

# 1 Erosion of somatic tissue identity with loss of the X-linked

## 2 intellectual disability factor KDM5C

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## 5 Abstract

It is currently unclear why mutations in numerous chromatin-modifying enzymes cause neurodevelopmental disorders (NDDs). While some chromatin regulators are important for neuron-specific gene expression, loss of repressive chromatin regulators can also lead to the aberrant transcription of tissue-specific genes outside of their intended context. Because very few studies have characterized the these aberrant, tissue-specific transcripts, the mechanisms behind their dysregulation and their functional consequences are largely unknown. Here, we explored this cellular identity crisis in NDDs by investigating the ectopic expression of germline (e.g. sperm and egg) genes in mice lacking lysine demethylase 5c (KDM5C, also known as JARID1C or SMCX), an eraser of histone 3 lysine 4 di and tri-methylation (H3K4me2/3). We found male *Kdm5c* knockout (-KO) mice, which recapitulate key behavioral phenotypes of Claes-Jensen X-linked intellectual disability (MRXSCJ, OMIM: 300534), aberrantly expresses many liver, muscle, ovary, and testis genes within the amygdala and hippocampus. Many genes expressed in the *Kdm5c*-KO brain were typically unique to germ cells, indicating an erosion of the soma-germline boundary. Using germ cell-depleted RNA sequencing datasets, we curated a a list of germline-enriched genes for unbiased characterization of KDM5C in germline gene repression. Germline genes are typically decommissioned in somatic lineages in the post-implantation epiblast, yet *Kdm5c*-KO epiblast-like cells (EpiLCs) aberrantly expressed key regulators of germline identity and meiosis, including *Dazl* and *Stra8*. Germline gene dysregualtion is sexually dimorphic, as female *Kdm5c*-KO EpiLCs were more prone to germline gene dysregulation than knockout males. We found KDM5C acts at a subset of germline gene promoters in EpiLCs to promote the initial placement of DNA methylation at CpG islands. However, germline genes can also become activated in *Kdm5c*-KO cells independent of direct KDM5C regulation. Intriguingly, many of the germline transcripts expressed in the *Kdm5c*-KO brain were late-stage spermatogenesis genes that are not directly bound by KDM5C. This suggests germline developmental programs can progress ectopically in the background of typical *Kdm5c*-KO development. Ultimately, this work characterizes a novel suppressor of germline gene transcription and links impaired soma-vs-germline demarcation to a chromatin-linked neurodevelopmental disorder.

## 30 Introduction

31 A single genome holds the instructions to generate the myriad of cell types found within the adult  
32 organism. This is, in part, accomplished by chromatin regulators that can either promote or impede lineage-  
33 specific gene expression through DNA and histone modifications<sup>1,2</sup>. Many chromatin regulators were initially  
34 identified for their roles in shaping cellular and tissue identity<sup>3–5</sup>. Recent advancements in next generation  
35 sequencing unexpectedly revealed mutations in many chromatin regulators cause neurodevelopmental  
36 disorders (NDDs)<sup>6</sup>. Several studies have suggested this connection between chromatin regulators and  
37 neurodevelopment is due to their regulation of brain-specific genes, such as orchestrating transcriptional  
38 programs for synaptic maturation<sup>7</sup> and transitioning between neuronal and glial fates during neural precursor  
39 differentiation<sup>8</sup>. However, loss of some chromatin regulators can also result in the ectopic transcription  
40 of tissue-specific genes outside of their target environment, such as the misexpression of liver-specific  
41 genes within adult neurons<sup>9</sup>. Very few studies have investigated this cellular identity crisis in chromatin-  
42 linked NDDs<sup>9,10</sup> and it is currently unknown if ectopic gene expression contributes to neurodevelopmental  
43 impairments.

44 To elucidate the role of tissue identity in chromatin-linked neurodevelopmental disorders, it is essential  
45 to first characterize the nature of genes dysregulated and the molecular mechanisms governing their de-  
46 repression. In this study, we characterized the aberrant expression of tissue-enriched genes with loss of  
47 lysine demethylase 5C (KDM5C). KDM5C, , also known as SMCX or JARID1C, is a chromatin regulator that  
48 can repress gene expression through erasure of histone 3 lysine 4 di- and trimethylation (H3K4me2/3)<sup>11</sup>,  
49 marks enriched at active gene promoters. Pathogenic mutations in *KDM5C* cause Intellectual Developmental  
50 Disorder, X-linked, Syndromic, Claes-Jensen Type (MRXSCJ, OMIM: 300534), whose predominant features  
51 include intellectual disability, seizures, aberrant aggression, and autistic behaviors<sup>12–14</sup>. *Kdm5c* knockout  
52 (-KO) mice recapitulate key MRXSCJ phenotypes, including hyperaggression, increased seizure propensity,  
53 and learning impairments<sup>10,15</sup>. Unexpectedly, RNA sequencing (RNA-seq) of the *Kdm5c*-KO hippocampus  
54 revealed ectopic expression of testis genes within the brain<sup>10</sup>. It is currently unknown what types of testis  
55 genes are dysregulated, when in *Kdm5c*-KO development this begins, and if other tissue-specific genes are  
56 also aberrantly expressed with KDM5C loss.

57 The testis contains both germ cells (cells that pass on their genetic material, e.g. sperm) and somatic  
58 cells (cells that perform all other bodily functions). Distinguishing between the germline and the soma is a key  
59 feature of multicellularity<sup>16</sup> and that typically occurs during early embryogenesis<sup>17</sup>. In mammals, chromatin  
60 regulators play a key role in decommissioning germline genes in somatic cells during the transition from naïve  
61 to primed pluripotency. Initially, repressive histone H2A lysine 119 monoubiquitination (H2AK119ub1)<sup>18</sup>,  
62 histone 3 lysine 9 trimethylation (H3K9me3)<sup>18,19</sup>, are placed at germline gene promoters in embryonic stem  
63 cells and are then decorated with DNA CpG methylation<sup>19–21</sup> in the post-implantation embryo. How KDM5C  
64 interacts with known germline gene silencers during this time point is currently unknown. Additionally, most

65 studies have focused on select genes important for early germ cell development rather than germline genes  
66 as a whole given the lack of a curated list of germline-enriched genes. Thus, it is unclear if the mechanism of  
67 repression differs for certain classes of germline genes, e.g. meiotic genes versus spermatid differentiation  
68 genes. Systematically characterizing KDM5C's role in germline gene repression during early embryogenesis  
69 will unveil key mechanisms underlying the demarcation between soma and germline identity and while also  
70 providing molecular footholds to test the impact of ectopic germline genes on neurodevelopment.

- 71 • Add females - KDM5C is a sexually dimorphic chromatin regulator, embryonic lethality makes it difficult  
72 to compare

73 To illuminate KDM5C's role in tissue identity, here we characterized the aberrant transcription of tissue-  
74 enriched genes within the *Kdm5c*-KO brain and epiblast-like stem cells (EpiLCs), an *in vitro* model of  
75 the post-implantation embryo. We observed general dysregulation of tissue-enriched genes in both the  
76 *Kdm5c*-KO brain and EpiLCs, including testis, liver, muscle, and ovary-enriched genes. Both the *Kdm5c*-KO  
77 amygdala and hippocampus had significant enrichment of testis genes. We demonstrated testis-enriched  
78 genes genes are germline genes and not somatic testis genes by. We found *Kdm5c*-KO EpiLCs aberrantly  
79 expressed key drivers of germline identity and meiosis, including *Dazl* and *Stra8*, while the *Kdm5c*-KO  
80 brain primarily expressed germline genes important for late spermatogenesis. Additionally, KDM5C was  
81 bound to only a subset of germline genes expressed in *Kdm5c*-KO EpiLCs, indicating germline-enriched  
82 mRNAs can be aberrantly transcribed through indirect mechanisms. Finally, we found KDM5C promotes the  
83 long-term silencing of germline genes in somatic cells by aiding the placement of DNA CpG methylation in  
84 EpiLCs. Thus, we propose KDM5C plays a fundamental role in the development of tissue identity during  
85 early embryogenesis, including the establishment of the soma-germline boundary.

## 86 Results

### 87 **Tissue-enriched genes, including testis genes, are aberrantly expressed in the** 88 ***Kdm5c*-KO brain**

89 Previous RNA sequencing (RNA-seq) in the adult hippocampus revealed ectopic expression of some testis  
90 genes within the *Kdm5c* knockout (-KO) brain<sup>10</sup>. Since tissue-enriched genes have not been systematically  
91 characterized in the *Kdm5c*-KO brain, if the testis is the only tissue type misexpressed. Therefore, we  
92 systematically assessed the expression of genes enriched in 17 mouse tissues<sup>22</sup> in our published mRNA-seq  
93 datasets of the adult amygdala and hippocampus in mice with constitutive knockout of *Kdm5c*<sup>23</sup>.

94 We found a large proportion of genes that are significantly upregulated within the *Kdm5c*-KO brain  
95 (DESeq2<sup>24</sup>, log2 fold change > 0.5, q < 0.1) are enriched in non-brain tissues in wild-type mice (Amygdala:  
96 35%, Hippocampus: 24%) (Figure 1A-B). The majority of tissue-enriched differentially expressed genes

97 (tissue-enriched DEGs) were testis genes (Figure 1A-C). Even though the testis has the largest total number  
98 of tissue-biased genes compared to any other tissue (2,496 genes), testis-biased DEGs were significantly  
99 enriched for both brain regions (Amygdala p = 1.83e-05, Odds Ratio = 5.13; Hippocampus p = 4.26e-11,  
100 Odds Ratio = 4.45, Fisher's Exact Test). One example of a testis-enriched DEG is *FK506 binding protein 6*  
101 (*Fkbp6*), a known regulator of piRNA expression and meiosis in germ cells<sup>25,26</sup> (Figure 1C).

102 Interestingly, we also observed significant enrichment of ovary-biased DEGs in both the amygdala and  
103 hippocampus (Amygdala p = 0.00574, Odds Ratio = 18.7; Hippocampus p = 0.048, Odds Ratio = 5.88,  
104 Fisher's Exact) (Figure 1D). Ovary-enriched DEGs included *Zygotic arrest 1* (*Zar1*), sequesters mRNAs  
105 in oocytes for meiotic maturation and early zygote development<sup>27</sup> (Figure 1D). Given the *Kdm5c*-KO mice  
106 we analyzed are male, this demonstrates ectopic expression of tissue-enriched genes is independent  
107 of organismal sex. Although not consistent across brain regions, we also found significant enrichment  
108 of DEGs biased towards two non-gonadal tissues - the liver (Amygdala p = 0.0398, Odds Ratio = 6.58,  
109 Fisher's Exact Test) and the muscle (Hippocampus p = 0.0104, Odds Ratio = 6.95, Fisher's Exact Test). An  
110 example of a liver-biased DEG dysregulated in both the hippocampus and amygdala is *Apolipoprotein C-I*  
111 (*Apoc1*), a lipoprotein metabolism and transport gene<sup>28</sup> (Figure 1E). These aberrantly expressed mRNAs are  
112 polyadenylated and spliced into mature transcripts (Figure 1C-E). Of note, we did not observe enrichment  
113 of brain-enriched genes (Amygdala p = 1; Hippocampus p = 0.74, Fisher's Exact), despite the fact these  
114 are brain samples and the brain has the second highest total number of tissue-enriched genes (708 genes).  
115 Together, these results suggest misexpression of tissue-enriched genes within the brain is a major effect of  
116 KDM5C loss.

