

1 The X-linked intellectual disability gene KDM5C is a
2 sex-biased brake against germline programs in somatic
3 lineages

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11 Abstract

12 Mutations in numerous chromatin-modifying enzymes cause neurodevelopmental disorders (NDDs). Loss
13 of repressive chromatin regulators can lead to the aberrant transcription of tissue-specific genes outside
14 of their intended context, however the mechanisms and consequences of their dysregulation are largely
15 unknown. Here, we examine how the X-linked intellectual disability gene lysine demethylase 5c (KDM5C), an
16 eraser of histone 3 lysine 4 di and tri-methylation (H3K4me2/3), contributes to tissue identity. We found male
17 *Kdm5c* knockout (-KO) mice, which recapitulate key human neurological phenotypes, aberrantly express
18 many liver, muscle, ovary, and testis genes within the amygdala and hippocampus. Gonad-enriched genes
19 misexpressed in the *Kdm5c*-KO brain are unique to germ cells, indicating an erosion of the soma-germline
20 boundary. Germline genes are typically decommissioned in somatic lineages in the post-implantation epiblast,
21 yet *Kdm5c*-KO epiblast-like cells (EpiLCs) aberrantly expressed key regulators of germline identity and
22 meiosis, including *Dazl* and *Stra8*. Characterizing germline gene misexpression in males and female mutants
23 revealed germline gene repression is sexually dimorphic, with female EpiLCs requiring a higher dose of
24 KDM5C to maintain germline gene suppression. Using a comprehensive list of mouse germline-enriched
25 genes, we found KDM5C is selectively recruited to a subset of germline gene promoters that contain CpG
26 islands (CGIs) to facilitate DNA CpG methylation during ESC to EpiLC differentiation. However, late-stage
27 spermatogenesis genes devoid of promoter CGIs can become expressed in *Kdm5c*-KO cells via ectopic
28 activation by RFX transcription factors. Together, these data demonstrate KDM5C's fundamental role in
29 tissue identity and indicate that KDM5C acts as a brake against runaway activation of germline developmental
30 programs in somatic lineages.

31 Introduction

32 A single genome holds the instructions to generate the myriad of cell types found within an organism.
33 This is, in part, accomplished by chromatin regulators that can either promote or impede lineage-specific
34 gene expression through DNA and histone modifications^{1–5}. Human genetic studies revealed mutations in
35 chromatin regulators are a major cause of neurodevelopmental disorders (NDDs)⁶ and many studies have
36 identified their importance for regulating brain-specific transcriptional programs. Loss of chromatin regulators
37 can also result in the ectopic expression of tissue-specific genes outside of their target environment, such
38 as the misexpression of liver-specific genes within adult neurons⁷. However, the mechanisms underlying
39 ectopic gene expression and its impact upon neurodevelopment are poorly understood.

40 To elucidate the role of tissue identity in chromatin-linked NDDs, it is essential to first characterize the
41 nature of the dysregulated genes and the molecular mechanisms governing their de-repression. Here we
42 focus on the X chromosome gene lysine demethylase 5C (KDM5C, also known as SMCX or JARID1C),
43 which erases histone 3 lysine 4 di- and trimethylation (H3K4me2/3), a permissive chromatin modification
44 enriched at gene promoters⁸. Pathogenic mutations in *KDM5C* cause Intellectual Developmental Disorder,
45 X-linked, Syndromic, Claes-Jensen Type (MRXSCJ, OMIM: 300534). MRXSCJ is more common and severe
46 in males and its neurological phenotypes include intellectual disability, seizures, aberrant aggression, and
47 autistic behaviors^{9–11}. Male *Kdm5c* knockout (-KO) mice recapitulate key MRXSCJ phenotypes, including
48 hyperaggression, increased seizure propensity, social deficits, and learning impairments^{12–14}. RNA sequenc-
49 ing (RNA-seq) of the *Kdm5c*-KO hippocampus revealed ectopic expression of some germline genes within
50 the brain¹³. However, it is unclear if other tissue-specific genes are aberrantly transcribed with KDM5C loss,
51 at what point in development germline gene misexpression begins, and what mechanisms underlie their
52 dysregulation.

53 Distinguishing between germ cells and somatic cells is a key feature of multicellularity¹⁵ that occurs
54 during early embryogenesis in many metazoans¹⁶. In mammals, chromatin regulators are crucial for
55 decommissioning germline genes during the transition from naïve to primed pluripotency. Initially, germline
56 gene promoters gain repressive histone H2A lysine 119 monoubiquitination (H2AK119ub1)¹⁷ and histone H3
57 lysine 9 trimethylation (H3K9me3)^{17,18} in embryonic stem cells (ESCs) and are then decorated with DNA
58 CpG methylation (CpGme) in post-implantation epiblast cells^{18–21}. The contribution of KDM5C to this process
59 remains unclear. Additionally, studies on germline gene repression have primarily been conducted in males
60 and focused on select marker genes, given the lack of a comprehensive list for germline-enriched genes.
61 Therefore, it is unknown if the mechanism of repression differs between sexes or for different classes of
62 germline genes, e.g. meiotic versus spermatid differentiation genes.

63 To illuminate KDM5C's role in tissue identity, here we characterized the aberrant expression of tissue-
64 enriched genes within the *Kdm5c*-KO brain and epiblast-like stem cells (EpiLCs), an *in vitro* model of the
65 post-implantation embryo. We curated a list of mouse germline-enriched genes, which enabled genome-wide

66 analysis of germline gene silencing mechanisms for the first time. Additionally, we characterized germline
67 transcripts expressed in male and female *Kdm5c* mutants to illuminate the impact of sex upon germline
68 gene suppression. Based on the data presented below, we propose KDM5C plays a fundamental, sexually
69 dimorphic role in the development of tissue identity during early embryogenesis, including the establishment
70 of the soma-germline boundary.

71 **Results**

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

73 Previous RNA sequencing (RNA-seq) of the adult male *Kdm5c*-KO hippocampus revealed ectopic
74 expression of some germline genes unique to the testis¹³. It is currently unknown if the testis is the only
75 tissue type misexpressed in the *Kdm5c*-KO brain. We thus systematically tested whether other tissue-specific
76 genes are misexpressed in the male brain with constitutive knockout of *Kdm5c* (*Kdm5c*^{-y}, 5CKO in figures)²²
77 by using a published list of mouse tissue-enriched genes²³.

78 We found a large proportion of significantly upregulated genes (DESeq2²⁴, log2 fold change > 0.5, q <
79 0.1) within the male *Kdm5c*-KO amygdala and hippocampus are non-brain, tissue-specific genes (Amygdala:
80 21/59 up DEGs, 35.59% ; Hippocampus: 48/183 up DEGs, 26.23%) (Figure 1A-B, Supplementary Table
81 1). For both the amygdala and hippocampus, the majority of tissue-enriched differentially expressed genes
82 (DEGs) were testis genes (Figure 1A-B). Even though the testis has the largest total number of tissue-
83 enriched genes (2,496 genes) compared to any other tissue, testis-enriched DEGs were significantly enriched
84 in both brain regions (Amygdala p = 1.83e-05, Odds Ratio = 5.13; Hippocampus p = 4.26e-11, Odds Ratio =
85 4.45, Fisher's Exact Test). An example of a testis-enriched gene misexpressed in the *Kdm5c*-KO brain is
86 *FK506 binding protein 6 (Fkbp6)*, a known regulator of PIWI-interacting RNAs (piRNAs) and meiosis^{25,26}
87 (Figure 1C).

