Loss Of Chromodomain of Male-Specific Lethal 3 (MSL3) Does Not Affect Spermatogenesis in Rodents

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Abstract:

Male-specific lethal (MSL3) is a member of the Dosage Compensation Complex in *Drosophila*. It is required for transcriptional upregulation of genes on the X chromosome in males to equal that of females. Even though the dosage complex is carried out differently in mammals, Msl3 is conserved in humans. Intriguingly, Msl3 is expressed in the undifferentiated cells from Drosophila to humans, including in spermatogonia of macaque and humans. During *Drosophila* oogenesis, Msl3 is required for meiotic entry. However, its role in meiotic entry in other organisms has not been explored. Using mouse spermatogenesis as a model system, we probed for the role of Msl3 in the meiotic entry. We found that MSL3 is expressed in mouse testes in meiotic cells in contrast to flies, primates, and humans. Further, using a newly generated MSL3 conditional knock-out mouse line, we found no spermatogenesis defects within the seminiferous tubules of the KOs.MSL3 mutants were also viable and fertile, suggesting that MSL3 is dispensable for rodent gametogenesis.

32 **Introduction:**

33 Gametes ensure the survival and propagation of all sexually reproducing organisms. Gametes
34 arise from germ cells that differentiate and undergo meiosis to produce haploid oocytes and
35 sperms. Meiosis is essential for reductional cell division to generate haploid gametes and genetic
36 diversity by promoting recombination. Thus, regulation of meiosis is critical for gametogenesis
37 and fertility. However, the regulation of meiotic entry remains incompletely understood.

38 Mammalian spermatogenesis is an excellent model system to determine regulators of meiotic
39 entry due to the presence of spermatogonial stem cells (SSCs) that self-renew and differentiate,
40 undergoing meiosis throughout the animal's life [1,2]. This is in contrast to oogenesis, where germ
41 cells directly differentiate into oocytes during embryonic development that then meiotically arrest
42 [3]. Spermatogenesis in mouse initiates with SSC, are also called Single spermatogonia (A_s),
43 which give rise to A paired spermatogonia (A_{paired}) and A aligned spermatogonia ($A_{aligned}$), which
44 make chains of 4, 8 and 16 Type A spermatogonia which eventually gives rise to type B
45 differentiated spermatogonia through mitotic divisions. Type B spermatogonia enter meiosis
46 prophase I when they initiate recombination. These type B spermatogonia then progress to a
47 second round of meiotic divisions to yield round spermatids. Round spermatids then go through
48 spermiogenesis, in which morphological changes occur, including the trading of histones for
49 protamines, to get elongated spermatids and eventually mature spermatozoa [4-6].

50 In mammals, the entry into meiosis is promoted by steroid signaling during oogenesis and
51 spermatogenesis. Retinoic acid (RA) from somatic cells surrounding the spermatogonia activates
52 the transcription factor *Stimulated by retinoic acid 8* (*Stra8*) in the germline. During
53 spermatogenesis, Stra8 fosters meiotic entry by promoting the transcription of a broad gene
54 expression program. However, *Stra8* is not sufficient to induce meiosis, suggesting a cell type-
55 specific chromatin landscape and/or factors that cooperate with STRA8 [7].

56 Male-specific lethal 3 (MSL3) is a member of the Male-specific lethal (MSL) complex which is
57 necessary for dosage compensation in *Drosophila*. The MSL complex promotes transcriptional
58 upregulation of genes on the X chromosome in males to equal the two copies from the two X
59 chromosomes in females. This transcriptional upregulation is essential to the survival of male
60 *Drosophila*. The complex is made up of several components, including MSL1, MSL2, MSL3, male-
61 absent on first (MOF), maleless (MLE), and two roX noncoding RNAs. MSL3's chromodomain
62 has been shown to be important for binding to X-linked genes by recognizing Histone 3 lysine 36
63 trimethylation marks (H3K36me3). Conversely, the MRG domain is important for interaction with

the Dosage Compensation Complex [8-10]. Thus, Msl3 and its Chromodomain are critical for Dosage compensation.

It was thought that some MSL complex components were not expressed in the females, thus precluding the formation of the MSL complex. However, Msl3 is expressed in a temporal manner during *Drosophila* oogenesis from the undifferentiated germline stem cells (GSC) to the differentiating cysts stages when the germ cell enters meiosis. It was found to be part of a transcriptional axis that promotes GSC differentiation and meiotic entry by recognizing H3K36me3 marks, presumably via its chromodomain. Intriguing, it has been reported that Msl3 is expressed in undifferentiated spermatogonia of primates and humans, suggesting it could have a potential function in the germline in mammals. In addition, large-scale analyses of the X chromosome in infertile men discovered Msl3 to be one of the genes associated with spermatogenic failure. Taken together, this suggested that Msl3 may be playing a role in the meiotic entry in mammals [11-13].