### 117 Germline genes are misexpressed in the *Kdm5c*-KO brain

118 The testis contains both germ cells (meiotic cells, e.g. spermatogonia) and somatic cells (non-meiotic,  
119 e.g. Leydig cells) that support hormone production and germline functions. To determine if *Kdm5c*-KO  
120 testis-enriched DEGs are somatic or germline genes, we first assessed their known functions through  
121 gene ontology analysis. We found *Kdm5c*-KO testis-enriched DEGs high enrichment of germline-relevant  
122 ontologies, including spermatid development (GO: 0007286, p.adjust = 6.2e-12) and sperm axoneme  
123 assembly (GO: 0007288, p.adjust = 2.45e-14) (Figure 2A).

124 To further validate if these testis DEGs are truly germline genes, we evaluated their expression in  
125 somatic versus germ cells within the testis. We first compared their expression in the testis without germ  
126 cells<sup>29</sup>, which was accomplished by heterozygous *W* and *Wv* mutations in the enzymatic domain of *c-Kit*  
127 (*Kit<sup>W/Wv</sup>*)<sup>30</sup>. Almost all *Kdm5c*-KO testis-enriched DEGs lost expression with germ cell depletion (Figure  
128 2B). We then assessed testis-enriched DEG expression in a published single cell RNA-seq dataset that  
129 identified cell type-specific markers within the testis<sup>31</sup>. We found some testis-enriched DEGs were classified  
130 as specific markers for different germ cell types (e.g. spermatogonia, spermatocytes, round spermatids, and  
131 elongating spermatids), yet none marked somatic cells (Figure 2C). Together, these data demonstrate that

132 the *Kdm5c*-KO brain aberrantly expresses germline genes, but not somatic testicular genes, reflecting an  
133 erosion between somatic versus germline identity.

134 We then wanted to characterize germline gene misexpression with *Kdm5c* loss more deeply, but lacked a  
135 comprehensive list of mouse germline-enriched genes. We thus generated a list of germline-enriched genes  
136 using RNA-seq datasets of *Kit<sup>W/Wv</sup>* mice that include males and females at embryonic day 12, 14, and 16<sup>32</sup>  
137 and adult male testes<sup>29</sup>. We defined genes as germline-enriched if their expression met the following criteria:  
138 1) their expression is greater than 1 FPKM in wild-type gonads 2) their expression in any non-gonadal tissue  
139 for adult wild type<sup>22</sup> does not exceed 20% of their maximum expression in the wild-type germline, and 3)  
140 their expression in the germ cell-depleted gonads, for any sex or time point, does not exceed 20% of their  
141 maximum expression in the wild-type germline. These criteria yielded 1,288 germline-enriched genes (Figure  
142 2D), which was hereafter used as a resource to globally assess germline gene misexpression with *Kdm5c*  
143 loss (Supplementary table 1).

144 ***Kdm5c*-KO epiblast-like cells aberrantly express master regulators of germline  
145 identity**

146 Germ cells are typically distinguished from somatic cells soon after the embryo implants into the uterine  
147 wall<sup>33,34</sup>. A subset of epiblast stem cells become the primordial germ cells (PGCs) while the remainder  
148 differentiate into the ectoderm, mesoderm, and endoderm to form the somatic tissues<sup>35</sup>. This developmental  
149 time point can be modeled *in vitro* through differentiation of embryonic stem cells (ESCs) into epiblast-like  
150 stem cells (EpiLCs) (Figure 3A, top)<sup>36,37</sup>. While some germline-enriched genes are also expressed in  
151 embryonic stem cells (ESCs) and in the 2-cell stage<sup>38–40</sup>, they are silenced as they differentiate into EpiLCs<sup>19</sup>.  
152 Therefore, we tested if KDM5C was necessary for silencing germline genes at this developmental stage by  
153 evaluating the impact of *Kdm5c* loss in EpiLCs.

154 We first identified *Kdm5c*-KO EpiLC DEGs in our previously published RNA-seq dataset<sup>41</sup> (DESeq2, log2  
155 fold change > 0.5, q < 0.1) and assessed tissue-enriched gene expression. Similar to the *Kdm5c*-KO brain,  
156 we observed general dysregulation of tissue-enriched genes, with the largest number of genes belonging to  
157 the brain and testis, although they were not significantly enriched (Figure 3B). Primed pluripotency genes,  
158 such as *Dnmt3b*, *Fgf5*, *Pou3f1*, and *Otx2*, were unaltered in *Kdm5c*-KO EpiLCs (Figure 3C). Furthermore,  
159 *Kdm5c*-KO cell morphology during ESC to EpiLC differentiation (Figure 3D) was normal, indicating KDM5C  
160 loss does not impair EpiLC formation.

161 To determine if germline DEGs are constitutively dysregulated or if they can change over the course  
162 of development, we compared EpiLC germline DEGs to those expressed in the *Kdm5c*-KO brain. We  
163 found the majority of germline DEGs were unique to either EpiLCs or the brain, with only *Cyct* shared  
164 across all tissue/cell types (Figure 3E-F). We found particularly high enrichment of meiosis-related gene  
165 ontologies in EpiLCs (Figure 3G), such as meiotic cell cycle process (GO: 1903046, p.adjust = 1.59e-08)

166 and meiotic nuclear division (GO: 0140013, p.adjust = 9.76e-09). While there was modest enrichment of  
167 meiotic gene ontologies in both brain regions, the *Kdm5c*-KO hippocampus primarily expressed late-stage  
168 spermatogenesis genes, such those involved in the sperm axoneme structure.

169 Notably, DEGs unique to *Kdm5c*-KO EpiLCs included key drivers of germline identity, such as *Stimulated*  
170 *by retinoic acid 8 (Stra8)* and *Deleted in azoospermia like (Dazl)* (Figure 3H). These genes are typically  
171 expressed during embryonic germ cell development to commit PGCs to the germline fate, but are also  
172 expressed later in life to trigger meiotic gene expression programs<sup>42-44</sup>. Of note, some germline genes,  
173 including *Dazl*, are also expressed in the two-cell embryo<sup>39,45</sup>. However, we did not see misexpression of  
174 two-cell embryo-specific genes, like *Duxf3 (Dux)* ( $q = 0.337$ ) and *Zscan4d* ( $q = 0.381$ ), indicating *Kdm5c*-KO  
175 in EpiLCs do not revert back to a 2-cell state (Figure 3H).

## 176 Females have increased sensitivity to germline gene misexpression with *Kdm5c* 177 loss

178 It is currently unknown if the misexpression of germline genes is influenced by chromosomal sex, as  
179 previous studies on germline gene repressors have been conducted exclusively in males. We explored the  
180 impact of sex upon germline gene misexpression by comparing their dysregulation in male *Kdm5c* hemizygous  
181 knockout (XY *Kdm5c*-KO), female homozygous knockout (XX *Kdm5c*-KO) and female heterozygous (XX  
182 *Kdm5c*-HET) EpiLCs.<sup>41</sup> We first identified differentially expressed genes (DEGs) compared to sex-matched  
183 wild-type controls (DESeq2<sup>24</sup>, log2 fold change > 0.5,  $q < 0.1$ ) and then filtered for germline-enriched genes.

184 Homozygous and heterozygous females expressed over double the number of germline-enriched genes  
185 than hemizygous males (Figure 4A). While the majority of germline DEGs in *Kdm5c*-KO males were also  
186 dysregulated in females (74%), there were also many male-specific and female-specific germline DEGs  
187 (Figure 4A-B). We compared the known functions of germline genes dysregulated in all samples (shared),  
188 only in females (XX only - XX *Kdm5c*-KO, XX *Kdm5c*-HET, or both), and only in males (XY only). Gene  
189 ontologies uniquely enriched in female-specific germline DEGs included meiotic (meiotic cell cycle) and  
190 flagellar (cilium movement) genes, while mitochondrial and cell signaling gene ontologies were enriched in  
191 male-specific DEGs (protein localization to mitochondrion).

192 Germline genes dysregulated in both sexes were also enriched for meiotic ontologies (meiotic nuclear  
193 division), as well as egg-specific genes (female gamete generation). The majority of these shared germline  
194 DEGs had a greater log2 fold change from wild-type in females compared to males (Figure 4D-F). The  
195 increased number of germline genes and degree of dysregulation in females could be caused by improper  
196 X chromosome inactivation (XCI), as the X chromosome is enriched for many testis-specific germline  
197 genes[XXX]. However, both shared and female-specific germline DEGs were not biased towards the X  
198 chromosome, with the majority of genes lying on autosomes instead (Figure 4G). Thus, while female EpiLCs  
199 have increased sensitivity to germline gene misexpression with KDM5C loss, it is likely independent of

200 potential XCI defects.

## 201 **Germline gene misexpression in *Kdm5c* mutants is independent of germ cell sex**

202 While many germline genes have roles in both the male and female germline, some display sex-biased  
203 expression or have functions unique to eggs and sperm. To comprehensively assess if germline gene  
204 sex corresponds with *Kdm5c* mutant sex, we filtered our list of germline-enriched genes for egg (XX) and  
205 sperm (XY) biased genes. We defined sex-biased genes as those whose expression in the opposite sex,  
206 at any time point, is no greater than 20% of the gene's maximum expression in a given sex. This yielded  
207 67 egg-biased, 1,024 sperm-biased, and 197 unbiased germline-enriched genes, which is consistent with  
208 the testis overall having a more unique transcriptome than the ovary<sup>22</sup> (Figure 4H). We found egg, sperm,  
209 and unbiased germline genes were dysregulated in all *Kdm5c* mutants (Figure 4I-L). *Kdm5c* mutant male  
210 and female-specific germline DEGs were also not biased to the corresponding germ cell sex, indicating  
211 differences between male and female germline gene dysregulation not due to sex-specific activation of  
212 sperm or egg-specific transcriptional programs. These results demonstrate sex influences the degree of  
213 gene misexpression with loss of KDM5C, but not the sex of germ cell-enriched genes.

## 214 **KDM5C binds to a subset of germline gene promoters during early embryogenesis**

215 Previous work suggests KDM5C represses germline genes during early development, as re-expression  
216 of KDM5C in knockout neuronal cultures fails to suppress two hippocampal germline DEGs<sup>10</sup>. KDM5C binds  
217 to several germline gene promoters in embryonic stem cells (ESCs) but is lost in neurons<sup>10</sup>. However, the  
218 lack of a comprehensive list of germline-enriched genes prohibited systematic characterization of KDM5C  
219 binding at germline gene promoters. Thus, it is currently unclear if KDM5C is enriched at germline gene  
220 promoters, what types of germline genes KDM5C regulates, and if its binding is maintained at any germline  
221 genes in neurons.

222 To address these questions, we analyzed KDM5C chromatin immunoprecipitation followed by DNA  
223 sequencing (ChIP-seq) datasets in EpiLCs<sup>41</sup> and primary forebrain neuron cultures (PNCs)<sup>15</sup>. EpiLCs had a  
224 higher total number of KDM5C peaks than PNCs (EpiLCs: 5,808, PNCs: 1,276, MACS2 q < 0.1 and fold  
225 enrichment > 1, removal of *Kdm5c*-KO false positive peaks) and KDM5C was primarily localized to gene  
226 promoters in both cell types (EpiLCs: 4,190, PNCs: 745 +/- 500bp from the TSS), although PNCs showed  
227 increased localization to non-promoter regions (Figure 4A).

