

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

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5 Abstract

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

28 Introduction

29 A single genome holds the instructions to generate the myriad of cell types found within the adult
30 organism. This is, in part, accomplished by chromatin regulators that can either promote or impede lineage-
31 specific gene expression through DNA and histone modifications^{1,2}. Many chromatin regulators were initially
32 identified for their roles in shaping cellular and tissue identity^{3–5}. Recent advancements in next generation
33 sequencing unexpectedly revealed mutations in many chromatin regulators cause neurodevelopmental
34 disorders (NDDs)⁶. Several studies have suggested this connection between chromatin regulators and
35 neurodevelopment is due to their regulation of brain-specific genes, such as orchestrating transcriptional
36 programs for synaptic maturation⁷ and transitioning between neuronal and glial fates during neural precursor
37 differentiation⁸. However, loss of some chromatin regulators can also result in the ectopic transcription
38 of tissue-specific genes outside of their target environment, such as the misexpression of liver-specific
39 genes within adult neurons⁹. Very few studies have investigated this cellular identity crisis in chromatin-
40 linked NDDs^{9,10} and it is currently unknown if ectopic gene expression contributes to neurodevelopmental
41 impairments.

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

55 The testis contains both germ cells (cells that pass on their genetic material, e.g. sperm) and somatic
56 cells (cells that perform all other bodily functions). Distinguishing between the germline and the soma is a key
57 feature of multicellularity¹⁶ and that typically occurs during early embryogenesis¹⁷. In mammals, chromatin
58 regulators play a key role in decommissioning germline genes in somatic cells during the transition from naïve
59 to primed pluripotency. Initially, repressive histone H2A lysine 119 monoubiquitination (H2AK119ub1)¹⁸,
60 histone 3 lysine 9 trimethylation (H3K9me3)^{18,19}, are placed at germline gene promoters in embryonic stem
61 cells and are then decorated with DNA CpG methylation^{19–21} in the post-implantation embryo. How KDM5C
62 interacts with known germline gene silencers during this time point is currently unknown. Additionally, most

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

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

82 Results

83 **Tissue-enriched genes, including testis genes, are aberrantly expressed in the** 84 ***Kdm5c*-KO brain**

85 Previous RNA sequencing (RNA-seq) in the adult hippocampus revealed ectopic expression of some testis
86 genes within the *Kdm5c* knockout (-KO) brain¹⁰. Since tissue-enriched genes have not been systematically
87 characterized in the *Kdm5c*-KO brain, if the testis is the only tissue type misexpressed. Therefore, we
88 systematically assessed the expression of genes enriched in 17 mouse tissues²² in our published mRNA-seq
89 datasets of the adult amygdala and hippocampus in mice with constitutive knockout of *Kdm5c*²³.

90 We found a large proportion of genes that are significantly upregulated within the *Kdm5c*-KO brain
91 (DESeq2²⁴, log2 fold change > 0.5, q < 0.1) are enriched in non-brain tissues in wild-type mice (Amygdala:
92 35%, Hippocampus: 24%) (Figure 1A-B). The majority of tissue-enriched differentially expressed genes
93 (tissue-enriched DEGs) were testis genes (Figure 1A-C). Even though the testis has the largest total number
94 of tissue-biased genes compared to any other tissue (2,496 genes), testis-biased DEGs were significantly
95 enriched for both brain regions (Amygdala p = 1.83e-05, Odds Ratio = 5.13; Hippocampus p = 4.26e-11,

96 Odds Ratio = 4.45, Fisher's Exact Test). One example of a testis-enriched DEG is *FK506 binding protein 6*
97 (*Fkbp6*), a known regulator of piRNA expression and meiosis in germ cells^{25,26} (Figure 1C).

98 Interestingly, we also observed significant enrichment of ovary-biased DEGs in both the amygdala and
99 hippocampus (Amygdala p = 0.00574, Odds Ratio = 18.7; Hippocampus p = 0.048, Odds Ratio = 5.88,
100 Fisher's Exact) (Figure 1D). Ovary-enriched DEGs included *Zygotic arrest 1 (Zar1)*, sequesters mRNAs
101 in oocytes for meiotic maturation and early zygote development²⁷ (Figure 1D). Given the *Kdm5c*-KO mice
102 we analyzed are male, this demonstrates ectopic expression of tissue-enriched genes is independent
103 of organismal sex. Although not consistent across brain regions, we also found significant enrichment
104 of DEGs biased towards two non-gonadal tissues - the liver (Amygdala p = 0.0398, Odds Ratio = 6.58,
105 Fisher's Exact Test) and the muscle (Hippocampus p = 0.0104, Odds Ratio = 6.95, Fisher's Exact Test). An
106 example of a liver-biased DEG dysregulated in both the hippocampus and amygdala is *Apolipoprotein C-I*
107 (*Apoc1*), a lipoprotein metabolism and transport gene²⁸ (Figure 1E). These aberrantly expressed mRNAs are
108 polyadenylated and spliced into mature transcripts (Figure 1C-E). Of note, we did not observe enrichment
109 of brain-enriched genes (Amygdala p = 1; Hippocampus p = 0.74, Fisher's Exact), despite the fact these
110 are brain samples and the brain has the second highest total number of tissue-enriched genes (708 genes).
111 Together, these results suggest misexpression of tissue-enriched genes within the brain is a major effect of
112 KDM5C loss.

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

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

120 To further validate if these testis DEGs are truly germline genes, we evaluated their expression in
121 somatic versus germ cells within the testis. We first compared their expression in the testis without germ
122 cells²⁹, which was accomplished by heterozygous *W* and *Wv* mutations in the enzymatic domain of *c-Kit*
123 (*Kit^{W/Wv}*)³⁰. Almost all *Kdm5c*-KO testis-enriched DEGs lost expression with germ cell depletion (Figure
124 2B). We then assessed testis-enriched DEG expression in a published single cell RNA-seq dataset that
125 identified cell type-specific markers within the testis³¹. We found some testis-enriched DEGs were classified
126 as specific markers for different germ cell types (e.g. spermatogonia, spermatocytes, round spermatids, and
127 elongating spermatids), yet none marked somatic cells (Figure 2C). Together, these data demonstrate that
128 the *Kdm5c*-KO brain aberrantly expresses germline genes, but not somatic testicular genes, reflecting an
129 erosion between somatic versus germline identity.

130 We then wanted to characterize germline gene misexpression with *Kdm5c* loss more deeply, but lacked a

131 comprehensive list of mouse germline-enriched genes. We thus generated a list of germline-enriched genes
132 using RNA-seq datasets of *Kit^{WWv}* mice that include males and females at embryonic day 12, 14, and 16³²
133 and adult male testes²⁹. We defined genes as germline-enriched if their expression met the following criteria:
134 1) their expression is greater than 1 FPKM in wild-type gonads 2) their expression in any non-gonadal tissue
135 for adult wild type²² does not exceed 20% of their maximum expression in the wild-type germline, and 3)
136 their expression in the germ cell-depleted gonads, for any sex or time point, does not exceed 20% of their
137 maximum expression in the wild-type germline. These criteria yielded 1,288 germline-enriched genes (Figure
138 2D), which was hereafter used as a resource to globally assess germline gene misexpression with *Kdm5c*
139 loss (Supplementary table 1).

