes (*Actrt1*, *4930557A04Rik*, *4930468A15Rik*), and one gene with both mitotic/early meiotic and postmeiotic enrichment (*1700013H16Rik*) (Namekawa *et al.* 2006; Chalmel *et al.* 2007; Achame *et al.* 2010). The 12 X-linked genes assayed in whole testis were a subset of the 32 genes with predominantly postmeiotic expression that were significantly overexpressed in the testes of sterile F_1 males in an earlier microarray experiment (Good *et al.* 2010). These genes were chosen to maximize the range of coverage on the X and the representation of both *musculus*[PWK] and *domesticus*[LEWES] alleles in X introgression genotypes (Figure 2A).

Primers were designed in Primer3 (v. 0.4.0) spanning at least one intron or across exon–UTR boundaries. Whenever possible, primers were placed in exons present in all splice variants listed in the Ensembl database (NCBIM37). All primer sequences were checked for SNPs in *musculus*[PWK] and *domesticus*[WSB] strains using the Wellcome Trust Sanger Institute database and BLASTed against the mouse G+T database using megablast. Sequences with multiple polymorphic sites or homology to nonspecific targets were discarded. Primer sequences and amplicon sizes are provided in Supporting Information, Table S1.

qRT–PCR

qRT–PCR was performed with PerfeCTa SYBR Green Fast-Mix for iQ (Quanta Biosciences) on a MyiQ2 light cycler (Bio-Rad). PCR efficiency for each primer pair was evaluated with standard curve analysis using an eight point dilution series from whole-testis cDNA. Efficiencies of 95–105% were obtained for all primers. For experimental assays, 16-μl reactions with a final concentration of 300 nM of each primer were run in 96-well plates for 40 cycles with annealing at 60°. We analyzed three to five (FACS cells) or three (testes) biological replicates per genotype with three technical replicates per sample. All standard deviations (SDs) for technical replicates were <0.30. For each locus, all genotypes from a single biological replicate were run on the same plate with appropriate negative controls; duplicate reactions for reference genes (*Ubc* or *beta-actin*) were included on each plate. Melt curve analysis was performed at the end of each run to check for multiple peaks indicative of nonspecific amplification. Single peaks were obtained for all loci. Crossing threshold (C_T) values were calculated automatically with iQ5 Optical System Software (v. 2.1). C_T values were normalized relative to *Ubc* or *beta-actin* values for each plate (ΔC_T). Because postmeiotic cells are depleted in the testes of sterile males (Good *et al.* 2008a), whole-testis

expression was normalized a second time relative to two postmeiotic autosomal genes, *Acrv1* and *Protamine 1*.

Choice of control genotypes for whole-testis expression assays

We analyzed expression in experimental genotypes relative to both the fertile F_1 (*domesticus*[LEWES] × *musculus*[PWK]) and mean expression in intrasubspecific controls (*musculus*[CZECHII] × *musculus*[PWK] and *domesticus*[WSB] × *domesticus*[LEWES]). There were minor differences in patterns of significance for individual genes but the results of analyses based on average relative expression (mean $\Delta\Delta C_T$) were the same (data not shown). We chose to present analyses with the fertile F_1 control because this eliminates any potential differences in X expression due to autosomal background.

Results

Validation of FACS purity

Based on Giemsa staining, mean percentage purity in the four targeted cell populations was 89–69% (leptotene/zygotene, $n = 6$ males, 89% SD 8.0%; pachytene/diplotene, $n = 4$, 80% SD 6.0%; secondary spermatocytes, $n = 4$, 69% SD 20%; round spermatids, $n = 6$, 85% SD 7.0%; Figure S1A). Lower purity in secondary spermatocyte samples was mainly due to contamination with round spermatids. Importantly, the cellular composition of targeted populations did not differ between reciprocal F_1 genotypes (ANOVA, $P \geq 0.6$). The relative expression of autosomal genes enriched at different spermatogenic time points corresponded to expectations for each cell population (Figure S1B).

X overexpression is consistent with defective MSCI and PSCR

If disrupted MSCI explains overexpression of the *musculus*[PWK] X in sterile F_1 males, then X-linked genes should be overexpressed only in meiotic cells subject to MSCI (*i.e.*, overexpressed in pachytene/diplotene cells but not in the leptotene/zygotene population). We would also expect to observe overexpression in secondary spermatocytes. Consistent with these predictions, six of the seven X-linked genes assayed (86%) were significantly overexpressed in pachytene/diplotene and secondary spermatocyte cells from sterile F_1 males, whereas expression in leptotene/zygotene cells did not differ between sterile and fertile genotypes for any gene (Figure 1). Across all seven genes, the average expression difference between genotypes was highly significant in the pachytene/diplotene population (PD, ANOVA, $F_{(63,1)} = 24.2, P < 0.0001$), moderately significant in secondary spermatocytes (SS, $F_{(62,1)} = 4.46, P = 0.04$), and nonsignificant in the leptotene/zygotene population (LZ, $F_{(60,1)} = 1.47, P = 0.2$) (Figure 1A). The same six X-linked genes were overexpressed in round spermatids (RS, Figure 1, B and C). Although genotype means for this population were not significantly different (RS, $F_{(62,1)} = 3.50, P = 0.07$), there

was a clear trend toward overexpression in the sterile F_1 (Figure 1A).

We found previously that a large number of X-linked genes are overexpressed in sterile F_1 male testes (Good *et al.* 2010). However, because progressive cell loss throughout spermatogenesis in sterile F_1 males could bias the cellular composition of the testis toward early spermatogenic cell types we excluded those genes normally expressed in mitotic/early meiotic cells and focused on postmeiotic genes only (*i.e.*, genes that escape PSCR). Here, using discrete cell populations, we show that both postmeiotic (Figure 1C) and mitotic/early meiotic genes (Figure 1B) are overexpressed in meiotic spermatocytes and in postmeiotic spermatids. Thus, both MSCI and PSCR appear to be disrupted and this likely affects the expression of a broad array of X-linked genes.