228 The majority of promoters bound by KDM5C in PNCs were also bound in EpiLCs (513 shared promoters),  
229 however a large portion of gene promoters were only bound by KDM5C in EpiLCs (3677 EpiLC only  
230 promoters) (Figure 4B). We then performed gene ontology analysis to compare the known functions of genes  
231 with KDM5C bound to their promoter in EpiLCs and PNCs. While there were no ontologies significantly  
232 enriched for genes only bound by KDM5C in PNCs, gene ontologies for genes shared between PNCs and

233 EpiLCs were enriched for functions involving nucleic acid turnover, such as deoxyribonucleotide metabolic  
234 process (GO:0009262, p.adjust = 8.28e-05) (Figure 4C). Germline-specific ontologies were only enriched in  
235 promoters unique to EpiLCs, such as meiotic nuclear division (GO: 0007127 p.adjust = 6.77e-16) and meiotic  
236 cell cycle process (GO:1903046, p.adjust = 5.05e-16) (Figure 3C). We then evaluated KDM5C binding  
237 around the transcription start site (TSS) of all germline-enriched genes. In EpiLCs, We observed modest  
238 KDM5C signal at about half of all germline genes (Figure 4D). Based on our ChIP-seq peak criteria, KDM5C  
239 was strongly bound to about 10% of germline gene promoters in EpiLCs (Figure 4E). In condordance with our  
240 gene ontology results, we did not observe KDM5C accumulation at any germline gene promtoers in PNCs  
241 (Figure 4D). Together, these results demonstrate KDM5C is significantly enriched at a subset of germline  
242 gene promoters in EpiLCs, including meiotic genes, but does not directly regulate germline genes in neurons.

243 Many germline-specific genes, including meiotic genes, are suppressed by the transcription factor  
244 heterodimers E2F6/DP1 and MGA/MAX, which respectively bind E2F and E-box motifs<sup>20,46-49</sup>. Thus, we  
245 identified transcription factor motifs enriched at KDM5C-bound or unbound germline gene promoters using  
246 HOMER<sup>50</sup> (TSS +/- 500 bp, q-value < 0.1). MAX and E2F6 binding sites were significantly enriched at  
247 germline genes bound by KDM5C in EpiLCs, but not at germline genes unbound by KDM5C (MAX q-value: ,  
248 E2F6 q-value:, E2F q-value: ) (Figure 4). One third of KDM5C-bound promoters contained the consensus  
249 sequence for either E2F6 (E2F, 5'-TCCCGC-3'), MGA (E-box, 5'-CACGTG-3'), or both, but only 17% of  
250 KDM5C-unbound genes contained these motifs (Figure 4). KDM5C-unbound germline genes were instead  
251 enriched for multiple RFX transcription factor binding sites. RFX transcription factors bind X-box motifs<sup>51</sup>  
252 to promote ciliogenesis<sup>52,53</sup>. Enriched RFX trancscription factors included RFX2, a central regulator of post-  
253 meiotic spermatogeneis<sup>54,55</sup>. Interestingly, RFX2 mRNA is upregulated in *Kdm5c*-KO EpiLCs, but is also not  
254 a direct target of KDM5C (Figure 4). Thus, distinct transcription factor programs regulate KDM5C-bound and  
255 unbound germline genes.

256 Finally, we compared KDM5C binding at promoters of RNA-seq DEGs to determine if the germline  
257 mRNAs de-repressed in *Kdm5c*-KO EpiLCs and brain are direct targets of KDM5C (Figure 4F). In EpiLCs,  
258 KDM5C was bound to about one third of EpiLC-specific and brain-specific germline DEGs (EpiLC only: 36%,  
259 Brain only: 33.3%). Again, we did not observe any KDM5C binding at germline gene promoters in PNCs,  
260 even for brain-specific DEGs (Figure 4H). Some notable differences in KDM5C binding for EpiLC-specific  
261 DEGs included *Dazl* and *Stra8*, two key drivers of germline identity discussed above. Although both mRNAs  
262 are expressed in *Kdm5c*-KO EpiLCs, KDM5C is only bound to *Dazl*'s promoter and not *Stra8*'s in EpiLCs  
263 (Figure 4F). In contrast to the unique DEGs, 3 out of the 4 genes dysregulated in both the brain and  
264 EpiLCs are bound by KDM5C around the TSS (Figure 4G). Altogether, this suggests KDM5C decomssions  
265 germline genes in EpiLCs but is no longer recruited to germline gene promoters in neurons. Furthermore, the  
266 majority of germline mRNAs expressed in *Kdm5c*-KO cells are dysregulated independent of direct KDM5C  
267 recruitment to their promoters.

268 **KDM5C promotes *de novo* DNA methylation at germline genes**

- 269 • if there are differences, say at germline gene CpG islands

270 In the early embryo, germline gene promoters are initially silenced by repressive histone modifications,  
271 including histone 2A lysine 119 monoubiquitination (H2AK119ub1) and histone 3 lysine 9 trimethylation  
272 (H3K9me3), and are then silenced long-term via DNA CpG methylation (CpGme)<sup>18,19,56</sup>. Our results above  
273 suggest KDM5C also acts during this time period, however how KDM5C interacts with other germline gene  
274 silencing mechanism is currently unclear. KDM5C is generally thought to suppress transcription through  
275 erasure of histone 3 lysine 4 di- and trimethylation (H3K4me2/3)<sup>11</sup>. However, KDM5C's catalytic activity was  
276 recently shown to not be required for suppressing *Dazl* in undifferentiated ESCs<sup>45</sup>. Since H3K4me3 impedes  
277 *de novo* CpGme placement<sup>57,58</sup>, KDM5C's catalytic activity may instead be required for CpGme-mediated,  
278 long-term silencing of germline genes. In support of this, CpGme is markedly reduced at two germline gene  
279 promoters in the *Kdm5c*-KO adult hippocampus<sup>10</sup>. Based on these observations, we hypothesized KDM5C  
280 erases H3K4me3 to promote the initial placement of CpGme at germline gene promoters in EpiLCs.

281 To test this hypothesis, we first characterized KDM5C's substrates, histone 3 lysine 4 di- and trimethylation  
282 (H3K4me2/3) in our previously published ChIP-seq datasets of the wild type and *Kdm5c*-KO amygdala<sup>23</sup>  
283 and EpiLCs<sup>41</sup>. In congruence with previous work in the *Kdm5c*-KO hippocampus<sup>10</sup>, we observed aberrant  
284 accumulation of H3K4me3 around the transcription start site (TSS) of germline genes in the *Kdm5c*-KO  
285 amygdala (Figure 5A). We additionally found a marked increase in H3K4me2 around the TSS of germline  
286 genes in *Kdm5c*-KO EpiLCs (Figure 5B).

287 We then evaluated KDM5C's role in germline gene silencing during ESC to EpiLC differentiation. We first  
288 characterized KDM5C's mRNA and protein expression at 0, 24, and 48 hours of differentiation (Figure 5C).  
289 While *Kdm5c* mRNA steadily decreased from 0 to 48 hours of differentiation (Figure 5D), KDM5C protein  
290 initially increased from 0 to 24 hours but then decreased to near knockout levels by 48 hours (Figure 5E).

- 291 • Germline genes are known to accumulate CpGme at (CGIs).  
292 – What percentage of germline genes have CGIs  
293 • Do differential methylation analysis for WT ESCs to WT EpiLCs  
294 – What percentage of germline genes significantly gain methylation (at CGI or at promoter)  
295 – Out of the ones that gain methylation, which are significantly reduced

296 To determine KDM5C's role in the initial placement of DNA methylation at germline gene promoters,  
297 we performed whole genome bisulfite sequencing (WGBS) in wild-type and *Kdm5c*-KO ESCs and 96 hour  
298 extended EpiLCs (exEpiLCs). Although wild-type cells accumulated high levels of DNA methylation at  
299 germline gene promoters over the course of ESCs to exEpiLC differentiation, DNA methylation was markedly  
300 reduced in *Kdm5c*-KO exEpiLCs (Figure 5F).

- 301 • discussion

302 • – However, CPG islands greatly determine KDM5C recruitment - KDM5C is known to be enriched at  
303 CGIs.)

304 • Altogether, this suggests KDM5C is dynamically regulated during ESC to EpiLC differentiation and  
305 promotes germline gene silencing via removal of H3K4me2/3 during early embryogenesis.

306 Discussion

307 In the above study, we demonstrate KDM5C's pivotal role in the development of tissue identity, resulting in  
308 substantial misexpression of tissue-enriched genes within the adult *Kdm5c*-KO hippocampus and amygdala.  
309 In addition to testis genes identified previously<sup>10</sup>, we found significant enrichment of muscle, liver, and even  
310 ovary-biased genes aberrantly expressed within the male *Kdm5c*-KO brain. Deeper characterization of  
311 testis-enriched DEGs revealed they were not somatic testis genes, but germline genes, thus demonstrating  
312 KDM5C's crucial role in establishing the soma-germline boundary. Given the majority of tissue-enriched  
313 DEGs are testis and ovary genes with no known brain functions, it is currently unknown if they impair  
314 *Kdm5c*-KO brain function and contribute to MRXSCJ-related phenotypes like intellectual disability and  
315 aberrant aggression. However, select liver and muscle-biased DEGs do have known roles within the brain,  
316 such as the liver-enriched lipid metabolism gene *Apolipoprotein C-I* (*Apoc1*)<sup>28</sup> that is highly expressed in the  
317 *Kdm5c*-KO amygdala and hippocampus. Intriguingly, *Apoc1* overexpression in the mouse brain can impair  
318 learning and memory<sup>59</sup> and is implicated in Alzheimer's disease in humans<sup>60</sup>.

319 We found a substantial portion of genes upregulated in *Kdm5c*-KO brain are germline genes with no  
320 known functions within the brain. Distinguishing the germline from the soma is a key feature of multicellularity  
321 and sexual reproduction<sup>16</sup>. Previous work characterizing chromatin regulators that silence germ cell-specific  
322 transcription has predominantly focused on their repression of key marker genes in embryonic stem cells  
323 (ESCs), such as *Dazl* and *Ddx4*<sup>18,19,49</sup>. To characterize KDM5C's role in germline gene repression at a  
324 genome-wide level throughout life, we curated a list of mouse germline-enriched genes using publically  
325 available germ cell-depleted RNA-seq datasets from *Kit<sup>W/Wv</sup>* mice<sup>29,32</sup>. This resource enabled us to identify 1)  
326 the extent of germline gene dysregulation in *Kdm5c*-KO cells, 2) the types of germline genes misexpressed  
327 at different developmental time points, and 3) which groups of germline genes are directly and indirectly  
328 regulated by KDM5C. This list of germline-enriched genes serves as a resource for future studies to  
329 systematically assess soma-germline dysregulation.

330 RNA-seq of epiblast-like cells (EpiLCs) revealed dysregulation of *Kdm5c*-KO tissue identity begins during  
331 early embryogenesis. *In vivo*, germline genes are typically decommissioned in epiblast stem cells and remain  
332 silenced as the epiblast differentiates into somatic tissues<sup>35</sup>. However, a small subset of epiblast stem cells  
333 will receive signals to reactivate germline gene expression to become the primordial germ cells (PGCs) that

334 will ultimately form the mature germline<sup>33,34</sup>. This process can be mimicked *in vitro* by differentiating EpiLCs  
335 into primordial germ cell-like cells (PGCLCs)<sup>36</sup>. Therefore, misexpression of germline genes in EpiLCs might  
336 suggest *Kdm5c*-KO ESCs are progressing beyond EpiLC differentiation and instead becoming PGCLCs. Yet,  
337 *Kdm5c*-KO EpiLCs properly express primed pluripotency genes, including *Otx2* - an epiblast stem cell marker  
338 that is known to repress differentiation into PGCs/PGCLCs<sup>61</sup>. Furthermore, we observed no difference in  
339 cellular morphology during *Kdm5c*-KO ESC to EpiLC differentiation. Proper EpiLC differentiation, together  
340 with *Kdm5c*-KO mice being viable, suggests germline gene expression is occurring ectopically in conjunction  
341 with typical developmental programs, rather than a complete shift to towards germline identity.

342 • XX vs XY

343 • While many germline genes have roles in both the male and female germline, some display sex-biased  
344 expression or have functions unique to eggs and sperm. Many of the germline genes characterized in  
345 the male *Kdm5c*-KO brain have sperm-specific functions. It is currently unclear if sperm genes are also  
346 dysregulated in female *Kdm5c* mutants, or if they would instead misexpress egg-biased genes.

