

1 Erosion of somatic tissue identity with loss of the X-linked intellectual  
2 disability factor KDM5C

3

4 **Abstract**

5 **Introduction**

6 A single genome holds the instructions to generate the myriad of cell types found within the adult organism. This is, in  
7 part, accomplished by chromatin regulators that can either promote or impede lineage-specific gene expression through DNA  
8 and histone modifications<sup>1,2</sup>. Although many chromatin regulators were initially identified for their roles in shaping cellular and  
9 tissue identity<sup>3,4</sup>, recent advancements in next generation sequencing unexpectedly revealed many neurodevelopmental  
10 disorders (NDDs) are caused by mutations in chromatin regulators<sup>5</sup>. Several studies have suggested this connection  
11 between chromatin regulators and neurodevelopment is due to their regulation of brain-specific genes, such as orchestrating  
12 transcriptional programs for synaptic maturation<sup>6</sup> and transitioning between neuronal and glial fates during neural precursor  
13 differentiation<sup>7</sup>. However, loss of some chromatin regulators can also result in the ectopic transcription of tissue-specific  
14 genes outside of their target environment<sup>3,4,8</sup>, such as the misexpression of liver-specific genes within adult neurons<sup>9</sup>. Very  
15 few studies have investigated the misexpression of tissue-specific genes in chromatin-linked NDDs<sup>9,10</sup> and it is currently  
16 unknown if this partial loss of brain identity contributes to neurodevelopmental impairments.

17 To elucidate the role of tissue identity in chromatin-linked neurodevelopmental disorders, it is essential to first characterize  
18 the types of genes dysregulated and the molecular mechanisms governing their de-repression. In this study, we characterized  
19 the aberrant expression of tissue-enriched genes with loss of lysine demethylase 5C (KDM5C). KDM5C, , also known  
20 as SMCX or JARID1C, is a chromatin regulator that can repress gene expression through erasure of histone 3 lysine 4  
21 di- and trimethylation (H3K4me2/3)<sup>11</sup>, marks enriched at active gene promoters. Pathogenic mutations in *KDM5C* cause  
22 Intellectual Developmental Disorder, X-linked, Syndromic, Claes-Jensen Type (MRXSCJ, OMIM: 300534), whose predominant  
23 features include intellectual disability, seizures, aberrant aggression, and autistic behaviors<sup>12–14</sup>. *Kdm5c* knockout (-KO)  
24 mice recapitulate key MRXSCJ patient phenotypes, including hyperaggression, increased seizure propensity, and learning  
25 impairments<sup>10,15</sup>. Unexpectedly, RNA sequencing (RNA-seq) of the *Kdm5c*-KO hippocampus revealed ectopic expression of  
26 testis genes within the brain<sup>10</sup>. It is currently unknown if this dysregulation of brain tissue identity further impairs *Kdm5c*-KO  
27 neurodevelopment and if ectopic gene expression within the *Kdm5c*-KO brain is unique to testis genes.

28 • one key process chrom reg are involved in are is distinguishing bw germ + soma cell identity

29 Distinguishing between the germline (cells that pass on their genetic material, e.g. sperm and eggs) and the soma (cells  
30 that perform all other bodily functions) is a key feature of multicellularity and occurs during early embryogenesis. In mammals,  
31 chromatin regulators play a key role in decommissioning germline genes in somatic cells during the transition from naïve to  
32 primed pluripotency by placing repressive histone H2A lysine 119 monoubiquitination (H2AK119ub1)<sup>16</sup>, histone 3 lysine 9

33 trimethylation (H3K9me3)<sup>16,17</sup>, and DNA CpG methylation<sup>17-19</sup> at germline gene promoters. Systematically characterizing  
34 KDM5C's role in germline gene repression during early embryogenesis will unveil key mechanisms underlying the demarcation  
35 between soma and germline identity and while also providing molecular footholds to test the impact of ectopic germline genes  
36 on neurodevelopment.

37 To illuminate KDM5C's role in tissue identity, here we characterized the aberrant transcription of tissue-enriched genes  
38 within the *Kdm5c*-KO brain and epiblast-like stem cells (EpiLCs), an *in vitro* model of the post-implantation embryo. We  
39 observed general dysregulation of tissue-enriched genes in both the *Kdm5c*-KO brain and EpiLCs, including misexpression  
40 of genes typically highly enriched within the testis, liver, muscle, and ovary. Both the *Kdm5c*-KO amygdala and hippocampus  
41 had significant enrichment of testis genes that are typically uniquely expressed in germ cells. While the *Kdm5c*-KO brain  
42 primarily expressed germline genes important for late spermatogenesis, *Kdm5c*-KO EpiLCs aberrantly expressed key drivers  
43 of germline identity and meiosis, including *Dazl* and *Stra8*. KDM5C was highly enriched at germline gene promoters in  
44 EpiLCs but only bound to a subset of germline genes expressed in *Kdm5c*-KO cells, indicating germline-enriched mRNAs  
45 can be aberrantly transcribed through indirect mechanisms. Finally, we found KDM5C promotes the long-term silencing of  
46 germline genes in somatic cells by aiding the placement of DNA methylation in EpiLCs through H3K4me2/3 removal. Thus,  
47 we propose KDM5C plays a fundamental role in the development of tissue identity during early embryogenesis, including the  
48 establishment of the soma-germline boundary.

## 49 Results

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

- 51 • note: should I compare amygdala and hippocampus? scandaglia only looked at hippocampus

52 Previous RNA sequencing (RNA-seq) in the adult hippocampus revealed ectopic expression of some testis genes within  
53 the *Kdm5c* knockout (-KO) brain<sup>10</sup>. Since tissue-enriched genes have not been systematically characterized in the *Kdm5c*-KO  
54 brain, it is currently unclear if this erosion of brain tissue identity is a major consequence of *Kdm5c* loss and if it is unique to  
55 testis-enriched genes. Therefore, we first globally assessed the expression of genes enriched in 17 mouse tissues<sup>20</sup> in our  
56 published mRNA-seq datasets of the adult amygdala and hippocampus in mice with constitutive knockout of *Kdm5c*<sup>21</sup>.

57 We found a large proportion of genes that are significantly upregulated within the *Kdm5c*-KO brain (DESeq2<sup>22</sup>, log2 fold  
58 change > 0.5, q < 0.1) are typically enriched in non-brain tissues (Amygdala: 35%, Hippocampus: 24%) (Figure 1A-B). The  
59 majority of tissue-enriched differentially expressed genes (tissue-enriched DEGs) were testis genes (Figure 1A-C). Even  
60 though the testis has the largest total number of tissue-biased genes compared to any other tissue (2,496 genes), testis-biased  
61 DEGs were significantly enriched for both brain regions (Amygdala p = 1.83e-05, Odds Ratio = 5.13; Hippocampus p =  
62 4.26e-11, Odds Ratio = 4.45, Fisher's Exact Test). One example of a testis-enriched DEG is *FK506 binding protein 6* (*Fkbp6*),  
63 a known regulator of piRNA expression and meiosis in germ cells<sup>23,24</sup> (Figure 1C).

64 In addition to the high enrichment of testis genes, we also identified aberrant expression of other tissue-enriched genes  
65 within the *Kdm5c*-KO brain. Although the *Kdm5c*-KO mice were male, we observed significant enrichment of ovary-biased  
66 DEGs in both the amygdala and hippocampus (Amygdala p = 0.00574, Odds Ratio = 18.7; Hippocampus p = 0.048, Odds  
67 Ratio = 5.88, Fisher's Exact) (Figure 1D). Ovary-enriched DEGs included *Zygotic arrest 1* (*Zar1*), which was recently shown  
68 to sequester mRNAs in oocytes for meiotic maturation and early zygote development<sup>25</sup> (Figure 1D). Although not consistent  
69 across brain regions, we also found significant enrichment of DEGS biased towards two non-gonadal tissues - the liver

70 (Amygdala p = 0.0398, Odds Ratio = 6.58, Fisher's Exact Test) and the muscle (Hippocampus p = 0.0104, Odds Ratio = 6.95, 71 Fisher's Exact Test). An example of a liver-biased DEG dysregulated in both the hippocampus and amygdala is *Apolipoprotein* 72 *C-I* (*Apoc1*), which is involved in lipoprotein metabolism (Figure 1E). Testis, ovary, and liver-enriched DEGs showed little to no 73 expression in the developing and adult wild-type brain, yet our mRNA-seq data indicate they are polyadenylated and spliced 74 into mature transcripts (Figure 1C-E). Of note, we did not observe enrichment of brain-enriched genes (Amygdala p = 1; 75 Hippocampus p = 0.74, Fisher's Exact), despite the fact these are brain samples and the brain has the second highest total 76 number of tissue-enriched genes (708 genes). Together, these results suggest misexpression of tissue-enriched genes within 77 the brain is a major effect of KDM5C loss.

78 **Germline genes are misexpressed in the *Kdm5c*-KO brain**

79 • Intriguingly, some of the ectopic testis transcripts identified within the *Kdm5c*-KO hippocampus have known functions 80 unique to germ cells<sup>10</sup>, suggesting KDM5C may play a role in demarcating somatic versus germline identity.

81 The testis contains both germ cells (meiotic cells, e.g. spermatogonia) and somatic cells (non-meiotic, e.g. Leydig cells) 82 that support hormone production and germline functions. Intriguingly, many *Kdm5c*-KO testis and ovary enriched-DEGs have 83 germline-specific functions, suggesting *Kdm5c*-KO cells fail to distinguish between the soma and germline. To test if this holds 84 true for all *Kdm5c*-KO testis-biased DEGs, we first assessed their known functions through gene ontology analysis. We 85 found *Kdm5c*-KO testis-enriched DEGs high enrichment of germline-relevant ontologies, including spermatid development 86 (GO: 0007286, p.adjust = 6.2e-12) and sperm axoneme assembly (GO: 0007288, p.adjust = 2.45e-14) (Figure 2A).

