

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

2 intellectual disability factor KDM5C

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5 Abstract

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

30 Introduction

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

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

58 The testis contains both germ cells (cells that pass on their genetic material, e.g. sperm) and somatic
59 cells (cells that perform all other bodily functions). Distinguishing between the germline and the soma is a
60 key feature of multicellularity¹⁶ that occurs during early embryogenesis in many metazoans¹⁷. In mammals,
61 chromatin regulators are crucial for decommissioning germline genes in somatic cells during the transition
62 from naïve to primed pluripotency. Initially, germline gene promoters gain repressive histone H2A lysine 119
63 monoubiquitination (H2AK119ub1)¹⁸ and histone 3 lysine 9 trimethylation (H3K9me3)^{18,19} in embryonic stem
64 cells and are then decorated with DNA CpG methylation in the post-implantation embryo^{19–21}. Currently,

most studies have focused on marker genes important for early germ cell development rather than germline genes as a whole given the lack of a curated list of germline-enriched genes. Thus, it is unclear if the mechanism of repression differs for certain classes of germline genes, e.g. meiotic genes versus spermatid differentiation genes. Furthermore, it is currently unknown if ectopic germline gene expression is influenced by chromosomal sex, as previous studies on germline gene repression have been exclusively in males. Sex is particularly pertinent in the case of sexually dimorphic chromatin regulators like KDM5C, which lies on the X chromosome and partially escapes X chromosome inactivation, resulting in 1.2-2-fold higher in females²²⁻²⁵. Thus, systematically characterizing KDM5C's role in germline gene repression during early embryogenesis, including its interaction with known silencing mechanisms, will unveil key mechanisms underlying the demarcation between soma and germline identity. These results will provide molecular footholds that can then be exploited to test the contribution of ectopic germline genes on neurodevelopment.

To illuminate KDM5C's role in tissue identity, here we characterized the aberrant transcription of tissue-enriched genes within the *Kdm5c*-KO brain and epiblast-like stem cells (EpiLCs), an *in vitro* model of the post-implantation embryo. We observed general dysregulation of tissue-enriched genes in both the *Kdm5c*-KO brain and EpiLCs, including testis, liver, muscle, and ovary-enriched genes. The *Kdm5c*-KO amygdala and hippocampus had significant enrichment of testis genes that are specific to germ cells and not somatic cells of the testis. We found *Kdm5c*-KO EpiLCs aberrantly expressed key drivers of germline identity and meiosis, including *Dazl* and *Stra8*, while the *Kdm5c*-KO brain primarily expressed germline genes important for late spermatogenesis. Female *Kdm5c*-KO EpiLCs expressed over double the number of germline genes than *Kdm5c*-KO males and germline genes dysregulated in both sexes were expressed more highly in females, demonstrating females have increased sensitivity to germline gene dysregulation. KDM5C was bound to only a subset of germline genes expressed in *Kdm5c*-KO EpiLCs, indicating germline-enriched mRNAs can be aberrantly transcribed through indirect mechanisms. Finally, we found KDM5C promotes the long-term silencing of germline genes in somatic cells by aiding the placement of DNA CpG methylation in EpiLCs. Thus, we propose KDM5C plays a fundamental role in the development of tissue identity during early embryogenesis, including the establishment of the soma-germline boundary.

Results

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

Previous RNA sequencing (RNA-seq) in the adult hippocampus revealed ectopic expression of some testis genes within the male *Kdm5c* knockout (-KO) brain¹⁰. Since tissue-enriched genes have not been systematically characterized in the *Kdm5c*-KO brain, if the testis is the only tissue type misexpressed. Therefore, we systematically assessed the expression of genes enriched in 17 mouse tissues²⁶ in our

98 published mRNA-seq datasets of the adult amygdala and hippocampus in mice with constitutive knockout of
99 *Kdm5c*²⁷.

100 We found a large proportion of genes that are significantly upregulated within the *Kdm5c*-KO brain
101 (DESeq2²⁸, log2 fold change > 0.5, q < 0.1) are enriched in non-brain tissues in wild-type mice (Amygdala:
102 35%, Hippocampus: 24%) (Figure 1A-B). The majority of tissue-enriched differentially expressed genes
103 (tissue-enriched DEGs) were testis genes (Figure 1A-C). Even though the testis has the largest total number
104 of tissue-biased genes compared to any other tissue (2,496 genes), testis-biased DEGs were significantly
105 enriched for both brain regions (Amygdala p = 1.83e-05, Odds Ratio = 5.13; Hippocampus p = 4.26e-11,
106 Odds Ratio = 4.45, Fisher's Exact Test). One example of a testis-enriched DEG is *FK506 binding protein 6*
107 (*Fkbp6*), a known regulator of piRNA expression and meiosis in germ cells^{29,30} (Figure 1C).

108 Interestingly, we also observed significant enrichment of ovary-biased DEGs in both the amygdala and
109 hippocampus (Amygdala p = 0.00574, Odds Ratio = 18.7; Hippocampus p = 0.048, Odds Ratio = 5.88,
110 Fisher's Exact) (Figure 1D). Ovary-enriched DEGs included *Zygotic arrest 1* (*Zar1*), sequesters mRNAs
111 in oocytes for meiotic maturation and early zygote development³¹ (Figure 1D). Given the *Kdm5c*-KO mice
112 we analyzed are male, this demonstrates ectopic expression of tissue-enriched genes is independent
113 of organismal sex. Although not consistent across brain regions, we also found significant enrichment
114 of DEGs biased towards two non-gonadal tissues - the liver (Amygdala p = 0.0398, Odds Ratio = 6.58,
115 Fisher's Exact Test) and the muscle (Hippocampus p = 0.0104, Odds Ratio = 6.95, Fisher's Exact Test). An
116 example of a liver-biased DEG dysregulated in both the hippocampus and amygdala is *Apolipoprotein C-I*
117 (*Apoc1*), a lipoprotein metabolism and transport gene³² (Figure 1E). These aberrantly expressed mRNAs are
118 polyadenylated and spliced into mature transcripts (Figure 1C-E). Of note, we did not observe enrichment
119 of brain-enriched genes (Amygdala p = 1; Hippocampus p = 0.74, Fisher's Exact), despite the fact these
120 are brain samples and the brain has the second highest total number of tissue-enriched genes (708 genes).
121 Together, these results suggest misexpression of tissue-enriched genes within the brain is a major effect of
122 KDM5C loss.

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

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

130 To further validate if these testis DEGs are truly germline genes, we evaluated their expression in
131 somatic versus germ cells within the testis. We first compared their expression in the testis without germ
132 cells³³, which was accomplished by heterozygous *W* and *Wv* mutations in the enzymatic domain of *c-Kit*

133 (*Kit*^{W/W^v})³⁴. Almost all *Kdm5c*-KO testis-enriched DEGs lost expression with germ cell depletion (Figure
134 2B). We then assessed testis-enriched DEG expression in a published single cell RNA-seq dataset that
135 identified cell type-specific markers within the testis³⁵. We found some testis-enriched DEGs were classified
136 as specific markers for different germ cell types (e.g. spermatogonia, spermatocytes, round spermatids, and
137 elongating spermatids), yet none marked somatic cells (Figure 2C). Together, these data demonstrate that
138 the *Kdm5c*-KO brain aberrantly expresses germline genes, but not somatic testicular genes, reflecting an
139 erosion between somatic versus germline identity.

