

1   **Title:** Stage-specific disruption of X chromosome expression during spermatogenesis in sterile  
2   house mouse hybrids

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16   **Data Availability:** The data reported in this paper are available through the National Center for  
17   Biotechnology Information Sequence Read Archive under accession numbers PRJNA296926  
18   (*domesticus* and *musculus* RNAseq data), PRJNA352861 (F1 hybrid RNAseq data),  
19   PRJNA732719 (lab strain whole genome sequence data). *Prdm9* sequences were deposited in  
20   Genbank under accession numbers MZ733983-MZ733986. Male reproductive phenotype data  
21   are available in Table S1.

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26 **Running Title:** Disruption of X chromosome expression in sterile hybrids

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## ABSTRACT

51 Hybrid sterility is a complex phenotype that can result from the breakdown of spermatogenesis  
52 at multiple developmental stages. Here, we disentangle two proposed hybrid male sterility  
53 mechanisms in the house mice, *Mus musculus domesticus* and *M. m. musculus*, by comparing  
54 patterns of gene expression in sterile F1 hybrids from a reciprocal cross. We found that hybrid  
55 males from both cross directions showed disrupted X chromosome expression during prophase  
56 of meiosis I consistent with a loss of Meiotic Sex Chromosome Inactivation (MSCI) and *Prdm9*-  
57 associated sterility, but that the degree of disruption was greater in mice with an *M. m. musculus*  
58 X chromosome consistent with previous studies. During postmeiotic development, gene  
59 expression on the X chromosome was only disrupted in one cross direction, suggesting that  
60 misexpression at this later stage was genotype-specific and not a simple downstream  
61 consequence of MSCI disruption which was observed in both reciprocal crosses. Instead,  
62 disrupted postmeiotic expression may depend on the magnitude of earlier disrupted MSCI, or  
63 the disruption of particular X-linked genes or gene networks. Alternatively, only hybrids with a  
64 potential deficit of *Sly* copies, a Y-linked ampliconic gene family, showed overexpression in  
65 postmeiotic cells, consistent with a previously proposed model of antagonistic coevolution  
66 between the X and Y-linked ampliconic genes contributing to disrupted expression late in  
67 spermatogenesis. The relative contributions of these two regulatory mechanisms and their  
68 impact on sterility phenotypes awaits further study. Our results further support the hypothesis  
69 that X-linked hybrid sterility in house mice has a variable genetic basis, and that genotype-  
70 specific disruption of gene regulation contributes to overexpression of the X chromosome at  
71 different stages of development. Overall, these findings underscore the critical role of epigenetic  
72 regulation of the X chromosome during spermatogenesis and suggest that these processes are  
73 prone to disruption in hybrids.

## INTRODUCTION

75 Hybrid sterility can result from the breakdown of gametogenesis at several developmental  
76 stages, from early divisions of mitotic cells, meiosis, to the differentiation of postmeiotic cells into  
77 mature gametes. After gamete production, hybrid fertility can also be reduced through  
78 mechanisms that impede fertilization, such as a failure of hybrid sperm to transfer or fertilize. In  
79 hybrid males, sterility is typically measured by quantitative traits such as testes weight and  
80 histology; sperm counts, motility, and morphology; and the ability to sire offspring. Often, these  
81 traits are correlated (White *et al.* 2011; Turner and Harr 2014; Larson *et al.* 2018b) and are  
82 evaluated as though they were a single phenotype, but that does not mean sterility arises from a  
83 single mechanism or genetic basis (Reed and Markow 2004; Campbell and Nachman 2014). To  
84 tease apart different mechanisms of hybrid sterility requires a developmental framework, where  
85 breakdown at different stages of spermatogenesis can be evaluated to understand, as a whole,  
86 the evolution of hybrid sterility (Larson *et al.* 2018a; Cutter and Bundus 2020).

87 Hybrid sterility is often a composite phenotype because it typically has a complex  
88 genetic basis that involves the negative epistatic interactions of multiple alleles, known as  
89 Dobzhansky-Muller Incompatibilities or DMIs (Dobzhansky 1937; Muller 1942, see also Bateson  
90 1909). Incompatible alleles can be polymorphic or have modifiers that affect their expression  
91 (Cutter 2012), so that the extent of reproductive isolation varies among individuals within or  
92 between populations (Good *et al.* 2008b; Sweigart and Flagel 2015; Case *et al.* 2016;  
93 Mandeville *et al.* 2017; Bracewell *et al.* 2017; Zuellig and Sweigart 2018). DMIs can also evolve  
94 early in the divergence process (Coughlan and Matute 2020) and are expected to accumulate  
95 over time so that many different epistatic combinations of alleles may contribute to hybrid  
96 breakdown (Moyle and Nakazato 2010; Wang *et al.* 2013). Gene flow between populations can  
97 also lead to recombination of incompatible alleles (Bank *et al.* 2012; Lindtke and Buerkle 2015),  
98 which can further complicate patterns of population-level variation in DMIs (Larson *et al.* 2018b;

99 Meiklejohn *et al.* 2018). For all these reasons, careful laboratory dissection of sterility  
100 phenotypes remains a critical component of understanding the genetic basis of speciation.

101 Between subspecies of house mice, *Mus musculus domesticus* and *M. m. musculus*  
102 (hereafter *domesticus* and *musculus*), the evolution of hybrid sterility appears to be due to a  
103 combination of several genetic factors. These subspecies diverged ~350-500 mya (Geraldes *et*  
104 *al.* 2011; Duvaux *et al.* 2011; Phifer-Rixey *et al.* 2020) and have come into secondary contact in  
105 a long hybrid zone in central Europe (Macholán *et al.* 2012; Phifer-Rixey and Nachman 2015).  
106 Female hybrids are generally more fertile than males (but see Suzuki and Nachman 2015) and  
107 hybrid male fertility varies considerably in the hybrid zone (Turner *et al.* 2012). In crosses  
108 between *domesticus* females and *musculus* males, hybrid male sterility depends on which  
109 individual genotypes are sampled, while crosses between *musculus* females and *domesticus*  
110 males typically produce sterile hybrid males (Vanlerberghe *et al.* 1986; Alibert *et al.* 1997;  
111 Britton-Davidian *et al.* 2005; Vyskočilová *et al.* 2005; Good *et al.* 2008b; Turner *et al.* 2012).  
112 There are many different autosomal regions that have been associated with hybrid  
113 sterility in house mice (e.g., Oka *et al.* 2007; Good *et al.* 2008a; White *et al.* 2011; Turner *et al.*  
114 2014; Turner and Harr 2014; Larson *et al.* 2018b; Schwahn *et al.* 2018; Morgan *et al.* 2020;  
115 Widmayer *et al.* 2020), but the primary genetic determinant of sterility in F1 hybrid males  
116 involves the rapid evolution of PRDM9 binding sites, the autosomal encoded protein that directs  
117 the location of recombination in mammals (Mihola *et al.* 2009; Mukaj *et al.* 2020). In F1 mouse  
118 hybrids, PRDM9 binds preferentially to ancestral binding sites, leading to the asymmetric  
119 formation of double strand breaks and autosomal asynapsis (Davies *et al.* 2016; Gregorova *et*  
120 *al.* 2018). When the number of asynapsed chromosomes in a cell reaches a threshold, it can  
121 trigger cell death and in the most severe cases, complete meiotic arrest (Bhattacharyya *et al.*  
122 2013). *Prdm9*-associated sterility is polymorphic, with alternative ‘fertile’ *Prdm9* alleles (Flachs  
123 *et al.* 2012; Mukaj *et al.* 2020) and is further modulated by epistatic interactions with a locus on

124 the *musculus* X chromosome (*Hstx2*, Storchová *et al.* 2004; Bhattacharyya *et al.* 2014; Lustyk *et*  
125 *al.* 2019). A characteristic signal of *Prdm9*-associated sterility is the overexpression of the X  
126 chromosome during early meiosis I (Good *et al.* 2010; Bhattacharyya *et al.* 2013; Campbell *et*  
127 *al.* 2013; Turner *et al.* 2014; Larson *et al.* 2017), a developmental stage where the X  
128 chromosome would normally be transcriptionally inactive known as Meiotic Sex Chromosome  
129 Inactivation (MSCI, Turner 2015). Whether disrupted MSCI is a byproduct, or an integral part of  
130 *Prdm9*-associated sterility is still unclear (Forejt *et al.* 2021), but it is a distinct regulatory  
131 phenotype of hybrid sterility at this developmental stage.

132 Hybrid male sterility in house mice may also be influenced by interactions among three  
133 ampliconic sex-linked gene families expressed in postmeiotic cells, *Slx* and *Slx/l1* (X  
134 chromosome) and *Sly* (Y chromosome, Ellis *et al.* 2011; Cocquet *et al.* 2012). *SLY* plays a  
135 central role in repressing the transcription of sex-linked genes, known as postmeiotic sex  
136 chromosome repression (PSCR), while *SLX/SLXL1* counteract the repression of *SLY* by  
137 competing for binding access to *SSTY1* at the promoter of thousands of postmeiotic genes  
138 (Moretti *et al.* 2020). *SLY* and *SLX/SLXL1* appear to compete through a copy-number arms  
139 race, with higher relative gene copies of *Sly* leading to the repression of other multicopy genes.  
140 Gene knockdowns of *Sly* (*i.e.*, *Sly*-deficient) result in the overexpression of the X chromosome  
141 and female-biased litters (Cocquet *et al.* 2009; Kruger *et al.* 2019), while knockdowns of  
142 *Slx/Slx/l1* (*i.e.*, *Slx*-deficient) result in a slight underexpression of the X chromosome and male-  
143 biased litters (Cocquet *et al.* 2010, 2012; Kruger *et al.* 2019). These genes have undergone a  
144 massive co-amplification across different mouse lineages, leading to different copy numbers in  
145 *domesticus* and *musculus* (Ellis *et al.* 2011; Morgan and Pardo-Manuel de Villena 2017). As a  
146 result, F1 hybrids between *musculus* females and *domesticus* males potentially have a deficit of  
147 *Sly* gene copies, while hybrids from the reciprocal cross have a deficit of *Slx/Slx/l1* gene copies  
148 (Good 2012). We previously demonstrated that the X chromosome in postmeiotic cells is

149 overexpressed in *Sly*-deficient hybrids, consistent with *Sly/Slx*-associated sterility (Larson *et al.*  
150 2017). We also observed overexpression in *Sly*-deficient hybrids of an ampliconic autosomal  
151 gene family, *a-takusan*, that is regulated by *SLY* (Moretti *et al.* 2017) and a slight  
152 underexpression of the X chromosome in *Slx*-deficient hybrids, consistent with *Sly* repression  
153 (Kruger *et al.* 2019). These results support a model of postmeiotic disruption of X chromosome  
154 expression and *Sly/Slx*-associated sterility.

155 Incompatibilities at each of these stages may produce similar sterility phenotypes, such  
156 as low testes weight and abnormal sperm morphology, making it difficult to tease apart their  
157 contribution to overall hybrid sterility and the maintenance of the house mouse hybrid zone. The  
158 disrupted expression of the X chromosome at different developmental stages suggests that  
159 hybrid sterility in these mice is a composite of multiple regulatory mechanisms (Larson *et al.*  
160 2017). However, because both *Prdm9* and *Sly/Slx* associated sterility are often asymmetric and  
161 depend on interactions with the *M. m. musculus* X chromosome it is possible that postmeiotic  
162 disruption of the X chromosome observed in some crosses is simply a downstream effect of  
163 disrupted MSCI and a cascade of disrupted X chromosome expression. In this study, we used  
164 an independent cross to help disentangle the effects of regulatory disruption at different  
165 developmental stages of spermatogenesis. We used strains of mice that produce subfertile  
166 hybrid males in both cross directions, but only offspring from *musculus* females and *domesticus*  
167 males have a *Sly* deficit. We found that both reciprocal hybrids showed disrupted MSCI,  
168 consistent with *Prdm9*-associated sterility. However, only the hybrids that had the greater  
169 disruption of MSCI and are *Sly*-deficient showed disrupted postmeiotic X chromosome  
170 expression, suggesting that postmeiotic disruption is genotype-specific. Collectively, these  
171 results further underscore the considerable genotypic and phenotypic (regulatory and  
172 reproductive) variably underlying F1 hybrid sterility between these closely-related mouse  
173 lineages.