88 Interestingly, we also observed significant enrichment of ovary-enriched genes in both the amygdala
89 and hippocampus (Amygdala p = 0.00574, Odds Ratio = 18.7; Hippocampus p = 0.048, Odds Ratio = 5.88,
90 Fisher's Exact Test) (Figure 1A-B). Ovary-enriched DEGs included *Zygotic arrest 1 (Zar1)*, which sequesters
91 mRNAs in oocytes for meiotic maturation²⁷ (Figure 1D). Given that the *Kdm5c*-KO mice we analyzed are
92 male, these data demonstrate that the ectopic expression of gonad-enriched genes is independent of
93 organismal sex.

94 Although not consistent across brain regions, we also found significant enrichment of genes biased
95 towards two non-gonadal tissues - the liver (Amygdala p = 0.04, Odds Ratio = 6.58, Fisher's Exact Test)
96 and muscle (Hippocampus p = 0.01, Odds Ratio = 6.95, Fisher's Exact Test) (Figure 1A-B). These include
97 *Apolipoprotein C-I (Apoc1)*, a lipoprotein metabolism and transport gene²⁸ (Figure 1E, see Discussion).

98 Our analysis of oligo(dT)-primed libraries²² indicates aberrantly expressed mRNAs are polyadenylated

99 and spliced into mature transcripts in the *Kdm5c*-KO brain (Figure 1C-E). Of note, we observed little to no
100 dysregulation of brain-enriched genes (Amygdala p = 1, Odds Ratio = 1.22; Hippocampus p = 0.74, Odds
101 Ratio = 1.22, Fisher's Exact Test), despite the fact these are brain samples and the brain has the second
102 highest total number of tissue-enriched genes (708 genes). Altogether, these results suggest the aberrant
103 expression of tissue-enriched genes within the brain is a major effect of KDM5C loss.

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

105 *Kdm5c*-KO brain expresses testicular germline genes¹³ (Figure 1), however the testis also contains
106 somatic cells that support hormone production and germline functions. To determine if *Kdm5c*-KO results
107 in ectopic expression of testicular somatic genes, we first evaluated the known functions of testicular
108 DEGs through gene ontology. We found *Kdm5c*-KO testis-enriched DEGs had high enrichment of germline-
109 relevant ontologies, including spermatid development (GO: 0007286, p.adjust = 6.2e-12) and sperm axoneme
110 assembly (GO: 0007288, p.adjust = 2.45e-14) (Figure 2A, Supplementary Table 1).

111 We then evaluated *Kdm5c*-KO testicular DEG expression in wild-type testes versus testes with germ cell
112 depletion²⁹, which was accomplished by heterozygous *W* and *Wv* mutations in the enzymatic domain of *c-Kit*
113 (*Kit*^{W/Wv})³⁰. Almost all *Kdm5c*-KO testis-enriched DEGs lost expression with germ cell depletion (Figure 2B).
114 We then assessed testis-enriched DEG expression in a published single cell RNA-seq dataset that identified
115 cell type-specific markers within the testis³¹. Some *Kdm5c*-KO testis-enriched DEGs were classified as
116 specific markers for different germ cell developmental stages (e.g. spermatogonia, spermatocytes, round
117 spermatids, and elongating spermatids), yet none marked somatic cells (Figure 2C). Together, these data
118 demonstrate that the *Kdm5c*-KO brain aberrantly expresses germline genes but not somatic testicular genes,
119 reflecting an erosion of the soma-germline boundary.

120 As of yet, research on germline gene silencing mechanisms has focused on a handful of key genes rather
121 than assessing germline gene suppression genome-wide, due to the lack of a comprehensive gene list.
122 We therefore generated a list of mouse germline-enriched genes using RNA-seq datasets of *Kit*^{W/Wv} mice
123 that included males and females at embryonic day 12, 14, and 16³² and adult male testes²⁹. We defined
124 genes as germline-enriched if their expression met the following criteria: 1) their expression is greater than
125 1 FPKM in wild-type gonads 2) their expression in any non-gonadal tissue of adult wild type mice²³ does
126 not exceed 20% of their maximum expression in the wild-type germline, and 3) their expression in the germ
127 cell-depleted gonads, for any sex or time point, does not exceed 20% of their maximum expression in the
128 wild-type germline. These criteria yielded 1,288 germline-enriched genes (Figure 2D), which was hereafter
129 used as a resource to globally characterize germline gene misexpression with *Kdm5c* loss (Supplementary
130 Table 2).

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

132 Germ cells are typically distinguished from somatic cells soon after the embryo implants into the uterine
133 wall^{33,34}, when germline genes are silenced in epiblast stem cells that will form the somatic tissues³⁵. This
134 developmental time point can be modeled *in vitro* through differentiation of naïve embryonic stem cells
135 (nESCs) into epiblast-like stem cells (EpiLCs) (Figure 3A)^{36,37}. While some germline-enriched genes are
136 also expressed in nESCs and in the 2-cell stage^{38–40}, they are silenced as they differentiate into EpiLCs^{18,19}.
137 Therefore, we tested if KDM5C was necessary for the initial silencing of germline genes in somatic lineages
138 by evaluating the impact of *Kdm5c* loss in male EpiLCs.

139 *Kdm5c*-KO cell morphology during ESC to EpiLC differentiation appeared normal (Figure 3B) and EpiLCs
140 properly expressed markers of primed pluripotency, such as *Dnmt3b*, *Fgf5*, *Pou3f1*, and *Otx2* (Figure 3C). We
141 then identified tissue-enriched DEGs in a RNA-seq dataset of wild-type and *Kdm5c*-KO EpiLCs⁴¹ (DESeq2,
142 log₂ fold change > 0.5, q < 0.1, Supplementary Table 3). Similar to the *Kdm5c*-KO brain, we observed
143 general dysregulation of tissue-enriched genes, with the largest number of genes belonging to the brain and
144 testis, although they were not significantly enriched (Figure 3D). Using our list of mouse germline-enriched
145 genes assembled above, we identified 68 germline genes misexpressed in male *Kdm5c*-KO EpiLCs.

146 We then compared EpiLC germline DEGs to those expressed in the *Kdm5c*-KO brain to determine if
147 germline genes are constitutively dysregulated or change over the course of development. The majority of
148 germline DEGs were unique to either EpiLCs or the brain, with only *D1Pas1* and *Cyct* shared across all
149 tissue/cell types (Figure 3E-F). EpiLC germline DEGs had particularly high enrichment of meiosis-related
150 gene ontologies when compared to the brain (Figure 3G, Supplementary Table 3), such as meiotic cell
151 cycle process (GO:1903046, p.adjust = 2.2e-07) and meiotic nuclear division (GO:0140013, p.adjust
152 = 1.37e-07). While there was modest enrichment of meiotic gene ontologies in both brain regions, the
153 *Kdm5c*-KO hippocampus primarily expressed late-stage spermatogenesis genes involved in sperm axoneme
154 assembly (GO:0007288, p.adjust = 0.00621) and sperm motility (GO:0097722, p.adjust = 0.00612).