140 We then wanted to characterize germline gene misexpression with *Kdm5c* loss more deeply, but lacked a
141 comprehensive list of mouse germline-enriched genes. We thus generated a list of germline-enriched genes
142 using RNA-seq datasets of *Kit*^{W/W^v} mice that include males and females at embryonic day 12, 14, and 16³⁶
143 and adult male testes³³. We defined genes as germline-enriched if their expression met the following criteria:
144 1) their expression is greater than 1 FPKM in wild-type gonads 2) their expression in any non-gonadal tissue
145 for adult wild type²⁶ does not exceed 20% of their maximum expression in the wild-type germline, and 3)
146 their expression in the germ cell-depleted gonads, for any sex or time point, does not exceed 20% of their
147 maximum expression in the wild-type germline. These criteria yielded 1,288 germline-enriched genes (Figure
148 2D), which was hereafter used as a resource to globally assess germline gene misexpression with *Kdm5c*
149 loss (Supplementary table 1).

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

152 Germ cells are typically distinguished from somatic cells soon after the embryo implants into the uterine
153 wall^{37,38}. A subset of epiblast stem cells become the primordial germ cells (PGCs) while the remainder
154 differentiate into the ectoderm, mesoderm, and endoderm to form the somatic tissues³⁹. This developmental
155 time point can be modeled *in vitro* through differentiation of embryonic stem cells (ESCs) into epiblast-like
156 stem cells (EpiLCs) (Figure 3A, top)^{40,41}. While some germline-enriched genes are also expressed in
157 embryonic stem cells (ESCs) and in the 2-cell stage^{42–44}, they are silenced as they differentiate into EpiLCs¹⁹.
158 Therefore, we tested if KDM5C was necessary for silencing germline genes at this developmental stage by
159 evaluating the impact of *Kdm5c* loss in EpiLCs.

160 We first identified *Kdm5c*-KO EpiLC DEGs in our previously published RNA-seq dataset⁴⁵ (DESeq2, log2
161 fold change > 0.5, q < 0.1) and assessed tissue-enriched gene expression. Similar to the *Kdm5c*-KO brain,
162 we observed general dysregulation of tissue-enriched genes, with the largest number of genes belonging to
163 the brain and testis, although they were not significantly enriched (Figure 3B). Primed pluripotency genes,
164 such as *Dnmt3b*, *Fgf5*, *Pou3f1*, and *Otx2*, were unaltered in *Kdm5c*-KO EpiLCs (Figure 3C). Furthermore,
165 *Kdm5c*-KO cell morphology during ESC to EpiLC differentiation (Figure 3D) was normal, indicating KDM5C
166 loss does not impair EpiLC formation.

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

175 Notably, DEGs unique to *Kdm5c*-KO EpiLCs included key drivers of germline identity, such as *Stimulated*
176 *by retinoic acid 8* (*Stra8*) and *Deleted in azoospermia like* (*Dazl*) (Figure 3H). These genes are typically
177 expressed during embryonic germ cell development to commit PGCs to the germline fate, but are also
178 expressed later in life to trigger meiotic gene expression programs^{46–48}. Of note, some germline genes,
179 including *Dazl*, are also expressed in the two-cell embryo^{43,49}. However, we did not see misexpression of
180 two-cell embryo-specific genes, like *Duxf3* (*Dux*) ($q = 0.337$) and *Zscan4d* ($q = 0.381$), indicating *Kdm5c*-KO
181 in EpiLCs do not revert back to a 2-cell state (Figure 3H).

182 **Females have increased sensitivity to germline gene misexpression with *Kdm5c* 183 loss**

184 It is currently unknown if the misexpression of germline genes is influenced by chromosomal sex, as
185 previous studies on germline gene repressors have been conducted exclusively in males. We explored the
186 impact of sex upon germline gene misexpression by comparing their dysregulation in male *Kdm5c* hemizygous
187 knockout (XY *Kdm5c*-KO), female homozygous knockout (XX *Kdm5c*-KO) and female heterozygous (XX
188 *Kdm5c*-HET) EpiLCs.⁴⁵ We first identified differentially expressed genes (DEGs) compared to sex-matched
189 wild-type controls (DESeq2²⁸, log2 fold change > 0.5, $q < 0.1$) and then filtered for germline-enriched genes.

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

198 Germline genes dysregulated in both sexes were also enriched for meiotic ontologies (meiotic nuclear
199 division), as well as egg-specific genes (female gamete generation). The majority of these shared germline
200 DEGs had a greater log2 fold change from wild-type in females compared to males (Figure 4D-F). The

201 increased number of germline genes and degree of dysregulation in females could be caused by improper
202 X chromosome inactivation (XCI), as the X chromosome is enriched for many testis-specific germline
203 genes[XXX]. However, both shared and female-specific germline DEGs were not biased towards the X
204 chromosome, with the majority of genes lying on autosomes instead (Figure 4G). Thus, while female EpiLCs
205 have increased sensitivity to germline gene misexpression with KDM5C loss, it is likely independent of
206 potential XCI defects.

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

208 While many germline genes have roles in both the male and female germline, some display sex-biased
209 expression or have functions unique to eggs and sperm. To comprehensively assess if germline gene
210 sex corresponds with *Kdm5c* mutant sex, we filtered our list of germline-enriched genes for egg (XX) and
211 sperm (XY) biased genes. We defined sex-biased genes as those whose expression in the opposite sex,
212 at any time point, is no greater than 20% of the gene's maximum expression in a given sex. This yielded
213 67 egg-biased, 1,024 sperm-biased, and 197 unbiased germline-enriched genes, which is consistent with
214 the testis overall having a more unique transcriptome than the ovary²⁶ (Figure 4H). We found egg, sperm,
215 and unbiased germline genes were dysregulated in all *Kdm5c* mutants (Figure 4I-L). *Kdm5c* mutant male
216 and female-specific germline DEGs were also not biased to the corresponding germ cell sex, indicating
217 differences between male and female germline gene dysregulation not due to sex-specific activation of
218 sperm or egg-specific transcriptional programs. These results demonstrate sex influences the degree of
219 gene misexpression with loss of KDM5C, but not the sex of germ cell-enriched genes.

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

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

228 To address these questions, we analyzed KDM5C chromatin immunoprecipitation followed by DNA
229 sequencing (ChIP-seq) datasets in EpiLCs⁴⁵ and primary forebrain neuron cultures (PNCs)¹⁵. EpiLCs had a
230 higher total number of KDM5C peaks than PNCs (EpiLCs: 5,808, PNCs: 1,276, MACS2 q < 0.1 and fold
231 enrichment > 1, removal of *Kdm5c*-KO false positive peaks) and KDM5C was primarily localized to gene
232 promoters in both cell types (EpiLCs: 4,190, PNCs: 745 +/- 500bp from the TSS), although PNCs showed
233 increased localization to non-promoter regions (Figure 4A).

234 The majority of promoters bound by KDM5C in PNCs were also bound in EpiLCs (513 shared promoters),
235 however a large portion of gene promoters were only bound by KDM5C in EpiLCs (3677 EpiLC only
236 promoters) (Figure 4B). We then performed gene ontology analysis to compare the known functions of genes
237 with KDM5C bound to their promoter in EpiLCs and PNCs. While there were no ontologies significantly
238 enriched for genes only bound by KDM5C in PNCs, gene ontologies for genes shared between PNCs and
239 EpiLCs were enriched for functions involving nucleic acid turnover, such as deoxyribonucleotide metabolic
240 process (GO:0009262, p.adjust = 8.28e-05) (Figure 4C). Germline-specific ontologies were only enriched in
241 promoters unique to EpiLCs, such as meiotic nuclear division (GO: 0007127 p.adjust = 6.77e-16) and meiotic
242 cell cycle process (GO:1903046, p.adjust = 5.05e-16) (Figure 3C). We then evaluated KDM5C binding
243 around the transcription start site (TSS) of all germline-enriched genes. In EpiLCs, We observed modest
244 KDM5C signal at about half of all germline genes (Figure 4D). Based on our ChIP-seq peak criteria, KDM5C
245 was strongly bound to about 10% of germline gene promoters in EpiLCs (Figure 4E). In condordance with our
246 gene ontology results, we did not observe KDM5C accumulation at any germline gene promtoers in PNCs
247 (Figure 4D). Together, these results demonstrate KDM5C is significantly enriched at a subset of germline
248 gene promoters in EpiLCs, including meiotic genes, but does not directly regulate germline genes in neurons.