347 • Motif analysis

348 – only 1/3 had max/e2f - Unlikely to be the major recruitment mechanism for KDM5C  
349 – KDM5C does not contain motif-specific binding  
350 – controls cilia in multiple cell types, initially identified in neurons. Shared axis that could contribute  
351 to NDDs

352 \* RFX Cilia in NDDs - <https://pubmed.ncbi.nlm.nih.gov/33658631/>  
353 – Enriched RFX transcription factors included the spermiogenesis regulator RFX2, whose mRNA is  
354 also significantly upregulated in *Kdm5c*-KO EpiLCs (Figure 4).  
355 – <https://www.nature.com/articles/srep20435>  
356 – <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4498915/>  
357 – <https://pubmed.ncbi.nlm.nih.gov/32725242/>

358 We then globally characterized KDM5C binding at germline-enriched gene promoters through KDM5C  
359 ChIP-seq in EpiLCs and primary hippocampus and cortex neuron cultures (PNCs).

360 Our analysis of KDM5C ChIP-seq data provided novel insights into direct and indirect mechanisms by  
361 which germline genes can be misexpressed in *Kdm5c*-KO cells.

362 While we observed high enrichment of KDM5C peaks at germline genes in EpiLCs, KDM5C was not  
363 bound to germline gene promoters in PNCs. This suggests dysregulation of germline genes in the mature  
364 *Kdm5c*-KO brain is due to loss of repression during embryogenesis, which is consistent with previous  
365 work that found introducing human KDM5C into *Kdm5c*-KO PNCs does not repress two aberrant germline  
366 transcripts<sup>10</sup>. Although enriched at germline gene promoters in EpiLCs, KDM5C was only bound to about a  
367 third of the germline genes aberrantly transcribed in *Kdm5c*-KO EpiLCs. One notable EpiLC DEG not bound

368 by KDM5C is *Stra8*, a transcription factor activated by retinoic acid signaling in germ cells to promote meiotic  
369 initiation<sup>62,63</sup>. However, retinoic acid can only activate *Stra8* expression when DAZL is present. Unlike *Stra8*,  
370 *Dazl* is a direct target of KDM5C in EpiCs and is both transcribed and translated in *Kdm5c*-KO EpiLCs.  
371 Expression of indirect DEGs like *Stra8* indicates germline genes can be aberrantly expressed in *Kdm5c*-KO  
372 cells through downstream activation by other ectopic germline programs. These ectopic germline programs  
373 appear to loosely mimic the trajectory of typical germline development, as germline genes important for early  
374 germ cell development and meiosis are expressed in *Kdm5c*-KO EpiLC while late spermatogenesis genes  
375 are expressed in the mature *Kdm5c*-KO brain. Altogether, this indicates once a subset of germline genes  
376 are activated due to loss of direct KDM5C repression in the early embryo, these ectopic germline programs  
377 can continue to progress in the background of *Kdm5c*-KO somatic development.

378 It is important to note that while we focused on KDM5C's regulation of germline genes, some germline-  
379 enriched genes are also expressed in naïve ESCs and at the 2-cell stage due to their roles in pluripotency and  
380 self-renewal. For example, *Dazl* is primarily known for committing PGCs to the germline fate and regulating  
381 the translation of germline-specific RNAs, but is also expressed at the 2-cell stage<sup>64</sup>, in naïve ESCs<sup>38</sup>, and in  
382 the inner cell mass<sup>38</sup>. Very recently, two screens of *Dazl*-repressors identified KDM5C as a direct regulator in  
383 ESCs<sup>45,65</sup>. Interestingly, one study found *Kdm5c*-KO ESCs also aberrantly expressed 2-cell-specific genes,  
384 indicating KDM5C negatively regulates totipotency<sup>45</sup>. However, out of the four regulators characterized,  
385 KDM5C was the only factor whose repression of *Dazl* was independent of the 2-cell specific transcription  
386 factor *Dux*<sup>45</sup>. Although expressed at the 2-cell stage and in ESCs, *Dazl* is typically silenced when ESCs  
387 differentiate into EpiLCs<sup>19</sup>. We found KDM5C also represses *Dazl* in EpiLCs, but *Kdm5c*-KO EpiLCs did  
388 not express 2-cell specific genes like *Dux* and *Zscan4c*. Together, this suggests the 2-cell-like state in  
389 *Kdm5c*-KO ESCs is an indirect consequence of KDM5C's primary role in germline gene repression.

390 *Dazl* and other germline-enriched genes are initially decommissioned in ESCs by repressive histone  
391 modifications and then *de novo* DNA CpG methylation (CpGme) in EpiLCs<sup>19–21,56,66,67</sup>. Unlike the previously  
392 characterized germline gene suppressors, KDM5C removes an active histone mark, histone 3 lysine 4 di-  
393 and trimethylation (H3K4me2/3)<sup>11</sup>. In this study, we observed a global increase in H3K4me2/3 in *Kdm5c*-KO  
394 EpiLCs and amygdala around the transcription start site (TSS) of germline-enriched genes. However,  
395 KDM5C's catalytic activity may not be required for germline gene silencing, as it was recently found to be  
396 dispensible for repressing *Dazl* in ESCs<sup>45</sup>. Although not necessary in ESCs, KDM5C's catalytic activity be  
397 required for CpGme in EpiLCs, as H3K4me3 impedes *de novo* CpGme placement<sup>57,58</sup>. This is supported  
398 by previous work in the *Kdm5c*-KO adult hippocampus, which found CpGme is significantly eroded at at  
399 least two germline promoters<sup>10</sup>. To elucidate the mechanism behind KDM5C-mediated silencing of germline  
400 genes, we characterized KDM5C in ESCs and EpiLCs and found its expression is dynamically regulated  
401 during differentiation. Furthermore, KDM5C acts during the transition from naïve to primed pluripotency to  
402 promote the initial placement of CpGme at CpG islands for the long-term silencing of germline genes.

403 The above work provides the mechanistic foundation for KDM5C's role in germline gene repression

404 and offers novel insight into how dysregulation in *Kdm5c*-KO tissue identity changes over the course of  
405 development. Although their impact upon neurodevelopment is currently unclear, ectopic germline transcripts  
406 are also found in models of other related neurodevelopmental disorders<sup>68</sup>, including Immunodeficiency,  
407 Centromere instability, Facial anomalies syndrome 1 (ICF1 OMIM: #242860)<sup>69,70</sup>, Kleefstra syndrome  
408 1 (OMIM: #610253)<sup>9</sup>, and MeCP2 duplication syndrome (MDS, OMIM: #300260)<sup>71</sup>. Like KDM5C, the  
409 chromatin regulators underlying these conditions, DNA methyltransferase 3b (DNMT3B), H3K9me1/2  
410 methyltransferases G9A/GLP, methyl-CpG -binding protein 2 (MECP2), primarily silence gene expression.  
411 Thus, KDM5C is among a growing cohort of chromatin-linked neurodevelopmental disorders that may have a  
412 similar underlying cause of germline versus soma dysregulation. However, further research is required to  
413 determine the ultimate impact of these germline genes and the extent to which this phenomenon occurs in  
414 humans.

415 • Last paragraph

416 – Thus, failure to fine-tune the expression of tissue-enriched, dosage-sensitive genes like *Apoc1*  
417 could be one route by which loss of brain tissue identity contributes to *Kdm5c*-KO impairments.

## 418 Materials and Methods

### 419 Classifying tissue-enriched and germline-enriched genes

420 Tissue-enriched differentially expresssd genes (DEGs) were determined by their classification in a previ-  
421 ously published dataset from 17 male and female mouse tissues<sup>22</sup>. This study defined tissue expression as  
422 greater than 1 Fragments Per Kilobase of transcript per Million mapped read (FPKM) and tissue enrichment  
423 as at least 4-fold higher expression than any other tissue.

424 We curated a list of germline-enriched genes using an RNA-seq dataset from wild-type and germline-  
425 depleted (*Kit*<sup>W/W<sup>v</sup></sup>) male and female mouse embryos from embryonic day 12, 14, and 16<sup>32</sup>, as well as adult  
426 male testes<sup>29</sup>. Germline-enriched genes met the following criteria: 1) their expression is greater than 1  
427 FPKM in wild-type germline 2) their expression in any wild-type somatic tissues<sup>22</sup> does not exceed 20%  
428 of maximum expression in wild-type germline, and 3) their expression in the germ cell-depleted (*Kit*<sup>W/W<sup>v</sup></sup>)  
429 germline, for any sex or time point, does not exceed 20% of maximum expression in wild-type germline.

### 430 Cell culture

431 We utilized our previously established cultures of male wild-type and *Kdm5c* knockout (-KO) embryonic  
432 stem cells<sup>41</sup>. Sex was confirmed by genotyping *Uba1/Uba1y* on the X and Y chromosomes with the following  
433 primers: 5'-TGGATGGTGTGCCAATG-3', 5'-CACCTGCACGTTGCCCTT-3'. Deletion of *Kdm5c* was

434 confirmed through the primers 5'-ATGCCCATATTAAGAGTCCCTG-3', 5'-TCTGCCTTGATGGGACTGTT-3',  
435 and 5'-GGTTCTAACACTCACATAGTG-3'.

436 Embryonic stem cells (ESCs) and epiblast-like cells were cultured using previously established  
437 methods<sup>37</sup>. Briefly, ESCs were initially cultured in primed ESC (pESC) media consisting of KnockOut  
438 DMEM (Gibco#10829–018), fetal bovine serum (Gibco#A5209501), KnockOut serum replacement  
439 (Invitrogen#10828–028), Glutamax (Gibco#35050-061), Anti-Anti (Gibco#15240-062), MEM Non-essential  
440 amino acids (Gibco#11140-050), and beta-mercaptoethanol (Sigma#M7522). They were then transitioned  
441 into ground-state, “naïve” ESCs (nESCs) by culturing for four passages in N2B27 media containing  
442 DMEM/F12 (Gibco#11330–032), Neurobasal media (Gibco#21103–049), Gluamax, Anti-Anti, N2 sup-  
443 plement (Invitrogen#17502048), and B27 supplement without vitamin A (Invitrogen#12587-010), and  
444 beta-mercaptoethanol. Both pESC and nESC media were supplemented with 3 μM GSK3 inhibitor  
445 CHIR99021 (Sigma #SML1046-5MG), 1 μM MEK inhibitor PD0325901 (Sigma #PZ0162-5MG), and 1,000  
446 units/mL leukemia inhibitory factor (LIF, Millipore#ESG1107).

447 nESCs were differentiated into epiblast-like cells (EpiLCs, 48 hours) and extendend EpiLCs (exEpiLCs,  
448 96 hours) by culturing in N2B27 media containing DMEM/F12, Neurobasal media, Gluamax, Anti-Anti,  
449 N2 supplement, B27 supplement (Invitrogen#17504044), beta-mercaptoethanol, fibroblast growth factor 2  
450 (FGF2, R&D Biotechne 233-FB), and activin A (R&D Biotechne 338AC050CF), as previously described<sup>37</sup>.

## 451 Immunocytochemistry (ICC)

452 ICC of DAZL in EpiLCs was performed by first growing cells on fibronectin-coated coverslips. Cells were  
453 then washed thrice with phosphobuffered saline (PBS), fixed in 4% paraformaldehyde, washed thrice in PBS,  
454 and blotted in PBS containing 0.3% Triton X-100, and 5% fetal bovine serum for 1 hour. They were then  
455 washed thrice with PBS and incubating in primary antibody (Rabbit anti DAZL, abcam ab34139, 1:200) in  
456 the blocking solution overnight at 4°C with gentle rocking. The next day, cells were rinsed thrice with PBS,  
457 and incubated in secondary antibody (Alexafluor 488 Invitrogen #710369, 1:1,000) with DAPI (1:1,000) in  
458 blocking buffer for 1 hour at room temperature. Coverslips were then rinsed thrice in PBS, and mounted onto  
459 slides using Prolong Gold (Invitrogen #P36930). Images were taken blinded for genotype, chosen based on  
460 similar levels of DAPI signal, and quantified via ImageJ before unblinding.

