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Deficiency of SYCP3-related XLR3 disrupts the initiation of meiotic sex chromosome  
inactivation in mouse spermatogenesis

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## 1 **Abstract**

2           In mammals, the X and Y chromosomes share only small regions of homology  
3 called pseudo-autosomal regions (PAR) where pairing and recombination in  
4 spermatocytes can occur. Consequently, the sex chromosomes remain largely  
5 unsynapsed during meiosis I and are sequestered in a nuclear compartment known as  
6 the XY body where they are transcriptionally silenced in a process called meiotic sex  
7 chromosome inactivation (MSCI). MSCI mirrors meiotic silencing of unpaired chromatin  
8 (MSUC), the sequestration and transcriptional repression of unpaired DNA observed  
9 widely in eukaryotes. MSCI is initiated by the assembly of the axial elements of the  
10 synaptonemal complex (SC) comprising the structural proteins SYCP2 and SYCP3  
11 followed by the ordered recruitment of DNA Damage Response (DDR) factors to effect  
12 gene silencing. However, the precise mechanism of how unsynapsed chromatin is  
13 detected in meiocytes is poorly understood. The sex chromosomes in eutherian  
14 mammals harbor multiple clusters of *SYCP3*-like amplicons comprising the *Xlr* gene  
15 family, only a handful of which have been functionally studied. We used a shRNA-  
16 transgenic mouse model to create a deficiency in the testis-expressed multicopy *Xlr3*  
17 genes to investigate their role in spermatogenesis. Here we show that knockdown of  
18 *Xlr3* in mice leads to spermatogenic defects and a skewed sex ratio that can be traced  
19 to MSCI breakdown. Spermatocytes deficient in *XLR3* form the XY body and the SC  
20 axial elements therein, but are compromised in their ability to recruit DDR components  
21 to the XY body.

22

## 23 **Author Summary**

24 A key event in the production of sperm is the pairing and synapsis of homologous  
25 chromosome pairs to facilitate genetic recombination during the first meiotic division.  
26 Chromosomal abnormalities that undermine pairing and synapsis in spermatocytes  
27 trigger a checkpoint that leads to removal of the abnormal cells via programmed cell  
28 death. In mammals, the sex chromosomes, X and Y, lack homology through most of  
29 their length and remain largely unpaired. To avoid triggering the checkpoint the X and Y  
30 are sequestered in a specialized nuclear compartment called the XY body. DNA  
31 damage response (DDR) proteins are recruited to the XY body in a highly ordered  
32 progression leading to repression of all gene transcription from the sex chromosomes.  
33 We show that knocking down expression of the X-linked *SYCP3*-like gene, *Xlr3*,  
34 disrupts sex chromosome gene silencing by interfering with recruitment of DDR factors,  
35 leading to compromised sperm production.

36

## 37 **Introduction**

38 In eukaryotes, genetic recombination between paired, homologous  
39 chromosomes is enabled by induction of DNA double-strand breaks (DSBs) and  
40 repaired by factors of the DNA Damage Response (DDR) during meiosis I. Unpaired  
41 chromosomes or chromosomal segments are also subject to DSBs, yet they undergo  
42 DDR repair mechanisms specific to unsynapsed chromatin [1]. These repair  
43 mechanisms typically involve sequestration of unsynapsed chromatin in defined nuclear

44 domains and subsequent transcriptional silencing and heterochromatinization in a  
45 process known as meiotic silencing of unpaired chromatin (MSUC) [2, 3].

46 In mammalian spermatogenesis, synapsis of the heterologous X and Y  
47 chromosomes is delayed compared to autosomes and is restricted to the pseudo-  
48 autosomal regions (PARs) [4, 5]. As a consequence, during the pachytene stage of  
49 prophase I a process consistent with MSUC occurs: a distinct nuclear compartment  
50 known as the XY body forms, wherein DSB repair depends on transcriptional  
51 inactivation of the sex chromosomes (MSCI) effected by DDR factors distinct from that  
52 of the synapsed autosomes [6]. MSCI initiates in pachynema and persists through  
53 meiosis and into spermiogenesis where the transcriptional repressed state is referred to  
54 as post-meiotic sex chromatin (PMSC) [7]. Most X and Y-linked mRNA-encoding genes  
55 are subject to silencing, although a few notable genes escape and are actively  
56 transcribed post-meiotically [8, 9]. Disruption of MSCI activates the pachytene meiotic  
57 checkpoint (PMC) and spermatocytes with active sex chromosome transcription  
58 undergo arrest and apoptosis [2].

59 The sensing of unsynapsed chromatin at the onset of homologous pairing is not  
60 well understood, but it appears to accompany the assembly of the synaptonemal  
61 complex (SC) and recruitment of the first DDR factors. After DSBs are induced,  
62 homologous chromosomes begin to pair and synapse as the SC assembles [10].  
63 Homologs are arranged in chromosome loops anchored at the axial elements (AE) of  
64 the SC. The AE are constituted by Synaptonemal Complex Proteins 2 and 3 (SYCP2,  
65 SYCP3) [11, 12], which are necessary for the early events of DDR including recruitment

66 of RPA, HORMAD1/2 and ATR to DSBs [13]. For reasons that are unclear, the DDR  
67 cascade in early pachynema is slightly different within the XY body: DSBs attract the  
68 damage sensors BRCA1 and TOPBP1, and ATR displaces from the axes into the loops  
69 [14-17] where it phosphorylates H2AX, leading to deposition of the repressive chromatin  
70 mark histone 3 lysine 9 trimethylation (H3K9me3) [18], sumoylation (SUMO1) [14] and  
71 ultimately to MSCI.

72         The precise role of SYCP3 in sensing unsynapsed chromatin is unknown.  
73 *SYCP3* is a highly conserved SC component found in the genomes of most metazoans,  
74 typically as a single copy autosomal gene [19]. However, in eutherian mammals  
75 numerous *SYCP3*-like amplicons are found across the X and Y chromosomes. The  
76 amplicons comprise the mammalian *X-linked Lymphocyte Regulated (Xlr)* superfamily  
77 [20] which includes: *SYCP3-like X-linked (Slx)* and *SYCP3-like Y-linked (Sly)* genes  
78 found in certain species of *Mus*; the *Xlr3*, 4 and 5 triad found in a variety of Rodentia,  
79 and the *FAM9A*, *B* and *C* genes in primates [21-24]. The function of most *Xlr* members  
80 is unknown, however Cocquet and co-workers have shown that *Slx/Slx1* and *Sly* are  
81 expressed in post-meiotic spermatids where they play antagonistic roles in the  
82 maintenance of PMSC [22, 25, 26]. Moreover, copy number variation of *Slx/Slx1* and  
83 *Sly* between species of *Mus* is thought to underlie male sterility in inter-subspecific  
84 hybrids underscoring the importance of the *Xlr* family members in spermatogenesis [27].

85         Here we investigate the function of the *Xlr3* genes utilizing a transgenic mouse  
86 capable of tissue-specific expression of a short-hairpin RNA targeting *Xlr3* mRNA.  
87 Unlike other *Xlr* family members, which are typically present in dozens of copies, the

88 *Xlr3* family consists of three closely-linked, protein-encoding paralogs, *Xlr3a*, *Xlr3b*, and  
89 *Xlr3c*. These genes are broadly expressed in mouse fetal and adult tissues and one  
90 paralog, *Xlr3b*, is imprinted in developing and adult mouse brain where it is expressed  
91 predominantly from the maternal X [28]. The three functional copies, hereafter referred  
92 to as *Xlr3*, encode a near-identical 26 kDa protein that is expressed in testis [21]. We  
93 show that *Xlr3* expression initiates during early meiotic prophase I where the protein  
94 localizes to the XY body in primary spermatocytes. Germ-cell specific knockdown of  
95 *Xlr3* mRNA in shRNA-transgenic males leads to partial disruption of spermatogenesis,  
96 reduced sperm count and offspring with a skewed sex ratio. Examination of sex-linked  
97 gene expression and localization of DDR factors points to a breakdown in the earliest  
98 stages of MSCI in the *Xlr3* knockdown males, implicating *Xlr3* as the earliest acting  
99 factor involved in XY body formation and the only known sex-linked factor implicated in  
100 MSCI.

101

## 102 **Results**

103 ***Xlr3* is a multicopy gene encoding an *Sycp3*-like *Cor1* domain.** *Xlr* superfamily  
104 genes are scattered in multicopy clusters across the mouse X chromosome. The  
105 *Xlr3a/b/c* paralogs map to a small cluster encompassed within ~250 kilobases on  
106 mouse XA7.3 (Fig.1A). *Xlr3a/b/c* encode near-identical proteins; XLR3B and XLR3C  
107 contain a single residue difference, while XLR3A contains eight amino acid substitutions  
108 compared to the other two paralogs (Fig.1B). Our current understanding of  
109 structure/function relationships of *Xlr* superfamily members is informed by structural

110 studies of the family progenitor, *Sycp3*. The N-terminus of SYCP3 binds DNA to  
111 facilitate the scaffolding of homologous chromosomes. Central coiled-coil helices are  
112 formed by the Cor1 domain, which tetramerizes to create SYCP3 fibers, while assembly  
113 of fibers into larger filaments is governed by the C-terminal region [29]. While the Cor1  
114 domain is the most highly conserved segment among *Xlr* superfamily members, they all  
115 possess a monopartite nuclear localization signal (RKRK) within the divergent N-  
116 terminus (Fig.1C).

117

118 ***Xlr3* is upregulated in early prophase I and colocalizes with the XY body in**  
119 **pachynema.** To quantify *Xlr3* transcript levels during spermatogenesis, quantitative  
120 reverse transcription PCR (qRT-PCR) using primers common to the three *Xlr3* mRNA  
121 paralogs (*a/b/c*) was used. In the testes of staged-prepubertal mice, *Xlr3* transcription  
122 begins in pre-meiotic stages, but increases sharply from 8.5 days post-partum (dpp),  
123 representing meiotic entry, through 10.5dpp, representing prophase I leptonema  
124 (Fig.2A). *Xlr3* transcript abundance peaks by 11.5dpp, at which point cells enter and  
125 progress through zygonema (Fig.2A).

126 While *Xlr3* transcripts are detected before meiotic entry, XLR3 protein is not  
127 detectable via immunoblot until 10.5-11.5dpp, where it appears restricted to the nucleus  
128 (Fig.2B). Thus, XLR3 translation and nuclear localization coincide with the appearance  
129 of DSBs during leptonema [6]. Through immunocytochemistry (ICC), we observed  
130 specific XLR3 protein subcellular localization to the XY body during pachynema (Fig.2C-  
131 D). In early pachynema, XLR3 closely associates with the sex chromosome axes,

132 including on the PAR (Fig.2C). By late pachynema, XLR3 appears to move away from  
133 the axes, but maintains association with the XY body, suggesting it translocates to the  
134 XY chromatin loops (Fig.2D).