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

142 Germ cells are typically distinguished from somatic cells soon after the embryo implants into the uterine
143 wall^{33,34}. A subset of epiblast stem cells become the primordial germ cells (PGCs) while the remainder
144 differentiate into the ectoderm, mesoderm, and endoderm to form the somatic tissues³⁵. This developmental
145 time point can be modeled *in vitro* through differentiation of embryonic stem cells (ESCs) into epiblast-like
146 stem cells (EpiLCs) (Figure 3A, top)^{36,37}. While some germline-enriched genes are also expressed in
147 embryonic stem cells (ESCs) and in the 2-cell stage^{38–40}, they are silenced as they differentiate into EpiLCs¹⁹.
148 Therefore, we tested if KDM5C was necessary for silencing germline genes at this developmental stage by
149 evaluating the impact of *Kdm5c* loss in EpiLCs.

150 We first identified *Kdm5c*-KO EpiLC DEGs in our previously published RNA-seq dataset⁴¹ (DESeq2, log2
151 fold change > 0.5, q < 0.1) and assessed tissue-enriched gene expression. Similar to the *Kdm5c*-KO brain,
152 we observed general dysregulation of tissue-enriched genes, with the largest number of genes belonging to
153 the brain and testis, although they were not significantly enriched (Figure 3B). Primed pluripotency genes,
154 such as *Dnmt3b*, *Fgf5*, *Pou3f1*, and *Otx2*, were unaltered in *Kdm5c*-KO EpiLCs (Figure 3C). Furthermore,
155 *Kdm5c*-KO cell morphology during ESC to EpiLC differentiation (Figure 3D) was normal, indicating KDM5C
156 loss does not impair EpiLC formation.

157 To determine if germline DEGs are constitutively dysregulated or if they can change over the course
158 of development, we compared EpiLC germline DEGs to those expressed in the *Kdm5c*-KO brain. We
159 found the majority of germline DEGs were unique to either EpiLCs or the brain, with only *Cyct* shared
160 across all tissue/cell types (Figure 3E-F). We found particularly high enrichment of meiosis-related gene
161 ontologies in EpiLCs (Figure 3G), such as meiotic cell cycle process (GO: 1903046, p.adjust = 1.59e-08)
162 and meiotic nuclear division (GO: 0140013, p.adjust = 9.76e-09). While there was modest enrichment of
163 meiotic gene ontologies in both brain regions, the *Kdm5c*-KO hippocampus primarily expressed late-stage
164 spermatogenesis genes, such those involved in the sperm axoneme structure.

165 Notably, DEGs unique to *Kdm5c*-KO EpiLCs included key drivers of germline identity, such as *Stimulated*
166 *by retinoic acid 8 (Stra8)* and *Deleted in azoospermia like (Dazl)* (Figure 3H). These genes are typically
167 expressed during embryonic germ cell development to commit PGCs to the germline fate, but are also
168 expressed later in life to trigger meiotic gene expression programs⁴²⁻⁴⁴. Of note, some germline genes,
169 including *Dazl*, are also expressed in the two-cell embryo^{39,45}. However, we did not see misexpression of
170 two-cell embryo-specific genes, like *Duxf3 (Dux)* ($q = 0.337$) and *Zscan4d* ($q = 0.381$), indicating *Kdm5c*-KO
171 in EpiLCs do not revert back to a 2-cell state (Figure 3H).

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

173 Previous work suggests KDM5C represses germline genes during early development, as re-expression
174 of KDM5C in knockout neuronal cultures fails to suppress two hippocampal germline DEGs¹⁰. KDM5C binds
175 to several germline gene promoters in embryonic stem cells (ESCs) but is lost in neurons¹⁰. However, the
176 lack of a comprehensive list of germline-enriched genes prohibited systematic characterization of KDM5C
177 binding at germline gene promoters. Thus, it is currently unclear if KDM5C is enriched at germline gene
178 promoters, what types of germline genes KDM5C regulates, and if its binding is maintained at any germline
179 genes in neurons.

180 To address these questions, we analyzed KDM5C chromatin immunoprecipitation followed by DNA
181 sequencing (ChIP-seq) datasets in EpiLCs⁴¹ and primary forebrain neuron cultures (PNCs)¹⁵. EpiLCs had a
182 higher total number of KDM5C peaks than PNCs (EpiLCs: 5,808, PNCs: 1,276, MACS2 $q < 0.1$ and fold
183 enrichment > 1 , removal of *Kdm5c*-KO false positive peaks) and KDM5C was primarily localized to gene
184 promoters in both cell types (EpiLCs: 4,190, PNCs: 745 +/- 500bp from the TSS), although PNCs showed
185 increased localization to non-promoter regions (Figure 4A).

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

199 We then compared KDM5C binding at promoters of RNA-seq DEGs to determine if the germline mRNAs

200 de-repressed in *Kdm5c*-KO EpiLCs and brain are direct targets of KDM5C (Figure 4F). In EpiLCs, KDM5C
201 was bound to about one third of EpiLC-specific and brain-specific germline DEGs (EpiLC only: 36%, Brain
202 only: 33.3%). Again, we did not observe any KDM5C binding at germline gene promoters in PNCs, even
203 for brain-specific DEGs (Figure 4H). Some notable differences in KDM5C binding for EpiLC-specific DEGs
204 included *Dazl* and *Stra8*, two key drivers of germline identity discussed above. Although both mRNAs are
205 expressed in *Kdm5c*-KO EpiLCs, KDM5C is only bound to *Dazl*'s promoter and not *Stra8*'s in EpiLCs (Figure
206 4F). In contrast to the unique DEGs, 3 out of the 4 genes dysregulated in both the brain and EpiLCs are bound
207 by KDM5C around the TSS (Figure 4G). Altogether, this suggests KDM5C decommissions germline genes
208 in EpiLCs but is no longer recruited to germline gene promoters in neurons. Furthermore, the majority of
209 germline mRNAs expressed in *Kdm5c*-KO cells are dysregulated independent of direct KDM5C recruitment
210 to their promoters.

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

212 In the early embryo, germline gene promoters are initially silenced by repressive histone modifications,
213 including histone 2A lysine 119 monoubiquitination (H2AK119ub1) and histone 3 lysine 9 trimethylation
214 (H3K9me3), and are then silenced long-term via DNA CpG methylation (CpGme)^{18,19,46}. Our results above
215 suggest KDM5C also acts during this time period, however how KDM5C interacts with other germline gene
216 silencing mechanism is currently unclear. KDM5C is generally thought to suppress transcription through
217 erasure of histone 3 lysine 4 di- and trimethylation (H3K4me2/3)¹¹. However, KDM5C's catalytic activity was
218 recently shown to not be required for suppressing *Dazl* in undifferentiated ESCs⁴⁵. Since H3K4me3 impedes
219 *de novo* CpGme placement^{47,48}, KDM5C's catalytic activity may instead be required for CpGme-mediated,
220 long-term silencing of germline genes. In support of this, CpGme is markedly reduced at two germline gene
221 promoters in the *Kdm5c*-KO adult hippocampus¹⁰. Based on these observations, we hypothesized KDM5C
222 erases H3K4me3 to promote the initial placement of CpGme at germline gene promoters in EpiLCs.

223 To test this hypothesis, we first characterized KDM5C's substrates, histone 3 lysine 4 di- and trimethylation
224 (H3K4me2/3) in our previously published ChIP-seq datasets of the wild type and *Kdm5c*-KO amygdala²³
225 and EpiLCs⁴¹. In congruence with previous work in the *Kdm5c*-KO hippocampus¹⁰, we observed aberrant
226 accumulation of H3K4me3 around the transcription start site (TSS) of germline genes in the *Kdm5c*-KO
227 amygdala (Figure 5A). We additionally found a marked increase in H3K4me2 around the TSS of germline
228 genes in *Kdm5c*-KO EpiLCs (Figure 5B).