174

175 **MATERIALS & METHODS**

176 **Crosses and reproductive phenotypes**

177 We used four inbred strains of wild-derived mice from two subspecies of *domesticus* (WSB/EiJ  
178 and LEWES/EiJ) and *musculus* (PWK/PhJ and CZECHII/EiJ). First, we generated intraspecific  
179 F1s between strains of *domesticus* (WSB females × LEWES males) and *musculus* (CZECHII  
180 females × PWK males). These mice served as parental controls for each species, but without  
181 the negative effects of inbreeding on male fertility. Second, we generated intersubspecific F1  
182 hybrids in reciprocal crosses between one strain of each subspecies (CZECHII females × WSB  
183 males and WSB females × CZECHII males, *hereafter*: ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup> and ♀ *dom*<sup>WSB</sup> ×  
184 ♂ *mus*<sup>CZII</sup>; throughout the manuscript we will indicate all crosses as female parent × male  
185 parent). We chose crosses involving CZECHII mice because F1 hybrid males from these  
186 crosses are subfertile in both directions of the cross (Good *et al.* 2008b; Larson *et al.* 2018b).  
187 This provided a direct contrast to other studies using strains that produce subfertile F1 hybrid  
188 males in only one cross direction, (*i.e.* PWK females × LEWES males, Good *et al.* 2010;  
189 Campbell *et al.* 2013; Mack *et al.* 2016; Larson *et al.* 2017), which allowed us to begin to isolate  
190 the effects of disrupted MSCI and imbalanced copy numbers of *Slx* and *Sly* on regulatory  
191 phenotypes. Experimental mice used in this study were obtained from breeding colonies  
192 established from mice purchased from The Jackson Laboratory (Bar Harbor, ME) in 2010 and  
193 were maintained at the University of Montana Department of Laboratory Animal Resources  
194 (IACUC protocol 002-13). One *domesticus* mouse had a sire from replacement stock of  
195 LEWES/EiJ ordered in 2013. The stock origin for each mouse is indicated in **Table S1**.  
196 We weaned experimental mice at ~21 days after birth and housed them in same sex  
197 sibling groups until males were individually isolated at 45 days. We euthanized males between

198 61 and 89 days old using CO<sub>2</sub> followed by cervical dislocation. Immediately after euthanasia we  
199 quantified male reproductive traits following previously described protocols (Good *et al.* 2008b,  
200 2008a). We weighed paired testes and seminal vesicles relative to body weight and isolated  
201 sperm by dicing the caudal epididymides in 1 mL of Dulbecco's PBS (Sigma) followed by a 10  
202 min incubation at 37°C. We estimated the proportion of motile sperm and total sperm numbers  
203 using 5 µL sperm suspensions (regular and heat-shocked, respectively) viewed in a Makler  
204 counting chamber on a light microscope over a fixed area and observation time. We fixed and  
205 stained 25 µL sperm suspensions and later counted 100 intact sperm to visually classify  
206 morphology. All samples were counted by a single individual (E.L.L.) while blind to genotype.  
207 We classified sperm as (1) normal with a long apical hook, (2) slightly abnormal with a  
208 shortened hook, (3) abnormal with a short hook and rounded shape, and (4) severely abnormal  
209 with an amorphous shape. We summarized sperm morphology using a weighted index that  
210 ranged from high (3) to low (0) following Oka *et al.* (2004) and Good *et al.* (2008a).

211

## 212 **RNA sequencing of spermatogenesis stages**

213 Testes are composed of at least eleven major cell types, with cell-specific patterns of gene  
214 expression (Margolin *et al.* 2014; Green *et al.* 2018). Whole testes expression patterns can be  
215 confounded by differences in cell composition between species, or between sterile and fertile  
216 hybrids (Good *et al.* 2010; Hunnicutt *et al.* 2021). To overcome these challenges, we used  
217 fluorescence activated cell sorting (FACS) to isolate highly enriched cell populations for three  
218 developmental stages of spermatogenesis: early prophase of meiosis I prior to MSCI  
219 (leptotene/zygotene cells), meiosis I after MSCI (diplotene cells) and postmeiotic development  
220 prior to spermiogenesis (round spermatids). Our complete FACS protocol, modified from Getun  
221 *et al.* (2011), is available on Github (<https://github.com/goodest-goodlab/good->

222 [protocols/tree/main/protocols/FACS](#)). We decapsulated the testes and disassociated them in a  
223 mixture of 1 mg/mL collagenase (Worthington Biochemical), GBSS (Sigma) and 1 mg/mL  
224 trypsin (Worthington Biochemical). We inactivated the trypsin with 0.16 mg/mL fetal calf serum  
225 (Sigma) and stained the cells with 0.36 mg/mL of Hoechst 33343 (Invitrogen) and 0.002 mg/mL  
226 propidium iodide. At each step, we incubated solutions in a mini shaker at 120 rpm at 33°C for  
227 15 min and added 0.004 mg/mL DNase to eliminate clumps. We filtered disassociated cells  
228 twice using a 40  $\mu$ m strainer and sorted cells on a FACS Aria IIu cell sorter (BD Biosciences) at  
229 the UM Center for Environmental Health Sciences Fluorescence Cytometry Core. FACS  
230 isolates cells based on size, granularity, and fluorescence (traits that change across different  
231 stages of spermatogenesis). We collected enriched cell populations in 15  $\mu$ L beta  
232 mercaptoethanol (Sigma) per mL of RLT lysis buffer (Qiagen) and extracted RNA from each cell  
233 type using a Qiagen RNeasy kit. We quantified our samples on a Bioanalzyer 2000 (Agilent)  
234 and prepared samples with RNA integrity (RIN) above 8 for sequencing using an Illumina  
235 Truseq Sample Prep Kit v2 in a design that avoided batch effects between cell populations and  
236 genotypes. We extracted RNA from a total of 21 mice, using the highest quality enriched cell  
237 populations to generate RNAseq libraries for three individuals per cell type, three cell types and  
238 four crosses (*domesticus*, *musculus* and their reciprocal F1 hybrids,  $n = 36$  RNAseq libraries).

239 We sequenced each library on an Illumina HiSeq 2500 (SE, 100 bp) at the University of  
240 Oregon Genomics and Cell Characterization Core Facility and on an Illumina HiSeq 2000 (PE,  
241 100 bp) and a NextSeq 500 (SE, 100 bp) at the University of Southern California Epigenome  
242 Center. While all of the RNAseq libraries in this study were prepared simultaneously, we  
243 previously published a subset of these data, the *domesticus* and *musculus* parent samples, as  
244 part of a study on the rate of molecular evolution in spermatogenesis (Larson *et al.* 2016). Here

245 we focus on comparisons between reciprocal F1 hybrids (unpublished data) and their parents,  
246 to disentangle the effects of different developmental stages on regulatory disruption in hybrids.

247

## 248 **Read mapping and differential expression analyses**

249 We trimmed reads using TRIMOMATIC v0.32 (Bolger *et al.* 2014) and mapped reads using  
250 TOPHAT v2.0.10 (Kim *et al.* 2013) to strain-specific pseudo-references for *domesticus*  
251 (WSB/EiJ) and *musculus* (PWK/PhJ) (Huang *et al.* 2014). These pseudo-references incorporate  
252 all known SNPs, indels and structural variants for these strains relative to the Genome  
253 Reference Consortium mouse build 38 (GRCm38), thereby minimizing mapping bias to the  
254 mouse reference genome, which is predominately *domesticus* (Yang *et al.* 2011). We used  
255 LAPELS v1.0.5 to translate our reads back into the GRCm38 coordinates and SUSPENDERS  
256 v0.2.4 to merge our alignments (Huang *et al.* 2014). We counted the number of reads that  
257 mapped to protein-coding genes (Ensembl release 78) using FEATURECOUNTS v1.4.4 (Liao  
258 *et al.* 2014). We counted reads that were 1) uniquely mapped to a single protein-coding gene  
259 and 2) mapped to multiple protein-coding genes. These two approaches were qualitatively the  
260 same, but by including multi-mapped reads we could account for the expression of multicopy  
261 gene families that are enriched on the mouse X chromosome, and in all cases we report these  
262 results.

263 We analyzed gene expression using Bioconductor v3.0 package edgeR v3.30.3  
264 (Robinson *et al.* 2010) in R v4.0.1 (R Core Team 2020). We normalized our data using the  
265 scaling factor method and restricted our analysis to genes with a minimum expression of FPKM  
266 > 1 in at least three samples. For all analyses, we tested alternative normalization methods  
267 (e.g., weighted trimmed mean of M-values) and found qualitatively similar results. We fit our  
268 data with a negative binomial generalized linear model with Cox-Reid tagwise dispersion  
269 estimates (McCarthy *et al.* 2012). Our model included cross and cell type as a single factor and

270 our design matrix contrasted different crosses for each cell type. To evaluate differential  
271 expression, we used likelihood ratio tests, dropping one coefficient from the design matrix and  
272 comparing that to the full model. For each contrast, we restricted our differentially expressed  
273 (DE) genes to genes that are expressed in the focal cell type (FPKM > 1 in 3/6 samples) and in  
274 all cases used a p-value adjusted for a false discovery rates (FDR) of 5% (Benjamini and  
275 Hochberg 1995). For all our RNAseq analysis, we focused on contrasts between each hybrid  
276 and their parental X chromosome, to account for potential mapping biases on the hemizygous X  
277 ( $\text{♀ } \text{mus}^{\text{CZII}} \times \text{♂ } \text{dom}^{\text{WSB}}$  vs. *musculus*;  $\text{♀ } \text{dom}^{\text{WSB}} \times \text{♂ } \text{mus}^{\text{CZII}}$  vs. *domesticus*) and contrasts  
278 between the two F1 hybrids ( $\text{♀ } \text{mus}^{\text{CZII}} \times \text{♂ } \text{dom}^{\text{WSB}}$  vs.  $\text{♀ } \text{dom}^{\text{WSB}} \times \text{♂ } \text{mus}^{\text{CZII}}$ ).

279 We used a sliding gene window to test for local enrichment of autosomal genes that  
280 were overexpressed in round spermatids of  $\text{♀ } \text{mus}^{\text{CZII}} \times \text{♂ } \text{dom}^{\text{WSB}}$  hybrids compared to  
281  $\text{♀ } \text{dom}^{\text{WSB}} \times \text{♂ } \text{mus}^{\text{CZI}}$  hybrids. We counted the proportion of genes that were up (+logFC) or  
282 down (-logFC) regulated within a given window and identified windows that fell outside of the  
283 99<sup>th</sup> quantile modeled with a Poisson distribution. We tested a range of window sizes (50-400  
284 genes/window) and found qualitatively similar results, so we used 250 genes/window. This  
285 method has been previously used to identify overexpression of an ampliconic autosomal gene  
286 family, *a-takusan* in sterile *musculus* x *domesticus* hybrids (Larson et al. 2017).

287

### 288 **Sequencing of *Prdm9* alleles**

289 We characterized the *Prdm9* Exon12 allele for each strain used in our study. For each strain, we  
290 extracted DNA from liver tissue of a single mouse using a Nucleospin Tissue Kit (Macherey-  
291 Nagel) and quantified the DNA with a QuantiFluor dsDNA System (Promega) on a Synergy HTX  
292 Multi-Mode Microplate Reader (Agilent). We amplified *Prdm9* Exon12 using the primers  
293 Exon12-L1 and Exon12-R (Mukaj et al. 2020), GoTaq Polymerase (Promega) and the following

294 protocol: an initial denaturation at 95°C for 2 min, followed by 41 cycles of 95°C for 30 s, 56°C  
295 for 30 s, and 72°C for 1 min, with a final extension step of 72°C for 5 min. We purified and  
296 sequenced amplicons at Genewiz (New Jersey, USA), using their hairpin sequencing. We  
297 manually cleaned and translated sequences in Geneious 9.1.8 (Biomatters) and aligned  
298 sequences using MAFFT v7.453 (Katoh and Standley 2013). We identified C-terminal zinc  
299 finger domains by searching sequences with hmmsearch for the Zf-C2H2 HMM profile  
300 (PF00096.27) from the Pfam database (HMMER v3.3.2; Mistry *et al.* 2021). We excluded the  
301 first nonvariant zinc finger domain then compared the -1, 3, and 6 positions within each domain  
302 (as in Oliver *et al.* 2009) to previously published *musculus* and *domesticus* alleles (Mukaj *et al.*  
303 2020).

304

### 305 **Gene Copy Number Estimates**

306 To estimate *Slx* and *Sly* gene copy numbers, we generated whole genome sequence data from  
307 a single mouse of each strain used in our study (*domesticus* WSB/EiJ, LEWES/EiJ and  
308 *musculus* PWK/PhJ and CZECHII/EiJ). For each sample, we prepared and sequenced libraries  
309 twice to increase unique read coverage. We extracted DNA from liver tissue using a Qiagen  
310 DNeasy kit and sent samples to Novogene for library preparation and sequencing on an Illumina  
311 NovaSeq 6000 (PE, 150bp). We trimmed reads with TRIMOMATIC v0.39 (Bolger *et al.* 2014),  
312 mapped our reads to the GRCm38 using BWA-MEM v0.7.17 (Li and Durbin 2009), and fixed  
313 mates and marked duplicates with Picard v2.18.29 (Broad Institute 2019). We merged the data  
314 from each sequencing effort resulting in 10-15X average genome-wide coverage.

315 To identify paralogs of ampliconic gene families, we extracted *Slx*, *Slx1*, and *Sly* gene  
316 sequences from the mouse reference GRCm38 using Ensembl release 102 (Yates *et al.* 2019).  
317 We performed Ensembl BLAT searches with these sequences against the GRCm38 mouse  
318 reference, allowing up to 1000 hits. We then extracted all BLAT hits with greater than or equal to

319 97% sequence identity and an e-value of 0.0 and considered these filtered BLAT hits to be gene  
320 family paralogs for downstream copy number estimation.

321 We estimated copy number using two methods based on relative coverage. First, we  
322 followed a similar approach as Morgan and Pardo-Manuel de Villena (2017) and used Mosdepth  
323 (Pedersen and Quinlan 2018) to estimate coverage in paralog regions and the average  
324 coverage across the whole genome. We estimated copy number by summing coverage across  
325 paralog regions and dividing this sum by half the genome-wide average coverage. We halved  
326 the average coverage because most of the mouse genome is diploid, while the sex  
327 chromosomes in males are haploid. We also used the approach implemented in AmpliCoNE  
328 (Vegesna *et al.* 2019), which estimates copy number from relative coverage using only regions  
329 that are considered informative based on repeat masking and mappability, while also controlling  
330 for GC content. AmpliCoNE was developed for estimating gene copy numbers on the human Y  
331 chromosome, so we made some modifications to account for the less complete assembly and  
332 annotation of the mouse sex chromosomes. Specifically, instead of relying on informative sites  
333 to differentiate copy numbers, we extracted all kmers of length 101bp from the *Slx*, *Slx1*, and  
334 *Sly* gene sequences and mapped these back to the mouse reference genome using Bowtie2,  
335 allowing up to 500 multiple mapping hits. For each gene, we identified the most frequent  
336 number of times ( $m$ ) kmers mapped to the mouse genome and kept only kmers that mapped  $m$   
337 times. We identified all locations where these kmers mapped with 2 or fewer mismatches and  
338 used these kmer start locations as the “informative sites” metric for AmpliCoNE.