Here, using published single-cell (sc) RNA-sequencing data [14] we found that MSL3 is expressed in the mouse testes but specifically in meiotic cells. This contrasts with flies, primates, and humans, where MSL3 is expressed in the undifferentiated cells before meiotic entry [11-13]. By generating a new MSL3 conditional knock-out consisting of loxP sites flanking the chromodomain of the MSL3 gene that is critical for its function. We found that conditional loss of MSL3 during mouse spermatogenesis is not sufficient to perturb spermatogenesis and fertility. In addition, MSL3 mutants were also viable and fertile, suggesting that MSL3 is dispensable for gametogenesis in general. Thus, unlike in Drosophila, Msl3 is dispensable for meiotic entry in rodents.

Materials & Methods

Animals

Mice used in this study were as follows: MSL3 floxed, Stra8-iCre and R26tdTomato. All mice described were maintained on a C57BL/6J background. The MSL3 floxed mouse line was designed using CRISPR/Cas9 technology by Cyagen. The MSL3 gene consists of 13 exons. Exons 2-4 of the chromodomain of MSL3 were targeted using CRISPR/Cas9. Lox*P* inserts flank exons 2-4, which upon Cre recombinase activity results in their removal from the gene and disruption of the chromodomain. The targeting vector was engineered with homology arms and the conditional knock-out region generated by BAC clone RP24-103L17 from the C57BL/6N

library as a template. Cas9 mRNA was injected into fertilized eggs along with MSL3 gRNA and the targeting vector. Founder mice were identified by PCR and bred to C57BL/6N to test germline transmission. The Stra8-iCre (stock #017490) and R26tdTomato (stock #007914) mouse lines are commercially available from JAX [15,16]. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee (IUACUC) at the University at Albany, SUNY. Genotyping of the mouse lines was conducted by PCR using tail genomic DNA. **iCre Primers:** *Fwd*-AGA TGC CAG GAC ATC AGG AAC CTG, *Rev*-ATC AGC CAC ACC AGA CAC AGA GAT, *Fwd control*-CTA GGC CAC AGA ATT GAA AGA TCT, *Rev control*-GTA GGT GGA AAT TCT AGC ATC ATC C. **MSL3^{flx} Primers:** *Fwd*-GAA TGC ATG GCT ATT AAG GGG AGG, *Rev*-GAA GTC ATT GGT CGG TAG ATT TGT C. **MSL3 KO Primers:** *MSL3 WT Fwd*-GCAGTAAGCAGCACCTGTCAT, *MSL3 WT Rev*-AGGAAAACTTGCAACATTGGTCT, *MSL3 KO Fwd*-TCCCGTCCAGAGGCTCTCAT, *MSL3 KO Rev*-GGAAAACTTGCAACATTGGTCTGA. **R26tdTomato Primers:** *IMR0920*-AAGGGAGCTGCAGTGGAGTA, *IMR09021*-CCGAAAATCTGTGGGAAGTC, *IMR9103*-GGCATTAAAGCAGCGTATCC, *IMR9105*-CTGTTCCTGTACGGCATGG.

Surface spreading and Immunofluorescence

Testes were excised from male mice euthanized at P17-20. Excised testes were placed in ice-cold PBS. Testes were then weighed to determine incubation time in hypotonic extraction buffer (HEB). **30 mg or less for 30 min, tubules from a pair of testes weighing 31 to 50 mg for 30 - 45 minutes, and larger testes for 60 minute**s. Testes were then placed back in ice cold PBS to remove the tunica albuginea and extract the seminiferous tubules. Seminiferous tubules were placed in HEB consisting of **30 mM Tris pH 8.2, 50 2 mM Sucrose, 17 mM Trisodium Citrate Dihydrate, 5 mM EDTA** and incubated according to their weight. Seminiferous tubules were then transferred back into a petri dish and separated into small clumps. Individual clumps were placed in 60 ul droplets of 100 mM sucrose pH 8.2 and pipetted repeatedly until the solution became turbid. Droplets of seminiferous tubules were placed in the corner of uncharged slides previously soaked in a fixative solution of 1% PFA and 0.15% triton X-100 pH 9.2 with a droplet of fixative remaining on the slide [17,18]. The droplet was allowed to spread along the slide then left to dry overnight in a humidified chamber at 4°C. Slides were then stored at -80°C until use.

For immunofluorescence analysis, the spreading nuclei were immunostained with mouse anti-SYCP3 (1:500), rabbit anti-SYCP1 (1:500) and mouse anti-γH2AX (1:500). Using SYCP3 to label axial/lateral element characteristics and DAPI-stained heterochromatin pattern, spermatocytes in spreading were classified as leptotene, zygotene, pachytene and diplotene.

128 **Histological and immunohistochemical analysis**

129 Testes were fixed in 4% formaldehyde embedded in OCT and sectioned, then stored at -80°C
130 until use. For immunofluorescence analysis, the following primary antibodies were used: Rabbit
131 anti-RFP (1:500 Rockland), Rabbit anti-Stra8 (1:250 ABCAM), mouse anti-SCP3 (1:500 ABCAM).