229 We then evaluated KDM5C's role in germline gene silencing during ESC to EpiLC differentiation. We
230 first characterized KDM5C's mRNA and protein expression at 0, 24, and 48 hours of differentiation (Figure
231 5C). While *Kdm5c* mRNA steadily decreased from 0 to 48 hours of differentiation (Figure 5D), KDM5C
232 protein initially increased from 0 to 24 hours but then decreased to near knockout levels by 48 hours (Figure
233 5E). To determine KDM5C's role in the initial placement of DNA methylation at germline gene promoters,
234 we performed whole genome bisulfite sequencing (WGBS) in wild-type and *Kdm5c*-KO ESCs and 96 hour

235 extended EpiLCs (exEpiLCs). Although wild-type cells accumulated high levels of DNA methylation at
236 germline gene promoters over the course of ESCs to exEpiLC differentiation, DNA methylation was markedly
237 reduced in *Kdm5c*-KO exEpiLCs (Figure 5F).

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

240 Discussion

241 In the above study, we demonstrate KDM5C's pivotal role in the development of tissue identity, resulting in
242 substantial misexpression of tissue-enriched genes within the adult *Kdm5c*-KO hippocampus and amygdala.
243 In addition to testis genes identified previously¹⁰, we found significant enrichment of muscle, liver, and
244 even ovary-biased genes aberrantly expressed within the male *Kdm5c*-KO brain. Given the majority of
245 tissue-enriched DEGs are testis and ovary genes with no known brain functions, it is currently unknown if
246 they impair *Kdm5c*-KO brain function and contribute to MRXSCJ-related phenotypes like intellectual disability
247 and aberrant aggression. However, select liver and muscle-biased DEGs do have known roles within the
248 brain, such as the liver-enriched lipid metabolism gene *Apolipoprotein C-I (Apoc1)*²⁸ that is highly expressed
249 in the *Kdm5c*-KO amygdala and hippocampus. Intriguingly, *Apoc1* overexpression in the mouse brain can
250 impair learning and memory⁴⁹ and is implicated in Alzheimer's disease in humans⁵⁰. Thus, failure to fine-tune
251 the expression of tissue-enriched, dosage-sensitive genes like *Apoc1* could be one route by which loss of
252 brain tissue identity contributes to *Kdm5c*-KO impairments.

253 We found a substantial portion of genes upregulated in *Kdm5c*-KO brain are germline genes with no
254 known fucntions within the brain. Distinguishing the germline (i.e. cells that pass on their genetic material
255 to the next generation) from the soma (i.e. all other cells of the body) is a key feature of multicellularity
256 and sexual reproduction. Previous work characterizing chromatin regualtors that silence germ cell-specific
257 transcription has predominatly focused on their repression of key marker genes in embryonic stem cells
258 (ESCs), such as *Dazl* and *Ddx4*^{18,19,51}. To globally characterize KDM5C's role in germline gene repression
259 throughout life, we curated a list of mouse germline-enriched genes using publically available germ cell-
260 depleted RNA-seq datasets from Kit^{W/Wv} mice^{29,32}. This resource enabled us to identify 1) the extent of
261 germline gene dysregulation in *Kdm5c*-KO cells, 2) the types of germline genes misexpressed at different
262 developmental time points, and 3) the categories of germline genes directly and indirectly regulated by
263 KDM5C.

264 RNA-seq of epiblast-like cells (EpiLCs) revealed dysregulation of *Kdm5c*-KO tissue identity begins during
265 early emrbyogenesis. *In vivo*, germline genes are typically decommissioned in epiblast stem cells and
266 remain silenced as the epiblast differentiates into the body's somatic tissues³⁵. However, a small subset of
267 epiblast stem cells will receive signals to reactivate germline gene expression to become the primordial germ
268 cells (PGCs) that will ultimately form the mature germline^{33,34}. This process can be mimicked *in vitro* by

269 differentiating EpiLCs into primordial germ cell-like cells (PGCLCs)³⁶. Therefore, misexpression of germline
270 genes in EpiLCs might suggest *Kdm5c*-KO ESCs are progressing beyond EpiLC differentiation and instead
271 becoming PGCLCs. Yet, *Kdm5c*-KO EpiLCs properly express primed pluripotency genes, including *Otx2*
272 - an epiblast stem cell marker that is known to repress epiblast and EpiLC differentiation into PGC and
273 PGCLCs⁵². Furthermore, we observed no difference in cellular morphology during *Kdm5c*-KO ESC to EpiLC
274 differentiation. Proper EpiLC differentiation, together with *Kdm5c*-KO mice being viable, suggests germline
275 gene expression is occurring ectopically in conjunction with typical developmental programs, rather than a
276 complete shift towards germline identity.

277 We then globally characterized KDM5C binding at germline-enriched gene promoters through KDM5C
278 ChIP-seq in EpiLCs and primary hippocampus and cortex neuron cultures (PNCs). While we observed
279 high enrichment of KDM5C peaks at germline genes in EpiLCs, KDM5C was not bound to germline gene
280 promoters in PNCs. This suggests dysregulation of germline genes in the mature *Kdm5c*-KO brain is due
281 to loss of repression during embryogenesis, which is consistent with previous work that found introducing
282 human KDM5C into *Kdm5c*-KO PNCs does not repress two aberrant germline transcripts¹⁰. Although
283 enriched at germline gene promoters in EpiLCs, KDM5C was only bound to about a third of the germline
284 genes aberrantly transcribed in *Kdm5c*-KO EpiLCs. One notable EpiLC DEG not bound by KDM5C is
285 *Stra8*, a transcription factor activated by retinoic acid signaling in germ cells to promote meiotic initiation^{53,54}.
286 However, retinoic acid can only activate *Stra8* expression when DAZL is present. Unlike *Stra8*, *Dazl* is a
287 direct target of KDM5C in EpiLCs and is both transcribed and translated in *Kdm5c*-KO EpiLCs. Expression of
288 indirect DEGs like *Stra8* indicates germline genes can be aberrantly expressed in *Kdm5c*-KO cells through
289 downstream activation by other ectopic germline programs. These ectopic germline programs appear to
290 loosely mimic the trajectory of typical germline development, as germline genes important for early germ
291 cell development and meiosis are expressed in *Kdm5c*-KO EpiLC while late spermatogenesis genes are
292 expressed in the mature *Kdm5c*-KO brain. Altogether, this indicates once a subset of germline genes are
293 activated due to loss of direct KDM5C repression in the early embryo, these ectopic germline programs can
294 continue to progress in the background of *Kdm5c*-KO somatic development.

