

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 In the above study, we demonstrate KDM5C's pivotal role in the development of tissue identity. We
301 first characterized the misexpression of tissue-enriched genes within the *Kdm5c*-KO brain and identified
302 substantial dysregulation of testis, liver, muscle, and ovary-enriched genes. Testis genes significantly
303 enriched within the *Kdm5c*-KO amygdala and hippocampus are specific to germ cells and not expressed
304 within testis somatic cells. *Kdm5c*-KO epiblast-like cells (EpiLCs) aberrantly express key drivers of germline
305 identity and meiosis, including *Dazl* and *Stra8*, while the *Kdm5c*-KO brain primarily expresses genes
306 important for late spermatogenesis. We demonstrated that while *Kdm5c* mutant sex did not influence
307 whether sperm or egg-specific genes were misexpressed, female EpiLCs are more sensitive to germline
308 gene de-repression. Germline-enriched mRNAs can become aberrantly expressed in *Kdm5c*-KO cells
309 indirect of KDM5C, as although KDM5C is enriched at germline gene promoters in EpiLCs, it is only bound
310 to a subset of germline-enriched DEGs. Finally, we found KDM5C is dynamically regulated during ESC to
311 EpiLC differentiation and promotes long-term germline gene silencing through DNA methylation at CpG
312 islands. Therefore, we propose KDM5C plays a fundamental role in the development of tissue identity
313 during early embryogenesis, including the establishment of the soma-germline boundary. By systematically
314 characterizing KDM5C's role in germline gene repression, including its interaction with known silencing
315 mechanisms, we unveiled unique repressive mechanisms governing distinct classes of germline gene in
316 somatic lineages. Furthermore, these data provide molecular footholds that can then be exploited to test the
317 ultimate contribution of ectopic germline gene expression upon neurodevelopment.

318 It is important to note that while we highlighted KDM5C's regulation of germline genes, some germline-
319 enriched genes are also expressed at the 2-cell stage and in naïve ESCs for their role in pluripotency and
320 self-renewal. For example, *Dazl* is primarily known for committing PGCs to the germline fate and regulating
321 the translation of germline-specific mRNAs, but is also expressed at the 2-cell stage⁶⁵, in naïve ESCs⁴¹,
322 and in the inner cell mass⁴¹. KDM5C may therefore negatively regulate totipotency, as KDM5C suppresses
323 *Dazl* in ESCs^{49,66} and *Kdm5c*-KO ESCs aberrantly express 2-cell-specific genes like *Zscan4c*⁴⁹. However,
324 misexpression of *Dazl* in *Kdm5c*-KO ESCs was independent of the 2-cell specific transcription factor *Dux*⁴⁹
325 and, unlike *Dazl*, KDM5C does not bind to the *Dux* promoter.

326 Although expressed in naïve ESCs, *Dazl* and other "self-renewal" germline genes are silenced during
327 differentiation into epiblast stem cells/EpiLCs^{17,44}. We found that while *Kdm5c*-KO EpiLCs also expressed
328 *Dazl*, they did not express 2-cell specific genes. Misexpression of germline genes in *Kdm5c*-KO EpiLCs could
329 indicate they are differentiating into primordial germ cell-like cells (PGCLCs)^{36,37,39}. Yet, *Kdm5c*-KO EpiLCs
330 had normal cellular morphology and properly expressed markers for primed pluripotency, including *Otx2*
331 which is known to repress EpiLC differentiation into PGCs/PGCLCs⁶⁷. Altogether, these data suggest *Kdm5c*-
332 KO germline gene misexpression occurs ectopically in conjunction with typical developmental programs and
333 the 2-cell-like state observed in *Kdm5c*-KO ESCs is an indirect consequence of KDM5C's primary role in

334 germline gene repression.

335 Although eggs and sperm employ the same transcriptional programs for shared functions, e.g. PGC
336 formation, meiosis, and genome defense, some germline genes are sex specific. We found *Kdm5c* mutant
337 males and females expressed both sperm and egg-biased genes, indicating the mechanism of derepression
338 is independent of a given germline gene's sex. However, organismal sex did greatly influence the degree of
339 germline gene dysregulation, as female *Kdm5c*-KO EpiLCs had over double the number of germline-enriched
340 DEGs compared to males. X chromosome inactivation (XCI) defects could explain why *Kdm5c* knockout
341 females are more prone to germline gene dysregulation, given the X chromosome harbors a large number
342 of spermatogenesis genes^{52,53} and loss of *Kdm5c* impairs XCI⁴⁵. However, female germline DEGs were
343 not significantly biased towards the X chromosome. Sex differences in germline gene suppression may be
344 instead connected to females having a higher dose of KDM5C than males, due to its partial escape from
345 XCI^{20–23}. Intriguingly, females with heterozygous loss of *Kdm5c* also had over double the number of germline
346 DEGs than hemizygous knockout males, even though their level of KDM5C should be roughly equivalent to
347 that of wild-type males. Altogether, these results suggest female EpiLCs are more prone to transitioning
348 to a germ cell-like state than males and require more KDM5C to maintain somatic cellular identity. Future
349 studies are required to illuminate if this phenomenon is unique to sexually dimorphic chromatin regulators or
350 a general feature of female cells.