132 Testes weight and Seminiferous Tubule Diameter Measurements

133 Average weights were calculated by weighing testes (n=3 for each genotype) immediately after
134 excision and averaged for each genotype. Average seminiferous tubule diameter measurements
135 were calculated by measuring 10 seminiferous tubules per animal per genotype and calculating
136 the average diameter of the 10 seminiferous tubules. This was repeated for a total of 3 animals
137 per genotype. The average of the 3 averages was taken for each genotype as the representative
138 weight.

139 **RNA-sequencing**

140 For whole RNA sequencing, testes were collected, and RNA extraction was performed using the
141 Thermo Fisher RNA PureLink Mini Kit. Sequencing was performed using Illumina Nextseq 500.

142 For Single-cell RNA sequencing, we utilized previously published data by Green at al. [14]
143 available through NCBI's Gene Expression Omnibus, GEO series accession number
144 GSE112393 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE112393). Files were
145 imported into the R package *Seurat 4.0.5* [19] for filtering. After filtering, mitochondrial genes
146 and RNA features, individual files were merged into one file representing all germ cell types.
147 Dimensionality reduction was performed using principal component analysis (PCA).

148 **Gene Ontology Term Analysis**

149 Gene Ontology term analysis was performed using the Panther Classification System.

150

151 **Results**

152 **MSL3 is expressed in mouse testes during meiotic stages via ScSeq**

153 To determine when MSL3 is expressed in mouse testes, we turned to single-cell RNA-sequencing
154 (scRNA-seq) analysis. Using previously published drop-seq data by Green et al. [14], we
155 reproduced a PCA clustering of testes germ cells. Markers used to identify spermatogonia were
156 as follows: Zbtb16, Uchl1, Stra8, and Kit [20-23]. Markers used to identify spermatocytes were as

157 follows: Sycp3, Tex101, Spag6 and H2afx [24-27]. Markers used to identify round/elongating
158 spermatids were as follows: Acrv1, Tssk1, Tnp1, Prm1 [28-31] (Fig. 2A,B). As continuous
159 transitions occur between cell states during spermatogenesis, they are represented by the
160 changes in expression patterns of marker genes. (Fig. 1B). After identifying germ cell populations,
161 we probed for MSL3 expression. We found that MSL3 is expressed in spermatocytes and
162 continues through round spermatids (fig.1C). MSL3 expression correlates with spermatocyte
163 markers Tbpl1 and Spag6 confirming its expression in this cell population [32] (fig.1D). Thus, Msl3
164 is expressed in meiotic stages during mouse spermatogenesis.

165 **iCre successfully recombines in Stra8+ cells**

166 To test iCre recombination efficacy in the testes, we mated Stra8iCre female mice with R26tdTom
167 males and looked for tdTomato expression in Stra8-positive cells. We collected the testes of
168 R26tdTomato/Stra8iCre males at P30. Testes were fixed, sectioned, and immunostained for
169 tdTomato and Stra8. Immunostaining showed colocalization between Stra8+ spermatogonia and
170 tdTomato+ cells in Stra8iCre/R26tdtomato mice but not R26tdTomato controls (fig. 2). This means
171 tdTomato/Rosa26 is only being expressed in Stra8iCre spermatogonia and that iCre successfully
172 recombines in Stra8+ cells. By counting the number of Stra8+ and tdTomato+ cells, we conclude
173 that Stra8iCre has a 99.4% efficacy in the testes.

174

175 **MSL3 mutant mice show normal testes morphology**

176 To determine if MSL3 has a role in mouse spermatogenesis, we generated a conditional Knock
177 out of MSL3 by targeting exons 2-4 of the Msl3 chromodomain with *loxP* upstream of exon 2 and
178 downstream of exon 4 (Fig.3). Under the Stra8 promoter, iCre expression results in excision of
179 exons 2-4 of Msl3 resulting in a nonfunctional chromodomain.
180 We first examined whether MSL3cKO (Stra8iCre$^{+/-}$; MSL3$^{flox/flox}$) mice displayed any gross
181 testicular morphological defects. Testes from MSL3 cKO and control littermates were removed at
182 P45, when males are sexually mature, and all cell types should be present in the testes. By
183 dissecting the testes, we found no significant difference in size, shape, or weight of the testes
184 between controls (0.056 g +/- 0.003) and mutants (0.059 g +/- 0.002) (Fig. 4A,B).
185 Microscopic examination of testes sections showed no morphological defects between mutants
186 and controls. Moreover, we found no significant difference between the size of seminiferous
187 tubules as indicated by diameter measurements between controls (114.6um +/-4.6) and mutants
188 (115.0um +/-6.7) (Fig. 4C). Both mutants and controls had rounded seminiferous tubules with

189 similar cellular organization and all cell types present from primary spermatogonia to elongated
190 spermatids based on the morphology of cells present within the seminiferous tubules.
191