339

#### 340 **Data Availability**

341 The data reported in this paper are available through the National Center for Biotechnology  
342 Information Sequence Read Archive under accession numbers PRJNA296926 (*domesticus* and  
343 *musculus* RNAseq data), PRJNA352861 (F1 hybrid RNAseq data), PRJNA732719 (lab strain

344 whole genome sequence data). *Prdm9* sequences were deposited in Genbank under accession  
345 numbers MZ733983-MZ733986. Male reproductive phenotype data are available in **Table S1**.

346

347 **RESULTS**

348 **Hybrid males from both cross directions were subfertile**

349 We found F1 hybrid males from crosses between *domesticus* (WSB) and *musculus* (CZECHII)  
350 were subfertile in both cross directions, but that ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup> hybrids had more severe  
351 abnormal sperm morphology. Overall, ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup> hybrids had lower fertility than both  
352 *domesticus* and *musculus*, with significantly smaller testes, lower sperm counts, and more  
353 abnormal sperm morphology, while ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup> hybrids had smaller testes and lower  
354 sperm counts, but after correcting for multiple tests these values were only significant in  
355 comparisons with *domesticus* (**Table 1**). The ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup> hybrids did have the most  
356 severely abnormal sperm morphology consistent with previous studies (Good *et al.* 2008b;  
357 Larson *et al.* 2018b). There were no significant differences in the relative seminal vesicle weight  
358 or the proportion of motile sperm across any comparisons.

359

360 **Table 1. Reproductive phenotypes of male mice used in this study.** The table summarizes  
361 the sample sizes for each cross (N) and the median (± standard error) trait value for five  
362 reproductive phenotypes. Arrows indicate whether the hybrids had significantly lower  
363 reproductive values relative to *domesticus* (closed arrows) or *musculus* (open arrows). Values in  
364 bold indicate traits that were significantly different between the two F1 hybrids. Testes and  
365 seminal vesicle weights are reported relative to body size. The sperm morphology index ranged  
366 from 3 (high quality sperm) to 0 (severally abnormal sperm). Significance was estimated using a  
367 Wilcoxon test with p-values FDR corrected for multiple comparisons.

Cross	N	Relative testis weight (mg/g)	Relative seminal vesicle weight (mg/g)	Proportion motile sperm	Sperm count (1x10 <sup>6</sup> )	Sperm head morphology index
<i>domesticus</i>	6	11.30 ± 0.34	5.18 ± 0.29	0.82 ± 0.04	14.8 ± 1.80	2.99 ± 0.01
<i>musculus</i>	5	9.53 ± 0.63	5.58 ± 1.30	0.87 ± 0.06	17.8 ± 2.50	3.00 ± 0.03
♀ <i>dom</i> <sup>WSB</sup> × ♂ <i>mus</i> <sup>CZII</sup>	6	▼▽6.28 ± 0.28	5.68 ± 0.54	0.83 ± 0.06	▼▽4.2 ± 0.72	▼▽1.29 ± 0.07
♀ <i>mus</i> <sup>CZII</sup> × ♂ <i>dom</i> <sup>WSB</sup>	4	▼6.46 ± 0.35	5.44 ± 0.27	0.65 ± 0.12	▼5.8 ± 2.70	▼▽0.66 ± 0.12

368

369 **Cell-specific gene expression**

370 For each cross, we generated between 14.7 and 26.8 million mapped fragments (paired or  
 371 unpaired reads) per cell type (738 million total, mean = 20.5 million). After filtering we retained  
 372 14,209 expressed protein-coding genes. Gene expression profiles clustered by cell type (**Fig**  
 373 **S1A**) and within each cell type, samples clustered by parental species with F1 hybrids  
 374 intermediate to the two parents (**Fig S1B-D**). Overall, the strong clustering by cell type and  
 375 cross, and the overall low variation among our samples (biological coefficient of variation =  
 376 0.1748), indicates our FACS approach generated high quality cell-specific data.

377

378 **Disrupted meiotic X inactivation in both subfertile hybrids**

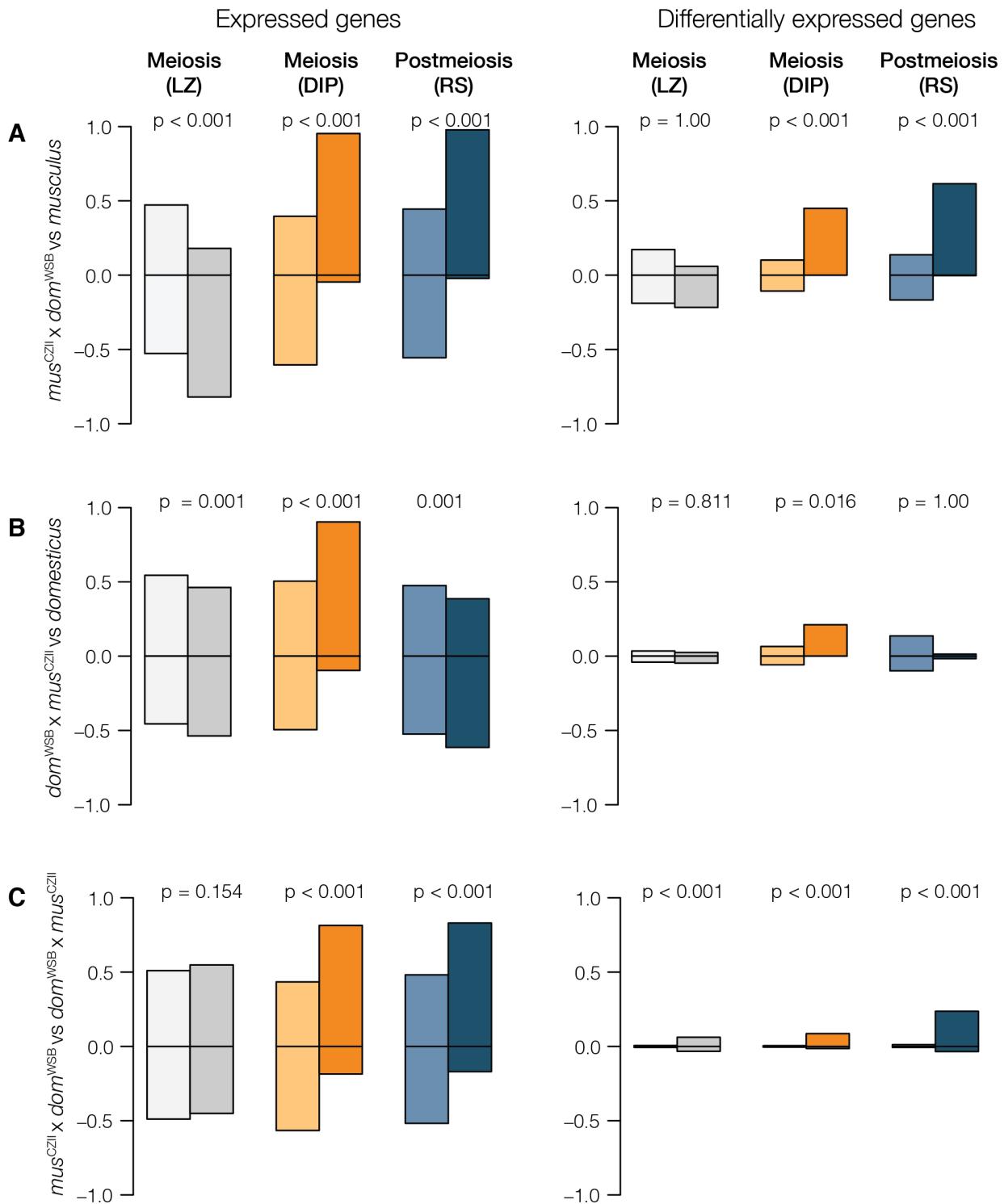
379 We found disrupted meiotic X chromosome inactivation (diplotene cells) in both subfertile  
 380 hybrids, but the disruption was more severe in ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup> hybrids. Consistent with  
 381 previous results (Larson *et al.* 2016, 2017), fertile *domesticus* and *musculus* males had very few  
 382 X-linked genes expressed in diplotene cells. In contrast, both F1 hybrids had elevated  
 383 expression of X-linked genes in diplotene cells (**Fig S2**), consistent with disrupted MSCI. In  
 384 comparisons between F1 hybrids and their parents with the same X chromosome (♀ *mus*<sup>CZII</sup> ×  
 385 ♂ *dom*<sup>WSB</sup> vs. *musculus*; ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup> vs. *domesticus*), F1 hybrids expressed more X-  
 386 linked genes and every differentially expressed (DE) gene was overexpressed in hybrids. In

387 contrast, there was no obvious asymmetry in expression on the autosomes (**Figs 1A, B**). When  
388 we compared the two hybrids,  $\text{♀ } \text{mus}^{\text{CZII}} \times \text{♂ } \text{dom}^{\text{WSB}}$  had higher X-linked expression and DE  
389 genes between the hybrids were largely overexpressed in  $\text{♀ } \text{mus}^{\text{CZII}} \times \text{♂ } \text{dom}^{\text{WSB}}$  hybrids (**Fig**  
390 **1C**), suggesting that disrupted MSCI was more severe in  $\text{♀ } \text{mus}^{\text{CZII}} \times \text{♂ } \text{dom}^{\text{WSB}}$  hybrids.

391

392 **Reciprocal hybrids have identical *Prdm9* genotypes**

393 We characterized all four strains for allelic variation within Exon12 of *Prdm9* and confirmed that  
394 both *musculus* strains (PWK and CZECHII) shared the same *msc1* ‘sterile’ allele and both  
395 *domesticus* strains (WSB and LEWES) shared the same ‘sterile’ *dom3* *Prdm9* allele (Forejt *et*  
396 *al.* 2021). Therefore, both  $\text{♀ } \text{mus}^{\text{CZII}} \times \text{♂ } \text{dom}^{\text{WSB}}$  and  $\text{♀ } \text{dom}^{\text{WSB}} \times \text{♂ } \text{mus}^{\text{CZII}}$  hybrids have the  
397 same *Prdm9* genotype at Exon12 (*msc1/dom3*).



398

399 **Fig 1.** Gene expression comparisons among hybrids and parents for autosomes (light colors)

400 and the X chromosome (dark colors). There are three contrasts: **A)** ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup>

401 hybrids compared to *musculus* , **B)** ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup> hybrids compared to *domesticus*, **C)**  
402 ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup> hybrids compared to ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup> hybrids. The first column  
403 shows the proportion of genes with higher or lower expression in a given contrast out of the total  
404 genes expressed in each cell type. The second column shows the proportion of those genes  
405 that are DE. Significant p-values indicate contrasts where there was a significant difference in  
406 the proportion of over or underexpressed genes on the X chromosome compared to the  
407 autosomes (Pearson's chi square test with FDR corrected p-values, Benjamini and Hochberg  
408 1995). LZ = leptotene/zygotene cells (meiosis before MSCI), DIP = diplotene cells (meiosis after  
409 MSCI), RS = round spermatids (postmeiosis).

410

#### 411 **Imbalanced *Sly* and *Slx/Slx1* copy numbers in reciprocal hybrids**

412 We estimated gene copy number for postmeiotic amplicon families in our mouse strains using  
413 two methods and found that *musculus* had higher copy number for *Sly*, *Slx*, and *Slx1* (**Table 2**).  
414 Our copy number estimates for *Sly* and *Slx* differed from what has been estimated using qPCR  
415 (Ellis *et al.* 2011) - we found higher copy numbers of *Sly* and lower copy numbers of *Slx*. Our  
416 estimates were closer to those from other studies that have used a computational approach to  
417 estimate copy number (Morgan and Pardo-Manuel de Villena 2017) and were similar to  
418 estimates for the *domesticus* Y chromosome assembly (Soh *et al.* 2014). Both our results and  
419 these other studies consistently found higher copy numbers in *musculus*, indicating there is an  
420 imbalance in *Sly* and *Slx/Slx1* copy numbers of F1 hybrids relative to parental strains.

421

422

423

424 **Table 2:** Copy number estimates for the *Sly* and *Slx/Slx1* gene families for the wild-derived  
425 mouse strains used in this study.

