

Erosion of somatic tissue identity with loss of the X-linked intellectual 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.
7 This is, in part, accomplished by chromatin regulators that can either promote or impede lineage-specific
8 gene expression through DNA and histone modifications^{1,2}. Although many chromatin regulators were initially
9 identified for their roles in shaping cellular and tissue identity^{3,4}, recent advancements in next generation
10 sequencing unexpectedly revealed many neurodevelopmental disorders (NDDs) are caused by mutations in
11 chromatin regulators⁵. Several studies have suggested this connection between chromatin regulators and
12 neurodevelopment is due to their regulation of brain-specific genes, such as orchestrating transcriptional
13 programs for synaptic maturation⁶ and transitioning between neuronal and glial fates during neural precursor
14 differentiation⁷. However, loss of some chromatin regulators can also result in the ectopic transcription of
15 tissue-specific genes outside of their target environment^{3,4,8}, such as the misexpression of liver-specific
16 genes within adult neurons⁹. Very few studies have investigated the misexpression of tissue-specific genes
17 in chromatin-linked NDDs^{9,10} and it is currently unknown if this partial loss of brain identity contributes to
18 neurodevelopmental impairments.

To elucidate the role of tissue identity in chromatin-linked neurodevelopmental disorders, it is essential to first characterize the types of genes dysregulated and the molecular mechanisms governing their depression. In this study, we characterized the aberrant expression of tissue-enriched genes with loss of lysine demethylase 5C (KDM5C). KDM5C, also known as SMCX or JARID1C, is a chromatin regulator that can repress gene expression through erasure of histone 3 lysine 4 di- and trimethylation (H3K4me2/3)¹¹, marks enriched at active gene promoters. Pathogenic mutations in *KDM5C* cause Intellectual Developmental Disorder, X-linked, Syndromic, Claes-Jensen Type (MRXSCJ, OMIM: 300534), whose predominant features include intellectual disability, seizures, aberrant aggression, and autistic behaviors^{12–14}. *Kdm5c* knockout (-KO) mice recapitulate key MRXSCJ patient phenotypes, including hyperaggression, increased seizure propensity, and learning impairments^{10,15}. Unexpectedly, RNA sequencing (RNA-seq) of the *Kdm5c*-KO hippocampus revealed ectopic expression of testis genes within the brain¹⁰. It is currently unknown if this

30 dysregulation of brain tissue identity further impairs *Kdm5c*-KO neurodevelopment and if ectopic gene
31 expression within the *Kdm5c*-KO brain is unique to testis genes.

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

33 Distinguishing between the germline (cells that pass on their genetic material, e.g. sperm and eggs) and
34 the soma (cells that perform all other bodily functions) is a key feature of multicellularity and occurs during
35 early embryogenesis. In mammals, chromatin regulators play a key role in decommissioning germline genes
36 in somatic cells during the transition from naïve to primed pluripotency by placing repressive histone H2A
37 lysine 119 monoubiquitination (H2AK119ub1)¹⁶, histone 3 lysine 9 trimethylation (H3K9me3)^{16,17}, and DNA
38 CpG methylation^{17–19} at germline gene promoters. Systematically characterizing KDM5C's role in germline
39 gene repression during early embryogenesis will unveil key mechanisms underlying the demarcation between
40 soma and germline identity and while also providing molecular footholds to test the impact of ectopic germline
41 genes on neurodevelopment.

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

56 **Results**

57 **Tissue-enriched genes, including testis genes, are aberrantly expressed in the** 58 ***Kdm5c*-KO brain**

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

60 Previous RNA sequencing (RNA-seq) in the adult hippocampus revealed ectopic expression of some testis
61 genes within the *Kdm5c* knockout (-KO) brain¹⁰. Since tissue-enriched genes have not been systematically

62 characterized in the *Kdm5c*-KO brain, it is currently unclear if this erosion of brain tissue identity is a major
63 consequence of *Kdm5c* loss and if it is unique to testis-enriched genes. Therefore, we first globally assessed
64 the expression of genes enriched in 17 mouse tissues²⁰ in our published mRNA-seq datasets of the adult
65 amygdala and hippocampus in mice with constitutive knockout of *Kdm5c*²¹.

66 We found a large proportion of genes that are significantly upregulated within the *Kdm5c*-KO brain
67 (DESeq2²², log2 fold change > 0.5, q < 0.1) are typically enriched in non-brain tissues (Amygdala: 35%,
68 Hippocampus: 24%) (Figure 1A-B). The majority of tissue-enriched differentially expressed genes (tissue-
69 enriched DEGs) were testis genes (Figure 1A-C). Even though the testis has the largest total number of
70 tissue-biased genes compared to any other tissue (2,496 genes), testis-biased DEGs were significantly
71 enriched for both brain regions (Amygdala p = 1.83e-05, Odds Ratio = 5.13; Hippocampus p = 4.26e-11,
72 Odds Ratio = 4.45, Fisher's Exact Test). One example of a testis-enriched DEG is *FK506 binding protein 6*
73 (*Fkbp6*), a known regulator of piRNA expression and meiosis in germ cells^{23,24} (Figure 1C).

74 In addition to the high enrichment of testis genes, we also identified aberrant expression of other
75 tissue-enriched genes within the *Kdm5c*-KO brain. Although the *Kdm5c*-KO mice were male, we observed
76 significant enrichment of ovary-biased DEGs in both the amygdala and hippocampus (Amygdala p = 0.00574,
77 Odds Ratio = 18.7; Hippocampus p = 0.048, Odds Ratio = 5.88, Fisher's Exact) (Figure 1D). Ovary-enriched
78 DEGs included *Zygotic arrest 1 (Zar1)*, which was recently shown to sequester mRNAs in oocytes for meiotic
79 maturation and early zygote development²⁵ (Figure 1D). Although not consistent across brain regions, we
80 also found significant enrichment of DEGS biased towards two non-gonadal tissues - the liver (Amygdala p =
81 0.0398, Odds Ratio = 6.58, Fisher's Exact Test) and the muscle (Hippocampus p = 0.0104, Odds Ratio =
82 6.95, Fisher's Exact Test). An example of a liver-biased DEG dysregulated in both the hippocampus and
83 amygdala is *Apolipoprotein C-I (Apoc1)*, which is involved in lipoprotein metabolism (Figure 1E). Testis,
84 ovary, and liver-enriched DEGs showed little to no expression in the developing and adult wild-type brain,
85 yet our mRNA-seq data indicate they are polyadenylated and spliced into mature transcripts (Figure 1C-E).
86 Of note, we did not observe enrichment of brain-enriched genes (Amygdala p = 1; Hippocampus p = 0.74,
87 Fisher's Exact), despite the fact these are brain samples and the brain has the second highest total number
88 of tissue-enriched genes (708 genes). Together, these results suggest misexpression of tissue-enriched
89 genes within the brain is a major effect of KDM5C loss.

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

91 • Intriguingly, some of the ectopic testis transcripts identified within the *Kdm5c*-KO hippocampus have
92 known functions unique to germ cells¹⁰, suggesting KDM5C may play a role in demarcating somatic
93 versus germline identity.

94 The testis contains both germ cells (meiotic cells, e.g. spermatogonia) and somatic cells (non-meiotic,
95 e.g. Leydig cells) that support hormone production and germline functions. Intriguingly, many *Kdm5c*-

96 KO testis and ovary enriched-DEGs have germline-specific functions, suggesting *Kdm5c*-KO cells fail to
97 distinguish between the soma and germline. To test if this holds true for all *Kdm5c*-KO testis-biased DEGs, we
98 first assessed their known functions through gene ontology analysis. We found *Kdm5c*-KO testis-enriched
99 DEGs high enrichment of germline-relevant ontologies, including spermatid development (GO: 0007286,
100 p.adjust = 6.2e-12) and sperm axoneme assembly (GO: 0007288, p.adjust = 2.45e-14) (Figure 2A).

