

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). Loss of repressive chromatin regulators can lead to the aberrant transcription of tissue-specific genes outside of their intended context, however the mechanisms behind their dysregulation and their functional consequences are largely unknown. Here, we explored this cellular identity crisis in NDDs by investigating the role of lysine demethylase 5c (KDM5C, also known as JARID1C or SMCX), an eraser of histone 3 lysine 4 di and tri-methylation (H3K4me2/3), in tissue identity. We found male *Kdm5c* knockout (-KO) mice, which recapitulate key behavioral phenotypes of Claes-Jensen X-linked intellectual disability, aberrantly expresses many liver, muscle, ovary, and testis genes within the amygdala and hippocampus. Gonad-enriched genes expressed in the *Kdm5c*-KO brain were typically unique to germ cells, indicating an erosion of the soma-germline boundary. We then 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*. Germline gene dysregulation was sexually dimorphic, as female *Kdm5c*-KO EpiLCs were more prone to germline gene misexpression than knockout males. We found KDM5C represses germline-specific transcription by binding to a subset of germline gene promoters in EpiLCs to facilitate DNA methylation of CpG islands. However, germline genes, particularly late-stage spermatogenesis genes, can also become activated in *Kdm5c*-KO cells independent of direct KDM5C binding. This suggests germline developmental programs can progress ectopically in the background of typical *Kdm5c*-KO development, due to downstream activation by germline transcription factors. These results define KDM5C's role in germline gene suppression and potentially link impaired soma-vs-germline demarcation to a chromatin-based neurodevelopmental disorder.

- 27 • Not sure if I like the last sentence, I think we should focus on what we did do versus what we could do
28 in the future

- 29 – Add something that we deepened the characterization of germline gene classes that use different
30 regulatory mechanisms (CpG islands vs not, meiotic vs late-stage, E2F6/MAX vs no)

31 Introduction

32 A single genome holds the instructions to generate the myriad of cell types found within the adult organism.
33 This is, in part, accomplished by chromatin regulators that can either promote or impede lineage-specific gene
34 expression through DNA and histone modifications^{1,2}. Many chromatin regulators were initially identified
35 for their roles in shaping cellular and tissue identity^{3–5}. Unexpectedly, human genetic studies revealed
36 mutations in chromatin regulators are a major cause of neurodevelopmental disorders (NDDs)⁶. Most studies
37 investigating this relationship have explored their regulation of brain-specific genes and chromatin marks.
38 However, loss of some chromatin regulators can also result in the ectopic transcription of tissue-specific
39 genes outside of their target environment, such as the misexpression of liver-specific genes within adult
40 neurons⁷. Very few studies have investigated this severe crisis in cellular identity in chromatin-linked NDDs^{7,8}
41 and it is currently unknown if these ectopic genes contribute to neurodevelopmental impairments.

42 To elucidate the role of tissue identity in chromatin-linked neurodevelopmental disorders, it is essential
43 to first characterize the nature of the dysregulated genes and the molecular mechanisms governing their
44 de-repression. We characterized lysine demethylase 5C (KDM5C), also known as SMCX or JARID1C, which
45 erases histone 3 lysine 4 di- and trimethylation (H3K4me2/3) found at active gene promoters⁹. Pathogenic
46 mutations in *KDM5C* cause Intellectual Developmental Disorder, X-linked, Syndromic, Claes-Jensen Type
47 (MRXSCJ, OMIM: 300534). MRXSCJ is more common and severe in males and its neurological phenotypes
48 include intellectual disability, seizures, aberrant aggression, and autistic behaviors^{10–12}. Male *Kdm5c*
49 knockout (-KO) mice recapitulate key MRXSCJ phenotypes, including hyperaggression, increased seizure
50 propensity, and learning impairments^{8,13}. RNA sequencing (RNA-seq) of the *Kdm5c*-KO hippocampus
51 revealed ectopic expression of testis genes within the brain⁸, however it is unknown what types of testis
52 genes are dysregulated, when in *Kdm5c*-KO development testis gene dysregulation begins, and if other
53 tissue-specific genes are also aberrantly transcribed with KDM5C loss.

54 Distinguishing between germ cells and somatic cells is a key feature of multicellularity¹⁴ that occurs
55 during early embryogenesis in many metazoans¹⁵. In mammals, chromatin regulators are crucial for
56 decommissioning germline genes in somatic cells during the transition from naïve to primed pluripotency.
57 Initially, germline gene promoters gain repressive histone H2A lysine 119 monoubiquitination (H2AK119ub1)¹⁶
58 and histone 3 lysine 9 trimethylation (H3K9me3)^{16,17} in embryonic stem cells and are then decorated with
59 DNA CpG methylation in the post-implantation embryo^{17–19}. The precise roles of KDM5C during this process
60 remains unclear. Additionally, studies on germline gene repression have primarily focused on marker genes
61 important for germ cell development rather than germline genes as a whole, given they lacked a curated
62 list of germline-enriched genes. Therefore, it is unknown if the mechanism of repression differs for certain

63 classes of germline genes, e.g. meiotic genes versus spermatid differentiation genes.

64 It is also unknown if ectopic germline gene expression is influenced by chromosomal sex, as previous
65 studies on germline gene repression have been exclusively in males. Sex is particularly pertinent in the case
66 of KDM5C, which lies on the X chromosome and partially escapes X chromosome inactivation, resulting in a
67 higher dosage in females²⁰⁻²³.

68 To illuminate KDM5C's role in tissue identity, here we characterized the aberrant transcription of tissue-
69 enriched genes within the *Kdm5c*-KO brain and epiblast-like stem cells (EpiLCs), an *in vitro* model of
70 the post-implantation embryo. We curated list of germline-enriched genes, which enabled genome-wide
71 analysis of germline gene silencing mechanisms. Based on the data presented below, we propose KDM5C
72 plays a fundamental role in the development of tissue identity during early embryogenesis, including the
73 establishment of the soma-germline boundary.

74 Results

75 **Tissue-enriched genes are aberrantly expressed in the *Kdm5c*-KO brain**

76 Previous RNA sequencing (RNA-seq) in the adult hippocampus revealed ectopic expression of some
77 testis genes within the male *Kdm5c* knockout (-KO) brain⁸. It is currently unclear if the testis is the only
78 tissue type misexpressed in the *Kdm5c*-KO brain. We thus characterized dysregulation of *Kdm5c*-KO brain
79 tissue identity by systematically assessing the expression of genes enriched in 17 mouse tissues²⁴, using
80 our published mRNA-seq datasets of the adult amygdala and hippocampus for male mice with constitutive
81 knockout of *Kdm5c*²⁵.

82 We found a large proportion of genes that are significantly upregulated within the *Kdm5c*-KO brain
83 (DESeq2²⁶, log2 fold change > 0.5, q < 0.1) are enriched in non-brain tissues in wild-type mice (Amygdala:
84 35%, Hippocampus: 24%) (Figure 1A-B). The majority of tissue-enriched differentially expressed genes
85 (tissue-enriched DEGs) were testis genes (Figure 1A-C). Even though the testis has the largest total number
86 of biased genes (2,496 genes) compared to any other tissue, testis-biased DEGs were significantly enriched
87 for both brain regions (Amygdala p = 1.83e-05, Odds Ratio = 5.13; Hippocampus p = 4.26e-11, Odds Ratio =
88 4.45, Fisher's Exact Test). One example of a testis-enriched gene misexpressed in the *Kdm5c*-KO brain is
89 *FK506 binding protein 6* (*Fkbp6*), a known regulator of piRNA expression and meiosis^{27,28} (Figure 1C).

90 Interestingly, we also observed significant enrichment of ovary-biased DEGs in both the amygdala and
91 hippocampus (Amygdala p = 0.00574, Odds Ratio = 18.7; Hippocampus p = 0.048, Odds Ratio = 5.88,
92 Fisher's Exact) (Figure 1D). Ovary-enriched DEGs included *Zygotic arrest 1* (*Zar1*), which sequesters mRNAs
93 in oocytes for meiotic maturation and early zygote development²⁹ (Figure 1D). Given the *Kdm5c*-KO mice
94 we analyzed are male, this demonstrates ectopic expression of tissue-enriched genes is independent of
95 organismal sex.

96 Although not consistent across brain regions, we also found significant enrichment of DEGs biased
97 towards two non-gonadal tissues - the liver (Amygdala p = 0.0398, Odds Ratio = 6.58, Fisher's Exact Test)
98 and the muscle (Hippocampus p = 0.0104, Odds Ratio = 6.95, Fisher's Exact Test). A liver-biased DEG
99 dysregulated in both the hippocampus and amygdala is *Apolipoprotein C-I* (*Apoc1*), a lipoprotein metabolism
100 and transport gene³⁰ that has been implicated in Alzheimer's disease³¹ (Figure 1E). For all tissue-enriched
101 DEGs, aberrantly expressed mRNAs are polyadenylated and spliced into mature transcripts (Figure 1C-E).
102 Of note, we observed little to no dysregulation of brain-enriched genes (Amygdala p = 1; Hippocampus p =
103 0.74, Fisher's Exact), despite the fact these are brain samples and the brain has the second highest total
104 number of tissue-enriched genes (708 genes). Together, these results suggest the aberrant expression of
105 tissue-enriched genes within the brain is a major effect of KDM5C loss.