351 It is yet unknown how KDM5C is recruited to germline gene promoters, given that KDM5C itself does
352 not contain domains for sequence-specific binding⁹. In HeLa cells and ESCs^{49,68}, KDM5C associates with
353 members of the polycomb repressive complex 1.6 (PRC1.6), which is recruited to germline gene promoters
354 through E2F6/DP1 and MGA/MAX heterodimers^{16,55}. While MAX and E2F6 motifs were enriched at KDM5C-
355 bound germline genes in EpiLCs, only about one-third of promoters contained their consensus sequence.
356 Additionally, some germline genes can become active in *Kdm5c*-KO cells independent of KDM5C binding to
357 their promoters. Germline genes unbound by KDM5C were primarily late-stage spermatogenesis genes and
358 significantly enriched for RFX transcription factors, including RFX2, a central regulator of spermiogenesis^{60,61}.
359 Another notable KDM5C-unbound DEG is *Stra8*, a meiotic transcription factor that is turned on in germ cells
360 with retionic acid signaling and DAZL expression^{69,70}. Misexpression of *Dazl* and *Rfx2* and their downstream
361 targets in *Kdm5c*-KO cells suggests that once activated, ectopic germline genes can then turn on other
362 aberrant germline programs to loosely mimic germ cell development.

363 Unlike the previously characterized germline gene suppressors that place repressive histone
364 modifications^{16,17,19,62,71–73}, KDM5C removes the active histone mark histone 3 lysine 4 di- and trimethylation
365 (H3K4me2/3)⁹. Yet, KDM5C's catalytic activity may not be required for germline gene silencing, as it
366 was recently found to be dispensible for repressing *Dazl* in ESCs⁴⁹. Emerging work indicates many
367 histone-modifying enzymes have non-catalytic functions that can influence gene expression, sometimes even
368 more potently than their catalytic roles^{74,75}. Although dispensible in ESCs, KDM5C's catalytic activity could
369 be necessary to silence germline genes in EpiLCs, as H3K4me3 impedes *de novo* CpGme placement^{63,64}.

370 In support of this, CpGme is significantly eroded at at least two germline promoters in the adult *Kdm5c*-KO
371 hippocampus⁸. We found *Kdm5c*-KO cells accumulate H3K4me2/3 around the TSS of germline genes
372 and have impaired CpGme placement at germline CpG islands. KDM5C's shifting role in germline gene
373 repression during the transition from naïve to primed pluripotency indicates chromatin modifiers can switch
374 between catalytic and non-catalytic gene regulatory mechanisms at the same loci over development.

- 375 • DNAme and CpG islands
- 376 – Loss of many chromatin regulators affects CpGme (Multiple, non-redundant silencing mech-
377 anisms) - seems to be the crux point of germline gene silencing
- 378 – Germline genes are methylated at promoter CGIs, which are typically not methylated for other
379 types of genes
- 380 – Because CGIs are typically resistant to CpGme (accurate?), germline CGIs may require a highly
381 repressive histone landscape to recruit sufficient DNMTs to these loci
- 382 – We found only 30% of germline-enriched genes have CGIs, but the majority of CGI-free germline
383 genes still gain CpGme around the TSS. Unclear what the repressive mechanism is for these
384 genes.
- 385 – CGIs highly determine KDM5C recruitment to germline gene promoters. - KDM5C is known to be
386 enriched at CGIs (in neurons? are these methylated? or is its germline CGI function different from
387 its somatic CGI function?).
- 388 – KDM5C loss impacts germline gene CGI methylation, but not really the non-CGI TSS methylation
- 389 * Other studies on germline gene repressors have shown they are important for CGIme, unclear
390 if they participate in non-CGI TSS CpGme
- 391 – Altogether demonstrates different classes of germline genes use distinct silencing mechanisms

392 The above work provides the mechanistic foundation for KDM5C's repression of germline-specific
393 transcription and offers novel insight into how dysregulation of *Kdm5c*-KO tissue identity changes over the
394 course of development. However, the contribution of these ectopic, tissue-specific genes towards *Kdm5c*-KO
395 neurological impairments is still unknown. In addition to germline genes, we also identified significant
396 enrichment of muscle, liver, and even ovary-biased transcripts within the male *Kdm5c*-KO brain. Intriguingly,
397 select liver and muscle-biased DEGs do have known roles within the brain, such as the liver-enriched
398 lipid metabolism gene *Apolipoprotein C-I* (*Apoc1*)³⁰. *APOC1* dysregulation is implicated in Alzheimer's
399 disease in humans³¹ and overexpression of *Apoc1* in the mouse brain can impair learning and memory⁷⁶.
400 KDM5C may therefore be crucial for neurodevelopment by fine-tuning the expression of tissue-enriched,
401 dosage-sensitive genes like *Apoc1*. Given germline genes have no known functions within the brain, their
402 impact upon neurodevelopment is currently unknown. Ectopic germline transcripts have been observed in a
403 variety of cancers^{77,78} and can drive brain tumor formation in *Drosophila*⁷⁹, indicating their dysregulation
404 may promote genome instability and cellular de-differentiation. Intriguingly, some models for other chromatin-

405 linked neurodevelopmental disorders also display impaired soma-germline demarcation^{7,80–83}. Like KDM5C,
406 the chromatin regulators underlying these conditions - DNA methyltransferase 3b (DNMT3B), H3K9me1/2
407 methyltransferases G9A/GLP, methyl-CpG -binding protein 2 (MECP2) - primarily silence gene expression.
408 Thus, KDM5C is among a growing cohort of chromatin-linked neurodevelopmental disorders that have a
409 similar underlying phenotype of germline versus soma dysregulation However, further research is required to
410 determine the impact of these germline genes and the extent to which this phenomenon occurs in humans.

411 Materials and Methods

412 Classifying tissue-enriched and germline-enriched genes

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

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

423 Cell culture

424 We utilized our previously established cultures of male wild-type and *Kdm5c* knockout (-KO) embryonic
425 stem cells⁴⁵. Sex was confirmed by genotyping *Uba1/Uba1y* on the X and Y chromosomes with the following
426 primers: 5'-TGGATGGTGTGCCAATG-3', 5'-CACCTGCACGTTGCCCTT-3'. Deletion of *Kdm5c* was
427 confirmed through the primers 5'-ATGCCCATATTAGAGTCCTG-3', 5'-TCTGCCCTGATGGACTGTT-3',
428 and 5'-GGTTCTCAACACTCACATAGTG-3'.