## 461 RNA sequencing (RNA-seq)

462 After ensuring read quality via FastQC (v0.11.8), reads were then mapped to the mm10 *Mus musculus*  
463 genome (Gencode) using STAR (v2.5.3a), during which we removed duplicates and kept only uniquely  
464 mapped reads. Count files were generated by FeatureCounts (Subread v1.5.0), and BAM files were  
465 converted to bigwigs using deeptools (v3.1.3) and visualized by the UCSC genome browser. RStudio (v3.6.0)  
466 was then used to analyze counts files by DESeq2 (v1.26.0)<sup>24</sup> to identify differentially expressed genes

467 (DEGs) with a q-value (p-adjusted via FDR/Benjamini–Hochberg correction) less than 0.1 and a log2 fold  
468 change greater than 0.5. For all DESeq2 analyses, log2 fold changes were calculated with IfcShrink using  
469 the ashr package<sup>72</sup>. MA-plots were generated by ggpubr (v0.6.0), and Eulerr diagrams were generated by  
470 eulerr (v6.1.1). Boxplots and scatterplots were generated by ggpubr (v0.6.0) and ggplot2 (v3.3.2). The Upset  
471 plot was generated via the package UpSetR (v1.4.0)<sup>73</sup>. Gene ontology (GO) analyses were performed by  
472 the R package enrichPlot (v1.16.2) using the biological processes setting and compareCluster.

### 473 Chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq)

474 ChIP-seq reads were aligned to mm10 using Bowtie1 (v1.1.2) allowing up to two mismatches. Only  
475 uniquely mapped reads were used for analysis. Peaks were called using MACS2 software (v2.2.9.1) using  
476 input BAM files for normalization, with filters for a q-value < 0.1 and a fold enrichment > 1. We removed  
477 “black-listed” genomic regions that often give aberrant signals. Common peak sets were obtained in R via  
478 DiffBind (v3.6.5). In the case of KDM5C ChIP-seq, *Kdm5c*-KO peaks were then subtracted from wild-type  
479 samples using bedtools (v2.25.0). Peak proximity to genome annotations was determined by ChIPSeeker  
480 (v1.32.1). Gene ontology (GO) analyses were performed by the R package enrichPlot (v1.16.2) using the  
481 biological processes setting and compareCluster. Enriched motifs were identified using HOMER<sup>50</sup>. Average  
482 binding across the genome was visualized using deeptools (v3.1.3). Bigwigs were visualized using the  
483 UCSC genome browser.

### 484 Whole genome bisulfite sequencing (WGBS)

485 Genomic DNA (gDNA) from naïve ESCs and extended EpiLCs was extracted using the Wizard Genomic  
486 DNA Purification Kit (Promega A1120), following the instructions for Tissue Culture Cells. gDNA was sent  
487 to Novogene for WGBS using the Illumina NovaSeq X Plus platform and sequenced for 150bp paired-end  
488 reads (PE150).

### 489 Data availability

### 490 Published datasets

491 All published datasets are available at the Gene Expression Omnibus (GEO) <https://www.ncbi.nlm.nih>  
492 .gov/geo. Published RNA-seq datasets analyzed in this study included the male wild-type and *Kdm5c*-KO  
493 adult amygdala and hippocampus<sup>23</sup> (available at GEO: GSE127722) and male wild-type and *Kdm5c*-KO  
494 EpiLCs<sup>41</sup> (available at GEO: GSE96797).

495 Previously published ChIP-seq experiments included KDM5C in wild-type and *Kdm5c*-KO EpiLCs<sup>41</sup> (avail-  
496 able at GEO: GSE96797) and mouse primary neuron cultures (PNCs) from the cortex and hippocampus<sup>15</sup>  
497 (available at GEO: GSE61036). ChIP-seq of histone 3 lysine 4 dimethylation in male wild-type and *Kdm5c*-KO

498 EpiLCs<sup>41</sup> is also available at GEO: GSE96797. ChIP-seq of histone 3 lysine 4 trimethylation in wild-type and  
499 *Kdm5c*-KO male amygdala<sup>23</sup> are available at GEO: GSE127817.

500 **Data analysis**

501 Scripts used to generate the results, tables, and figures of this study are available via a GitHub repository:  
502 XXX

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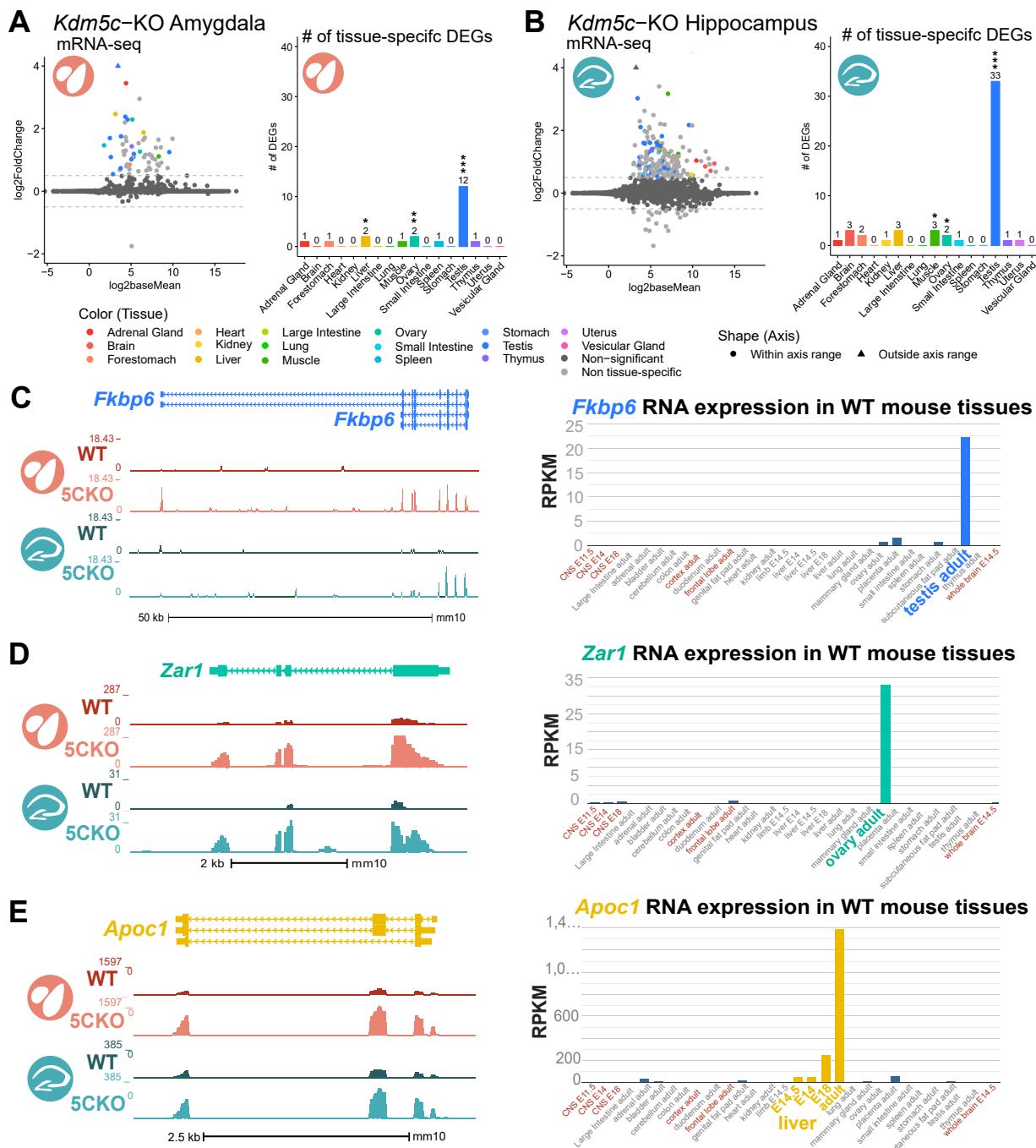
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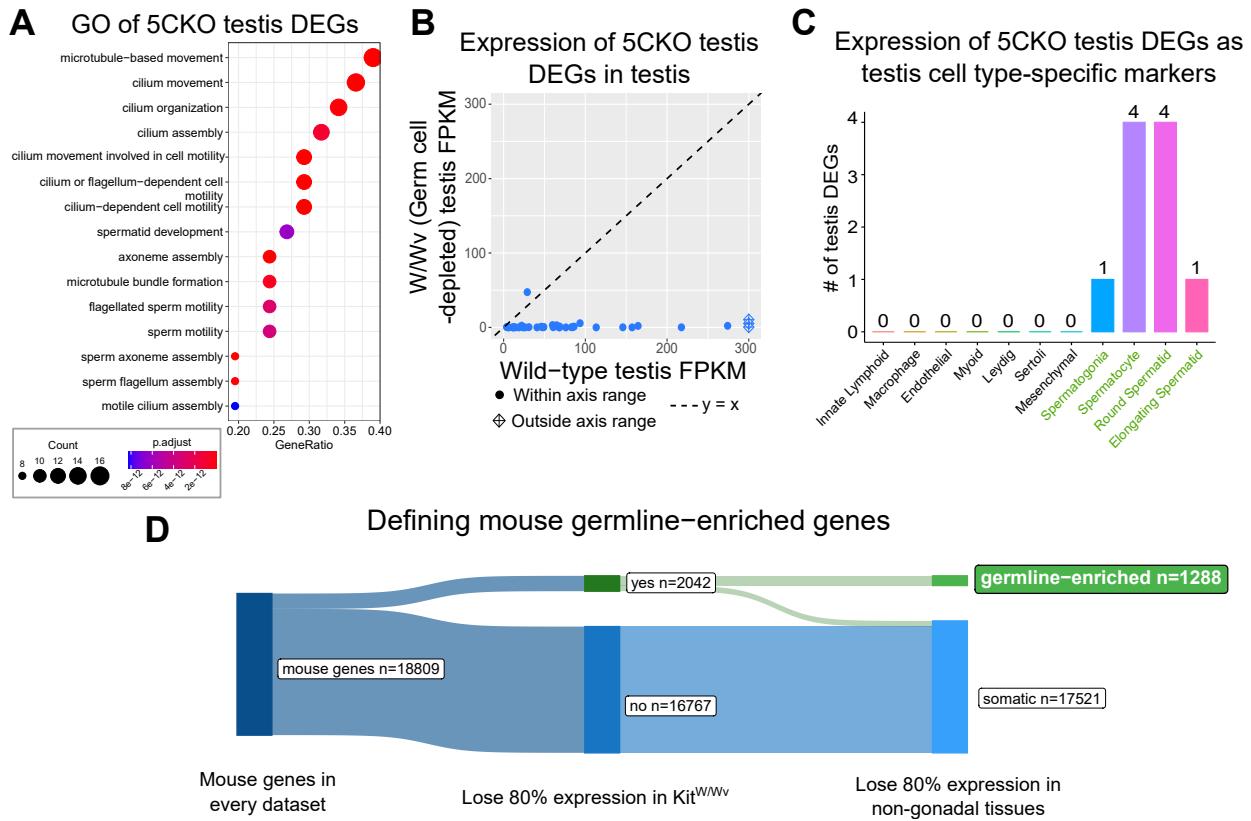
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659 **Figures and Tables**