295 It is important to note that while we focused on KDM5C's regulation of germline genes, some germline-
296 enriched genes are also expressed in naïve ESCs and at the 2-cell stage due to their roles in pluripotency and
297 self-renewal. For example, *Dazl* is primarily known for committing PGCs to the germline fate and regulating
298 the translation of germline-specific RNAs, but is also expressed at the 2-cell stage⁵⁵, in naïve ESCs³⁸, and in
299 the inner cell mass³⁸. Very recently, two screens of *Dazl*-repressors identified KDM5C as a direct regulator in
300 ESCs^{45,56}. Interestingly, one study found *Kdm5c*-KO ESCs also aberrantly expressed 2-cell-specific genes,
301 indicating KDM5C negatively regulates totipotency⁴⁵. However, out of the four regulators characterized,
302 KDM5C was the only factor whose repression of *Dazl* was independent of the 2-cell specific transcription
303 factor *Dux*⁴⁵. Although expressed at the 2-cell stage and in ESCs, *Dazl* is typically silenced when ESCs
304 differentiate into EpiLCs¹⁹. We found KDM5C also represses *Dazl* in EpiLCs, but *Kdm5c*-KO EpiLCs did

305 not express 2-cell specific genes like *Dux* and *Zscan4c*. Together, this suggests the 2-cell-like state in
306 *Kdm5c*-KO ESCs is an indirect consequence of KDM5C's primary role in germline gene repression.

307 *Dazl* and other germline-enriched genes are initially decommissioned in ESCs by repressive histone
308 modifications and then *de novo* DNA CpG methylation (CpGme) in EpiLCs^{19–21,46,57,58}. Unlike the previously
309 characterized germline gene suppressors, KDM5C removes an active histone mark, histone 3 lysine 4 di-
310 and trimethylation (H3K4me2/3)¹¹. In this study, we observed a global increase in H3K4me2/3 in *Kdm5c*-KO
311 EpiLCs and amygdala around the transcription start site (TSS) of germline-enriched genes. However,
312 KDM5C's catalytic activity may not be required for germline gene silencing, as it was recently found to be
313 dispensible for repressing *Dazl* in ESCs⁴⁵. Although not necessary in ESCs, KDM5C's catalytic activity be
314 required for CpGme in EpiLCs, as H3K4me3 impedes *de novo* CpGme placement^{47,48}. This is supported
315 by previous work in the *Kdm5c*-KO adult hippocampus, which found CpGme is significantly eroded at at
316 least two germline promoters¹⁰. To elucidate the mechanism behind KDM5C-mediated silencing of germline
317 genes, we characterized KDM5C in ESCs and EpiLCs and found its expression is dynamically regulated
318 during differentiation. Furthermore, KDM5C acts during the transition from naïve to primed pluripotency to
319 promote the initial placement of CpGme at CpG islands for the long-term silencing of germline genes.

320 The above work provides the mechanistic foundation for KDM5C's role in germline gene repression
321 and offers novel insight into how dysregulation in *Kdm5c*-KO tissue identity changes over the course of
322 development. Although their impact upon neurodevelopment is currently unclear, ectopic germline transcripts
323 are also found in models of other related neurodevelopmental disorders⁵⁹, including Immunodeficiency,
324 Centromere instability, Facial anomalies syndrome 1 (ICF1 OMIM: #242860)^{60,61}, Kleefstra syndrome
325 1 (OMIM: #610253)⁹, and MeCP2 duplication syndrome (MDS, OMIM: #300260)⁶². Like KDM5C, the
326 chromatin regulators underlying these conditions, DNA methyltransferase 3b (DNMT3B), H3K9me1/2
327 methyltransferases G9A/GLP, methyl-CpG -binding protein 2 (MECP2), primarily silence gene expression.
328 Thus, KDM5C is among a growing cohort of chromatin-linked neurodevelopmental disorders that may have a
329 similar underlying cause of germline versus soma dysregulation. However, further research is required to
330 determine the ultimate impact of these germline genes and the extent to which this phenomenon occurs in
331 humans.

332 Materials and Methods

333 Classifying tissue-enriched and germline-enriched genes

334 Tissue-enriched differentially expresssd genes (DEGs) were determined by their classification in a previ-
335 ously published dataset from 17 male and female mouse tissues²². This study defined tissue expression as
336 greater than 1 Fragments Per Kilobase of transcript per Million mapped read (FPKM) and tissue enrichment
337 as at least 4-fold higher expression than any other tissue.

338 We curated a list of germline-enriched genes using an RNA-seq dataset from wild-type and germline-depleted (Kit^{W/W^v}) male and female mouse embryos from embryonic day 12, 14, and 16³², as well as adult male testes²⁹. Germline-enriched genes met the following criteria: 1) their expression is greater than 1 FPKM in wild-type germline 2) their expression in any wild-type somatic tissues²² does not exceed 20% of maximum expression in wild-type germline, and 3) their expression in the germ cell-depleted (Kit^{W/W^v}) germline, for any sex or time point, does not exceed 20% of maximum expression in wild-type germline.

344 Cell culture

345 We utilized our previously established cultures of male wild-type and *Kdm5c* knockout (-KO) embryonic stem cells⁴¹. Sex was confirmed by genotyping *Uba1* on the X and Y chromosomes with the following primers: 5'-TGGATGGTGTGCCAATG-3', 5'-CACCTGCACGTTGCCCTT-3'. Deletion of *Kdm5c* was confirmed through the primers 5'-ATGCCCATATTAAGAGTCCTG-3', 5'-TCTGCCTTGATGGGACTGTT-3', and 5'-GGTTCTCAACACTCACATAGTG-3'.

350 Embryonic stem cells (ESCs) were initially cultured in primed ESC (pESC) media consisting of KnockOut DMEM (Gibco#10829-018), fetal bovine serum (Gibco#A5209501), KnockOut serum replacement (Invitrogen#10828-028), Glutamax (Gibco#35050-061), Anti-Anti (Gibco#15240-062), MEM Non-essential amino acids (Gibco#11140-050), and beta-mercaptoethanol (Sigma#M7522). They were then transitioned into ground-state "naïve" ESCs (nESCs) by culturing for four passages in N2B27 media containing DMEM/F12 (Gibco#11330-032), Neurobasal media (Gibco#21103-049), Gluamax, Anti-Anti, N2 supplement (Invitrogen#17502048), and B27 supplement without vitamin A (Invitrogen#12587-010), and beta-mercaptoethanol. Both pESC and nESC media were supplemented with 3 μ M GSK3 inhibitor CHIR99021 (Sigma #SML1046-5MG), 1 μ M MEK inhibitor PD0325901 (Sigma #PZ0162-5MG), and 1,000 units/mL leukemia inhibitory factor (LIF, Millipore#ESG1107).

360 nESCs were differentiated into epiblast-like cells (EpiLCs, 48 hours) and extended EpiLCs (exEpiLCs, 361 96 hours) by culturing in N2B27 media containing DMEM/F12, Neurobasal media, Gluamax, Anti-Anti, 362 N2 supplement, B27 supplement (Invitrogen#17504044), beta-mercaptoethanol, fibroblast growth factor 2 363 (FGF2, R&D Biotechne 233-FB), and activin A (R&D Biotechne 338AC050CF), as previously described³⁷.

364 Immunocytochemistry (ICC)

365 ICC of DAZL in EpiLCs was performed by first growing cells on coverslips. Cells were then washed thrice 366 with phosphobuffered saline (PBS), fixed in 4% paraformaldehyde, washed thrice in PBS, and blocked in 367 PBS containing 0.3% Triton X-100, and 5% fetal bovine serum for 1 hour. They were then washed thrice 368 with PBS and incubating in primary antibody (Rabbit anti DAZL, abcam ab34139, 1:200) in the blocking 369 solution overnight at 4 °C with gentle rocking. The next day, cells were rinsed thrice with PBS, and incubated 370 in secondary antibody (Alexafluor 488 Invitrogen #710369, 1:1,000) with DAPI (1:1,000) in blocking buffer

371 for 1 hour at room temperature. Coverslips were then rinsed thrice in PBS, and mounted onto slides using
372 Prolong Gold (Invitrogen #P36930). Images were taken blinded for genotype, chosen based on similar levels
373 of DAPI signal, and quantified via ImageJ before unblinding.