135

136 **shRNA-*Xlr3* activity significantly reduces *Xlr3* abundance in mouse testis.**

137 Upregulation in early prophase I and localization to the XY body suggests a possible  
138 role for *Xlr3* in sex chromosome regulation in meiosis I. To explore this possibility, we  
139 created a mouse model in which *Xlr3* function is abrogated through RNA interference  
140 (RNAi) in an approach similar to that described by Cocquet *et al.* [22, 25]. To knock  
141 down *Xlr3* post-transcriptionally, we designed a construct containing a short hairpin  
142 RNA (shRNA) with sequence complementary to the *Xlr3* exon3/4 region (Fig. 3A) that is  
143 invariant among the *Xlr3* paralogs but divergent from *Xlr4* and *Xlr5* at several sites (Fig.  
144 S2A). A floxed stop cassette between the *ROSA26* promoter and the *Xlr3*-shRNA  
145 transgene was excised by crossing to a *Ddx4-Vasa* Cre recombinase mouse, allowing  
146 initiation of expression of the shRNA in spermatogonia [30] (Fig. S1). Efficacy of the  
147 shRNA knockdown was assessed via qRT-PCR using primers targeting exon 3 of *Xlr3*,  
148 the site of shRNA binding, and primers further downstream at exon 6. *Xlr3* transcript  
149 abundance was decreased by approximately 50% in shRNA-*Xlr3* mouse testis  
150 compared to that of age-matched wild type males (Fig. 3B). Immunofluorescence (IF)  
151 was used to quantify the effect of this knock down on XLR3 protein levels in  
152 spermatocytes. Using  $\gamma$ H2AX as a marker for the XY body, we quantified the XLR3  
153 signal overlap in wild type and shRNA-*Xlr3* pachytene spermatocytes. Compared to the



154 wild type, shRNA-*Xlr3* cells had on average approximately 50% of the IF signal (Fig.  
155 3C), suggesting the reduction of protein product is directly proportional to that of *Xlr3*  
156 transcript levels.

157 To verify specificity of the shRNA to *Xlr3*, we used qRT-PCR to assay mRNA  
158 levels of the most closely related *Xlr* family members, *Xlr4* and *Xlr5*. Transcript levels of  
159 these genes were unaffected in the testis of shRNA-*Xlr3* transgenic mice (Fig. S2B,C).  
160 To assess potential induction of a viral immune response due to the expression of  
161 double-stranded RNA in the transgenic mice, we assayed expression of 2',5'-  
162 *Oligoadenylate synthetase 1b* (*Oas1b*), which is part of the interferon viral response  
163 pathway [22, 31, 32]. Again, there was no significant difference in transcript level of this  
164 gene, indicating our shRNA did not induce an interferon response (Fig. S2D). Taken  
165 together, these data indicate we have achieved specificity of the targeted shRNA to *Xlr3*  
166 reduction in testes.

167

168 ***Xlr3* knock down leads to germ cell loss and a skewed sex ratio.** Initially, both male  
169 and female shRNA-*Xlr3* transgenic mice appeared to be fully fertile, as they produced  
170 offspring with the same frequency and litter size as wild type sibling controls (Fig. 4A-B).  
171 However, a thorough examination of the transgenic males revealed a significant  
172 reduction in the sperm count (49.3% reduction) and normalized testes weight (14.7%  
173 reduction) at 14 weeks of age compared to wild type siblings (Fig. 4C-D). Histological  
174 cross-sections of the testes of shRNA-*Xlr3* transgenic males revealed the presence of  
175 disorganized seminiferous tubules and a significant decrease in average tubule

176 diameter (50% reduction) suggestive of either germ cell or Sertoli cell loss (Fig. 4E-G).  
177 To distinguish between these two possible causes of tubule defects, we assayed  
178 expression of the Sertoli cell marker, *Sox9* (Rebourcet et al., 2014, 2017) to test for  
179 Sertoli cell loss. There was no difference in *Sox9* expression between transgenic testis  
180 and wild type, suggesting Sertoli cell loss has not occurred (Fig. S2E). However, a two-  
181 fold increase in germ cell apoptotic figures was observed in tubules of shRNA-*Xlr3*  
182 transgenic males compared to wild type (Fig. 4H-J). Overall, the *Xlr3* knock down leads  
183 to significant germ cell loss, but not to an extent to subvert fertility.

184         During the course of fertility assessment, we observed a significant skew (60:40)  
185 towards female offspring produced by the shRNA-*Xlr3* males across 400 individuals  
186 (Fig. 5A). To determine if sex chromosome nondisjunction (ND) events in the transgenic  
187 males were leading to excess daughters (i.e.  $X^{\text{mat}}$  monosomics), we mated shRNA-*Xlr3*  
188 males on a C57BL/6J background to wild type C3H/HeJ females to trace the parental  
189 origin of the X chromosome via strain-specific sequence polymorphisms (Fig. 5B). While  
190 the same 60:40 skew toward females was observed in the inter-strain cross, only 2% of  
191 hybrid offspring were found to be  $39,X^{\text{mat}}$ , attributable to sex chromosome ND (Fig. 5C).  
192 While this rate of ND is significantly higher compared to spontaneous events (0.01%)  
193 [33], ND does not fully account for the observed increase in female offspring, indicating  
194 the *Xlr3* knockdown enhances X sperm or compromises Y sperm function.

195

196 ***Xlr3* knock down leads to disruption of meiotic sex chromosome inactivation.**

197 RNAi knockdown of the *Xlr* superfamily members *Slx* and *Sly* also leads to sex

198 chromosome transmission distortion and either enhancement (*Slx* knockdown) or  
199 disruption (*Sly* knockdown) of normal transcriptional repression of post-meiotic sex  
200 chromatin (PMSC) [22, 25]. Given the localization of XLR3 to the XY body in  
201 pachynema, we examined the status of MSCI in shRNA-*Xlr3* mice. To quantify changes  
202 in relative abundance of sex-linked transcripts, we assayed several genes by qRT-PCR  
203 at two time points. Since steady-state transcript levels are reflective of both  
204 transcriptional output and mRNA half-life, we determined potential changes in transcript  
205 levels in transgenics in reference to a normalized ratio of wild type transcript levels from  
206 9.5dpp, the day before XLR3 protein is detected, and 14.5dpp, (Fig. 2B) the earliest  
207 point at which MSCI is detected [34]. We observed a statistically significant upregulation  
208 of several genes across the X chromosome (*Rhox13*, *Hprt*, *Fmr1*, *Rps6ka6*, *Tcp11x2*),  
209 as well as the “pachytene-lethal” Y-linked gene *Zfy2* [10, 35] (Fig. 6A-B).

210         Since qRT-PCR measures abundance of transcripts, but cannot differentiate  
211 between stable transcripts and those actively transcribed at any one time point, we used  
212 RNA fluorescence *in situ* hybridization (RNA-FISH) to verify active transcription from the  
213 XY body during pachynema. Using a probe for *Fmr1*, one of the X-linked qRT-PCR  
214 targets (Fig. 6A-B), we confirmed there are no nascent transcripts observable within the  
215 XY body (0/40 cells) in wild type spermatocytes that maintain active MSCI regulation  
216 (Fig. 6C). However, approximately 50% of spermatocytes from shRNA-*Xlr3*  
217 homozygotes showed colocalization of the *Fmr1* signal with the  $\gamma$ H2AX XY body marker  
218 (29/60 cells), indicative of MSCI disruption commensurate with the extent of *Xlr3* knock  
219 down (Fig. 6C).

220

221 **XLR3 marks the XY body to recruit DDR and chromatin regulators.** Since XLR3  
222 production and nuclear localization coincides with the appearance of DSBs in meiotic  
223 prophase I, it is important to place XLR3 localization to the XY body in relation to the  
224 sequential recruitment of DDR factors that bring about MSCI. BRCA1 recruitment to sex  
225 chromosome DSBs in late zygonema/early pachynema leads to localization of ATR  
226 kinase to sex chromosome axes (Turner et al., 2004). Subsequently, ATR translocates  
227 from the axes to chromatin loops in early to mid-pachynema where it phosphorylates  
228 H2AX [16, 17, 36], and is followed by the later accumulation of SUMO1 [37, 38], and  
229 H3K9me3 [18] by diplonema.

230 We assessed the sequential localization of XY body markers via ICC from later to  
231 earlier stages (from top to bottom Fig. 8). In late-pachytene spermatocytes (18.5 dpp) of  
232 the *shRNA-Xlr3* males we detected a spectrum of signal intensity for SUMO1 (Fig. 7A-  
233 C). While some spermatocytes showed staining equivalent to wild type, many in the  
234 same spreads showed severely diminished staining. The average corrected total  
235 fluorescence (CTF) of the anti-SUMO1 signal was half the intensity in *shRNA-Xlr3* cells  
236 compared that of WT cells (Fig. 7A-C). Contrarily, the accumulation of H3K9me3 at the  
237 XY body, another late pachytene signal, was found to be elevated on average in  
238 *shRNA-Xlr3* cells compared to wild type, again showing a spectrum of signal (Fig. 7D-  
239 F). These results suggest that XLR3 plays a role in regulating the epigenetic landscape  
240 of the XY body, resulting in higher levels of H3K9me3 at the XY body at the end of  
241 pachynema [18].

242           In mid-pachytene spermatocytes (16.5 dpp), the average signal intensity for  
243  $\gamma$ H2AX at the XY body in shRNA-*Xlr3* transgenic mice is half that of wild type, again  
244 showing cell-to-cell differences from normal to weak staining (Fig. 7G-I). Likewise, ATR  
245 was observed to localize appropriately to the XY body, i.e. along the chromosomal axes  
246 and chromatin loops, but at approximately 50% average intensity in shRNA-*Xlr3*  
247 compared to wild type cells (Fig. J-L). Finally, BRCA1, the earliest known mark on the  
248 XY body preceding recruitment of ATR to the XY body [17], localized to the  
249 chromosomal axes of the XY body in early pachytene spermatocytes (13.5 dpp) in both  
250 transgenic and WT testis, but at half the level in shRNA-*Xlr3* transgenic cells compared  
251 to WT cells, again showing cell-to-cell differences in the knockdown (Fig. M-O). These  
252 results suggest that XLR3 localization is a necessary pre-condition for the recruitment of  
253 BRCA1 to the XY body, placing XLR3 as one of the earliest known factors leading to the  
254 demarcation of the XY body and the only known sex-linked factor essential in MSCI.