101 To further validate if these testis DEGs are truly germline genes, we then evaluated their expression in
102 somatic versus germ cells within the testis. We first compared their expression in the testis with germ cell
103 depletion²⁶, which was accomplished by heterozygous *W* and *Wv* mutations in the enzymatic domain of
104 *c-Kit* (*Kit*^{W/Wv}) that prevent the maturation of germ cells²⁷. Almost all *Kdm5c*-KO testis-enriched DEGs lost
105 expression with germ cell depletion (Figure 2B). The only testis-enriched DEG that did not show considerable
106 downregulation with germline depletion was *FK506 binding protein 6* (*Fkbp6*), the aforementioned testis
107 gene that regulates piRNA expression and meiosis in germ cells^{23,24}. We then assessed testis-enriched
108 DEG expression in a published single cell RNA-seq dataset that identified cell type-specific markers within
109 the testis²⁸. We found that while some testis-enriched DEGs were classified as specific markers for different
110 germ cell types (e.g. spermatogonia, spermatocytes, round spermatids, and elongating spermatids) none
111 marked somatic cells (Figure 2C). Together, these data suggest the somatic *Kdm5c*-KO brain aberrantly
112 expresses germline genes.

113 We then wanted to more deeply characterize germline gene misexpression with *Kdm5c* loss, but lacked
114 a comprehensive list of mouse germline-enriched genes. To facilitate downstream analyses, we generated a
115 curated list of germline-enriched genes using currently available RNA-seq datasets in *Kit*^{W/Wv} mice. Wild-type
116 and *Kit*^{W/Wv} datasets included males and females at embryonic day 12, 14, and 16²⁹, as well as adult
117 male testes²⁶. We defined genes as germline-enriched if their expression met the following criteria: 1)
118 their expression is greater than 1 FPKM in wild-type gonads 2) their expression in any adult wild-type,
119 non-gonadal tissue²⁰ does not exceed 20% of their maximum expression in the wild-type germline, and
120 3) their expression in the germ cell-depleted germline, for any sex or time point, does not exceed 20% of
121 their maximum expression in the wild-type germline. These criteria yielded 1,288 germline-enriched genes
122 (Figure 2D), which was hereafter used as a resource for assessing germline gene misexpression with *Kdm5c*
123 loss (Supplementary table 1).

124 ***Kdm5c*-KO epiblast-like cells aberrantly express master regulators of germline 125 identity**

126 Misexpression of germline genes in the adult brain suggests *Kdm5c*-KO cells fail to demarcate between
127 germline and somatic identity. Germ cells are typically distinguished from somatic cells soon after the embryo
128 implants into the uterine wall^{30,31} when a subset of epiblast stem cells become the primordial germ cells
129 (PGCs) while the remainder differentiate into the ectoderm, mesoderm, and endoderm to form the somatic

130 tissues³². This developmental time point can be modeled *in vitro* through differentiation of embryonic stem
131 cells (ESCs) into post-implantation epiblast-like stem cells (EpiLCs) (Figure 3A, top). Previous studies have
132 demonstrated that while some germline-enriched genes are also expressed in embryonic stem cells (ESCs)
133 and in the 2-cell stage^{33–35}, they are silenced as they differentiate into EpiLCs¹⁷. Therefore, we assessed if
134 KDM5C was necessary for embryonic silencing of germline genes by evaluating the impact of *Kdm5c* loss in
135 EpiLCs.

136 We first identified *Kdm5c*-KO EpiLC DEGs in of our previously published RNA-seq dataset³⁶ (DESeq2,
137 log2 fold change > 0.5, q < 0.1) and assessed tissue-enriched gene expression. Similar to the *Kdm5c*-KO
138 brain, we observed general dysregulation of tissue-enriched genes, with the largest number of genes
139 belonging to the brain and testis, although they were not significantly enriched (Figure 3B). Using the curated
140 list of germline genes generated above, we found *Kdm5c*-KO EpiLCs aberrantly expressed 54 germline-
141 enriched genes, including the previously characterized hippocampal DEG¹⁰ *Cytochrome C, testis-specific*
142 (*Cyct*) (Figure 3C). Although we observed aberrant expression of many tissue-enriched genes, we did not
143 observe any significant difference in primed pluripotency genes (Figure 3D) or gross changes in *Kdm5c*-KO
144 cell morpholgy during differentiation (Figure 3E), indicating KDM5C loss does not impair EpiLC formation.

145 We then compared EpiLC germline DEGs to those expressed in the *Kdm5c*-KO brain to determine
146 if all germline DEGs, like *Cyct*, are constitutively dysregulated or if they can change over the course of
147 development. We found this was primarily not the case, as the majority of germline DEGs expressed
148 in EpiLCs and the brain were unique, with only *Cyct* shared across all sequencing datasets (Figure 3F).
149 We then compared the known functions of EpiLC and brain germline DEGs and found particularly high
150 enrichment of meiosis-related gene ontologies in EpiLCs (Figure 3G), such as meiotic cell cycle process (GO:
151 1903046, p.adjust = 1.59e-08) and meiotic nuclear division (GO: 0140013, p.adjust = 9.76e-09). While there
152 was modest enrichment of meiotic gene ontologies in both brain regions, the *Kdm5c*-KO hippocampus
153 primarily expressed late-stage spermatogenesis genes, such those involved in the sperm axoneme structure.

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

161 We were particularly interested in the aberrant transcription of *Dazl*, since it is essential for germ cell
162 development and promotes the translation of germline mRNAs⁴¹. A significant portion of germline transcripts
163 misexpressed in *Kdm5c*-KO EpiLCs are known binding targets of DAZL, including *Stra8*⁴² (p = 1.698e-07,
164 Fisher's Exact Test). This suggests expression of DAZL protein could promote the translation of other
165 aberrant germline transcripts, influencing their ability to impact *Kdm5c*-KO cellular function. We thus tested

166 DAZL protein expression in *Kdm5c*-KO EpiLCs through immunocytochemistry (Figure 3I). We observed
167 about 25% of *Kdm5c*-KO EpiLCs expressed DAZL protein and it was localized to the cytoplasm ($p = 0.0015$,
168 Welch's t-test), consistent with the pattern of DAZL expression in spermatogonia⁴². Altogether these results
169 suggest tissue-specific genes are misexpressed during *Kdm5c*-KO embryogenesis, including key drivers of
170 germline identity that can be translated into protein.

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

172 • KDM5C may also be involved in this early decommissioning of germline genes, as re-expression of
173 KDM5C in knockout neurons fails to suppress their dysregulation¹⁰.

174 Previous work suggests KDM5C represses germline genes during early development, as re-expression
175 of KDM5C in knockout neuronal cultures fails to suppress hippocampal germline DEGs and KDM5C is not
176 bound to their promoters in neurons¹⁰. There is some evidence KDM5C binds to select germline gene
177 promoters in ESCs¹⁰, including *Dazl*^{40,43}. As KDM5C's binding at germline gene promoters has not been
178 systematically characterized, it is currently unclear if KDM5C is enriched at germline gene promoters, what
179 types of germline genes KDM5C regulates, and if its binding is maintained at any germline genes in neurons.

180 To further characterize the mechanism behind *Kdm5c*-KO germline gene misexpression, we analyzed
181 KDM5C chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq) datasets in EpiLCs³⁶ and
182 primary neuron cultures (PNCs) from the cortex and hippocampus¹⁵. EpiLCs had a higher total number of
183 KDM5C peaks than PNCs (EpiLCs: 5,808, PNCs: 1,276, MACS2 q < 0.1 and fold enrichment > 1, removal
184 of *Kdm5c*-KO false positive peaks) and KDM5C was primarily localized to gene promoters in both cell types
185 (EpiLCs: 4,190, PNCs: 745 +/- 500bp from the TSS), although PNCs showed increased localization to
186 non-promoter regions (Figure 4A).