374 **RNA sequencing (RNA-seq)**

375 After ensuring read quality via FastQC (v0.11.8), reads were then mapped to the mm10 *Mus musculus*
376 genome (Gencode) using STAR (v2.5.3a), during which we removed duplicates and kept only uniquely
377 mapped reads. Count files were generated by FeatureCounts (Subread v1.5.0), and BAM files were
378 converted to bigwigs using deeptools (v3.1.3) and visualized by the UCSC genome browser. RStudio (v3.6.0)
379 was then used to analyze counts files by DESeq2 (v1.26.0)²⁴ to identify differentially expressed genes
380 (DEGs) with a q-value (p-adjusted via FDR/Benjamini–Hochberg correction) less than 0.1 and a log2 fold
381 change greater than 0.5. For all DESeq2 analyses, log2 fold changes were calculated with IfcShrink using
382 the ashr package⁶³. MA-plots were generated by ggpibr (v0.6.0), and Eulerr diagrams were generated by
383 eulerr (v6.1.1). Boxplots and scatterplots were generated by ggpibr (v0.6.0) and ggplot2 (v3.3.2). The Upset
384 plot was generated via the package UpSetR (v1.4.0)⁶⁴. Gene ontology (GO) analyses were performed by
385 the R package enrichPlot (v1.16.2) using the biological processes setting and compareCluster.

386 **Chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq)**

387 ChIP-seq reads were aligned to mm10 using Bowtie1 (v1.1.2) allowing up to two mismatches. Only
388 uniquely mapped reads were used for analysis. Peaks were called using MACS2 software (v2.2.9.1) using
389 input BAM files for normalization, with filters for a q-value < 0.1 and a fold enrichment > 1. We removed
390 “black-listed” genomic regions that often give aberrant signals. Common peak sets were obtained in R via
391 DiffBind (v3.6.5). In the case of KDM5C ChIP-seq, *Kdm5c*-KO peaks were then subtracted from wild-type
392 samples using bedtools (v2.25.0). Peak proximity to genome annotations was determined by ChIPSeeker
393 (v1.32.1). Gene ontology (GO) analyses were performed by the R package enrichPlot (v1.16.2) using the
394 biological processes setting and compareCluster. Enriched motifs were identified using HOMER⁶⁵. Average
395 binding across the genome was visualized using deeptools (v3.1.3). Bigwigs were visualized using the
396 UCSC genome browser.

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

398 **Data availability**

399 **Published datasets**

400 All published datasets are available at the Gene Expression Omnibus (GEO) <https://www.ncbi.nlm.nih>
401 .gov/geo. Published RNA-seq datasets analyzed in this study included the male wild-type and *Kdm5c*-KO

402 adult amygdala and hippocampus²³ (available at GEO: GSE127722) and male wild-type and *Kdm5c*-KO
403 EpiLCs⁴¹ (available at GEO: GSE96797).

404 Previously published ChIP-seq experiments included KDM5C in wild-type and *Kdm5c*-KO EpiLCs⁴¹ (avail-
405 able at GEO: GSE96797) and mouse primary neuron cultures (PNCs) from the cortex and hippocampus¹⁵
406 (available at GEO: GSE61036). ChIP-seq of histone 3 lysine 4 dimethylation in male wild-type and *Kdm5c*-KO
407 EpiLCs⁴¹ is also available at GEO: GSE96797. ChIP-seq of histone 3 lysine 4 trimethylation in wild-type and
408 *Kdm5c*-KO male amygdala²³ are available at GEO: GSE127817.

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545 spermatogonial progenitors. eLife 9, e56523. <https://doi.org/10.7554/eLife.56523>.

549 **Figures and Tables**

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

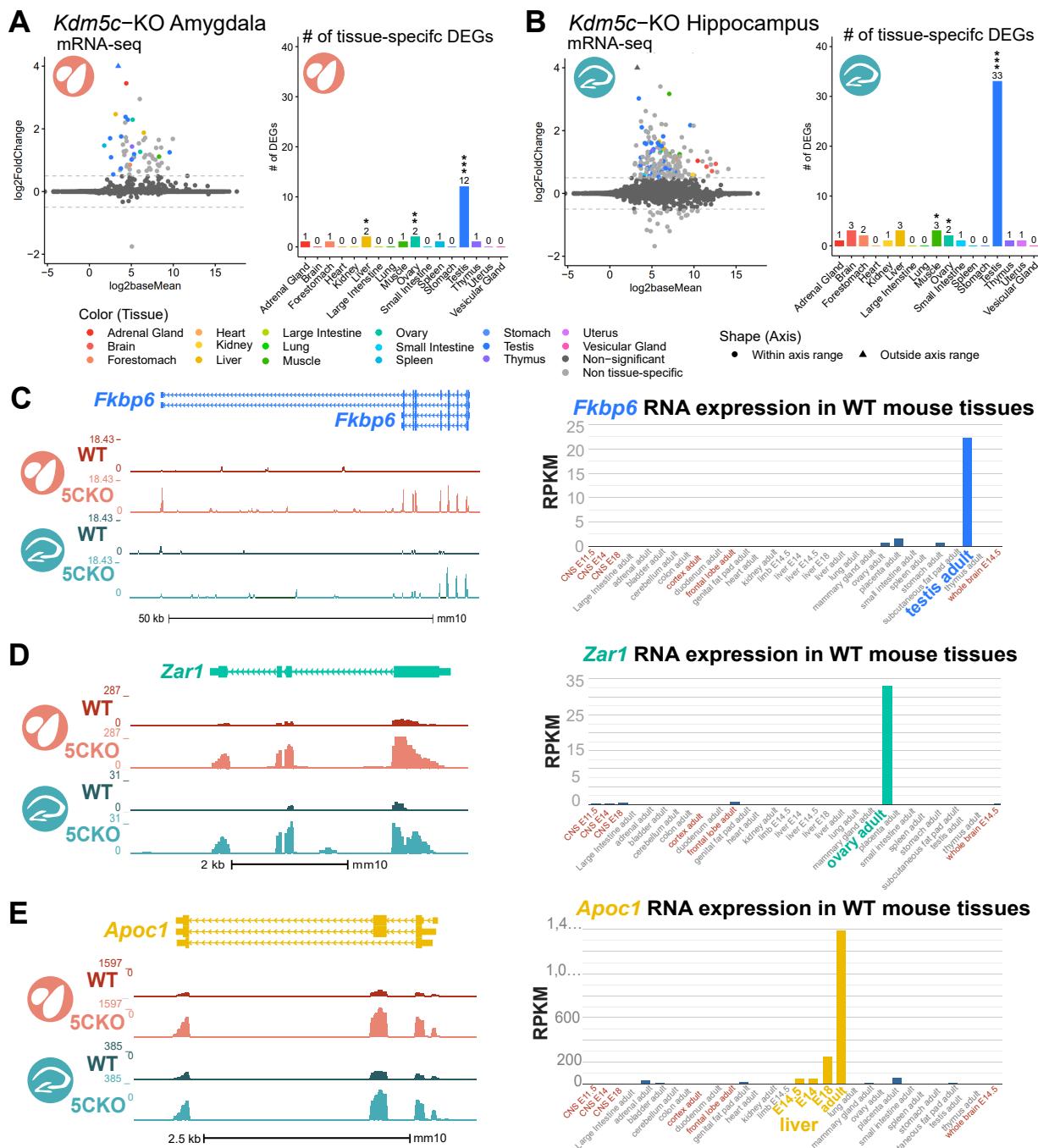


Figure 1: Tissue-enriched genes are misexpressed in the *Kdm5c*-KO brain. **A-B.** Expression of tissue-enriched genes (Li et al 2017) in the male *Kdm5c*-KO amygdala (A) and hippocampus (B). Left - MA plot of mRNA-sequencing. Right - Number of tissue-enriched differentially expressed genes (DEGs). *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