255

## 256 **Discussion**

257           The first identification of an *X-linked lymphocyte regulated (Xlr)* gene came from  
258 the isolation of a B lymphocyte expressed cDNA that was an early candidate for a  
259 mouse X-linked immunodeficiency syndrome (*xid*) [39]. *Xlr* (a.k.a. pM1) was ultimately  
260 dismissed as *xid*, but deeper genomic characterization of this multicopy gene led to the  
261 discovery of other *Xlr* homologs with broad expression profiles scattered across the  
262 murine X and Y [21, 40, 41]. The *Xlr* name, with a few exceptions, has largely persisted  
263 despite the recognition that *SYCP3* is the ancestral source gene of this family [42, 43].

264 Sex-linked *SYCP3*-like genes can be found in the published genome sequences of a  
265 variety of eutherian mammals (e.g. *Canis lupus familiaris* LOC102152531). The broad  
266 distribution of these genes across Eutheria raises the question: Why have duplicate  
267 copies of *SYCP3* moved to the sex chromosomes where they have multiplied and  
268 diversified in the course of eutherian divergence?

269         At least some members of the *Xlr* family seem to have evolved under the  
270 influence of sexual antagonism as suggested by functional studies of the *Slx/Slx11* and  
271 *Sly* genes [22, 25, 26, 44]. *Slx/Slx11* and *Sly*, X-linked and Y-linked respectively, are  
272 multicopy genes found in certain species of *Mus* that are expressed predominantly in  
273 post-meiotic spermatids [42, 45]. Using a shRNA transgenic approach, upon which ours  
274 was modeled, Cocquet and coworkers [22, 25] showed knockdown of *Sly* results in  
275 subfertility, a female-skewed sex ratio, and relaxation of PMSC leading to up-regulation  
276 of X-linked genes. *Slx/Slx11* knockdown males are also subfertile, but with the opposite  
277 effect observed in the *Sly* knockdown: PMSC repression is enhanced when *Slx/Slx11*  
278 expression is reduced, leading to inappropriate silencing of several otherwise active X-  
279 linked genes and an offspring sex ratio skewed toward males. Curiously, full fertility is  
280 restored in double knockdown males expressing both anti-*Slx/Slx11* and anti-*Sly*  
281 shRNAs [25], indicating that Y-linked copies are acting in conflict with X-linked copies  
282 [26]. Good and coworkers demonstrated that sterility in F1 hybrid males from the mating  
283 of two sub-species of *Mus musculus* is associated with a breakdown in PMSC, showing  
284 broad up-regulation of sex-linked genes [46]. The two sub-species differ significantly in  
285 copy number of the *Slx/Sly* genes; in hybrid males, the presence of a higher *Slx* copy

286 number compared to *Sly* leads to the up-regulation of sex-linked genes, which is  
287 consistent with the induced *Slx/Sly* imbalance and PMSC failure in the knockdown  
288 model [25]. Recently, it was shown that SLX/SLXL1 compete with SLY in binding to the  
289 H3K4me3-reader, SSTY1, in spermatid nuclei, mediating their respective activities as  
290 transcriptional up- or down-regulators [26].

291 Unlike the *Slx/Slx1/Sly* clusters, which are restricted to the genus *Mus*, the  
292 *Xlr3/4/5* cluster appears to be more ancient, having homologs with shared local synteny  
293 in dog, pig and alpaca. The broad distribution of these genes across eutherian lineages  
294 and our observations that knockdown of *Xlr3* results in disruption of BRCA1 and ATR  
295 localization to the XY body suggest a fundamental role for these genes in the initiation  
296 of MSCI in eutherian male meiosis.

297 Marsupials share a common origin for the sex chromosomes with Eutheria [47],  
298 and likewise share many features of MSCI in spermatogenesis, including formation of  
299 an XY body and localization of BRCA1 and ATR to the sex chromosomes early in  
300 meiotic prophase I [48, 49]. Unlike eutherians, the marsupial X and Y chromosomes  
301 lack a PAR and do not pair during meiosis I; no synaptonemal complex is formed but  
302 the sex chromosomes are held together within the XY body by an SYCP3-enriched  
303 structure called the “dense plate” [50]. The emergence of *Xlr* genes at the base of the  
304 eutherian lineage, therefore, suggests they were recruited into an established MSCI  
305 mechanism present in the therian common ancestor.

306 We envision two possible scenarios underlying the co-optation of a sex-linked  
307 *SYCP3* duplicate, *Xlr*, into MSCI. Like *Slx/Sly*, the earliest *Xlr*'s could have evolved as a

308 consequence of the persistent intragenomic conflict between the sex chromosomes [51,  
309 52]. Supporting this scenario is the observation that knockdown of *Xlr3* results in an  
310 offspring sex ratio skewed toward females (Fig. 5A). However, it is difficult to reconcile  
311 this result with the observation that knockdown of the only other X-linked *Xlr* members  
312 with known function, *Slx/Slx1*, skews toward male offspring. In other words, normal  
313 *Slx/Slx1* function appears to drives X chromosome transmission, while normal *Sly*  
314 function drives Y transmission. *Xlr3* appears to function more similarly to the Y-linked  
315 *Sly*, by promoting sex chromosome transcriptional silencing and, ostensibly, Y-  
316 chromosome transmission. While it is possible that *Xlr3* evolved as a suppressor of X  
317 meiotic drive, it is more likely that the sex ratio skew resulting from *Xlr3* knockdown is a  
318 byproduct of disrupted MSCI.

319         Alternatively, *Xlr* genes may have evolved to augment or adapt an ancestral  
320 therian MSCI mechanism to address an emergent feature of the sex chromosomes in  
321 Eutheria. The emergence of *Xlr* genes at the base of the eutherian lineage coincides  
322 with the addition of a large segment of autosomal DNA to the sex chromosomes after  
323 the divergence of Eutheria from Marsupialia [53, 54]. While this added segment likely  
324 paired initially, it is clear that the Y-borne segment underwent rapid attrition during the  
325 divergence of placental mammals [55]. We propose that *Xlr3* evolved to accommodate  
326 silencing of the emergent unpaired sex chromosome DNA. The factors that govern  
327 meiotic silencing of unpaired chromatin (MSUC) are drawn from a limited pool and,  
328 hence, are highly dosage sensitive [56-58]. If unpaired chromatin exceeds a certain  
329 threshold, transcriptional silencing is disrupted, leading to meiocyte loss or the



330 production of aneuploid gametes [14, 59]. Likewise, it is clear that, like SLX/SLXL1/SLY  
331 in PMSC, XLR3 involvement in MSC1 is dosage sensitive. Our finding that ~50%  
332 knockdown of *Xlr3* results in ~50% loss of sperm suggests that the compromised  
333 expression of *Xlr3* in the shRNA transgenics lies at a critical threshold. If a minimal  
334 threshold of XLR3 expression is met, MSC1 succeeds and meioocyte loss is averted; but  
335 if XLR3 levels are just below the threshold, MSC1 is abrogated and the spermatocytes  
336 undergo apoptosis. Spermatocytes from shRNA-*Xlr3* transgenic mice reveal a range of  
337 phenotypes that model these predictions: from cells with XY body MSC1 marks well  
338 below the levels of wild type, likely contributing to the increased apoptotic figures  
339 observed; to cells with normal MSC1 mark intensity that presumably go on to produce  
340 viable sperm (Fig. 4H-J, Fig. 7). It should be noted that the fact that *Xlr3* genes are  
341 themselves subject to MSC1 lends an additional dynamic to the phenotypic variance  
342 seen in the knockdown.

343         The apparent dosage sensitivity of *Xlr* family members may also offer an  
344 explanation for why one *Xlr3* paralog, *Xlr3b*, is imprinted [28]. In females, the paternal  
345 allele of *Xlr3b* is repressed in both fetal and adult tissues, including ovary. Imprinting of  
346 the paternal copy of *Xlr3b* may serve to lower overall *Xlr3* expression in oocytes below  
347 the threshold where it might induce chromatin silencing inappropriately. Aneuploid  
348 mouse models exhibiting unpaired chromatin in females and/or unpaired chromatin  
349 apart from the sex chromosomes in males may shed light on this hypothesis.

350

351

## 352 **Materials and methods**

### 353 **Ethics statement**

354 All mouse procedures were approved by the Institutional Animal Care and Use  
355 Committee of the University of Connecticut.

356

### 357 **Sequence homology and alignments**

358 Sequences were downloaded from the PFAM database (Family: Cor1  
359 [PF04803]). Percent identity matrices (PIMs) were generated using ClustalW. Protein  
360 domain alignments and corresponding graphics were generated using NCBI's  
361 constraint-based multiple alignment tool (COBALT) [60]. The *Xlr3/4/5* locus map was  
362 drawn to scale based on the UCSC genome browser view of identified regions (mm10).

363

### 364 **Animal husbandry & transgenesis**

365 Mice were housed under climate-controlled conditions with a 12-h light/dark cycle  
366 and provided standard food and water ad libitum. A floxed stop containing, short hairpin  
367 RNA (shRNA) complementary to all *Xlr3* transcripts was cloned into the ROSA-PAS  
368 gene targeting vector [61]. This vector was electroporated into C57BL/6J (RRID:IMSR  
369 000664) mouse embryonic stem cells (mESCs) and targeted clones were screened by  
370 long range PCR and Southern analysis. Two independent clones (B2 and B11) were  
371 microinjected into B6(Cg)-Tyrc-2J/J (C57BL/6J albino) host embryos (RRID:IMSR  
372 JAX:000058). Germline transmission was achieved through multiple male chimaeras  
373 from clone B2, and a stable line was bred through three stable male founders by

374 backcrossing with C57BL/6J wild-type female mice. All experiments were performed on  
375 the 6th to 13th generation, male and female offspring (Fig. S1). The C57BL/6J-  
376 Gt(ROSA)26Sor<sup>tm1</sup>(shRNA:Xi3)/Lgr<sup>+</sup>/Lgr<sup>+</sup>J mouse line will be available for distribution  
377 from The Jackson Laboratory as stock #034383. As described in Fig. S1, B6.Cg-  
378 Tg(Pgk1-flpo)10Sykr/J (RRID:IMSR 011065) and FVB-Tg(Ddx4-cre)1Dcas/J  
379 (RRID:IMSR 006954) were used in this study, along with C3H/HeJ (RRID:IMSR  
380 000659) mice.

381

### 382 **Tissue collection, testis weighing, and sperm counting**

383 Tissues collected from euthanized mice were fixed as described in the following  
384 sections or snap frozen in liquid nitrogen and stored at -80°C. Testis weights were taken  
385 from fresh tissue before freezing or fixation. Sperm counting was performed as  
386 described by Handel (Handel and O'Brien,  
387 <https://phenome.jax.org/projects/Handel1/protocol>).