249 Many germline-specific genes, including meiotic genes, are suppressed by the transcription factor
250 heterodimers E2F6/DP1 and MGA/MAX, which respectively bind E2F and E-box motifs^{20,50-53}. Thus, we
251 identified transcription factor motifs enriched at KDM5C-bound or unbound germline gene promoters using
252 HOMER⁵⁴ (TSS +/- 500 bp, q-value < 0.1). MAX and E2F6 binding sites were significantly enriched at
253 germline genes bound by KDM5C in EpiLCs, but not at germline genes unbound by KDM5C (MAX q-value: ,
254 E2F6 q-value:, E2F q-value:) (Figure 4). One third of KDM5C-bound promoters contained the consensus
255 sequence for either E2F6 (E2F, 5'-TCCCGC-3'), MGA (E-box, 5'-CACGTG-3'), or both, but only 17% of
256 KDM5C-unbound genes contained these motifs (Figure 4). KDM5C-unbound germline genes were intstead
257 enriched for multiple RFX transcription factor binding sites. RFX transcription factors bind X-box motifs⁵⁵
258 to promote ciliogenesis^{56,57}. Enriched RFX tranccription factors included RFX2, a central regulator of post-
259 meiotic spermatogeneis^{58,59}. Interestingly, RFX2 mRNA is upregualted in *Kdm5c*-KO EpiLCs, but is also not
260 a direct target of KDM5C (Figure 4). Thus, distinct transcription factor programs regulate KDM5C-bound and
261 unbound germline genes.

262 Finally, we compared KDM5C binding at promoters of RNA-seq DEGs to determine if the germline
263 mRNAs de-repressed in *Kdm5c*-KO EpiLCs and brain are direct targets of KDM5C (Figure 4F). In EpiLCs,
264 KDM5C was bound to about one third of EpiLC-specific and brain-specific germline DEGs (EpiLC only: 36%,
265 Brain only: 33.3%). Again, we did not observe any KDM5C binding at germline gene promoters in PNCs,
266 even for brain-specific DEGs (Figure 4H). Some notable differences in KDM5C binding for EpiLC-specific
267 DEGs included *Dazl* and *Stra8*, two key drivers of germline identity discussed above. Although both mRNAs
268 are expressed in *Kdm5c*-KO EpiLCs, KDM5C is only bound to *Dazl*'s promoter and not *Stra8*'s in EpiLCs
269 (Figure 4F). In contrast to the unique DEGs, 3 out of the 4 genes dysregulated in both the brain and

270 EpiLCs are bound by KDM5C around the TSS (Figure 4G). Altogether, this suggests KDM5C decommissions
271 germline genes in EpiLCs but is no longer recruited to germline gene promoters in neurons. Furthermore, the
272 majority of germline mRNAs expressed in *Kdm5c*-KO cells are dysregulated independent of direct KDM5C
273 recruitment to their promoters.

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

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

276 In the early embryo, germline gene promoters are initially silenced by repressive histone modifications,
277 including histone 2A lysine 119 monoubiquitination (H2AK119ub1) and histone 3 lysine 9 trimethylation
278 (H3K9me3), and are then silenced long-term via DNA CpG methylation (CpGme)^{18,19,60}. Our results above
279 suggest KDM5C also acts during this time period, however how KDM5C interacts with other germline gene
280 silencing mechanism is currently unclear. KDM5C is generally thought to suppress transcription through
281 erasure of histone 3 lysine 4 di- and trimethylation (H3K4me2/3)¹¹. However, KDM5C's catalytic activity was
282 recently shown to not be required for suppressing *Dazl* in undifferentiated ESCs⁴⁹. Since H3K4me3 impedes
283 *de novo* CpGme placement^{61,62}, KDM5C's catalytic activity may instead be required for CpGme-mediated,
284 long-term silencing of germline genes. In support of this, CpGme is markedly reduced at two germline gene
285 promoters in the *Kdm5c*-KO adult hippocampus¹⁰. Based on these observations, we hypothesized KDM5C
286 erases H3K4me3 to promote the initial placement of CpGme at germline gene promoters in EpiLCs.

287 To test this hypothesis, we first characterized KDM5C's substrates, histone 3 lysine 4 di- and trimethylation
288 (H3K4me2/3) in our previously published ChIP-seq datasets of the wild type and *Kdm5c*-KO amygdala²⁷
289 and EpiLCs⁴⁵. In congruence with previous work in the *Kdm5c*-KO hippocampus¹⁰, we observed aberrant
290 accumulation of H3K4me3 around the transcription start site (TSS) of germline genes in the *Kdm5c*-KO
291 amygdala (Figure 5A). We additionally found a marked increase in H3K4me2 around the TSS of germline
292 genes in *Kdm5c*-KO EpiLCs (Figure 5B).

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

297 • Germline genes are known to accumulate CpGme at (CGIs).

298 – What percentage of germline genes have CGIs

299 • Do differential methylation analysis for WT ESCs to WT EpiLCs

300 – What percentage of germline genes significantly gain methylation (at CGI or at promoter)

301 – Out of the ones that gain methylation, which are significantly reduced

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

- 307 • discussion
- 308 • – However, CPG islands greatly determine KDM5C recruitment - KDM5C is known to be enriched at
309 CGIs.)
- 310 • Altogether, this suggests KDM5C is dynamically regulated during ESC to EpiLC differentiation and
311 promotes germline gene silencing via removal of H3K4me2/3 during early embryogenesis.

312 Discussion

313 In the above study, we demonstrate KDM5C's pivotal role in the development of tissue identity, resulting in
314 substantial misexpression of tissue-enriched genes within the adult *Kdm5c*-KO hippocampus and amygdala.
315 In addition to testis genes identified previously¹⁰, we found significant enrichment of muscle, liver, and even
316 ovary-biased genes aberrantly expressed within the male *Kdm5c*-KO brain. Deeper characterization of
317 testis-enriched DEGs revealed they were not somatic testis genes, but germline genes, thus demonstrating
318 KDM5C's crucial role in establishing the soma-germline boundary. Given the majority of tissue-enriched
319 DEGs are testis and ovary genes with no known brain functions, it is currently unknown if they impair
320 *Kdm5c*-KO brain function and contribute to MRXSCJ-related phenotypes like intellectual disability and
321 aberrant aggression. However, select liver and muscle-biased DEGs do have known roles within the brain,
322 such as the liver-enriched lipid metabolism gene *Apolipoprotein C-I (Apoc1)*³² that is highly expressed in the
323 *Kdm5c*-KO amygdala and hippocampus. Intriguingly, *Apoc1* overexpression in the mouse brain can impair
324 learning and memory⁶³ and is implicated in Alzheimer's disease in humans⁶⁴.