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

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

113 To further validate if these testis DEGs are truly germline genes, we assessed their expression in somatic
114 versus germ cells within the testis. We first compared their expression in wild-type testes to those without
115 germ cells³², which was accomplished by heterozygous *W* and *Wv* mutations in the enzymatic domain of
116 *c-Kit* (*Kit*^{W/Wv})³³. Almost all *Kdm5c*-KO testis-enriched DEGs lost expression with germ cell depletion (Figure
117 2B). We then assessed testis-enriched DEG expression in a published single cell RNA-seq dataset that
118 identified cell type-specific markers within the testis³⁴. Some *Kdm5c*-KO testis-enriched DEGs were classified
119 as specific markers for different germ cell types (e.g. spermatogonia, spermatocytes, round spermatids, and
120 elongating spermatids), yet none marked somatic cells (Figure 2C). Together, these data demonstrate that
121 the *Kdm5c*-KO brain aberrantly expresses germline genes, but not somatic testicular genes, reflecting an
122 erosion of the soma-germline boundary.

123 We then wanted to characterize germline gene misexpression with *Kdm5c* loss more deeply, but lacked
124 a comprehensive list of mouse germline genes. We therefore generated a list of germline-enriched genes
125 using RNA-seq datasets of *Kit*^{W/Wv} mice that included males and females at embryonic day 12, 14, and 16³⁵
126 and adult male testes³². We defined genes as germline-enriched if their expression met the following criteria:
127 1) their expression is greater than 1 FPKM in wild-type gonads 2) their expression in any non-gonadal tissue
128 of adult wild type mice²⁴ does not exceed 20% of their maximum expression in the wild-type germline, and
129 3) their expression in the germ cell-depleted gonads, for any sex or time point, does not exceed 20% of
130 their maximum expression in the wild-type germline. These criteria yielded 1,288 germline-enriched genes

131 (Figure 2D), which was hereafter used as a resource to globally assess germline gene misexpression with
132 *Kdm5c* loss (Supplementary table 1).

133 ***Kdm5c*-KO epiblast-like cells aberrantly express key regulators of germline identity**

134 Germ cells are typically distinguished from somatic cells soon after the embryo implants into the uterine
135 wall^{36,37}. A subset of epiblast stem cells become the primordial germ cells (PGCs) while the remainder
136 differentiate into the ectoderm, mesoderm, and endoderm to form the somatic tissues³⁸. This developmental
137 time point can be modeled *in vitro* through differentiation of embryonic stem cells (ESCs) into epiblast-like
138 stem cells (EpiLCs) (Figure 3A, top)^{39,40}. While some germline-enriched genes are also expressed in
139 naïve embryonic stem cells (ESCs) and in the 2-cell stage^{41–43}, they are silenced as they differentiate into
140 EpiLCs^{17,44}. Therefore, we tested if KDM5C was necessary for silencing germline genes at this developmental
141 stage by evaluating the impact of *Kdm5c* loss in male EpiLCs.

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

149 We next compared EpiLC germline DEGs to those expressed in the *Kdm5c*-KO brain to determine if
150 germline DEGs are constitutively dysregulated or change over the course of development. We found the
151 majority of germline DEGs were unique to either EpiLCs or the brain, with only *CycT* shared across all
152 tissue/cell types (Figure 3E-F). EpiLCs had particularly high enrichment of meiosis-related gene ontologies
153 (Figure 3G), such as meiotic cell cycle process (GO:1903046, p.adjust = 1.59e-08) and meiotic nuclear
154 division (GO:0140013, p.adjust = 9.76e-09). While there was modest enrichment of meiotic gene ontologies
155 in both brain regions, the *Kdm5c*-KO hippocampus primarily expressed late-stage spermatogenesis genes
156 involved in sperm axoneme assembly (GO:0007288, p.adjust = 0.00621) and sperm motility (GO:0097722,
157 p.adjust = 0.00612).

158 Notably, DEGs unique to *Kdm5c*-KO EpiLCs included key drivers of germline identity, such as *Stimulated*
159 *by retinoic acid 8* (*Stra8*) and *Deleted in azoospermia like* (*Dazl*) (Figure 3H). These genes are typically
160 expressed during embryonic germ cell development to commit PGCs to the germline fate, but are also
161 expressed later in life to trigger meiotic gene expression programs^{46–48}. Of note, some germline genes,
162 including *Dazl*, are also expressed in the two-cell embryo^{42,49}. However, we did not see misexpression of
163 two-cell stage-specific genes, like *Duxf3* (*Dux*) (q = 0.337) and *Zscan4d* (q = 0.381), indicating *Kdm5c*-KO in
164 EpiLCs do not revert back to a 2-cell-like state (Figure 3H). Altogether, these results demonstrate that while
165 the *Kdm5c*-KO brain primarily expresses spermatogenesis genes, *Kdm5c*-KO EpiLCs express key drivers of

166 germline identity and meiosis.

167 **Female epiblast-like cells have increased sensitivity to germline gene misexpression**
168 **with *Kdm5c* loss**

169 It is currently unknown if the misexpression of germline genes is influenced by sex, as previous studies
170 on germline gene repressors have focused on males^{16–18,50,51}. We explored the impact of sex upon germline
171 gene misexpression by comparing their dysregulation in male *Kdm5c* hemizygous knockout (XY *Kdm5c*-
172 KO), female homozygous knockout (XX *Kdm5c*-KO), and female heterozygous knockout (XX *Kdm5c*-HET)
173 EpiLCs.⁴⁵.

174 Homozygous and heterozygous females expressed over double the number of germline-enriched genes
175 than hemizygous males (Figure 4A). While the majority of germline DEGs in *Kdm5c*-KO males were also
176 dysregulated in females (74%), there were also many male-specific and female-specific germline DEGs
177 (Figure 4B), such as *Tktl2* and *Esx1*. We compared the known functions of germline genes dysregulated
178 only in females (XX only - XX *Kdm5c*-KO, XX *Kdm5c*-HET, or both), only in males (XY only), or in all samples
179 (shared). Female-specific germline DEGs were enriched for meiotic (GO:0051321 meiotic cell cycle) and
180 flagellar (GO:0003341 cilium movement) functions, male-specific DEGs had mitochondrial and cell signaling
181 gene functions (GO:0070585 protein localization to mitochondrion). Germline transcripts expressed in
182 both sexes were enriched for meiotic (GO:0140013 meiotic nuclear division) and egg-specific functions
183 (GO:0007292 female gamete generation).

184 The majority of germline genes expressed in both sexes had a greater log2 fold change in females
185 compared to males (Figure 4D). This increased degree of dysregulation in females, along with the increased
186 total number of germline genes, indicates females are more sensitive to losing KDM5C-mediated suppression
187 of germline genes. Female sensitivity could be due to improper X chromosome inactivation (XCI) in *Kdm5c*
188 mutants⁴⁵, as many spermatogenesis genes lie on the X chromosome^{52,53}. However, both shared and
189 female-specific germline DEGs were not biased towards the X chromosome and the majority of female
190 DEGs instead lie on autosomes (Figure 4G). Thus, while female EpiLCs are more prone to germline gene
191 misexpression with KDM5C loss, it is likely independent of potential defects in XCI.

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

193 While many germline genes act in both the male and female germline, some display sex-biased expression
194 or have functions unique to eggs and sperm. Therefore, we wondered if *Kdm5c* mutant males express
195 sperm genes while mutant females express egg genes. To comprehensively assess whether germline
196 gene sex corresponds with *Kdm5c* mutant sex, we filtered our list of germline-enriched genes for egg and
197 sperm-biased genes. We defined germ cell sex-biased genes as those whose expression in the opposite
198 sex, at any time point, is no greater than 20% of the gene's maximum expression in a given sex. This yielded

199 67 egg-biased, 1,024 sperm-biased, and 197 unbiased germline-enriched genes (Figure 4H). We found
200 egg, sperm, and unbiased germline genes were dysregulated in all *Kdm5c* mutants (Figure 4I-J). Germline
201 genes dysregulated exclusively in *Kdm5c* mutant males and females were also not biased towards their
202 corresponding germ cell sex. This indicates sex differences in germline gene dysregulation is not due to
203 sex-specific activation of sperm or egg transcriptional programs. These results demonstrate that the sex of
204 *Kdm5c* mutant cells influences the degree of germline gene, independent of germline gene sex.