87 To further validate if these testis DEGs are truly germline genes, we then evaluated their expression in somatic versus germ 88 cells within the testis. We first compared their expression in the testis with germ cell depletion<sup>26</sup>, which was accomplished by 89 heterozygous *W* and *Wv* mutations in the enzymatic domain of *c-Kit* (*Kit*<sup>W/Wv</sup>) that prevent the maturation of germ cells<sup>27</sup>. 90 Almost all *Kdm5c*-KO testis-enriched DEGs lost expression with germ cell depletion (Figure 2B). The only testis-enriched 91 DEG that did not show considerable downregulation with germline depletion was *FK506 binding protein 6* (*Fkbp6*), the 92 aforementioned testis gene that regulates piRNA expression and meiosis in germ cells<sup>23,24</sup>. We then assessed testis- 93 enriched DEG expression in a published single cell RNA-seq dataset that identified cell type-specific markers within the 94 testis<sup>28</sup>. We found that while some testis-enriched DEGs were classified as specific markers for different germ cell types 95 (e.g. spermatogonia, spermatocytes, round spermatids, and elongating spermatids) none marked somatic cells (Figure 2C). 96 Together, these data suggest the somatic *Kdm5c*-KO brain aberrantly expresses germline genes.

97 We then wanted to more deeply characterize germline gene misexpression with *Kdm5c* loss, but lacked a comprehensive 98 list of mouse germline-enriched genes. To facilitate downstream analyses, we generated a curated list of germline-enriched 99 genes using currently available RNA-seq datasets in *Kit*<sup>W/Wv</sup> mice. Wild-type and *Kit*<sup>W/Wv</sup> datasets included males and females 100 at embryonic day 12, 14, and 16<sup>29</sup>, as well as adult male testes<sup>26</sup>. We defined genes as germline-enriched if their expression 101 met the following criteria: 1) their expression is greater than 1 FPKM in wild-type gonads 2) their expression in any adult 102 wild-type, non-gonadal tissue<sup>20</sup> does not exceed 20% of their maximum expression in the wild-type germline, and 3) their 103 expression in the germ cell-depleted germline, for any sex or time point, does not exceed 20% of their maximum expression 104 in the wild-type germline. These criteria yielded 1,288 germline-enriched genes (Figure 2D), which was hereafter used as a 105 resource for assessing germline gene misexpression with *Kdm5c* loss (Supplementary table 1).

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

107 Misexpression of germline genes in the adult brain suggests *Kdm5c*-KO cells fail to demarcate between germline and  
108 somatic identity. Germ cells are typically distinguished from somatic cells soon after the embryo implants into the uterine  
109 wall<sup>30,31</sup> when a subset of epiblast stem cells become the primordial germ cells (PGCs) while the remainder differentiate into  
110 the ectoderm, mesoderm, and endoderm to form the somatic tissues<sup>32</sup>. This developmental time point can be modeled *in*  
111 *vitro* through differentiation of embryonic stem cells (ESCs) into post-implantation epiblast-like stem cells (EpiLCs) (Figure 3A,  
112 top). Previous studies have demonstrated that while some germline-enriched genes are also expressed in embryonic stem  
113 cells (ESCs) and in the 2-cell stage<sup>33–35</sup>, they are silenced as they differentiate into EpiLCs<sup>17</sup>. Therefore, we assessed if  
114 KDM5C was necessary for embryonic silencing of germline genes by evaluating the impact of *Kdm5c* loss in EpiLCs.

115 We first identified *Kdm5c*-KO EpiLC DEGs in of our previously published RNA-seq dataset<sup>36</sup> (DESeq2, log2 fold change  
116 > 0.5, q < 0.1) and assessed tissue-enriched gene expression. Similar to the *Kdm5c*-KO brain, we observed general  
117 dysregulation of tissue-enriched genes, with the largest number of genes belonging to the brain and testis, although they were  
118 not significantly enriched (Figure 3B). Using the curated list of germline genes generated above, we found *Kdm5c*-KO EpiLCs  
119 aberrantly expressed 54 germline-enriched genes, including the previously characterized hippocampal DEG<sup>10</sup> *Cytochrome*  
120 *C, testis-specific (Cyct)* (Figure 3C). Although we observed aberrant expression of many tissue-enriched genes, we did not  
121 observe any significant difference in primed pluripotency genes (Figure 3D) or gross changes in *Kdm5c*-KO cell morpholgy  
122 during differentiation (Figure 3E), indicating KDM5C loss does not impair EpiLC formation.

123 We then compared EpiLC germline DEGs to those expressed in the *Kdm5c*-KO brain to determine if all germline DEGs,  
124 like *Cyct*, are constitutively dysregulated or if they can change over the course of development. We found this was primarily  
125 not the case, as the majority of germline DEGs expressed in EpiLCs and the brain were unique, with only *Cyct* shared across  
126 all sequencing datasets (Figure 3F). We then compared the known functions of EpiLC and brain germline DEGs and found  
127 particularly high enrichment of meiosis-related gene ontologies in EpiLCs (Figure 3G), such as meiotic cell cycle process (GO:  
128 1903046, p.adjust = 1.59e-08) and meiotic nuclear division (GO: 0140013, p.adjust = 9.76e-09). While there was modest  
129 enrichment of meiotic gene ontologies in both brain regions, the *Kdm5c*-KO hippocampus primarily expressed late-stage  
130 spermatogenesis genes, such those involved in the sperm axoneme structure.

131 Interestingly, DEGs unique to *Kdm5c*-KO EpiLCs included key drivers of germline identity, such as *Stimulated by retinoic*  
132 *acid 8 (Stra8)* and *Deleted in azoospermia like (Dazl)* (Figure 3H). These genes are typically expressed during embryonic  
133 germ cell development to commit PGCs to the germline fate, but are also expressed later in life to trigger meiotic gene  
134 expression programs<sup>37–39</sup>. Of note, some germline genes, including *Dazl*, are also expressed in the two-cell embryo<sup>34,40</sup>.  
135 However, we did not see misexpression of two-cell embryo-specific genes, like *Duxf3 (Dux)* (q = 0.337) and *Zscan4d* (q =  
136 0.381), indicating *Kdm5c*-KO in EpiLCs do not revert back to a 2-cell state (Figure 3H).

137 We were particularly interested in the aberrant transcription of *Dazl*, since it is essential for germ cell development and  
138 promotes the translation of germline mRNAs<sup>41</sup>. A significant portion of germline transcripts misexpressed in *Kdm5c*-KO  
139 EpiLCs are known binding targets of DAZL, including *Stra8*<sup>42</sup> (p = 1.698e-07, Fisher's Exact Test). This suggests expression  
140 of DAZL protein could promote the translation of other aberrant germline transcripts, influencing their ability to impact  
141 *Kdm5c*-KO cellular function. We thus tested DAZL protein expression in *Kdm5c*-KO EpiLCs through immunocytochemistry  
142 (Figure 3I). We observed about 25% of *Kdm5c*-KO EpiLCs expressed DAZL protein and it was localized to the cytoplasm (p =  
143 0.0015, Welch's t-test), consistent with the pattern of DAZL expression in spermatogonia<sup>42</sup>. Altogether these results suggest  
144 tissue-specific genes are misexpressed during *Kdm5c*-KO embryogenesis, including key drivers of germline identity that can  
145 be translated into protein.

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

147 • KDM5C may also be involved in this early decommissioning of germline genes, as re-expression of KDM5C in knockout  
148 neurons fails to suppress their dysregulation<sup>10</sup>.

149 Previous work suggests KDM5C represses germline genes during early development, as re-expression of KDM5C in  
150 knockout neuronal cultures fails to suppress hippocampal germline DEGs and KDM5C is not bound to their promoters in  
151 neurons<sup>10</sup>. There is some evidence KDM5C binds to select germline gene promoters in ESCs<sup>10</sup>, including *Dazl*<sup>40,43</sup>. As  
152 KDM5C's binding at germline gene promoters has not been systematically characterized, it is currently unclear if KDM5C is  
153 enriched at germline gene promoters, what types of germline genes KDM5C regulates, and if its binding is maintained at any  
154 germline genes in neurons.

155 To further characterize the mechanism behind *Kdm5c*-KO germline gene misexpression, we analyzed KDM5C chromatin  
156 immunoprecipitation followed by DNA sequencing (ChIP-seq) datasets in EpiLCs<sup>36</sup> and primary neuron cultures (PNCs) from  
157 the cortex and hippocampus<sup>15</sup>. EpiLCs had a higher total number of KDM5C peaks than PNCs (EpiLCs: 5,808, PNCs: 1,276,  
158 MACS2 q < 0.1 and fold enrichment > 1, removal of *Kdm5c*-KO false positive peaks) and KDM5C was primarily localized to  
159 gene promoters in both cell types (EpiLCs: 4,190, PNCs: 745 +/- 500bp from the TSS), although PNCs showed increased  
160 localization to non-promoter regions (Figure 4A).

161 The majority of promoters bound by KDM5C in PNCs were also bound in EpiLCs (513 shared promoters), however  
162 a large portion of gene promoters were only bound by KDM5C in EpiLCs (3677 EpiLC only promoters) (Figure 4B). We  
163 then performed gene ontology analysis to compare the known functions of genes with KDM5C bound to their promoter in  
164 EpiLCs and PNCs. While there were no ontologies significantly enriched for genes only bound by KDM5C in PNCs, gene  
165 ontologies for genes shared between PNCs and EpiLCs were enriched for functions involving nucleic acid turnover, such  
166 as deoxyribonucleotide metabolic process (GO:0009262, p.adjust = 8.28e-05) (Figure 4C). Germline-specific ontologies  
167 were only enriched in promoters unique to EpiLCs, such as meiotic nuclear division (GO: 0007127 p.adjust = 6.77e-16)  
168 and meiotic cell cycle process (GO:1903046, p.adjust = 5.05e-16) (Figure 3C). We then evaluated KDM5C binding around  
169 the transcription start site (TSS) of all germline-enriched genes. While KDM5C was bound to a subset of germline gene  
170 promoters in EpiLCs, it was not bound to any in PNCs (Figure 4D). Together, this suggests KDM5C is significantly enriched at  
171 a subset of germline gene promoters in EpiLCs, including meiotic genes, but does not regulate germline genes in neurons.