155 Notably, DEGs unique to *Kdm5c*-KO EpiLCs included key drivers of germline identity, such as *Stimulated*
156 *by retinoic acid 8* (*Stra8*: log₂ fold change = 3.73, q = 2.17e-39) and *Deleted in azoospermia like* (*Dazl*:
157 log₂ fold change = 3.36, q = 3.19e-12) (Figure 3H). These genes are typically expressed when a subset
158 of epiblast stem cells become primordial germ cells (PGCs) and then again in mature germ cells to trigger
159 meiotic gene expression programs^{42–44}. Of note, some germline genes, including *Dazl*, are also expressed
160 in the two-cell embryo^{39,45}. However, we did not see derepression of two-cell stage-specific genes, like
161 *Duxf3* (*Dux*) (log₂ fold change = -0.282, q = 0.337) and *Zscan4d* (log₂ fold change = 0.25, q = 0.381) (Figure
162 3H, Supplementary Table 3), indicating *Kdm5c*-KO EpiLCs do not revert back to a 2-cell state. Altogether,
163 *Kdm5c*-KO EpiLCs express key drivers of germline identity and meiosis while the brain primarily expresses
164 spermiogenesis genes, indicating germline gene misexpression mirrors germline development during the
165 progression of somatic development.

166 **Female epiblast-like cells have heightened germline gene misexpression with *Kdm5c***
167 **loss**

168 It is currently unknown if the misexpression of germline genes is influenced by sex, as previous studies
169 on germline gene repressors have focused on male cells^{17,18,20,46,47}. Sex is particularly pertinent in the case
170 of KDM5C because it partially escapes X chromosome inactivation (XCI), resulting in a higher dosage in
171 females^{48–51}. We therefore explored the impact of chromosomal sex upon germline gene suppression by
172 comparing their dysregulation in male *Kdm5c* hemizygous knockout (*Kdm5c*^{-y}, XY *Kdm5c*-KO, XY 5CKO),
173 female homozygous knockout (*Kdm5c*^{-/-}, XX *Kdm5c*-KO, XX 5CKO), and female heterozygous knockout
174 (*Kdm5c*^{-/+}, XX *Kdm5c*-HET, XX 5CHET) EpiLCs⁴¹.

175 In EpiLCs, homozygous and heterozygous *Kdm5c* knockout females expressed over double the number
176 of germline-enriched genes than hemizygous males (Figure 4A, Supplementary Table 3). While the majority
177 of germline DEGs in *Kdm5c*-KO males were also dysregulated in females (74%), many were sex-specific,
178 such as *Tktl2* and *Esx1* (Figure 4B). We then compared the known functions of germline genes dysregulated
179 uniquely in males and females or misexpressed in all samples (Figure 4C, Supplementary Table 3). Female-
180 specific germline DEGs were enriched for meiotic (GO:0051321 - meiotic cell cycle) and flagellar (GO:
181 0003341 - cilium movement) functions, while male-specific DEGs had roles in mitochondrial and cell signaling
182 (GO:0070585 - protein localization to mitochondrion).

183 The majority of germline genes expressed in both sexes were more highly dysregulated in females
184 compared to males (Figure 4D-F). This increased degree of dysregulation in females, along with the
185 increased total number of germline genes, indicates females are more sensitive to losing KDM5C-mediated
186 germline gene suppression. Heightened germline gene dysregulation in females could be due to impaired
187 XCI in *Kdm5c* mutants⁴¹, as many spermatogenesis genes lie on the X chromosome^{52,53}. However, female
188 germline DEGs were not biased towards the X chromosome ($p = 1$, Odds Ratio = 0.96, Fisher's Exact Test)
189 and females had a similar overall proportion of germline DEGs belonging to the X chromosome as males
190 (XY *Kdm5c*-KO - 10.29%, XX *Kdm5c*-HET - 7.43%, XX *Kdm5c*-KO - 10.59%) (Figure 4G). The majority of
191 germline DEGs instead lie on autosomes for both male and female *Kdm5c* mutants (Figure 4G). Thus, while
192 female EpiLCs are more prone to germline gene misexpression with KDM5C loss, it is likely independent of
193 XCI defects.

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

195 Although many germline genes have shared functions in the male and female germline, e.g. PGC
196 formation, meiosis, and genome defense, some have unique or sex-biased expression. Therefore, we
197 wondered if *Kdm5c* mutant males would primarily express sperm genes while mutant females would primarily
198 express egg genes. To comprehensively assess whether germline gene sex corresponds with *Kdm5c*
199 mutant sex, we first filtered our list of germline-enriched genes for egg and sperm-biased genes (Figure 4,

200 Supplementary Table 2). We defined germ cell sex-biased genes as those whose expression in the opposite
201 sex, at any time point, is no greater than 20% of the gene's maximum expression in a given sex. This
202 criteria yielded 67 egg-biased, 1,024 sperm-biased, and 197 unbiased germline-enriched genes. We found
203 regardless of sex, egg, sperm, and unbiased germline genes were dysregulated in all *Kdm5c* mutants at
204 similar proportions (Figure 4I-J). Furthermore, germline genes dysregulated exclusively in either male or
205 female mutants were also not biased towards their corresponding germ cell sex (Figure 4I). Altogether, these
206 results demonstrate sex differences in germline gene dysregulation is not due to sex-specific activation of
207 sperm or egg transcriptional programs.

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 not in
210 neurons^{13,54}. However, due to the lack of a comprehensive list of germline-enriched genes, it is unclear if
211 KDM5C is enriched at germline gene promoters, what types of germline genes KDM5C regulates, and if its
212 binding is maintained at any germline genes in neurons.

213 To address these questions, we analyzed KDM5C chromatin immunoprecipitation followed by DNA
214 sequencing (ChIP-seq) datasets in EpiLCs⁴¹ and primary forebrain neuron cultures (PNCs)¹² (MACS2 q <
215 0.1, fold enrichment > 1, and removal of false-positive *Kdm5c*-KO peaks). EpiLCs had a higher total number
216 of high-confidence KDM5C peaks than PNCs (EpiLCs: 5,808, PNCs: 1,276). KDM5C was primarily localized
217 to gene promoters in both cell types (promoters = transcription start site (TSS) ± 500 bp, EpiLCs: 4,190,
218 PNCs: 745), although PNCs showed increased localization to non-promoter regions (Figure 5A).

219 The majority of promoters bound by KDM5C in PNCs were also bound in EpiLCs (513 shared promoters),
220 however a large portion of gene promoters were bound by KDM5C only in EpiLCs (3,677 EpiLC only
221 promoters) (Figure 5B). Genes bound by KDM5C in both PNCs and EpiLCs were enriched for functions
222 involving nucleic acid turnover, such as deoxyribonucleotide metabolic process (GO:0009262, p.adjust =
223 8.28e-05) (Figure 5C, Supplementary Table 4). Germline ontologies were enriched only in EpiLC-specific,
224 KDM5C-bound promoters, such as meiotic nuclear division (GO: 0007127 p.adjust = 6.77e-16) (Figure 5C).
225 There were no significant ontologies for PNC-specific KDM5C target genes. Using our mouse germline gene
226 list, we observed evident KDM5C signal around the TSS of many germline genes in EpiLCs, but not in PNCs
227 (Figure 5D). Based on our ChIP-seq peak cut-off criteria, KDM5C was highly enriched at 211 germline gene
228 promoters in EpiLCs (16.4% of all germline genes) (Figure 5E, Supplementary Table 2). Of note, KDM5C
229 was only bound to about one third of RNA-seq DEG promoters unique to EpiLCs or the brain (EpiLC only
230 DEGs: 34.9%, Brain only DEGs: 30%) (Supplementary Figure 1A-C). Representative examples of EpiLC
231 DEGs bound and unbound by KDM5C in EpiLCs are *Dazl* and *Stra8*, respectively (Figure 5F). However,
232 the four of the five germline genes dysregulated in both EpiLCs and the brain were bound by KDM5C in
233 EpiLCs (*D1Pas1*, *Hsf2bp*, *Cyct*, and *Stk31*) (Supplementary Figure 1A). Together, these results demonstrate
234 KDM5C is recruited to a subset of germline genes in EpiLCs, including meiotic genes, but does not directly

235 regulate germline genes in neurons. Furthermore, the majority of germline mRNAs expressed in *Kdm5c*-KO
236 cells are dysregulated independent of direct KDM5C recruitment to their gene promoters, however genes
237 dysregulated across *Kdm5c*-KO development are often direct KDM5C targets.