325 We found a substantial portion of genes upregulated in *Kdm5c*-KO brain are germline genes with no
326 known functions within the brain. Distinguishing the germline from the soma is a key feature of multicellularity
327 and sexual reproduction¹⁶. Previous work characterizing chromatin regulators that silence germ cell-specific
328 transcription has predominantly focused on their repression of key marker genes in embryonic stem cells
329 (ESCs), such as *Dazl* and *Ddx4*^{18,19,53}. To characterize KDM5C's role in germline gene repression at a
330 genome-wide level throughout life, we curated a list of mouse germline-enriched genes using publicly
331 available germ cell-depleted RNA-seq datasets from *Kit^{W/W^v}* mice^{33,36}. This resource enabled us to identify 1)
332 the extent of germline gene dysregulation in *Kdm5c*-KO cells, 2) the types of germline genes misexpressed
333 at different developmental time points, and 3) which groups of germline genes are directly and indirectly

334 regulated by KDM5C. This list of germline-enriched genes serves as a resource for future studies to
335 systematically assess soma-germline dysregulation.

336 RNA-seq of epiblast-like cells (EpiLCs) revealed dysregulation of *Kdm5c*-KO tissue identity begins during
337 early embryogenesis. *In vivo*, germline genes are typically decommissioned in epiblast stem cells and remain
338 silenced as the epiblast differentiates into somatic tissues³⁹. However, a small subset of epiblast stem cells
339 will receive signals to reactivate germline gene expression to become the primordial germ cells (PGCs) that
340 will ultimately form the mature germline^{37,38}. This process can be mimicked *in vitro* by differentiating EpiLCs
341 into primordial germ cell-like cells (PGCLCs)⁴⁰. Therefore, misexpression of germline genes in EpiLCs might
342 suggest *Kdm5c*-KO ESCs are progressing beyond EpiLC differentiation and instead becoming PGCLCs. Yet,
343 *Kdm5c*-KO EpiLCs properly express primed pluripotency genes, including *Otx2* - an epiblast stem cell marker
344 that is known to repress differentiation into PGCs/PGCLCs⁶⁵. Furthermore, we observed no difference in
345 cellular morphology during *Kdm5c*-KO ESC to EpiLC differentiation. Proper EpiLC differentiation, together
346 with *Kdm5c*-KO mice being viable, suggests germline gene expression is occurring ectopically in conjunction
347 with typical developmental programs, rather than a complete shift to towards germline identity.

348 • XX vs XY

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

353 • Motif analysis

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

358 * RFX Cilia in NDDs - <https://pubmed.ncbi.nlm.nih.gov/33658631/>

359 – Enriched RFX transcription factors included the spermiogenesis regulator RFX2, whose mRNA is
360 also significantly upregulated in *Kdm5c*-KO EpiLCs (Figure 4).

361 – <https://www.nature.com/articles/srep20435>

362 – <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4498915/>

363 – <https://pubmed.ncbi.nlm.nih.gov/32725242/>

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

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

368 While we observed high enrichment of KDM5C peaks at germline genes in EpiLCs, KDM5C was not
369 bound to germline gene promoters in PNCs. This suggests dysregulation of germline genes in the mature
370 *Kdm5c*-KO brain is due to loss of repression during embryogenesis, which is consistent with previous
371 work that found introducing human KDM5C into *Kdm5c*-KO PNCs does not repress two aberrant germline
372 transcripts¹⁰. Although enriched at germline gene promoters in EpiLCs, KDM5C was only bound to about a
373 third of the germline genes aberrantly transcribed in *Kdm5c*-KO EpiLCs. One notable EpiLC DEG not bound
374 by KDM5C is *Stra8*, a transcription factor activated by retinoic acid signaling in germ cells to promote meiotic
375 initiation^{66,67}. However, retinoic acid can only activate *Stra8* expression when DAZL is present. Unlike *Stra8*,
376 *Dazl* is a direct target of KDM5C in EpiCs and is both transcribed and translated in *Kdm5c*-KO EpiLCs.
377 Expression of indirect DEGs like *Stra8* indicates germline genes can be aberrantly expressed in *Kdm5c*-KO
378 cells through downstream activation by other ectopic germline programs. These ectopic germline programs
379 appear to loosely mimic the trajectory of typical germline development, as germline genes important for early
380 germ cell development and meiosis are expressed in *Kdm5c*-KO EpiLC while late spermatogenesis genes
381 are expressed in the mature *Kdm5c*-KO brain. Altogether, this indicates once a subset of germline genes
382 are activated due to loss of direct KDM5C repression in the early embryo, these ectopic germline programs
383 can continue to progress in the background of *Kdm5c*-KO somatic development.

384 It is important to note that while we focused on KDM5C's regulation of germline genes, some germline-
385 enriched genes are also expressed in naïve ESCs and at the 2-cell stage due to their roles in pluripotency and
386 self-renewal. For example, *Dazl* is primarily known for committing PGCs to the germline fate and regulating
387 the translation of germline-specific RNAs, but is also expressed at the 2-cell stage⁶⁸, in naïve ESCs⁴², and in
388 the inner cell mass⁴². Very recently, two screens of *Dazl*-repressors identified KDM5C as a direct regulator in
389 ESCs^{49,69}. Interestingly, one study found *Kdm5c*-KO ESCs also aberrantly expressed 2-cell-specific genes,
390 indicating KDM5C negatively regulates totipotency⁴⁹. However, out of the four regulators characterized,
391 KDM5C was the only factor whose repression of *Dazl* was independent of the 2-cell specific transcription
392 factor *Dux*⁴⁹. Although expressed at the 2-cell stage and in ESCs, *Dazl* is typically silenced when ESCs
393 differentiate into EpiLCs¹⁹. We found KDM5C also represses *Dazl* in EpiLCs, but *Kdm5c*-KO EpiLCs did
394 not express 2-cell specific genes like *Dux* and *Zscan4c*. Together, this suggests the 2-cell-like state in
395 *Kdm5c*-KO ESCs is an indirect consequence of KDM5C's primary role in germline gene repression.

396 *Dazl* and other germline-enriched genes are initially decommissioned in ESCs by repressive histone
397 modifications and then *de novo* DNA CpG methylation (CpGme) in EpiLCs^{19–21,60,70,71}. Unlike the previously
398 characterized germline gene suppressors, KDM5C removes an active histone mark, histone 3 lysine 4 di-
399 and trimethylation (H3K4me2/3)¹¹. In this study, we observed a global increase in H3K4me2/3 in *Kdm5c*-KO
400 EpiLCs and amygdala around the transcription start site (TSS) of germline-enriched genes. However,
401 KDM5C's catalytic activity may not be required for germline gene silencing, as it was recently found to be
402 dispensible for repressing *Dazl* in ESCs⁴⁹. Although not necessary in ESCs, KDM5C's catalytic activity be
403 required for CpGme in EpiLCs, as H3K4me3 impedes *de novo* CpGme placement^{61,62}. This is supported

404 by previous work in the *Kdm5C*-KO adult hippocampus, which found CpGme is significantly eroded at at
405 least two germline promoters¹⁰. To elucidate the mechanism behind KDM5C-mediated silencing of germline
406 genes, we characterized KDM5C in ESCs and EpiLCs and found its expression is dynamically regulated
407 during differentiation. Furthermore, KDM5C acts during the transition from naïve to primed pluripotency to
408 promote the initial placement of CpGme at CpG islands for the long-term silencing of germline genes.

409 The above work provides the mechanistic foundation for KDM5C's role in germline gene repression
410 and offers novel insight into how dysregulation in *Kdm5c*-KO tissue identity changes over the course of
411 development. Although their impact upon neurodevelopment is currently unclear, ectopic germline transcripts
412 are also found in models of other related neurodevelopmental disorders⁷², including Immunodeficiency,
413 Centromere instability, Facial anomalies syndrome 1 (ICF1 OMIM: #242860)^{73,74}, Kleefstra syndrome
414 1 (OMIM: #610253)⁹, and MeCP2 duplication syndrome (MDS, OMIM: #300260)⁷⁵. Like KDM5C, the
415 chromatin regulators underlying these conditions, DNA methyltransferase 3b (DNMT3B), H3K9me1/2
416 methyltransferases G9A/GLP, methyl-CpG -binding protein 2 (MECP2), primarily silence gene expression.
417 Thus, KDM5C is among a growing cohort of chromatin-linked neurodevelopmental disorders that may have a
418 similar underlying cause of germline versus soma dysregulation. However, further research is required to
419 determine the ultimate impact of these germline genes and the extent to which this phenomenon occurs in
420 humans.