172 We then compared KDM5C binding at RNA-seq DEG promoters to determine if the germline mRNAs expressed in the  
173 *Kdm5c*-KO brain and EpiLCs are direct targets of KDM5C (Figure 4E). About one third of EpiLC-specific and brain-specific  
174 (hippocampus, amygdala, or both) germline DEGs were bound by KDM5C in EpiLCs (EpiLC only: 36%, Brain only: 33.3%).  
175 Some notable differences in KDM5C binding for EpiLC-specific DEGs included *Dazl* and *Stra8*, two key drivers of germline  
176 identity discussed above. Although both mRNAs are expressed in *Kdm5c*-KO EpiLCs, KDM5C is only bound to *Dazl*'s  
177 promoter and not *Stra8*'s in EpiLCs (Figure 4F). In contrast to the unique DEGs, 3 out of the 4 genes dysregulated in both  
178 the brain and EpiLCs are bound by KDM5C around the TSS (Figure 4G). Again, we did not observe any KDM5C binding  
179 at germline gene promoters in PNCs, even for brain-specific DEGs (Figure 4H). Altogether, this suggests the majority of  
180 germline mRNAs expressed in *Kdm5c*-KO cells are dysregulated independent of direct KDM5C recruitment to their promoters  
181 during embryogenesis.

182 **notes:** - do Direct vs indirect DEGs motif analysis

183 **KDM5C erases H3K4me3 to promote long-term repression of germline genes via DNA methylation**

184     Although KDM5C is generally thought to suppress transcription through erasure of histone 3 lysine 4 di- and trimethylation  
185 (H3K4me2/3)<sup>11</sup>, recent studies in ESCs have suggested KDM5C's repression *Dazl* is independent of its catalytic activity<sup>40</sup>.  
186 Somatic repression of germline genes is typically established during the transition between naïve and primed pluripotency,  
187 which modeled *in vitro* as ESCs to EpiLC differentiation. In ESCs, chromatin regulators place repressive histone modifications  
188 at germline gene promoters, including histone 2A 119 monoubiquitination (H2AK119ub1) and histone 3 lysine 9 trimethylation  
189 (H3K9me3)<sup>16,17,44</sup>. Germline genes are then silenced long-term in EpiLCs by *de novo* placement of DNA CpG methylation  
190 (DNAm)<sup>17</sup>. Although KDM5C's catalytic activity is not required to suppress *Dazl* in ESCs, KDM5C may promote long-term  
191 germline gene silencing via H3K4me3 removal since H3K4me3 can impede DNAm placement<sup>45,46</sup> and DNAm is lost at  
192 select germline gene promoters in the hippocampus<sup>10</sup>. Because KDM5C's role in germline gene repression has only been  
193 characterized in ESCs and in the mature brain, it is currently unclear to what extent KDM5C is involved during transition  
194 between ESCs and EpiLCs and if its catalytic activity is required for long-term silencing.

195     To elucidate KDM5C's role in germline gene silencing, we first characterized KDM5C's substrates, histone 3 lysine 4  
196 di- and trimethylation (H3K4me2/3) in our previously published ChIP-seq datasets in wild type and *Kdm5c*-KO amygdala<sup>21</sup>  
197 and EpiLCs<sup>36</sup>. In congruence with previous work in the *Kdm5c*-KO hippocampus<sup>10</sup>, we observed aberrant accumulation of  
198 H3K4me3 around the transcription start site (TSS) of germline genes in the *Kdm5c*-KO amygdala (Figure 5A). We additionally  
199 found a marked increase in H3K4me2 around the TSS of germline genes in *Kdm5c*-KO EpiLCs (Figure 5B).

200     We then evaluated KDM5C's embryonic role in germline gene silencing during ESC to EpiLC differentiation. We first  
201 characterized KDM5C's mRNA and protein expression at 0, 24, and 48 hours of differentiation (Figure 5C). While *Kdm5c*  
202 mRNA steadily decreased from 0 to 48 hours of differentiation (Figure 5D), KDM5C protein initially increased from 0 to  
203 24 hours but then decreased to near knockout levels by 48 hours (Figure 5E). We then performed whole genome bisulfite  
204 sequencing in wild-type and *Kdm5c*-KO ESCs and 96 hour extended EpiLCs (exEpiLCs) to determine KDM5C's role in the  
205 initial placement of DNA methylation at germline gene promoters. While wild-type cells accumulated high levels of DNA  
206 methylation at germline gene promoters over the course of ESCs to exEpiLC differentiation, DNA methylation was markedly  
207 reduced in *Kdm5c*-KO exEpiLCs (Figure 5F).

- 208     • Catalytic activity  
209     • Altogether, this suggests KDM5C is dynamically regulated during ESC to EpiLC differentiation and promotes germline  
210 gene silencing via removal of H3K4me2/3 during early embryogenesis.  
211     • **note:** should I test if increase in H3K4me2 and H3K4me3 was highest in *Kdm5c*-KO cells at genes bound by KDM5C  
212 at their promoter in EpiLCs? Don't think it's that impactful

213 **Experimental Procedures**

214 **Discussion**

215     In the above study, we demonstrate KDM5C's pivotal role in the development of tissue identity, resulting in substantial  
216 misexpression of tissue-enriched genes within the adult *Kdm5c*-KO hippocampus and amygdala. We defined tissue-enriched  
217 genes based on the criteria used in a previously published dataset of 17 C57/Bl6J mouse tissues<sup>20</sup>, which defined genes as

218 tissue-enriched if they had more than 4-fold higher expression when compared to any other tissue. In addition to testis genes  
219 identified previously<sup>10</sup>, we found significant enrichment of muscle, liver, and even ovary-biased genes aberrantly expressed  
220 within the male *Kdm5c*-KO brain. It is currently unclear what the impact of these tissue-enriched genes are upon *Kdm5c*-KO  
221 brain function and if they ultimately contribute to MRXSCJ-related phenotypes. While the majority of tissue-enriched DEGs  
222 were testis and ovary genes with no known brain functions, select liver and muscle-biased DEGs do have known roles in  
223 the wild-type brain. For example, *Apolipoprotein C-I* (*Apoc1*) is a lipid transport gene highly enriched within the liver[XXX],  
224 but is lowly expressed in the wild-type brain. Intriguingly, *Apoc1* overexpression in the mouse brain can impair learning and  
225 memory<sup>47</sup> and is implicated in Alzheimer's disease in humans<sup>48</sup>. Our results suggest KDM5C fine-tunes the expression of  
226 tissue-enriched genes like *Apoc1* to match the level required for proper brain function. However, futher studies are required to  
227 detrmine if these genes contribute to *Kdm5c*-KO neuronal impairments and MRXSCJ pathology.

228 A substantial portion of genes upregulated in *Kdm5c*-KO brain are testis genes that have no known function within the  
229 brain. Through the use of publically available RNA-seq datasets, we demonstrated these testis-enriched DEGs are typically  
230 unique to germ cells. Misexpression of germline genes in the brain suggests *Kdm5c*-KOs fail to demarcate between somatic  
231 and germline lineages, a key feature of multicellularity and sexual reproduction. Previous studies have demonstrated germline  
232 genes are decommissioned in somatic cells by multiple chromatin-modifying enzymes, but have primarily focused on the  
233 repression of key marker genes. To globally characterize KDM5C's role in germline gene repression, we curated a list of  
234 mouse germline-enriched genes using publically available germ cell-depleted RNA-seq datasets. This resource enabled us to  
235 identify 1) the extent of germline gene dysregulation in *Kdm5c*-KO cells, 2) the types of germline genes misexpressed at  
236 different developmental time points, and 3) which types of germline genes are directly or indirectly regulated by KDM5C.

237 We found this dysregulation in tissue identity likely begins during early emrbyogenesis, as germline genes and other  
238 tissue-enriched genes are aberrantly expressed in *Kdm5c*-KO epiblast-like cells (EpiLCs), an *in vitro* model of the post-  
239 implantation embryo. *In vivo*, germline genes are typically decommissioned in epiblast stem cells and remain silenced as the  
240 epiblast differentiates into the body's somatic tissues[]. However, a small subset of epiblast stem cells will receive signals  
241 to reactivate germline gene expression to become the primordial germ cells (PGCs) that will ultimately form the mature  
242 germline[]. This process can be mimicked *in vitro* by differentiating EpiLCs into primordial germ cell-like cells (PGCLCs)[XXX].  
243 Thus, misexpression of germline genes in *Kdm5c*-KO EpiLCs might suggest they are progressing beyond EpiLC differentiation  
244 and becoming PGCLCs. Yet, *Kdm5c*-KO EpiLCs had proper expression of EpiLC marker genes for primed pluripotency and  
245 we observed no difference in cellular morphology during ESC to EpiLC differentiation. Furthermore, we saw no significant  
246 change in *Otx2* expression, an epiblast stem cell marker that is known to repress epiblast and EpiLC differentiation into  
247 PGC and PGCLCs<sup>zhangOTX2RestrictsEntry2018?</sup>. This, in conjunction with *Kdm5c*-KO mice being viable, suggests germline gene  
248 expression is occurring ectopically in conjunction with typical developmental programs, rather than a complete shift to germline  
249 identity.

250 • Only a small number of germline genes were shared between *Kdm5c*-KO EpiLCs and in the brain.