192 **Formation of synaptonemal complex in the absence of functional MSL3**

193 scRNA-Seq analysis showed that MSL3 is expressed in primary spermatocytes (Fig.1), and
194 previous work suggested that MSL3 is necessary for transcriptional upregulation of synaptonemal
195 complex proteins [10]. To determine if Msl3 is required for meiosis, we examined the meiotic
196 prophase-I using marker for synapsis that is promoted by synaptonemal complex proteins. We
197 prepared Meiotic chromosome spreads from both control and MSL3 cKO testes from P17-21 male
198 mutants. Using an antibody against SYCP3, one of the lateral elements of the synaptonemal
199 complex, we characterized primary spermatocytes as in leptonema, zygonema, pachynema or
200 diplonema by way of immunofluorescent staining. Chromosome spreads from both controls and
201 MSL3 cKO showed spermatocytes at all stages of prophase I, suggesting that both genotypes
202 can complete prophase-I. Counts of 100 spermatocytes in both mutants and controls also showed
203 that there were no significant differences between the distribution of spermatocytes between
204 genotypes. (Fig. 5). This was determined by the change in localization of SYCP3 from a more
205 diffuse/widespread expression in leptonema to a more condensed expression with strong puncta
206 at the centromeric region in diplonema. Taken together this suggested that MSL3 is not required
207 for the formation of the synaptonemal complex.

208

209 **MSL3 mutants are fertile and produce normal litter sizes**

210 Histological analysis of MSL3 cKO seminiferous tubules showed the presence of all cell types,
211 from primary spermatogonia to elongated spermatids. The presence of elongated spermatids
212 means cells can initiate spermiogenesis or the differentiation of spermatids leading to mature
213 spermatozoa. MSL3 cKO males were thus mated with WT females to examine their fertility along
214 with littermate controls. Each MSL3 cKO male was paired with 2 C57B/6J females and allowed
215 to mate for 2 weeks. MSL3 cKO males successfully impregnated both females and produced
216 litters of comparable sizes to that of controls. MSL3 cKO offspring were also viable (Table 1).
217 Thus, we conclude that MSL3 is not necessary for spermatogenesis.

218

219

220 **Loss of Msl3 shows no gross disruptions in the transcriptome**

221 As MSL3 mutants show no defects in the spermatogenic process, we asked if upregulation of
222 other genes may compensate for a potential function of MSL3. To do this, we performed whole
223 RNA-sequencing on MSL3 cKO mutants and wildtype controls (n=3 for each genotype). We
224 performed differential gene expression (DGE) analysis on the sequenced testes to check for any
225 differentially expressed genes between MSL3 cKOs and controls.

226 Read counts for MSL3 between genotypes were visualized using the Interactive Genomics
227 Viewer. We found few reads that mapped for exons 2-4 in our MSL3 cKOs (Fig. 6A) consistent
228 with the deletion of the chromo domain. Notably few read counts were observed in our cKOs,
229 these may reflect MSL3 expression in Sertoli cells or in some spermatogonial cells that escaped
230 recombination.

231 Differential expression analysis between control and mutant samples reveled the upregulation of
232 seven genes and downregulation of 44 genes in MSL3 cKOs (Fig. 6B) (significant genes were
233 determined by $\log_2$ fold change >0.5 and <-0.5 and a p-adj. value <0.05). Of the 7 upregulated
234 genes, 3 of them with the highest Log_2 fold change (0.5 or greater) (Cyp2c55, A1CF, Pyroxd2)
235 have enzymatic and RNA metabolic functions; however, there is not known function for these
236 genes in the testes or spermatogenesis specifically. Of the 44 significantly down-regulated genes,
237 there were 18 with a Log_2 fold change of -0.5 or lower. GO term analysis on genes with a Log_2
238 fold change of -0.5 or lower and 0.5 or greater did not reveal convergence on any commonly
239 known pathway. Thus, based on our analysis, the absence of functional chromodomain in MSL3
240 does not alter the meiotic program during mouse spermatogenesis.

241

242 **Discussion**

243 Our efforts to uncover a functional role of Msl3 in murine spermatogenesis revealed it's
244 dispensability in this context. Notably, there are expression pattern differences between rodents
245 and primates and humans. In humans, Msl3 was shown to be a marker of spermatogonial stem
246 cells (SSC), particularly State 0 SSCs, in fetal-infant testes [12]. Similarly, in macaque monkeys,
247 Msl3 is expressed in Spermatogonia state 1 (SPG1) [13]. Our study suggests that, in mice, Msl3
248 expression is mostly restricted to the spermatocyte stage and only in a small portion of
249 spermatogonia (Figure 1C). In this study, we ablated Msl3 downstream of Stra8, which marks the

differentiation phase of spermatogonia; we cannot exclude that Msl3 may be potentially critical prior to the differentiation of spermatogonia.