238 Many germline-specific genes are suppressed by the polycomb repressive complex 1.6 (PRC1.6), which
239 contains the transcription factor heterodimers E2F6/DP1 and MGA/MAX that respectively bind E2F and
240 E-box motifs within germline gene promoters^{17,18,20,40,46,47,55–57}. PRC1.6 members may recruit KDM5C to
241 germline gene promoters¹³, given their association with KDM5C in HeLa cells and ESCs^{45,58}. We thus
242 used HOMER⁵⁹ to identify transcription factor motifs enriched at KDM5C-bound or unbound germline gene
243 promoters (TSS ± 500 bp, q-value < 0.1, Supplementary Table 4). MAX and E2F6 binding sites were
244 significantly enriched at germline genes bound by KDM5C in EpiLCs (MAX q-value: 0.0068, E2F6 q-value:
245 0.0673, E2F q-value: 0.0917), but not at germline genes unbound by KDM5C (Figure 5G). One third of
246 KDM5C-bound promoters contained the consensus sequence for either E2F6 (E2F, 5'-TCCCGC-3'), MGA
247 (E-box, 5'-CACGTG-3'), or both, but only 17% of KDM5C-unbound genes contained these motifs (Figure 5H).
248 KDM5C-unbound germline genes were instead enriched for multiple RFX transcription factor binding sites
249 (RFX q-value < 0.0001, RFX2 q-value < 0.0001, RFX5 q-value < 0.0001) (Figure 5I, Supplementary figure
250 1D). RFX transcription factors bind X-box motifs⁶⁰ to promote ciliogenesis^{61,62} and among them is RFX2, a
251 central regulator of post-meiotic spermatogenesis^{63,64}. Although *Rfx2* is also not a direct target of KDM5C
252 (Supplementary Figure 1E), RFX2 mRNA is derepressed in *Kdm5c*-KO EpiLCs (Figure 5J). Thus, RFX2 is a
253 candidate transcription factor for driving the ectopic expression of many KDM5C-unbound germline genes in
254 *Kdm5c*-KO cells.

255 **KDM5C is recruited to CpG islands at germline promoters to facilitate *de novo* DNA
256 methylation**

257 Previous work found two germline gene promoters have a marked reduction in DNA CpG methylation
258 (CpGme) in the adult *Kdm5c*-KO hippocampus¹³. Since histone H3K4me2/3 impede *de novo* CpGme^{65,66},
259 KDM5C's removal of H3K4me2/3 may be required to suppress germline genes. However, KDM5C's catalytic
260 activity was recently shown to be dispensable for suppressing *Dazl* in undifferentiated ESCs⁴⁵. To reconcile
261 these observations, we hypothesized KDM5C erases H3K4me2/3 to promote the initial placement of CpGme
262 at germline gene promoters in EpiLCs.

263 To test this hypothesis, we first characterized KDM5C's expression as naïve ESCs differentiate into
264 EpiLCs (Figure 6A). While *Kdm5c* mRNA steadily decreased from 0 to 48 hours of differentiation (Figure
265 6B), KDM5C protein initially increased from 0 to 24 hours and then decreased to near knockout levels by 48
266 hours (Figure 6C). We then characterized KDM5C's substrates (H3K4me2/3) at germline gene promoters
267 with *Kdm5c* loss using published ChIP-seq datasets^{22,41}. *Kdm5c*-KO samples showed a marked increase in
268 H3K4me2 in EpiLCs (Figure 6D) and H3K4me3 in the amygdala (Figure 6E) around the TSS of germline

269 genes. Together, these data suggest KDM5C acts during the transition between ESCs and EpiLCs to remove
270 H3K4me2/3 at germline gene promoters.

271 Germline genes accumulate CpG methylation (CpGme) at CpG islands (CGIs) during the transition
272 from naïve to primed pluripotency^{19,21,67}. We first examined how many of our germline-enriched genes had
273 promoter CGIs (TSS ± 500 bp) using the UCSC genome browser⁶⁸. Notably, out of 1,288 germline-enriched
274 genes, only 356 (27.64%) had promoter CGIs (Figure 6F, Supplementary Table 2). CGI-containing germline
275 genes had higher enrichment of meiotic gene ontologies compared to CGI-free genes, including meiotic
276 nuclear division (GO:0140013, p.adjust = 2.17e-12) and meiosis I (GO:0007127, p.adjust = 3.91e-10)
277 (Figure 6G, Supplementary Table 5). Germline genes with promoter CGIs were more highly expressed than
278 CGI-free genes across spermatogenesis stages, with highest expression in meiotic spermatocytes (Figure
279 6H). Contrastingly, CGI-free genes only displayed substantial expression in post-meiotic round spermatids
280 (Figure 6H). Although only a minor portion of germline gene promoters contained CGIs, CGIs strongly
281 determined KDM5C's recruitment to germline genes ($p = 2.37e-67$, Odds Ratio = 17.8, Fisher's Exact Test),
282 with 79.15% of KDM5C-bound germline gene promoters harboring CGIs (Figure 6F).

283 To assess how KDM5C loss impacts initial CpGme placement at germline gene promoters, we performed
284 whole genome bisulfite sequencing (WGBS) in male wild-type and *Kdm5c*-KO ESCs and 96-hour extend
285 EpiLCs (exEpiLCs), when germline genes reach peak methylation levels¹⁸ (Figure 6I). We first identified
286 which germline gene promoters significantly gained CpGme in wild-type cells during nESC to exEpiLCs
287 differentiation (methylKit⁶⁹, $q < 0.01$, $|methylation\ difference| > 25\%$, TSS ± 500 bp). In wild-type cells, the
288 majority of germline genes gained substantial CpGme at their promoter during differentiation (60.08%),
289 regardless if their promoter contained a CGI (Figure 6J, Supplementary Table 5).

290 We then identified promoters differentially methylated in wild-type versus *Kdm5c*-KO exEpiLCs (methylKit,
291 $q < 0.01$, $|methylation\ difference| > 25\%$, TSS ± 500 bp, Supplementary Table 5). Of the 48,882 promoters
292 assessed, 274 promoters were significantly hypomethylated and 377 promoters were significantly hyper-
293 methylated with KDM5C loss (Supplementary Figure 2A). Many promoters hyper- and hypomethylated
294 in *Kdm5c*-KO exEpiLCs belonged to genes with unknown functions. However, 10.22% of hypomethyl-
295 ated promoters belonged to germline genes and germline-relevant ontologies like meiotic nuclear division
296 (GO:0140013, p.adjust = 0.012) are significantly enriched (Supplementary Figure 2B, Supplementary Table
297 5). Approximately half of all germline gene promoters hypomethylated in *Kdm5c*-KO exEpiLCs are direct
298 targets of KDM5C in EpiLCs (13 out of 28 hypomethylated promoters).