429 Embryonic stem cells (ESCs) and epiblast-like cells were cultured using previously established
430 methods⁴⁰. Briefly, ESCs were initially cultured in primed ESC (pESC) media consisting of KnockOut
431 DMEM (Gibco#10829–018), fetal bovine serum (Gibco#A5209501), KnockOut serum replacement
432 (Invitrogen#10828–028), Glutamax (Gibco#35050-061), Anti-Anti (Gibco#15240-062), MEM Non-essential
433 amino acids (Gibco#11140-050), and beta-mercaptoethanol (Sigma#M7522). They were then transitioned
434 into ground-state, “naïve” ESCs (nESCs) by culturing for four passages in N2B27 media containing
435 DMEM/F12 (Gibco#11330–032), Neurobasal media (Gibco#21103–049), Gluamax, Anti-Anti, N2 sup-
436 plement (Invitrogen#17502048), and B27 supplement without vitamin A (Invitrogen#12587-010), and

437 beta-mercaptoethanol. Both pESC and nESC media were supplemented with 3 μ M GSK3 inhibitor
438 CHIR99021 (Sigma #SML1046-5MG), 1 μ M MEK inhibitor PD0325901 (Sigma #PZ0162-5MG), and 1,000
439 units/mL leukemia inhibitory factor (LIF, Millipore#ESG1107).

440 nESCs were differentiated into epiblast-like cells (EpiLCs, 48 hours) and extendend EpiLCs (exEpiLCs,
441 96 hours) by culturing in N2B27 media containing DMEM/F12, Neurobasal media, Gluamax, Anti-Anti,
442 N2 supplement, B27 supplement (Invitrogen#17504044), beta-mercaptoethanol, fibroblast growth factor 2
443 (FGF2, R&D Biotechne 233-FB), and activin A (R&D Biotechne 338AC050CF), as previously described⁴⁰.

444 **Immunocytochemistry (ICC)**

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

454 **RNA sequencing (RNA-seq)**

455 After ensuring read quality via FastQC (v0.11.8), reads were then mapped to the mm10 *Mus musculus*
456 genome (Gencode) using STAR (v2.5.3a), during which we removed duplicates and kept only uniquely
457 mapped reads. Count files were generated by FeatureCounts (Subread v1.5.0), and BAM files were
458 converted to bigwigs using deeptools (v3.1.3) and visualized by the UCSC genome browser. RStudio (v3.6.0)
459 was then used to analyze counts files by DESeq2 (v1.26.0)²⁶ to identify differentially expressed genes
460 (DEGs) with a q-value (p-adjusted via FDR/Benjamini–Hochberg correction) less than 0.1 and a log2 fold
461 change greater than 0.5. For all DESeq2 analyses, log2 fold changes were calculated with IfcShrink using
462 the ashr package⁸⁴. MA-plots were generated by ggpubr (v0.6.0), and Eulerr diagrams were generated by
463 eulerr (v6.1.1). Boxplots and scatterplots were generated by ggpubr (v0.6.0) and ggplot2 (v3.3.2). The Upset
464 plot was generated via the package UpSetR (v1.4.0)⁸⁵. Gene ontology (GO) analyses were performed by
465 the R package enrichPlot (v1.16.2) using the biological processes setting and compareCluster.

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

467 ChIP-seq reads were aligned to mm10 using Bowtie1 (v1.1.2) allowing up to two mismatches. Only
468 uniquely mapped reads were used for analysis. Peaks were called using MACS2 software (v2.2.9.1) using

469 input BAM files for normalization, with filters for a q-value < 0.1 and a fold enrichment > 1. We removed
470 “black-listed” genomic regions that often give aberrant signals. Common peak sets were obtained in R via
471 DiffBind (v3.6.5). In the case of KDM5C ChIP-seq, *Kdm5c*-KO peaks were then subtracted from wild-type
472 samples using bedtools (v2.25.0). Peak proximity to genome annotations was determined by ChIPSeeker
473 (v1.32.1). Gene ontology (GO) analyses were performed by the R package enrichPlot (v1.16.2) using the
474 biological processes setting and compareCluster. Enriched motifs were identified using HOMER⁵⁶. Average
475 binding across the genome was visualized using deeptools (v3.1.3). Bigwigs were visualized using the
476 UCSC genome browser.

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

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

482 **Data availability**

483 **Published datasets**

484 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
485 adult amygdala and hippocampus²⁵ (available at GEO: GSE127722) and male wild-type and *Kdm5c*-KO
486 EpiLCs⁴⁵ (available at GEO: GSE96797).