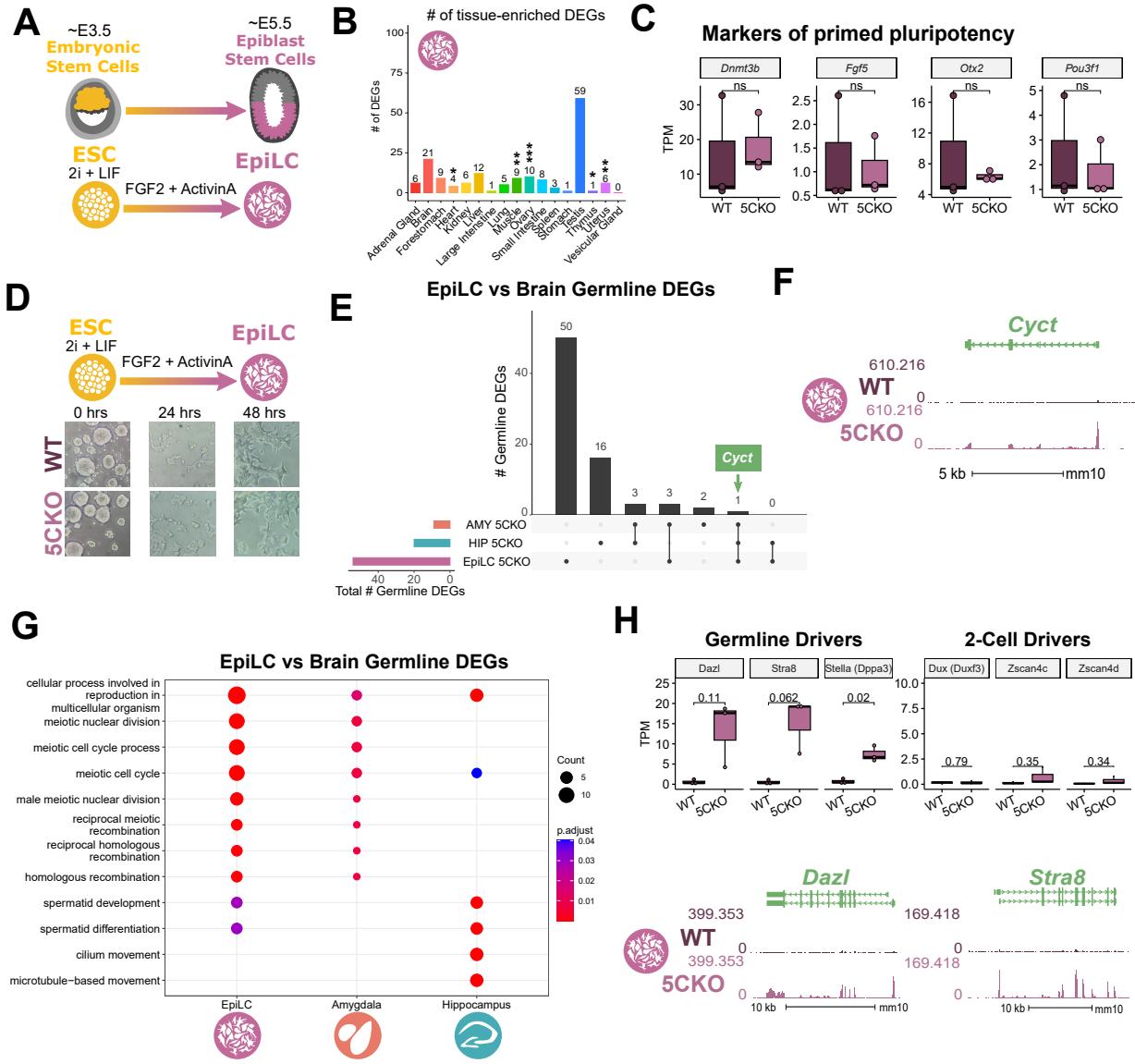
- 660     • Supplementary table 1: list of all germline genes.
- 661       – Columns to include:
- 662           \* KDM5C bound vs not
- 663           \* DEG in EpiLC, brain, both, neither (separate columns?)



**Figure 1: Tissue-enriched genes are misexpressed in the *Kdm5c*-KO brain.** **A-B.** Expression of tissue-enriched genes (Li et al 2017) in the male *Kdm5c*-KO amygdala (A) and hippocampus (B). Left - MA plot of mRNA-sequencing. Right - Number of tissue-enriched differentially expressed genes (DEGs). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , Fisher's exact test. **C.** Left - Average bigwigs of an example aberrantly expressed testis-enriched DEG, *FK506 binding protein 6* (*Fkbp6*) in the wild-type (WT) and *Kdm5c*-KO (5CKO) amygdala (red) and hippocampus (teal). Right - Expression of *Cyct* in wild-type tissues from NCBI Gene, with testis highlighted in blue and brain tissues highlighted in red. **D.** Left - Average bigwigs of an example ovary-enriched DEG, *Zygotic arrest 1* (*Zar1*). Right - Expression of *Zar1* in wild-type tissues from NCBI Gene, with ovary highlighted in teal and brain tissues highlighted in red. **E.** Left - Average bigwigs of an example liver-enriched DEG, *Apolipoprotein C-I* (*Apoc1*). Right - Expression of *Apoc1* in wild-type tissues from NCBI Gene, with liver highlighted in orange and brain tissues highlighted in red.



**Figure 2: Aberrant transcription of germline genes in the *Kdm5c*-KO in the brain.** **A.** enrichPlot gene ontology (GO) of *Kdm5c*-KO amygdala and hippocampus testis-enriched DEGs **B.** Expression of testis DEGs in wild-type (WT) testis versus germ cell-depleted (W/Wv) testis (Mueller et al 2013). Expression is in Fragments Per Kilobase of transcript per Million mapped reads (FPKM). **C.** Number of testis DEGs that were classified as cell-type specific markers in a single cell RNA-seq dataset of the testis (Green et al 2018). Germline cell types are highlighted in green, somatic cell types in black. **D.** Sankey diagram of mouse genes filtered for germline enrichment based on their expression in wild-type and germline-depleted mice and in adult mouse non-gonadal tissues (Li et al 2017).



**Figure 3: *Kdm5c*-KO epiblast-like cells express key drivers of germline identity**

**A.** Top - Diagram of *in vivo* differentiation of embryonic stem cells (ESCs) of the inner cell mass into epiblast stem cells. Bottom - *in vitro* differentiation of ESCs into epiblast-like cells (EpiLCs).

**B.** Number of tissue-enriched differentially expressed genes (DEGs) in *Kdm5c*-KO EpiLCs. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , Fisher's exact test.

**C.** Average bigwigs of an example germline gene, *Cyct*, that is dysregulated *Kdm5c*-KO EpiLCs.

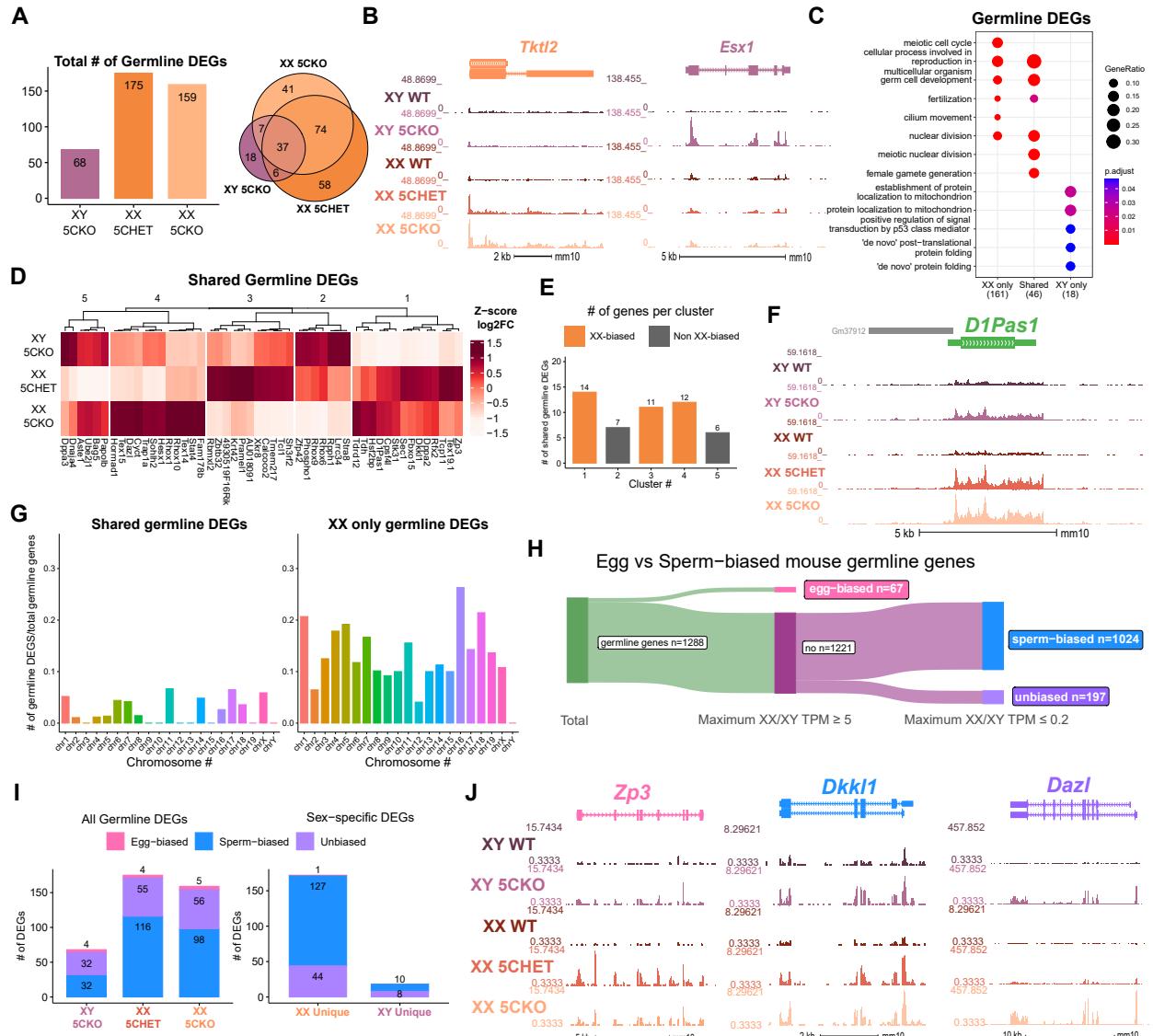
**D.** No significant difference in primed pluripotency marker expression in wild-type versus *Kdm5c*-KO EpiLCs. Welch's t-test, expression in transcripts per million (TPM).

**E.** Representative images of wild-type (WT) and *Kdm5c*-KO cells during ESC to EpiLC differentiation. Brightfield images taken at 20X.