		Mosdepth			AmpliCoNE		
		<i>Sly</i>	<i>Slx</i>	<i>Slx1</i>	<i>Sly</i>	<i>Slx</i>	<i>Slx1</i>
<i>musculus</i>	CZECHII	206	51	34	217	62	38
	PWK	192	48	34	213	50	38
<i>domesticus</i>	LEWES	152	16	22	134	15	20
	WSB	155	13	29	127	13	25

426

427 **Postmeiotic disruption in *Sly*-deficient hybrids**

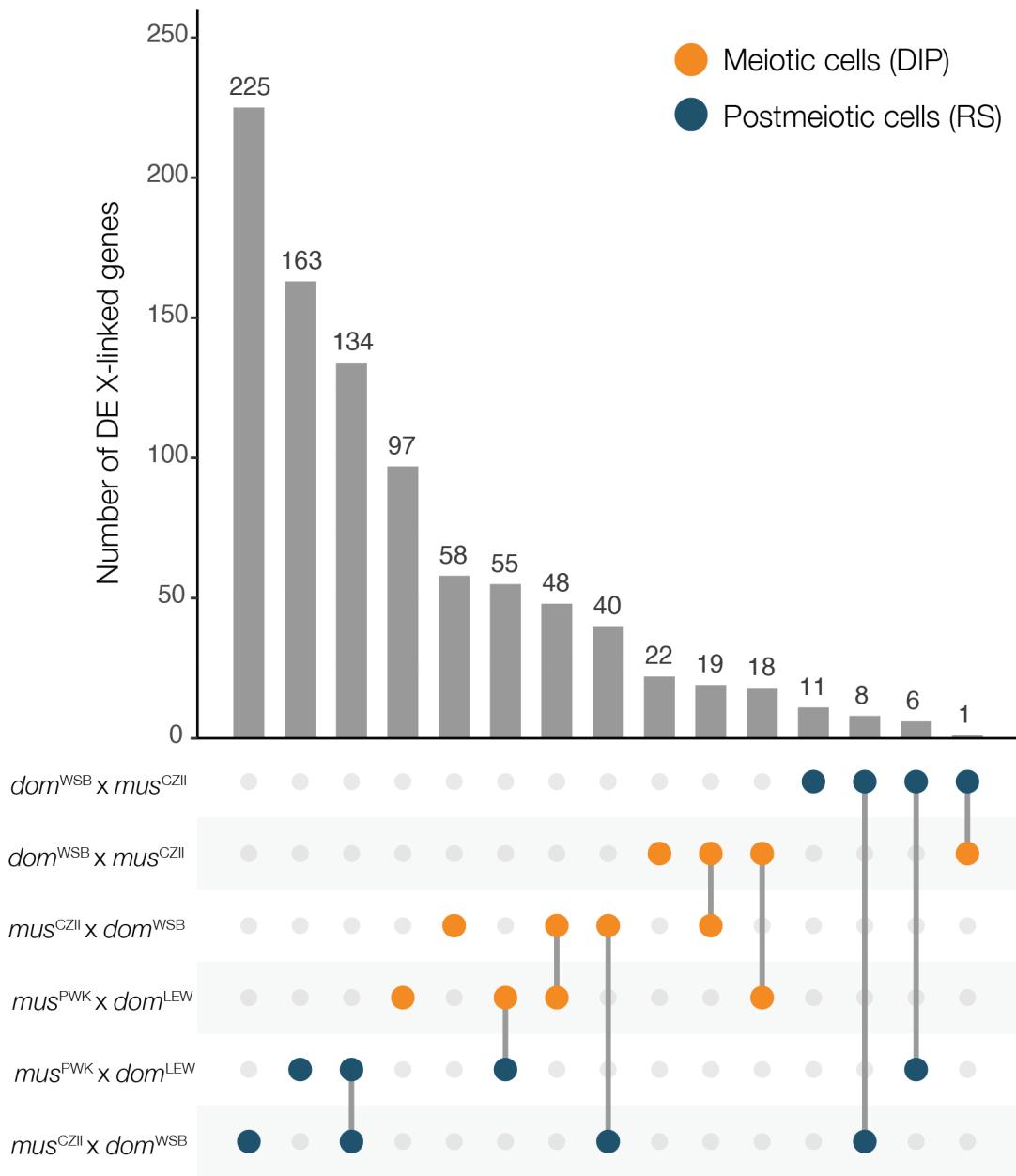
428 The X chromosome was overexpressed in postmeiotic round spermatids of ♀ *mus*<sup>CZII</sup> ×  
429 ♂ *dom*<sup>WSB</sup> hybrids (*Sly*-deficient), but not in ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup> hybrids (*Slx*-deficient). Nearly  
430 all of the X-linked postmeiotic genes in ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup> hybrids were overexpressed  
431 relative to *musculus* and more than half of these were DE (Fig 1A). In contrast, ♀ *dom*<sup>WSB</sup> ×  
432 ♂ *mus*<sup>CZII</sup> hybrids had genes that were both over- and under-expressed relative to *domesticus*  
433 and DE genes tended to be expressed at lower levels (although this pattern wasn't significant)  
434 (Fig 1B). There were no clear asymmetries on the autosomes for either hybrid relative to their  
435 parent. Given that both hybrids showed some degree of disrupted expression in meiotic cells,  
436 this suggests postmeiotic disruption is not a simple downstream consequence of earlier MSCI  
437 disruption, but is either an independent mechanism for postmeiotic disruption in *Sly*-deficient  
438 hybrids or there is a threshold of disrupted MSCI required to lead to downstream disruption.  
439 SLX/SLXL1 and SLY compete for interaction with SSTY1 at promotors to regulate a  
440 suite of postmeiotic multicopy genes, including autosomal gene families *a-takusan* and *Speer*

441 (Moretti *et al.* 2017, 2020). To test if we could detect misexpression of these autosomal gene  
442 families in our *Sly*-deficient hybrids, we used a sliding gene-window analysis (250  
443 genes/window) to identify genomic regions with clusters of over or under-expressed genes  
444 between our reciprocal F1 hybrids. We found two small gene windows on chromosomes 5 and 8  
445 that exceeded our threshold for overexpressed gene-windows (99th quantile modeled with a  
446 Poisson distribution) (**Fig S3**), but these regions did not overlap with any known multicopy gene  
447 families. We did not detect any large gene-windows that were consistently overexpressed in  
448 *Sly*-deficient hybrids as we did in crosses between *mus*<sup>PWK</sup> x *dom*<sup>LEW</sup> (Larson *et al.* 2017).  
449

#### 450 **Comparison of patterns of disrupted X expression across different hybrid genotypes**

451 Finally, we used previously published data from Larson *et al.* 2017 to compare overlap in X-  
452 linked DE genes between reciprocal subfertile hybrids in this study (♀ *mus*<sup>CZII</sup> x ♂ *dom*<sup>WSB</sup>,  
453 ♀ *dom*<sup>WSB</sup> x ♂ *mus*<sup>CZII</sup>) with other subfertile hybrids (♀ *mus*<sup>PWK</sup> x ♂ *dom*<sup>LEW</sup>). We found the  
454 greatest number of X-linked DE genes in postmeiotic round spermatids of ♀ *mus*<sup>CZII</sup> x ♂ *dom*<sup>WSB</sup>  
455 hybrids, and many of these same genes were also DE in ♀ *mus*<sup>PWK</sup> x ♂ *dom*<sup>LEW</sup> hybrids (**Fig 2**).  
456 The second highest number of X-linked DE genes were in meiotic cells (diplotene) of *mus*<sup>PWK</sup> x  
457 *dom*<sup>LEW</sup> hybrids, and a subset of these genes were also DE in ♀ *mus*<sup>CZII</sup> x ♂ *dom*<sup>WSB</sup> hybrids.  
458 There were approximately half as many DE meiotic genes in ♀ *dom*<sup>WSB</sup> x ♂ *mus*<sup>CZII</sup> hybrids, but  
459 nearly all of these were also misexpressed in the meiotic cells of the other two *mus* x *dom*  
460 hybrids. There were very few X-linked DE genes in the postmeiotic cells of ♀ *dom*<sup>WSB</sup> x  
461 ♂ *mus*<sup>CZII</sup> hybrids, though these genes did tend to overlap with DE postmeiotic genes in the  
462 other two subfertile hybrids.

463



464

465 **Fig 2. Number of X-linked DE genes across multiple subfertile hybrids.** The dots indicate a  
 466 contrast between a subfertile hybrid and its respective parental X chromosome and the barplot  
 467 indicates the number of X-linked DE genes in that contrast. When there are two contrasts listed  
 468 and a line connecting them it indicates the number of DE X-linked genes that are overlapping  
 469 between the two contrasts. DIP = diplotene cells (orange), RS = round spermatids (blue).

470

471

## DISCUSSION

472 **Disrupted meiotic X inactivation in reciprocal F1 hybrids**

473 Disruption of spermatogenesis during early meiosis has been linked to PRDM9, a protein that  
474 directs the location of meiotic recombination (Mihola *et al.* 2009). Divergence at PRDM9 DNA-  
475 binding sites can lead to incomplete meiotic synapsis of homologous chromosomes  
476 (Bhattacharyya *et al.* 2013; Davies *et al.* 2016; Gregorova *et al.* 2018), and associated  
477 disruption of MSCI (Good *et al.* 2010; Bhattacharyya *et al.* 2013; Campbell *et al.* 2013; Turner *et*  
478 *al.* 2014; Mack *et al.* 2016; Larson *et al.* 2017). We found disrupted MSCI in reciprocal subfertile  
479 hybrids, consistent with *Prdm9*-associated sterility in both F1 hybrids. Overall, the disruption  
480 was greater in ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup> hybrids, but meiotic arrest was not complete in either  
481 cross, suggesting variation in the mechanisms that contribute to *Prdm9*-associated sterility.

482 PRDM9 defines where meiotic recombination will occur by adding histone marks that  
483 guide SPO11 protein to induce double-strand breaks, which are repaired as either crossovers or  
484 non-crossovers (Baudat *et al.* 2010; Myers *et al.* 2010; Parvanov *et al.* 2010). The C-terminal  
485 zinc finger domain of PRDM9 determines its binding affinity to a particular site, but *Prdm9*  
486 binding sites evolve very rapidly due to biased gene conversion. If one homolog has a mutation  
487 at a PRDM9 binding site, then PRDM9 will bind preferentially to the other homolog with the  
488 ancestral binding site, causing double strand break formation in only one chromosome. This  
489 break will be repaired using the mutated strand as a template, thus mutations at PRDM9 binding  
490 sites are rapidly incorporated into both homologs, leading to the erosion of PRDM9 binding sites  
491 over time (Myers *et al.* 2010; Baker *et al.* 2015). The same mechanism is what leads to  
492 autosomal asynapsis in hybrids (Smagulova *et al.* 2016; Davies *et al.* 2016; Gregorova *et al.*  
493 2018). When hybrids are heterozygous at *Prdm9* and at PRDM9 binding-sites throughout the  
494 genome, PRDM9 binds preferentially to its ancestral binding sites, leading to asymmetric

495 formation of double strand breaks, and the failure of autosomes to properly synapse.  
496 Asynapsed autosomes interfere with normal MSCI leading to the overexpression of the X  
497 chromosome, although the exact mechanism is still unknown (Forejt *et al.* 2021). Consistent  
498 with this model, we found reciprocal F1 hybrids both had disrupted MSCI and we found the  
499 same X-linked genes had disrupted meiotic X expression in both crosses, although there were  
500 slightly more disrupted genes in *mus*<sup>PWK</sup> x *dom*<sup>LEW</sup> hybrids (**Fig 2**). This suggests asymmetric  
501 PRDM9 binding occurs in both cross directions.

502 *Prdm9*-associated sterility is also influenced by an interaction with the *Hstx2* locus, a  
503 ~2.7 Mb region in the middle of the X chromosome (Storchová *et al.* 2004; Bhattacharyya *et al.*  
504 2014; Lustyk *et al.* 2019). Complete meiotic arrest typically only occurs in F1 mice with a  
505 *musculus* *Hstx2* allele (*i.e.*, *musculus* X chromosome), while F1 mice with a *domesticus* X  
506 chromosome may vary from subfertile to nearly fully fertile (Dzur-Gejdosova *et al.* 2012; Flachs  
507 *et al.* 2012; Mukaj *et al.* 2020). The *Hstx2* locus harbors a gene, *Meir1* that controls  
508 recombination rates, and is a strong candidate for directly modulating PRDM9 binding (Dumont  
509 and Payseur 2011; Balcova *et al.* 2016). This model predicts that sterility and disrupted gene  
510 expression will be the most severe in F1 hybrids with a *musculus* X chromosome. When we  
511 have examined expression in enriched cell populations, hybrids from *musculus* x *domesticus*  
512 crosses were subfertile and had disrupted MSCI (♀ *mus*<sup>CZII</sup> x ♂ *dom*<sup>WSB</sup>, this study; *mus*<sup>PWK</sup> x  
513 *dom*<sup>LEW</sup>, Larson *et al.* 2017), while some reciprocal hybrids were fertile with normal MSCI  
514 (*dom*<sup>LEW</sup> x *mus*<sup>PWK</sup>, Larson *et al.* 2017). Indeed, we found that disrupted MSCI was much less  
515 severe in ♀ *dom*<sup>WSB</sup> x ♂ *mus*<sup>CZII</sup> hybrids (**Fig 2**), consistent with the idea that the *musculus* X  
516 chromosome is required for more severe meiotic disruption.

517 The severity of sterility in *musculus* x *domesticus* crosses appears to depend on allelic  
518 variation at *Prdm9* (Chromosome 17) and/or *Hstx2* (X chromosome). The PRDM9 C-terminal

519 zinc finger domain is composed of repeats that are polymorphic within each subspecies (Board  
520 *et al.* 2014; Kono *et al.* 2014; Vara *et al.* 2019) and ‘fertile’ and ‘sterile’ alleles have been  
521 described in both *musculus* and *domesticus* (Flachs *et al.* 2012; Mukaj *et al.* 2020). The strains  
522 we used in this study appear to have identical *Prdm9* alleles to those that have been described  
523 as ‘sterile’ in other studies (Mukaj *et al.* 2020; see also Forejt *et al.* 2021). Thus, despite both  
524 reciprocal hybrids having identical *Prdm9* genotypes (*msc1/dom3*), ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup>  
525 produce some sperm with normal morphology (Table 1), suggesting that other loci must  
526 modulate *Prdm9*-associated sterility in this cross. In *musculus* PWK × *domesticus* B6 hybrids  
527 with two ‘sterile’ *Prdm9* alleles, partial fertility appears to be associated with allelic variation on  
528 the X chromosome (Flachs *et al.* 2014), possibly at the *Hstx2* locus. Allelic variation on the X  
529 chromosome may also explain why complete meiotic arrest was not found in crosses with wild-  
530 derived strains in this study (♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup>), by Larson *et al.* (2017; ♀ *mus*<sup>PWK</sup> ×  
531 ♂ *dom*<sup>LEW</sup>), and in some other *musculus* × *domesticus* crosses with two ‘sterile’ *Prdm9* alleles  
532 (Mukaj *et al.* 2020).