205 • note: the edited last sentence ended with “independent of germ cell sex”, but I think if it’s read
206 out of context that sounds like we tested KDM5C in the four core genotypes. But I’m not sure if
207 “germline gene sex” is confusing

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

209 KDM5C binds to the promoters of several germline genes in embryonic stem cells (ESCs) but its binding
210 is absent in neurons⁸. However, the lack of a comprehensive list of germline-enriched genes prohibited
211 systematic characterization of KDM5C binding at germline gene promoters. Thus, it is unclear if KDM5C is
212 enriched at germline gene promoters, what types of germline genes KDM5C regulates, and if its binding is
213 maintained at any germline genes in neurons.

214 To address these questions, we analyzed KDM5C chromatin immunoprecipitation followed by DNA
215 sequencing (ChIP-seq) datasets in EpiLCs⁴⁵ and primary forebrain neuron cultures (PNCs)¹³. EpiLCs had a
216 higher total number of KDM5C peaks than PNCs (EpiLCs: 5,808, PNCs: 1,276, MACS2 q < 0.1 and fold
217 enrichment > 1, removal of *Kdm5c*-KO false positive peaks) and KDM5C was primarily localized to gene
218 promoters in both cell types (EpiLCs: 4,190, PNCs: 745 +/- 500bp from the TSS), although PNCs showed
219 increased localization to non-promoter regions (Figure 5A).

220 The majority of promoters bound by KDM5C in PNCs were also bound in EpiLCs (513 shared promoters),
221 however a large portion of gene promoters were only bound by KDM5C in EpiLCs (3677 EpiLC only
222 promoters) (Figure 5B). Genes bound by KDM5C in both PNCs and EpiLCs were enriched for functions
223 involving nucleic acid turnover, such as deoxyribonucleotide metabolic process (GO:0009262, p.adjust =
224 8.28e-05) (Figure 5C). Germline-specific ontologies were only enriched in promoters unique to EpiLCs, such
225 as meiotic nuclear division (GO: 0007127 p.adjust = 6.77e-16) and meiotic cell cycle process (GO:1903046,
226 p.adjust = 5.05e-16) (Figure 5C). There were no ontologies significantly enriched for genes only bound
227 by KDM5C in PNCs. We next evaluated KDM5C binding around the transcription start site (TSS) of all
228 germline-enriched genes. In EpiLCs, we observed modest KDM5C signal at about half of all germline genes
229 (Figure 5D). Based on our ChIP-seq peak cut-off criteria, KDM5C was strongly bound to about 10% of
230 germline gene promoters in EpiLCs (Figure 5E). One notable gene that lacked KDM5C binding was *Stra8*,
231 even though its mRNA is expressed in *Kdm5c*-KO EpiLCs (Figure 5K). In EpiLCs, KDM5C was only bound
232 to about one third of *Kdm5c*-KO RNA-seq DEG promoters (EpiLC only DEGs: 36%, Brain only DEGs:

233 33.3%), but 3 out of the 4 genes dysregulated in both the brain and EpiLCs (Supplementary figure XXX).
234 In condordance with our gene ontology results, we did not observe KDM5C accumulation at any germline
235 gene promtoers in PNCs (Figure 5D). Together, these results demonstrate KDM5C is recruited to a subset of
236 germline genes in EpiLCs, including meiotic genes, but does not directly regulate germline genes in neurons.
237 Furthermore, the majority of germline mRNAs expressed in *Kdm5c*-KO cells are dysregulated independent
238 of direct KDM5C binding to their promoters.

239 Many germline-specific genes are suppressed by the transcription factor heterodimers E2F/DP1 and
240 MGA/MAX, which respectively bind E2F and E-box motifs^{18,50,51,54,55}. To elucidate if KDM5C is recruited to
241 germline gene promoters by a similar mechanism, we used HOMER to identify transcription factor motifs
242 enriched at KDM5C-bound or unbound germline gene promoters⁵⁶ (TSS +/- 500 bp, q-value < 0.1). MAX
243 and E2F6 binding sites were significantly enriched at germline genes bound by KDM5C in EpiLCs, but
244 not at germline genes unbound by KDM5C (MAX q-value: 0.0068, E2F6 q-value: 0.0673, E2F q-value:
245 0.0917) (Figure 5F). One third of KDM5C-bound promoters contained the consensus sequence for either
246 E2F6 (E2F, 5'-TCCCGC-3'), MGA (E-box, 5'-CACGTG-3'), or both, but only 17% of KDM5C-unbound genes
247 contained these motifs (Figure 5G). KDM5C-unbound germline genes were intstead enriched for multiple
248 RFX transcription factor binding sites (RFX q-value < 0.0001, RFX2 q-value < 0.0001, RFX5 q-value < 0.0001)
249 (Figure 5H). RFX transcription factors bind X-box motifs⁵⁷ to promote ciliogenesis^{58,59} and among them is
250 RFX2, a central regulator of post-meiotic spermatogeneis^{60,61}. Interestingly, RFX2 mRNA is derepressed in
251 *Kdm5c*-KO EpiLCs (Figure 5I), however it is not a direct target of KDM5C (Supplementary figure XXX). Thus,
252 RFX2 is a candidate transcription factor for driving the ectopic expression of KDM5C-unbound germline
253 genes in *Kdm5c*-KO cells.

254 **KDM5C promotes *de novo* DNA methylation at germline genes (if there are differ-
255 ences, say at germline gene CpG islands)**

256 In the early embryo, germline gene promoters are initially decorated with repressive histone modifications
257 and are then silenced long-term via DNA CpG methylation (CpGme)^{16,17,44,62}. Our results above indicate
258 KDM5C also acts at germline gene promoters during this time period. However, how KDM5C interacts with
259 other germline gene silencing mechanisms is currently unclear. KDM5C is generally thought to supress
260 transcription through erasure of histone 3 lysine 4 di- and trimethylation (H3K4me2/3)⁹, yet KDM5C's
261 catalytic activity was recently shown to be dispensible for suppressing *Dazl* in undifferentiated ESCs⁴⁹. Since
262 H3K4me3 impedes *de novo* CpGme placement^{63,64}, KDM5C's catalytic activity may instead be required later
263 in development for long-term silencing of germline genes. In support of this, CpGme is markedly reduced at
264 two germline gene promoters in the *Kdm5c*-KO adult hippocampus⁸.

265 Based on the above observations, we hypothesized KDM5C erases H3K4me3 to promote the initial
266 placement of CpGme at germline gene promoters in EpiLCs. To test this hypothesis, we first characterized

267 KDM5C's substrates, histone 3 lysine 4 di- and trimethylation (H3K4me2/3) in our previously published ChIP-
268 seq datasets of the wild type and *Kdm5c*-KO amygdala²⁵ and EpiLCs⁴⁵. In congruence with previous work in
269 the *Kdm5c*-KO hippocampus⁸, we observed aberrant accumulation of H3K4me3 around the transcription
270 start site (TSS) of germline genes in the *Kdm5c*-KO amygdala (Figure 6A). We additionally found a marked
271 increase in H3K4me2 around the TSS of germline genes in *Kdm5c*-KO EpiLCs (Figure 6B).

272 We then evaluated KDM5C's role in germline gene silencing during ESC to EpiLC differentiation. We first
273 characterized KDM5C's mRNA and protein expression at 0, 24, and 48 hours of differentiation into EpiLCs
274 (Figure 6C). While *Kdm5c* mRNA steadily decreased from 0 to 48 hours of differentiation (Figure 6D), KDM5C
275 protein initially increased from 0 to 24 hours but then decreased to near knockout levels by 48 hours (Figure
276 6E).

277 To determine KDM5C's role in the initial placement of DNA methylation at germline gene promoters,
278 we performed whole genome bisulfite sequencing (WGBS) in wild-type and *Kdm5c*-KO ESCs and 96 hour
279 extended EpiLCs (exEpiLCs).

280 • Germline genes are known to accumulate CpGme at (CGIs) during the transition from naive to primed
281 pluripotency.

282 • We first identified the number of germline genes with CGIs neighboring their promoter.

283 – Found XXX% of germline genes had CGIs, XXX% of which were direct KDM5C targets in EpiLCs
284 (Eulerr).

285 • We then curated a list of germline genes that significantly gained CpGme in wild-type exEpiLCs
286 compared to ESCs.

287 – Example gene bedgraph

288 – Majority of CGI germline genes?

289 • Out of the CGI genes, which had significantly reduced CpGme in 5CKO (heatmap of % methylation)

290 – Highlight interesting genes affected vs unaffected by KDM5C (especially if same/different from
291 E2F6, PRC1.6, Setdb1 targets)

292 – CGIs that never gain DNAme in WT

293 – Although wild-type cells accumulated high levels of DNA methylation at germline gene promoters
294 over the course of ESCs to exEpiLC differentiation, DNA methylation was markedly reduced in
295 *Kdm5c*-KO exEpiLCs (Figure 6F).