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

493 **Data analysis**

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

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677 **Figures and Tables**

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

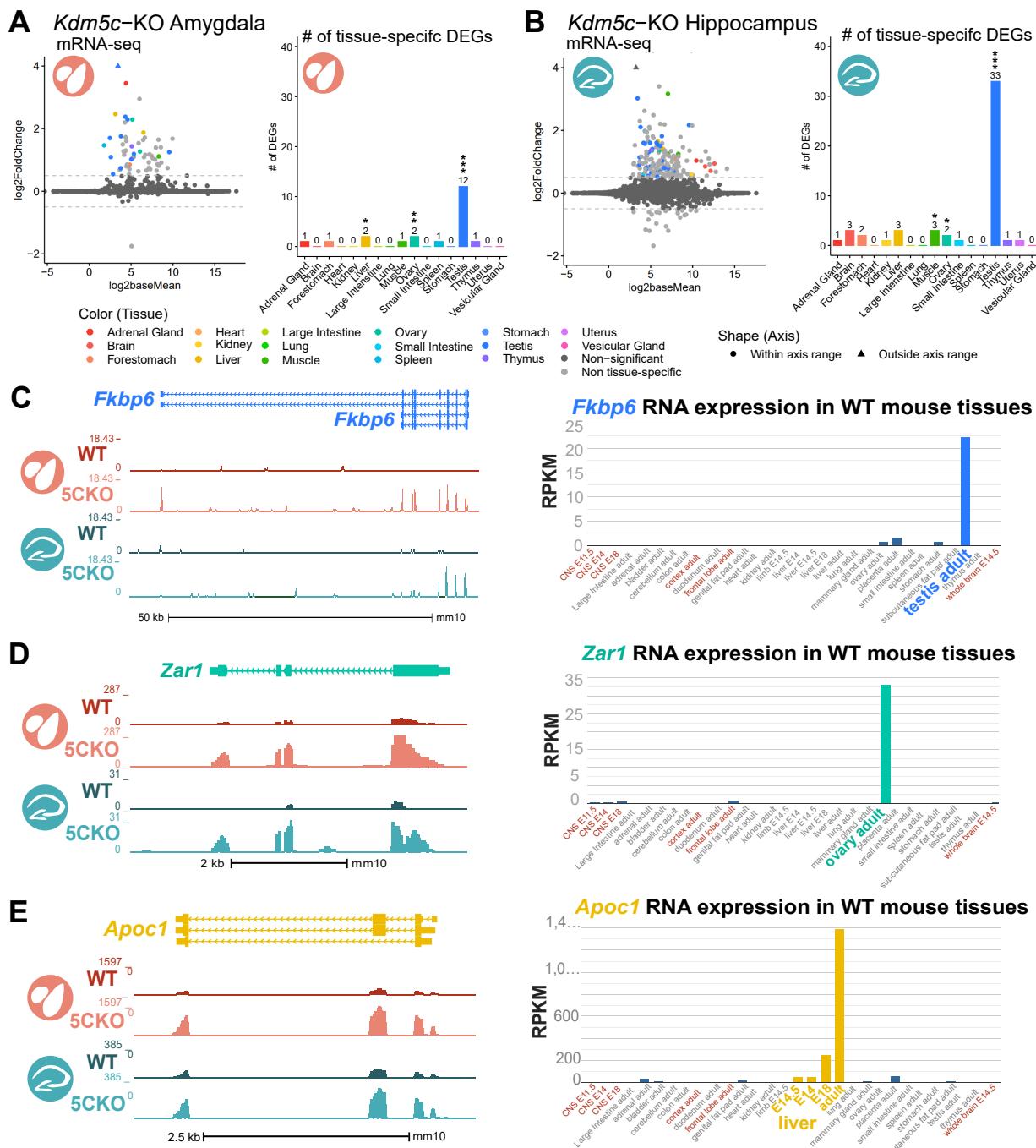


Figure 1: Tissue-enriched genes are misexpressed in the *Kdm5c*-KO brain. **A-B.** Expression of tissue-enriched genes (Li et al 2017) in the male *Kdm5c*-KO amygdala (A) and hippocampus (B). Left - MA plot of mRNA-sequencing. Right - Number of tissue-enriched differentially expressed genes (DEGs). *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