Overexpression of X-linked genes is not limited to *musculus*[PWK] alleles

We used a series of *musculus*[PWK] X introgression lines (Good *et al.* 2008b; Campbell *et al.* 2012) to test the hypothesis that disrupted MSCI in hybrid males results from local mismatch between loci in the *domesticus*[LEWES] autosomal genome and *cis*-acting sequences distributed along the *musculus*[PWK] X. If MSCI is disrupted in *cis*, only *musculus*[PWK] alleles should be overexpressed. We did not find support for this hypothesis: there was no relationship between whole-testis expression and allelic species identity in X introgression F_1 males (ANOVA, $F_{(250,1)} = 0.4, P = 0.5$). There was also no effect of the origin of the Y chromosome ($F_{(19,1)} = 0.3, P = 0.6$). Instead, most instances of overexpression were concentrated in two genotypes (3 and 7) with a *musculus*[PWK] introgression on the proximal X (Figure 2B and Figure S2). Across all 12 genes assayed in this experiment, the average difference in expression between these two genotypes and controls was highly significant (Dunnett *post hoc* on ANOVA, genotype 3, $P = 0.005$, genotype 7, $P = 0.003$; Figure 2A). Nonetheless, overexpression was consistently most pronounced in the F_1 with the complete *musculus*[PWK] X (Figure 2B and Figure S2). These results indicate that *cis*-regulatory differences between the *musculus*[PWK] and *domesticus*[LEWES] X chromosomes do not fully account for X overexpression in sterile hybrids and suggest that proper regulation of MSCI depends in part on a *trans*-acting locus or loci on the proximal part of the X.

Strong association between X overexpression and sterility phenotypes

Although all X introgression F_1's used in this study have subfertile reproductive phenotypes, variance in the severity of these deficits is large and is mostly explained by the location of *musculus*[PWK] introgressions on the X (Campbell *et al.* 2012). Notably, the interval on the proximal X associated with overexpression in genotypes 3 and 7 contains QTL for which *musculus*[PWK] alleles have negative effects on testis weight and sperm head morphology on an F_1 background (Campbell *et al.* 2012).
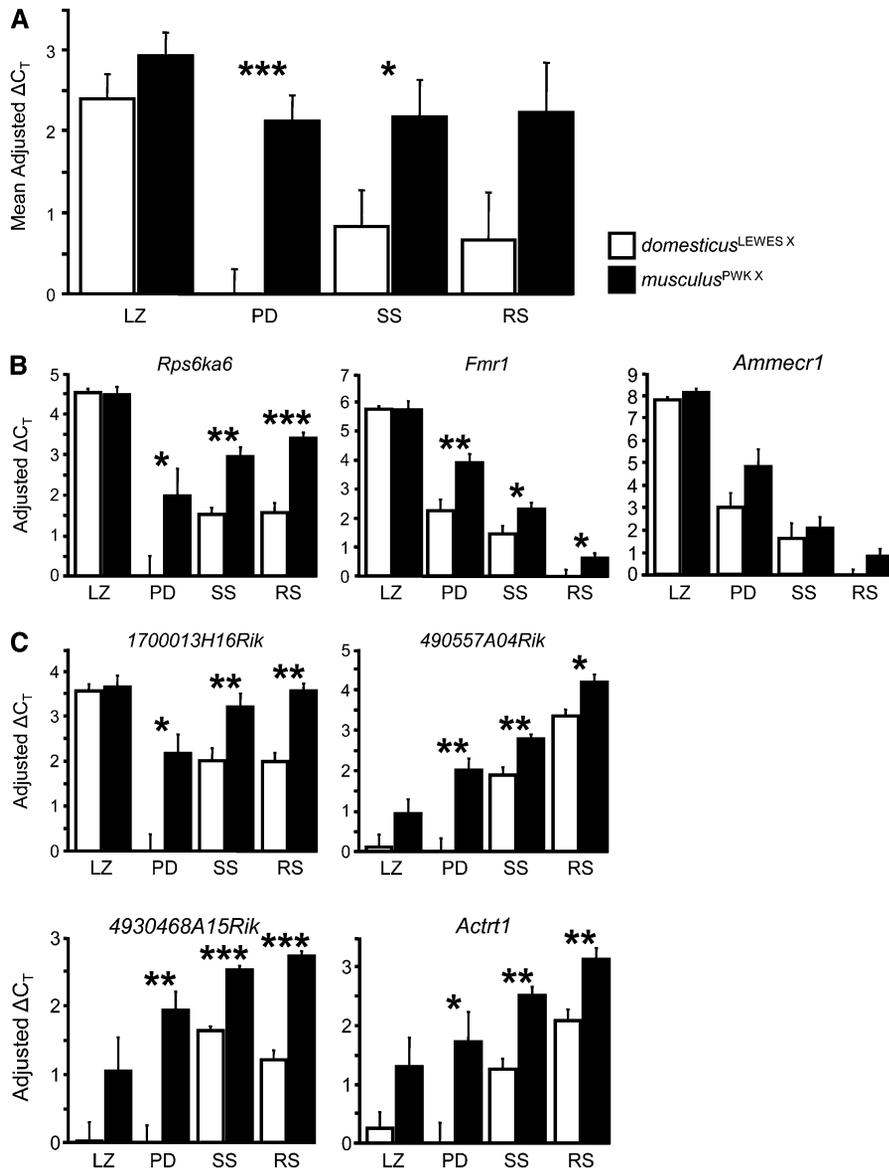
Figure 1 Overexpression of X-linked genes in sterile F$_1$ hybrid males is coincident with the onset of MSCI in primary spermatocytes and persists in postmeiotic round spermatids. (A–C) Expression in fertile F$_1$ (open bars, *domesticus*[LEWES] X chromosome) and sterile F$_1$ (solid bars, *musculus*[PWK] X chromosome) hybrid males in four germ cell populations in which the X is normally expressed (LZ, spermatogonia/early prophase I), silenced by MSCI (PD, pachytene/diplotene; SS, secondary spermatocytes), and repressed by PSCR (RS, round spermatids). Expression was normalized relative to *Ubc* (ΔC_T) and adjusted to produce positive values by setting the lowest expression level to zero. Data are presented as genotype means +1 SE; pairwise differences between genotypes were tested with ANOVA, (*) $P \leq 0.05$, (**) $P \leq 0.005$, (***) $P \leq 0.0001$. (A) Mean expression of the seven X-linked genes shown in B and C. (B) Expression of three X-linked genes that are normally highly expressed prior to MSCI in spermatogonia and early prophase I primary spermatocytes and do not escape PSCR. (C) Expression of four X-linked genes that are normally moderately expressed in postmeiotic round spermatids. Note that *1700013H16Rik* is also normally enriched in spermatogonia (Namekawa *et al.* 2006).