299 Promoters that showed the most robust loss of CpGme in *Kdm5c*-KO exEpiLCs (lowest q-values) harbored
300 CGIs (Figure 6K). CGI promoters, but not CGI-free promoters, had a significant reduction in CpGme with
301 KDM5C loss as a whole (Figure 6L) (Non-CGI promoters $p = 0.0846$, CGI promoters $p = 0.0081$, Mann-
302 Whitney U test). Significantly hypomethylated promoters included germline genes consistently dysregulated
303 across multiple *Kdm5c*-KO RNA-seq datasets¹³, such as *D1Pas1* (methylation difference = -60.03%, q-value
304 = 3.26e-153) and *Naa11* (methylation difference = -42.45%, q-value = 1.44e-38) (Figure 6M). Unexpectedly,

305 we observed only a modest reduction in CpGme at *Dazl*'s promoter (methylation difference = -6.525%,
306 q-value = 0.0159) (Figure 6N). Altogether, these results demonstrate KDM5C is recruited to germline gene
307 CGIs in EpiLCs to promote CpGme at those promoters. Furthermore, our data suggest while KDM5C's
308 catalytic activity is required for the repression of some germline genes, CpGme can be placed at others even
309 with elevated H3K4me2/3 around the TSS.

310 Discussion

311 In the above study, we demonstrate KDM5C's pivotal role in the development of tissue identity. We first
312 characterized tissue-enriched genes expressed within the mouse *Kdm5c*-KO brain and identified substantial
313 derepression of testis, liver, muscle, and ovary-enriched genes. Testis genes significantly enriched within the
314 *Kdm5c*-KO amygdala and hippocampus are specific to the germline and absent in somatic cells. *Kdm5c*-
315 KO epiblast-like cells (EpiLCs) aberrantly express key drivers of germline identity and meiosis, including
316 *Dazl* and *Stra8*, while the adult brain primarily expresses genes important for late spermatogenesis. We
317 demonstrated that although sex did not influence whether sperm or egg-specific genes were misexpressed,
318 female EpiLCs have heightened germline gene de-repression with KDM5C loss. Germline genes can become
319 aberrantly expressed in *Kdm5c*-KO cells via indirect mechanisms, such as activation through ectopic RFX
320 transcription factors. Finally, we found KDM5C is dynamically regulated during ESC to EpiLC differentiation
321 to promote long-term germline gene silencing through CGI DNA methylation. Therefore, we propose KDM5C
322 plays a fundamental role in the development of tissue identity during early embryogenesis, including the
323 establishment of the soma-germline boundary. By systematically characterizing KDM5C's role in germline
324 gene repression, we unveiled distinct mechanisms governing the misexpression of distinct germline gene
325 classes in somatic lineages. Ultimately, these data provide molecular footholds which can be exploited to
326 test the overarching contribution of ectopic germline gene expression upon neurodevelopment.

327 By comparing *Kdm5c* mutant males and females, we revealed germline gene suppression is sexually
328 dimorphic. Female EpiLCs are more severely impacted by loss of KDM5C-mediated germline gene sup-
329 pression, yet this difference is not due to the large number of germline genes on the X chromosome^{52,53}.
330 Heightened germline gene misexpression in females may be related to females having a higher dose of
331 KDM5C than males, due to its escape from XCI^{48–51}. Intriguingly, heterozygous knockout females (*Kdm5c*^{-/+})
332 also had over double the number of germline DEGs than hemizygous knockout males (*Kdm5c*^{+/Y}), even
333 though their expression of KDM5C should be roughly equivalent to that of wild-type males (*Kdm5c*^{+/Y}). Males
334 could partially compensate for KDM5C's loss via the Y-chromosome homolog, KDM5D⁸. However, KDM5D
335 has not been reported to regulate germline gene expression. Nevertheless, these results demonstrate
336 germline gene silencing mechanisms differ between males and females, which warrants further study to
337 elucidate the biological ramifications and underlying mechanisms.

338 We found KDM5C is largely dispensable for promoting normal gene expression during development, yet

339 is critical for suppressing ectopic developmental programs. While some germline genes, such as *Dazl*, are
340 also expressed in the 2-cell stage, the inner cell mass, and naïve ESCs, they are silenced in epiblast stem
341 cells/EpiLCs^{18,40,45,70,71}. Our data suggest the 2-cell-like state reported in *Kdm5c*-KO ESCs⁴⁵ likely reflects
342 KDM5C's primary role in germline gene repression (Figure 3). Germline gene misexpression in *Kdm5c*-
343 KO EpiLCs may indicate they are differentiating into primordial germ cell-like cells (PGCLCs)^{33,34,36}. Yet,
344 *Kdm5c*-KO EpiLCs had normal cellular morphology and properly expressed markers for primed pluripotency,
345 including *Otx2* which blocks EpiLC differentiation into PGCs/PGCLCs⁷². In addition to unimpaired EpiLC
346 differentiation, *Kdm5c*-KO gross brain morphology is overall normal¹² and hardly any brain-specific genes
347 were significantly dysregulated in the amygdala and hippocampus (Figure 1). Thus, ectopic germline gene
348 expression occurs in conjunction with overall proper somatic differentiation in *Kdm5c*-KO animals.

349 Our work provides novel insight into the cross-talk between H3K4me2/3 and CpGme, which are gen-
350 erally mutually exclusive⁷³. In EpiLCs, loss of KDM5C binding at a subset of germline gene promoters,
351 e.g. *D1Pas1*, strongly impaired promoter CGI methylation and resulted in their long-lasting de-repression
352 into adulthood. Removal of H3K4me2/3 at CGIs is a plausible mechanism for KDM5C-mediated germline
353 gene suppression^{13,54}, given H3K4me2/3 repell DNMT3 activity^{65,66}. However, emerging work indicates
354 many histone-modifying enzymes have non-catalytic functions that influence gene expression, sometimes
355 even more potently than their catalytic roles^{74,75}. Indeed, KDM5C's catalytic activity was recently found to be
356 dispensable for repressing *Dazl* in ESCs⁴⁵. In our study, *Dazl*'s promoter still gained CpGme in *Kdm5c*-KO
357 exEpiLCs, even with elevated H3K4me2. *Dazl* and a few other germline genes employ multiple repressive
358 mechanisms to facilitate CpGme, such as DNMT3A/B recruitment via E2F6 and MGA^{17,18,46,47}. Thus, while
359 some germline CGIs require KDM5C-mediated H3K4me removal to overcome promoter CGI escape from
360 CpGme^{73,76}, others do not. These results also suggest the requirement for KDM5C's catalytic activity can
361 change depending upon the locus and developmental stage. Further experiments are required to determine
362 if catalytically inactive KDM5C can suppress germline genes at later developmental stages.

363 By generating a comprehensive list of mouse germline-enriched genes, we revealed distinct derepressive
364 mechanisms governing early versus late-stage germline programs. Previous work on germline gene silencing
365 has focused on genes with promoter CGIs^{19,73}, and indeed the majority of KDM5C targets in EpiLCs were
366 germ cell identity genes harboring CGIs. However, over 70% of germline-enriched gene promoters lacked
367 CGIs, including the many KDM5C-unbound germline genes that are de-repressed in *Kdm5c*-KO cells. CGI-
368 free, KDM5C-unbound germline genes were primarily late-stage spermatogenesis genes and significantly
369 enriched for RFX2 binding sites, a central regulator of spermiogenesis^{63,64}. These data suggest that once
370 activated during early embryogenesis, drivers of germline gene expression like *Rfx2*, *Stra8*, and *Dazl* turn
371 on downstream germline programs, ultimately culminating in the expression of spermiogenesis genes in
372 the adult *Kdm5c*-KO brain. Therefore, we propose KDM5C is recruited via promoter CGIs to act as a brake
373 against runaway activation of germline-specific programs. Future studies should address how KDM5C is
374 targeted to CGIs.