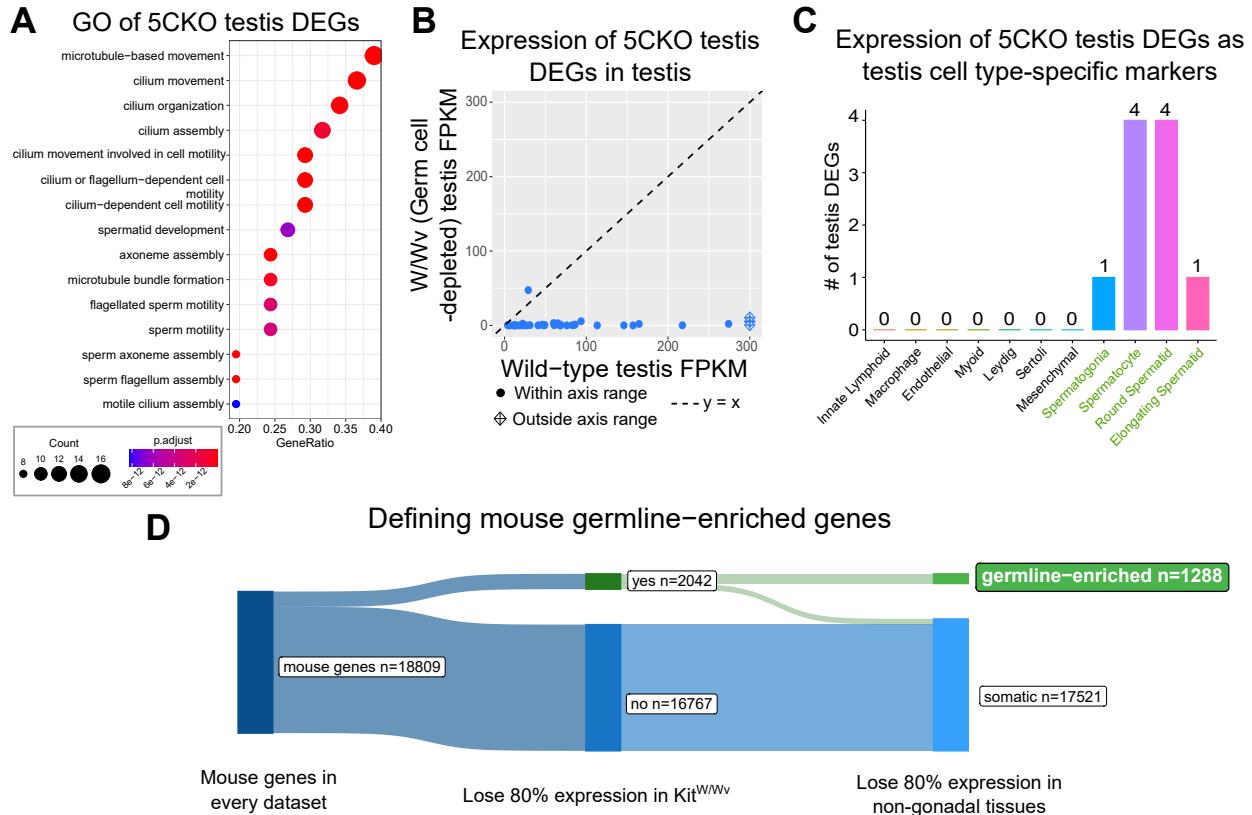


Figure 2: Aberrant transcription of germline genes in the *Kdm5c*-KO in the brain. **A.** enrichPlot gene ontology (GO) of *Kdm5c*-KO amygdala and hippocampus testis-enriched DEGs **B.** Expression of testis DEGs in wild-type (WT) testis versus germ cell-depleted (W/Wv) testis (Mueller et al 2013). Expression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

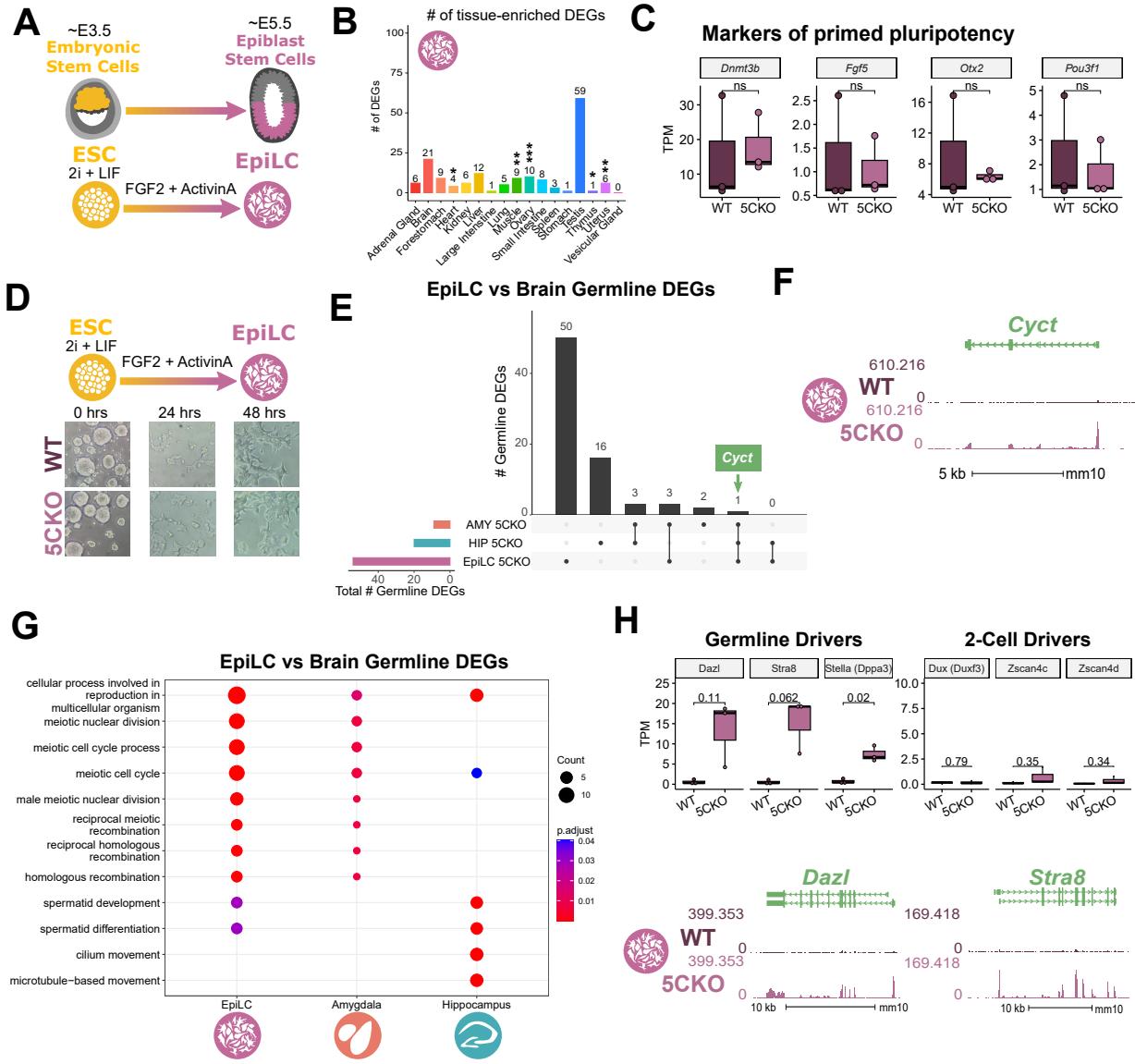


Figure 3: *Kdm5c*-KO epiblast-like cells express key drivers of germline identity

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. Percentage of DAZL-positive cells normalized to number of DAPI-positive cells, p-value for Welch's t-test.