296 • Non-CGI germline genes, any significant changes at their promoter? (Heatmap again?)

297 • Altogether, this suggests KDM5C is dynamically regulated during ESC to EpiLC differentiation and
298 promotes germline gene silencing via DNA methylation during early embryogenesis.

299 **Discussion**

300 We observed general dysregulation of tissue-enriched genes in both the *Kdm5c*-KO brain and EpiLCs,
301 including testis, liver, muscle, and ovary-enriched genes. The *Kdm5c*-KO amygdala and hippocampus had
302 significant enrichment of testis genes that are specific to germ cells and not somatic cells of the testis. We
303 found *Kdm5c*-KO EpiLCs aberrantly expressed key drivers of germline identity and meiosis, including *Dazl*
304 and *Stra8*, while the *Kdm5c*-KO brain primarily expressed germline genes important for late spermatogenesis.
305 Female *Kdm5c*-KO EpiLCs expressed over double the number of germline genes than *Kdm5c*-KO males and
306 germline genes dysregulated in both sexes were expressed more highly in females, demonstrating females
307 have increased sensitivity to germline gene dysregulation. KDM5C was bound to only a subset of germline
308 genes expressed in *Kdm5c*-KO EpiLCs, indicating germline-enriched mRNAs can be aberrantly transcribed
309 through indirect mechanisms. Finally, we found KDM5C promotes the long-term silencing of germline genes
310 in somatic cells by aiding the placement of DNA CpG methylation in EpiLCs. - Change: we propose KDM5C
311 plays a fundamental role in the development of tissue identity during early embryogenesis, including the
312 establishment of the soma-germline boundary.

- 313 • Thus, systematically characterizing KDM5C's role in germline gene repression during early embryogen-
314 esis, including its interaction with known silencing mechanisms, will unveil key mechanisms underlying
315 the demarcation between soma and germline identity. These results will provide molecular footholds
316 that can then be exploited to test the contribution of ectopic germline genes on neurodevelopment.

317 In the above study, we demonstrate KDM5C's pivotal role in the development of tissue identity, resulting in
318 substantial misexpression of tissue-enriched genes within the adult *Kdm5c*-KO hippocampus and amygdala.
319 In addition to testis genes identified previously⁸, we found significant enrichment of muscle, liver, and
320 even ovary-biased genes aberrantly expressed within the male *Kdm5c*-KO brain. Given the majority of
321 tissue-enriched DEGs have no known brain functions, it is currently unknown if they impair *Kdm5c*-KO
322 neurodevelopment and contribute to MRXSCJ-related phenotypes like intellectual disability and aberrant
323 aggression. However, select liver and muscle-biased DEGs do have known roles within the brain, such as the
324 liver-enriched lipid metabolism gene *Apolipoprotein C-I* (*Apoc1*)³⁰ that is highly expressed in the *Kdm5c*-KO
325 amygdala and hippocampus. Intriguingly, *Apoc1* overexpression in the mouse brain can impair learning and
326 memory⁶⁵ and is implicated in Alzheimer's disease in humans³¹, however further investigation is required to
327 determine its impact upon *Kdm5c*-KO phenotypes.

328 Deeper characterization of testis-enriched DEGs revealed they were not somatic testis genes but instead
329 germline genes, thus demonstrating KDM5C's crucial role in establishing the soma-germline boundary.
330 Distinguishing the germline from the soma is a key feature of multicellularity and sexual reproduction¹⁴.
331 Previous work characterizing repressors of germ cell-specific transcription has predominately focused on
332 their repression of key marker genes in embryonic stem cells (ESCs), such as *Dazl* and *Ddx4*^{16,17,51}. To
333 characterize KDM5C's role in germline gene repression throughout life at a genome-wide level, we curated a

334 list of germline-enriched genes using publically available germ cell-depleted RNA-seq datasets from Kit^{W/Wv}
335 mice^{32,35}. This resource enabled us to identify 1) the extent of germline gene dysregulation in *Kdm5c*-KO
336 cells, 2) the types of germline genes misexpressed at different developmental time points, 3) the impact of
337 germ cell sex upon germline gene misexpression, and 4) which groups of germline genes are directly and
338 indirectly regulated by KDM5C. This list of germline-enriched genes serves as a resource for future studies
339 to systematically assess soma-germline dysregulation.

340 Analysis of epiblast-like cells (EpiLCs) revealed dysregulation of *Kdm5c*-KO tissue identity begins during
341 early embryogenesis. *In vivo*, germline genes are typically fully decommissioned in epiblast stem cells and
342 remain silenced as the epiblast differentiates into somatic tissues³⁸. However, a small subset of epiblast
343 stem cells will reactivate germline genes to become the primordial germ cells (PGCs) that will ultimately form
344 the mature germline^{36,37}. This process can be mimicked *in vitro* by differentiating EpiLCs into primordial
345 germ cell-like cells (PGCLCs)³⁹. Thus, misexpression of germline genes in *Kdm5c*-KO EpiLCs might suggest
346 ESCs are progressing beyond EpiLC differentiation and instead becoming PGCLCs. Yet, *Kdm5c*-KO EpiLCs
347 properly express genes for primed pluripotency, including *Otx2* - an epiblast stem cell marker that is known to
348 repress differentiation into PGCs/PGCLCs⁶⁶. Furthermore, we observed no difference in cellular morphology
349 during *Kdm5c*-KO ESC to EpiLC differentiation. Proper EpiLC differentiation, coupled with *Kdm5c*-KO
350 mice being viable, suggests germline gene expression is occurring ectopically in conjunction with typical
351 developmental programs, rather than a complete shift to towards germline identity.

352 While many genes involved in early germline development and meiosis are common between males
353 and females, some germline genes display sex-biased expression or perform functions unique to eggs and
354 sperm. We found both males and females expressed both sperm and egg biased genes, indicating the
355 mechanisms underlying germline gene repression are independent of germ cell sex. However, we found
356 organismal sex did greatly influence the manifestation of germline gene dysregulation, as *Kdm5c*-KO females
357 had over double the number of germline-enriched DEGs compared to male EpiLCs. Knockout of KDM5C in
358 females is embryonic lethal, likely due to impaired X chromosome inactivation (XCI)⁴⁵. XCI defects could
359 explain why *Kdm5c*-KO females are more prone to germline gene dysregulation, given the X chromosome
360 harbors a large number of spermatogenesis genes^{52,53}. However, ectopic germline transcripts, including
361 those unique to females, were not significantly biased towards the X chromosome. Sex differences in germline
362 gene suppression may be instead connected to females having a higher dose of KDM5C than males, due to
363 its partial escape from XCI^{20–23}. Intriguingly, females heterozygous for *Kdm5c* loss also had over double the
364 number of germline DEGs than males, even though their XCI is largely preserved and their expression of
365 KDM5C should be roughly equivalent to that of wild-type males. Altogether, this suggests female EpiLCs are
366 more prone to transitioning to a germ cell-like state than males and require more KDM5C to maintain somatic
367 cellular identity. Future studies investigating sex differences in germline gene repression are required to
368 illuminate if this phenomenon is unique to sexually dimorphic chromatin regulators like KDM5C or a general
369 feature of female cells.

370 Our analysis of KDM5C ChIP-seq data provided novel insights into direct and indirect mechanisms by
371 which germline genes can be misexpressed in *Kdm5c*-KO cells. While we observed high enrichment of
372 KDM5C peaks at germline genes in EpiLCs, KDM5C was not bound to germline gene promoters in PNCs.
373 This suggests dysregulation of germline genes in the mature *Kdm5c*-KO brain is due to loss of repression
374 during embryogenesis, which is consistent with previous work that found introducing human KDM5C into
375 *Kdm5c*-KO PNCs does not repress two aberrant germline transcripts⁸. It is unclear how KDM5C is recruited
376 to germline genes given that KDM5C itself does not contain domains for sequence-specific binding. In HeLa
377 cells and ESCs^{49,67}, KDM5C associates with members of the polycomb repressive complex 1.6 (PRC1.6),
378 which is recruited to germline gene promoters through E2F6/DP1 and MGA/MAX heterodimers^{16,55}. While
379 MAX and E2F6 motifs were enriched at KDM5C-bound promoters in EpiLCs, only about one third of KDM5C-
380 bound germline genes contained their consensus sequence at their promoter. Thus, other, unknown factors
381 likely facilitate KDM5C's recruitment to germline genes.