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

424 Materials and Methods

425 Classifying tissue-enriched and germline-enriched genes

426 Tissue-enriched differentially expresssd genes (DEGs) were determined by their classification in a previ-
427 ously published dataset from 17 male and female mouse tissues²⁶. This study defined tissue expression as
428 greater than 1 Fragments Per Kilobase of transcript per Million mapped read (FPKM) and tissue enrichment
429 as at least 4-fold higher expression than any other tissue.

430 We curated a list of germline-enriched genes using an RNA-seq dataset from wild-type and germline-
431 depleted (*Kit^{W/Wv}*) male and female mouse embryos from embryonic day 12, 14, and 16³⁶, as well as adult
432 male testes³³. Germline-enriched genes met the following criteria: 1) their expression is greater than 1
433 FPKM in wild-type germline 2) their expression in any wild-type somatic tissues²⁶ does not exceed 20%
434 of maximum expression in wild-type germline, and 3) their expression in the germ cell-depleted (*Kit^{W/Wv}*)
435 germline, for any sex or time point, does not exceed 20% of maximum expression in wild-type germline.

436 **Cell culture**

437 We utilized our previously established cultures of male wild-type and *Kdm5c* knockout (-KO) embryonic
438 stem cells⁴⁵. Sex was confirmed by genotyping *Uba1/Uba1y* on the X and Y chromosomes with the following
439 primers: 5'-TGGATGGTGTGCCAATG-3', 5'-CACCTGCACGTTGCCCT-3'. Deletion of *Kdm5c* was
440 confirmed through the primers 5'-ATGCCCATATTAAGAGTCCTG-3', 5'-TCTGCCTTGATGGGACTGTT-3',
441 and 5'-GGTTCTAACACTCACATAGTG-3'.

442 Embryonic stem cells (ESCs) and epiblast-like cells were cultured using previously established
443 methods⁴¹. Briefly, ESCs were initially cultured in primed ESC (pESC) media consisting of KnockOut
444 DMEM (Gibco#10829-018), fetal bovine serum (Gibco#A5209501), KnockOut serum replacement
445 (Invitrogen#10828-028), Glutamax (Gibco#35050-061), Anti-Anti (Gibco#15240-062), MEM Non-essential
446 amino acids (Gibco#11140-050), and beta-mercaptoethanol (Sigma#M7522). They were then transitioned
447 into ground-state, "naïve" ESCs (nESCs) by culturing for four passages in N2B27 media containing
448 DMEM/F12 (Gibco#11330-032), Neurobasal media (Gibco#21103-049), Gluamax, Anti-Anti, N2 sup-
449 plement (Invitrogen#17502048), and B27 supplement without vitamin A (Invitrogen#12587-010), and
450 beta-mercaptoethanol. Both pESC and nESC media were supplemented with 3 μM GSK3 inhibitor
451 CHIR99021 (Sigma #SML1046-5MG), 1 μM MEK inhibitor PD0325901 (Sigma #PZ0162-5MG), and 1,000
452 units/mL leukemia inhibitory factor (LIF, Millipore#ESG1107).

453 nESCs were differentiated into epiblast-like cells (EpiLCs, 48 hours) and extended EpiLCs (exEpiLCs,
454 96 hours) by culturing in N2B27 media containing DMEM/F12, Neurobasal media, Gluamax, Anti-Anti,
455 N2 supplement, B27 supplement (Invitrogen#17504044), beta-mercaptoethanol, fibroblast growth factor 2
456 (FGF2, R&D Biotechne 233-FB), and activin A (R&D Biotechne 338AC050CF), as previously described⁴¹.

457 **Immunocytochemistry (ICC)**

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

467 **RNA sequencing (RNA-seq)**

468 After ensuring read quality via FastQC (v0.11.8), reads were then mapped to the mm10 *Mus musculus*
469 genome (Gencode) using STAR (v2.5.3a), during which we removed duplicates and kept only uniquely
470 mapped reads. Count files were generated by FeatureCounts (Subread v1.5.0), and BAM files were
471 converted to bigwigs using deeptools (v3.1.3) and visualized by the UCSC genome browser. RStudio (v3.6.0)
472 was then used to analyze counts files by DESeq2 (v1.26.0)²⁸ to identify differentially expressed genes
473 (DEGs) with a q-value (p-adjusted via FDR/Benjamini–Hochberg correction) less than 0.1 and a log2 fold
474 change greater than 0.5. For all DESeq2 analyses, log2 fold changes were calculated with IfcShrink using
475 the ashr package⁷⁶. MA-plots were generated by ggpibr (v0.6.0), and Eulerr diagrams were generated by
476 eulerr (v6.1.1). Boxplots and scatterplots were generated by ggpibr (v0.6.0) and ggplot2 (v3.3.2). The Upset
477 plot was generated via the package UpSetR (v1.4.0)⁷⁷. Gene ontology (GO) analyses were performed by
478 the R package enrichPlot (v1.16.2) using the biological processes setting and compareCluster.

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

480 ChIP-seq reads were aligned to mm10 using Bowtie1 (v1.1.2) allowing up to two mismatches. Only
481 uniquely mapped reads were used for analysis. Peaks were called using MACS2 software (v2.2.9.1) using
482 input BAM files for normalization, with filters for a q-value < 0.1 and a fold enrichment > 1. We removed
483 “black-listed” genomic regions that often give aberrant signals. Common peak sets were obtained in R via
484 DiffBind (v3.6.5). In the case of KDM5C ChIP-seq, *Kdm5c*-KO peaks were then subtracted from wild-type
485 samples using bedtools (v2.25.0). Peak proximity to genome annotations was determined by ChIPSeeker
486 (v1.32.1). Gene ontology (GO) analyses were performed by the R package enrichPlot (v1.16.2) using the
487 biological processes setting and compareCluster. Enriched motifs were identified using HOMER⁵⁴. Average
488 binding across the genome was visualized using deeptools (v3.1.3). Bigwigs were visualized using the
489 UCSC genome browser.

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

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

495 **Data availability**

496 **Published datasets**

497 All published datasets are available at the Gene Expression Omnibus (GEO) <https://www.ncbi.nlm.nih.gov/geo>. Published RNA-seq datasets analyzed in this study included the male wild-type and *Kdm5c*-KO
498 adult amygdala and hippocampus²⁷ (available at GEO: GSE127722) and male wild-type and *Kdm5c*-KO
499 EpiLCs⁴⁵ (available at GEO: GSE96797).

500 Previously published ChIP-seq experiments included KDM5C in wild-type and *Kdm5c*-KO EpiLCs⁴⁵ (avail-
501 able at GEO: GSE96797) and mouse primary neuron cultures (PNCs) from the cortex and hippocampus¹⁵
502 (available at GEO: GSE61036). ChIP-seq of histone 3 lysine 4 dimethylation in male wild-type and *Kdm5c*-KO
503 EpiLCs⁴⁵ is also available at GEO: GSE96797. ChIP-seq of histone 3 lysine 4 trimethylation in wild-type and
504 *Kdm5c*-KO male amygdala²⁷ are available at GEO: GSE127817.