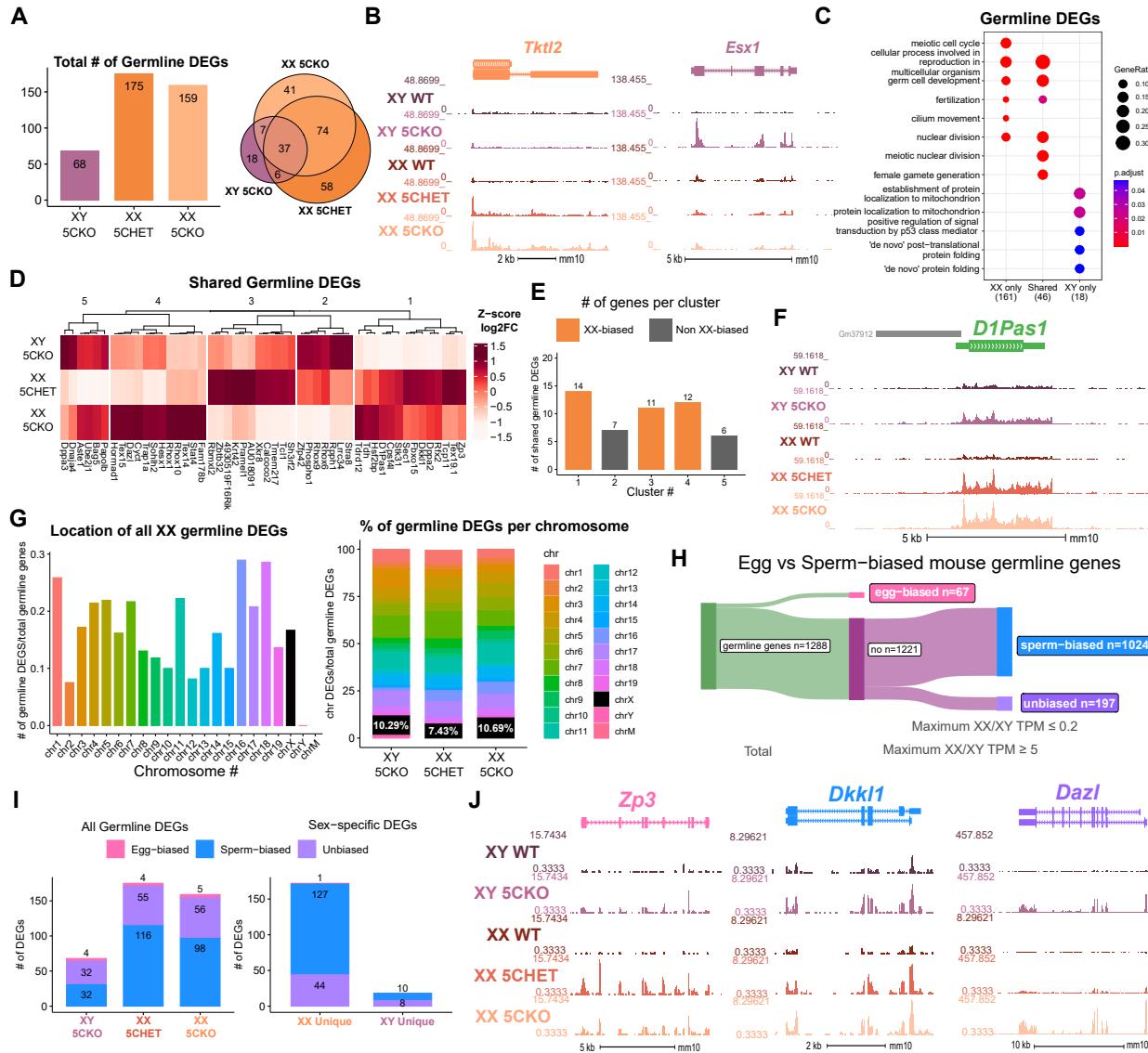


Figure 4: Chromosomal sex influences Kdm5c-KO germline gene misexpression. **A-B.** Expression of tissue-enriched genes (Li et al 2017) in the male Kdm5c-KO amygdala (A) and hippocampus (B). Left - MA plot of mRNA-sequencing. Right - Number of tissue-enriched differentially expressed genes (DEGs). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, 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.