251 dysregulation of tissue identity

252 • In line with previous work on other chromatin regulators, we found dysregulation of *Kdm5c*-KO tissue identity began  
253 during early embryogenesis. While *Kdm5c*-KO epiblast-like cells (EpiLCs)

254 • KDM5C represses germline genes during early embryogenesis

255 – Found this dysregulation in tissue identity begins during *Kdm5c*-KO early embryogenesis

- 256 – EpiLC express early regulators, Brain late sperm - program seems to be continuing across development  
257 – Importantly, even though germline genes are misexpressed, markers of EpiLC differentiation and EpiLC morphology  
258 is unimpaired.
- 259 \* Epiblast can become primordial germ cells  
260 \* Otx2 is expressed in EpiLCs and is known to repress PGC identity. It's properly expressed in *Kdm5c*-KO  
261 EpiLCs, further supporting they aren't just becoming PGCs <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6485399/>  
262 \* Germline Program happening in the background of typical development.
- 264 • One of the genes misexpressed in *Kdm5c*-KO EpiLCs is Dazl, *Deleted in azoospermia like (Dazl)*, a key regulator of  
265 germline development that promotes the translation of germline-specific transcripts[XXX].  
266 – 2 other studies of dazl regulators in ESCs also found KDM5C represses dazl.  
267 – While crucial for germ cell development, Dazl is also expressed in ESCs and at the 2-cell stage.  
268 – Some genes important for germ cell, ESC, and 2-cell development given they are important for self-renewal.  
269  
270 – We did not observe dysregulation of 2-cell-specific regulators like Dux.  
271 – We found Dazl was expressed in *Kdm5c*-KO EpiLCs, when *Dazl* is typically decommissioned. This indicates  
272 KDM5C loss results in long-term ectopic expression.  
273 – We additionally found DAZL protein is ectopically expressed and localized to the cytoplasm. Given that a significant  
274 number of KDM5C-KO germline DEGs are DAZL targets, it may promote the ectopic germline program.  
275 – Very recently two other studies identified KDM5C represses Dazl in ESCs.  
276 – \* KDM5C was very recently shown to repress *Deleted in azoospermia like (Dazl)*, a key regulator of germline  
277 development, in mouse embryonic stem cells (ESCs)<sup>40,43</sup>. However, KDM5C binding and *Kdm5c*-KO germline  
278 gene misexpression has yet to be globally characterized during early embryogenesis. Given that *Dazl* and  
279 other germline-enriched genes can also be expressed in ESCs and at the 2-cell stage, it is unclear if KDM5C  
280 has a direct role in the long-term germline gene silencing that occurs in the post-implantation epiblast.
- 281 • We globally characterized KDM5C's repression of germline genes during early embryogenesis and in mature neurons.  
282 – In line with previous work, we found KDM5C did not regulate germline genes in neurons.  
283 – KDM5C-bound genes in EpiLCs were enriched for germline ontologies, suggesting a major role of KDM5C during  
284 embryogenesis  
285 – While KDM5C directly binds some germline genes, including *Dazl*, many of the genes dysregulated in *Kdm5c*-KO  
286 were not direct targets.  
287 \* Stra8 notable exception  
288 \* Brain genes, including late-stage spermatogenesis genes  
289 \* Suggests germline genes can be dysregulated direct and indirect of KDM5C regulation  
290 \* Further supports germline programs can be ectopically activated  
291 \* Talk about different motifs if we do see differences there and if it explains direct vs indirect dysregulation

- 292 · Could also mention PRC1.6/KDM5C binds pcgf6 in ESCs

293 • We demonstrated KDM5C is important for the transition between histone-mediated to DNAme-mediated silencing of  
294 germline genes during the transition from naïve to primed pluripotency.

295 – Loss of DNAme can last throughout life at at least two germline gene promoters (hippocampus barco)

296 – KDM5C could be important for DNAme is that KDM5C erases H3K4me3 which can impede CpGme.

297 \* In support of this, KDM5C is highly enriched at the CpG islands near germline TSS that are methylated in  
298 EpiLCs

299 – However, Recently KDM5C's catalytic activity was found to be unnecessary for dazl suppression in ESCs.

300  
301 – Since DNAme is not placed until EpiLC stage, KDM5C's catalytic activity may be required for long-term silencing  
302 of germline genes.

303 – This would be the first (?) example in which removal of an active mark is required for germline gene repression.

304 • In summary, we demonstrate loss of the X-linked intellectual disability gene KDM5C leads to widespread dysregulation  
305 of brain tissue identity, including misexpression of germline genes in the somatic brain.

306 – In EpiLCs, KDM5C directly represses key drivers of germline identity like Dazl, likely through PRC1.6 recruitment  
307 and promoting CpG methylation. However, *Kdm5c*-KO also ectopically expresses germline genes activated  
308 indirectly, including *Stra8*.

309  
310 – The germline developmental program to some extent continues ectopically during *Kdm5c*-KO development,  
311 resulting in aberrant transcription of late stage spermatogenesis genes later in life.

312 – These results define KDM5C's role in the demarcation between soma and germline identity and offer a window  
313 into potential targets to assess the deleterious effects these ectopic genes on neurodevelopment.

## 314 Discussion notes

315 • Our results indicate KDM5C is a pivotal repressor of germline programs in somatic cells.

316 • Studies on other chromatin regulators have focused on key marker genes to identify germline gene misexpression,  
317 such as *Dazl*.

318 • Using a germline-depleted mouse model, we curated a list of mouse germline-enriched genes to globally assess  
319 germline gene dysregulation.

320 • Many of the genes dysregulated in the mature *Kdm5c*-KO brain were important for late stages of spermatogenesis,  
321 such as those important for sperm flagellar structure. Contrastingly, *Kdm5c*-KO EpiLCs aberrantly expressed key drivers  
322 of germ cell identity, including *Dazl* and *Stra8*.

323 • While a significant portion of KDM5C-bound promoters are germline genes, many germline genes expressed during  
324 *Kdm5c*-KO embryogenesis are not directly bound by kdm5c.

- 325 • One notable example is Stra8, a transcription factor important for germ cell specific transcription and meiotic initiation
- 326 •
- 327 While Directly binds to a subset of germline genes during early embryogenesis - KDM5C binds to a subset of germline
- 328 genes during early embryogenesis
- 329 • The including the demarcation between soma and germline fates.
- 330 the *Kdm5c*-KO brain also Previous work identified testis-enriched genes in *Kdm5c* genes
- 331 –
- 332 – However unlike the gonadal-biased DEGs,
- 333 • The demarcation of the germline versus soma is a key feature of multicellularity and sexually dimorphic reproduction
- 334 • Anything known about tissue-biased gene expression in other H3K4me regulators?
- 335 • Our data suggests the germline developmental program is occurring ectopically as *Kdm5c*-KOs progresses through
- 336 somatic tissue development
- 337 • tissue-biased gene expression:
- 338 • Altogether, these data indicate that while some germline genes are misexpressed due to direct loss of KDM5C binding
- 339 during emryogenesis, secondary downstream mechanisms can also promote their aberrant transcription.
- 340 – This shift from meiotic genes to later spermatogenesis genes in the hippocampus suggests the germline devel-
- 341 opmental program could occur ectopically as *Kdm5c*-KO cells progresses through somatic tissue development.
- 342 **note: this is strengthened by the ChIP-seq data since KDM5C is not directly bound to many brain/flagellar**
- 343 **DEGs. This point might be stronger in the ChIPseq figure**
- 344 • KDM5C is dynamically regulated during the window of embryonic germline gene silencing, our EpiLC ChIP-seq is likely
- 345 catching the tail end of KDM5C's main involvement.
- 346 • Papers to read/reference:
- 347 – Reconstitution of the Mouse Germ Cell Specification Pathway in Culture by Pluripotent Stem Cells: [https://www.cell.com/fulltext/S0092-8674\(11\)00771-9](https://www.cell.com/fulltext/S0092-8674(11)00771-9)
- 348 – 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/>

## 351 References

- 352 1. Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. *Nature* 403, 41–45. <https://doi.org/10.1038/47412>.
- 353
- 354 2. Jenuwein, T., and Allis, C.D. (2001). Translating the Histone Code. *Science* 293, 1074–1080. <https://doi.org/10.1126/science.1063127>.