While MSL3 is important for transcriptionally upregulating synaptonemal complex components during *Drosophila* oogenesis, it does not appear to have any effect on synaptonemal complex formation during mouse spermatogenesis. Using MSL3 conditional knock-out mouse model, we observed no noticeable defects or interruptions in spermatogenesis. The MSL3 gene consists of a chromodomain and an MRG domain. Our conditional knockout has exons 2-4 of the chromodomain removed upon Cre recombinase activity. It is possible that the two domains serve redundant functions resulting in the lack of any observable phenotype in our mouse models. The MRG gene family, consisting of MRG15 and MRGX, is involved in cellular proliferation and cycle progression [33]. In *Drosophila*, the MRG domain mediates MSL3's incorporation into the Dosage Compensation Complex [34]. The chromatin-binding protein MORF-related gene on chromosome 15 (MRG15) is involved in pre-mRNA splicing at the round spermatid stage [35]. The diverse involvement of MRG genes leaves open this possibility of a role of the MRG domain in MSL3 function. It may be responsible for chromatin binding and HAT activity in transcriptionally active genes. However, our current mouse model does not allow us to explore this idea. To further confirm that MSL3is a dispensable gene for rodent spermatogenesis and rule out a redundant function between the two domains, it would be beneficial to design a new MSL3 mouse model with a functionally inactive chromodomain and MRG domains

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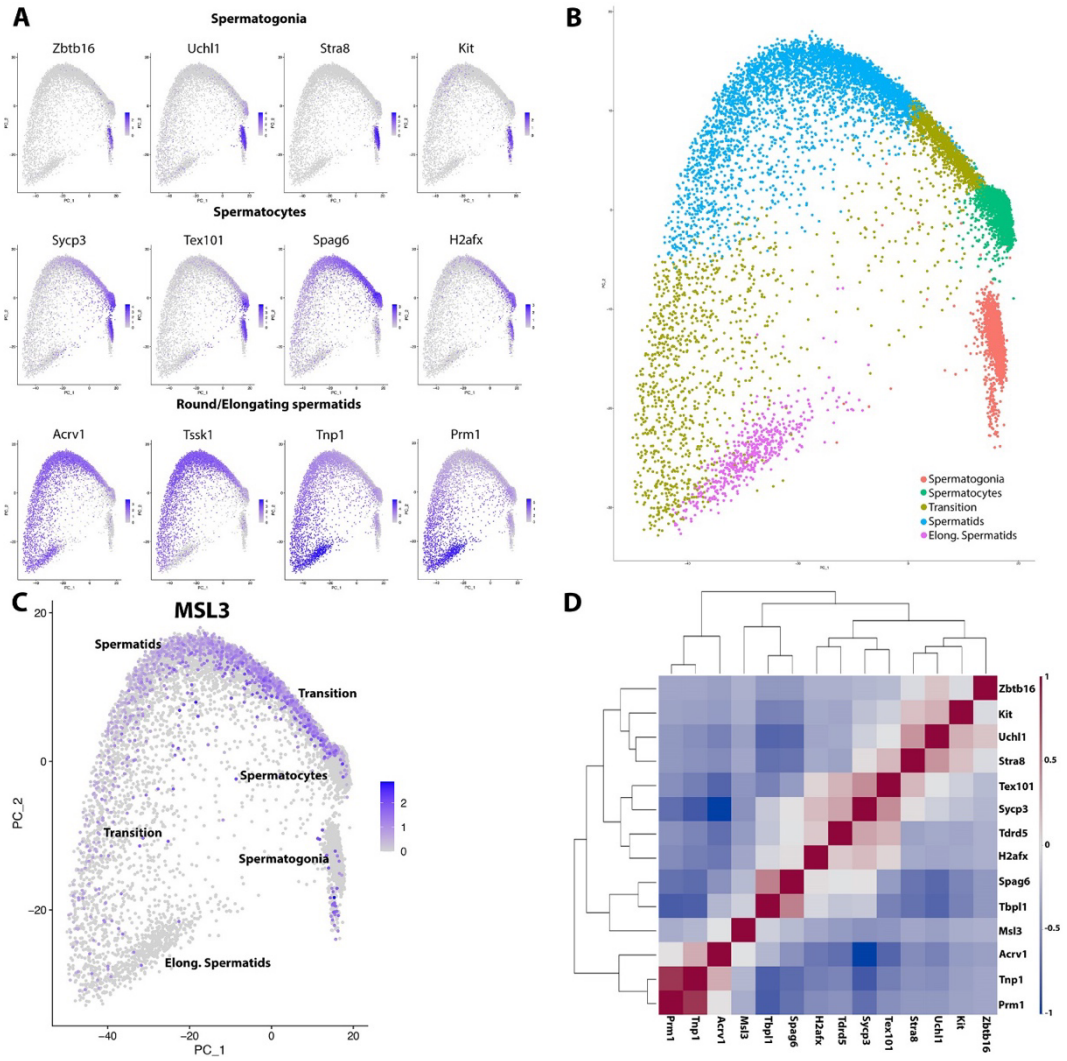


Figure 1. Single-cell RNA sequencing of mouse testes. A) PCA plot of different germ cell population markers for spermatogonia, spermatocytes and round/elongating spermatids to identify germ cell type of each cluster. B) Clusters grouped into spermatogonia, transition states, spermatocytes and spermatids based on the marker genes they expressed to establish trajectory of spermatogenesis. C) MSL3 is expressed in spermatocytes and continues into spermatids then ceases as spermatids transition into elongating spermatids. D) Correlation map of different marker genes showing that MSL3 expression most closely correlates with that of Tbpl1 and Spag6 which are markers of spermatocytes.