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

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

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

212 **KDM5C erases H3K4me3 to promote long-term repression of germline genes via 213 DNA methylation**

214 Although KDM5C is generally thought to suppress transcription through erasure of histone 3 lysine 4 di-
215 and trimethylation (H3K4me2/3)¹¹, recent studies in ESCs have suggested KDM5C's repression *Dazl* is
216 independent of its catalytic activity⁴⁰. Somatic repression of germline genes is typically established during the
217 transition between naïve and primed pluripotency, which modeled *in vitro* as ESCs to EpiLC differentiation.
218 In ESCs, chromatin regulators place repressive histone modifications at germline gene promoters, including
219 histone 2A 119 monoubiquitination (H2AK119ub1) and histone 3 lysine 9 trimethylation (H3K9me3)^{16,17,44}.
220 Germline genes are then silenced long-term in EpiLCs by *de novo* placement of DNA CpG methylation
221 (DNAm)¹⁷. Although KDM5C's catalytic activity is not required to suppress *Dazl* in ESCs, KDM5C may
222 promote long-term germline gene silencing via H3K4me3 removal since H3K4me3 can impede DNAm
223 placement^{45,46} and DNAm is lost at select germline gene promoters in the hippocampus¹⁰. Because
224 KDM5C's role in germline gene repression has only been characterized in ESCs and in the mature brain, it is
225 currently unclear to what extent KDM5C is involved during transition between ESCs and EpiLCs and if its
226 catalytic activity is required for long-term silencing.

227 To elucidate KDM5C's role in germline gene silencing, we first characterized KDM5C's substrates, histone
228 3 lysine 4 di- and trimethylation (H3K4me2/3) in our previously published ChIP-seq datasets in wild type and
229 *Kdm5c*-KO amygdala²¹ and EpiLCs³⁶. In congruence with previous work in the *Kdm5c*-KO hippocampus¹⁰,
230 we observed aberrant accumulation of H3K4me3 around the transcription start site (TSS) of germline genes
231 in the *Kdm5c*-KO amygdala (Figure 5A). We additionally found a marked increase in H3K4me2 around the
232 TSS of germline genes in *Kdm5c*-KO EpiLCs (Figure 5B).

233 We then evaluated KDM5C's embryonic role in germline gene silencing during ESC to EpiLC differentiation.

234 We first characterized KDM5C's mRNA and protein expression at 0, 24, and 48 hours of differentiation
235 (Figure 5C). While *Kdm5c* mRNA steadily decreased from 0 to 48 hours of differentiation (Figure 5D), KDM5C
236 protein initially increased from 0 to 24 hours but then decreased to near knockout levels by 48 hours (Figure
237 5E). We then performed whole genome bisulfite sequencing in wild-type and *Kdm5c*-KO ESCs and 96 hour
238 extended EpiLCs (exEpiLCs) to determine KDM5C's role in the initial placement of DNA methylation at
239 germline gene promoters. While wild-type cells accumulated high levels of DNA methylation at germline
240 gene promoters over the course of ESCs to exEpiLC differentiation, DNA methylation was markedly reduced
241 in *Kdm5c*-KO exEpiLCs (Figure 5F).

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

247 Discussion

248 In the above study, we demonstrate KDM5C's pivotal role in the development of tissue identity, resulting in
249 substantial misexpression of tissue-enriched genes within the adult *Kdm5c*-KO hippocampus and amygdala.
250 We defined tissue-enriched genes based on the criteria used in a previously published dataset of 17 C57/Bl6J
251 mouse tissues²⁰, which defined genes as tissue-enriched if they had more than 4-fold higher expression
252 when compared to any other tissue. In addition to testis genes identified previously¹⁰, we found significant
253 enrichment of muscle, liver, and even ovary-biased genes aberrantly expressed within the male *Kdm5c*-KO
254 brain. It is currently unclear what the impact of these tissue-enriched genes are upon *Kdm5c*-KO brain
255 function and if they ultimately contribute to MRXSCJ-related phenotypes. While the majority of tissue-
256 enriched DEGs were testis and ovary genes with no known brain functions, select liver and muscle-biased
257 DEGs do have known roles in the wild-type brain. For example, *Apolipoprotein C-I* (*Apoc1*) is a lipid transport
258 gene highly enriched within the liver, but is also lowly expressed in the wild-type brain. Intriguingly, *Apoc1*
259 overexpression in the mouse brain can impair learning and memory⁴⁷ and is implicated in Alzheimer's
260 disease in humans⁴⁸. Our results suggest KDM5C fine-tunes the expression of tissue-enriched genes like
261 *Apoc1* to match the level required for proper brain function. However, further studies are required to determine
262 if these genes contribute to *Kdm5c*-KO neuronal impairments and MRXSCJ pathology.

263 A substantial portion of genes upregulated in *Kdm5c*-KO brain are testis genes that have no known
264 function within the brain. Through the use of publicly available RNA-seq datasets, we demonstrated these
265 testis-enriched DEGs are typically unique to germ cells. Misexpression of germline genes in the brain sug-
266 gests *Kdm5c*-KOs fail to demarcate between somatic and germline lineages, a key feature of multicellularity

267 and sexual reproduction. Previous studies have demonstrated germline genes are decommissioned in
268 somatic cells by multiple chromatin-modifying enzymes, but have primarily focused on the repression of
269 key marker genes. To globally characterize KDM5C's role in germline gene repression, we curated a list
270 of mouse germline-enriched genes using publically available germ cell-depleted RNA-seq datasets. This
271 resource enabled us to identify 1) the extent of germline gene dysregulation in *Kdm5c*-KO cells, 2) the types
272 of germline genes misexpressed at different developmental time points, and 3) which types of germline
273 genes are directly or indirectly regulated by KDM5C.

274 Dysregulation of *Kdm5c*-KO tissue identity begins during early embryogenesis, as germline and other
275 tissue-enriched genes are aberrantly expressed in epiblast-like cells (EpiLCs). *In vivo*, germline genes
276 are typically decommissioned in epiblast stem cells and remain silenced as the epiblast differentiates
277 into the body's somatic tissues³². However, a small subset of epiblast stem cells will receive signals to
278 reactivate germline gene expression to become the primordial germ cells (PGCs) that will ultimately form
279 the mature germline^{30,31}. This process can be mimicked *in vitro* by differentiating EpiLCs into primordial
280 germ cell-like cells (PGCLCs)⁴⁹. Thus, misexpression of germline genes in *Kdm5c*-KO EpiLCs might
281 suggest they are progressing beyond EpiLC differentiation and becoming PGCLCs. Yet, *Kdm5c*-KO EpiLCs
282 had proper expression of EpiLC marker genes for primed pluripotency and we observed no difference in
283 cellular morphology during ESC to EpiLC differentiation. Furthermore, we saw no significant change in *Otx2*
284 expression, an epiblast stem cell marker that is known to repress epiblast and EpiLC differentiation into PGC
285 and PGCLCs⁵⁰. This, in conjunction with *Kdm5c*-KO mice being viable, suggests germline gene expression
286 is occurring ectopically in conjunction with typical developmental programs, rather than a complete shift to
287 germline identity.