506 **Data analysis**

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

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673 **Figures and Tables**

- 674 • Supplementary table 1: list of all germline genes.
- 675 – Columns to include:
- 676 * KDM5C bound vs not
- 677 * 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/W_v) testis (Mueller et al 2013). Expression is in Fragments Per Kilobase of transcript per Million mapped reads (FPKM). **C.** Number of testis DEGs that were classified as cell-type specific markers in a single cell RNA-seq dataset of the testis (Green et al 2018). Germline cell types are highlighted in green, somatic cell types in black. **D.** Sankey diagram of mouse genes filtered for germline enrichment based on their expression in wild-type and germline-depleted mice and in adult mouse non-gonadal tissues (Li et al 2017).

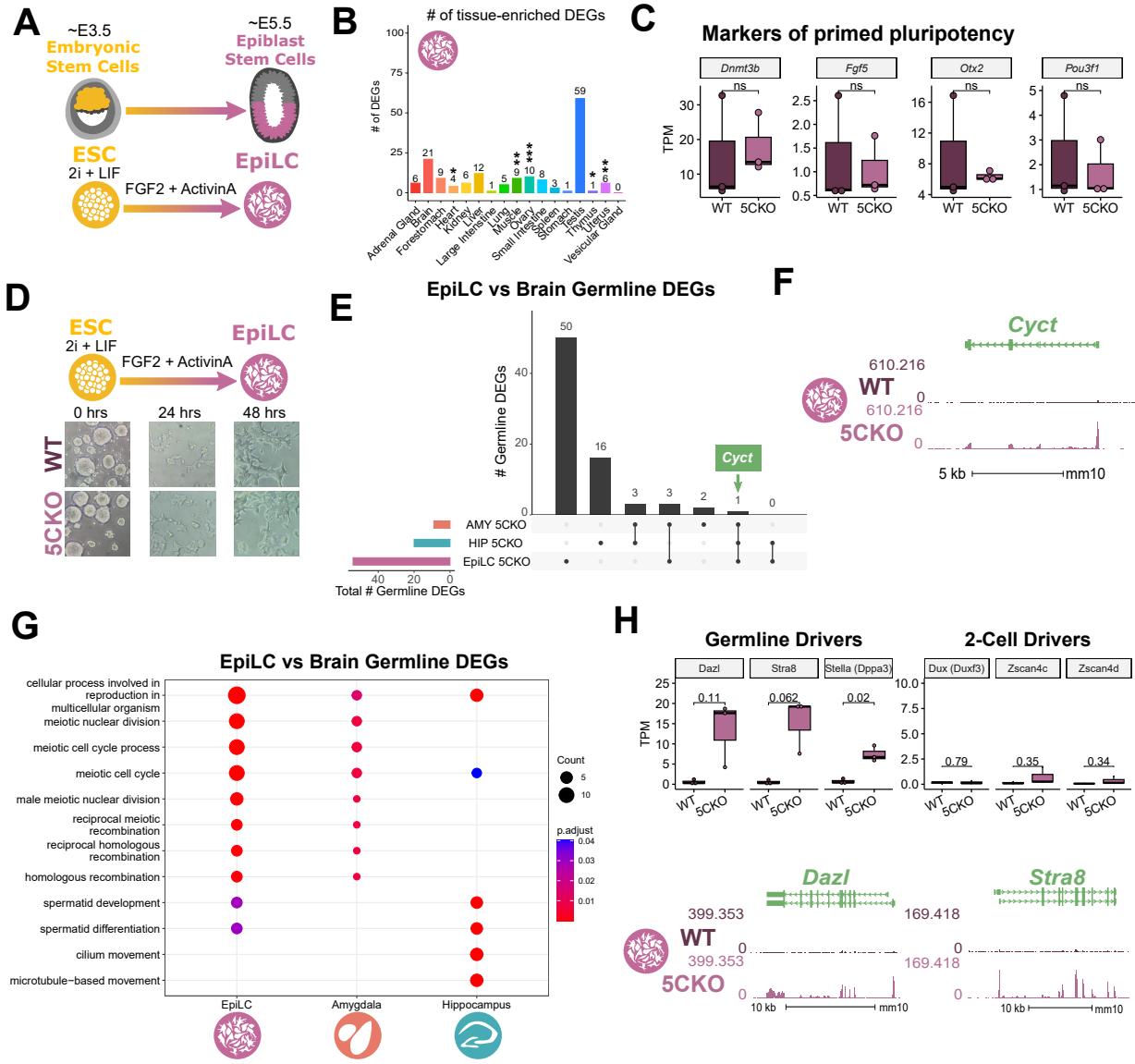


Figure 3: *Kdm5c*-KO epiblast-like cells express key drivers of germline identity