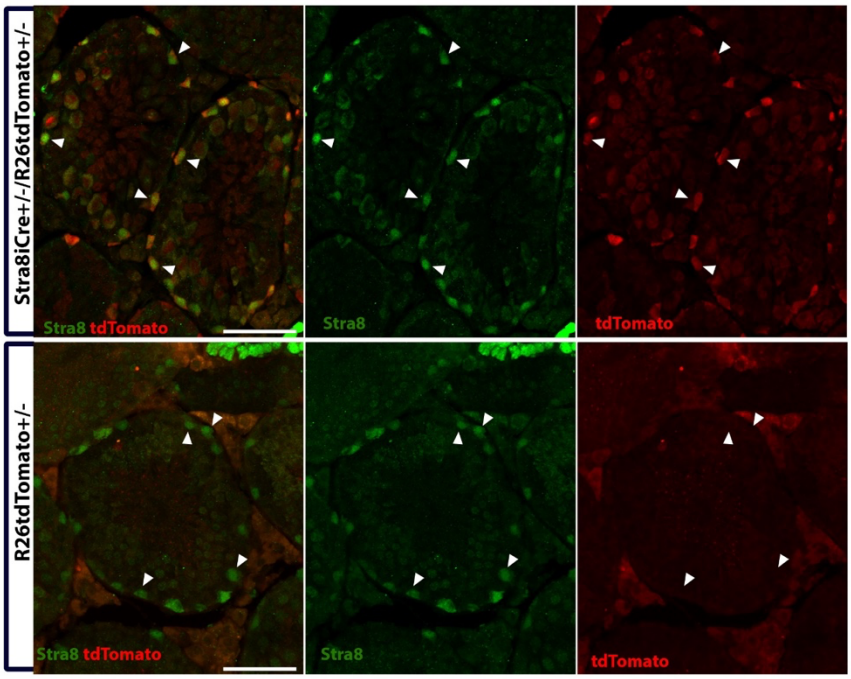


Figure 2. Testes Immunofluorescence staining for Stra8 and tdTomato. Stra8 in green, tdTomato in red. Immunofluorescence staining shows that in Stra8iCre+/-R26tdTomato+/- mutants, stra8 and tdTomato staining colocalize, while in tdTomato+/- controls, Stra8 positive cells are negative for tdTomato. This confirms that tdTomato is only expressed in Stra8iCre cells. Scale bar = 50μm.