388

### 389 **PCR genotyping**

390 DNA was extracted from ear punches by Proteinase K digestion. All primers used  
391 in this study are listed in Supplementary Table 1 with respective annealing  
392 temperatures. To amplify the shRNA-*Xi3* template, PrimeSTAR GXL (Clontech)  
393 enzyme system was used as per manufacturer instructions (cycling conditions: 98°C for  
394 10s, 60°C for 15s and 68°C for 10s/kB – products 3-4kB for 30s and <1kB for 10s, for  
395 35 cycles). Products were visualized on 1% agarose Tris-acetate-EDTA gels. All other

396 polymerase chain reactions (PCR) were performed using the GoTaq (Promega) enzyme  
397 system as per manufacturer instructions (95°C for 2 min, then 95°C for 30s, annealing  
398 as specified for 30s, 72°C for 1 min repeated for 35 cycles, then 72°C for 5 min) and  
399 products were visualized on 1-2% agarose and Tris-borate-EDTA gels.

400

### 401 **Xlr3 antibody generation**

402 The first 18 residues common to both XLR3A and XLR3B, and one amino acid  
403 difference from XLR3C, were used for peptide synthesis in rabbit immunization by  
404 Biosynthesis Inc. Following initial immunization, two rabbits received five biweekly  
405 boosters. Affinity purification of total IgG sera was performed using an AminoLink resin  
406 column (Thermo) and purified antibodies were complemented with pre-immune sera.

407

### 408 **Western blotting**

409 Whole testis lysate was made by homogenization in RIPA buffer (50mM Tris-HCl  
410 pH 7.4, 150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1mM  
411 EDTA, and 1mM PMSF), incubation on ice for 30 minutes, and centrifugation. Nuclear  
412 and cytoplasmic testis fractions were prepared from fresh tissue using a dounce  
413 homogenizer in Harvest Buffer (10mM HEPES pH 7.9, 25mM KCl, 2M sucrose, 1mM  
414 EDTA, 0.5mM spermidine, 1x protease inhibitor, 10% glycerol). Protein lysates were  
415 resolved by 12% SDS-PAGE and transferred to PVDF membrane (GE Healthcare  
416 Amersham™) using a Trans-Blot® SD Semi-Dry Transfer Cell (BioRad). The membrane  
417 was blocked with 5% milk in Tris-buffered saline (TBS), incubated in primary antibody

418 overnight, followed by secondary antibody for 45 min. Signal was detected by  
419 chemiluminescence (Western Lightning, Perkin Elmer) on autoradiography film.  
420 Antibodies used in this study are listed in Supplementary Table 2.

421

### 422 **Tissue fixation, sectioning, and staining**

423 Testes were dissected from adult mice and fixed as described previously [62].  
424 Paraffin embedding was performed by the University of Connecticut Veterinary and  
425 Medical Diagnostic Laboratory. Sections were sliced to 6  $\mu\text{m}$  on a rotary microtome for  
426 slide mounting. Hematoxylin and eosin (H&E) regressive staining was performed with  
427 Modified Harris Hematoxylin and Eosin Y 1% stock solution (Ricca Chemical Company)  
428 and used following manufacturer's instructions. Apoptosis detection was performed  
429 using the DeadEnd™ Colorimetric Apoptosis Detection System (Promega) following the  
430 manufacturer's instructions for fixed tissue slides. Imaging was performed on a light field  
431 microscope at 10x magnification.

432

### 433 **Spermatocyte chromosome spreads and immunocytochemistry**

434 Spermatocyte spreads were made as previously described [63] with several  
435 modifications. Testes were dissected from 13.5-18.5 dpp mice, detunicated, and free  
436 tubules were suspended in 1x phosphate buffered saline (PBS). Tubules were broken  
437 up and cells were released by needle disruption. A single cell suspension was made by  
438 putting the tubule mixture through a 70  $\mu\text{m}$  filter. Cells were placed in hypotonic solution  
439 of 2x HEB, centrifuged, and resuspended in fixative (1% PFA, 0.15% Triton X-100, and

440 13 mM DTT). Cells were collected by centrifugation and resuspended in 3.4 M sucrose,  
441 then applied to slides under 50-75% humidity. Slides remained in humidity for  
442 approximately 2 hours to allow cells to spread, then were washed in 1x PBS, and air  
443 dried.

444 Spermatocyte spreads were permeabilized with 0.5% Triton X-100 and blocked  
445 with 10% goat serum. Slides were incubated with diluted primary antibody at 4°C  
446 overnight and subsequently with diluted secondary antibody at 4°C for 30 minutes.  
447 Antibodies used in this study are listed in Supplementary Table 2. 4'6'-diamidino-2-  
448 phenylindole (DAPI) diluted 1:5 in Vectashield mounting media (Vector Labs) was used  
449 as a counterstain. Imaging was performed using an oil-immersion 100x objective of the  
450 Olympus IX 71 (DeltaVision) fluorescent microscope with DAPI, FITC, and TRITC  
451 channels. Images were captured and deconvolved using the softWoRx software  
452 (Applied Precision, LLC). Exposure times were set for particular antibody combinations  
453 and maintained for all slides imaged. Quantification of signal was accomplished by  
454 outlining cells and signals to take area and intensity measurements using ImageJ  
455 (Schindelin et al., 2015). Corrected total fluorescence (CTF) was calculated using the  
456 following formula:

$$457 \quad CTF = \text{integrated density} - (\text{area} \times \text{mean background fluorescence}).$$

458

#### 459 **RNA isolation and quantitative reverse transcription PCR (qRT-PCR)**

460 RNA was extracted from frozen tissue using the NucleoSpin RNA kit (Machery-  
461 Nagel) as described by the manufacturer. cDNA was synthesized from total RNA using

462 the QSCRIPT cDNA Supermix (Quanta Biosciences) and qRT-PCR was carried out in  
463 triplicate using the iTAQ Universal SYBR Green SuperMix (BioRad). Primers for these  
464 reactions are listed in Supplementary Table 1 and all reactions were carried out under  
465 the following cycling conditions: initial denaturation at 95°C for 3 mins, 40 cycles (95°C  
466 for 5 sec 58°C for 30 sec), a final extension of 65°C for 5 sec. CT and melting curve  
467 results were calculated by the CFX Manager 3.1 software (BioRad). Results were  
468 normalized to *β-actin* or *Gapdh* using the  $\Delta\Delta C_t$  method [64].

469

#### 470 **RNA fluorescence *in situ* hybridization (FISH)**

471 RNA FISH was performed as described by [65]. Probes were generated using  
472 primers listed in Supplementary Table 1 from the G135P65476A4 BAC as described in  
473 [8] and were labeled using the DIG-Nick Translation Mix (Roche) as per manufacturer's  
474 instructions. Following RNA FISH, slides were fixed in 4% paraformaldehyde and  
475 processed for IF as above.

476

#### 477 **Graphical and statistical data presentation**

478 All boxplot and bar graphs were produced using Rstudio (Rstudio Team, 2020).  
479 In the case of bar graphs, all error bars represent the mean  $\pm$  confidence interval (CI). In  
480 the case of boxplots, unfilled dots represent outliers and colored dots represent  
481 individual observations. Normal data distribution was tested using the Kolmogorov-  
482 Smirnov test. To determine statistical significance, if data were normally distributed, a T-

483 test was applied; if data were not normally distributed, a Wilcox Test was applied. All  
484 sample sizes are indicated with the relevant tests.

485

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491

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737

## 738 **Figure Legends**

739 **Figure 1. *Xlr3* is a family within the *Xlr* superfamily with X-linked copies and a**  
740 **Cor1 protein domain. A)** The three open reading frame-containing functional *Xlr3*  
741 copies are located on *Mus musculus* X7A.3. Diagram based on mm10 genome build. **B)**  
742 Percent identity matrices of the functional *Xlr3* paralogs at the mRNA (left) and protein  
743 (right) levels generated using ClustalW. **C)** The protein structure encoded by the XLR  
744 A/B/C paralogs have a COR1 protein domain and similar domain organization  
745 compared to other XLR superfamily members from the N- to C-terminal regions. Color  
746 indicates residue conservation wherein gray indicates in/del sites, and red or blue  
747 indicate high or low side chain similarity, respectively. Diagram generated with amino  
748 acid sequences aligned by COBALT (NCBI).

749

750 **Figure 2. *Xlr3* expression is regulated stage-specifically in mouse**  
751 **spermatogenesis and localizes to the XY body during meiosis I. A)** *Xlr3* mRNA  
752 expression increases during pre-leptotene stages (8.5-9.5 dpp) and is three-fold higher  
753 in zygotene (11.5 dpp) than 6.5 dpp. **B)** The XLR3 protein is only observed in the  
754 nuclear fraction from 10.5 dpp and strongly at 11.5 dpp through leptoneuma/zygonema.  
755 **C-D)** Immunocytochemistry of mouse spermatocyte chromosome spreads with DAPI  
756 (grey), SYCP3 (magenta), and XLR3 (yellow) reveals XLR3 localization. **C)** During early  
757 pachytene, XLR3 colocalizes with SYCP3 on the X and Y chromosome axes, indicated

758 with a dotted circle. **D)** By late pachytene, XLR3 moves to form a cloud around the XY  
759 bivalent, indicated with a dotted circle. Scale bar = 6  $\mu$ m.

760

761 **Figure 3. The shRNA-*Xlr3* targets transcripts at exon 3 and transgenic mice**

762 **display *Xlr3* knock down. A)** Diagram of *Xlr3* gene sequence and the location of

763 shRNA-*Xlr3* targeting. Gray and black boxes represent UTRs and ORF regions,

764 respectively. **B)** *Xlr3* mRNA is knocked down ~50% at 9.5 dpp in shRNA-*Xlr3* compared

765 to age-matched wild type mice. Expression levels were assessed by qRT-PCR of whole

766 testis cDNA. Two primers were used to measure the transcript at the location of shRNA

767 targeting, exon 3 (Paired t-test, df=5, p-value=0.0001016\*\*\*), and downstream, exon 6

768 (Paired t-test, df=5, p-value=2.725e-05\*\*\*). Error bars represent mean C.I. **C)** XLR3

769 signal on the XY body is reduced in 17.5dpp spermatocytes. Protein levels were

770 assessed by immunofluorescence of surface spread spermatocytes from shRNA-*Xlr3*

771 and age-matched wild type cells (Wilcox Test: W=646, p-value=1.019e-14\*\*\*\*).