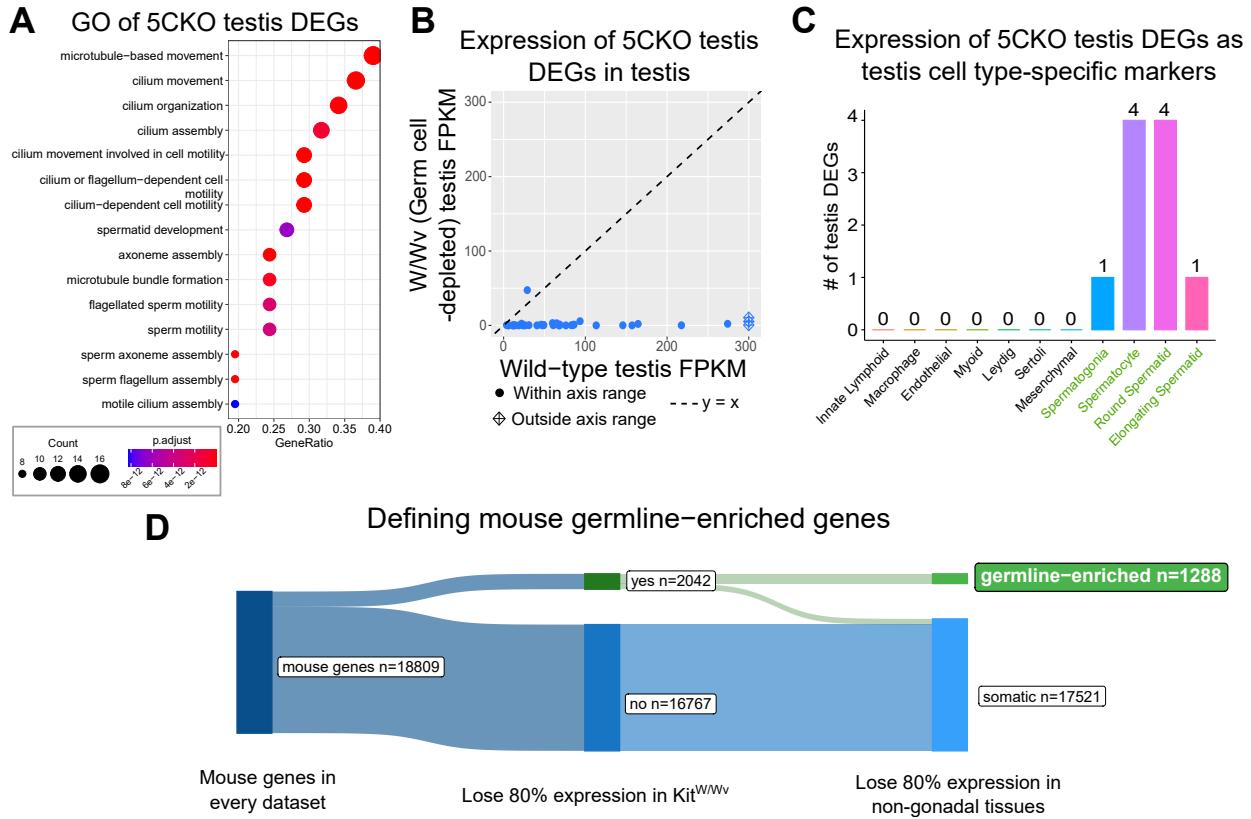


Figure 2: Aberrant transcription of germline genes in the *Kdm5c*-KO in the brain. **A.** enrichPlot gene ontology (GO) of *Kdm5c*-KO amygdala and hippocampus testis-enriched DEGs **B.** Expression of testis DEGs in wild-type (WT) testis versus germ cell-depleted (W/Wv) testis (Mueller et al 2013). Expression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