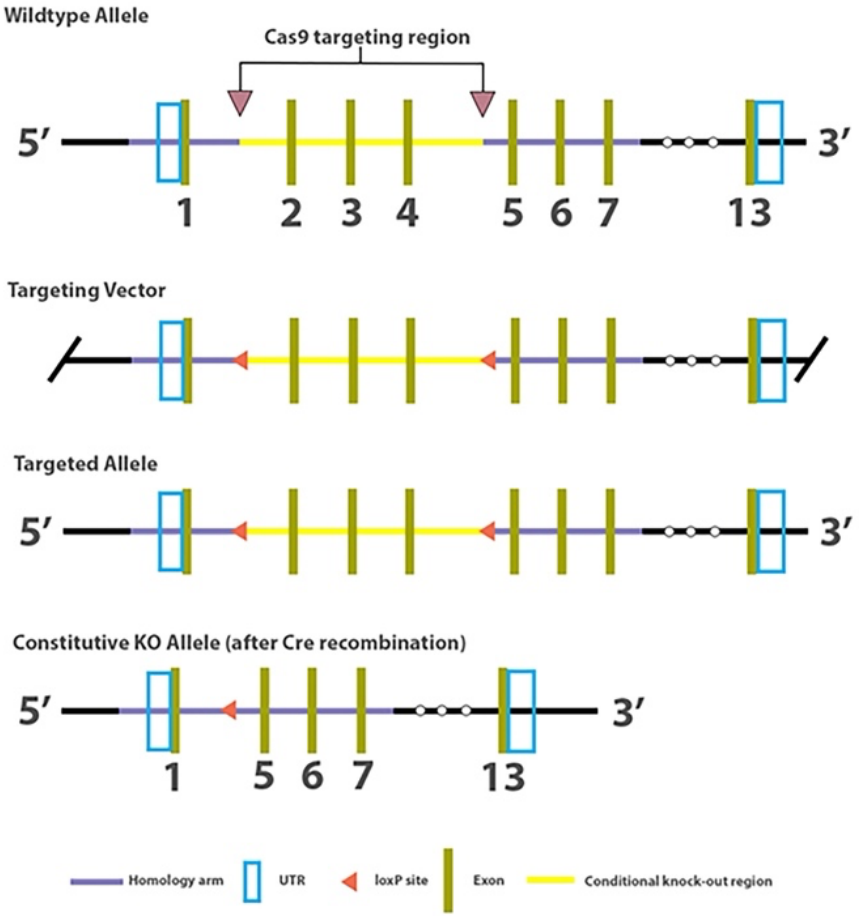
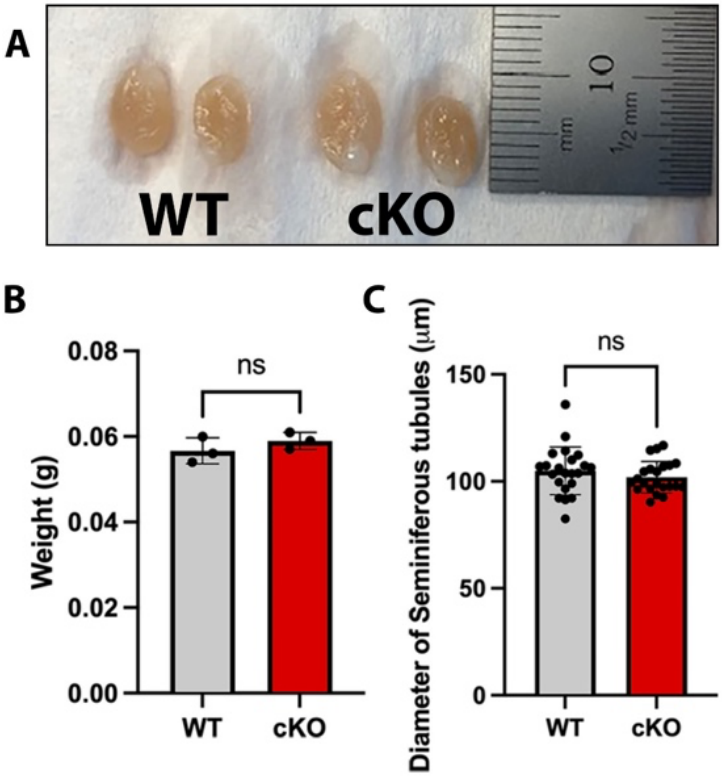
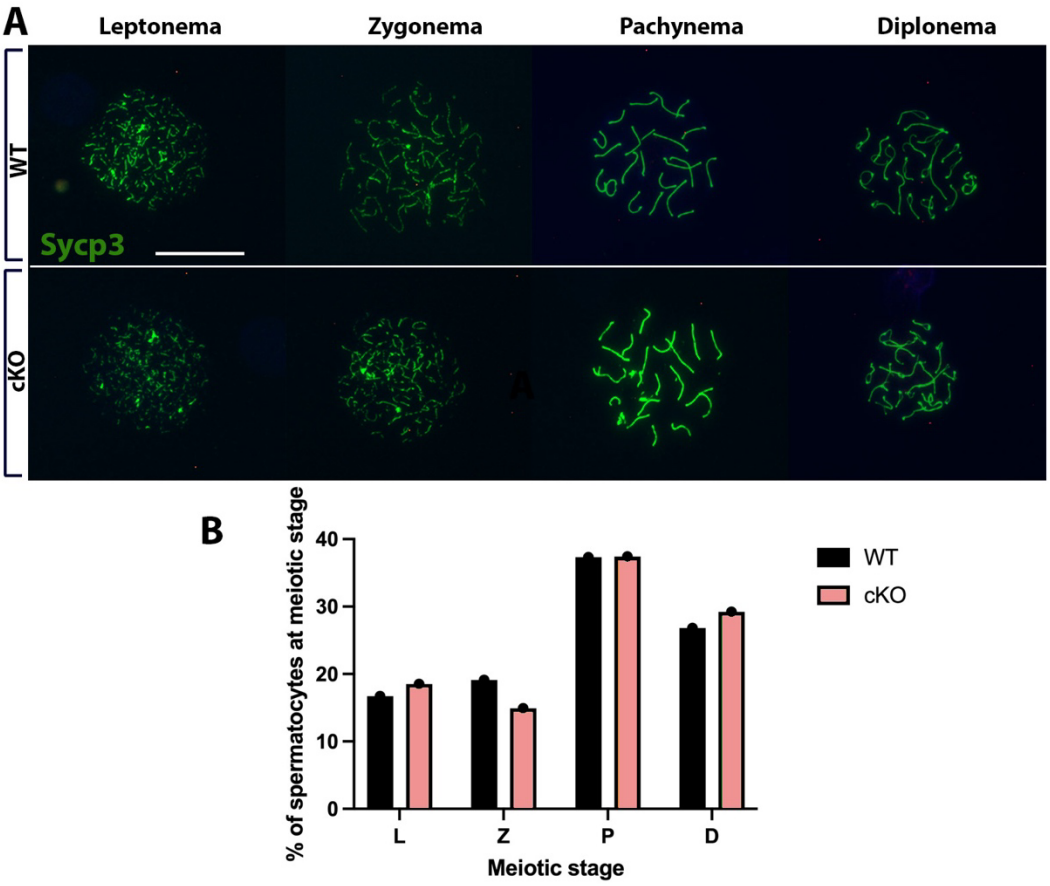


Figure 3. Design strategy adopted or MSL3 conditional knock-out mouse line generation. Crispr/Cas9 was used to insert loxP sites upstream of exon 2 and downstream of exon 4 of the MSL3 chromodomain. Upon Cre recombinase activity, exons 2-4 are excised from the MSL3 gene generating a constitutive knockout allele.