375 The above work provides the mechanistic foundation for KDM5C-mediated repression of tissue and
376 germline-specific genes. However, the contribution of these ectopic, tissue-specific genes towards neurolog-
377 ical impairments is still unknown. In addition to germline genes, we also identified significant enrichment
378 of muscle and liver-enriched transcripts within the *Kdm5c*-KO brain. Intriguingly, select liver and muscle-
379 enriched DEGs do have known roles within the brain, such as the liver-enriched lipid metabolism gene
380 *Apolipoprotein C-I (Apoc1)*²⁸. *APOC1* dysregulation is implicated in Alzheimer's disease in humans⁷⁷ and
381 overexpression of *Apoc1* in the mouse brain can impair learning and memory⁷⁸. KDM5C may therefore be
382 crucial for neurodevelopment by fine-tuning the expression of tissue-enriched, dosage-sensitive genes like
383 *Apoc1*.

384 Given that germline genes have no known functions within the brain, their impact upon neurodevelopment
385 is currently unknown. In *C. elegans*, somatic misexpression of germline genes via loss of *Retinoblastoma*
386 (*Rb*) homologs results in enhanced piRNA signaling and ectopic P granule formation in neurons^{79,80}. Ectopic
387 testicular germline transcripts have also been observed in a variety of cancers, including brain tumors in
388 *Drosophila* and mammals^{81,82} and shown to promote cancer progression^{83–85}. Intriguingly, mouse models
389 and human cells for other chromatin-linked NDDs also display impaired soma-germline demarcation^{86–88},
390 such as mutations in DNA methyltransferase 3b (DNMT3B), H3K9me1/2 methyltransferases G9A/GLP,
391 and methyl-CpG -binding protein 2 (MECP2). Recently, the transcription factor ZMYM2 (ZNF198), whose
392 mutation causes a NDD (OMIM #619522), was also shown to repress germline genes by promoting H3K4me
393 removal and CpGme⁸⁹. Thus, KDM5C is among a growing cohort of neurodevelopmental disorders with
394 erosion of the germline-soma boundary. Further research is required to determine the impact of these
395 germline genes upon neuronal functions and the extent to which this phenomenon occurs in humans.

396 Materials and Methods

397 Classifying tissue-enriched and germline-enriched genes

398 Tissue-enriched differentially expresssd genes (DEGs) were determined by their classification in a previ-
399 ously published dataset from 17 male and female mouse tissues²³. This study defined tissue expression as
400 greater than 1 Fragments Per Kilobase of transcript per Million mapped read (FPKM) and tissue enrichment
401 as at least 4-fold higher expression than any other tissue.

402 We curated a list of germline-enriched genes using an RNA-seq dataset from wild-type and germline-
403 depleted (Kit^{W/W^v}) male and female mouse embryos from embryonic day 12, 14, and 16³², as well as adult
404 male testes²⁹. Germline-enriched genes met the following criteria: 1) their expression is greater than 1
405 FPKM in wild-type germline 2) their expression in any wild-type somatic tissues²³ does not exceed 20%
406 of maximum expression in wild-type germline, and 3) their expression in the germ cell-depleted (Kit^{W/W^v})
407 germline, for any sex or time point, does not exceed 20% of maximum expression in wild-type germline. We

408 defined sperm and egg-biased genes as those whose expression in the opposite sex, at any time point, is no
409 greater than 20% of the gene's maximum expression in a given sex. Genes that did not meet this threshold
410 for either sex were classified as 'unbiased'.

411 **Cell culture**

412 We utilized our previously established cultures of male wild-type and *Kdm5c* knockout (-KO)
413 embryonic stem cells⁴¹. Sex was confirmed by genotyping *Uba1/Uba1y* on the X and Y chromo-
414 somes with the following primers: 5'-TGGATGGTGTGCCAATG-3', 5'-CACCTGCACGTTGCCCTT-
415 3'. Deletion of *Kdm5c* exons 11 and 12, which destabilize KDM5C protein¹², was confirmed
416 through the primers 5'-ATGCCCATATTAAGAGTCCCTG-3', 5'-TCTGCCTTGATGGACTGTT-3', and
417 5'-GGTTCTAACACTCACATAGTG-3'.

418 Embryonic stem cells (ESCs) and epiblast-like cells were cultured using previously established
419 methods³⁷. Briefly, ESCs were initially cultured in primed ESC (pESC) media consisting of KnockOut
420 DMEM (Gibco#10829–018), fetal bovine serum (Gibco#A5209501), KnockOut serum replacement
421 (Invitrogen#10828–028), Glutamax (Gibco#35050-061), Anti-Anti (Gibco#15240-062), MEM Non-essential
422 amino acids (Gibco#11140-050), and beta-mercaptoethanol (Sigma#M7522). They were then transitioned
423 into ground-state, "naïve" ESCs (nESCs) by culturing for four passages in N2B27 media containing
424 DMEM/F12 (Gibco#11330–032), Neurobasal media (Gibco#21103–049), Gluamax (Gibco#35050-061),
425 Anti-Anti (Gibco#15240-062), N2 supplement (Invitrogen#17502048), and B27 supplement without vitamin
426 A (Invitrogen#12587-010), and beta-mercaptoethanol (Sigma#M7522). Both pESC and nESC media
427 were supplemented with 3 µM GSK3 inhibitor CHIR99021 (Sigma #SML1046-5MG), 1 µM MEK inhibitor
428 PD0325901 (Sigma #PZ0162-5MG), and 1,000 units/mL leukemia inhibitory factor (LIF, Millipore#ESG1107).

429 nESCs were differentiated into epiblast-like cells (EpiLCs, 48 hours) and extendend EpiLCs (exEpiLCs,
430 96 hours) by culturing in N2B27 media containing DMEM/F12, Neurobasal media, Gluamax, Anti-Anti, N2
431 supplement, B27 supplement (Invitrogen#17504044), and beta-mercaptoethanol supplemented with 10
432 ng/mL fibroblast growth factor 2 (FGF2, R&D Biotechne 233-FB), and 20 ng/mL activin A (R&D Biotechne
433 338AC050CF), as previously described³⁷.

434 **Real time quantitative PCR (RT-qPCR)**

435 nESCs were differentiated into EpiLCs as described above. Cells were lysed with Tri-reagent BD (Sigma
436 #T3809) at 0, 24, and 48 hours of differentiation. RNA was phase separated with 0.1 µL/µL 1-bromo-3-
437 chloropropane (Sigma #B9673) and then precipitated with with isopropanol (Sigma #I9516) and ethanol puri-
438 fied. For each sample, 2 µg of RNA was reverse transcribed using the ProtoScript II Reverse transcriptase kit
439 from New England Biolabs (NEB #M0368S) and primed with oligo dT. Expression of *Kdm5c* was detected us-
440 ing the primers 5'-CCCATGGAGGCCAGAGAATAAG-3' 5'-CTCAGCGGATAAGAGAATTGCTAC-3' and nor-

441 malized to TBP using the primers 5'-TTCAGAGGATGCTCTAGGAAAGA-3' 5'-CTGTGGAGTAAGTCCTGTGCC-
442 3' with the Power SYBR™ Green PCR Master Mix (ThermoFisher #4367659).