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

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

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

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

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

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

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

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

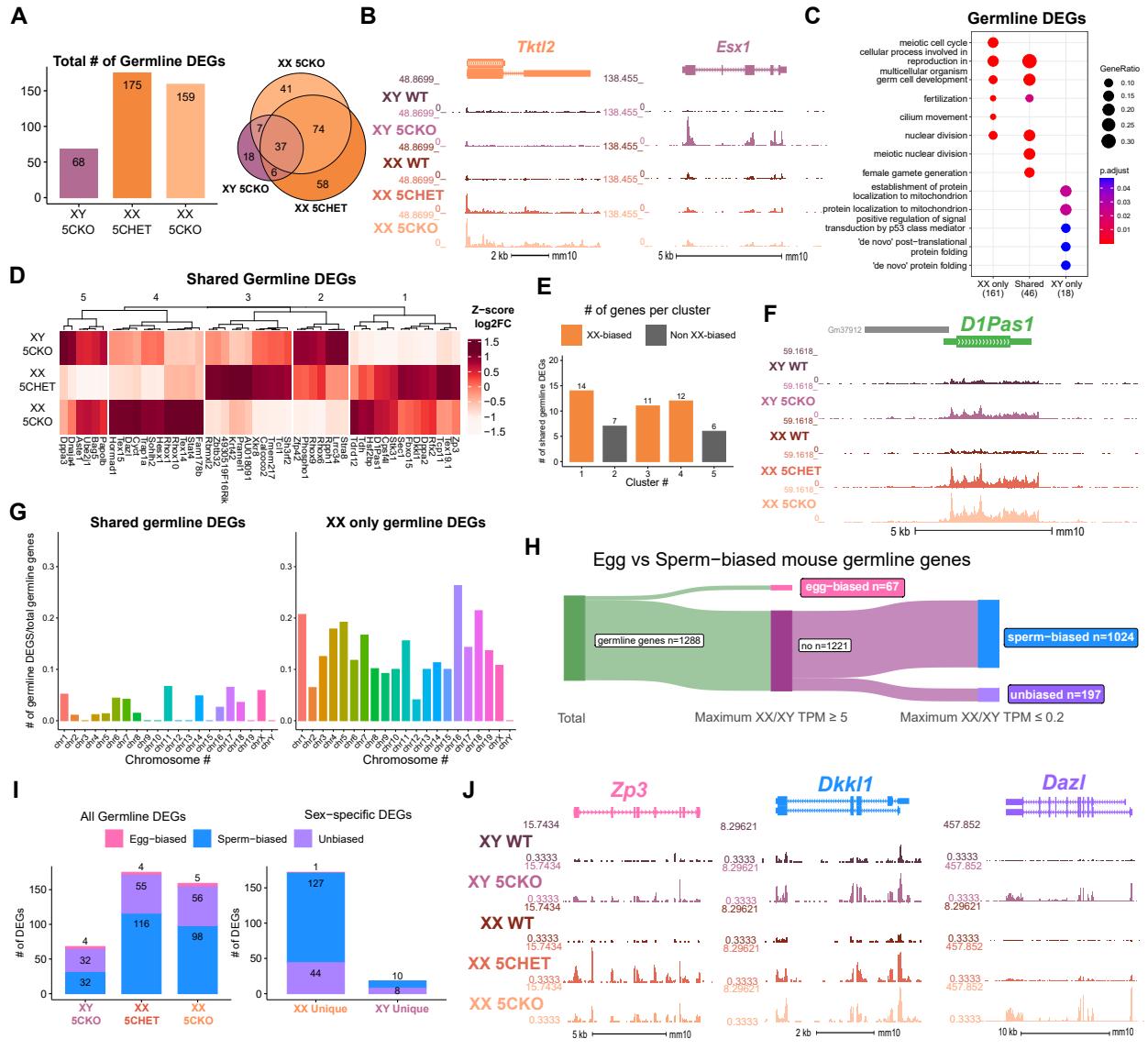


Figure 4: Chromosomal sex influences *Kdm5c*-KO germline gene misexpression. **A-B.** Expression of tissue-enriched genes (Li et al 2017) in the male *Kdm5c*-KO amygdala (A) and hippocampus (B). Left - MA plot of mRNA-seq. Right - Number of tissue-enriched differentially expressed genes (DEGs). * $p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

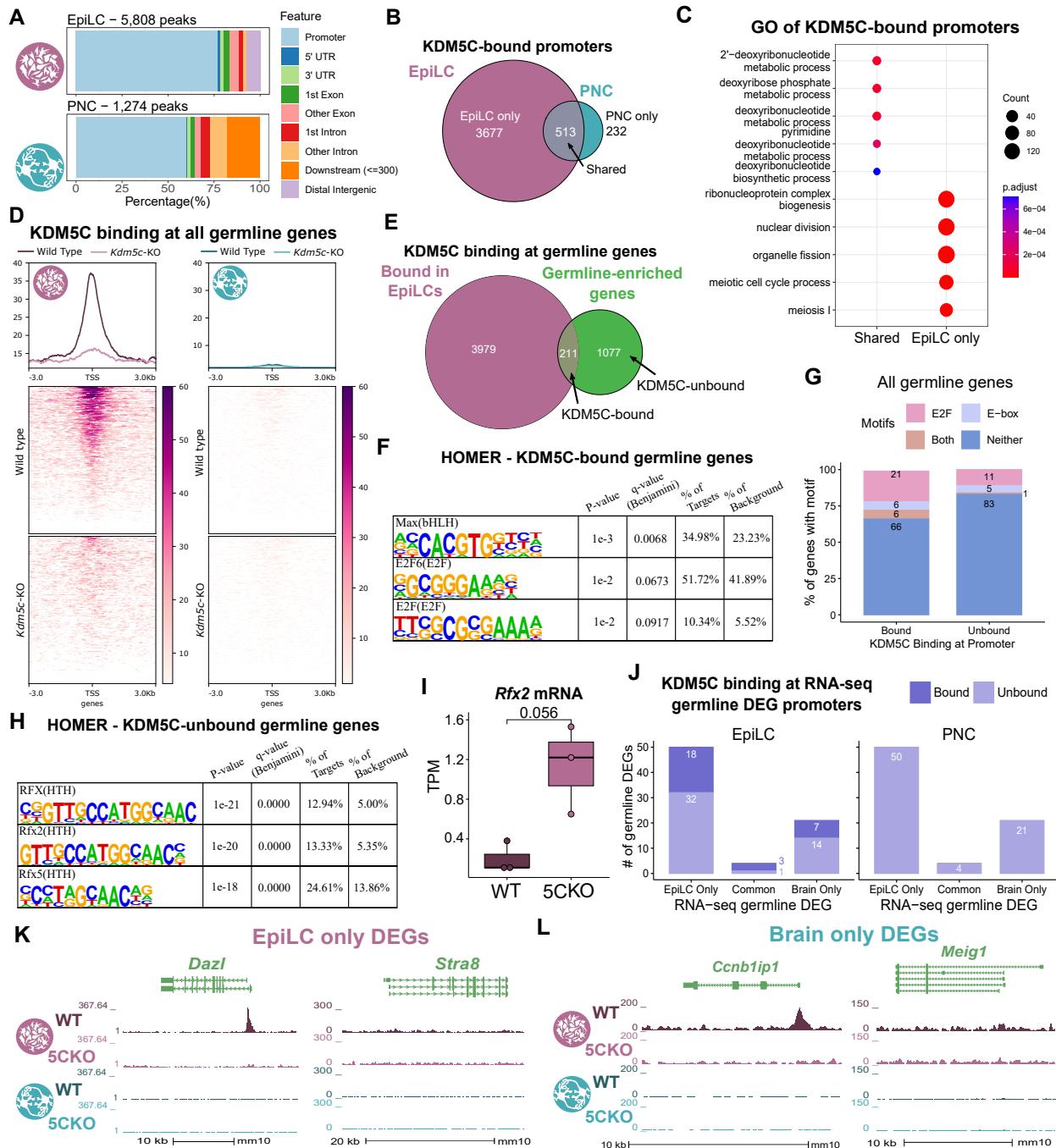
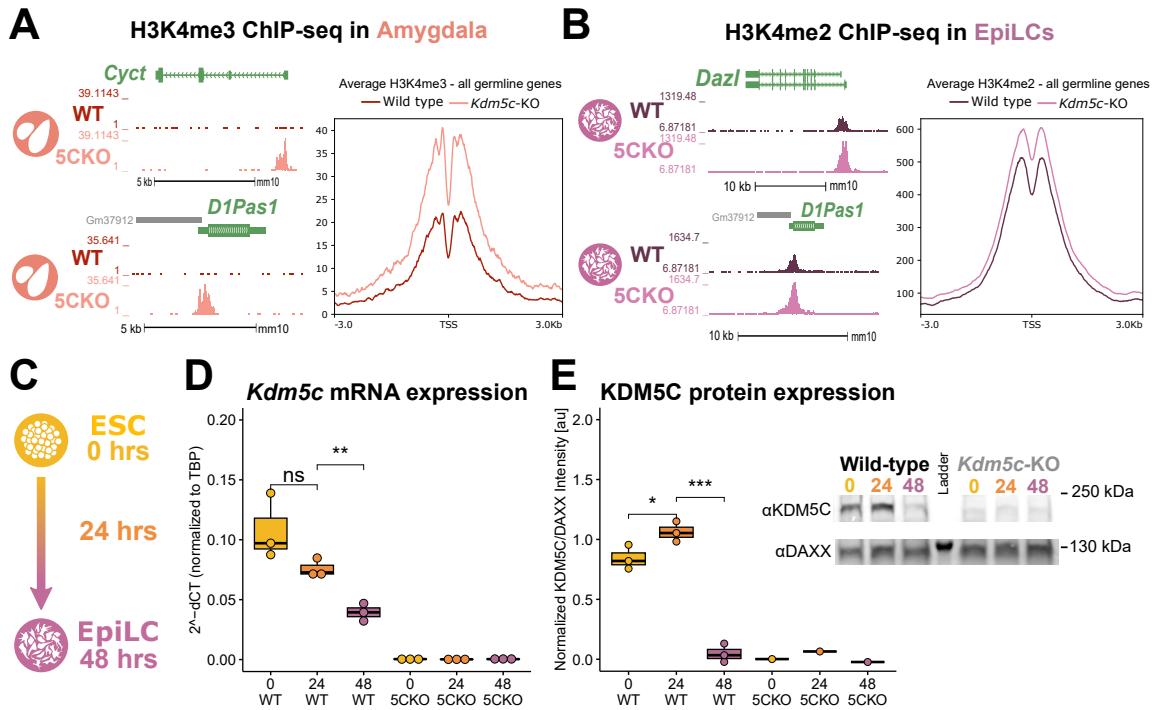