A.) Figure 4. Image of MSL3 wildtype (WT) (left) and MSL3 cKO (right) testes at P45. Image shows that they are of similar size and shape. B.) Bar graph showing the weight of pairs of testes for MSL3 WT and MSL3 cKO mice. C.) Bar graph showing the average diameter of the seminiferous tubules for MSL3 WT and MSL3 cKO mice. Average diameter of 10 seminiferous tubules for each genotype is represented by each dot. n=3 for each genotype



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461 **Figure 5. A.) Meiotic chromosome spread of spermatocytes** from P17-21 MSL3 WT and cKO testes
462 stained with antibody against SYCP3. Meiosis prophase I was examined between mutants and controls.
463 Spermatocytes were characterized as in leptonema, zygonema, pachynema or diplonema. Scale
464 bar=25um B.) 209 spermatocytes were counted for WT controls and 195 for Msl3 cKO. Subphases
465 of meiosis prophase I show no significant differences between MSL3 WT and MSL3 cKO.

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472 **Table 1**

Genotype	# of Breeders	# of Litters	Avg. litter size
MSL3 WT	**3**	**6**	**8.5 ± 1.05**
MSL3 cKO	**3**	**6**	**8.17 ± 1**

473

474 **Table 1**.) MSL3 cKO males and littermate controls were mated with 2 C57B/6 WT females each. Males
475 were given 2 weeks to impregnate females and removed at the conclusion of the 2 week period. All males
476 successfully impregnated respective female. The average litter size of MSL3 cKO males (8.17± 1) was not
477 significantly different from that of littermate MSL3 WT controls (8.5± 1.05) (p=0.5826).

478

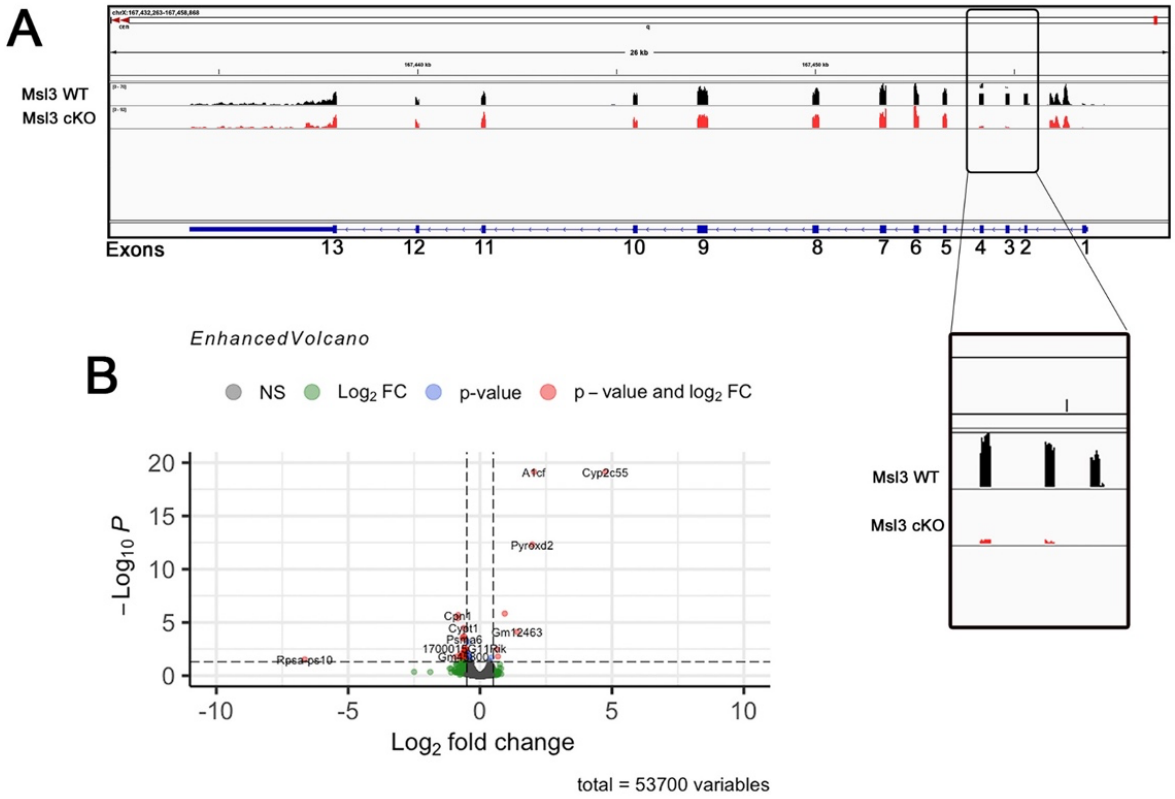

Figure 6 A) Read counts between MSL3 WT and MSL3 cKO show little to no reads for exons 2-4, validating their excision from the gene in our cKO model. B.) Volcano plot showing significantly upregulated and downregulated genes in our MSL3 cKO model.