533 Allelic variation at *Prdm9* could also explain the subfertility of ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup>  
534 hybrids, in the absence of the *musculus* X chromosome. F1 *domesticus* × *musculus* hybrids can  
535 be subfertile when both *Prdm9* alleles are ‘sterile’ (Flachs *et al.* 2012). The combination of two  
536 sterile *Prdm9* alleles and heterozygous PRDM9 binding sites throughout the genome may be  
537 sufficient to disrupt MSCI in ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup> hybrids. However, it is unclear why MSCI  
538 would be disrupted in ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup> hybrids, but not ♀ *dom*<sup>LEW</sup> × ♂ *mus*<sup>PWK</sup> hybrids,  
539 which also have two sterile *Prdm9* alleles. It is also unknown to what extent *Prdm9* contributes  
540 to the sterility phenotypes in ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup> hybrids, given that other autosomal sterility  
541 factors have been mapped in ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup> hybrids to chromosomes 2, 8 and 9 (Larson  
542 *et al.* 2018b).

543 In addition to allelic variation, the outcome of *Prdm9*-associated sterility is likely to be  
544 variable across cells within an individual. *Prdm9*-induced autosomal asynapsis is a threshold  
545 response. If a sufficiently large number of autosomes fail to pair (asynapsis rates > 60%) it  
546 leads to full meiotic arrest, while lower rates of asynapsis may lead to intermediate levels of  
547 meiotic disruption (Bhattacharyya *et al.* 2013; Mukaj *et al.* 2020). While MSCI is disrupted in  
548 ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup> hybrids, the X chromosome still had lower expression in meiosis  
549 compared to other cell types (**Fig S2**) and a similar pattern was found for disrupted MSCI in  
550 ♀ *mus*<sup>PWK</sup> × ♂ *dom*<sup>LEW</sup> hybrids (Larson *et al.* 2017). This suggests cell-to-cell variation in the  
551 occurrence or magnitude of disrupted MSCI which may contribute to the range of sperm  
552 morphologies found in these hybrids - from severely impaired to apparently normal (**Table 1**).  
553

554 **Asymmetric disruption of postmeiotic expression suggests genotype-specific hybrid  
555 sterility regulatory phenotypes**

556 Postmeiotic disruption of X chromosome expression was observed in ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup>  
557 hybrids but not in reciprocal ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup> hybrids (**Fig 1A, 1B**). Both F1 hybrids had  
558 earlier disruption of MSCI, which suggests that postmeiotic overexpression of the X  
559 chromosome is not a simple downstream consequence of disruption at earlier developmental  
560 timepoints. It is possible that downstream postmeiotic disruption depends on the magnitude of  
561 disrupted MSCI, or the disruption of particular X-linked genes or gene networks. Consistent with  
562 this, ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup> hybrids had a higher proportion of disrupted X-linked genes in  
563 meiosis.

564 Asymmetric postmeiotic disruption is also consistent with antagonistic coevolution of X-  
565 and Y-linked multicopy gene families that leads to overexpression only in one cross direction.  
566 We found the X chromosome was overexpressed in postmeiotic cells of F1 hybrids that had a

567 deficit of the Y-linked gene family *Sly* ( $\text{♀ } \text{mus}^{\text{CZII}} \times \text{♂ } \text{dom}^{\text{WSB}}$  hybrids), but not in reciprocal F1  
568 hybrids that had a deficit of the X-linked gene family *Slx/Slx1* ( $\text{♀ } \text{dom}^{\text{WSB}} \times \text{♂ } \text{mus}^{\text{CZII}}$   
569 hybrids)(**Fig 1A, 1B**). *Sly* and *Slx/Slx1* play a major role in suppressing or promoting  
570 postmeiotic expression of multi-copy sex-linked and interacting autosomal genes (Mueller *et al.*  
571 2008, 2013, Kruger *et al.* 2019, Moretti *et al.* 2020). Imbalanced copy numbers of these genes  
572 in F1 hybrids may also disrupt postmeiotic expression networks. This could happen either  
573 independently of upstream meiotic disruption, or there may be an interaction among X-linked  
574 regulatory networks at different stages of development.

575 *Slx/Slx1* originated from a single copy autosomal gene (*Sycp3*) that was transposed to  
576 the X chromosome (*Slx1* then *Slx*) and eventually a copy emerged on the Y chromosome (*Sly*)  
577 (Kruger *et al.* 2019) . Since their origin, these genes, additional sex-linked ampliconic genes,  
578 and associated autosomal ampliconic genes have undergone a massive co-amplification in  
579 different mouse lineages leading to divergent copy numbers in *domesticus* and *musculus* (see  
580 Table 2; Ellis *et al.* 2011; Turner *et al.* 2014; Soh *et al.* 2014; Morgan and Pardo-Manuel de  
581 Villena 2017). *Slx* and *Sly* appear to coevolve in a copy number arms race for interaction with  
582 *SSTY1* at the promoter of thousands of postmeiotic genes (Moretti *et al.* 2020). Knockdown of  
583 *Sly* expression or duplications of *Slx/Slx1* (i.e., *Sly*-deficient) leads to increased transmission of  
584 the X chromosome, abnormal sperm morphology, and upregulation of multicopy genes on the  
585 sex chromosomes (Cocquet *et al.* 2009, Kruger *et al.* 2019), as well as upregulation of the  
586 autosomal *Speer* (Chr 5) and *a-takusan* (Chr 14) gene families (Moretti *et al.* 2020). Knockdown  
587 of *Slx/Slx1* expression (i.e. *Slx*-deficient) suppresses postmeiotic multicopy gene expression  
588 and leads to increased transmission of the Y chromosome and mild sperm abnormalities  
589 (Cocquet *et al.* 2010, 2012; Kruger *et al.* 2019). Reciprocal F1 hybrids between *domesticus* (*Sly*  
590 130, *Slx/Slx1* 35, *Sly/Slx* ratio: 3.7) and *musculus* (*Sly* 215, *Slx/Slx1* 100, *Sly/Slx* ratio: 2.15)

591 mirror these knockdown experiments: F1 hybrids from *musculus* × *domesticus* are *Sly*-deficient  
592 (130 *Sly*, 100 *Slx*, *Sly*/*Slx* ratio: 1.3) and F1 hybrids from *domesticus* × *musculus* are *Slx*-  
593 deficient (215 *Sly*, 35 *Slx*, *Sly*/*Slx* ratio: 6.1, **Table 2**).

594 Consistent with a *Sly*/*Slx* imbalance, we found *Sly*-deficient ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup>  
595 hybrids overexpressed the X chromosome in postmeiotic cells (**Fig 1A**). We saw the same  
596 overexpression of the X chromosome in an independent contrast of *Sly*-deficient *mus*<sup>PWK</sup> ×  
597 *dom*<sup>LEW</sup> hybrids (Larson *et al.* 2017). The same X-linked genes were overexpressed in both  
598 crosses, though there were more upregulated X-linked genes in ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup> hybrids  
599 (**Fig 2**). In contrast, we found very few X-linked DE genes in *Slx*-deficient ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup>  
600 hybrids, and there was no asymmetry in the expression of DE genes in ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup>  
601 hybrids - genes were both up and down regulated relative to the *domesticus* X chromosome  
602 (**Fig 1B**). If anything, X-linked postmeiotic genes tended to be underexpressed in ♀ *dom*<sup>WSB</sup> ×  
603 ♂ *mus*<sup>CZII</sup> hybrids relative to the *domesticus* X chromosome, but, unlike in *Slx*-deficient  
604 ♀ *dom*<sup>LEW</sup> × ♂ *mus*<sup>PWK</sup> hybrids (Larson *et al.* 2017), this pattern was not significant. Still, the  
605 handful of X-linked postmeiotic genes that were overexpressed in ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup> hybrids  
606 were also upregulated in both *musculus* × *domesticus* hybrids (**Fig 2**).

607 In contrast to results from *mus*<sup>PWK</sup> × *dom*<sup>LEW</sup> hybrids (Larson *et al.* 2017), we did not find  
608 co-overexpression of ampliconic autosomal genes families (*Speer* or *a-takusan*) in ♀ *mus*<sup>CZII</sup> ×  
609 ♂ *dom*<sup>WSB</sup> hybrids. The overexpression of these gene families in *Sly*-deficient hybrids was one of  
610 the strongest arguments for an independent mechanism of disrupted X expression in *mus*<sup>PWK</sup> ×  
611 *dom*<sup>LEW</sup> hybrids. This lack of agreement makes it difficult to disentangle disrupted regulatory  
612 dynamics of *Sly* and *Slx/Slx/1* from possible downstream disruption of PRDM9 in this cross.

613 However, the clear differences in postmeiotic expression between ♀ *mus*<sup>CZII</sup> × ♂ *dom*<sup>WSB</sup> hybrids  
614 and ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup> hybrids indicates that postmeiotic disruption is genotype specific.

615 Whether or not postmeiotic sex chromosome overexpression contributes to sterility  
616 phenotypes in wild hybrids is still unknown. In knockdown studies, *Sly* and *Slx/Slx/1* have a  
617 major impact on sperm morphology (Cocquet *et al.* 2009, 2010, 2012; Kruger *et al.* 2019), but  
618 the extent to which these genes might contribute to hybrid sterility phenotypes in wild mice is  
619 still unclear (Campbell *et al.* 2013). In knockdown studies, *Sly*-deficient mice have severe sperm  
620 deformities (Cocquet *et al.* 2009) and biased X chromosome transmission, while *Slx*-deficient  
621 mice tend to have more typical sperm (but see Kruger *et al.* 2019) and biased Y chromosome  
622 transmission (Cocquet *et al.* 2010, 2012). The severe sperm deformities in *Sly*-deficient mice  
623 appear to particularly affect Y-bearing sperm, decreasing their mobility and providing a direct  
624 mechanism for how sperm morphology contributes to sex ratio skews (Rathje *et al.* 2019).  
625 However, it is still unclear if the imbalance manifested in mouse hybrids is sufficient to induce a  
626 regulatory misexpression phenotype. In F1 hybrids, the imbalanced copy number of *Sly* and  
627 *Slx/Slx/1* is certainly less severe in magnitude as total knockdown experiments. If there is a  
628 threshold of imbalance required for *Sly* or *Slx/Slx/1* to successfully outcompete the other  
629 (Moretti *et al.* 2020), we may not see the same impacts on sperm morphology or sex ratio  
630 distortion in wild hybrids. In general, we do find that *Sly*-deficient F1 *musculus* × *domesticus*  
631 hybrids have severely abnormal sperm morphology (see Table 1 and Larson *et al.* 2017), while  
632 *Slx*-deficient F1 *domesticus* × *musculus* hybrids tend to have more moderate sperm head  
633 abnormalities (Larson *et al.* 2017). Similar patterns have been observed in Y introgression lines  
634 that mismatch the *musculus* and *domesticus* X and Y chromosomes (Campbell and Nachman  
635 2014). In this study, ♀ *dom*<sup>WSB</sup> × ♂ *mus*<sup>CZII</sup> hybrids also have severely abnormal sperm head  
636 morphology (Table 1), but there are clear autosomal contributions to these abnormalities

637 (Larson *et al.* 2018b). To our knowledge, sex ratio distortion has not been documented in wild-  
638 derived crosses, though there is some evidence that it might occur in the mouse hybrid zone  
639 (Macholán *et al.* 2008, but see 2019).

640

#### 641 **Conclusions**

642 The elegant *Prdm9* incompatibility model is likely the single most important mechanism  
643 of F1 hybrid male sterility in house mice. We find evidence for *Prdm9*-associated disruption of  
644 meiosis in subfertile hybrids from reciprocal crosses of two wild-derived strains. We also find  
645 evidence that factors outside of *Prdm9* and *Hstx2* contribute to disrupted expression in F1  
646 hybrids, providing support for the idea that hybrid sterility is a composite phenotype and likely  
647 polygenic (Campbell and Nachman 2014; Larson *et al.* 2017). Other factors such as autosomal  
648 incompatibilities and postmeiotic X-Y interactions are likely to be important contributions to  
649 overall hybrid sterility. Indeed, the variation we found in the extent and timing of disrupted X  
650 expression among different F1 hybrids may reflect interactions among disrupted meiotic and  
651 postmeiotic gene networks.

652 The mouse hybrid zone is a relatively recent contact that stretches across central  
653 Europe, with a fairly narrow width (Phifer-Rixey and Nachman 2015). Despite the recency of  
654 contact and the proximity of parental species, there are few F1 hybrids found in the center of the  
655 zone. Instead, the mouse hybrid zone is composed predominantly of advanced generation  
656 hybrids and backcrosses (Turner *et al.* 2012; Janoušek *et al.* 2012; Turner and Harr 2014), and  
657 hybrid males vary considerably in their fertility (Turner *et al.* 2012). *Prdm9*-associated sterility is  
658 strongest in an F1 background with a *musculus* X chromosome and depends on a combination  
659 of sterile *Prdm9* alleles (Forejt *et al.* 2021). Stretches of conspecific genomic regions, which are  
660 typical for backcrosses and advanced generation hybrids, can rescue meiotic synapsis

661 (Gregorova *et al.* 2018). As a result, it is very unlikely that *Prdm9* alone can explain the reduced  
662 gene flow between *musculus* and *domesticus* in nature.