288 • change first sentence: Other chromatin regulators involved in germline gene repression act in ESCs
289 and EpiLCs. We observed this is true for KDM5C

290 We then globally characterized KDM5C binding at germline-enriched gene promoters through analysis
291 of KDM5C ChIP-seq in EpiLCs and primary hippocampus and cortex neuron cultures (PNCs). While we
292 observed high enrichment of KDM5C peaks at germline genes in EpiLCs, KDM5C was not bound to germline
293 gene promoters in PNCs. This suggests dysregulation of germline genes in the mature *Kdm5c*-KO brain is
294 due to loss of repression during embryogenesis, which is consistent with previous work that found introducing
295 human KDM5C into *Kdm5c*-KO PNCs does not repress germline transcripts¹⁰. Although KDM5C is enriched
296 at germline gene promoters in EpiLCs, KDM5C was only bound to about a third of EpiLC germline-enriched
297 DEGs. One notable DEG not bound by KDM5C is *Stra8*, a transcription factor activated by retinoic acid
298 signaling in germ cells that promotes meiotic initiation^{51,52}. Retinoic acid can only activate *Stra8* expression
299 when DAZL is present, which is a direct target of KDM5C and is aberrantly transcribed and translated in
300 *Kdm5c*-KO EpiLCs. This indicates germline genes can be aberrantly expressed in *Kdm5c*-KO cells indirectly
301 of KDM5C regulation through activation by other ectopic germline programs. Consistent with this idea, many

302 *Kdm5c*-KO EpiLC germline DEGs are important for early germ cell development and meiosis while those
303 expressed in the mature *Kdm5c*-KO brain are involved in late sperm development. Altogether, this indicates
304 ectopic germline programs are, to some extent, progressing through germ cell developmental stages over
305 the course of *Kdm5c*-KO development.

- 306 • Talk about different motifs if we do see differences there and if it explains direct vs indirect dysregulation
307 – Could also mention PRC1.6/KDM5C binds pcgf6 in ESCs
308 • Maybe talk about other regulators/if anything is known about them long term? Or save for 2nd paper.
309 – another germline repressor: <https://pubmed.ncbi.nlm.nih.gov/35345626/>
310 • 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>

312 It is important to note that while we focused on KDM5C's regulation of germline genes, some germline-
313 enriched genes are also expressed in naïve ESCs and at the 2-cell stage due to their roles in pluripotency
314 and self-renewal. For example, although primarily known for committing PGCs to the germline fate and
315 regulating the translation of germline-specific RNAs, *Dazl* is also expressed in naïve ESCs *in vitro*³³, the
316 inner cell mass *in vivo*³³, and at the 2-cell stage⁵³, but is silenced when cells transition from naïve to primed
317 pluripotency during ESC to EpiLC differentiation¹⁷. Very recently, two screens of *Dazl*-repressors in ESCs
318 identified KDM5C as a direct regulator of *Dazl* expression^{40,43}. Interestingly, one screen found *Kdm5c*-KO
319 ESCs also aberrantly expressed 2-cell-specific genes, indicating KDM5C negatively regulates totipotency in
320 ESCs⁴⁰. We found that while KDM5C also represses *Dazl* expression in EpiLCs, *Kdm5c*-KO EpiLCs do not
321 express 2-cell specific genes like *Dux* and *Zscan4c*. Out of the four 2-cell regulators characterized in ESCs,
322 KDM5C was the only factor whose repression of *Dazl* was independent of *Dux* expression⁴⁰. Together, this
323 suggests the 2-cell-like state in *Kdm5c*-KO ESCs is an indirect consequence of KDM5C's primary role in
324 germline gene repression, including germline genes that are also involved in pluripotency and self-renewal.

- 325 • We demonstrated KDM5C is important for the transition between histone-mediated to DNAme-mediated
326 silencing of germline genes during the transition from naïve to primed pluripotency.
327 – Loss of DNAme can last throughout life at at least two germline gene promoters (hippocampus
328 barco)
329 – KDM5C could be important for DNAme is that KDM5C erases H3K4me3 which can impede
330 CpGme.
331 * In support of this, KDM5C is highly enriched at the CpG islands near germline TSS that are
332 methylated in EpiLCs
333 – However, Recently KDM5C's catalytic activity was found to be unnecessary for dazl suppression in
334 ESCs.

335

- 336 – Since DNAm is not placed until EpiLC stage, KDM5C's catalytic activity may be required for
337 long-term silencing of germline genes.
- 338 – This would be the first (?) example in which removal of an active mark is required for germline
339 gene repression.
- 340 • In summary, we demonstrate loss of the X-linked intellectual disability gene KDM5C leads to widespread
341 dysregulation of brain tissue identity, including misexpression of germline genes in the somatic brain.
- 342 – In EpiLCs, KDM5C directly represses key drivers of germline identity like *Dazl*, likely through
343 PRC1.6 recruitment and promoting CpG methylation. However, *Kdm5c*-KO also ectopically
344 expresses germline genes activated indirectly, including *Stra8*.
- 345
- 346 – The germline developmental program to some extent continues ectopically during *Kdm5c*-KO
347 development, resulting in aberrant transcription of late stage spermatogenesis genes later in life.
- 348 – These results define KDM5C's role in the demarcation between soma and germline identity and
349 offer a window into potential targets to assess the deleterious effects these ectopic genes on
350 neurodevelopment.

351 **Discussion notes**

- 352 • We additionally found *Kdm5c*-KO EpiLCs ectopically express DAZL protein that is localized to the
353 cytoplasm, similar to its morphology in spermatogonia⁴². **note: maybe just put in results.** Could
354 move around depending upon if I get pheno working.
- 355 • end with something like: this indicates kdm5c not only modulates pluripotency and self-renewal in
356 ESCs, but also has a role in long-term silencing of germline genes
- 357 – then transition into the long term silencing mechanism paragraph
- 358 • KDM5C is dynamically regulated during the window of embryonic germline gene silencing, our EpiLC
359 ChIP-seq is likely catching the tail end of KDM5C's main involvement.
- 360 • Our results indicate KDM5C is a pivotal repressor of germline programs in somatic cells.
- 361 • Studies on other chromatin regulators have focused on key marker genes to identify germline gene
362 misexpression, such as *Dazl*.
- 363 • Using a germline-depleted mouse model, we curated a list of mouse germline-enriched genes to
364 globally assess germline gene dysregulation.
- 365 • Many of the genes dysregulated in the mature *Kdm5c*-KO brain were important for late stages of
366 spermatogenesis, such as those important for sperm flagellar structure. Contrastingly, *Kdm5c*-KO
367 EpiLCs aberrantly expressed key drivers of germ cell identity, including *Dazl* and *Stra8*.

- 368 • While a significant portion of KDM5C-bound promoters are germline genes, many germline genes
369 expressed during *Kdm5c*-KO embryogenesis are not directly bound by *kdm5c*.
- 370 • One notable example is *Stra8*, a transcription factor important for germ cell specific transcription and
371 meiotic initiation
- 372 • The including the demarcation between soma and germline fates.
- 373 the *Kdm5c*-KO brain also Previous work identified testis-enriched genes in *Kdm5c* genes
- 374 –
- 375 – However unlike the gonadal-biased DEGs,
- 376 • The demarcation of the germline versus soma is a key feature of multicellularity and sexually dimorphic
377 reproduction
- 378 • Anything known about tissue-biased gene expression in other H3K4me regulators?
- 379 • Our data suggests the germline developmental program is occurring ectopically as *Kdm5c*-KOs pro-
380 gresses through somatic tissue development
- 381 • tissue-biased gene expression:
- 382 • Altogether, these data indicate that while some germline genes are misexpressed due to direct loss of
383 KDM5C binding during emryogenesis, secondary downstream mechanisms can also promote their
384 aberrant transcription.
- 385 • Papers to read/reference:
- 386 – Reconstitution of the Mouse Germ Cell Specification Pathway in Culture by Pluripotent Stem Cells:
387 [https://www.cell.com/fulltext/S0092-8674\(11\)00771-9](https://www.cell.com/fulltext/S0092-8674(11)00771-9)
- 388 – 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/>

390 Materials and Methods

391 Classifying tissue-enriched and germline-enriched genes

392 Tissue-enriched differentially expressd genes were determined by their classification in a previously
393 published list of genes enriched in 17 male and female mouse tissues²⁰. This study defined expression as
394 greater than 1 Fragments Per Kilobase of transcript per Million mapped read (FPKM) and tissue enrichment
395 as at least 4-fold higher expression than any other tissue.