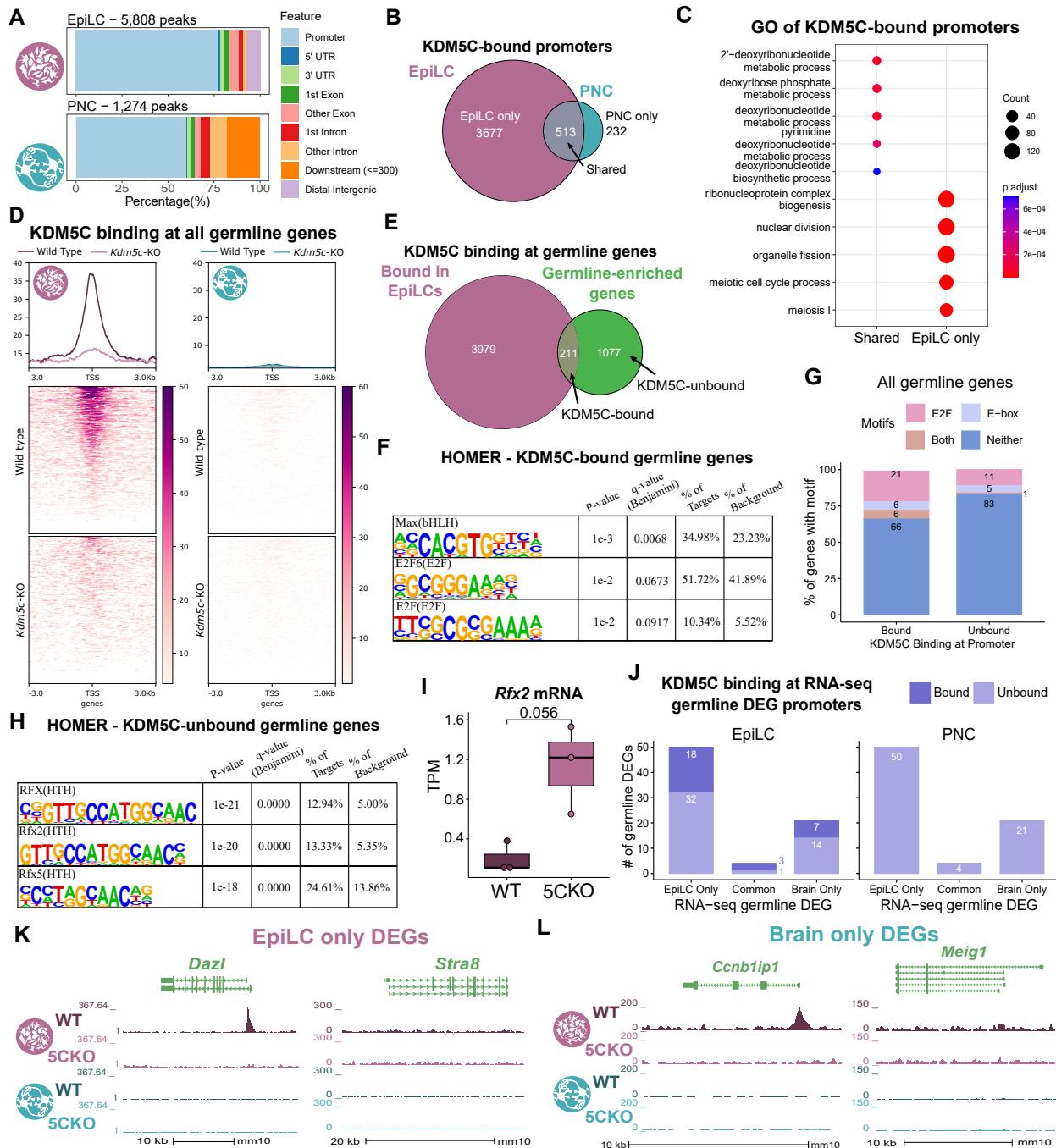


Figure 5: KDM5C binds to a subset of germline gene promoters during early embryogenesis **A.** ChIPseeker localization of KDM5C peaks at different genomic regions in EpiLCs (top) and hippocampal and cortex primary neuron cultures (PNCs, bottom). **B.** Overlap of genes with KDM5C bound to their promoters 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