663 Studies of differential introgression in the mouse hybrid zone have consistently found the  
664 X chromosome to have restricted introgression, as well as a number of different autosomal  
665 regions (Tucker *et al.* 1992; Payseur *et al.* 2004; Macholán *et al.* 2007, 2011; Teeter *et al.* 2010;  
666 Janoušek *et al.* 2012; Turner and Harr 2014). Restricted introgression can point to regions of  
667 the genome that contribute to reproductive barriers. While there is some evidence for premating  
668 barriers between *musculus* and *domesticus* (Smadja and Ganem 2002, 2007; Bímová *et al.*  
669 2011; Loire *et al.* 2017), the singular phenotype in all studies of these subspecies is the reduced  
670 fertility of hybrid males. Indeed, mapping studies have identified multiple regions of the X  
671 chromosome (Oka *et al.* 2004; Good *et al.* 2008a; Dufková *et al.* 2011; Turner *et al.* 2014;  
672 Turner and Harr 2014; Morgan *et al.* 2020) and numerous autosomal regions contributing to  
673 sterility in F1 hybrids (Larson *et al.* 2018b), F2 crosses and backcrosses (Good *et al.* 2008a;  
674 White *et al.* 2011; Turner *et al.* 2014; Schwahn *et al.* 2018; Morgan *et al.* 2020) and wild hybrids  
675 (Turner and Harr 2014). There is also evidence that XY mismatch contributing to abnormal  
676 sperm morphology (Campbell and Nachman 2014; Martinová *et al.* 2019b, 2019a), and  
677 patterns of directional introgression of the *musculus* Y chromosome into *domesticus*  
678 backgrounds (Macholán *et al.* 2008, 2019; Ďureje *et al.* 2012), consistent with postmeiotic X and  
679 Y chromosome conflict.

680 Complex hybrid incompatibilities, involving many genes, both autosomal and sex-linked,  
681 are a common feature of hybrid male sterility (Coughlan and Matute 2020). The multigenic  
682 nature of hybrid male sterility in house mice, and the availability of wild-derived strains makes  
683 this an excellent system to identify the genetic basis of hybrid sterility (Forejt *et al.* 2021) and  
684 relate these incompatibilities directly to reproductive isolation between natural populations.

685

686

## AUTHOR CONTRIBUTIONS

687 JMG conceived of the study and designed the experiments. ELL, EEKK, KEH, DV and SK  
688 performed experiments and collected data. ELL, EEKK, and KEH analyzed data. ELL and JMG  
689 wrote the manuscript with feedback from all co-authors.

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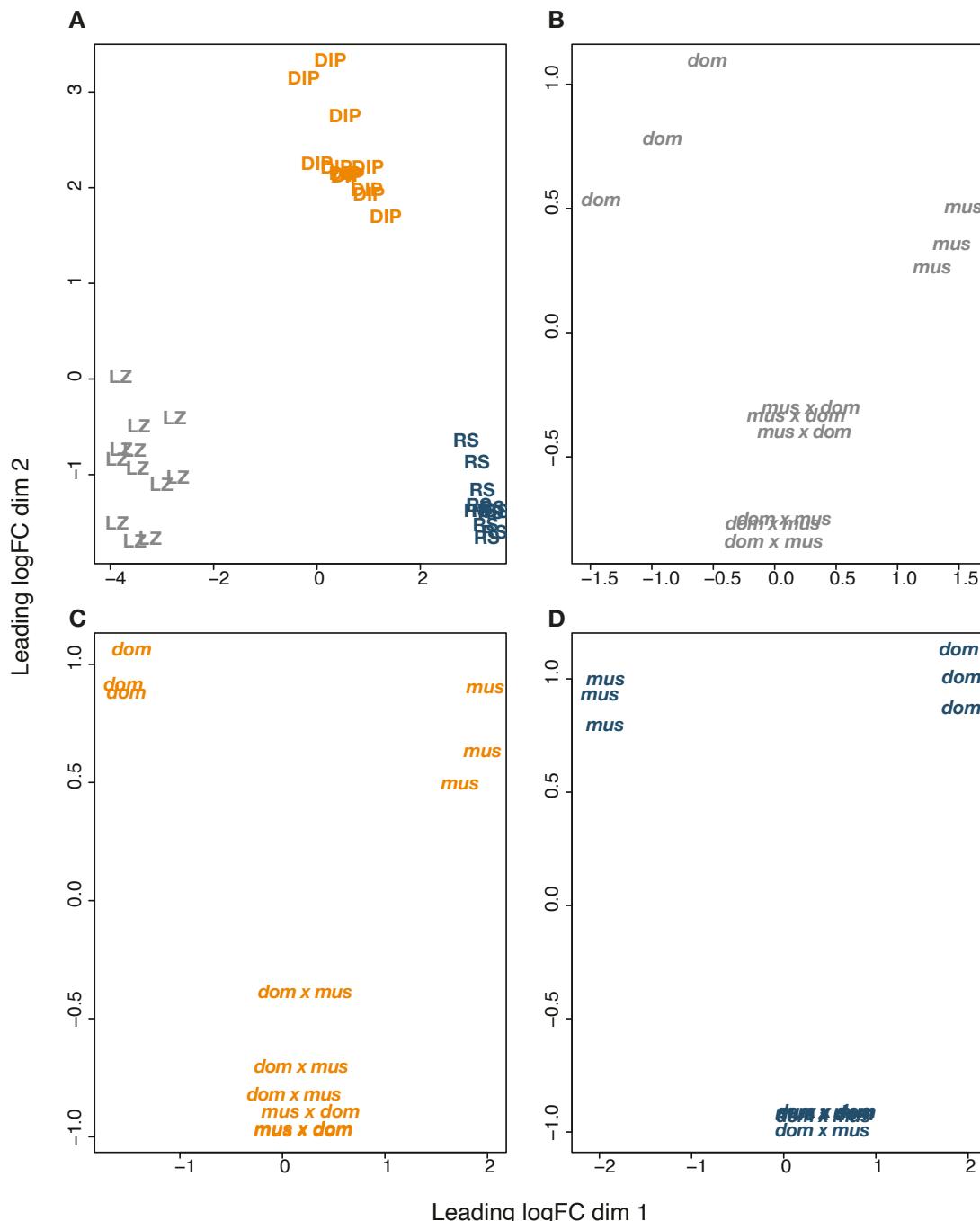
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701 conclusions or recommendations expressed in this material are those of the author(s) and do  
702 not necessarily reflect the views of the National Science Foundation or the National Institutes of  
703 Health.

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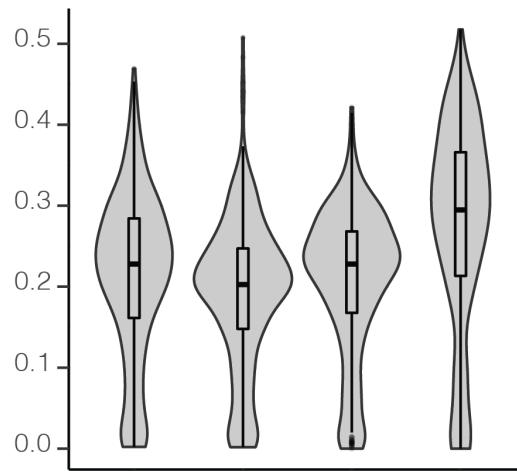
## SUPPLEMENTARY DATA

706 **Table S1: Table of individual male reproductive phenotypes (.csv).** Table includes each  
707 individual mouse ID (e.g. CCPP 21.1M stands for dam x sire, litter number, individual number  
708 and sex; CC = CZECHII, PP = PWK, WW = WSB, LL = LEWES), cross type, dates the mice  
709 were born and phenotype, their age at phenotyping, measures of body size (weight, body  
710 length, tail length, right hind foot, left ear length), weights of paired testes and seminiferous  
711 vesicles, counts of motile and nonmotile sperm, counts of total sperm, and counts of sperm  
712 head morphology categories.

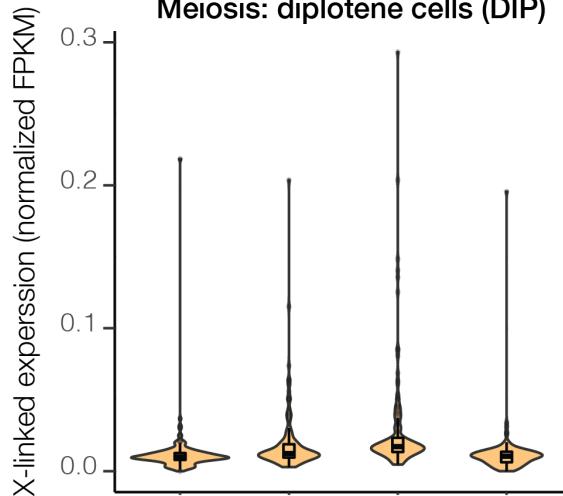


715 **Figure S1. Clustering of gene expression profiles.** Multidimensional scaling plots (MDS) of  
 716 the Euclidean distance among gene expression profiles. Distance approximates the typical log2  
 717 fold changes between samples. **A)** RNAseq profiles cluster overall by cell type. LZ =  
 718 leptotene/zygotene cells (gray), DIP = diplotene cells (orange), RS = round spermatids (blue).  
 719 **B-D)** Within each cell type, RNAseq profiles cluster by subspecies, with F1 hybrids intermediate  
 720 to the two parental subspecies. **B)** LZ = leptotene/zygotene cells (gray). **C)** DIP = diplotene cells  
 721 (orange). **D)** RS = round spermatids (blue)

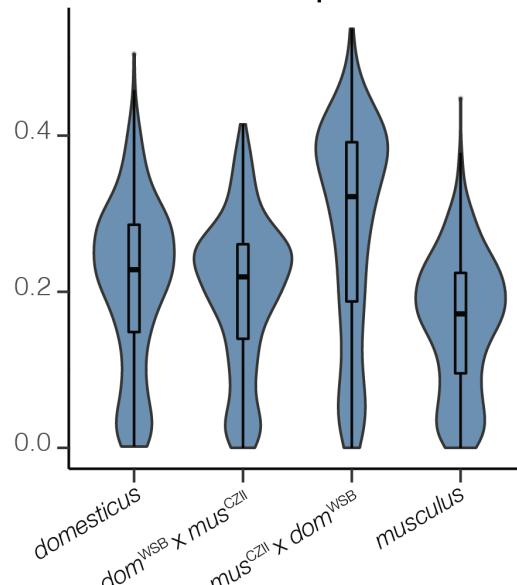
Meiosis: leptotene/zygotene cells (LZ)



Meiosis: diplotene cells (DIP)



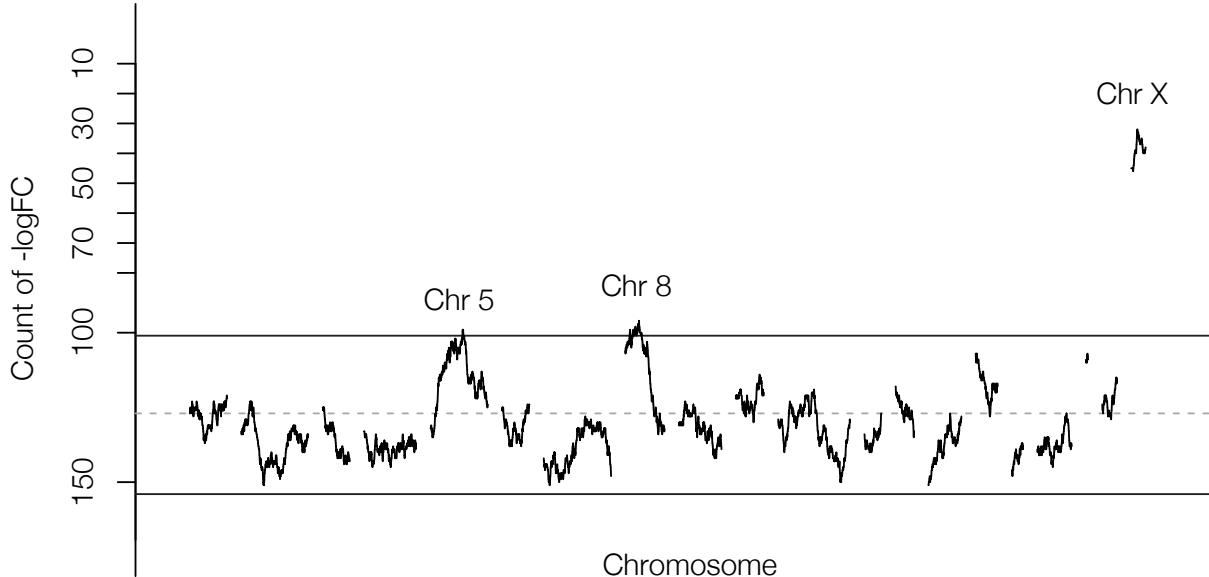
Postmeiosis: round spermatids cells (RS)



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**FigS2. X chromosome expression across different cell types and crosses.** The distribution of X-linked gene expression in normalized FPKM values (values range 0 to 1). The violin plots show the density of genes with a given expression level and the boxplots depict the median values and quartiles. Gene expression was restricted to genes that have an FPKM > 1 in at least 3 samples per cell type. X-linked expression was elevated in diplotene cells of both hybrids and in round spermatids of  $\text{♀ } \text{mus}^{\text{CZII}} \times \text{♂ } \text{dom}^{\text{WSB}}$  hybrids.

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**Figure S3. Spatial patterns of postmeiotic expression between subfertile hybrids.** Sliding-gene windows (250 genes) for counts of underexpressed genes in postmeiotic cells (round spermatids) between  $\text{♀ } \text{mus}^{\text{CZII}} \times \text{♂ } \text{dom}^{\text{WSB}}$  hybrids and  $\text{♀ } \text{dom}^{\text{WSB}} \times \text{♂ } \text{mus}^{\text{CZII}}$  hybrids. Solid lines represent the 99th quantile modeled with a Poisson distribution. Note the Y-axis is plotted so that underexpressed genes fall below the 99th quantile and overexpressed genes are above the 99th quantile. Chromosomes 5 and 8 had relatively small windows of genes overexpressed in *Sly*-deficient  $\text{♀ } \text{mus}^{\text{CZII}} \times \text{♂ } \text{dom}^{\text{WSB}}$  hybrids, but these windows did not coincide with known multicopy gene families (*Speer/a-takusan*).