This prompted us to ask whether there was a correlation between the degree of X overexpression and the severity of reproductive deficits.

Using phenotypic data for the males assayed in this study, we found highly significant negative relationships between average X overexpression and body mass-corrected testis weight (RTW, $R^2 = 0.33$, $P = 0.003$), sperm count ($R^2 = 0.34$, $P = 0.003$), and the percentage of normal sperm ($R^2 = 0.71$, $P < 0.0001$; Figure 3). This pattern suggests that hybrid male sterility and disrupted MSCI share a common genetic basis on the X.

Discussion

We present evidence that MSCI is defective in sterile hybrid house mice with a *M. m. musculus* X chromosome. Persistent X overexpression in round spermatids indicates that postmeiotic sex chromatin repression is also incomplete. These results, together with a strong association between X overexpression and the severity of sterility phenotypes, provide support for the longstanding hypothesis that failed X inactivation during spermatogenesis could contribute to hybrid male sterility (Lifschytz and Lindsley 1972; Forejt 1996; Presgraves 2008). While most work on the genetic mechanisms of mammalian MSCI has focused on autosomal genes, the finding that MSCI is disrupted in F$_1$ males with a *musculus*[PWK] X chromosome but is normal in the reciprocal F$_1$ indicates that MSCI-essential loci may exist on the X chromosome. The significant increase in X expression in males with a large *musculus*[PWK] introgression on the proximal X suggests that this interval contains a *trans*-acting locus or loci important for MSCI. We discuss alternative mechanisms that could cause MSCI and PSCR defects in sterile hybrids, and the relationship between MSCI and the large role of the X chromosome in the origins of reproductive isolation.
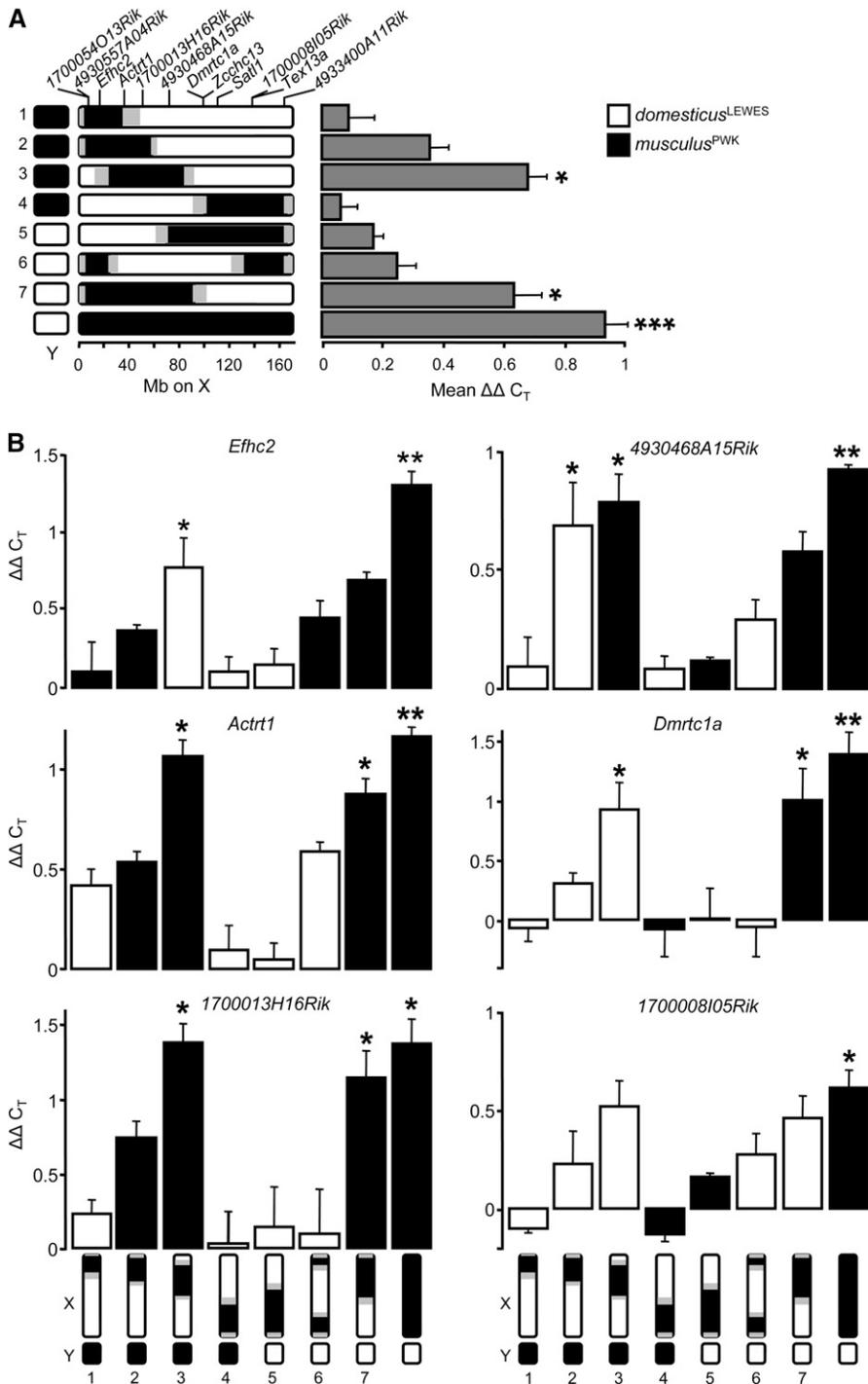
Figure 2 X overexpression in F_1 hybrid male testes is associated with a *musculus*[PWK] introgression on the proximal X. (A) Sex chromosome genotypes and mean X overexpression in experimental males. X introgression genotypes are numbered 1–7; regions of uncertainty between *domesticus*[LEWES] (open) and *musculus*[PWK] (solid) recombination break points on the X are shown shaded; all genotypes share the same F_1 autosomal background. Expression was normalized relative to *beta-actin*, *Acrv1*, and *Protamine 1* (ΔC_T); mean $\Delta\Delta C_T$ is the difference in expression between experimental genotypes and the fertile F_1, averaged across the 12 X-linked genes indicated on the left side. Error bars are +1 SE. Tests for significant differences between experimental genotypes and control were performed on ΔC_T values with ANOVA followed by Dunnett *post hoc* tests, (*) $P < 0.01$, (***) $P < 0.0001$. (B) Overexpression of individual X-linked genes does not require a *musculus*[PWK] allele. Data are presented as genotype means (± 1 SE) for six representative genes. Sex chromosome genotypes are shown on the *x*-axis. Solid bars denote the *musculus*[PWK] allele at a given locus; open bars denote the *domesticus*[LEWES] allele. Significance was tested as in 2A, (*) $P \leq 0.05$, (**) $P \leq 0.001$. See also Figure S2.