772

773 **Figure 4. *Xlr3* knock down mice are fertile but have spermatogenic defects. A)**

774 Across 50 shRNA-*Xlr3* sired litters, the number of pups produced per litter is not

775 significantly (n.s.) different from that of wild type counterparts (Wilcox Test, W=135, p-

776 value=0.7778) and **B)** there is no significant difference in the time to litter (T-test,

777 t=0.2017, df=52.434, p-value=0.8409). **C)** Upon further examination of a cohort of 14-

778 week old males, significantly reduced sperm count (Paired t-test, df=6.1897, p-

779 value=0.0005\*\*\*) was observed, as well as **D)** significantly reduced testis weight as

780 percent of body weight (Paired t-test,  $df=6.8775$ ,  $p\text{-value}=0.03966^*$ ). **E,F**) H&E staining  
781 revealed that shRNA-*Xlr3* testes (**F**) have several disorganized seminiferous tubules,  
782 indicated by arrows, that are not present in any wild type section (**E**). **G**) The tubules of  
783 these mice are also significantly smaller in diameter than those of their wild type  
784 counterparts (T-test,  $df=9694$ ,  $p\text{-value}=6.889e-06^{****}$ ). **H-J**) These phenotypes may be  
785 due to (**H**) an increased number of apoptotic cells per tubule (T-test,  $df=3$ ,  $p\text{-}$   
786  $value=0.006456^{**}$ ), which was observed through TUNEL stained wild type (**I**) and  
787 shRNA-*Xlr3* (**J**) testes. Apoptotic cells are stained dark brown. Error bars represent  
788 mean  $\pm$  C.I. Scale bars = 20  $\mu\text{m}$ .

789

790 **Figure 5. *Xlr3* knock down alters offspring sex ratios and increases XY non-**  
791 **disjunction. A)** shRNA-*Xlr3* males produce a significantly increased percentage of  
792 female offspring than expected (400 offspring, binomial distribution test,  $p=0.5$ ,  $p\text{-}$   
793  $value=0.0003238$ ) compared to the expected 50:50 sex ratio. **B)** To identify XY  
794 nondisjunction, shRNA-*Xlr3*/shRNA-*Xlr3*•C57 males were mated to wild type C3H  
795 females to use polymorphism genotyping. We observed the production of  $\sim 2\%$  hybrid  
796 39,X offspring (box). **C)** ND events (X monosomy) were identified by PCR and *MseI*  
797 restriction digest. Ladder (MW) is shown to the left and X-specific allele is indicated to  
798 the right.

799

800 **Figure 6. *Xlr3* knock down disrupts MSCI. A)** Map (ideogram of mm10) of X-linked  
801 targets. **B)** To detect transcript abundance of the X-linked targets and *Zfy2* during



802 pachytene (P) when the XY body should be silent, transcript levels from 9.5 dpp  
803 (leptotene, N=3) and 14.5 dpp (pachytene, N=3) testes were measured by qRT-PCR  
804 and normalized to those of age-matched wild type mice. All targets were significantly  
805 upregulated by the knock down of *Xlr3* (One-sided T-test, *Zfy2*:  $t=-8.3929$ ,  $df=2.4548$ ,  $p$ -  
806  $value=0.003624$ , *Rhox13*,  $t=-2.8488$ ,  $df=2.1466$ ,  $p$ -value=0.04812, *Hprt*,  $t=-4.2463$ ,  
807  $df=2.0958$ ,  $p$ -value=0.02358, *Fmr1*,  $t=-40.279$ ,  $df=2.9412$ ,  $p$ -value=1.984e-05, *Rps6ka6*:  
808  $t=-13.992$ ,  $df=2.8365$ ,  $p$ -value=0.0005269, *Tcp11x2*:  $t=-5.8356$ ,  $df=2.5986$ ,  $p$ -  
809  $value=0.007383$ ). **C)** RNA FISH of 17.5dpp spermatocytes using *Fmr1* probe (yellow),  
810  $\gamma$ H2Ax as an XY body marker (magenta) and DAPI as a counterstain (grey). No overlap  
811 of *Fmr1* and  $\gamma$ H2Ax was observed in the wild type cells (top), while signal overlap was  
812 observed in the shRNA-*Xlr3* transgenic cells (bottom). Far right panel is zoom of boxed  
813 inset shown in merge images. Scale bars = 6  $\mu$ m.

814

815 **Figure 7. XY body MSCI DDR factors and chromatin modifications are diminished**  
816 **in shRNA-*Xlr3* pachytene spermatocytes.** Surface spread images were subjected to  
817 ICC with an antibody against SYCP3 (magenta). **A-E)** DDR factor signal intensity  
818 (yellow) in shRNA-*Xlr3* spermatocytes is equal to that observed in wild type cells (left)  
819 and is diminished in some knockdown cells (middle). Overall, the average shRNA-*Xlr3*  
820 population DDR signal corrected total fluorescence (CTF) were significantly decreased  
821 (right). **A-C)** SUMO1 signal across the XY body loops (Wilcox Test:  $W = 188$ ,  $p$ -value =  
822 0.000497\*\*\*). **D-F)** H3K9me3 signal across the XY body loops (T test:  $t = 4.6677$ ,  $df =$   
823 62.88,  $p$ -value = 1.644e-5\*\*\*), **G-I)**  $\gamma$ H2Ax signal across the XY body loops (Wilcox Test:

824 W = 169, p-value = 5.298e-10\*\*\*\*), **J-L**) ATR signal across the XY body axes and loops  
825 (Wilcox Test: W = 384, p-value = 5.713e-07\*\*\*\*), **M-O**) BRCA1 signal across the XY  
826 body axes (Wilcox Test: W = 1575, p-value = 3.995e-05\*\*\*\*). Images are deconvolved  
827 quick projections. Scale bars = 10  $\mu$ m.  
828

Figure 1

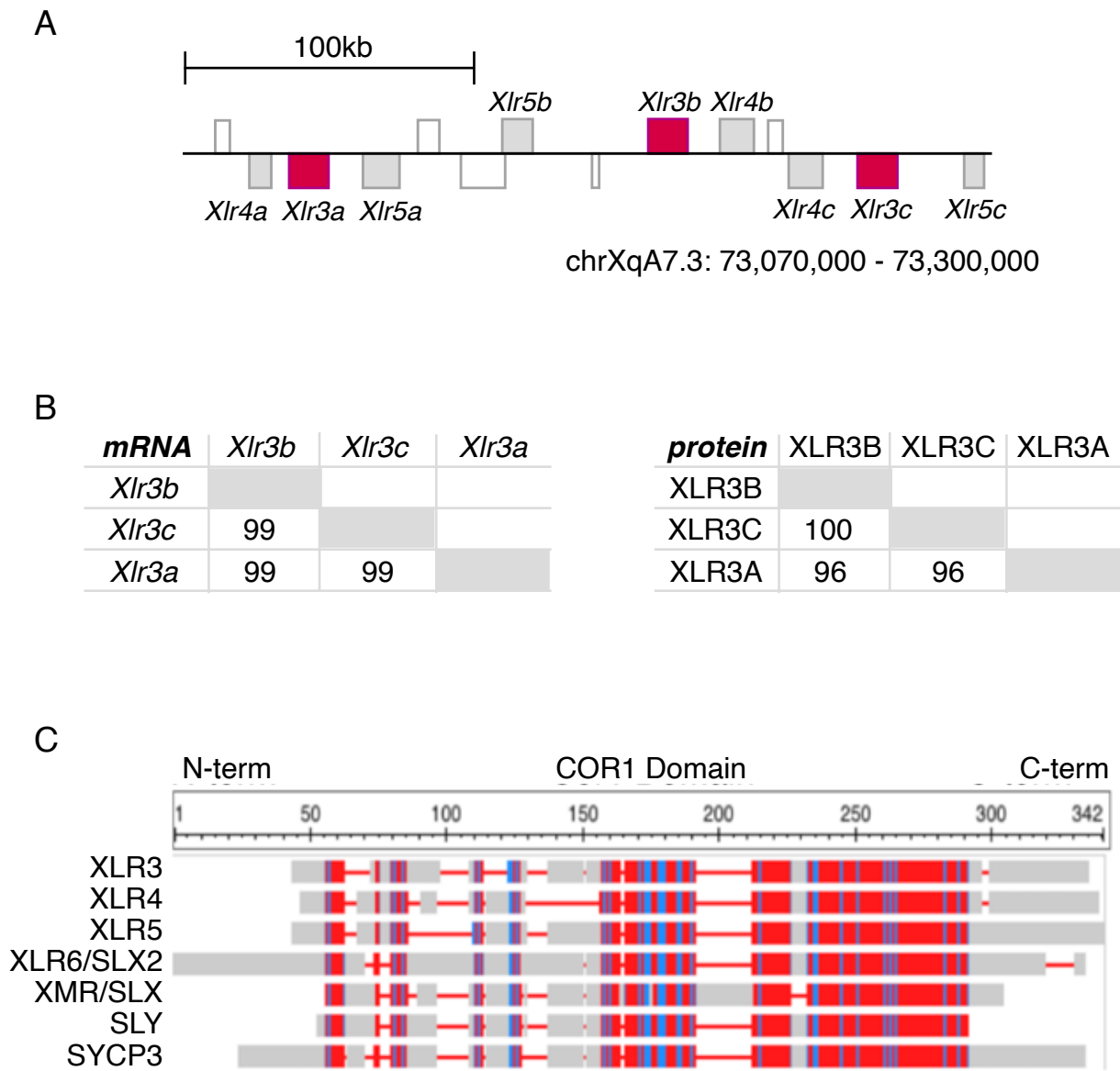
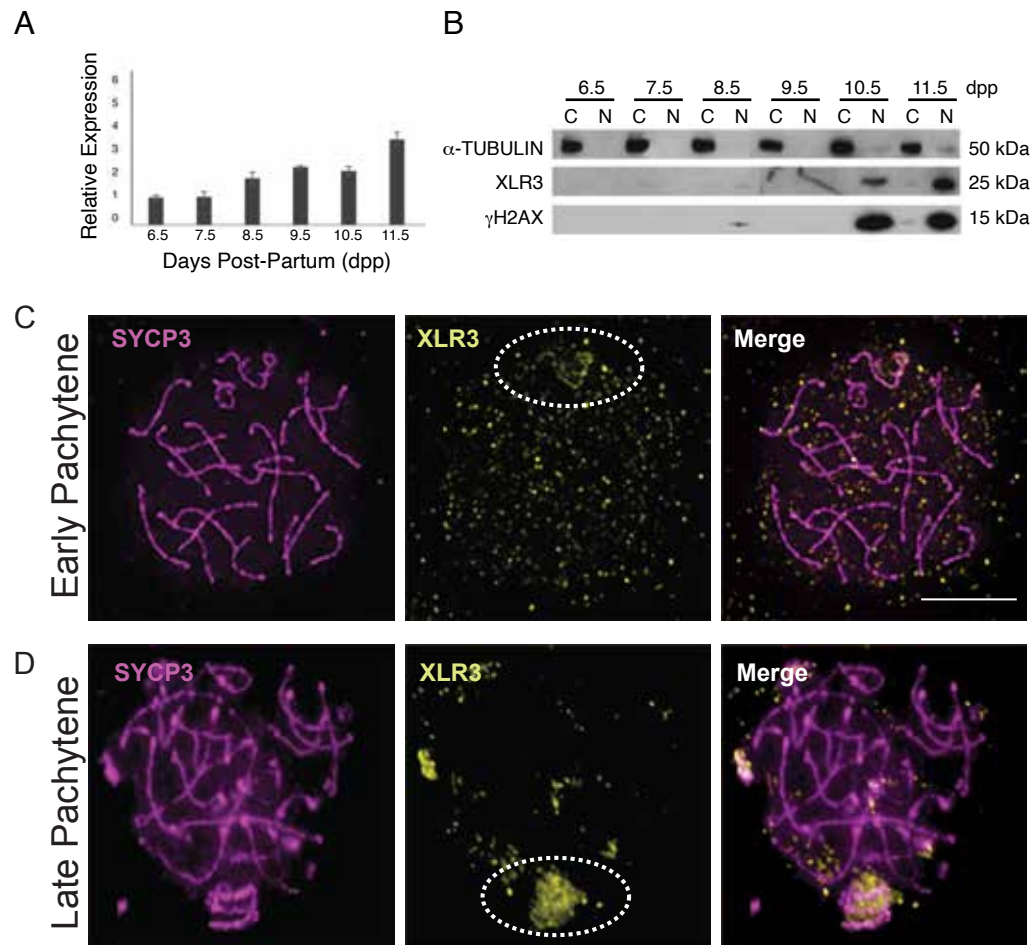
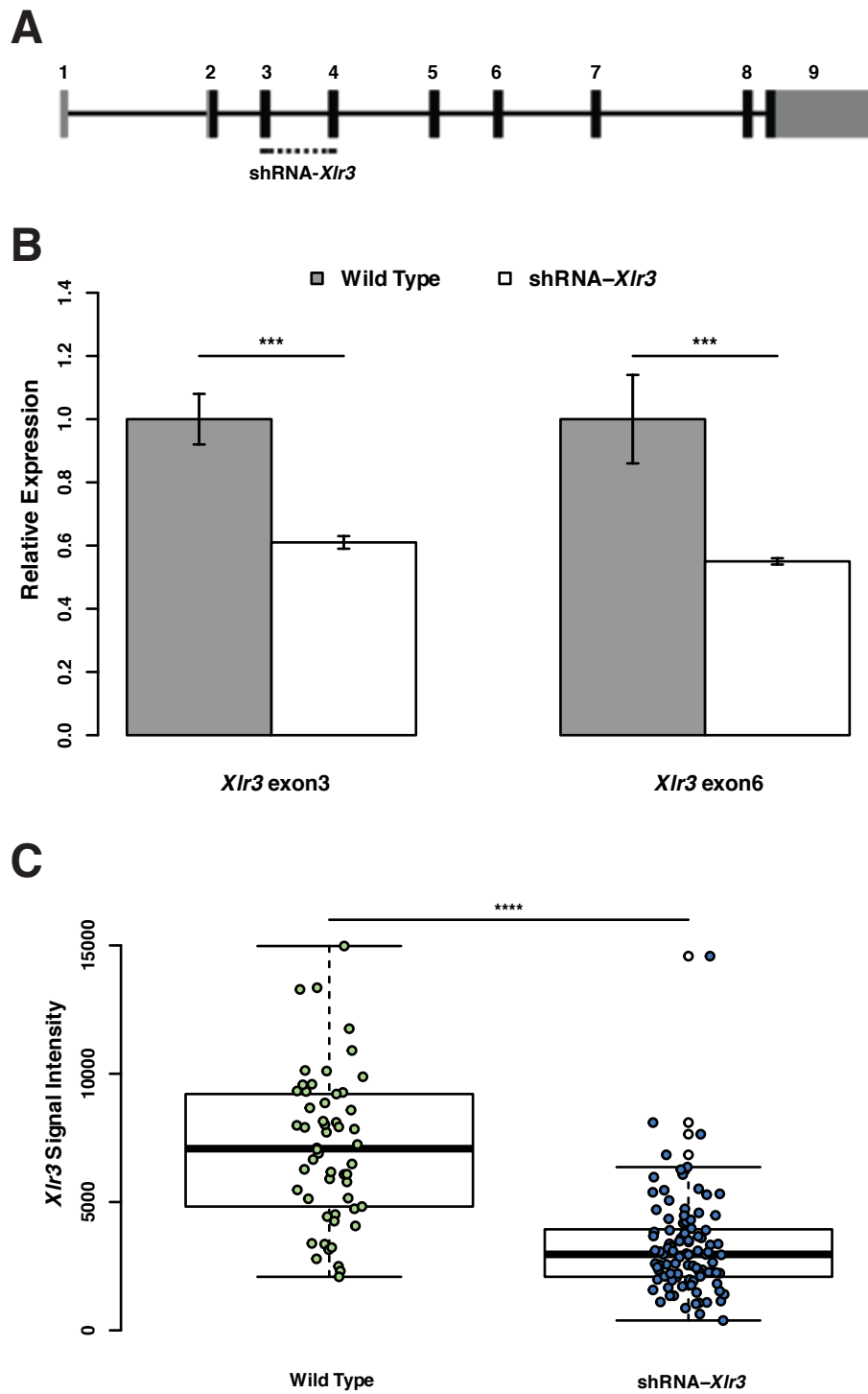