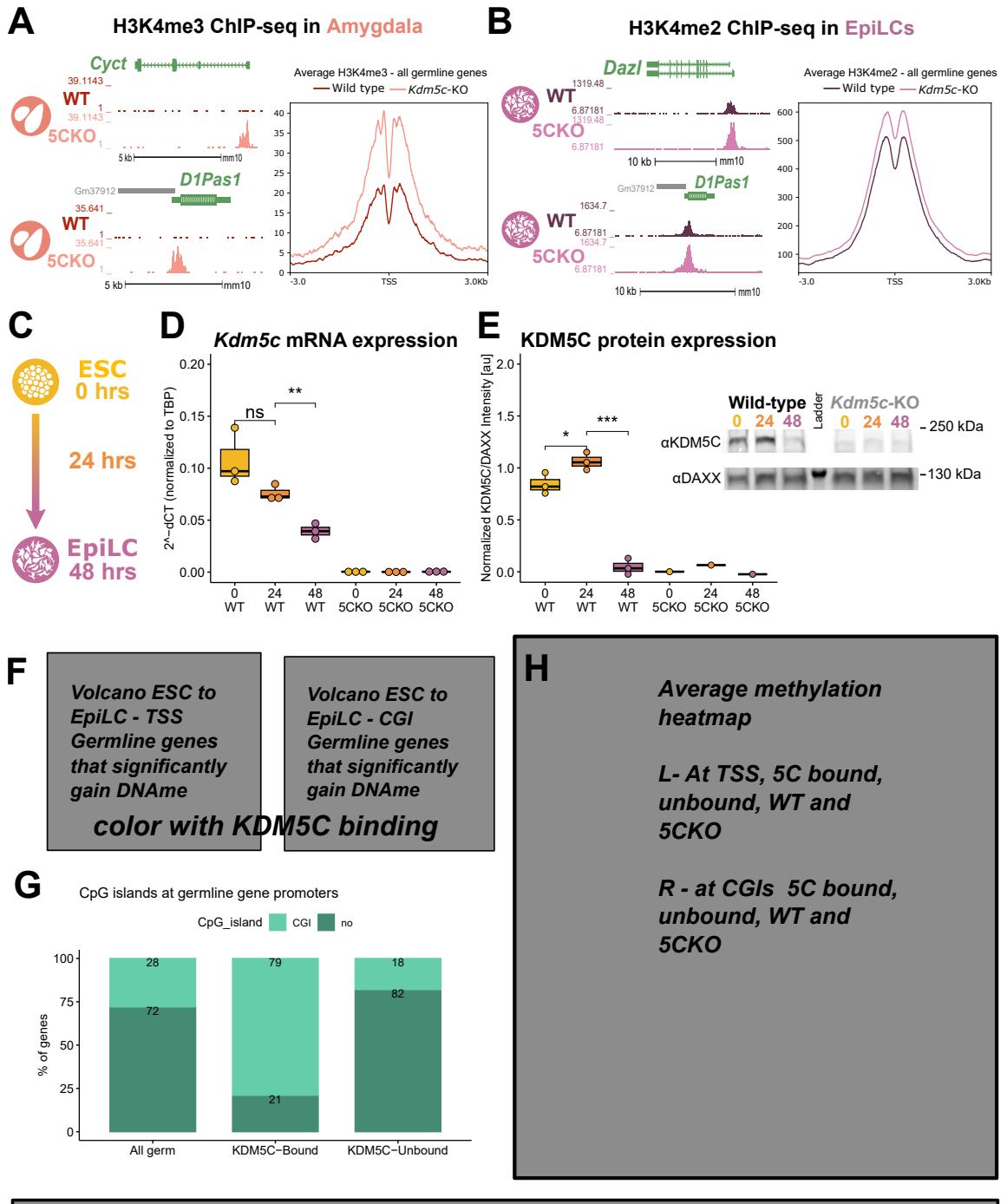


Figure 6: KDM5C's catalytic activity promotes long-term silencing of germline genes via DNA methylation. **A.** Left - Bigwigs of representative histone 3 lysine 4 trimethylation (H3K4me3) ChIP-seq peaks at two germline genes in the wild-type (WT) and *Kdm5c*-KO (5CKO) adult amygdala. Right - Average 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

682 Notes

683 Things to do

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

688 Dazl

689 We were particularly interested in the aberrant transcription of *Dazl*, since it is essential for germ cell
690 development and promotes the translation of germline mRNAs⁸⁶. A significant portion of germline transcripts
691 misexpressed in *Kdm5c*-KO EpiLCs are known binding targets of DAZL, including *Stra8*⁸⁷ ($p = 1.698e-07$,
692 Fisher's Exact Test). This suggests expression of DAZL protein could promote the translation of other
693 aberrant germline transcripts, influencing their ability to impact *Kdm5c*-KO cellular function. We thus tested
694 DAZL protein expression in *Kdm5c*-KO EpiLCs through immunocytochemistry (Figure 3I). We observed
695 about 25% of *Kdm5c*-KO EpiLCs expressed DAZL protein and it was localized to the cytoplasm ($p = 0.0015$,
696 Welch's t-test), consistent with the pattern of DAZL expression in spermatogonia⁸⁷. Altogether these results
697 suggest tissue-specific genes are misexpressed during *Kdm5c*-KO embryogenesis, including key drivers of
698 germline identity that can be translated into protein.

- 699 • We additionally found *Kdm5c*-KO EpiLCs ectopically express DAZL protein that is localized to the
700 cytoplasm, similar to its morphology in spermatogonia⁸⁷. **note: maybe just put in results.** Could
701 move around depending upon if I get pheno working.

702 Discussion notes

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

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

- 739 • Altogether, these data indicate that while some germline genes are misexpressed due to direct loss of
740 KDM5C binding during emryogenesis, secondary downstream mechanisms can also promote their
741 aberrant transcription.
- 742 • Papers to read/reference:
743 – Reconstitution of the Mouse Germ Cell Specification Pathway in Culture by Pluripotent Stem Cells:
744 [https://www.cell.com/fulltext/S0092-8674\(11\)00771-9](https://www.cell.com/fulltext/S0092-8674(11)00771-9)
745 – 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/>

747 **Figure outline:**

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

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

760 **Figure 3: *Kdm5c*-KO epiblast-like cells express key drivers regulators of germline identity** * A) ESC
761 to EpiLC differentiation Left - Morphology is unchanged, * B) 5CKO EpiLCs express EpiLC differentiation
762 genes similar to WT lvs * C) Male EpiLCs express germline genes (example Cyct again) * Overlap between
763 brain and EpiLC germline genes - show they're mostly unique * GO of Brain and EpiLC germline genes
764 (meiotic enriched) * Bigwigs or TPM of master regulators * Show that while some are also 2-cell genes
765 (Dazl), 2-cell specific genes aren't dysregulated (Zscan4). Important point because published KDM5C dazl
766 paper is saying KDM5C is a 2-cell regulator, but as far as I can tell only genes shared between germline and
767 2-cell are dysregulated.

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

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

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

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

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

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

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

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

812 **Germline gene repression background:**

813 Interestingly, some of the ectopic testis transcripts identified in the *Kdm5c*-KO brain are typically ex-
814 pressed in germ cells⁸. Unlike somatic cells, germ cells (e.g. sperm and eggs) undergo meiosis and pass on
815 their genetic material to the next generation. The germline and the soma are typically distinguished during
816 early embryogenesis, when germline genes are silenced in epiblast stem cells soon after implantation and
817 only reactivated in a subset to form the germline. Chromatin regulators play a key role in decommissioning
818 germline genes as the embryo transitions from naïve to primed pluripotency by placing repressive histone
819 H2A lysine 119 monoubiquitination (H2AK119ub1)¹⁶, histone 3 lysine 9 trimethylation (H3K9me3)^{16,17}, and
820 DNA CpG methylation^{17–19} at germline gene promoters. KDM5C may also be involved in this early decom-
821 missioning of germline genes, as re-expression of KDM5C in neurons fails to suppress their dysregulation⁸.
822 In support of this, KDM5C was very recently shown to repress *Deleted in azoospermia like (Dazl)*, a key
823 regulator of germline development, in mouse embryonic stem cells (ESCs)^{49,66}. In support of this, two
824 independent screens in mouse embryonic stem cells (ESCs) recently identified KDM5C as a repressor of
825 *Deleted in azoospermia like (Dazl)*, a key regulator of germline development. However, KDM5C's role in
826 embryonic germline gene repression is currently unclear, given that Dazl is also expressed in ESCs and in
827 the 2-cell stage and germline gene misexpression has yet to be globally characterized during *Kdm5c*-KO
828 embryogenesis.