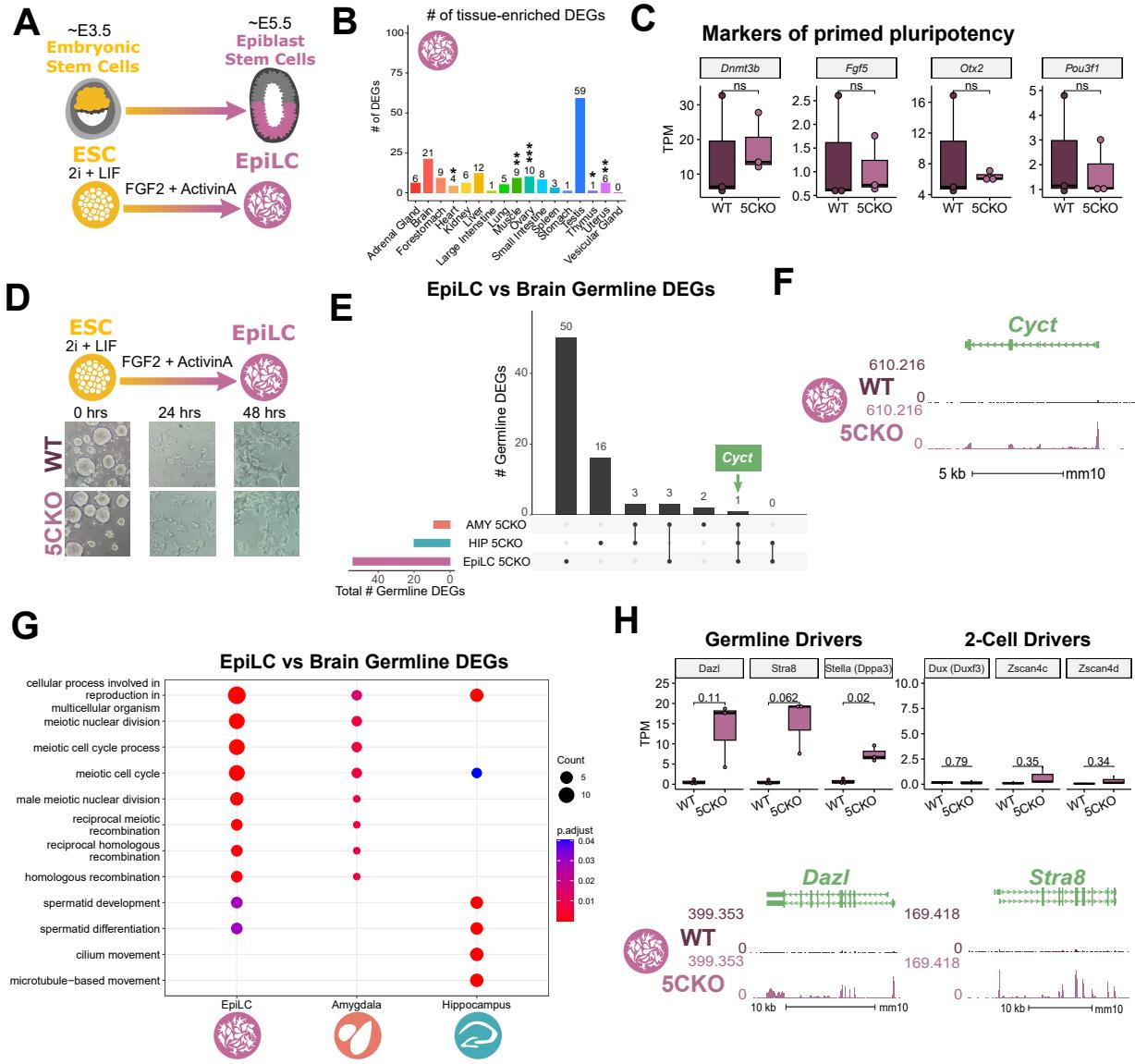


Figure 3: Kdm5c-KO epiblast-like cells express key drivers of germline identity

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

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

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

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

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

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

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

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

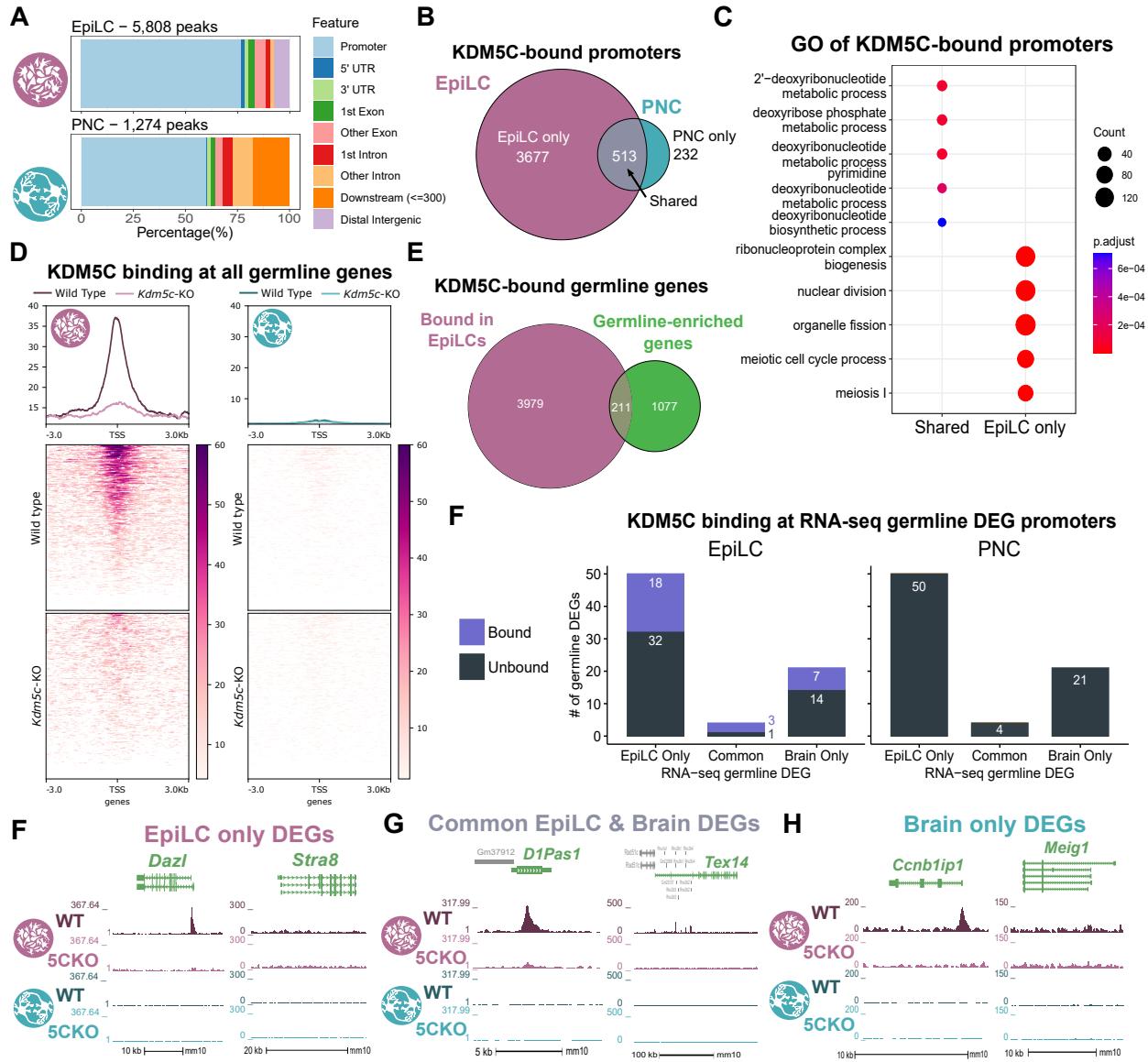
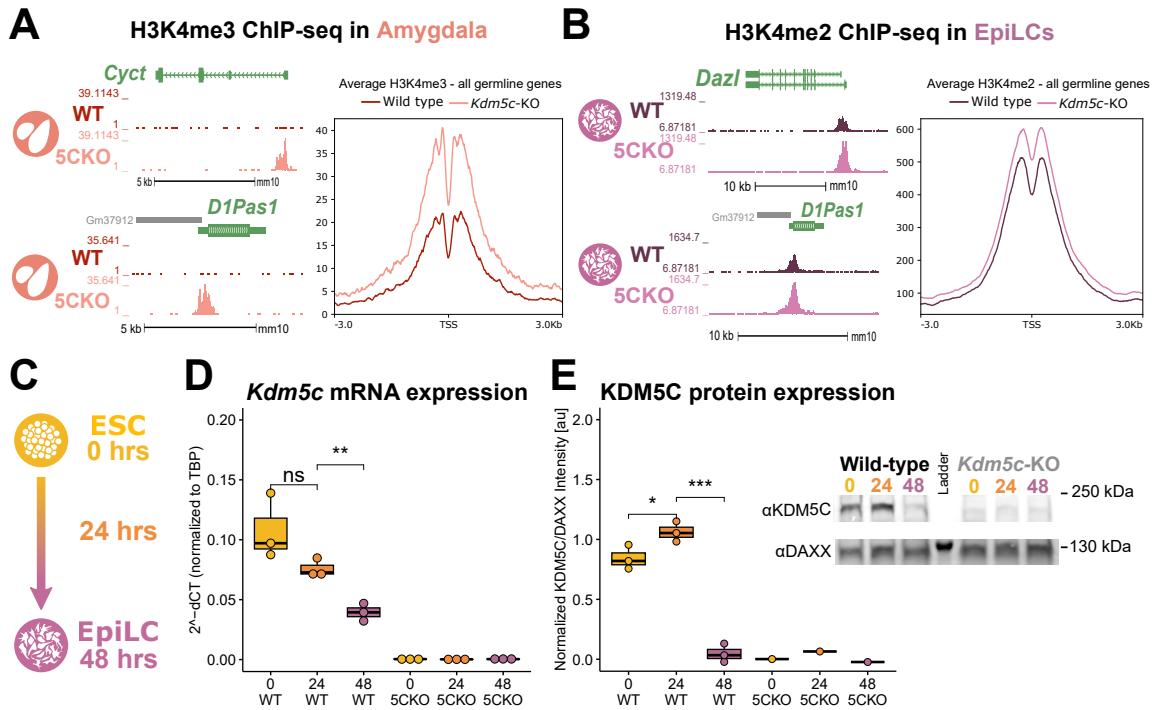