## REFERENCES

745 Alibert, P., F. Fel-Clair, K. Manolakou, J. Britton-Davidian, and J.-C. Auffray, 1997  
746 Developmental stability, fitness, and trait size in laboratory hybrids between European  
747 subspecies of the house mouse. *Evolution* 51: 1284–1295.

748 Baker, C. L., S. Kajita, M. Walker, R. L. Saxl, N. Raghupathy *et al.*, 2015 PRDM9 drives  
749 evolutionary erosion of hotspots in *Mus musculus* through haplotype-specific initiation of  
750 meiotic recombination. *PLoS Genet.* 11: e1004916.

751 Balcova, M., B. Faltusova, V. Gergelits, T. Bhattacharyya, O. Mihola *et al.*, 2016 Hybrid sterility  
752 locus on chromosome X controls meiotic recombination rate in mouse. *PLoS Genet.* 12:  
753 e1005906.

754 Bank, C., R. Bürger, and J. Hermisson, 2012 The limits to parapatric speciation: Dobzhansky-  
755 Muller incompatibilities in a continent-island model. *Genetics* 191: 845–863.

756 Bateson, W., 1909 Heredity and variation in modern lights, pp. 85–101 in *Darwin and Modern  
757 Science*, edited by A. C. Seward. Cambridge University Press, Cambridge, UK.

758 Baudat, F., J. Buard, C. Grey, A. Fledel-Alon, C. Ober *et al.*, 2010 PRDM9 is a major  
759 determinant of meiotic recombination hotspots in humans and mice. *Science* 327: 836–840.

760 Benjamini, Y., and Y. Hochberg, 1995 Controlling the false discovery rate: a practical and  
761 powerful approach to multiple testing. *J. R. Stat. Soc. Series B Stat. Methodol.* 57: 289–  
762 300.

763 Bhattacharyya, T., S. Gregorova, O. Mihola, M. Anger, J. Sebestova *et al.*, 2013 Mechanistic  
764 basis of infertility of mouse intersubspecific hybrids. *Proc. Natl. Acad. Sci. U. S. A.* 110:  
765 E468–77.

766 Bhattacharyya, T., R. Reifova, S. Gregorova, P. Simecek, V. Gergelits *et al.*, 2014 X  
767 chromosome control of meiotic chromosome synapsis in mouse inter-subspecific hybrids.  
768 *PLoS Genet.* 10: e1004088.

769 Bímová, B. V., M. Macholán, S. J. E. Baird, P. Munclinger, P. Dufková *et al.*, 2011  
770 Reinforcement selection acting on the European house mouse hybrid zone. *Mol. Ecol.* 20:  
771 2403–2424.

772 Bolger, A. M., M. Lohse, and B. Usadel, 2014 Trimmomatic: a flexible trimmer for Illumina  
773 sequence data. *Bioinformatics* 30: 2114–2120.

774 Bracewell, R. R., B. J. Bentz, B. T. Sullivan, and J. M. Good, 2017 Rapid neo-sex chromosome  
775 evolution and incipient speciation in a major forest pest. *Nat. Commun.* 8: 1593.

776 Britton-Davidian, J., F. Fel-Clair, J. Lopez, P. Alibert, and P. Boursot, 2005 Postzygotic isolation  
777 between the two European subspecies of the house mouse: estimates from fertility. *Biol. J.  
778 Linn. Soc. Lond.* 84: 379–393.

779 Broad Institute, 2019 *Picard Tools*.

780 Buard, J., E. Rivals, D. Dunoyer de Segonzac, C. Garres, P. Caminade *et al.*, 2014 Diversity of  
781 Prdm9 zinc finger array in wild mice unravels new facets of the evolutionary turnover of this  
782 coding minisatellite. *PLoS One* 9: e85021.

783 Campbell, P., J. M. Good, and M. W. Nachman, 2013 Meiotic sex chromosome inactivation is  
784 disrupted in sterile hybrid male house mice. *Genetics* 193: 819–828.

785 Campbell, P., and M. W. Nachman, 2014 X–Y interactions underlie sperm head abnormality in  
786 hybrid male house mice. *Genetics* 196: 1231–1240.

787 Case, A. L., F. R. Finseth, C. M. Barr, and L. Fishman, 2016 Selfish evolution of cytonuclear  
788 hybrid incompatibility in *Mimulus*. *Proc. Biol. Sci.* 283: 20161493.

789 Cocquet, J., P. J. I. Ellis, S. K. Mahadevaiah, N. A. Affara, D. Vaiman *et al.*, 2012 A genetic  
790 basis for a postmeiotic X versus Y chromosome intragenomic conflict in the mouse. *PLoS  
791 Genet.* 8: e1002900.

792 Cocquet, J., P. J. I. Ellis, Y. Yamauchi, S. K. Mahadevaiah, N. A. Affara *et al.*, 2009 The  
793 multicity gene Sly represses the sex chromosomes in the male mouse germline after  
794 meiosis. *PLoS Biol.* 7: e1000244.

795 Cocquet, J., P. J. I. Ellis, Y. Yamauchi, J. M. Riel, T. P. S. Karacs *et al.*, 2010 Deficiency in the  
796 multicity Sycp3-like X-linked genes Slx and Slxl1 causes major defects in spermatid  
797 differentiation. *Mol. Biol. Cell* 21: 3497–3505.

798 Coughlan, J. M., and D. R. Matute, 2020 The importance of intrinsic postzygotic barriers  
799 throughout the speciation process. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 375: 20190533.

800 Cutter, A. D., 2012 The polymorphic prelude to Bateson-Dobzhansky-Muller incompatibilities.  
801 *Trends Ecol. Evol.* 27: 209–218.

802 Cutter, A. D., and J. D. Bundus, 2020 Speciation and the developmental alarm clock. *Elife* 9:  
803 e56276.

804 Davies, B., E. Hatton, N. Altemose, J. G. Hussin, F. Pratto *et al.*, 2016 Re-engineering the zinc  
805 fingers of PRDM9 reverses hybrid sterility in mice. *Nature* 530: 171–176.

806 Dobzhansky, T., 1937 *Genetics and the Origin of Species*. Columbia University Press.

807 Dufková, P., M. Macholán, and J. Piálek, 2011 Inference of selection and stochastic effects in  
808 the house mouse hybrid zone. *Evolution* 65: 993–1010.

809 Dumont, B. L., and B. A. Payseur, 2011 Genetic analysis of genome-scale recombination rate  
810 evolution in house mice. *PLoS Genet.* 7: e1002116.

811 Ďureje, L., M. Macholán, S. J. E. Baird, and J. Piálek, 2012 The mouse hybrid zone in Central  
812 Europe: from morphology to molecules. *J. Vertebr. Biol.* 61: 308–318.

813 Duvaux, L., K. Belkhir, M. Boulesteix, and P. Boursot, 2011 Isolation and gene flow: inferring the  
814 speciation history of European house mice. *Mol. Ecol.* 20: 5248–5264.

815 Dzur-Gejdosova, M., P. Simecek, S. Gregorova, T. Bhattacharyya, and J. Forejt, 2012  
816 Dissecting the genetic architecture of F1 hybrid sterility in house mice. *Evolution* 66: 3321–  
817 3335.

818 Ellis, P. J. I., J. Bacon, and N. A. Affara, 2011 Association of Sly with sex-linked gene  
819 amplification during mouse evolution: a side effect of genomic conflict in spermatids? *Hum.*  
820 *Mol. Genet.* 20: 3010–3021.

821 Flachs, P., T. Bhattacharyya, O. Mihola, J. Piálek, J. Forejt *et al.*, 2014 Prdm9 incompatibility  
822 controls oligospermia and delayed fertility but no selfish transmission in mouse  
823 intersubspecific hybrids. *PLoS One* 9: e95806.

824 Flachs, P., O. Mihola, P. Simeček, S. Gregorová, J. C. Schimenti *et al.*, 2012 Interallelic and  
825 intergenic incompatibilities of the Prdm9 (Hst1) gene in mouse hybrid sterility. *PLoS Genet.*  
826 8: e1003044.

827 Forejt, J., P. Jansa, and E. Parvanov, 2021 Hybrid sterility genes in mice (*Mus musculus*): a  
828 peculiar case of PRDM9 incompatibility. *Trends Genet.*

829 Geraldes, A., P. Basset, K. L. Smith, and M. W. Nachman, 2011 Higher differentiation among  
830 subspecies of the house mouse (*Mus musculus*) in genomic regions with low  
831 recombination. *Mol. Ecol.* 20: 4722–4736.

832 Getun, I. V., B. Torres, and P. R. J. Bois, 2011 Flow cytometry purification of mouse meiotic  
833 cells. *J. Vis. Exp.* 50: e2602.

834 Good, J. M., 2012 The conflict within and the escalating war between the sex chromosomes.  
835 *PLoS Genet.* 8: e1002955.

836 Good, J. M., M. D. Dean, and M. W. Nachman, 2008a A complex genetic basis to X-linked  
837 hybrid male sterility between two species of house mice. *Genetics* 179: 2213–2228.

838 Good, J. M., T. Giger, M. D. Dean, and M. W. Nachman, 2010 Widespread over-expression of  
839 the X chromosome in sterile F1 hybrid mice. *PLoS Genet.* 6: e1001148.

840 Good, J. M., M. A. Handel, and M. W. Nachman, 2008b Asymmetry and polymorphism of hybrid  
841 male sterility during the early stages of speciation in house mice. *Evolution* 62: 50–65.

842 Green, C. D., Q. Ma, G. L. Manske, A. N. Shami, X. Zheng *et al.*, 2018 A comprehensive  
843 roadmap of murine spermatogenesis defined by single-cell RNA-Seq. *Dev. Cell* 46: 651–  
844 667.e10.

845 Gregorova, S., V. Gergelits, I. Chvatalova, T. Bhattacharyya, B. Valiskova *et al.*, 2018  
846 Modulation of Prdm9-controlled meiotic chromosome asynapsis overrides hybrid sterility in  
847 mice. *Elife* 7: e34282.

848 Huang, S., J. Holt, C.-Y. Kao, L. McMillan, and W. Wang, 2014 A novel multi-alignment pipeline  
849 for high-throughput sequencing data. *Database* 2014.:.

850 Hunnicutt, K. E., J. M. Good, and E. L. Larson, 2021 Unraveling patterns of disrupted gene  
851 expression across a complex tissue. *bioRxiv* 2021.07.08.451646.

852 Janoušek, V., L. Wang, K. Luzynski, P. Dufková, M. M. Vyskočilová *et al.*, 2012 Genome-wide  
853 architecture of reproductive isolation in a naturally occurring hybrid zone between *Mus*  
854 *musculus musculus* and *M. m. domesticus*. *Mol. Ecol.* 21: 3032–3047.

855 Katoh, K., and D. M. Standley, 2013 MAFFT multiple sequence alignment software version 7:  
856 improvements in performance and usability. *Mol. Biol. Evol.* 30: 772–780.

857 Kim, D., G. Pertea, C. Trapnell, H. Pimentel, R. Kelley *et al.*, 2013 TopHat2: accurate alignment  
858 of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.*  
859 14: R36.

860 Kono, H., M. Tamura, N. Osada, H. Suzuki, K. Abe *et al.*, 2014 Prdm9 polymorphism unveils  
861 mouse evolutionary tracks. *DNA Res.* 21: 315–326.

862 Kruger, A. N., M. A. Brogley, J. L. Huizinga, J. M. Kidd, D. G. de Rooij *et al.*, 2019 A  
863 neofunctionalized X-Linked ampliconic gene family is essential for male fertility and equal  
864 sex ratio in mice. *Curr. Biol.* 29: 3699–3706.e5.

865 Larson, E. L., S. Keeble, D. Vanderpool, M. D. Dean, and J. M. Good, 2017 The composite  
866 regulatory basis of the large X-effect in mouse speciation. *Mol. Biol. Evol.* 34: 282–295.

867 Larson, E. L., E. E. K. Kopania, and J. M. Good, 2018a Spermatogenesis and the evolution of  
868 mammalian sex chromosomes. *Trends Genet.* 34: 722–732.

869 Larson, E. L., D. Vanderpool, S. Keeble, M. Zhou, B. A. J. Sarver *et al.*, 2016 Contrasting levels  
870 of molecular evolution on the mouse X chromosome. *Genetics* 203: 1841–1857.

871 Larson, E. L., D. Vanderpool, B. A. J. Sarver, C. Callahan, S. Keeble *et al.*, 2018b The evolution  
872 of polymorphic hybrid incompatibilities in house mice. *Genetics* 209: 845–859.

873 Liao, Y., G. K. Smyth, and W. Shi, 2014 featureCounts: an efficient general purpose program for  
874 assigning sequence reads to genomic features. *Bioinformatics* 30: 923–930.

875 Li, H., and R. Durbin, 2009 Fast and accurate short read alignment with Burrows-Wheeler  
876 transform. *Bioinformatics* 25: 1754–1760.

877 Lindtke, D., and C. A. Buerkle, 2015 The genetic architecture of hybrid incompatibilities and  
878 their effect on barriers to introgression in secondary contact. *Evolution* 69: 1987–2004.

879 Loire, E., S. Tusso, P. Caminade, D. Severac, P. Boursot *et al.*, 2017 Do changes in gene  
880 expression contribute to sexual isolation and reinforcement in the house mouse? *Mol. Ecol.*  
881 26: 5189–5202.

882 Lustyk, D., S. Kinský, K. K. Ullrich, M. Yancoskie, L. Kašíková *et al.*, 2019 Genomic structure of  
883 Hstx2 modifier of Prdm9-dependent hybrid male sterility in mice. *Genetics* 213: 1047–1063.