382 We also found germline genes can become activated in *Kdm5c*-KO cells independent of direct loss of
383 KDM5C suppression, given that KDM5C was only bound to about a third of germline-enriched DEGs. One
384 notable EpiLC DEG not bound by KDM5C is *Stra8*, a transcription factor activated by retinoic acid signaling in
385 germ cells to promote meiotic initiation^{68,69}. However, retinoic acid can only activate *Stra8* expression when
386 DAZL is present. Unlike *Stra8*, *Dazl* is a direct target of KDM5C in EpiLCs and is transcribed *Kdm5c*-KO
387 EpiLCs. Expression of indirect DEGs like *Stra8* indicates germline genes can be aberrantly expressed
388 in *Kdm5c*-KO cells through downstream activation by other ectopic germline programs. In support of
389 this, germline genes unbound by KDM5C were significantly enriched for RFX transcription factors. RFX
390 transcription factors bind to X-box motifs⁵⁷ to promote the transcription of cilia and flagellar genes^{58,59}. One
391 of the top RFX members enriched included RFX2, a central regulator of spermiogenesis^{60,61} whose mRNA
392 is also expressed in *Kdm5c*-KO EpiLCs. Intriguingly, *Kdm5c*-KO EpiLCs primarily expressed germline genes
393 involved in early germ cell development and meiosis while the mature *Kdm5c*-KO brain expressed late-stage
394 spermatogenesis genes, including known RFX2 targets. Altogether, these data suggest once activated due
395 to loss of direct KDM5C repression, ectopic germline programs can loosely mimic germline development and
396 progress in the background of *Kdm5c*-KO somatic development.

397 It is important to note that while we focused on KDM5C's regulation of germline genes, some germline-
398 enriched genes are also expressed in naïve ESCs and at the 2-cell stage due to their roles in pluripotency and
399 self-renewal. For example, *Dazl* is primarily known for committing PGCs to the germline fate and regulating
400 the translation of germline-specific RNAs, but is also expressed at the 2-cell stage⁷⁰, in naïve ESCs⁴¹, and in
401 the inner cell mass⁴¹. Very recently, two screens of *Dazl*-repressors identified KDM5C as a direct regulator in
402 ESCs^{49,71}. Interestingly, one study found *Kdm5c*-KO ESCs also aberrantly expressed 2-cell-specific genes,
403 indicating KDM5C negatively regulates totipotency⁴⁹. However, out of the four regulators characterized,
404 KDM5C was the only factor whose repression of *Dazl* was independent of the 2-cell specific transcription
405 factor *Dux*⁴⁹. Although expressed at the 2-cell stage and in ESCs, *Dazl* is typically silenced when ESCs

406 differentiate into EpiLCs¹⁷. We found KDM5C also represses *Dazl* in EpiLCs, but *Kdm5c*-KO EpiLCs did
407 not express 2-cell specific genes like *Dux* and *Zscan4c*. Together, this suggests the 2-cell-like state in
408 *Kdm5c*-KO ESCs is an indirect consequence of KDM5C's primary role in germline gene repression.

409 *Dazl* and other germline-enriched genes are initially decommissioned in ESCs by repressive histone
410 modifications and then *de novo* DNA CpG methylation (CpGme) in EpiLCs^{17–19,62,72,73}. Unlike the previously
411 characterized germline gene suppressors, KDM5C removes an active histone mark, histone 3 lysine 4 di-
412 and trimethylation (H3K4me2/3)⁹. In this study, we observed a global increase in H3K4me2/3 in *Kdm5c*-KO
413 EpiLCs and amygdala around the transcription start site (TSS) of germline-enriched genes. However,
414 KDM5C's catalytic activity may not be required for germline gene silencing, as it was recently found to be
415 dispensible for repressing *Dazl* in ESCs⁴⁹. Although not necessary in ESCs, KDM5C's catalytic activity be
416 required for CpGme in EpiLCs, as H3K4me3 impedes *de novo* CpGme placement^{63,64}. This is supported
417 by previous work in the *Kdm5c*-KO adult hippocampus, which found CpGme is significantly eroded at at
418 least two germline promoters⁸. To elucidate the mechanism behind KDM5C-mediated silencing of germline
419 genes, we characterized KDM5C in ESCs and EpiLCs and found its expression is dynamically regulated
420 during differentiation. Furthermore, KDM5C acts during the transition from naïve to primed pluripotency to
421 promote the initial placement of CpGme at CpG islands for the long-term silencing of germline genes.

422 • CPG islands greatly determine KDM5C recruitment - KDM5C is previously known to be enriched at
423 CGIs. CGIs not typically methylated, germline genes exception. KDM5C promotes this process.

424 The above work provides the mechanistic foundation for KDM5C's role in germline gene repression
425 and offers novel insight into how dysregulation in *Kdm5c*-KO tissue identity changes over the course of
426 development. However, the contribution of ectopic, tissue-specific genes towards *Kdm5c*-KO neurological
427 impairments is still unknown. KDM5C may be crucial for neurodevelopment by fine-tuning the expression of
428 tissue-enriched, dosage-sensitive genes, such as *Apoc1*. KDM5C could also ensure tissue-specific timing of
429 transcription factor family members, like RFX genes that are broadly required for cilia and flagella formation,
430 including neuronal cilia^{58,59,74,75}. While their impact upon neurodevelopment is currently unclear, ectopic
431 germline transcripts are also found in models of other related neurodevelopmental disorders⁷⁶, including
432 Immunodeficiency, Centromere instability, Facial anomalies syndrome 1 (ICF1 OMIM: #242860)^{77,78}, Kleefstra
433 syndrome 1 (OMIM: #610253)⁷, and MeCP2 duplication syndrome (MDS, OMIM: #300260)⁷⁹. Like KDM5C,
434 the chromatin regulators underlying these conditions, DNA methyltransferase 3b (DNMT3B), H3K9me1/2
435 methyltransferases G9A/GLP, methyl-CpG-binding protein 2 (MECP2), primarily silence gene expression.
436 Thus, KDM5C is among a growing cohort of chromatin-linked neurodevelopmental disorders that have
437 a similar underlying phenotype of germline versus soma dysregulation. Further research is required to
438 determine the ultimate impact of these germline genes and the extent to which this phenomenon occurs in
439 humans.

440 **Materials and Methods**

441 **Classifying tissue-enriched and germline-enriched genes**

442 Tissue-enriched differentially expresssd genes (DEGs) were determined by their classification in a previ-
443 ously published dataset from 17 male and female mouse tissues²⁴. This study defined tissue expression as
444 greater than 1 Fragments Per Kilobase of transcript per Million mapped read (FPKM) and tissue enrichment
445 as at least 4-fold higher expression than any other tissue.

446 We curated a list of germline-enriched genes using an RNA-seq dataset from wild-type and germline-
447 depleted (Kit^{W/W^v}) male and female mouse embryos from embryonic day 12, 14, and 16³⁵, as well as adult
448 male testes³². Germline-enriched genes met the following criteria: 1) their expression is greater than 1
449 FPKM in wild-type germline 2) their expression in any wild-type somatic tissues²⁴ does not exceed 20%
450 of maximum expression in wild-type germline, and 3) their expression in the germ cell-depleted (Kit^{W/W^v})
451 germline, for any sex or time point, does not exceed 20% of maximum expression in wild-type germline.

452 **Cell culture**

453 We utilized our previously established cultures of male wild-type and *Kdm5c* knockout (-KO) embryonic
454 stem cells⁴⁵. Sex was confirmed by genotyping *Uba1/Uba1y* on the X and Y chromosomes with the following
455 primers: 5'-TGGATGGTGTGCCAATG-3', 5'-CACCTGCACGTTGCCCTT-3'. Deletion of *Kdm5c* was
456 confirmed through the primers 5'-ATGCCCATATTAAGAGTCCTG-3', 5'-TCTGCCTTGATGGACTGTT-3',
457 and 5'-GGTTCTAACACTCACATAGTG-3'.

458 Embryonic stem cells (ESCs) and epiblast-like cells were cultured using previously established
459 methods⁴⁰. Briefly, ESCs were initially cultured in primed ESC (pESC) media consisting of KnockOut
460 DMEM (Gibco#10829–018), fetal bovine serum (Gibco#A5209501), KnockOut serum replacement
461 (Invitrogen#10828–028), Glutamax (Gibco#35050-061), Anti-Anti (Gibco#15240-062), MEM Non-essential
462 amino acids (Gibco#11140-050), and beta-mercaptoethanol (Sigma#M7522). They were then transitioned
463 into ground-state, “naïve” ESCs (nESCs) by culturing for four passages in N2B27 media containing
464 DMEM/F12 (Gibco#11330–032), Neurobasal media (Gibco#21103–049), Gluamax, Anti-Anti, N2 sup-
465 plement (Invitrogen#17502048), and B27 supplement without vitamin A (Invitrogen#12587-010), and
466 beta-mercaptoethanol. Both pESC and nESC media were supplemented with 3 µM GSK3 inhibitor
467 CHIR99021 (Sigma #SML1046-5MG), 1 µM MEK inhibitor PD0325901 (Sigma #PZ0162-5MG), and 1,000
468 units/mL leukemia inhibitory factor (LIF, Millipore#ESG1107).