Figure 4: KDM5C binds to a subset of germline gene promoters during early embryogenesis **A.** ChIPseeker localization of KDM5C peaks at different genomic regions in EpiLCs (top) and hippocampal and cortex primary neuron cultures (PNCs, bottom). **B.** Overlap of genes with KDM5C bound to their promoters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

554 Notes

555 Things to do

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

560 Dazl

561 We were particularly interested in the aberrant transcription of *Dazl*, since it is essential for germ cell
562 development and promotes the translation of germline mRNAs⁶⁶. A significant portion of germline transcripts
563 misexpressed in *Kdm5c*-KO EpiLCs are known binding targets of DAZL, including *Stra8*⁶⁷ ($p = 1.698e-07$,
564 Fisher's Exact Test). This suggests expression of DAZL protein could promote the translation of other
565 aberrant germline transcripts, influencing their ability to impact *Kdm5c*-KO cellular function. We thus tested
566 DAZL protein expression in *Kdm5c*-KO EpiLCs through immunocytochemistry (Figure 3I). We observed
567 about 25% of *Kdm5c*-KO EpiLCs expressed DAZL protein and it was localized to the cytoplasm ($p = 0.0015$,
568 Welch's t-test), consistent with the pattern of DAZL expression in spermatogonia⁶⁷. Altogether these results
569 suggest tissue-specific genes are misexpressed during *Kdm5c*-KO embryogenesis, including key drivers of
570 germline identity that can be translated into protein.

- 571 • We additionally found *Kdm5c*-KO EpiLCs ectopically express DAZL protein that is localized to the
572 cytoplasm, similar to its morphology in spermatogonia⁶⁷. **note: maybe just put in results.** Could
573 move around depending upon if I get pheno working.

574 Discussion notes

- 575 • For other paper:
 - 576 – for methods: Heatmaps of gene expression were generated using the base R functions scale and
577 hclust and visualized using the R package ComplexHeatmap (v2.12.1).
 - 578 – * 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>
- 580 • Talk about different motifs if we do see differences there and if it explains direct vs indirect dysregulation
 - 581 – Could also mention PRC1.6/KDM5C binds pcgf6 in ESCs
- 582 • Maybe talk about other regulators/if anything is known about them long term? Or save for 2nd paper.

- 583 – another germline repressor: <https://pubmed.ncbi.nlm.nih.gov/35345626/>
- 584 • end with something like: this indicates kdm5c not only modulates pluripotency and self-renewal in
585 ESCs, but also has a role in long-term silencing of germline genes
- 586 – then transition into the long term silencing mechanism paragraph
- 587 • KDM5C is dynamically regulated during the window of embryonic germline gene silencing, our EpiLC
588 ChIP-seq is likely catching the tail end of KDM5C's main involvement.
- 589 • Our results indicate KDM5C is a pivotal repressor of germline programs in somatic cells.
- 590 • Studies on other chromatin regulators have focused on key marker genes to identify germline gene
591 misexpression, such as *Dazl*.
- 592 • Using a germline-depleted mouse model, we curated a list of mouse germline-enriched genes to
593 globally assess germline gene dysregulation.
- 594 • Many of the genes dysregulated in the mature *Kdm5c*-KO brain were important for late stages of
595 spermatogenesis, such as those important for sperm flagellar structure. Contrastingly, *Kdm5c*-KO
596 EpiLCs aberrantly expressed key drivers of germ cell identity, including *Dazl* and *Stra8*.
- 597 • While a significant portion of KDM5C-bound promoters are germline genes, many germline genes
598 expressed during *Kdm5c*-KO embryogenesis are not directly bound by kdm5c.
- 599 • One notable example is *Stra8*, a transcription factor important for germ cell specific transcription and
600 meiotic initiation
- 601 • The including the demarcation between soma and germline fates.
- 602 the *Kdm5c*-KO brain also Previous work identified testis-enriched genes in *Kdm5c* genes
- 603 –
- 604 – However unlike the gonadal-biased DEGs,
- 605 • The demarcation of the germline versus soma is a key feature of multicellularity and sexually dimorphic
606 reproduction
- 607 • Anything known about tissue-biased gene expression in other H3K4me regulators?
- 608 • Our data suggests the germline developmental program is occurring ectopically as *Kdm5c*-KOs pro-
609 gresses through somatic tissue development
- 610 • tissue-biased gene expression:

- 611 • Altogether, these data indicate that while some germline genes are misexpressed due to direct loss of
612 KDM5C binding during emryogenesis, secondary downstream mechanisms can also promote their
613 aberrant transcription.
- 614 • Papers to read/reference:
615 – Reconstitution of the Mouse Germ Cell Specification Pathway in Culture by Pluripotent Stem Cells:
616 [https://www.cell.com/fulltext/S0092-8674\(11\)00771-9](https://www.cell.com/fulltext/S0092-8674(11)00771-9)
617 – 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/>

619 **Figure outline:**

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

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

632 **Figure 3: *Kdm5c*-KO epiblast-like cells express key drivers regulators of germline identity** * A) ESC
633 to EpiLC differentiation Left - Morphology is unchanged, * B) 5CKO EpiLCs express EpiLC differentiation
634 genes similar to WT lvs * C) Male EpiLCs express germline genes (example Cyct again) * Overlap between
635 brain and EpiLC germline genes - show they're mostly unique * GO of Brain and EpiLC germline genes
636 (meiotic enriched) * Bigwigs or TPM of master regulators * Show that while some are also 2-cell genes
637 (Dazl), 2-cell specific genes aren't dysregulated (Zscan4). Important point because published KDM5C dazl
638 paper is saying KDM5C is a 2-cell regulator, but as far as I can tell only genes shared between germline and
639 2-cell are dysregulated.

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

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

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

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

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

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

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

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

684 **Germline gene repression background:**

685 Interestingly, some of the ectopic testis transcripts identified in the *Kdm5c*-KO brain are typically ex-
686 pressed in germ cells¹⁰. Unlike somatic cells, germ cells (e.g. sperm and eggs) undergo meiosis and pass
687 on their genetic material to the next generation. The germline and the soma are typically distinguished during
688 early embryogenesis, when germline genes are silenced in epiblast stem cells soon after implantation and
689 only reactivated in a subset to form the germline. Chromatin regulators play a key role in decommissioning
690 germline genes as the embryo transitions from naïve to primed pluripotency by placing repressive histone
691 H2A lysine 119 monoubiquitination (H2AK119ub1)¹⁸, histone 3 lysine 9 trimethylation (H3K9me3)^{18,19}, and
692 DNA CpG methylation^{19–21} at germline gene promoters. KDM5C may also be involved in this early decom-
693 missioning of germline genes, as re-expression of KDM5C in neurons fails to suppress their dysregulation¹⁰.
694 In support of this, KDM5C was very recently shown to repress *Deleted in azoospermia like (Dazl)*, a key
695 regulator of germline development, in mouse embryonic stem cells (ESCs)^{45,56}. In support of this, two
696 independent screens in mouse embryonic stem cells (ESCs) recently identified KDM5C as a repressor of
697 *Deleted in azoospermia like (Dazl)*, a key regulator of germline development. However, KDM5C's role in
698 embryonic germline gene repression is currently unclear, given that Dazl is also expressed in ESCs and in
699 the 2-cell stage and germline gene misexpression has yet to be globally characterized during *Kdm5c*-KO
700 embryogenesis.