- 356 3. Lewis, E.B. (1978). A gene complex controlling segmentation in *Drosophila*. *Nature* *276*, 565–570. <https://doi.org/10.1038/276565a0>.
- 357 4. Kennison, J.A., and Tamkun, J.W. (1988). Dosage-dependent modifiers of polycomb and antennapedia mutations in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* *85*, 8136–8140. <https://doi.org/10.1073/pnas.85.21.8136>.
- 359 5. Gabriele, M., Lopez Tobon, A., D'Agostino, G., and Testa, G. (2018). The chromatin basis of neurodevelopmental disorders: Rethinking dysfunction along the molecular and temporal axes. *Prog Neuropsychopharmacol Biol Psychiatry* *84*, 306–327. <https://doi.org/10.1016/j.pnpbp.2017.12.013>.
- 361 6. Zhou, Z., Hong, E.J., Cohen, S., Zhao, W.-N., Ho, H.-Y.H., Schmidt, L., Chen, W.G., Lin, Y., Savner, E., Griffith, E.C., et al. (2006). Brain-specific phosphorylation of MeCP2 regulates activity-dependent Bdnf transcription, dendritic growth, and spine maturation. *Neuron* *52*, 255–269. <https://doi.org/10.1016/j.neuron.2006.09.037>.
- 363 7. Hirabayashi, Y., Suzki, N., Tsuboi, M., Endo, T.A., Toyoda, T., Shinga, J., Koseki, H., Vidal, M., and Gotoh, Y. (2009). Polycomb Limits the Neurogenic Competence of Neural Precursor Cells to Promote Astrocytic Fate Transition. *Neuron* *63*, 600–613. <https://doi.org/10.1016/j.neuron.2009.08.021>.
- 365 8. Kassis, J.A., Kennison, J.A., and Tamkun, J.W. (2017). Polycomb and Trithorax Group Genes in *Drosophila*. *Genetics* *206*, 1699–1725. <https://doi.org/10.1534/genetics.115.185116>.
- 367 9. Schaefer, A., Sampath, S.C., Intrator, A., Min, A., Gertler, T.S., Surmeier, D.J., Tarakhovsky, A., and Greengard, P. (2009). Control of cognition and adaptive behavior by the GLP/G9a epigenetic suppressor complex. *Neuron* *64*, 678–691. <https://doi.org/10.1016/j.neuron.2009.11.019>.
- 369 10. Scandaglia, M., Lopez-Atalaya, J.P., Medrano-Fernandez, A., Lopez-Cascales, M.T., Del Blanco, B., Lipinski, M., Benito, E., Olivares, R., Iwase, S., Shi, Y., et al. (2017). Loss of Kdm5c Causes Spurious Transcription and Prevents the Fine-Tuning of Activity-Regulated Enhancers in Neurons. *Cell Rep* *21*, 47–59. <https://doi.org/10.1016/j.celrep.2017.09.014>.
- 371 11. Iwase, S., Lan, F., Bayliss, P., De La Torre-Ubieta, L., Huarte, M., Qi, H.H., Whetstine, J.R., Bonni, A., Roberts, T.M., and Shi, Y. (2007). The X-Linked Mental Retardation Gene SMCX/JARID1C Defines a Family of Histone H3 Lysine 4 Demethylases. *Cell* *128*, 1077–1088. <https://doi.org/10.1016/j.cell.2007.02.017>.
- 373 12. Claes, S., Devriendt, K., Van Goethem, G., Roelen, L., Meireleire, J., Raeymaekers, P., Cassiman, J.J., and Fryns, J.P. (2000). Novel syndromic form of X-linked complicated spastic paraplegia. *Am J Med Genet* *94*, 1–4.
- 375 13. Jensen, L.R., Amende, M., Gurok, U., Moser, B., Gimmel, V., Tzschach, A., Janecke, A.R., Tariverdian, G., Chelly, J., Fryns, J.P., et al. (2005). Mutations in the JARID1C gene, which is involved in transcriptional regulation and chromatin remodeling, cause X-linked mental retardation. *Am J Hum Genet* *76*, 227–236. <https://doi.org/10.1086/427563>.
- 377 14. Carmignac, V., Nambot, S., Lehalle, D., Callier, P., Moortgat, S., Benoit, V., Ghoumid, J., Delobel, B., Smol, T., Thuillier, C., et al. (2020). Further delineation of the female phenotype with KDM5C disease causing variants: 19 new individuals and review of the literature. *Clin Genet* *98*, 43–55. <https://doi.org/10.1111/cge.13755>.
- 379 15. Iwase, S., Brookes, E., Agarwal, S., Badeaux, A.I., Ito, H., Vallianatos, C.N., Tomassy, G.S., Kasza, T., Lin, G., Thompson, A., et al. (2016). A Mouse Model of X-linked Intellectual Disability Associated with Impaired Removal of Histone Methylation. *Cell Reports* *14*, 1000–1009. <https://doi.org/10.1016/j.celrep.2015.12.091>.
- 381 16. Endoh, M., Endo, T.A., Shinga, J., Hayashi, K., Farcas, A., Ma, K.W., Ito, S., Sharif, J., Endoh, T., Onaga, N., et al. (2017). PCGF6-PRC1 suppresses premature differentiation of mouse embryonic stem cells by regulating germ cell-related genes. *Elife* *6*. <https://doi.org/10.7554/elife.21064>.

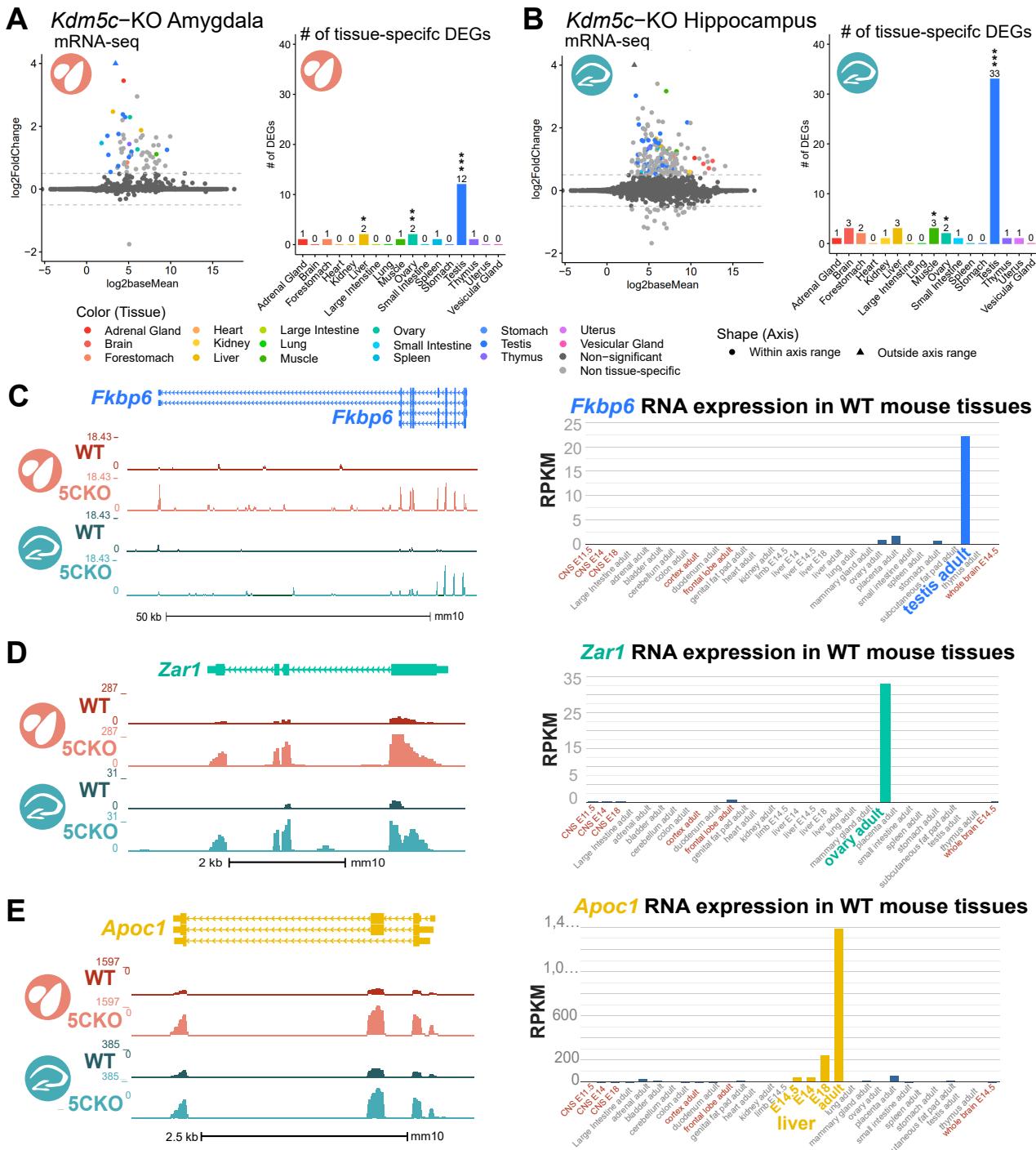
- 384 17. Mochizuki, K., Sharif, J., Shirane, K., Uranishi, K., Bogutz, A.B., Janssen, S.M., Suzuki, A., Okuda, A., Koseki, H.,  
385 and Lorincz, M.C. (2021). Repression of germline genes by PRC1.6 and SETDB1 in the early embryo precedes DNA  
methylation-mediated silencing. *Nat Commun* *12*, 7020. <https://doi.org/10.1038/s41467-021-27345-x>.
- 386 18. Velasco, G., Hubé, F., Rollin, J., Neuillet, D., Philippe, C., Bouzinba-Segard, H., Galvani, A., Viegas-Péquignot, E., and  
387 Francastel, C. (2010). Dnmt3b recruitment through E2F6 transcriptional repressor mediates germ-line gene silencing  
in murine somatic tissues. *Proc Natl Acad Sci U S A* *107*, 9281–9286. <https://doi.org/10.1073/pnas.1000473107>.
- 388 19. Hackett, J.A., Reddington, J.P., Nestor, C.E., Dunican, D.S., Branco, M.R., Reichmann, J., Reik, W., Surani, M.A.,  
389 Adams, I.R., and Meehan, R.R. (2012). Promoter DNA methylation couples genome-defence mechanisms to epigenetic  
reprogramming in the mouse germline. *Development* *139*, 3623–3632. <https://doi.org/10.1242/dev.081661>.
- 390 20. Li, B., Qing, T., Zhu, J., Wen, Z., Yu, Y., Fukumura, R., Zheng, Y., Gondo, Y., and Shi, L. (2017). A Comprehensive  
391 Mouse Transcriptomic BodyMap across 17 Tissues by RNA-seq. *Sci Rep* *7*, 4200. <https://doi.org/10.1038/s41598-017-04520-z>.
- 392 21. Vallianatos, C.N., Raines, B., Porter, R.S., Bonefas, K.M., Wu, M.C., Garay, P.M., Collette, K.M., Seo, Y.A., Dou, Y.,  
393 Keegan, C.E., et al. (2020). Mutually suppressive roles of KMT2A and KDM5C in behaviour, neuronal structure, and  
histone H3K4 methylation. *Commun Biol* *3*, 278. <https://doi.org/10.1038/s42003-020-1001-6>.
- 394 22. Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data  
395 with DESeq2. *Genome Biol* *15*, 550. <https://doi.org/10.1186/s13059-014-0550-8>.
- 396 23. Crackower, M.A., Kolas, N.K., Noguchi, J., Sarao, R., Kikuchi, K., Kaneko, H., Kobayashi, E., Kawai, Y., Kozieradzki, I.,  
397 Landers, R., et al. (2003). Essential Role of Fkbp6 in Male Fertility and Homologous Chromosome Pairing in Meiosis.  
*Science* *300*, 1291–1295. <https://doi.org/10.1126/science.1083022>.
- 398 24. Xiol, J., Cora, E., Koglgruber, R., Chuma, S., Subramanian, S., Hosokawa, M., Reuter, M., Yang, Z., Berninger, P.,  
399 Palencia, A., et al. (2012). A Role for Fkbp6 and the Chaperone Machinery in piRNA Amplification and Transposon  
Silencing. *Molecular Cell* *47*, 970–979. <https://doi.org/10.1016/j.molcel.2012.07.019>.
- 400 25. Cheng, S., Altmeppen, G., So, C., Welp, L.M., Penir, S., Ruhwedel, T., Menelaou, K., Harasimov, K., Stützer, A.,  
Blayney, M., et al. (2022). Mammalian oocytes store mRNAs in a mitochondria-associated membraneless compartment.  
401 *Science* *378*, eabq4835. <https://doi.org/10.1126/science.abq4835>.
- 402 26. Mueller, J.L., Skaletsky, H., Brown, L.G., Zaghlul, S., Rock, S., Graves, T., Auger, K., Warren, W.C., Wilson, R.K., and  
403 Page, D.C. (2013). Independent specialization of the human and mouse X chromosomes for the male germ line. *Nat Genet* *45*, 1083–1087. <https://doi.org/10.1038/ng.2705>.
- 404 27. Handel, M.A., and Eppig, J.J. (1979). Sertoli Cell Differentiation in the Testes of Mice Genetically Deficient in Germ  
405 Cells. *Biology of Reproduction* *20*, 1031–1038. <https://doi.org/10.1095/biolreprod20.5.1031>.
- 406 28. Green, C.D., Ma, Q., Manske, G.L., Shami, A.N., Zheng, X., Marini, S., Moritz, L., Sultan, C., Gurczynski, S.J., Moore,  
407 B.B., et al. (2018). A Comprehensive Roadmap of Murine Spermatogenesis Defined by Single-Cell RNA-Seq. *Dev Cell* *46*, 651–667.e10. <https://doi.org/10.1016/j.devcel.2018.07.025>.
- 408 29. Soh, Y.Q., Junker, J.P., Gill, M.E., Mueller, J.L., van Oudenaarden, A., and Page, D.C. (2015). A Gene Regulatory  
409 Program for Meiotic Prophase in the Fetal Ovary. *PLoS Genet* *11*, e1005531. <https://doi.org/10.1371/journal.pgen.1005531>.