Disruption of MSCI

Transcriptional silencing of the sex chromosomes in early pachytene is essential for mammalian male fertility (Royo *et al.* 2010). In lab mouse mutants, primary spermatocytes with continued expression of the X, or the Y, or even specific Y-linked genes (*e.g.*, *Zfy2*), are eliminated by apoptosis before the end of pachytene (Turner *et al.* 2005, 2006; Royo *et al.* 2010). Thus, global failure of MSCI causes meiotic arrest and complete absence of postmeiotic cells. In sterile F_1 hybrid males, X overexpression coincident with the onset of MSCI

strongly suggests that this process is disrupted. However, both the reproductive phenotypes and the pattern of X overexpression in these hybrids indicate that failure of MSCI is not complete. Some germ cells progress through meiosis and a smaller number survive spermiogenesis, albeit with severe abnormalities in sperm differentiation (Good *et al.* 2008a; Campbell *et al.* 2012). Likewise, while the difference in average X expression between the sterile and fertile F_1 is most extreme in the late prophase I (PD) cells in which MSCI is in effect (Figure 1A), for any individual gene, the level of
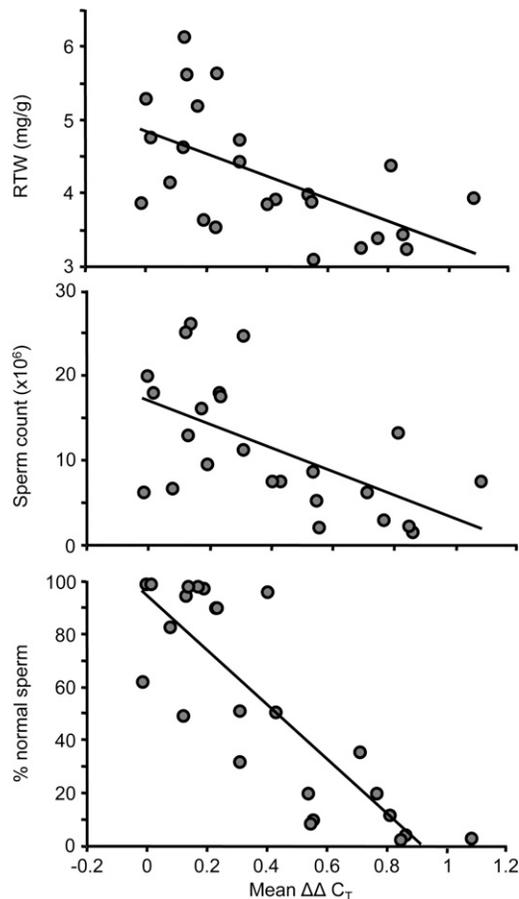
Figure 3 Hybrid males with more X overexpression have worse reproductive phenotypes. Significant negative correlations between X overexpression (mean $\Delta\Delta C_T$) and relative testis weight (RTW, $R^2 = 0.33$, $P = 0.003$), sperm count ($R^2 = 0.34$, $P = 0.003$), and the percentage of normal sperm ($R^2 = 0.71$, $P < 0.0001$), measured in the 24 F_1 males used in the whole-testis expression study (see Figure 2A for genotypes). Mean $\Delta\Delta C_T$ is the difference in expression between experimental genotypes and the fertile F_1, averaged across the 12 X-linked genes shown in Figure 2A.

expression in the sterile F_1 is lower in PD cells than in the cell population in which that gene is normally expressed (Figure 1, B and C).

There are at least three possible explanations for the pattern of incomplete disruption of MSCI. First, "leaky" MSCI in all or most primary spermatocytes results in moderate levels of X overexpression that are not sufficient to trigger the apoptotic response. The fact that failed silencing of several Y-linked genes does not cause meiotic arrest in transgenic lab mice suggests that misexpression of some genes can be tolerated (Royo *et al.* 2010). However, a large number of X-linked genes are overexpressed in sterile F_1 testes (Good *et al.* 2010). Given that all protein-coding genes on the X are subject to MSCI, it is likely that even moderate overexpression of many X-linked genes would be toxic to primary spermatocytes. A second alternative is that MSCI fails completely in a subset of cells, and these are eliminated before the first meiotic division. In this case, overexpression in secondary spermatocytes could be a direct

consequence of disrupted localization of heterochromatin proteins to the sex chromosomes in primary spermatocytes, or an indirect effect of an unhealthy cellular environment. Finally, because the late prophase I population was enriched for both pachytene and diplotene cells, the data are also consistent with a scenario in which MSCI is initiated normally but is not properly maintained. Quantification of stage-specific meiotic cell loss, together with immunostaining for epigenetic markers of the sex body, heterochromatin, and transcriptional repression, will pinpoint the timing and cellular distribution of MSCI defects in sterile hybrids.