396 We curated a list of germline-enriched genes using an RNA-seq dataset from wild-type and germline-
397 depleted ($Kit^{W/Wv}$) male and female mouse embryos from embryonic day 12, 14, and 16²⁹, as well as adult
398 male testes²⁶. Germline-enriched genes met the following criteria: 1) their expression is greater than 1
399 FPKM in wild-type germline 2) their expression in any wild-type somatic tissues²⁰ does not exceed 20%
400 of maximum expression in wild-type germline, and 3) their expression in the germ cell-depleted ($Kit^{W/Wv}$)
401 germline, for any sex or time point, does not exceed 20% of maximum expression in wild-type germline.

402 Cell culture

403 We utilized our previously established cultures of male wild-type and *Kdm5c* knockout (-KO) embryonic
404 stem cells³⁶. Sex was confirmed by genotyping *Uba1* on the X and Y chromosomes with the following
405 primers: 5'-TGGATGGTGTGCCAATG-3', 5'-CACCTGCACGTTGCCCTT-3'. Deletion of *Kdm5c* was
406 confirmed through the primers 5'-ATGCCCATATTAAGAGTCCTG-3', 5'-TCTGCCTTGATGGGACTGTT-3',
407 and 5'-GGTTCTCAACACTCACATAGTG-3'.

408 Embryonic stem cells (ESCs) were initially cultured in primed ESC (pESC) media consisting of Knock-
409 Out DMEM (Gibco#10829-018), fetal bovine serum (Gibco#A5209501), KnockOut serum replacement
410 (Invitrogen#10828-028), Glutamax (Gibco#35050-061), Anti-Anti (Gibco#15240-062), MEM Non-essential
411 amino acids (Gibco#11140-050), and beta-mercaptoethanol (Sigma#M7522). They were then transitioned
412 into ground-state "naive" ESCs (nESCs) by culturing for four passages in N2B27 media containing DMEM/F12
413 (Gibco#11330-032), Neurobasal media (Gibco#21103-049), Gluamax, Anti-Anti, N2 supplement (Invitro-
414 gen#17502048), and B27 supplement without vitamin A (Invitrogen#12587-010), and beta-mercaptoethanol.
415 Both pESC and nESC media were supplemented with the GSK3 inhibitor CHIR99021 (Sigma #SML1046-
416 5MG), the MEK inhibitor PD0325901 (Sigma #PZ0162-5MG), and leukemia inhibitory factor (LIF, Milli-
417 pore#ESG1107).

418 nESCs were differentiated into epiblast-like cells (EpiLCs, 48 hours) and extendend EpiLCs (exEpiLCs,
419 96 hours) by culturing in N2B27 media containing DMEM/F12, Neurobasal media, Gluamax, Anti-Anti,
420 N2 supplement, B27 supplement (Invitrogen#17504044), beta-mercaptoethanol, fibroblast growth factor 2
421 (FGF2, R&D Biotechne 233-FB), and activin A (R&D Biotechne 338AC050CF).

422 Immunocytochemistry (ICC)

423 ICC of DAZL in EpiLCs was performed by washing cells thrice with phosphobuffered saline (PBS), fixing
424 cells in 4% paraformaldehyde, washing thrice in PBS, blocking in PBS containing 0.3% Triton X-100, and 5%
425 fetal bovine serum for 1 hour, washing thrice with PBS, and incubating in primary antibody (Rabbit anti DAZL,
426 abcam ab34139, 1:200) in the blocking solution overnight at 4C. The next day cells were rinsed thrice with
427 PBS, incubated in secondary antibody (Alexafluor 488 Invitrogen #710369, 1:1000) in blocking buffer, rinsed
428 thrice in PBS, and then imaged. Images were taken blinded for genotype, chosen based on similar levels of

429 DAPI signal, and then quantified via ImageJ.

430 RNA sequencing

431 Published datasets

432 All published datasets are available at the Gene Expression Omnibus (GEO) [https://www.ncbi.nlm.nih.g](https://www.ncbi.nlm.nih.gov/geo/)
433 ov/geo. Previously published RNA sequencing datasets analyzed in this study included the male wild-type
434 and *Kdm5c*-KO adult amygdala and hippocampus²¹ (available at GEO: GSE127722) and male wild-type and
435 *Kdm5c*-KO EpiLCs³⁶ (available at GSE: GSE96797).

436 Alignment and analysis

437 After ensuring read quality via FastQC (v0.11.8), reads were then mapped to the mm10 *Mus musculus*
438 genome (Gencode) using STAR (v2.5.3a), during which we removed duplicates and kept only uniquely
439 mapped reads. Count files were generated by FeatureCounts (Subread v1.5.0), and BAM files were
440 converted to bigwigs using deeptools (v3.1.3) and visualized by the UCSC genome browser. RStudio (v3.6.0)
441 was then used to analyze counts files by DESeq2 (v1.26.0)²² to identify differentially expressed genes
442 (DEGs) with a q-value (p-adjusted via FDR/Benjamini–Hochberg correction) less than 0.1 and a log2 fold
443 change greater than 0.5. For all DESeq2 analyses, log2 fold changes were calculated with IfcShrink using
444 the ashR package⁵⁴. MA-plots were generated by ggpahr (v0.4.0), and Eulerr diagrams were generated by
445 eulerr (v6.1.1). Boxplots and scatterplots were generated by ggpahr (v0.6.0) and ggplot2 (v3.3.2). Heatmaps
446 of gene expression were generated using the base R functions scale and hclust and visualized using the R
447 package ComplexHeatmap (v2.12.1). The Upset plot was generated via the package UpSetR (v1.4.0)⁵⁵.
448 Gene ontology (GO) analyses were performed by the R package enrichPlot (v1.16.2) using the biological
449 processes setting.

450 Chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq)

451 We analyzed our previously published KDM5C ChIP-seq experiments in primary neuron cultures from
452 the cortex and hippocampus¹⁵ (available at GEO: GSE61036) and EpiLCs³⁶ (available at GEO: GSE96797).
453 ChIP-seq of histone 3 lysine 4 dimethylation in male EpiLCs³⁶ is also available at GEO: GSE96797. ChIP-seq
454 of histone 3 lysine 4 trimethylation in the male amygdala²¹ are available at GEO: GSE127817.

455 ChIP-seq reads were aligned to mm10 using Bowtie1 (v1.1.2) allowing up to two mismatches. Only
456 uniquely mapped reads were used for analysis. Peaks were called using MACS2 software (v2.1.0.20140616)
457 using input BAM files for normalization, with filters for a q-value < 0.1 and a fold enrichment > 1. Common
458 peak sets were obtained in R via DiffBind (v3.6.5), and count tables for the common peaks were generated
459 with the Bedtools multicov command. We removed “black-listed” genomic regions that often give aberrant

460 signals. Peak proximity to genome annotations was determined by ChIPSeeker (v1.32.1). Enriched motifs
461 were identified using HOMER⁵⁶. Gene ontology (GO) analyses were performed by the R package enrichPlot
462 (v1.16.2) using the biological processes setting. Average binding across the genome was visualized using
463 deeptools (v3.1.3). Bigwigs were visualized using the UCSC genome browser.

464 **Whole genome bisulfite sequencing (WGBS)**

465 **Data availability**

466 **Acknowledgements**

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580 **Figures and Tables**

- 581 • Supplementary table 1: list of all germline genes.
- 582 – Columns to include:
- 583 * KDM5C bound vs not
- 584 * DEG in EpiLC, brain, both, neither (separate columns?)