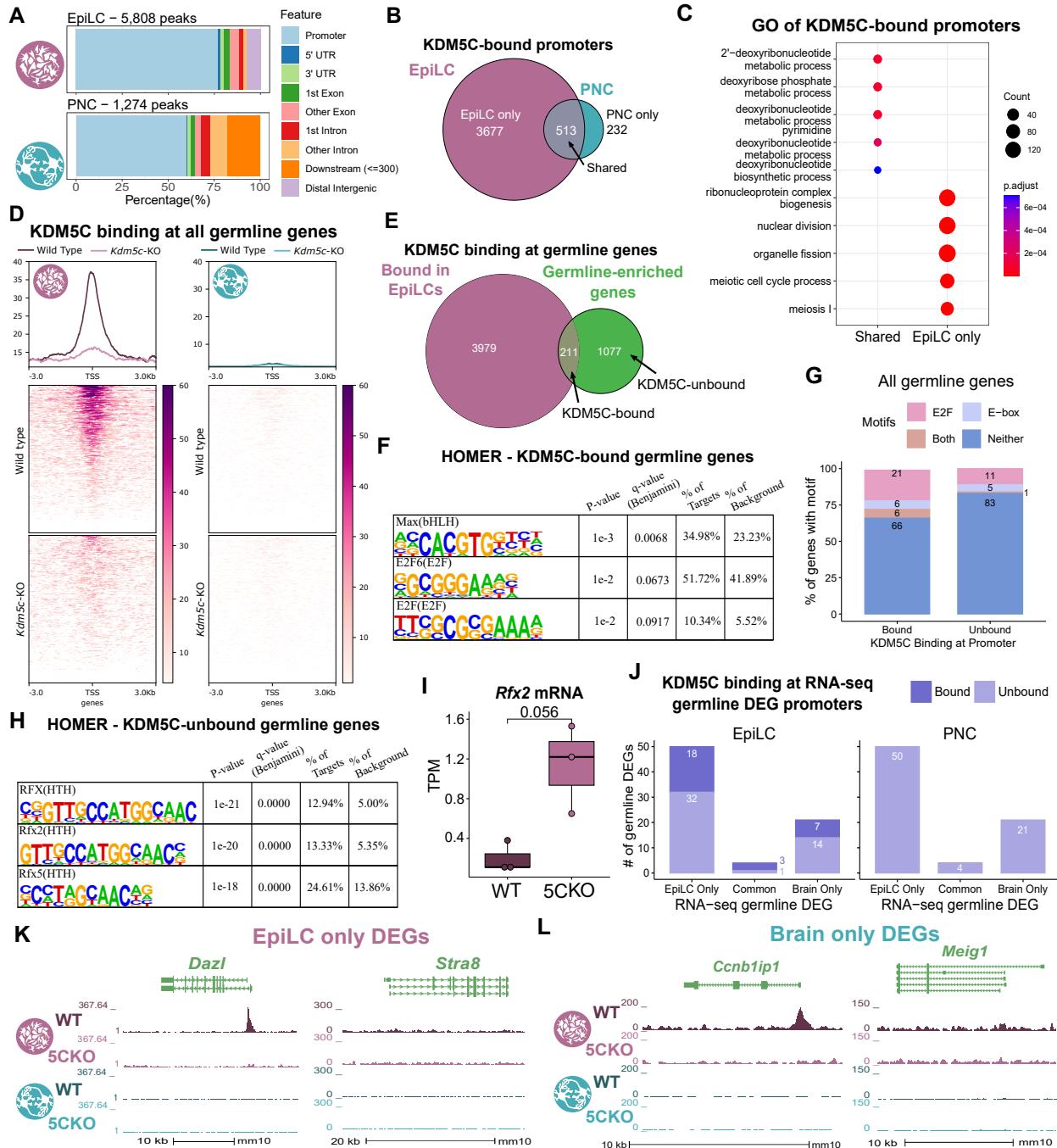
**F.** Upset plot displaying the overlap of germline DEGs expressed in *Kdm5c*-KO EpiLCs, amygdala (AMY), and hippocampus (HIP) RNA-seq datasets.

**G.** enrichPlot comparing enriched biological process gene ontologies for *Kdm5c*-KO EpiLC, amygdala, and hippocampus germline DEGs.

**H.** Top left - Example germline identity DEGs unique to EpiLCs. Top right - Example 2-cell genes that are not dysregulated in *Kdm5c*-KO EpiLCs. p-values for Welch's t-test. Bottom - Average bigwigs of *Dazl* and *Stra8* expression in wild-type and *Kdm5c*-KO EpiLCs. Immunocytochemistry of DAZL in male wild-type (WT) and *Kdm5c*-KO (5CKO) EpiLCs. Percentage of DAZL-positive cells normalized to number of DAPI-positive cells, p-value for Welch's t-test.

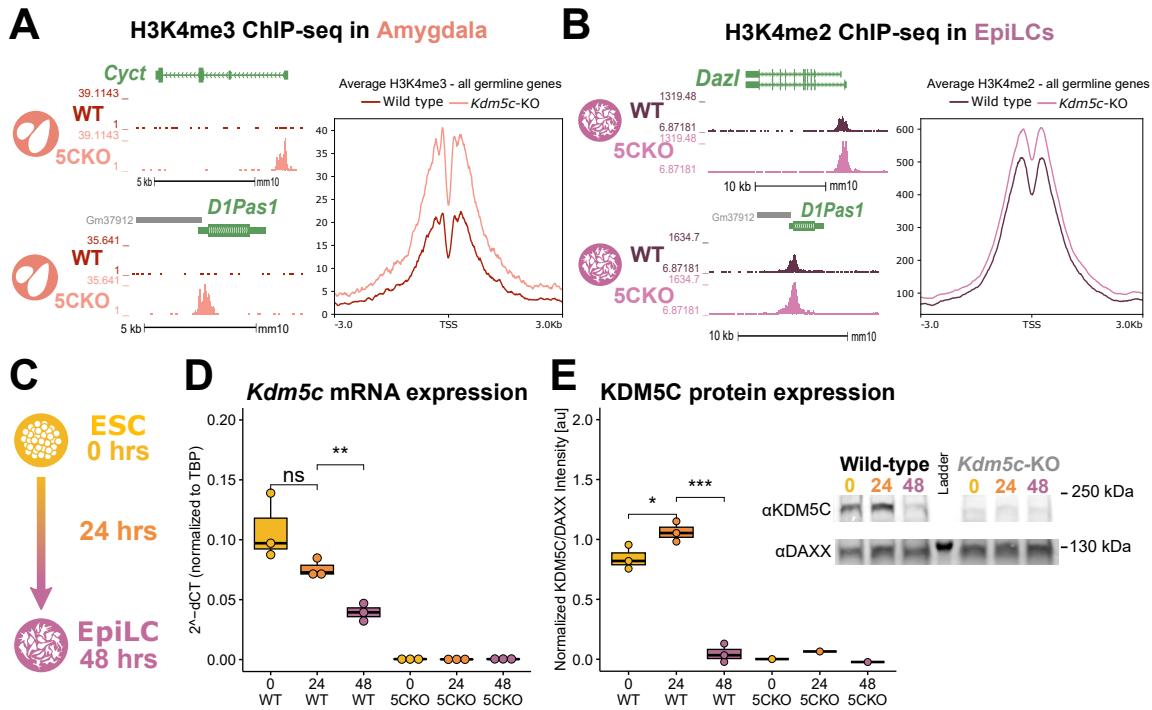


**Figure 4: Chromosomal sex influences *Kdm5c*-KO germline gene misexpression. A-B.** Expression of tissue-enriched genes (Li et al 2017) in the male *Kdm5c*-KO amygdala (A) and hippocampus (B). Left - MA plot of mRNA-seq. Right - Number of tissue-enriched differentially expressed genes (DEGs). \*  $p<0.05$ , \*\*  $p<0.01$ , \*\*\*  $p<0.001$ , Fisher's exact test. **C.** Left - Average bigwigs of an example aberrantly expressed testis-enriched DEG, *FK506 binding protein 6* (*Fkbp6*) in the wild-type (WT) and *Kdm5c*-KO (5CKO) amygdala (red) and hippocampus (teal). Right - Expression of *Cyct* in wild-type tissues from NCBI Gene, with testis highlighted in blue and brain tissues highlighted in red. **D.** Left - Average bigwigs of an example ovary-enriched DEG, *Zygotic arrest 1* (*Zar1*). Right - Expression of *Zar1* in wild-type tissues from NCBI Gene, with ovary highlighted in teal and brain tissues highlighted in red. **E.** Left - Average bigwigs of an example liver-enriched DEG, *Apolipoprotein C-I* (*Apoc1*). Right - Expression of *Apoc1* in wild-type tissues from NCBI Gene, with liver highlighted in orange and brain tissues highlighted in red.



**Figure 5: KDM5C binds to a subset of germline gene promoters during early embryogenesis** **A.** ChIPseeker localization of KDM5C peaks at different genomic regions in EpiLCs (top) and hippocampal and cortex primary neuron cultures (PNCs, bottom). **B.** Overlap of genes with KDM5C bound to their promoters in EpiLCs (purple) and PNCs (blue). **C.** Gene ontology (GO) comparison of genes with KDM5C bound to their promoter in EpiLCs and PNCs. Genes were classified as either bound in EpiLCs only (EpiLC only), unique to PNCs (PNC only, no significant ontologies) or bound in both PNCs and EpiLCs (shared). **D.** Average KDM5C binding around the transcription start site (TSS) of all germline-enriched genes in EpiLCs (left) and PNCs (right). **E.** KDM5C binding at the promoters of RNA-seq germline DEGs. Genes were classified as either only dysregulated in EpiLCs (EpiLC only), genes dysregulated in the hippocampus or amygdala but not EpiLCs (brain only), or genes dysregulated in both EpiLCs and the brain (common). (Legend continued on next page.)

**Figure 5: KDM5C binds to a subset of germline gene promoters during early embryogenesis.** (Legend continued.) **F.** Example KDM5C ChIP-seq bigwigs of DEGs unique to EpiLCs. Although both are expressed in *Kdm5c*-KO EpiLCs, KDM5C is only bound to the *Dazl* promoter and not the *Stra8* promoter in EpiLCs. **G.** Example KDM5C ChIP-seq bigwigs of RNA-seq DEGs common between the brain and EpiLCs. KDM5C is bound to the *D1Pas1* promoter but not the *XXX* promoter in EpiLCs. **H.** Example KDM5C ChIP-seq bigwigs of RNA-seq DEGs unique to the brain. KDM5C is bound to the *XXX* promoter but not the *Meig1* promoter



**F**

**G**

**Figure 6: KDM5C's catalytic activity promotes long-term silencing of germline genes via DNA methylation.** **A.** Left - Bigwigs of representative histone 3 lysine 4 trimethylation (H3K4me3) ChIP-seq peaks at two germline genes in the wild-type (WT) and *Kdm5c*-KO (5CKO) adult amygdala. Right - Average H3K4me3 at the transcripton start site (TSS) of all germline-enriched genes in wild-type (dark red) and *Kdm5c*-KO (light red) amygdala. **B.** Left - Bigwigs of representative histone 3 lysine 4 dimethylation (H3K4me2) ChIP-seq peaks at representative germline genes in wild-type and *Kdm5c*-KO EpiLCs. Right - Average H3K4me2 at the TSS of all germline-enriched genes in wild-type (dark purple) and *Kdm5c*-KO (light purple) EpiLCs. **C.** Diagram of embryonic stem cell (ESC) to epiblast-like cell (EpiLC) differentiation protocol and collection time points for RNA and protein. **D.** Real time quantitative PCR (RT-qPCR) of *Kdm5c* RNA expression, calculated in comparision to TBP expression ( $2^{-\Delta\Delta CT}$ ). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , Welch's t-test. **E.** KDM5C protein expression normalized to DAXX. Quantified intensity using ImageJ (artificial units - au). Right - representative lanes of Western blot for KDM5C and DAXX. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , Welch's t-test **F.** XXX **G.** XXX

## 664 Notes

### 665 Things to do

- 666     • Move dazl to new figure if other staining works
- 667     • Use Ddx4 staining (with dazl?). If not should add RNA to diagram because its a key germline gene.
- 668     • Motif analysis
  - 669         – Discussion - talk about motifs

### 670 Dazl

671    We were particularly interested in the aberrant transcription of *Dazl*, since it is essential for germ cell  
672 development and promotes the translation of germline mRNAs<sup>74</sup>. A significant portion of germline transcripts  
673 misexpressed in *Kdm5c*-KO EpiLCs are known binding targets of DAZL, including *Stra8*<sup>75</sup> ( $p = 1.698e-07$ ,  
674 Fisher's Exact Test). This suggests expression of DAZL protein could promote the translation of other  
675 aberrant germline transcripts, influencing their ability to impact *Kdm5c*-KO cellular function. We thus tested  
676 DAZL protein expression in *Kdm5c*-KO EpiLCs through immunocytochemistry (Figure 3I). We observed  
677 about 25% of *Kdm5c*-KO EpiLCs expressed DAZL protein and it was localized to the cytoplasm ( $p = 0.0015$ ,  
678 Welch's t-test), consistent with the pattern of DAZL expression in spermatogonia<sup>75</sup>. Altogether these results  
679 suggest tissue-specific genes are misexpressed during *Kdm5c*-KO embryogenesis, including key drivers of  
680 germline identity that can be translated into protein.