What are the causes and consequences of MSCI disruption in hybrid males?

In the *musculus*[PWK] × *domesticus*[LEWES] cross, both male sterility and defective MSCI segregate with the *musculus*[PWK] X chromosome. Therefore, incompatibilities explaining these phenotypes must involve X-linked loci whose allelic identity, or distribution, differs between *musculus*[PWK] and *domesticus*[LEWES]. These findings raise two key questions: (1) Is defective MSCI the primary cause of hybrid male sterility; in other words, are the genes underlying defective MSCI and the genes underlying hybrid male sterility the same in this cross? and (2) Is defective MSCI a direct consequence of mismatch between loci that regulate this process, or is it a secondary consequence of earlier-acting incompatibilities? The strong association between X overexpression and male reproductive defects hints at a common genetic basis. However, evaluating this hypothesis awaits a better understanding of the genetic basis of MSCI defects. Therefore, we focus our discussion on the alternatives raised in the second question.

Although several autosomal genes essential for the initiation and progression of MSCI have been identified (*e.g.*, *H2ax*, Fernandez-Capetillo *et al.* 2003; *Brca1*, Turner *et al.* 2004; *MDC1*, Ichijima *et al.* 2011), it is not known whether these loci interact with specific sequences on the X. In fact, evidence that MSCI is a special case of meiotic silencing of unsynapsed chromatin (MSUC), a generalized response to asynapsis at the zygotene/pachytene transition (Baarends *et al.* 2005; Turner *et al.* 2005, 2006), argues against the necessity of MSCI-essential loci on the X. However, based on the finding that most micro(mi)RNAs on the mouse X are highly expressed when all X-linked protein coding genes are silenced by MSCI (Song *et al.* 2009), Yan and McCarrey (2009) proposed that these MSCI-escaping miRNAs play a key role in regulating X inactivation in the male germ line. Preliminary support for this hypothesis is provided by an association between failed MSCI and disproportionate downregulation of miRNAs on the X *vs.* the autosomes in male lab mice lacking AGO4, a key protein in the miRNA pathway (Modzelewski *et al.* 2012).

The expression patterns in the seven X introgression F_1's assayed in this study demonstrate a strong association between X overexpression and an interval on the proximal half

of the *musculus*[PWK] X between ∼37.1 and 68.7 Mb. If hybrid defects in MSCI are caused by incompatibilities between loci that control this process, then this interval contains a locus (or loci) whose *trans*-acting effects are essential to MSCI. Notably, while this interval contains ∼15% of protein-coding genes on the X, it contains ∼40% of X-linked miRNAs. As such, *musculus*[PWK]-derived miRNAs on the proximal X are promising candidates for targeted fine-scale mapping of the genetic basis of disrupted MSCI in hybrid males.

A nonmutually exclusive alternative is that MSCI defects are a consequence of genetic incompatibilities that act earlier in meiosis. In reproductively normal males, the intricate process of recombination immediately precedes MSCI. Recombination is initiated at leptotene by the formation of DNA double-strand breaks (DSBs) required for synapsis between homologous chromosomes, and culminates at the zygotene to pachytene transition when autosomal synapsis and DSB repair is complete. Notably, in lab mouse mutants with extensive autosomal asynapsis at pachytene, the sex chromosomes are not properly condensed or silenced and meiosis arrests midway through pachytene (Mahadevaiah *et al.* 2008; Burgoyne *et al.* 2009; Homolka *et al.* 2012). The link between autosomal asynapsis and failed MSCI may be explained by the integral role of several DNA damage response proteins in both DSB repair and initiation of MSCI (*e.g.*, γH2AX, Fernandez-Capetillo *et al.* 2003; BRCA1, Turner *et al.* 2004). Asynapsis delays DSB repair and can promote the formation of additional breaks; persistent unrepaired DSBs on asynapsed autosomes sequester BRCA1 (Mahadevaiah *et al.* 2008). Burgoyne and colleagues proposed that MSCI fails under these conditions because this key protein is unavailable (Mahadevaiah *et al.* 2008; Burgoyne *et al.* 2009). A recent report of excess asynapsis in *musculus*[PWK] × C57BL/6J pachytene spermatocytes (Modzelewski *et al.* 2012) indicates that this phenotype may exist in the *musculus*[PWK] × *domesticus*[LEWES] cross, a hypothesis that we plan to evaluate by quantifying asynapsis, DSBs, and BRCA1 distribution in pachytene chromosome spreads.

Postmeiotic overexpression

In normal postmeiotic cells, dozens of multicopy genes on both sex chromosomes (Mueller *et al.* 2008; Reynard *et al.* 2009), together with ∼13% of single copy genes on the X (Namekawa *et al.* 2006), are expressed in round spermatids. Nevertheless, chromosome-wide expression on the X is substantially lower than that on the autosomes, and both sex chromosomes remain condensed and are enriched for epigenetic markers of transcriptional repression (Namekawa *et al.* 2006; Turner *et al.* 2006). In the sterile F_1 males, two of three mitotic/early meiotic X-linked genes (*Rps6ka6*, *Fmr1*) were significantly overexpressed in round spermatids, although the signal of overexpression was stronger in postmeiotic genes (*4930557A04Rik*, *4930468A15Rik*, *Actrt1*, *1700013H16Rik*). These data suggest that PSCR is also defective in the sterile F_1 males.

PSCR is thought to depend on MSCI at the epigenetic level: repressive histone marks established on the sex chromosomes at pachytene are transmitted through the two meiotic divisions (Namekawa *et al.* 2006; Turner *et al.* 2006). Thus, defective PSCR in the sterile F_1 may be a secondary consequence of disrupted MSCI. It is possible, however, that X overexpression in meiotic cells *vs.* postmeiotic cells is caused by distinct genetic incompatibilities between *domesticus*[LEWES]-derived loci and loci on the *musculus*[PWK] X.