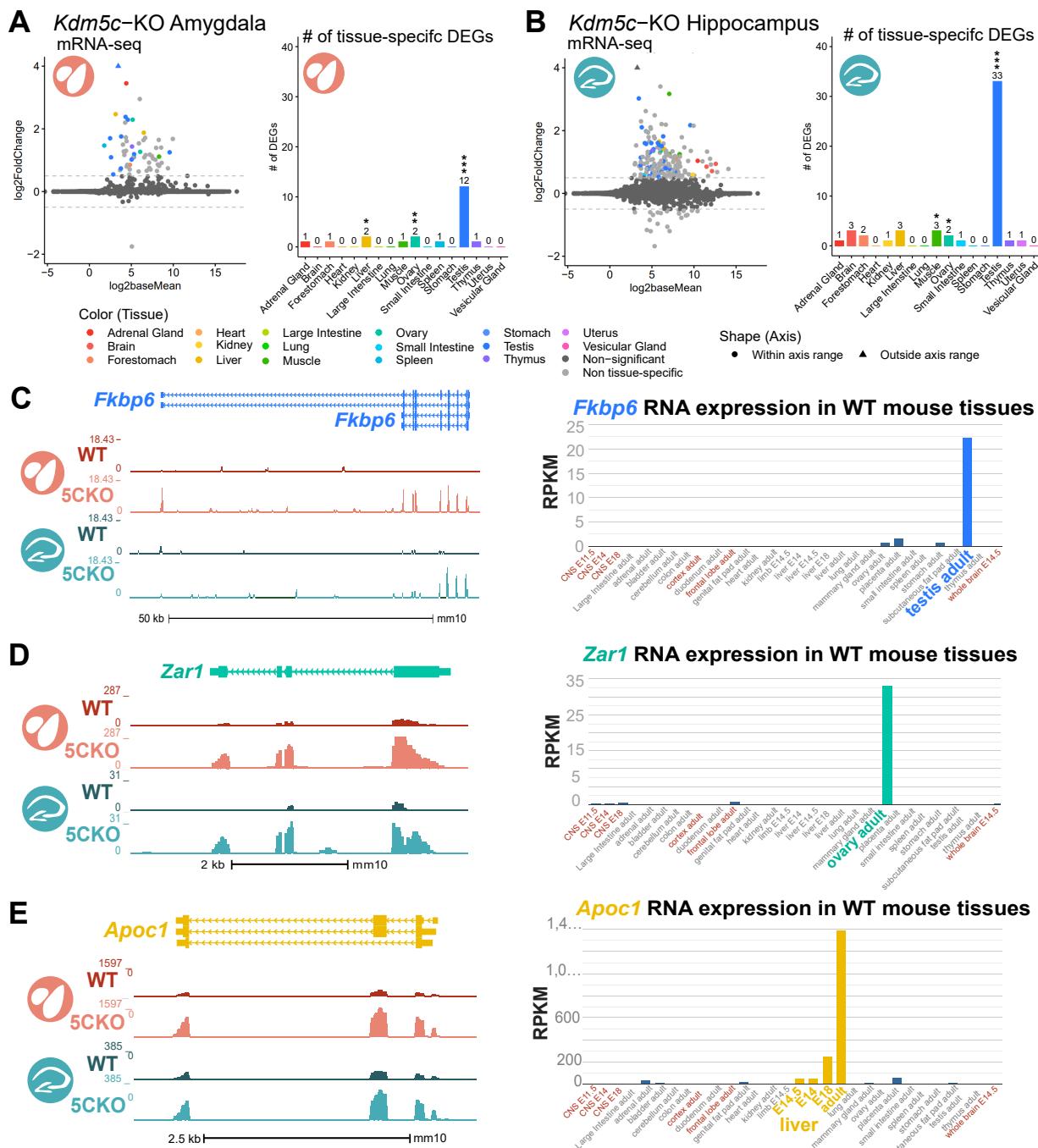


Figure 1: Tissue-enriched genes are misexpressed in the *Kdm5c*-KO brain. **A-B.** Expression of tissue-enriched genes (Li et al 2017) in the male *Kdm5c*-KO amygdala (A) and hippocampus (B). Left - MA plot of mRNA-sequencing. Right - Number of tissue-enriched differentially expressed genes (DEGs). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, 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.

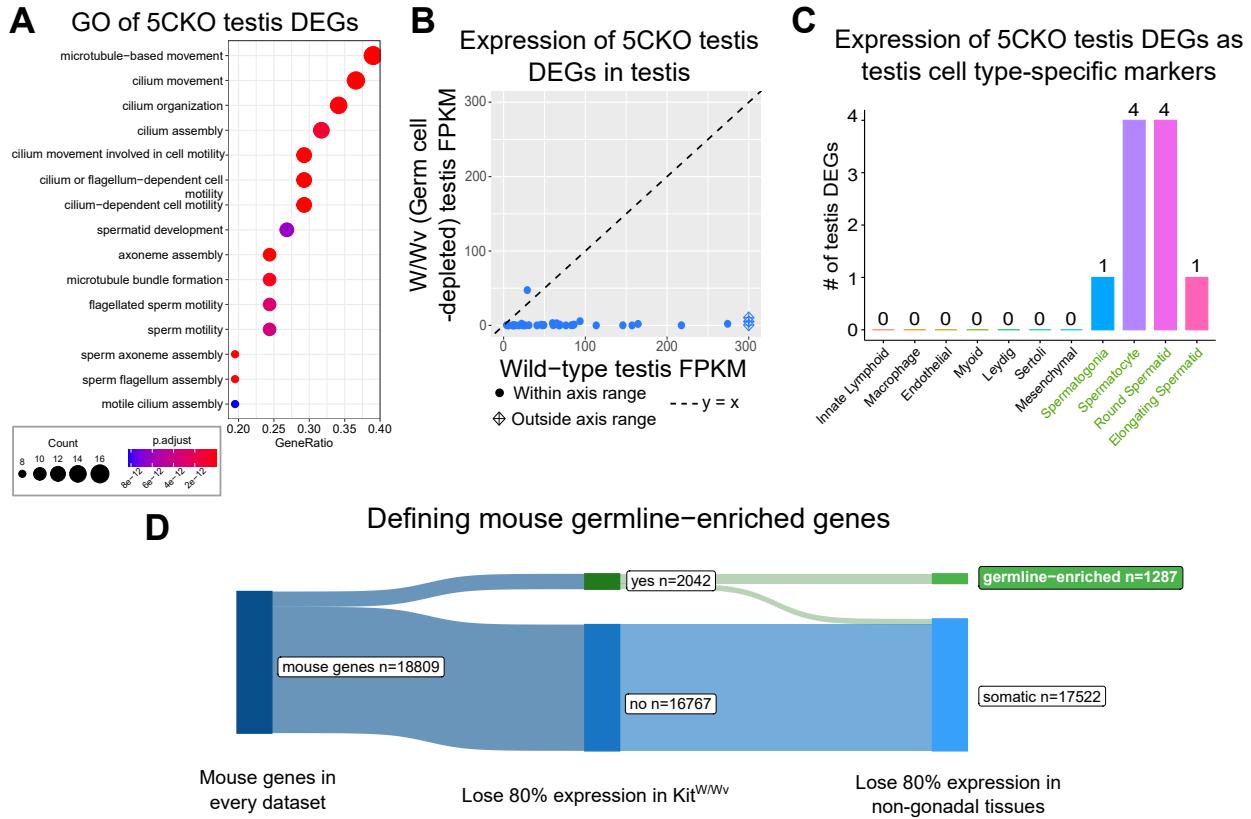


Figure 2: Aberrant transcription of germline genes in the *Kdm5c*-KO in the brain. **A.** enrichPlot gene ontology (GO) of *Kdm5c*-KO amygdala and hippocampus testis-enriched DEGs **B.** Expression of testis DEGs in wild-type (WT) testis versus germ cell-depleted (W/Wv) testis (Mueller et al 2013). Expression 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).