469 nESCs were differentiated into epiblast-like cells (EpiLCs, 48 hours) and extendend EpiLCs (exEpiLCs,
470 96 hours) by culturing in N2B27 media containing DMEM/F12, Neurobasal media, Gluamax, Anti-Anti,
471 N2 supplement, B27 supplement (Invitrogen#17504044), beta-mercaptoethanol, fibroblast growth factor 2
472 (FGF2, R&D Biotechne 233-FB), and activin A (R&D Biotechne 338AC050CF), as previously described⁴⁰.

473 **Immunocytochemistry (ICC)**

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

483 **RNA sequencing (RNA-seq)**

484 After ensuring read quality via FastQC (v0.11.8), reads were then mapped to the mm10 *Mus musculus*
485 genome (Gencode) using STAR (v2.5.3a), during which we removed duplicates and kept only uniquely
486 mapped reads. Count files were generated by FeatureCounts (Subread v1.5.0), and BAM files were
487 converted to bigwigs using deeptools (v3.1.3) and visualized by the UCSC genome browser. RStudio (v3.6.0)
488 was then used to analyze counts files by DESeq2 (v1.26.0)²⁶ to identify differentially expressed genes
489 (DEGs) with a q-value (p-adjusted via FDR/Benjamini–Hochberg correction) less than 0.1 and a log2 fold
490 change greater than 0.5. For all DESeq2 analyses, log2 fold changes were calculated with IfcShrink using
491 the ashr package⁸⁰. MA-plots were generated by ggpibr (v0.6.0), and Eulerr diagrams were generated by
492 eulerr (v6.1.1). Boxplots and scatterplots were generated by ggpibr (v0.6.0) and ggplot2 (v3.3.2). The Upset
493 plot was generated via the package UpSetR (v1.4.0)⁸¹. Gene ontology (GO) analyses were performed by
494 the R package enrichPlot (v1.16.2) using the biological processes setting and compareCluster.

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

496 ChIP-seq reads were aligned to mm10 using Bowtie1 (v1.1.2) allowing up to two mismatches. Only
497 uniquely mapped reads were used for analysis. Peaks were called using MACS2 software (v2.2.9.1) using
498 input BAM files for normalization, with filters for a q-value < 0.1 and a fold enrichment > 1. We removed
499 “black-listed” genomic regions that often give aberrant signals. Common peak sets were obtained in R via
500 DiffBind (v3.6.5). In the case of KDM5C ChIP-seq, *Kdm5c*-KO peaks were then subtracted from wild-type
501 samples using bedtools (v2.25.0). Peak proximity to genome annotations was determined by ChIPSeeker
502 (v1.32.1). Gene ontology (GO) analyses were performed by the R package enrichPlot (v1.16.2) using the
503 biological processes setting and compareCluster. Enriched motifs were identified using HOMER⁵⁶. Average
504 binding across the genome was visualized using deeptools (v3.1.3). Bigwigs were visualized using the
505 UCSC genome browser.

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

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

511 **Data availability**

512 **Published datasets**

513 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
514 adult amygdala and hippocampus²⁵ (available at GEO: GSE127722) and male wild-type and *Kdm5c*-KO
515 EpiLCs⁴⁵ (available at GEO: GSE96797).

516 Previously published ChIP-seq experiments included KDM5C in wild-type and *Kdm5c*-KO EpiLCs⁴⁵ (avail-
517 able at GEO: GSE96797) and mouse primary neuron cultures (PNCs) from the cortex and hippocampus¹³
518 (available at GEO: GSE61036). ChIP-seq of histone 3 lysine 4 dimethylation in male wild-type and *Kdm5c*-KO
519 EpiLCs⁴⁵ is also available at GEO: GSE96797. ChIP-seq of histone 3 lysine 4 trimethylation in wild-type and
520 *Kdm5c*-KO male amygdala²⁵ are available at GEO: GSE127817.

522 **Data analysis**

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

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698 **Figures and Tables**

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



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



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

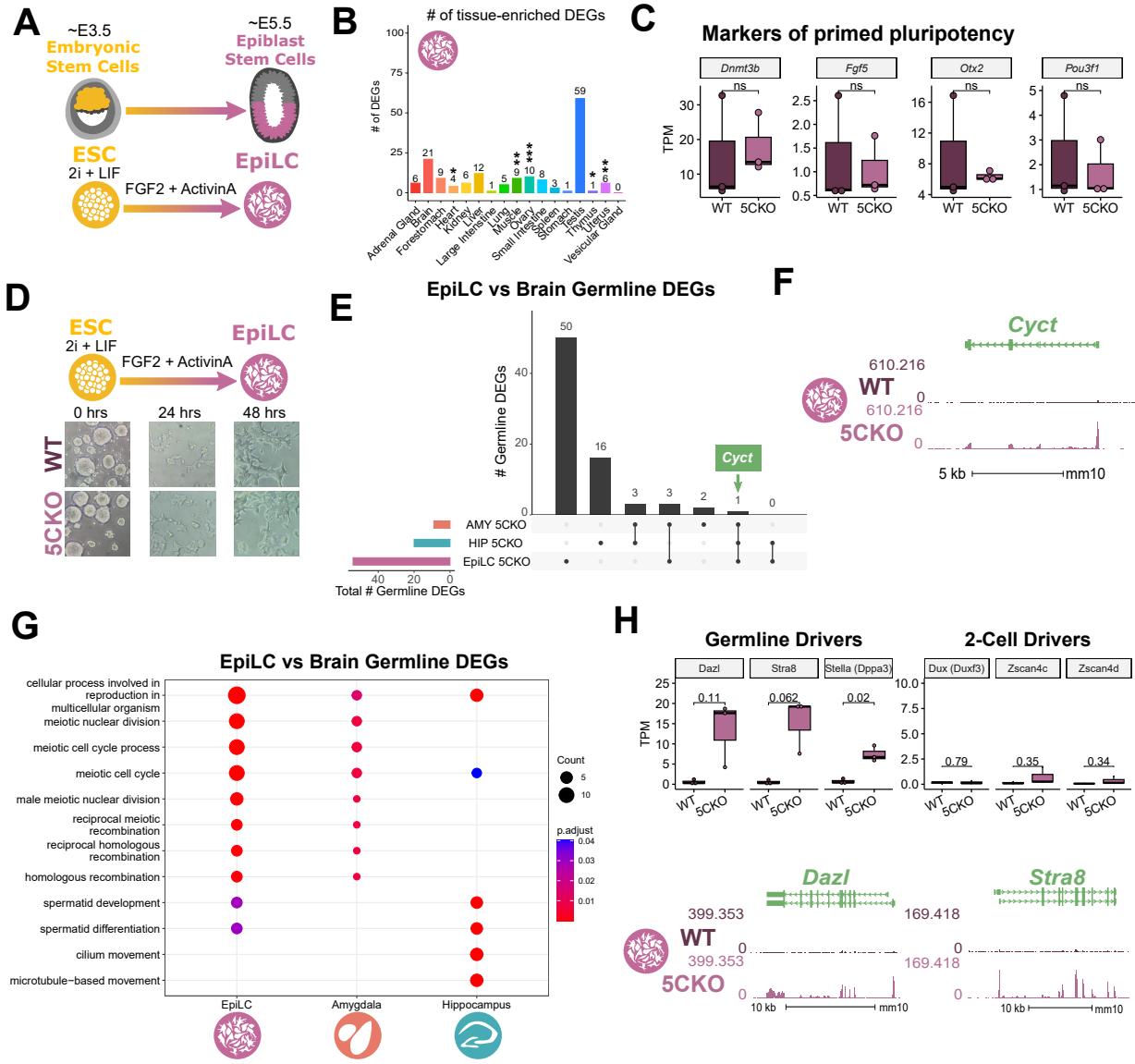


Figure 3: *Kdm5c*-KO epiblast-like cells express key drivers of germline identity