884 Macholán, M., S. J. E. Baird, P. Dufková, P. Munclinger, B. V. Bímová *et al.*, 2011 Assessing  
885 multilocus introgression patterns: a case study on the mouse X chromosome in central  
886 Europe. *Evolution* 65: 1428–1446.

887 Macholán, M., S. J. E. Baird, A. Fornůsková, I. Martincová, P. Rubík *et al.*, 2019 Widespread  
888 introgression of the *Mus musculus musculus* Y chromosome in Central Europe. *bioRxiv*  
889 2019.12.23.887471.

890 Macholán, M., S. J. E. Baird, P. Munclinger, P. Dufková, B. Bímová *et al.*, 2008 Genetic conflict  
891 outweighs heterogametic incompatibility in the mouse hybrid zone? *BMC Evol. Biol.* 8: 271.

892 Macholán, M., S. J. E. Baird, J. Piálek, and P. Munclinger, 2012 *Evolution of the House Mouse*.  
893 Cambridge University Press.

894 Macholán, M., P. Munclinger, M. Sugerková, P. Dufková, B. Bímová *et al.*, 2007 Genetic  
895 analysis of autosomal and X-linked markers across a mouse hybrid zone. *Evolution* 61:  
896 746–771.

897 Mack, K. L., P. Campbell, and M. W. Nachman, 2016 Gene regulation and speciation in house  
898 mice. *Genome Res.* 26: 451–461.

899 Mandeville, E. G., T. L. Parchman, K. G. Thompson, R. I. Compton, K. R. Gelwicks *et al.*, 2017  
900 Inconsistent reproductive isolation revealed by interactions between *Catostomus* fish  
901 species. *Evol Lett* 1: 255–268.

902 Margolin, G., P. P. Khil, J. Kim, M. A. Bellani, and R. D. Camerini-Otero, 2014 Integrated  
903 transcriptome analysis of mouse spermatogenesis. *BMC Genomics* 15: 39.

904 Martincová, I., L. Ďureje, S. J. E. Baird, and J. Piálek, 2019a Sperm quality parameters are  
905 increased and asymmetric in house mouse hybrids. *bioRxiv* 666511.

906 Martincová, I., L. Ďureje, J. Kreisinger, M. Macholán, and J. Piálek, 2019b Phenotypic effects of  
907 the Y chromosome are variable and structured in hybrids among house mouse recombinant  
908 lines. *Ecol. Evol.* 9: 6124–6137.

909 McCarthy, D. J., Y. Chen, and G. K. Smyth, 2012 Differential expression analysis of multifactor  
910 RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res.* 40: 4288–  
911 4297.

912 Meiklejohn, C. D., E. L. Landeen, K. E. Gordon, T. Rzatkiewicz, S. B. Kingan *et al.*, 2018 Gene  
913 flow mediates the role of sex chromosome meiotic drive during complex speciation. *Elife* 7:  
914 e35468.

915 Mihola, O., Z. Trachtulec, C. Vlcek, J. C. Schimenti, and J. Forejt, 2009 A mouse speciation  
916 gene encodes a meiotic histone H3 methyltransferase. *Science* 323: 373–375.

917 Mistry, J., S. Chuguransky, L. Williams, M. Qureshi, G. A. Salazar *et al.*, 2021 Pfam: The protein  
918 families database in 2021. *Nucleic Acids Res.* 49: D412–D419.

919 Moretti, C., M. Blanco, C. Italy-Radio, M.-E. Serrentino, C. Gobé *et al.*, 2020 Battle of the sex  
920 chromosomes: competition between X and Y chromosome-encoded proteins for partner  
921 interaction and chromatin occupancy drives multicity gene expression and evolution in  
922 Murid rodents. *Mol. Biol. Evol.* 37: 3453–3468.

923 Moretti, C., M.-E. Serrentino, C. Italy-Radio, M. Delessard, T. A. Soboleva *et al.*, 2017 SLY  
924 regulates genes involved in chromatin remodeling and interacts with TBL1XR1 during  
925 sperm differentiation. *Cell Death Differ.* 24: 1029–1044.

926 Morgan, K., B. Harr, M. A. White, B. A. Payseur, and L. M. Turner, 2020 Disrupted gene  
927 networks in subfertile hybrid house mice. *Mol. Biol. Evol.* 37: 1547–1562.

928 Morgan, A. P., and F. Pardo-Manuel de Villena, 2017 Sequence and structural diversity of  
929 mouse Y chromosomes. *Mol. Biol. Evol.* 34: 3186–3204.

930 Moyle, L. C., and T. Nakazato, 2010 Hybrid incompatibility “snowballs” between *Solanum*  
931 species. *Science* 329: 1521–1523.

932 Mueller, J. L., S. K. Mahadevaiah, P. J. Park, P. E. Warburton, D. C. Page *et al.*, 2008 The  
933 mouse X chromosome is enriched for multicity testis genes showing postmeiotic  
934 expression. *Nat. Genet.* 40: 794–799.

935 Mueller, J. L., H. Skaletsky, L. G. Brown, S. Zaghlul, S. Rock *et al.*, 2013 Independent  
936 specialization of the human and mouse X chromosomes for the male germ line. *Nat. Genet.*  
937 45: 1083–1087.

938 Mukaj, A., J. Piálek, V. Fotopulosova, A. P. Morgan, L. Odenthal-Hesse *et al.*, 2020 *Prdm9*  
939 intersubspecific interactions in hybrid male sterility of house mouse. *Mol. Biol. Evol.* 37:  
940 3423–3438.

941 Muller, H., 1942 Isolating mechanisms, evolution, and temperature. *Biol. Symp.* 6: 71–125.

942 Myers, S., R. Bowden, A. Tumian, R. E. Bontrop, C. Freeman *et al.*, 2010 Drive against hotspot  
943 motifs in primates implicates the *PRDM9* gene in meiotic recombination. *Science* 327: 876–  
944 879.

945 Oka, A., T. Aoto, Y. Totsuka, R. Takahashi, M. Ueda *et al.*, 2007 Disruption of genetic  
946 interaction between two autosomal regions and the X chromosome causes reproductive  
947 isolation between mouse strains derived from different subspecies. *Genetics* 175: 185–197.

948 Oka, A., A. Mita, N. Sakurai-Yamatani, H. Yamamoto, N. Takagi *et al.*, 2004 Hybrid breakdown  
949 caused by substitution of the X chromosome between two mouse subspecies. *Genetics*  
950 166: 913–924.

951 Oliver, P. L., L. Goodstadt, J. J. Bayes, Z. Birtle, K. C. Roach *et al.*, 2009 Accelerated evolution  
952 of the *Prdm9* speciation gene across diverse metazoan taxa. *PLoS Genet.* 5: e1000753.

953 Parvanov, E. D., P. M. Petkov, and K. Paigen, 2010 *Prdm9* controls activation of mammalian  
954 recombination hotspots. *Science* 327: 835.

955 Payseur, B. A., J. G. Krenz, and M. W. Nachman, 2004 Differential patterns of introgression  
956 across the X chromosome in a hybrid zone between two species of house mice. *Evolution*  
957 58: 2064–2078.

958 Pedersen, B. S., and A. R. Quinlan, 2018 Mosdepth: quick coverage calculation for genomes  
959 and exomes. *Bioinformatics* 34: 867–868.

960 Phifer-Rixey, M., B. Harr, and J. Hey, 2020 Further resolution of the house mouse (*Mus*  
961 *musculus*) phylogeny by integration over isolation-with-migration histories. *BMC Evol. Biol.*  
962 20: 120.

963 Phifer-Rixey, M., and M. W. Nachman, 2015 Insights into mammalian biology from the wild  
964 house mouse *Mus musculus*. *Elife* 4: e05959.

965 Rathje, C. C., E. E. P. Johnson, D. Drage, C. Patinotti, G. Silvestri *et al.*, 2019 Differential sperm  
966 motility mediates the sex ratio drive shaping mouse sex chromosome evolution. *Curr. Biol.*  
967 29: 3692–3698.e4.

968 R Core Team, 2020 *R: A language and environment for statistical computing*. R Foundation for  
969 Statistical Computing, Vienna, Austria.

970 Reed, L. K., and T. A. Markow, 2004 Early events in speciation: polymorphism for hybrid male  
971 sterility in *Drosophila*. *Proc. Natl. Acad. Sci. U. S. A.* 101: 9009–9012.

972 Robinson, M. D., D. J. McCarthy, and G. K. Smyth, 2010 edgeR: a Bioconductor package for  
973 differential expression analysis of digital gene expression data. *Bioinformatics* 26: 139–140.

974 Schwahn, D. J., R. J. Wang, M. A. White, and B. A. Payseur, 2018 Genetic dissection of hybrid  
975 male sterility across stages of spermatogenesis. *Genetics* 210: 1453–1465.

976 Smadja, C., and G. Ganem, 2007 Divergence of odorant signals within and between the two  
977 European subspecies of the house mouse. *Behav. Ecol.* 19: 223–230.

978 Smadja, C., and G. Ganem, 2002 Subspecies recognition in the house mouse: a study of two  
979 populations from the border of a hybrid zone. *Behav. Ecol.* 13: 312–320.

980 Smagulova, F., K. Brick, Y. Pu, R. D. Camerini-Otero, and G. V. Petukhova, 2016 The  
981 evolutionary turnover of recombination hot spots contributes to speciation in mice. *Genes*  
982 *Dev.* 30: 266–280.

983 Soh, Y. Q. S., J. Alföldi, T. Pyntikova, L. G. Brown, T. Graves *et al.*, 2014 Sequencing the  
984 mouse Y chromosome reveals convergent gene acquisition and amplification on both sex  
985 chromosomes. *Cell* 159: 800–813.

986 Storchová, R., S. Gregorová, D. Buckiová, V. Kyselová, P. Divina *et al.*, 2004 Genetic analysis  
987 of X-linked hybrid sterility in the house mouse. *Mamm. Genome* 15: 515–524.

988 Suzuki, T. A., and M. W. Nachman, 2015 Speciation and reduced hybrid female fertility in house  
989 mice. *Evolution* 69: 2468–2481.

990 Sweigart, A. L., and L. E. Flagel, 2015 Evidence of natural selection acting on a polymorphic  
991 hybrid incompatibility locus in *Mimulus*. *Genetics* 199: 543–554.

992 Teeter, K. C., L. M. Thibodeau, Z. Gompert, C. A. Buerkle, M. W. Nachman *et al.*, 2010 The  
993 variable genomic architecture of isolation between hybridizing species of house mice.  
994 *Evolution* 64: 472–485.

995 Tucker, P. K., R. D. Sage, J. Warner, A. C. Wilson, and E. M. Eicher, 1992 Abrupt cline for sex  
996 chromosomes in a hybrid zone between two species of mice. *Evolution* 46: 1146–1163.

997 Turner, J. M. A., 2015 Meiotic silencing in mammals. *Annu. Rev. Genet.* 49: 395–412.

998 Turner, L. M., and B. Harr, 2014 Genome-wide mapping in a house mouse hybrid zone reveals  
999 hybrid sterility loci and Dobzhansky-Muller interactions. *Elife* 3: e02504.

1000 Turner, L. M., D. J. Schwahn, and B. Harr, 2012 Reduced male fertility is common but highly  
1001 variable in form and severity in a natural house mouse hybrid zone. *Evolution* 66: 443–458.

1002 Turner, L. M., M. A. White, D. Tautz, and B. A. Payseur, 2014 Genomic networks of hybrid  
1003 sterility. *PLoS Genet.* 10: e1004162.

1004 Vanlerberghe, F., B. Dod, P. Boursot, M. Bellis, and F. Bonhomme, 1986 Absence of Y-  
1005 chromosome introgression across the hybrid zone between *Mus musculus domesticus* and  
1006 *Mus musculus musculus*. *Genet. Res.* 48: 191–197.

1007 Vara, C., L. Capilla, L. Ferretti, A. Ledda, R. A. Sánchez-Guillén *et al.*, 2019 PRDM9 diversity at  
1008 fine geographical scale reveals contrasting evolutionary patterns and functional constraints  
1009 in natural populations of house mice. *Mol. Biol. Evol.* 36: 1686–1700.

1010 Vigesna, R., M. Tomaszkiewicz, P. Medvedev, and K. D. Makova, 2019 Dosage regulation, and  
1011 variation in gene expression and copy number of human Y chromosome ampliconic genes.  
1012 *PLoS Genet.* 15: e1008369.

1013 Vyskočilová, M., Z. Trachulec, J. Forejt, and J. Piálek, 2005 Does geography matter in hybrid  
1014 sterility in house mice? *Biol. J. Linn. Soc. Lond.* 84: 663–674.

1015 Wang, R. J., C. Ané, and B. A. Payseur, 2013 The evolution of hybrid incompatibilities along a  
1016 phylogeny. *Evolution* 67: 2905–2922.

1017 White, M. A., B. Steffy, T. Wiltshire, and B. A. Payseur, 2011 Genetic dissection of a key  
1018 reproductive barrier between nascent species of house mice. *Genetics* 189: 289–304.

1019 Widmayer, S. J., M. A. Handel, and D. L. Aylor, 2020 Age and genetic background modify  
1020 hybrid male sterility in house mice. *Genetics* 216: 585–597.

1021 Yang, H., J. R. Wang, J. P. Didion, R. J. Buus, T. A. Bell *et al.*, 2011 Subspecific origin and  
1022 haplotype diversity in the laboratory mouse. *Nat. Genet.* 43: 648–655.

1023 Yates, A. D., P. Achuthan, W. Akanni, J. Allen, J. Allen *et al.*, 2019 Ensembl 2020. *Nucleic  
1024 Acids Res.* 48: D682–D688.

1025 Zuellig, M. P., and A. L. Sweigart, 2018 A two-locus hybrid incompatibility is widespread,  
1026 polymorphic, and active in natural populations of *Mimulus*. *Evolution* 72: 2394–2405.

1027

1028