- 681     • We additionally found *Kdm5c*-KO EpiLCs ectopically express DAZL protein that is localized to the  
682         cytoplasm, similar to its morphology in spermatogonia<sup>75</sup>. **note: maybe just put in results.** Could  
683         move around depending upon if I get pheno working.

### 684 Discussion notes

- 685     • For other paper:
  - 686         – for methods: Heatmaps of gene expression were generated using the base R functions scale and  
687             hclust and visualized using the R package ComplexHeatmap (v2.12.1).
  - 688         –     \* Might be good to look at for retinoic acid paper (WT germ expression): <https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0005654>
- 690     • Talk about different motifs if we do see differences there and if it explains direct vs indirect dysregulation
  - 691         – Could also mention PRC1.6/KDM5C binds pcgf6 in ESCs
- 692     • Maybe talk about other regulators/if anything is known about them long term? Or save for 2nd paper.

- 693           – another germline repressor: <https://pubmed.ncbi.nlm.nih.gov/35345626/>
- 694       • end with something like: this indicates kdm5c not only modulates pluripotency and self-renewal in  
695        ESCs, but also has a role in long-term silencing of germline genes
- 696           – then transition into the long term silencing mechanism paragraph
- 697       • KDM5C is dynamically regulated during the window of embryonic germline gene silencing, our EpiLC  
698        ChIP-seq is likely catching the tail end of KDM5C's main involvement.
- 699       • Our results indicate KDM5C is a pivotal repressor of germline programs in somatic cells.
- 700       • Studies on other chromatin regulators have focused on key marker genes to identify germline gene  
701        misexpression, such as *Dazl*.
- 702       • Using a germline-depleted mouse model, we curated a list of mouse germline-enriched genes to  
703        globally assess germline gene dysregulation.
- 704       • Many of the genes dysregulated in the mature *Kdm5c*-KO brain were important for late stages of  
705        spermatogenesis, such as those important for sperm flagellar structure. Contrastingly, *Kdm5c*-KO  
706        EpiLCs aberrantly expressed key drivers of germ cell identity, including *Dazl* and *Stra8*.
- 707       • While a significant portion of KDM5C-bound promoters are germline genes, many germline genes  
708        expressed during *Kdm5c*-KO embryogenesis are not directly bound by kdm5c.
- 709       • One notable example is *Stra8*, a transcription factor important for germ cell specific transcription and  
710        meiotic initiation
- 711       • The including the demarcation between soma and germline fates.
- 712       the *Kdm5c*-KO brain also Previous work identified testis-enriched genes in *Kdm5c* genes
- 713           –
- 714           – However unlike the gonadal-biased DEGs,
- 715       • The demarcation of the germline versus soma is a key feature of multicellularity and sexually dimorphic  
716        reproduction
- 717       • Anything known about tissue-biased gene expression in other H3K4me regulators?
- 718       • Our data suggests the germline developmental program is occurring ectopically as *Kdm5c*-KOs pro-  
719        gresses through somatic tissue development
- 720       • tissue-biased gene expression:

- 721 • Altogether, these data indicate that while some germline genes are misexpressed due to direct loss of  
722 KDM5C binding during emryogenesis, secondary downstream mechanisms can also promote their  
723 aberrant transcription.
- 724 • Papers to read/reference:  
725 – Reconstitution of the Mouse Germ Cell Specification Pathway in Culture by Pluripotent Stem Cells:  
726 [https://www.cell.com/fulltext/S0092-8674\(11\)00771-9](https://www.cell.com/fulltext/S0092-8674(11)00771-9)  
727 – two cell gene list used by Suzuki et al Max paper is based on 2 cell sequencing: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3395470/>

729 **Figure outline:**

730 **Figure 1: Misexpression of tissue-specific genes in the *Kdm5c*-KO brain** \* MA-plot and bar graphs of  
731 tissue-enriched genes \* Example testis-specific genes (NCBI and bigwigs) \* An example ovary tissue-specific  
732 gene \* An example muscle/liver tissue-specific gene (NCBI and bigwigs)

733 **Figure 2: The male *Kdm5c*-KO brain expresses male and female germline-enriched genes** \* Gene  
734 ontology of testis DEGs in the amygdala and hippocampus - ontologies are germline ontologies \* Expression  
735 of testis DEGs in germline-depleted testis (this is adult testis data) \* scRNASeq of testis - # of testis DEGs that  
736 are germline-specific markers \* Although far fewer, 5CKO brain also expresses ovary-enriched genes(NCBI  
737 and bigwigs of Zar1) \* These ovary enriched genes are also germline specific (NCBI/Li tissues are in adult  
738 ovary, so it would be best to show they're oocyte-specific in adult ovary. But I don't think there's a published  
739 adult female W/Wv dataset. Could try looking at scRNASeq or just do TPM in embryonic W/Wv data since  
740 oocytes are developed at this point? Or both?) \* Defining what is/isn't a germline gene, and which are  
741 male/female biased using embryonic W/Wv data

742 **Figure 3: *Kdm5c*-KO epiblast-like cells express key drivers regulators of germline identity** \* A) ESC  
743 to EpiLC differentiation Left - Morphology is unchanged, \* B) 5CKO EpiLCs express EpiLC differentiation  
744 genes similar to WT lvs \* C) Male EpiLCs express germline genes (example Cyct again) \* Overlap between  
745 brain and EpiLC germline genes - show they're mostly unique \* GO of Brain and EpiLC germline genes  
746 (meiotic enriched) \* Bigwigs or TPM of master regulators \* Show that while some are also 2-cell genes  
747 (Dazl), 2-cell specific genes aren't dysregulated (Zscan4). Important point because published KDM5C dazl  
748 paper is saying KDM5C is a 2-cell regulator, but as far as I can tell only genes shared between germline and  
749 2-cell are dysregulated.

750 Staining of Dazl (+ Stra8 if I can get it to work)

751 **Figure 4: Loss of KDM5C's catalytic activity impairs DNAme placement and long-term silencing of  
752 germline genes** \* Increase in H3K4me3 in *Kdm5c*-KO amygdala at germline genes \* Increase in H3K4me2  
753 in EpiLCs at germline genes \* *Kdm5c* binding in EpiLCs vs PNCs to show that germline repression is  
754 happening in early embryo \* Previous studies only looked at ESCs, unknown if catalytic activity is required

755 for long-term repression, especially since DNA methylation is placed later). KDM5C RNA and protein ESC →  
756 EpiLC (increasing then decreasing) \* RNA expression of germline genes with catalytic dead rescue (Ilakkia)  
757 \* DNA methylation in WT and 5CKO EpiLCs (Ilakkia)

758 **Figure 5: Ectopic, germline-like phenotypes in Kdm5c-KO ESCs/EpiLCs** \* Sycp3 staining \* DDX4  
759 staining and repression of retrotransposons \* Cilia??

760 Gaps in knowledge addressed: \* Are other tissue-enriched genes dysregulated, or only testis, germline  
761 genes? \* Curating a robust list of male and female germline genes \* Should talk about 2-cell genes  
762 vs germline genes - way to systematically categorize? \* Mechanism behind long-term germline gene  
763 misexpression \* Recent evidence suggests loss of KDM5C in ESCs express some germline genes \* Unclear  
764 if catalytic activity is required for long-term silencing \* Unclear if their dysregulation lasts throughout life or  
765 the same between brain or not \* When in development does it begin? - Recent evidence suggests some  
766 germline genes expressed in 5CKO ESCs but unclear if their dysregulation lasts throughout differentiation  
767 and if the identity of germline genes are different compared to the brain \* Are there functional consequences  
768 to germline gene misexpression?

769 Introduction: \* Chromatin regulators are important for cellular identity \* H3K4me1-3 linked to active  
770 gene promoters and enhancers \* Surprisingly, mutations in Chromatin regulators lead to many NDDs  
771 (including many H3K4 regulators) \* Recent studies have shown some chromatin regulators are important  
772 for regulating neuron-specific gene expression/chromatin stat\_compare\_means \* However, loss of some  
773 chromatin regulators can also lead to ectopic expression of tissue-enriched genes \* Very few studies have  
774 looked at these genes and it's unclear if these genes contribute to NDD impairments. \* Necessary to  
775 first characterize the mechanism behind their derepression to identify molecular footholds into testing their  
776 contribution to neuronal impairments and potential for therapeutic intervention

- 777 • Loss of KDM5C can result in the misexpression of genes typically only found in the testis
- 778 – Misexpression of tissue-enriched genes hasn't been systematically characterized - Unclear if  
779 these genes are exceptions or if other tissue-specific genes are dysregulated
- 780 – Interestingly, these genes (Cyct, D1pas1) typically function in the germline
- 781 – Germ cells (meiotic cells) are typically distinguished from somatic cells very early on in embryogen-  
782 esis and is a key feature of multicellularity
- 783 – Chromatin regulators are very important for decommissioning germline genes and act successively  
784 the embryo implants into the uterine wall
- 785 – Most studies have focused on ESCs, which have a similar transcriptome to germ cells / 2-cells
- 786 – recently, KDM5C was shown to repress DAZL in ESCs, independent of its catalytic activity
- 787 – However, DNA methylation is lost in the mature 5CKO brain, DNA methylation is placed later  
788 and it's Unclear if it's required for long-term repression (maybe too specific, just trying to go  
789 into the fact that the mechanism is partially understood but unclear)

- 790 – Systematic characterization of ectopic germline genes hasn't been done  
791 \* unknown if other germline-enriched genes are dysregulated, including oocyte-specific genes  
792 \* Crucially, it's unknown if misexpression of the germline program leads to functional conse-  
793 quences in 5CKO cells.

794 **Germline gene repression background:**

795 Interestingly, some of the ectopic testis transcripts identified in the *Kdm5c*-KO brain are typically ex-  
796 pressed in germ cells<sup>10</sup>. Unlike somatic cells, germ cells (e.g. sperm and eggs) undergo meiosis and pass  
797 on their genetic material to the next generation. The germline and the soma are typically distinguished during  
798 early embryogenesis, when germline genes are silenced in epiblast stem cells soon after implantation and  
799 only reactivated in a subset to form the germline. Chromatin regulators play a key role in decommissioning  
800 germline genes as the embryo transitions from naïve to primed pluripotency by placing repressive histone  
801 H2A lysine 119 monoubiquitination (H2AK119ub1)<sup>18</sup>, histone 3 lysine 9 trimethylation (H3K9me3)<sup>18,19</sup>, and  
802 DNA CpG methylation<sup>19–21</sup> at germline gene promoters. KDM5C may also be involved in this early decom-  
803 missioning of germline genes, as re-expression of KDM5C in neurons fails to suppress their dysregulation<sup>10</sup>.  
804 In support of this, KDM5C was very recently shown to repress *Deleted in azoospermia like (Dazl)*, a key  
805 regulator of germline development, in mouse embryonic stem cells (ESCs)<sup>45,65</sup>. In support of this, two  
806 independent screens in mouse embryonic stem cells (ESCs) recently identified KDM5C as a repressor of  
807 *Deleted in azoospermia like (Dazl)*, a key regulator of germline development. However, KDM5C's role in  
808 embryonic germline gene repression is currently unclear, given that Dazl is also expressed in ESCs and in  
809 the 2-cell stage and germline gene misexpression has yet to be globally characterized during *Kdm5c*-KO  
810 embryogenesis.