- 410 30. Magnúsdóttir, E., and Surani, M.A. (2014). How to make a primordial germ cell. *Development* *141*, 245–252.  
411 <https://doi.org/10.1242/dev.098269>.
- 412 31. Günesdogan, U., Magnúsdóttir, E., and Surani, M.A. (2014). Primordial germ cell specification: A context-dependent  
413 cellular differentiation event [corrected]. *Philos Trans R Soc Lond B Biol Sci* *369*. <https://doi.org/10.1098/rstb.2013.0543>.
- 414 32. Bardot, E.S., and Hadjantonakis, A.-K. (2020). Mouse gastrulation: Coordination of tissue patterning, specification and  
415 diversification of cell fate. *Mechanisms of Development* *163*, 103617. <https://doi.org/10.1016/j.mod.2020.103617>.
- 416 33. Welling, M., Chen, H., Muñoz, J., Musheev, M.U., Kester, L., Junker, J.P., Mischerikow, N., Arbab, M., Kuijk, E.,  
417 Silberstein, L., et al. (2015). DAZL regulates Tet1 translation in murine embryonic stem cells. *EMBO Reports* *16*,  
791–802. <https://doi.org/10.15252/embr.201540538>.
- 418 34. Macfarlan, T.S., Gifford, W.D., Driscoll, S., Lettieri, K., Rowe, H.M., Bonanomi, D., Firth, A., Singer, O., Trono, D., and  
419 Pfaff, S.L. (2012). Embryonic stem cell potency fluctuates with endogenous retrovirus activity. *Nature* *487*, 57–63.  
<https://doi.org/10.1038/nature11244>.
- 420 35. Suzuki, A., Hirasaki, M., Hishida, T., Wu, J., Okamura, D., Ueda, A., Nishimoto, M., Nakachi, Y., Mizuno, Y., Okazaki,  
421 Y., et al. (2016). Loss of MAX results in meiotic entry in mouse embryonic and germline stem cells. *Nat Commun* *7*,  
11056. <https://doi.org/10.1038/ncomms11056>.
- 422 36. Samanta, M.K., Gayen, S., Harris, C., Maclary, E., Murata-Nakamura, Y., Malcore, R.M., Porter, R.S., Garay, P.M.,  
423 Vallianatos, C.N., Samollow, P.B., et al. (2022). Activation of Xist by an evolutionarily conserved function of KDM5C  
demethylase. *Nat Commun* *13*, 2602. <https://doi.org/10.1038/s41467-022-30352-1>.
- 424 37. Koubova, J., Menke, D.B., Zhou, Q., Capel, B., Griswold, M.D., and Page, D.C. (2006). Retinoic acid regulates  
sex-specific timing of meiotic initiation in mice. *Proc. Natl. Acad. Sci. U.S.A.* *103*, 2474–2479. <https://doi.org/10.1073/pnas.0510813103>.
- 426 38. Lin, Y., Gill, M.E., Koubova, J., and Page, D.C. (2008). Germ Cell-Intrinsic and -Extrinsic Factors Govern Meiotic  
427 Initiation in Mouse Embryos. *Science* *322*, 1685–1687. <https://doi.org/10.1126/science.1166340>.
- 428 39. Endo, T., Mikedis, M.M., Nicholls, P.K., Page, D.C., and De Rooij, D.G. (2019). Retinoic Acid and Germ Cell  
429 Development in the Ovary and Testis. *Biomolecules* *9*, 775. <https://doi.org/10.3390/biom9120775>.
- 430 40. Gupta, N., Yakhou, L., Albert, J.R., Azogui, A., Ferry, L., Kirsh, O., Miura, F., Battault, S., Yamaguchi, K., Laisné,  
431 M., et al. (2023). A genome-wide screen reveals new regulators of the 2-cell-like cell state. *Nat Struct Mol Biol*.  
<https://doi.org/10.1038/s41594-023-01038-z>.
- 432 41. Li, H., Liang, Z., Yang, J., Wang, D., Wang, H., Zhu, M., Geng, B., and Xu, E.Y. (2019). DAZL is a master translational  
433 regulator of murine spermatogenesis. *Natl Sci Rev* *6*, 455–468. <https://doi.org/10.1093/nsr/nwy163>.
- 434 42. Mikedis, M.M., Fan, Y., Nicholls, P.K., Endo, T., Jackson, E.K., Cobb, S.A., De Rooij, D.G., and Page, D.C. (2020).  
DAZL mediates a broad translational program regulating expansion and differentiation of spermatogonial progenitors.  
435 *eLife* *9*, e56523. <https://doi.org/10.7554/eLife.56523>.
- 436 43. Al Adhami, H., Vallet, J., Schaal, C., Schumacher, P., Bardet, A.F., Dumas, M., Chicher, J., Hammann, P., Daujat,  
437 S., and Weber, M. (2023). Systematic identification of factors involved in the silencing of germline genes in mouse  
embryonic stem cells. *Nucleic Acids Research* *51*, 3130–3149. <https://doi.org/10.1093/nar/gkad071>.