Figure 2



## Figure 3



**Figure 4**

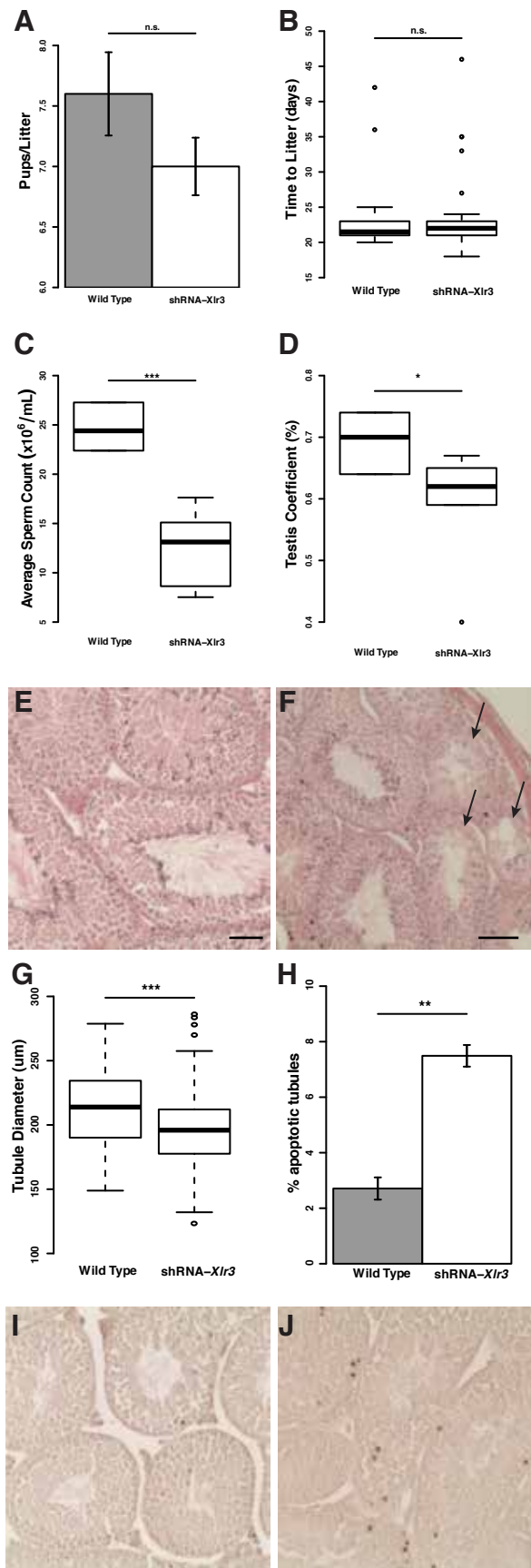
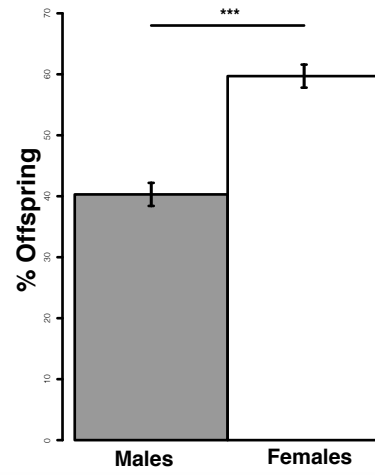
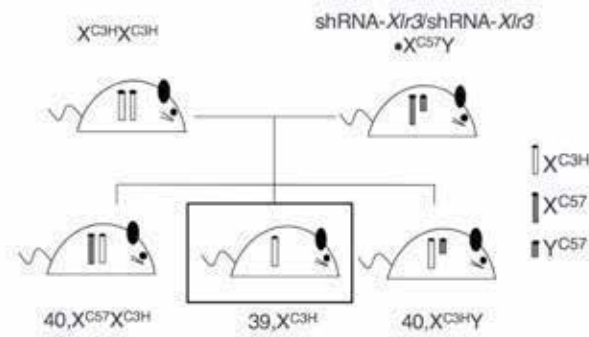


Figure 5

A



B



C

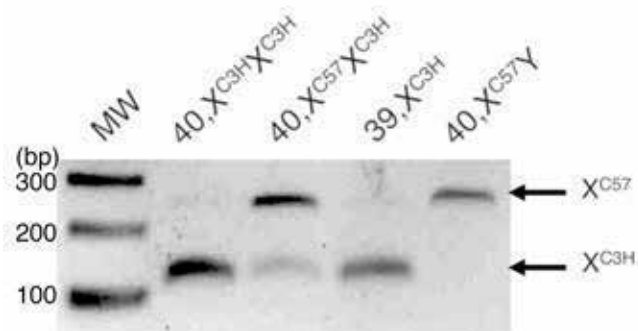
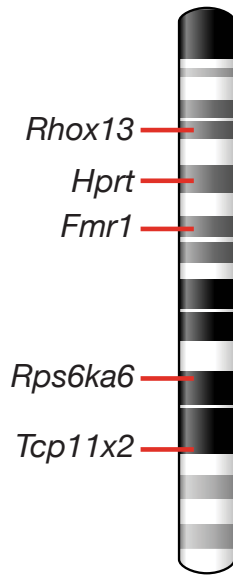
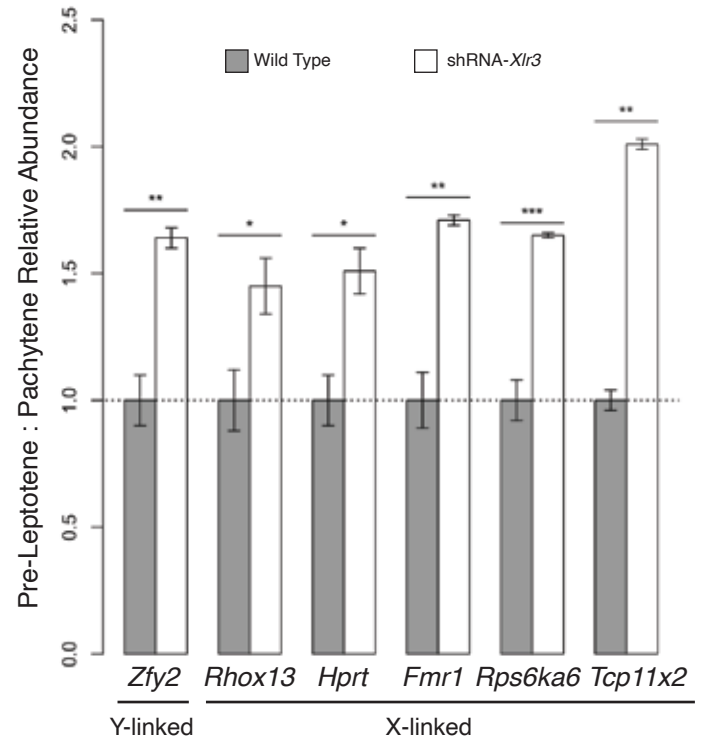