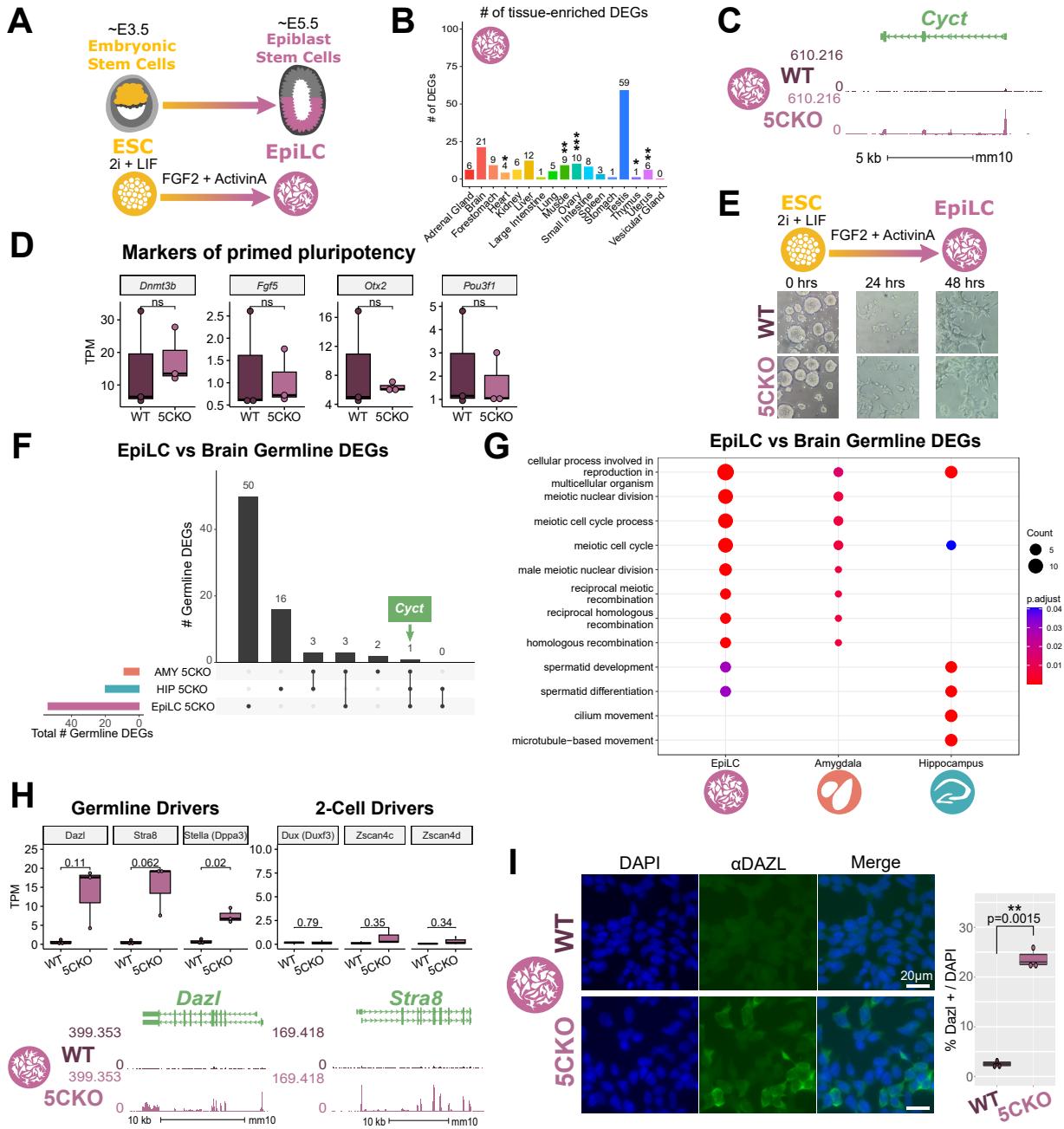


Figure 3: Kdm5c-KO epiblast-like cells express key drivers of germline identity **A.** Top - Diagram of *in vivo* differentiation of embryonic stem cells (ESCs) of the inner cell mass into epiblast stem cells. Bottom - *in vitro* differentiation of ESCs into epiblast-like cells (EpiLCs). **B.** Number 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. **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.

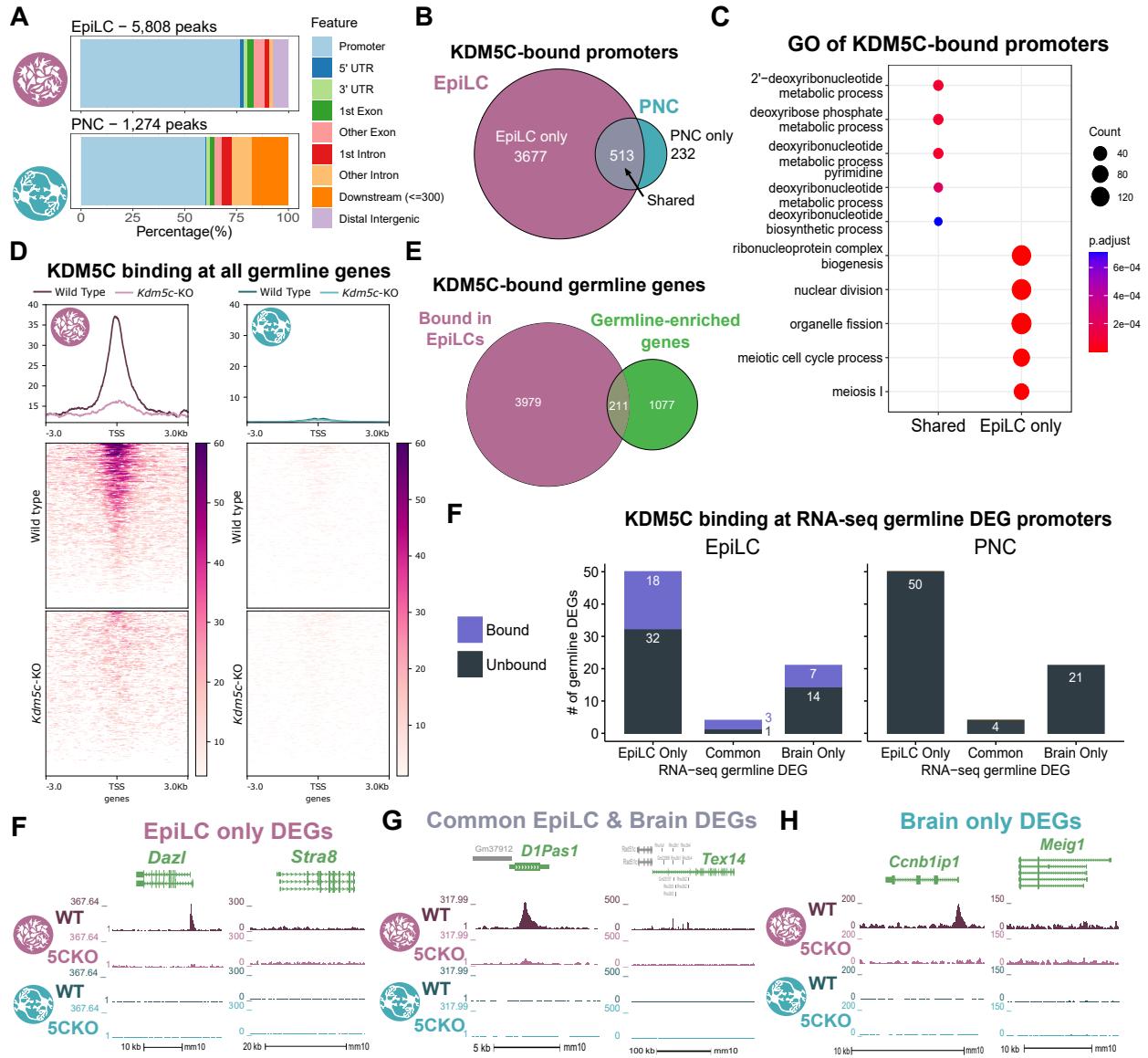
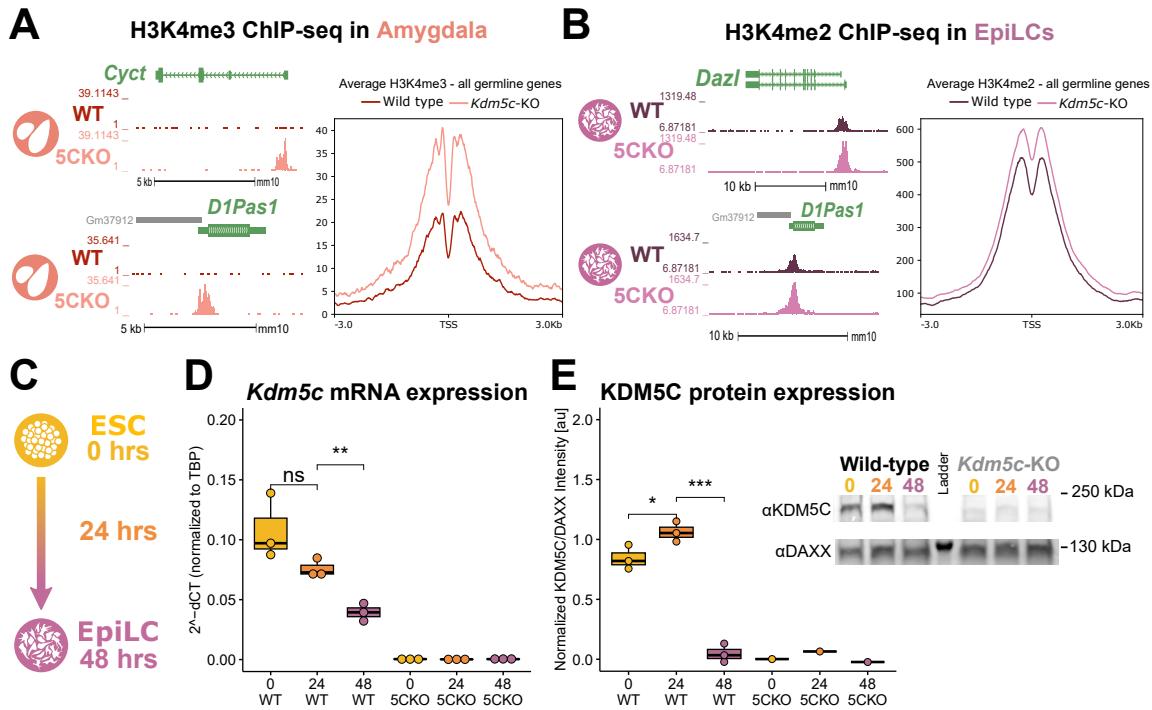