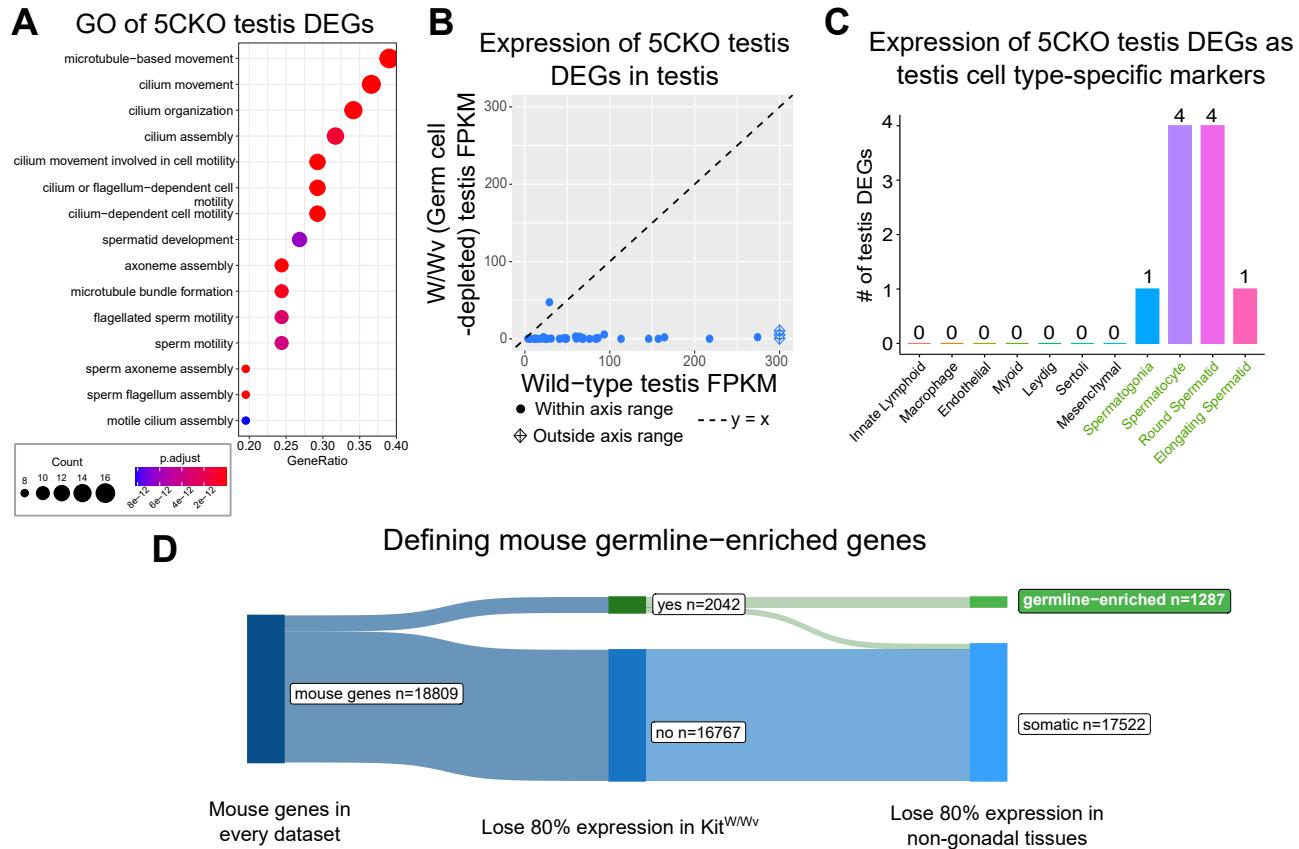
- 438 44. Liu, M., Zhu, Y., Xing, F., Liu, S., Xia, Y., Jiang, Q., and Qin, J. (2020). The polycomb group protein PCGF6  
439 mediates germline gene silencing by recruiting histone-modifying proteins to target gene promoters. *J Biol Chem* **295**,  
9712–9724. <https://doi.org/10.1074/jbc.RA119.012121>.
- 440 45. Otani, J., Nankumo, T., Arita, K., Inamoto, S., Ariyoshi, M., and Shirakawa, M. (2009). Structural basis for recognition  
441 of H3K4 methylation status by the DNA methyltransferase 3A ATRX–DNMT3–DNMT3L domain. *EMBO Reports* **10**,  
1235–1241. <https://doi.org/10.1038/embor.2009.218>.
- 442 46. Guo, X., Wang, L., Li, J., Ding, Z., Xiao, J., Yin, X., He, S., Shi, P., Dong, L., Li, G., et al. (2015). Structural insight into  
443 autoinhibition and histone H3-induced activation of DNMT3A. *Nature* **517**, 640–644. <https://doi.org/10.1038/nature13>  
899.
- 444 47. Abildayeva, K., Berbée, J.F.P., Blokland, A., Jansen, P.J., Hoek, F.J., Meijer, O., Lütjohann, D., Gautier, T., Pillot, T., De  
445 Vente, J., et al. (2008). Human apolipoprotein C-I expression in mice impairs learning and memory functions. *Journal of Lipid Research* **49**, 856–869. <https://doi.org/10.1194/jlr.M700518-JLR200>.
- 446 48. Leduc, V., Jasmin-Bélanger, S., and Poirier, J. (2010). APOE and cholesterol homeostasis in Alzheimer's disease.  
447 *Trends in Molecular Medicine* **16**, 469–477. <https://doi.org/10.1016/j.molmed.2010.07.008>.

448 **Figures and Tables**

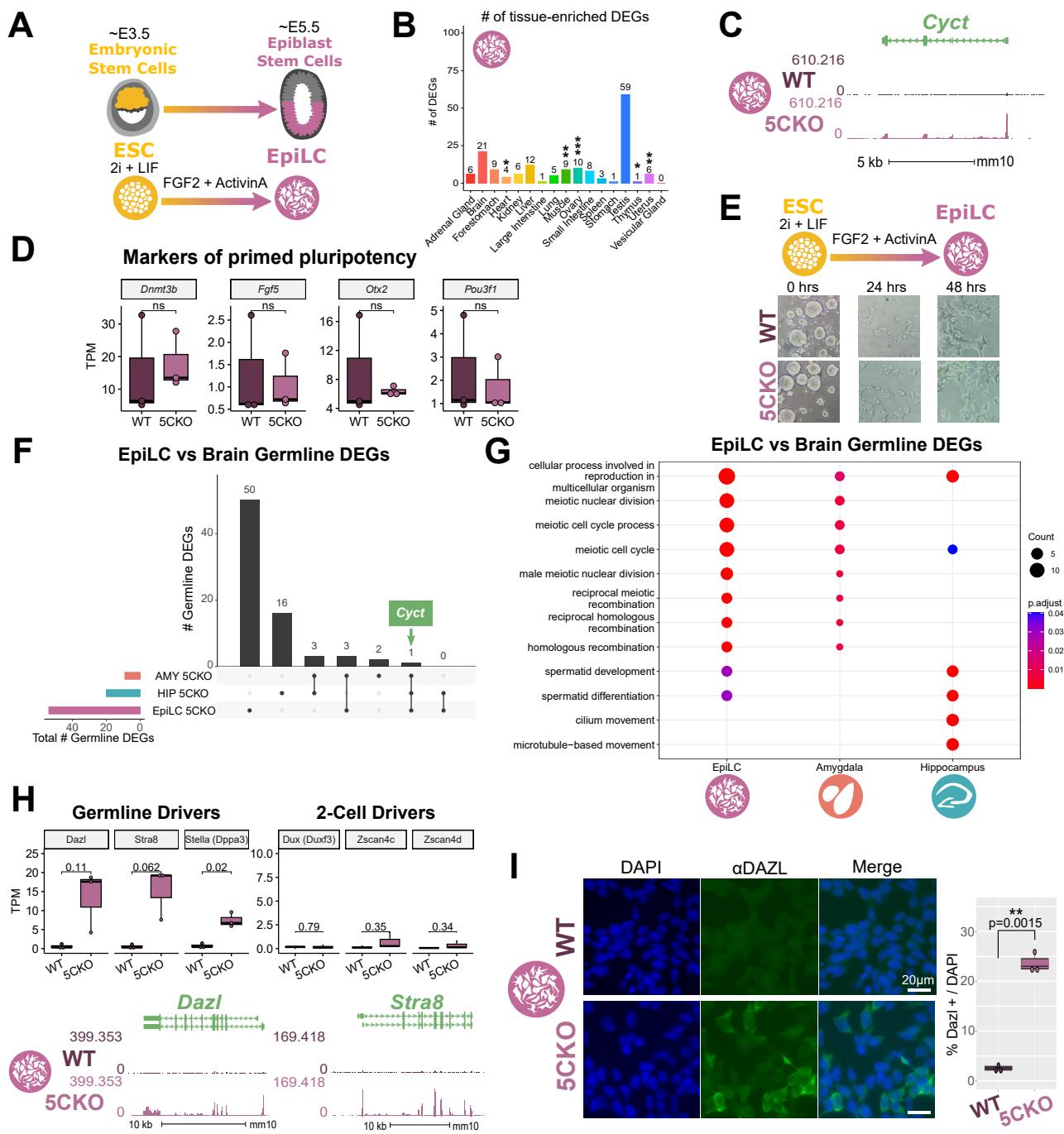
- 449     • Supplementary table 1: list of all germline genes.
- 450        – Columns to include:
- 451           \* KDM5C bound vs not
- 452           \* DEG in EpiLC, brain, both, neither (separate columns?)



**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-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 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/W<sub>v</sub>) 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).

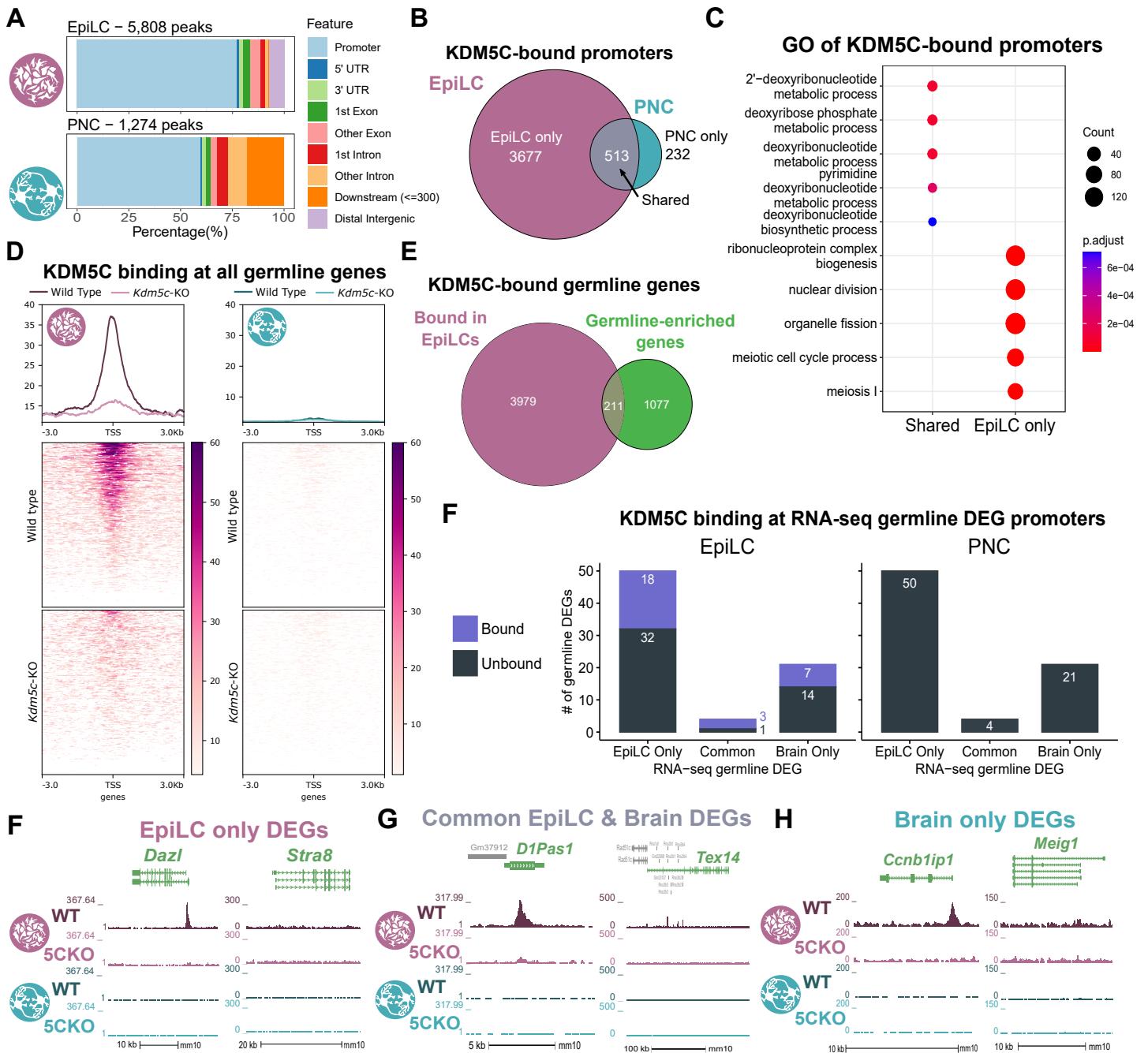
**E.** Representative images of wild-type (WT) and *Kdm5c*-KO cells during ESC to EpiLC differentiation. Brightfield images taken at 20X.