Figure 5: KDM5C binds to a subset of germline gene promoters during early embryogenesis **A.** ChIPseeker localization of KDM5C peaks at different genomic regions in EpiLCs (top) and hippocampal and cortex primary neuron cultures (PNCs, bottom). **B.** Overlap of genes with KDM5C bound to their promoters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

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



F

G

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

678 Notes

679 Things to do

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

684 **Dazl**

685 We were particularly interested in the aberrant transcription of *Dazl*, since it is essential for germ cell
686 development and promotes the translation of germline mRNAs⁷⁸. A significant portion of germline transcripts
687 misexpressed in *Kdm5c*-KO EpiLCs are known binding targets of DAZL, including *Stra8*⁷⁹ ($p = 1.698e-07$,
688 Fisher's Exact Test). This suggests expression of DAZL protein could promote the translation of other
689 aberrant germline transcripts, influencing their ability to impact *Kdm5c*-KO cellular function. We thus tested
690 DAZL protein expression in *Kdm5c*-KO EpiLCs through immunocytochemistry (Figure 3I). We observed
691 about 25% of *Kdm5c*-KO EpiLCs expressed DAZL protein and it was localized to the cytoplasm ($p = 0.0015$,
692 Welch's t-test), consistent with the pattern of DAZL expression in spermatogonia⁷⁹. Altogether these results
693 suggest tissue-specific genes are misexpressed during *Kdm5c*-KO embryogenesis, including key drivers of
694 germline identity that can be translated into protein.

- 695 • We additionally found *Kdm5c*-KO EpiLCs ectopically express DAZL protein that is localized to the
696 cytoplasm, similar to its morphology in spermatogonia⁷⁹. **note: maybe just put in results.** Could
697 move around depending upon if I get pheno working.

698 Discussion notes

- 699 • For other paper:
 - 700 – for methods: Heatmaps of gene expression were generated using the base R functions scale and
701 hclust and visualized using the R package ComplexHeatmap (v2.12.1).
 - 702 – * 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>
- 704 • Talk about different motifs if we do see differences there and if it explains direct vs indirect dysregulation
 - 705 – Could also mention PRC1.6/KDM5C binds pcgf6 in ESCs
- 706 • Maybe talk about other regulators/if anything is known about them long term? Or save for 2nd paper.

- 707 – another germline repressor: <https://pubmed.ncbi.nlm.nih.gov/35345626/>
- 708 • end with something like: this indicates kdm5c not only modulates pluripotency and self-renewal in
709 ESCs, but also has a role in long-term silencing of germline genes
- 710 – then transition into the long term silencing mechanism paragraph
- 711 • KDM5C is dynamically regulated during the window of embryonic germline gene silencing, our EpiLC
712 ChIP-seq is likely catching the tail end of KDM5C's main involvement.
- 713 • Our results indicate KDM5C is a pivotal repressor of germline programs in somatic cells.
- 714 • Studies on other chromatin regulators have focused on key marker genes to identify germline gene
715 misexpression, such as *Dazl*.
- 716 • Using a germline-depleted mouse model, we curated a list of mouse germline-enriched genes to
717 globally assess germline gene dysregulation.
- 718 • Many of the genes dysregulated in the mature *Kdm5c*-KO brain were important for late stages of
719 spermatogenesis, such as those important for sperm flagellar structure. Contrastingly, *Kdm5c*-KO
720 EpiLCs aberrantly expressed key drivers of germ cell identity, including *Dazl* and *Stra8*.
- 721 • While a significant portion of KDM5C-bound promoters are germline genes, many germline genes
722 expressed during *Kdm5c*-KO embryogenesis are not directly bound by kdm5c.
- 723 • One notable example is *Stra8*, a transcription factor important for germ cell specific transcription and
724 meiotic initiation
- 725 • The including the demarcation between soma and germline fates.
- 726 the *Kdm5c*-KO brain also Previous work identified testis-enriched genes in *Kdm5c* genes
- 727 –
- 728 – However unlike the gonadal-biased DEGs,
- 729 • The demarcation of the germline versus soma is a key feature of multicellularity and sexually dimorphic
730 reproduction
- 731 • Anything known about tissue-biased gene expression in other H3K4me regulators?
- 732 • Our data suggests the germline developmental program is occurring ectopically as *Kdm5c*-KOs pro-
733 gresses through somatic tissue development
- 734 • tissue-biased gene expression:

- 735 • Altogether, these data indicate that while some germline genes are misexpressed due to direct loss of
736 KDM5C binding during emryogenesis, secondary downstream mechanisms can also promote their
737 aberrant transcription.
- 738 • Papers to read/reference:
739 – Reconstitution of the Mouse Germ Cell Specification Pathway in Culture by Pluripotent Stem Cells:
740 [https://www.cell.com/fulltext/S0092-8674\(11\)00771-9](https://www.cell.com/fulltext/S0092-8674(11)00771-9)
741 – 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/>

743 **Figure outline:**

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

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

756 **Figure 3: *Kdm5c*-KO epiblast-like cells express key drivers regulators of germline identity** * A) ESC
757 to EpiLC differentiation Left - Morphology is unchanged, * B) 5CKO EpiLCs express EpiLC differentiation
758 genes similar to WT lvs * C) Male EpiLCs express germline genes (example Cyct again) * Overlap between
759 brain and EpiLC germline genes - show they're mostly unique * GO of Brain and EpiLC germline genes
760 (meiotic enriched) * Bigwigs or TPM of master regulators * Show that while some are also 2-cell genes
761 (Dazl), 2-cell specific genes aren't dysregulated (Zscan4). Important point because published KDM5C dazl
762 paper is saying KDM5C is a 2-cell regulator, but as far as I can tell only genes shared between germline and
763 2-cell are dysregulated.

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

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

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

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

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

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

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

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

808 **Germline gene repression background:**

809 Interestingly, some of the ectopic testis transcripts identified in the *Kdm5c*-KO brain are typically ex-
810 pressed in germ cells¹⁰. Unlike somatic cells, germ cells (e.g. sperm and eggs) undergo meiosis and pass
811 on their genetic material to the next generation. The germline and the soma are typically distinguished during
812 early embryogenesis, when germline genes are silenced in epiblast stem cells soon after implantation and
813 only reactivated in a subset to form the germline. Chromatin regulators play a key role in decommissioning
814 germline genes as the embryo transitions from naïve to primed pluripotency by placing repressive histone
815 H2A lysine 119 monoubiquitination (H2AK119ub1)¹⁸, histone 3 lysine 9 trimethylation (H3K9me3)^{18,19}, and
816 DNA CpG methylation^{19–21} at germline gene promoters. KDM5C may also be involved in this early decom-
817 missioning of germline genes, as re-expression of KDM5C in neurons fails to suppress their dysregulation¹⁰.
818 In support of this, KDM5C was very recently shown to repress *Deleted in azoospermia like (Dazl)*, a key
819 regulator of germline development, in mouse embryonic stem cells (ESCs)^{49,69}. In support of this, two
820 independent screens in mouse embryonic stem cells (ESCs) recently identified KDM5C as a repressor of
821 *Deleted in azoospermia like (Dazl)*, a key regulator of germline development. However, KDM5C's role in
822 embryonic germline gene repression is currently unclear, given that Dazl is also expressed in ESCs and in
823 the 2-cell stage and germline gene misexpression has yet to be globally characterized during *Kdm5c*-KO
824 embryogenesis.