Figure 6

A



B



C

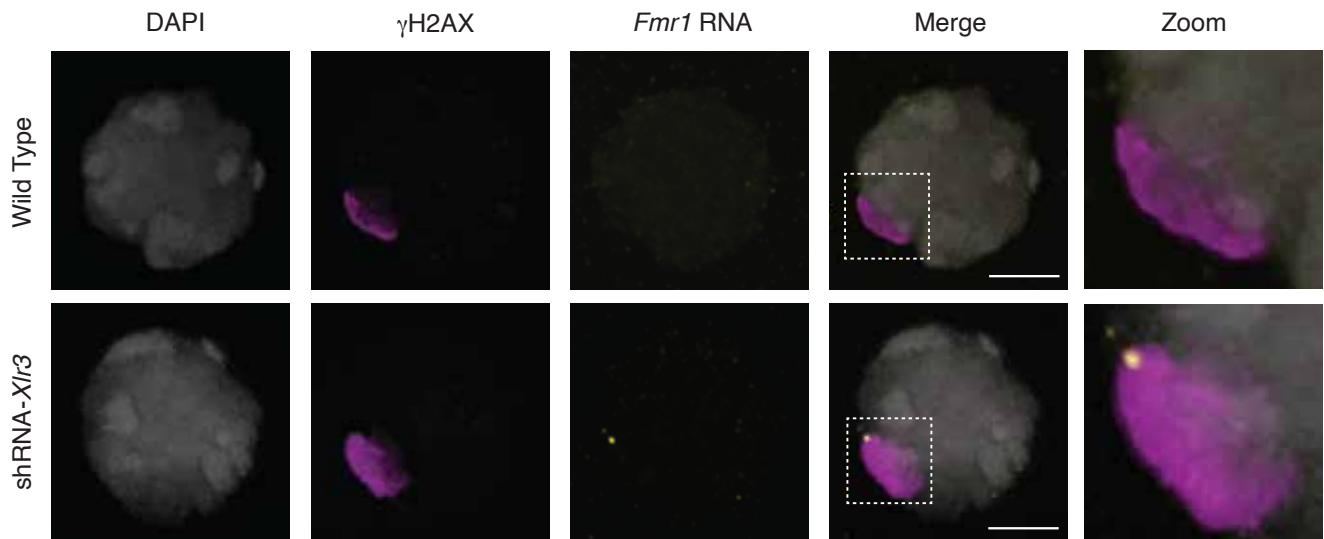
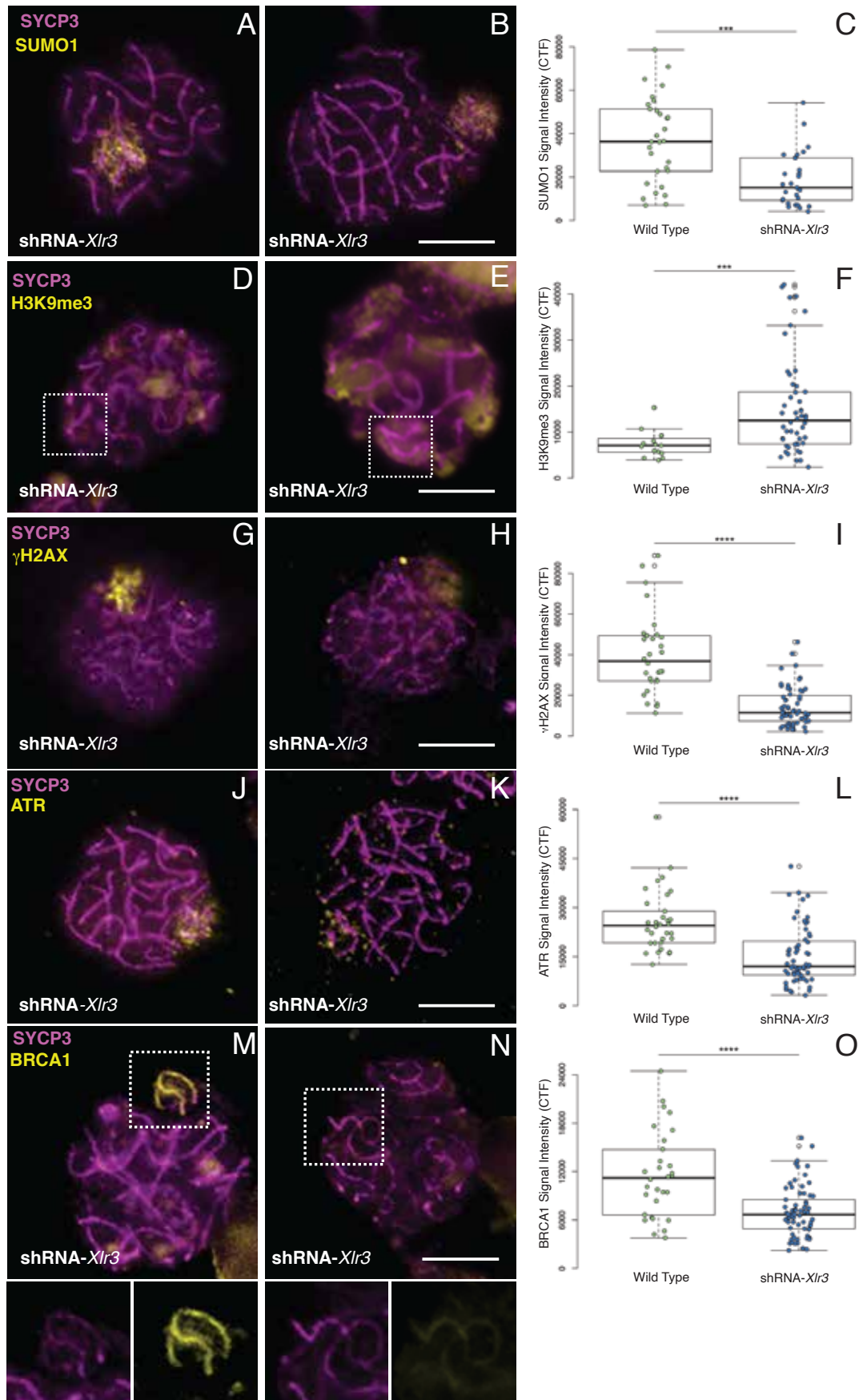




Figure 7



1 **Supplementary Figure 1. Breeding schematic of shRNA-*Xlr3* transgenic mouse.** 1)

2 The linearized vector was electroporated into cells and incorporated into the *ROSA26*  
3 locus on chromosome 6 by homologous recombination. Chimeric mice were produced by  
4 blastocyst incorporation. 2) Mice with full-length construct were crossed to mice  
5 expressing flippase to recombine the *FRT* sites surrounding the neomycin cassette. 3)  
6 Mice with shortened construct were crossed to *Ddx4-Vasa-Cre* mice expressing Cre  
7 recombinase in germ cells. The stop cassette was excised and shRNA-*Xlr3* was  
8 expressed in a tissue-specific manner. 4) Ubiquitously expressing shRNA-*Xlr3* mice were  
9 generated by breeding mice with the active construct in the germ line.

10

11 **Supplementary Figure 2. Off-target effects as a result of shRNA-*Xlr3* activation**

12 **were not observed. A)** Alignment and conservation of *Xlr3/4/5* mRNA sequences at  
13 the site of shRNA targeting generated by CLC Sequence Viewer 7 (Qiagen). **B)** *Xlr4* (T-  
14 test:  $t=-0.0217$ ,  $df=11.953$ ,  $p\text{-value}=0.983$ ), **C)** *Xlr5* (T-test:  $t=-2.7428$ ,  $df=10.204$ ,  $p$ -  
15  $value=0.7893$ ), **D)** *Oas1b* (T-test:  $t=-0.68427$ ,  $df=11$ ,  $p\text{-value}=0.508$ ), **E)** and *Sox9* (T-  
16 test:  $t=0.44358$ ,  $df=9.5631$ ,  $p\text{-value}=0.6672$ ) transcription levels as measured by qRT-  
17 PCR are not significantly (n.s) different between wild type and shRNA-*Xlr3* testis total  
18 cDNA. Error bars represent mean C.I.