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

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

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

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

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

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

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

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

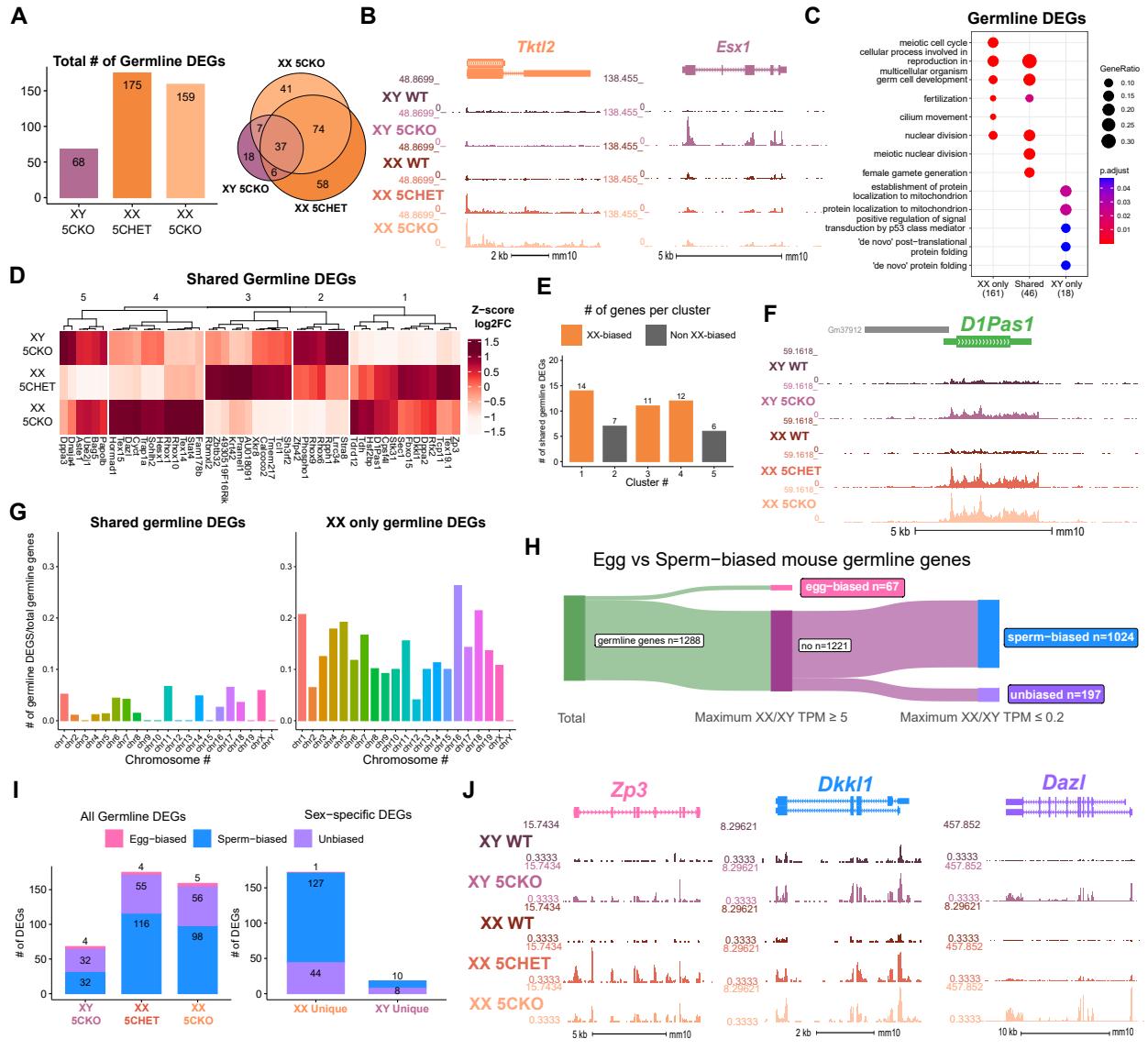


Figure 4: Chromosomal sex influences *Kdm5c*-KO germline gene misexpression. **A-B.** Expression of tissue-enriched genes (Li et al 2017) in the male *Kdm5c*-KO amygdala (A) and hippocampus (B). Left - MA plot of mRNA-seq. Right - Number of tissue-enriched differentially expressed genes (DEGs). * $p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

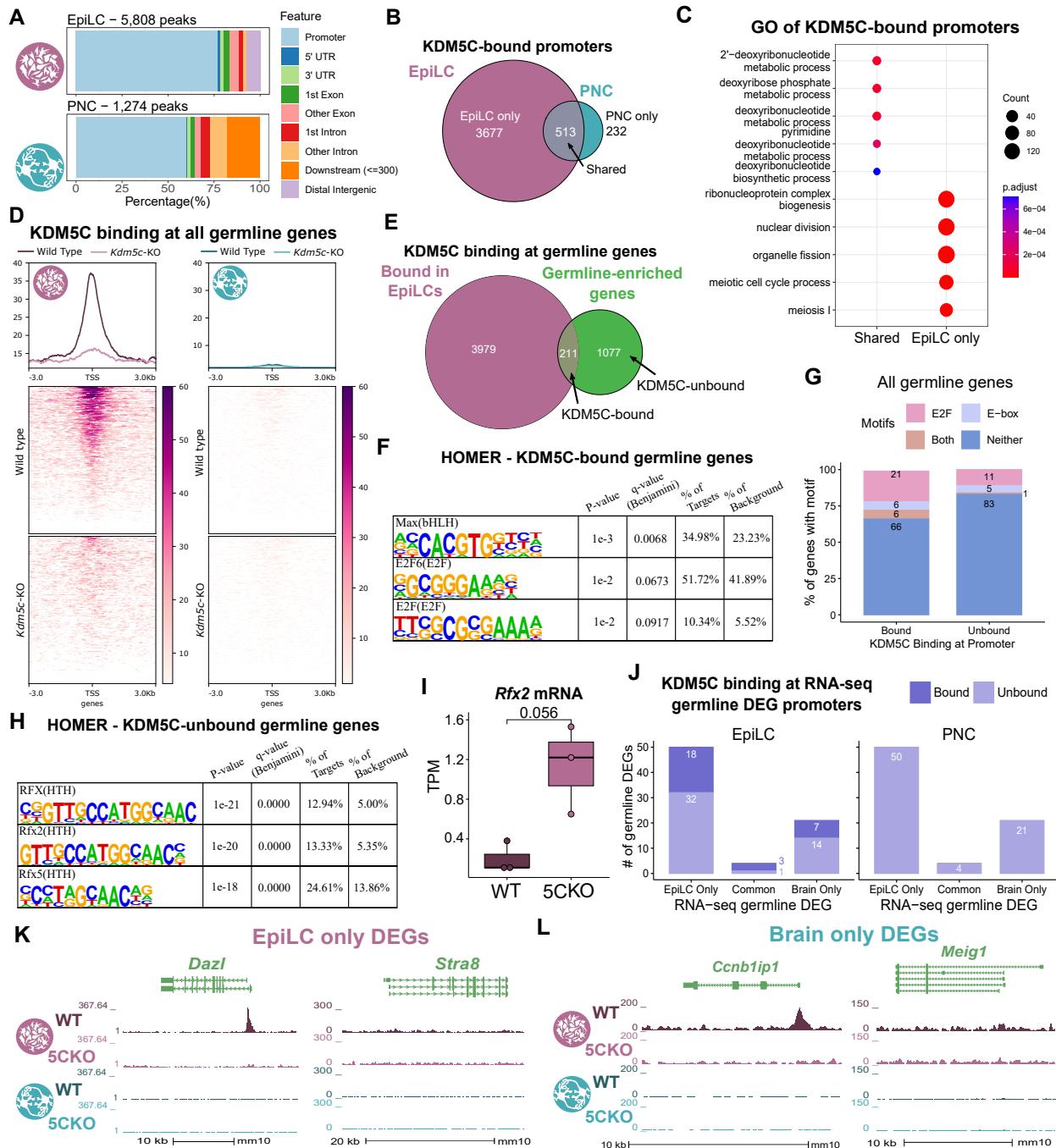
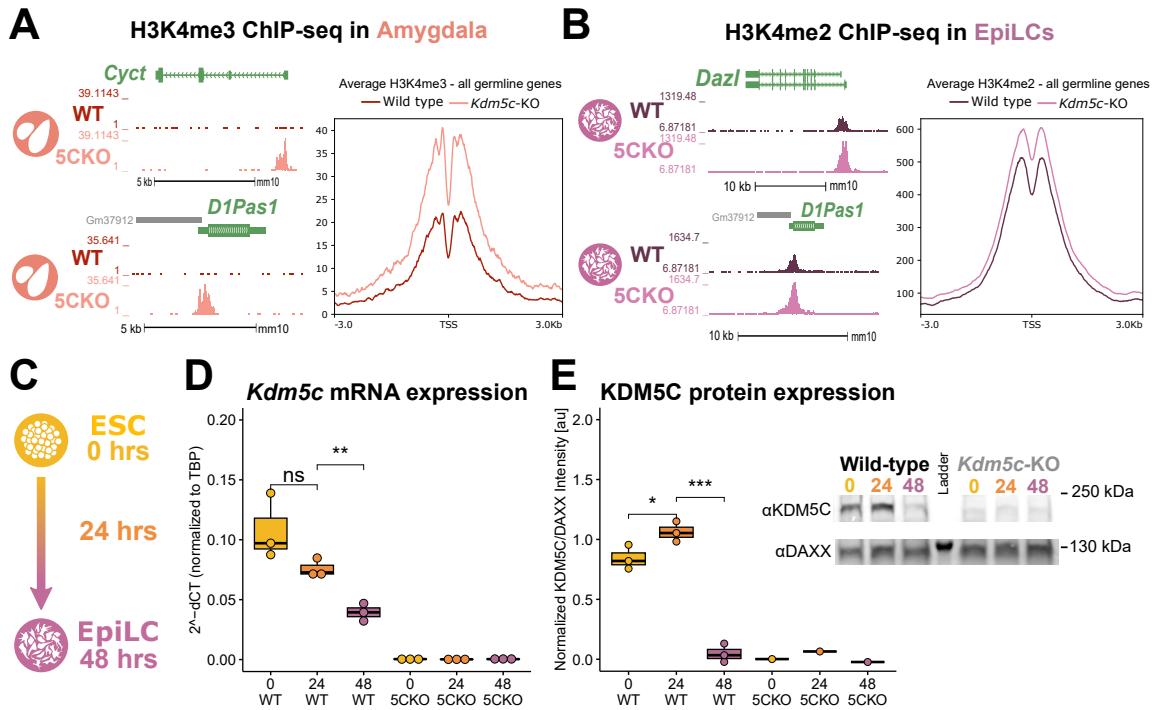