Several lab mouse mutants with normal MSCI exhibit global derepression of the sex chromosomes in postmeiotic spermatids (reviewed in Montellier *et al.* 2011). In particular, the multicopy Y-linked gene, *Sly*, is essential for normal sperm differentiation and is a key regulator of PSCR (Reynard *et al.* 2009; Cocquet *et al.* 2009). *Sly*-deficient male lab mice are subfertile, produce female-biased litters, and exhibit disruption of PSCR and widespread overexpression of X-linked postmeiotic genes, including the multicopy *Slx* and *Slxl1* genes that are closely related to *Sly* (Ellis *et al.* 2005; Cocquet *et al.* 2009). In contrast, *Slx/Slxl1*-deficiency results in reduced male fertility and male-biased litters, but does not disrupt PSCR (Cocquet *et al.* 2012). Simultaneous ablation of all three genes significantly improves reproductive phenotypes and restores sex ratio parity and PSCR, suggesting that *Slx/Slxl1* and *Sly* are involved in an intragenomic conflict (Cocquet *et al.* 2012). These antagonistic interactions appear to underlie the dramatic copy number expansion of *Slx/Slxl1*, *Sly*, and several other X-linked genes in house mice (Ellis *et al.* 2011).

The *M. m. musculus* X and Y carry at least twice as many copies of *Slx/Slxl1* and *Sly*, respectively, as the *M. m. domesticus* X and Y (Scavetta and Tautz 2010; Ellis *et al.* 2011). Thus, the ratio of *Slx/Slxl1* to *Sly* gene copies is unbalanced in hybrids, and sterile F_1 males with a *musculus*[PWK] X and *domesticus*[LEWES] Y are potentially *Sly* deficient. Given phenotypic similarities between sterile F_1 hybrids and *Sly*-deficient lab mice (*i.e.*, X overexpression and sperm abnormalities), Cocquet and colleagues proposed that *Slx/Slxl1:Sly* copy number imbalance contributes to hybrid male sterility in the *musculus*[PWK] × *domesticus*[LEWES] cross (Cocquet *et al.* 2012; see also Ellis *et al.* 2011). Unfortunately, the distribution of *musculus*- *vs.* *domesticus*-derived *Sly*, *Slx*, and *Slxl1* genotypes among the X introgression F_1's used in this study precludes a statistical test for the effect of copy number imbalance on X expression: only genotype 7 carries the mismatch between *musculus*-derived *Slx* and *Slxl1* and *domesticus*-derived *Sly* (Figure 2A). However, the observation that genotype 7 and genotype 3, which carries *musculus*-derived *Slxl1* and *Sly*, exhibit comparable levels of X overexpression (Figure 2A) and reproductive deficits (Campbell *et al.* 2012), suggests that any negative effect of *Slx/Slxl1:Sly* copy number imbalance is outweighed by interactions between other loci on the *musculus*[PWK] X and loci in the *domesticus*[LEWES] autosomal genome.

Conclusions

Intrinsic fitness deficits in hybrids are caused by negative interactions between loci that function normally on the

genetic background in which they evolved (Bateson 1909; Dobzhansky 1937; Muller 1942). In species with a heterogametic sex, these incompatibilities often involve the X chromosome; identifying the genetic mechanisms and evolutionary processes that cause this pattern is a major focus in speciation genetics. Here we show that sterility in hybrid males is associated with disruption of MSCI. Thus, X-linked genetic incompatibilities can have epigenetic consequences. Moreover, we find that a proximal portion of the *M. m. musculus* X chromosome is involved, suggesting that disrupted MSCI in sterile males reflects mismatch between *M. m. musculus* alleles at X-linked loci essential for MSCI and *M. m. domesticus* alleles at autosomal loci that regulate this process.

Given that MSCI evolved ~180 million years ago in the common ancestor of eutherian and metatherian mammals (Kumar and Hedges 1998; Potrzebowski *et al.* 2008) and is an essential component of male fitness, it might seem surprising that loci involved in this process should vary between house mouse subspecies that diverged less than half a million years ago (Geraldes *et al.* 2011). However, it has been proposed that MSCI evolved as a genome defense against selfish genetic elements such as transposons or sex ratio distorters (Kelly and Aramayo 2007; Meiklejohn and Tao 2010; Presgraves 2010), both of which accumulate preferentially on the X. Under this scenario, it is plausible that antagonistic coevolution between loci that regulate MSCI and selfish genetic elements, whose transmission depends on MSCI escape, could produce functional divergence over short evolutionary timescales. Thus, identification of X-linked loci required for MSCI promises insight into the proposed relationship between intragenomic conflict and the process of speciation (Presgraves 2010; Cocquet *et al.* 2012).

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GENETICS

Meiotic Sex Chromosome Inactivation Is Disrupted in Sterile Hybrid Male House Mice