19

## Figure S1

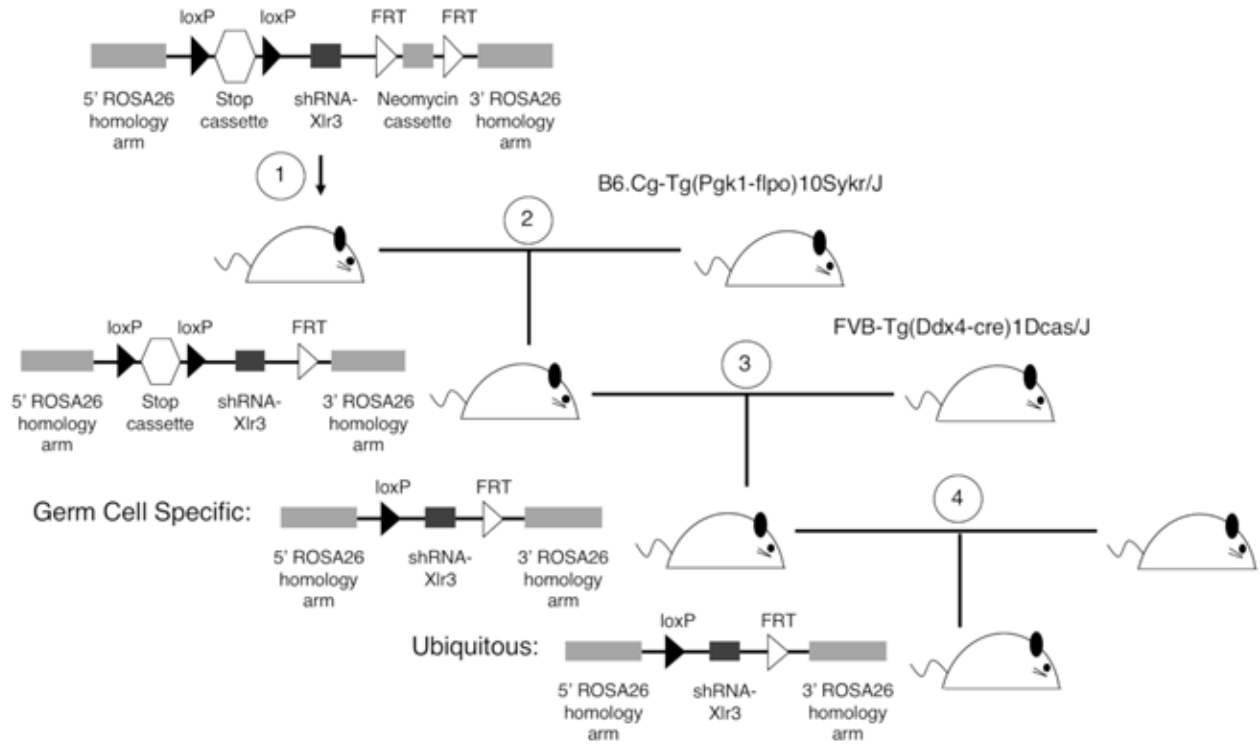
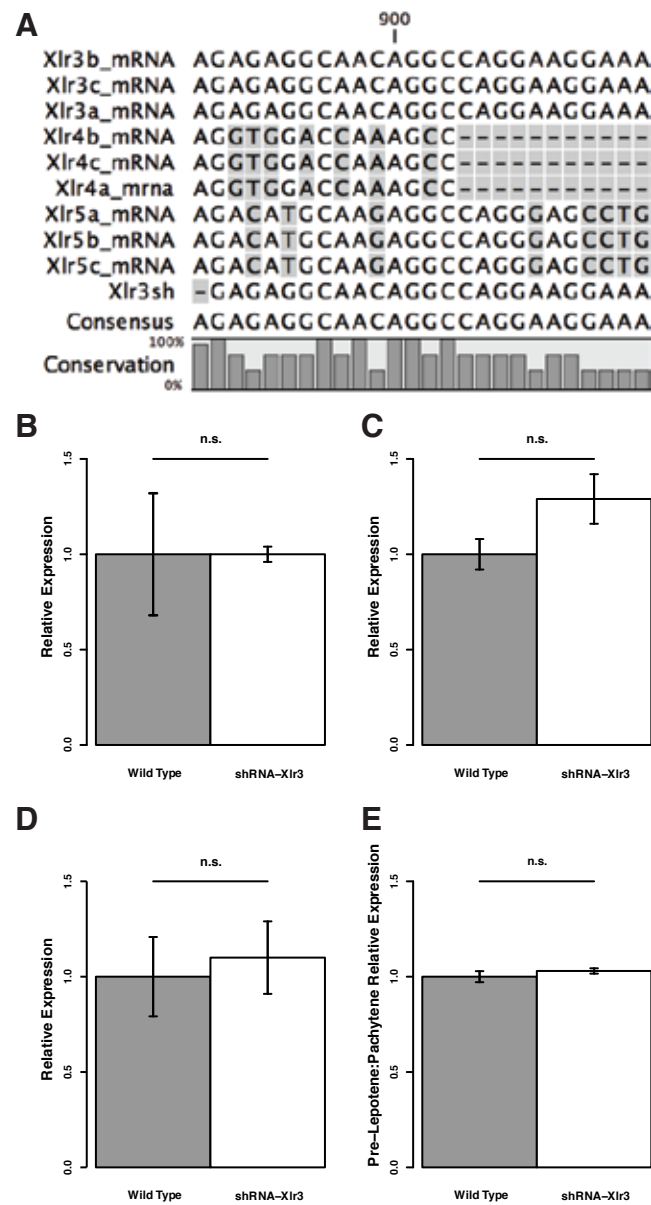


Figure S2



**Supplementary Table 1.** Primers used in this study

Target	Forward	Sequence 5'-3'	Reverse	Sequence 5'-3'	Tm
5' shRNA-Xlr3	rosa05	CCGCCTAAAGAAGAGGCTGTGCTTTGG	Xlr3sh_s_5	CCCCCTGAACCTGAAACATA	60
3' shRNA-Xlr3	neof	AGGATCTCCTGTCATCTCACCTTGCTCCT	Rosa11	GGGCAATCTGGGAAGGTTCCCTTAAGAA	60
shRNA-Xlr3	Xlr3sh_F	CCTCTTCCCCTCGTGATCTG	Xlr3sh_R4	ACTTCCCGACAAAACCGAAA	58
Internal control <sup>1</sup>	oIMR7338	CTAGGCCACAGAATTGAAAGATCT	oIMR7339	GTAGGTGGAAATTCTAGCATCATCC	58
Flpo <sup>1</sup>	oIMR1130	ATAGCAGCTTTGCTCCTTCG	10671	TGGCTCATCACCTTCCTCTT	58
Ddx4-Cre <sup>1</sup>	oIMR7643	CACGTGCAGCCGTTTAAGCCGCGT	oIMR7644	TTCCCATTCTAAACAACACCCTGAA	58
Actin B	ActB_F	ACACCCGCCACCAGTTCG	ActB_R	CGATGGAGGGGAATACAGCC	58
Gapdh	Gapdh_mus_F2	ACTCCACTCACGGCAAATTC	Gapdh_mus_R2	GTGGTTCACACCCATCACAA	58
Oas1b	Oas1b_F2	GCCCAACAAGCTCTTCCTAAA	Oas1b_R2	CTCAAACGTCACCTCCCCT	58
Xlr3 exon 3	Xlr3abcde_F	AAAGGAAGGCCACTGACAC	Xlr3abcde_R	TGTTGCCTCTCTGTTCCCTGA	58
Xlr3 exon 6	Xlr3ex6_F	ACTTCGGATGCATACAAACTCA	Xlr3ex6_R	AGTACCTCCAGTTTCTCCAAGT	58
Xlr4	Xlr4bc_F	GACTCCTGCTCTGCCATCTAAT	Xlr4bc_R	CTTCGCTCATGCTGGACTTT	58
Xlr5	Xlr5_F	AGCAGAATTCAAGGCAGGAG	Xlr5_R2	AGCTTGGTTTCATGGTCCTC	58
Zfy2 <sup>2</sup>	Zfy2_F	CTTAATTCAGACATTTTAACTTCCA	Zfy2_R	ATCACTTGTTCAAATGTCCTACATT	58
Rhox13 <sup>3</sup>	Rhox13_F	GCTCATCCAGGTCCTCACTT	Rhox13_R	TCCTCCTTGCCTCCACAAT	58
Hprt <sup>4</sup>	Hprt_F	GTTAAGCAGTACAGCCCCAAA	Hprt_R	AGGGCATATCCAACAACAAACT	58
Fmr1 <sup>5</sup>	Fmr1_F	GGAAAAGCCAGACAGCGTAG	Fmr1_R	CCTGTGCCATCTTGCCTACT	58
Fmr1 <sup>6</sup>	Fmr1probe_F1	CTGTCAGCAGGCAGCTTTTACATCCTGT	Fmr1probe_R1	CTTGTGCGTGGACAGCATTTTGAGAGTA	65
Fmr1 <sup>6</sup>	Fmr1probe_F2	ATGCCACCAAGTTCCTACCTTCCAATA	Fmr1probe_R2	GTGACAAATATCTCCTCCAACCCCAACA	65
Rsp6ka6 <sup>5</sup>	Rsp6ka6_F	AAGAACGCAGCAACGGTTAT	Rsp6ka6_R	AAACTGGCTCTCCCTCTTCC	58
Tcp11x2 <sup>5</sup>	1700008I05 Rik-F	AAAGCCAATTCGTGGAGACAAT	1700008I05 Rik-R	TGGGAGAGATGCAGAATATCCA	58
SMCY	SMCY_F	TGAACTGCCTGCTATGCTAC	SMCY_R	GCCTCAGATTCCAATGCTG	60
DXMit130	MIT130-1	TTCATATCGCCCCAACCTAC	MIT130_R	TATTTTGAAACCTCTGCCATTT	56

<sup>1</sup> from Jax<sup>2</sup> from Cocquet et al 2009<sup>3</sup> from Modzelewski et al 2012<sup>4</sup> from Date et al 2012<sup>5</sup> from Campbell et al 2013<sup>6</sup> from Muller et al 2008

**Supplementary Table 2. Antibodies used in this study**

Antibody	Concentration	Supplier	Catalog #/RRID
Rabbit polyclonal anti-Xlr3	ICC 1:20 Western 1:2,000	Custom generated by Biosynthesis Inc to peptide MSSRKRKATSTAGRHSRM	N/A
Mouse monoclonal anti-Sycp3	ICC 1:200	Santa Cruz	sc-74569 RRID:AB_2197353
Rabbit polyclonal anti-Sycp3	ICC 1:100	Thermo Fisher	PA1-16764 RRID:AB_568727
Mouse monoclonal anti-gH2aX (ser139)	ICC 1:200	Thermo Fisher	14-9865-82 RRID:AB_2573048
Rabbit polyclonal anti-Sumo1	ICC 1:200	Thermo Fisher	PA5-17352 RRID:AB_10977036
Rabbit polyclonal anti-H3K9me3	ICC 1:180	Thermo Fisher	49-1008 RRID:AB_2533859
Rabbit polyclonal anti- $\alpha$ -tubulin	Western 1:40,000	Abcam	Ab15246 RRID:AB_301787
anti-rabbit IgG HRP	Western 1:2,000	Abcam	Ab6721 RRID:AB_955447
anti-mouse IgG HRP	Western 1:100	Abcam	Ab6728 RRID:AB_955440
Goat anti-Mouse Alex Fluor® 568	ICC 1:200	Invitrogen	A11004 RRID:AB_2534072
Goat anti-Rabbit Alex Fluor® 488	ICC 1:200 RNA FISH 1:200	Invitrogen	A27034 RRID:AB_2536097
Anti-DIG Fluorescein	RNA FISH 1:200	Roche	11207741910 RRID:AB_514498
Rabbit anti FITC	RNA FISH 1:200	Invitrogen	71-1900 RRID:AB_2533978