Figure 4: KDM5C binds to a subset of germline gene promoters during early embryogenesis **A.** ChIPseeker localization of KDM5C peaks at different genomic regions in EpiLCs (top) and hippocampal and cortex primary neuron cultures (PNCs, bottom). **B.** Overlap of genes with KDM5C bound to their promoters 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



F

G

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 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

585 **Notes**

586 **Figure outline:**

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

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

599 **Figure 3: *Kdm5c*-KO epiblast-like cells express key drivers regulators of germline identity** * A) ESC
600 to EpiLC differentiation Left - Morphology is unchanged, * B) 5CKO EpiLCs express EpiLC differentiation
601 genes similar to WT lvs * C) Male EpiLCs express germline genes (example Cyct again) * Overlap between
602 brain and EpiLC germline genes - show they're mostly unique * GO of Brain and EpiLC germline genes
603 (meiotic enriched) * Bigwigs or TPM of master regulators * Show that while some are also 2-cell genes
604 (Dazl), 2-cell specific genes aren't dysregulated (Zscan4). Important point because published KDM5C dazl
605 paper is saying KDM5C is a 2-cell regulator, but as far as I can tell only genes shared between germline and
606 2-cell are dysregulated.

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

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

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

617 Gaps in knowledge addressed: * Are other tissue-enriched genes dysregulated, or only testis, germline
618 genes? * Curating a robust list of male and female germline genes * Should talk about 2-cell genes
619 vs germline genes - way to systematically categorize? * Mechanism behind long-term germline gene

620 misexpression * Recent evidence suggests loss of KDM5C in ESCs express some germline genes * Unclear
621 if catalytic activity is required for long-term silencing * Unclear if their dysregulation lasts throughout life or
622 the same between brain or not * When in development does it begin? - Recent evidence suggests some
623 germline genes expressed in 5CKO ESCs but unclear if their dysregulation lasts throughout differentiation
624 and if the identity of germline genes are different compared to the brain * Are there functional consequences
625 to germline gene misexpression?

626 Introduction: * Chromatin regulators are important for cellular identity * H3K4me1-3 linked to active
627 gene promoters and enhancers * Surprisingly, mutations in Chromatin regulators lead to many NDDs
628 (including many H3K4 regulators) * Recent studies have shown some chromatin regulators are important
629 for regulating neuron-specific gene expression/chromatin stat_compare_means * However, loss of some
630 chromatin regulators can also lead to ectopic expression of tissue-enriched genes * Very few studies have
631 looked at these genes and it's unclear if these genes contribute to NDD impairments. * Necessary to
632 first characterize the mechanism behind their derepression to identify molecular footholds into testing their
633 contribution to neuronal impairments and potential for therapeutic intervention

- 634 • Loss of KDM5C can result in the misexpression of genes typically only found in the testis
 - 635 – Misexpression of tissue-enriched genes hasn't been systematically characterized - Unclear if
636 these genes are exceptions or if other tissue-specific genes are dysregulated
 - 637 – Interestingly, these genes (Cyct, D1pas1) typically function in the germline
 - 638 – Germ cells (meiotic cells) are typically distinguished from somatic cells very early on in embryogenesis and is a key feature of multicellularity
 - 639 – Chromatin regulators are very important for decommissioning germline genes and act successively
640 the embryo implants into the uterine wall
 - 642 * Most studies have focused on ESCs, which have a similar transcriptome to germ cells / 2-cells
 - 643 * recently, KDM5C was shown to repress DAZL in ESCs, independent of its catalytic activity
 - 644 * However, DNA methylation is lost in the mature 5CKO brain, DNA methylation is placed later
645 and it's Unclear if it's required for long-term repression (maybe too specific, just trying to go
646 into the fact that the mechanism is partially understood but unclear)
 - 647 – Systematic characterization of ectopic germline genes hasn't been done
 - 648 * unknown if other germline-enriched genes are dysregulated, including oocyte-specific genes
 - 649 * Crucially, it's unknown if misexpression of the germline program leads to functional conse-
650 quences in 5CKO cells.

651 **Germline gene repression background:**

652 Interestingly, some of the ectopic testis transcripts identified in the *Kdm5c*-KO brain are typically ex-
653 pressed in germ cells¹⁰. Unlike somatic cells, germ cells (e.g. sperm and eggs) undergo meiosis and pass
654 on their genetic material to the next generation. The germline and the soma are typically distinguished during

655 early embryogenesis, when germline genes are silenced in epiblast stem cells soon after implantation and
656 only reactivated in a subset to form the germline. Chromatin regulators play a key role in decommissioning
657 germline genes as the embryo transitions from naïve to primed pluripotency by placing repressive histone
658 H2A lysine 119 monoubiquitination (H2AK119ub1)¹⁶, histone 3 lysine 9 trimethylation (H3K9me3)^{16,17}, and
659 DNA CpG methylation^{17–19} at germline gene promoters. KDM5C may also be involved in this early decom-
660 missioning of germline genes, as re-expression of KDM5C in neurons fails to suppress their dysregulation¹⁰.
661 In support of this, KDM5C was very recently shown to repress *Deleted in azoospermia like (Dazl)*, a key
662 regulator of germline development, in mouse embryonic stem cells (ESCs)^{40,43}. In support of this, two
663 independent screens in mouse embryonic stem cells (ESCs) recently identified KDM5C as a repressor of
664 *Deleted in azoospermia like (Dazl)*, a key regulator of germline development. However, KDM5C's role in
665 embryonic germline gene repression is currently unclear, given that Dazl is also expressed in ESCs and in
666 the 2-cell stage and germline gene misexpression has yet to be globally characterized during *Kdm5c*-KO
667 embryogenesis.