Figure 5: KDM5C binds to a subset of germline gene promoters during early embryogenesis **A.** ChIPseeker localization of KDM5C peaks at different genomic regions in EpiLCs (top) and hippocampal and cortex primary neuron cultures (PNCs, bottom). **B.** Overlap of genes with KDM5C bound to their promoters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

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



F

G

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

703 Notes

704 Things to do

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

709 Dazl

710 We were particularly interested in the aberrant transcription of *Dazl*, since it is essential for germ cell
711 development and promotes the translation of germline mRNAs⁸². A significant portion of germline transcripts
712 misexpressed in *Kdm5c*-KO EpiLCs are known binding targets of DAZL, including *Stra8*⁸³ ($p = 1.698e-07$,
713 Fisher's Exact Test). This suggests expression of DAZL protein could promote the translation of other
714 aberrant germline transcripts, influencing their ability to impact *Kdm5c*-KO cellular function. We thus tested
715 DAZL protein expression in *Kdm5c*-KO EpiLCs through immunocytochemistry (Figure 3I). We observed
716 about 25% of *Kdm5c*-KO EpiLCs expressed DAZL protein and it was localized to the cytoplasm ($p = 0.0015$,
717 Welch's t-test), consistent with the pattern of DAZL expression in spermatogonia⁸³. Altogether these results
718 suggest tissue-specific genes are misexpressed during *Kdm5c*-KO embryogenesis, including key drivers of
719 germline identity that can be translated into protein.

- 720 • We additionally found *Kdm5c*-KO EpiLCs ectopically express DAZL protein that is localized to the
721 cytoplasm, similar to its morphology in spermatogonia⁸³. **note: maybe just put in results.** Could
722 move around depending upon if I get pheno working.

723 Discussion notes

- 724 • For other paper:
 - 725 – for methods: Heatmaps of gene expression were generated using the base R functions scale and
726 hclust and visualized using the R package ComplexHeatmap (v2.12.1).
 - 727 – * 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>
- 729 • Talk about different motifs if we do see differences there and if it explains direct vs indirect dysregulation
 - 730 – Could also mention PRC1.6/KDM5C binds pcgf6 in ESCs
- 731 • Maybe talk about other regulators/if anything is known about them long term? Or save for 2nd paper.

- 732 – another germline repressor: <https://pubmed.ncbi.nlm.nih.gov/35345626/>
- 733 • end with something like: this indicates kdm5c not only modulates pluripotency and self-renewal in
734 ESCs, but also has a role in long-term silencing of germline genes
- 735 – then transition into the long term silencing mechanism paragraph
- 736 • KDM5C is dynamically regulated during the window of embryonic germline gene silencing, our EpiLC
737 ChIP-seq is likely catching the tail end of KDM5C's main involvement.
- 738 • Our results indicate KDM5C is a pivotal repressor of germline programs in somatic cells.
- 739 • Studies on other chromatin regulators have focused on key marker genes to identify germline gene
740 misexpression, such as *Dazl*.
- 741 • Using a germline-depleted mouse model, we curated a list of mouse germline-enriched genes to
742 globally assess germline gene dysregulation.
- 743 • Many of the genes dysregulated in the mature *Kdm5c*-KO brain were important for late stages of
744 spermatogenesis, such as those important for sperm flagellar structure. Contrastingly, *Kdm5c*-KO
745 EpiLCs aberrantly expressed key drivers of germ cell identity, including *Dazl* and *Stra8*.
- 746 • While a significant portion of KDM5C-bound promoters are germline genes, many germline genes
747 expressed during *Kdm5c*-KO embryogenesis are not directly bound by kdm5c.
- 748 • One notable example is *Stra8*, a transcription factor important for germ cell specific transcription and
749 meiotic initiation
- 750 • The including the demarcation between soma and germline fates.
- 751 the *Kdm5c*-KO brain also Previous work identified testis-enriched genes in *Kdm5c* genes
- 752 –
- 753 – However unlike the gonadal-biased DEGs,
- 754 • The demarcation of the germline versus soma is a key feature of multicellularity and sexually dimorphic
755 reproduction
- 756 • Anything known about tissue-biased gene expression in other H3K4me regulators?
- 757 • Our data suggests the germline developmental program is occurring ectopically as *Kdm5c*-KOs pro-
758 gresses through somatic tissue development
- 759 • tissue-biased gene expression:

- 760 • Altogether, these data indicate that while some germline genes are misexpressed due to direct loss of
761 KDM5C binding during emryogenesis, secondary downstream mechanisms can also promote their
762 aberrant transcription.
- 763 • Papers to read/reference:
764 – Reconstitution of the Mouse Germ Cell Specification Pathway in Culture by Pluripotent Stem Cells:
765 [https://www.cell.com/fulltext/S0092-8674\(11\)00771-9](https://www.cell.com/fulltext/S0092-8674(11)00771-9)
766 – 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/>

768 **Figure outline:**

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

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

781 **Figure 3: *Kdm5c*-KO epiblast-like cells express key drivers regulators of germline identity** * A) ESC
782 to EpiLC differentiation Left - Morphology is unchanged, * B) 5CKO EpiLCs express EpiLC differentiation
783 genes similar to WT lvs * C) Male EpiLCs express germline genes (example Cyct again) * Overlap between
784 brain and EpiLC germline genes - show they're mostly unique * GO of Brain and EpiLC germline genes
785 (meiotic enriched) * Bigwigs or TPM of master regulators * Show that while some are also 2-cell genes
786 (Dazl), 2-cell specific genes aren't dysregulated (Zscan4). Important point because published KDM5C dazl
787 paper is saying KDM5C is a 2-cell regulator, but as far as I can tell only genes shared between germline and
788 2-cell are dysregulated.

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

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

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

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

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

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

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

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

833 **Germline gene repression background:**

834 Interestingly, some of the ectopic testis transcripts identified in the *Kdm5c*-KO brain are typically ex-
835 pressed in germ cells⁸. Unlike somatic cells, germ cells (e.g. sperm and eggs) undergo meiosis and pass on
836 their genetic material to the next generation. The germline and the soma are typically distinguished during
837 early embryogenesis, when germline genes are silenced in epiblast stem cells soon after implantation and
838 only reactivated in a subset to form the germline. Chromatin regulators play a key role in decommissioning
839 germline genes as the embryo transitions from naïve to primed pluripotency by placing repressive histone
840 H2A lysine 119 monoubiquitination (H2AK119ub1)¹⁶, histone 3 lysine 9 trimethylation (H3K9me3)^{16,17}, and
841 DNA CpG methylation^{17–19} at germline gene promoters. KDM5C may also be involved in this early decom-
842 missioning of germline genes, as re-expression of KDM5C in neurons fails to suppress their dysregulation⁸.
843 In support of this, KDM5C was very recently shown to repress *Deleted in azoospermia like (Dazl)*, a key
844 regulator of germline development, in mouse embryonic stem cells (ESCs)^{49,71}. In support of this, two
845 independent screens in mouse embryonic stem cells (ESCs) recently identified KDM5C as a repressor of
846 *Deleted in azoospermia like (Dazl)*, a key regulator of germline development. However, KDM5C's role in
847 embryonic germline gene repression is currently unclear, given that Dazl is also expressed in ESCs and in
848 the 2-cell stage and germline gene misexpression has yet to be globally characterized during *Kdm5c*-KO
849 embryogenesis.