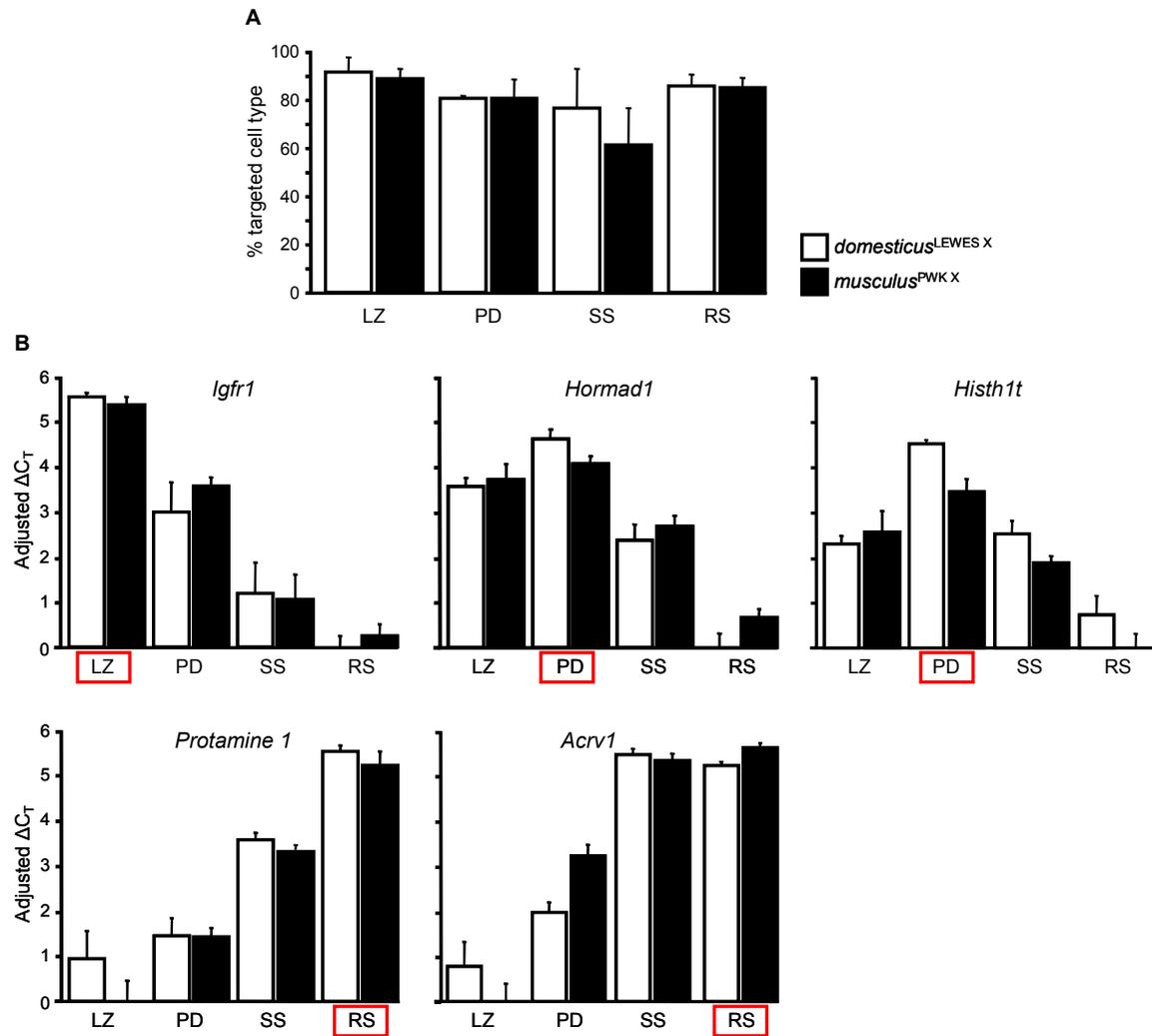Polly Campbell, Jeffrey M. Good, and Michael W. Nachman

Figure S1 Validations of germ cell population purity. (A) Percentage of targeted cell types based on Giemsa staining for spermatogonia/early prophase I (LZ), pachytene/diplotene (PD), secondary spermatocyte (SS), and round spermatid (RS) populations from reciprocal F_1 males (white bars, *domesticus*[LEWES] X chromosome; black bars, *musculus*[PWK] X chromosome). Data are presented as genotype means + 1 SE. (B) Autosomal genes that are expressed in different germ cell types are enriched in the correct germ cell populations from reciprocal F_1 males. The cell population in which each gene should be most highly expressed is indicated with a red box. All genes were enriched in the expected cell populations with the exception of *Acrv1*, which was equally enriched in the SS and RS populations. Expression was normalized relative to *Ubc* (ΔC_T) and adjusted to produce positive values by setting the lowest expression level to zero. Data are presented as genotype means + 1 SE.

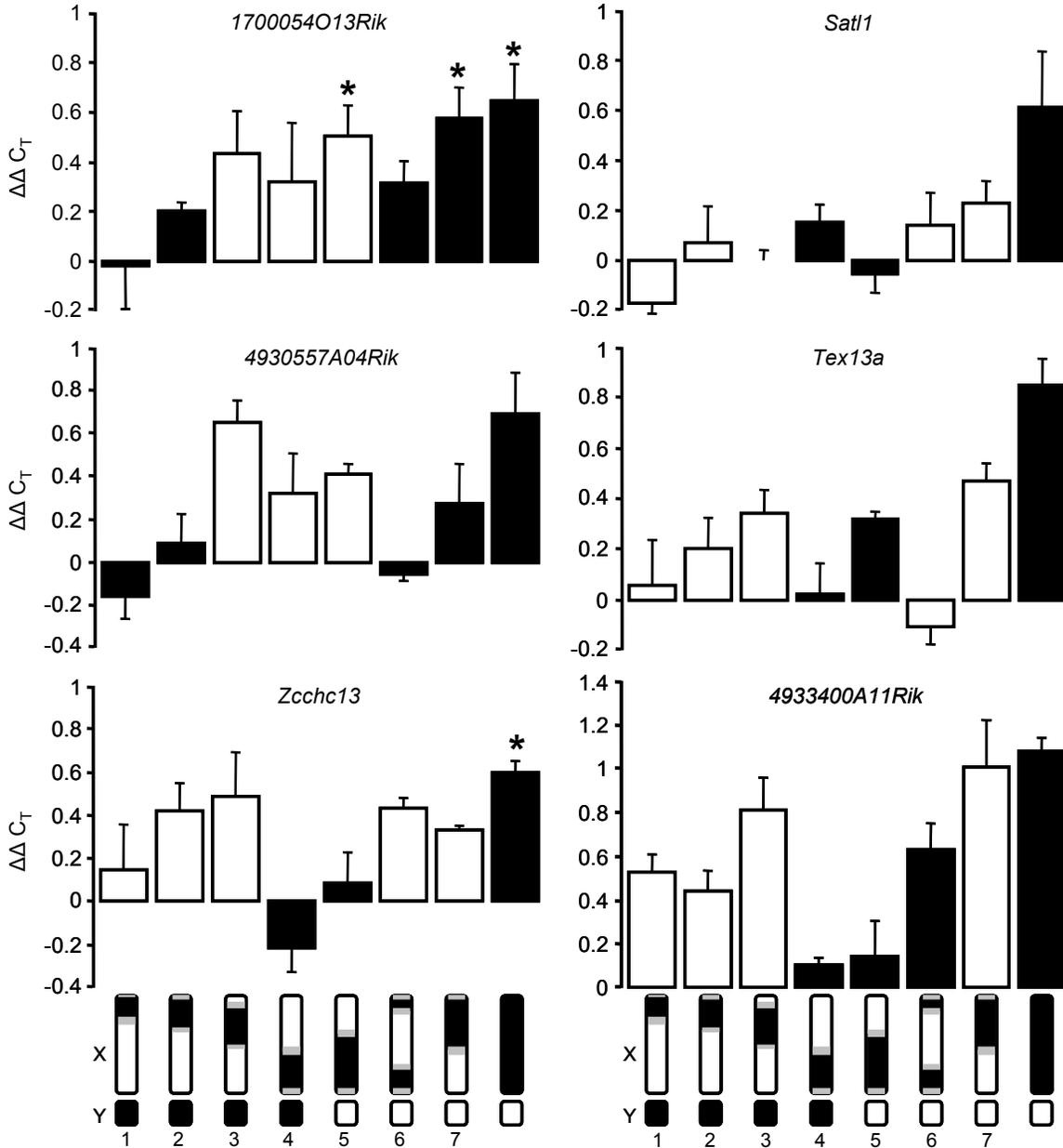P. Campbell, J. M. Good, and M. W. Nachman