**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.

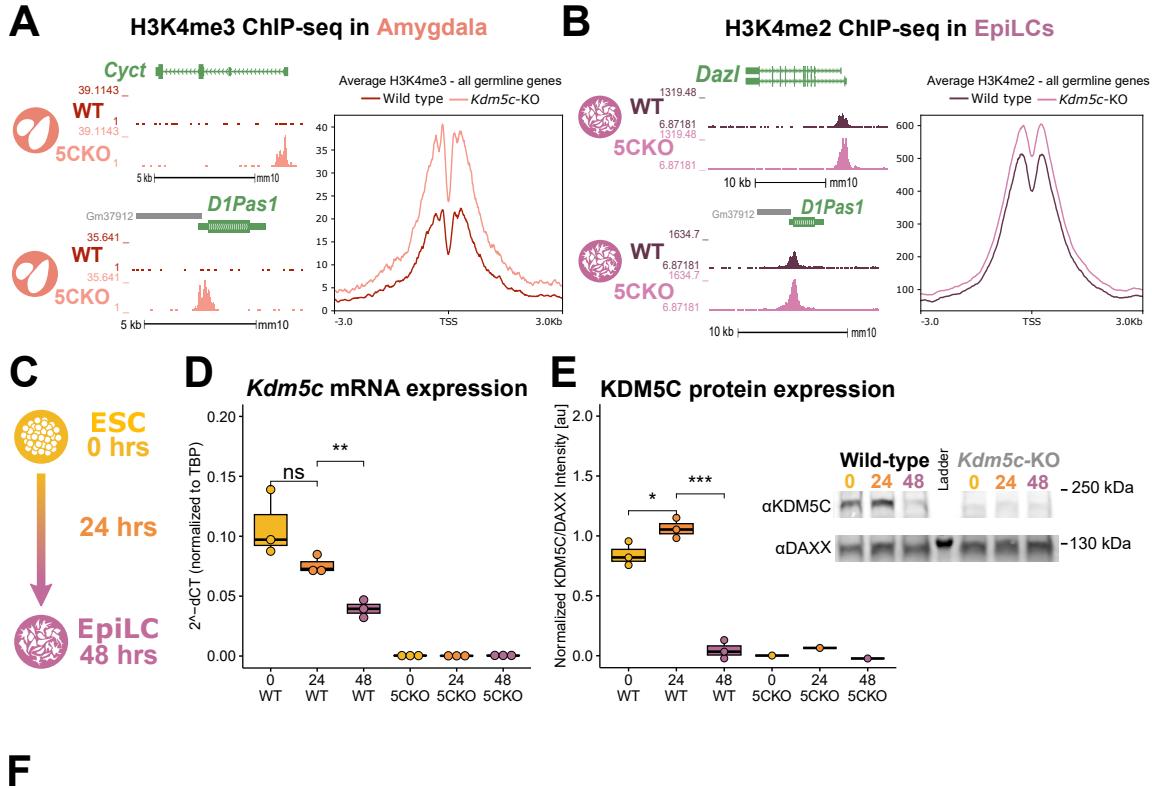
**H.** Top left - Example germline identity DEGs unique to EpiLCs. Top right - Example 2-cell genes that are not dysregulated in *Kdm5c*-KO EpiLCs. p-values for Welch's t-test. Bottom - Average bigwigs of *Dazl* and *Stra8* expression in wild-type and *Kdm5c*-KO EpiLCs.

**I.** 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: 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 4: 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



**Figure 5: 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 transcription 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 comparison 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

453 **Notes**

454 **Figure outline:**

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

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

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

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

474     **Figure 4: Loss of KDM5C's catalytic activity impairs DNAme placement and long-term silencing of germline  
475     genes** \* Increase in H3K4me3 in *Kdm5c*-KO amygdala at germline genes \* Increase in H3K4me2 in EpiLCs at germline  
476     genes \* *Kdm5c* binding in EpiLCs vs PNCs to show that germline repression is happening in early embryo \* Previous studies  
477     only looked at ESCs, unknown if catalytic activity is required for long-term repression, especially since DNA methylation is  
478     placed later). KDM5C RNA and protein ESC → EpiLC (increasing then decreasing) \* RNA expression of germline genes with  
479     catalytic dead rescue (Ilakkiya) \* DNA methylation in WT and 5CKO EpiLCs (Ilakkiya)

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

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

490     Introduction: \* Chromatin regulators are important for cellular identity \* H3K4me1-3 linked to active gene promoters and  
491     enhancers \* Surprisingly, mutations in Chromatin regulators lead to many NDDs (including many H3K4 regulators) \* Recent

492 studies have shown some chromatin regulators are important for regulating neuron-specific gene expression/chromatin  
493 stat\_compare\_means \* However, loss of some chromatin regulators can also lead to ectopic expression of tissue-enriched  
494 genes \* Very few studies have looked at these genes and it's unclear if these genes contribute to NDD impairments. \*  
495 Necessary to first characterize the mechanism behind their derepression to identify molecular footholds into testing their  
496 contribution to neuronal impairments and potential for therapeutic intervention

- 497 • Loss of KDM5C can result in the misexpression of genes typically only found in the testis
- 498     – Misexpression of tissue-enriched genes hasn't been systematically characterized - Unclear if these genes are  
499         exceptions or if other tissue-specific genes are dysregulated
- 500     – Interestingly, these genes (Cyct, D1pas1) typically function in the germline
- 501     – Germ cells (meiotic cells) are typically distinguished from somatic cells very early on in embryogenesis and is a  
502         key feature of multicellularity
- 503     – Chromatin regulators are very important for decommissioning germline genes and act successively the embryo  
504         implants into the uterine wall
- 505         \* Most studies have focused on ESCs, which have a similar transcriptome to germ cells / 2-cells
- 506         \* recently, KDM5C was shown to repress DAZL in ESCs, independent of its catalytic activity
- 507         \* However, DNA methylation is lost in the mature 5CKO brain, DNA methylation is placed later and it's unclear if  
508             it's required for long-term repression (maybe too specific, just trying to go into the fact that the mechanism is  
509             partially understood but unclear)
- 510     – Systematic characterization of ectopic germline genes hasn't been done
- 511         \* unknown if other germline-enriched genes are dysregulated, including oocyte-specific genes
- 512         \* Crucially, it's unknown if misexpression of the germline program leads to functional consequences in 5CKO  
513         cells.

514 **Germline gene repression background:**

515 Interestingly, some of the ectopic testis transcripts identified in the *Kdm5c*-KO brain are typically expressed in germ cells<sup>10</sup>.  
516 Unlike somatic cells, germ cells (e.g. sperm and eggs) undergo meiosis and pass on their genetic material to the next genera-  
517 tion. The germline and the soma are typically distinguished during early embryogenesis, when germline genes are silenced  
518 in epiblast stem cells soon after implantation and only reactivated in a subset to form the germline. Chromatin regulators  
519 play a key role in decommissioning germline genes as the embryo transitions from naïve to primed pluripotency by placing  
520 repressive histone H2A lysine 119 monoubiquitination (H2AK119ub1)<sup>16</sup>, histone 3 lysine 9 trimethylation (H3K9me3)<sup>16,17</sup>,  
521 and DNA CpG methylation<sup>17-19</sup> at germline gene promoters. KDM5C may also be involved in this early decommissioning of  
522 germline genes, as re-expression of KDM5C in neurons fails to suppress their dysregulation<sup>10</sup>. In support of this, KDM5C  
523 was very recently shown to repress *Deleted in azoospermia like* (*Dazl*), a key regulator of germline development, in mouse  
524 embryonic stem cells (ESCs)<sup>40,43</sup>. In support of this, two independent screens in mouse embryonic stem cells (ESCs) recently  
525 identified KDM5C as a repressor of *Deleted in azoospermia like* (*Dazl*), a key regulator of germline development. However,  
526 KDM5C's role in embryonic germline gene repression is currently unclear, given that *Dazl* is also expressed in ESCs and in  
527 the 2-cell stage and germline gene misexpression has yet to be globally characterized during *Kdm5c*-KO embryogenesis.