443 **Western Blot**

444 Total protein was extracted during nESC to EpiLC differentiation at 0, 24, and 48 hours by sonicating cells
445 at 20% amplitude for 15 seconds in 2X SDS sample buffer, then boiling at 100 °C for 10 minutes. Proteins
446 were separated on a 7.5% SDS page gel, transferred overnight onto a fluorescent membrane, blotted for
447 rabbit anti-KDM5C (in house, 1:500) and mouse anti-DAXX (Santa Cruz #(H-7): sc-8043, 1:500), and then
448 imaged using the LiCor Odyssey CLx system. Band intensity was quantified using ImageJ.

449 **RNA sequencing (RNA-seq) data analysis**

450 After ensuring read quality via FastQC (v0.11.8), reads were then mapped to the mm10 *Mus musculus*
451 genome (Gencode) using STAR (v2.5.3a), during which we removed duplicates and kept only uniquely
452 mapped reads. Count files were generated by FeatureCounts (Subread v1.5.0), and BAM files were
453 converted to bigwigs using deeptools (v3.1.3) and visualized by the UCSC genome browser⁶⁸. RStudio
454 (v3.6.0) was then used to analyze counts files by DESeq2 (v1.26.0)²⁴ to identify differentially expressed
455 genes (DEGs) with a q-value (p-adjusted via FDR/Benjamini–Hochberg correction) less than 0.1 and a log2
456 fold change greater than 0.5. For all DESeq2 analyses, log2 fold changes were calculated with IfcShrink
457 using the ashR package⁹⁰. MA-plots were generated by ggpubr (v0.6.0), and Eulerr diagrams were generated
458 by eulerr (v6.1.1). Boxplots and scatterplots were generated by ggpubr (v0.6.0) and ggplot2 (v3.3.2). The
459 Upset plot was generated via the package UpSetR (v1.4.0)⁹¹. Gene ontology (GO) analyses were performed
460 by the R package enrichPlot (v1.16.2) using the biological processes setting and compareCluster.

461 **Chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq) data analysis**

462 ChIP-seq reads were aligned to mm10 using Bowtie1 (v1.1.2) allowing up to two mismatches. Only
463 uniquely mapped reads were used for analysis. Peaks were called using MACS2 software (v2.2.9.1) using
464 input BAM files for normalization, with filters for a q-value < 0.1 and a fold enrichment > 1. We removed
465 “black-listed” genomic regions that often give aberrant signals. Common peak sets were obtained in R via
466 DiffBind⁹² (v3.6.5). In the case of KDM5C ChIP-seq, *Kdm5c*-KO false-positive peaks were then removed from
467 wild-type samples using bedtools (v2.25.0). Peak proximity to genomic loci was determined by ChIPSeeker⁹³
468 (v1.32.1). Gene ontology (GO) analyses were performed by the R package enrichPlot (v1.16.2) using the
469 biological processes setting and compareCluster. Enriched motifs were identified using HOMER⁵⁹ to search
470 for known motifs within 500 base pairs upstream and downstream of the transcription start site. Average binding
471 across genes was visualized using deeptools (v3.1.3). Bigwigs were visualized using the UCSC genome
472 browser⁶⁸.

473 **CpG island (CGI) analysis**

474 Locations of CpG islands were determined through the mm10 UCSC genome browser CpG island track⁶⁸,
475 which classified CGIs as regions that have greater than 50% GC content, are larger than 200 base pairs,
476 and have a ratio of CG dinucleotides observed over the expected amount greater than 0.6. CGI genomic
477 coordinates were then annotated using ChIPseeker⁹³ (v1.32.1) and filtered for ones that lie within promoters
478 of germline-enriched genes (TSS ± 500).

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

480 Genomic DNA (gDNA) from male naïve ESCs and extended EpiLCs was extracted using the Wizard
481 Genomic DNA Purification Kit (Promega A1120), following the instructions for Tissue Culture Cells. gDNA
482 from two wild-types and two *Kdm5c*-KOs of each cell type was sent to Novogene for WGBS using the
483 Illumina NovaSeq X Plus platform and sequenced for 150 bp paired-end reads (PE150). All samples had
484 greater than 99% bisulfite conversion rates. Reads were adapter and quality trimmed with Trim Galore
485 (v0.6.10) and aligned to the mm10 genome using Bismark⁹⁴ (v0.22.1). Analysis of differential methylation at
486 gene promoters was performed using methylKit⁶⁹ (v1.28.0) with a minimum coverage of 3 paired reads, a
487 percentage greater than 25% or less than -25%, and q-value less than 0.01. methylKit was also used to
488 calculate average percentage methylation at germline gene promoters. Methylation bedgraph tracks were
489 generated via Bismark and visualized using the UCSC genome browser⁶⁸.

490 **Data availability**

491 **WGBS in wild-type and *Kdm5c*-KO ESCs and exEpiLCs**

492 Raw fastq files are deposited in the Sequence Read Archive (SRA) <https://www.ncbi.nlm.nih.gov/sra>
493 under the bioProject PRJNA1165148.

494 **Published datasets**

495 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
496 adult amygdala and hippocampus²², available at GEO: GSE127722. Male and female wild-type, *Kdm5c*-KO,
497 and *Kdm5c*-HET EpiLCs⁴¹ are available at GEO: GSE96797.

499 Previously published ChIP-seq experiments included KDM5C binding in wild-type and *Kdm5c*-KO
500 EpiLCs⁴¹ (available at GEO: GSE96797) and mouse primary neuron cultures (PNCs) from the cortex
501 and hippocampus¹² (available at GEO: GSE61036). ChIP-seq of histone 3 lysine 4 dimethylation (H3K4me2)
502 in male wild-type and *Kdm5c*-KO EpiLCs⁴¹ is also available at GEO: GSE96797. ChIP-seq of histone 3 lysine
503 4 trimethylation (H3K4me3) in wild-type and *Kdm5c*-KO male amygdala²² are available at GEO: GSE127817.

504 **Data analysis**

505 Scripts used to generate the results, tables, and figures of this study are available via the GitHub
506 repository: https://github.com/kbonefas/KDM5C_Germ_Mechanism

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522 **Author contributions**

523 K.M.B. and S.I. conceived the study and designed the experiments. I.V. generated the ESC and exEpiLC
524 WGBS data. K.M.B performed all data analysis and all other experiments. The manuscript was written by
525 K.M.B and S.I., and edited by K.M.B, S.I., and I.V.