Figure S2 Relative expression of six X-linked genes in the testes of F_1 males. $\Delta\Delta C_T$ is the normalized difference in expression between experimental genotypes (shown on the X axis) and the fertile F_1. X introgression genotypes are numbered 1-7; black bars denote the *musculus*[PWK] allele at a given locus, white bars denote the *domesticus*[LEWES] allele. Error bars are +/- 1 SE. Tests for significant differences between experimental and control genotypes were performed on ΔC_T values with ANOVA followed by Dunnett *post hoc* tests, * $P \leq 0.05$.

Table S1   Primers used in this study

Gene	Chr.	Primer name	Primer sequence	Amplicon size (bp)	Reference
4930557A04Rik	X	A04Rik F	KGCTGATGAGACTCCAGGAT	163	*a*
		A04Rik R	TCCACCAACAAGGGAGTCAT		
Efhc2	X	Efhc2 F	CATCCTGGGTTGCTTTTGAT	136	*a*
		Efhc2 R	TGAATGGTGTCATCTTCAAGG		
Actrt1	X	Actrt1 F	CTCAAAAATGGTCTGCAACAGC	164	*b*
		Actrt1 R	TCTTGATAGGGGTTCCCTCAAA		
1700013H16Rik	X	H16Rik F	GCAATGGCACAGCTTAATGA	68	*a*
		H16Rik R	TCCTCATTCCATTGCTCAAA		
Fmr1	X	Fmr1 F	GGAAAAGCCAGACAGCGTAG	165	*a*
		Fmr1 R	CCTGTGCCATCTTGCCTACT		
4930468A15Rik	X	A15Rik F	CACTGGCAATGGCAACTCTA	162	*a*
		A15Rik R	GCCTGCGATAACTCCTGGTA		
Dmrtc1a	X	Dmrtc1a F	GAGACGCCTGGTTGAGAGAC	104	*a*
		Dmrtc1a R	TCTTTCCAGTGCGAACTCCT		
Zcchc13	X	Zcchc13 F	GTGAACTGCAGCAAGACGAG	100	*a*
		Zcchc13 R	GGGGTTACTAAGCGGTAGCC		
Rps6ka6	X	Rps6ka6 F	AAGAACGCAGCAACGGTTAT	94	*a*
		Rps6ka6 R	AAACTGGCTCTCCCTCTTCC		
Satl1	X	Satl1 F	AACCATTGGATTTGCCATGT	153	*a*
		Satl1 R	TCGGTGTTGATGGCTATCTG		
1700008I05Rik	X	I05Rik F	AAAGCCAATTCGTGGAGACAAT	192	*b*
		I05Rik R	TGGGAGAGATGCAGAATATCCA		
Tex13a (*1700025D03Rik*)	X	Tex13a F	TGTCACCAGACCTGAAGCAG	169	*a*
		Tex13a R	CTTACTGCATTCGGGAGAGC		
Ammecr1	X	Ammecr1 F	GCTTTTCTGCTCGGTGTCTC	143	*a*
		Ammecr1 R	CAACCTCCGGTAGGTAGGTG		
4933400A11Rik	X	A11Rik F	AAATGGGTATCAGGCAGCAC	170	*a*
		A11Rik R	CAGTGGGCCATTAAGCATGT		
Acrv1	9	Acrv1 F	TGAGTACACCACTTCCAAGCA	60	*c*
		Acrv1 R	AAGCACATGTGTGGCAATTT		
Protamine 1 (Prm1)	16	Prot1 F	ACAAAATTCCACCTGCTCACA	129	*d*
		Prot1 R	CATCTGCTCCTGCTTTTGCT		*a*

Gene	Chr.	Primer name	Primer sequence	Amplicon size (bp)	Reference
Hormad1	3	Hormad1 F	CTGCTGACACCAAGAAAGCA	148	*a*
		Hormad1 R	CCTGGTGGTTGGTAATCTGG		
Hist1h1t (H1t)	13	Hist1h1t F	GTTGGCTCTCCTTTAGTTTTCACTT	128	*a*
		Hist1h1t R	GCCTTTTAGATGAAGGTTTCTCCT		
Igf1r	7	Igf1r F	CGGTGACTTCTGCTCAAATG	150	*a*
		Igf1r R	AATGGCGGATCTTCACGTAG		
Ubc	5	Ubc F	AGCCCAGTGTTACCACCAAG	118	*a*
		Ubc R	CTAAGACACCTCCCCCATCA		
beta-actin (actb1)	5	B-actin F	CTGTATTCCCCTCCATCGTG	167	*a*
		B-actin R	CTTCTCCATGTCGTCCCAGT		

a, this study; *b*, Coquet *et al.* 2009; *c*, Åkerfelt *et al.* 2008; *d*, Okada *et al.* 2007

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