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715 **Figures and Tables**

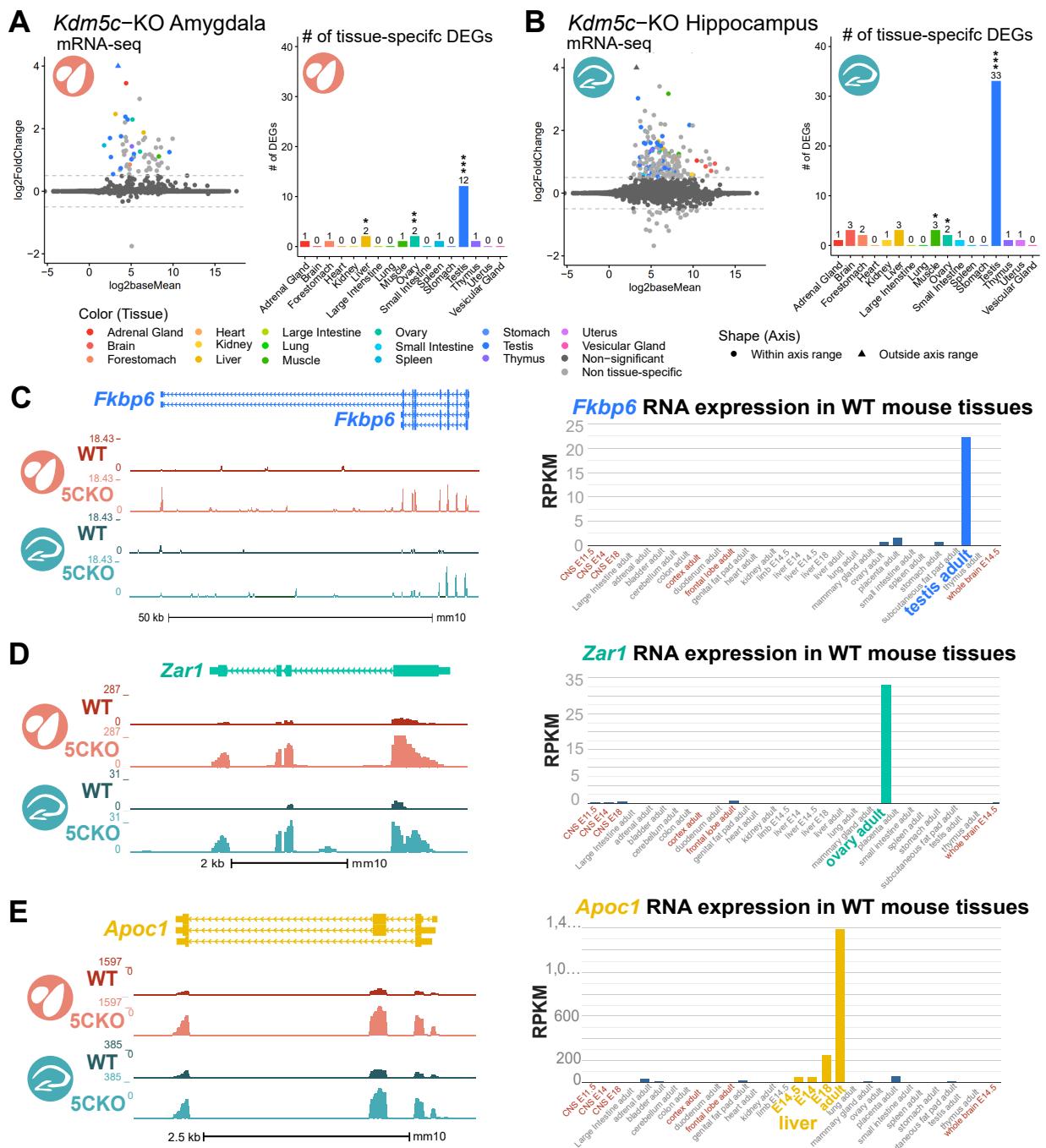


Figure 1: Tissue-enriched genes are misexpressed in the *Kdm5c*-KO brain. **A-B.** Expression of tissue-enriched genes (Li et al 2017) in the male *Kdm5c*-KO amygdala (A) and hippocampus (B). Left - MA plot of mRNA-sequencing. Right - Number of tissue-enriched differentially expressed genes (DEGs). * p<0.05, ** p<0.01, *** p<0.001, Fisher's Exact Test. **C.** Left - UCSC browser view 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) (Average, n = 4). Right - Expression of *Cyct* in wild-type tissues from NCBI Gene, with testis highlighted in blue and brain tissues highlighted in red. **D.** Left - UCSC browser view 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 - UCSC browser view 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.

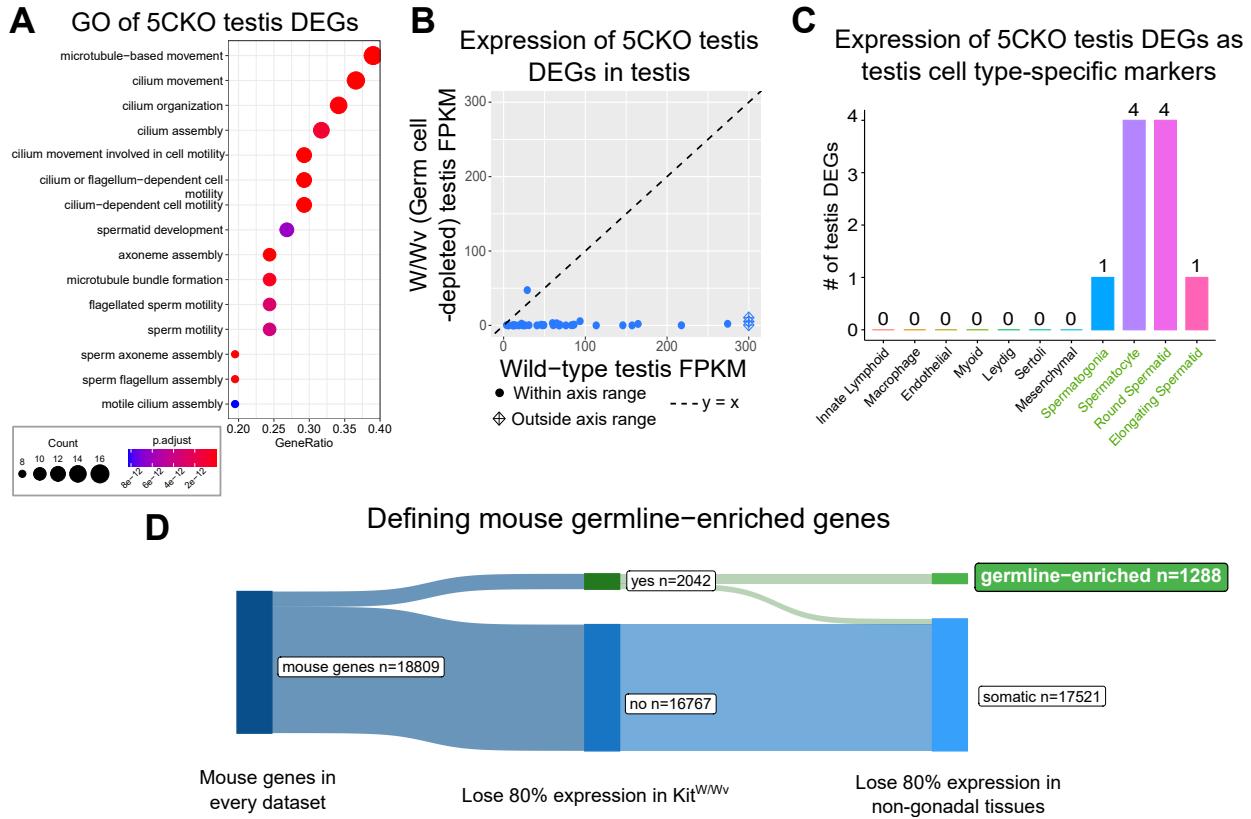


Figure 2: Aberrant transcription of germline genes in the *Kdm5c*-KO in the brain. **A.** enrichPlot gene ontology (GO) of *Kdm5c*-KO amygdala and hippocampus testis-enriched DEGs **B.** Expression of testis DEGs in wild-type (WT) testis versus germ cell-depleted (W/Wv) testis (Mueller et al 2013). Expression 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 W/Wv mice and in adult mouse non-gonadal tissues (Li et al 2017).

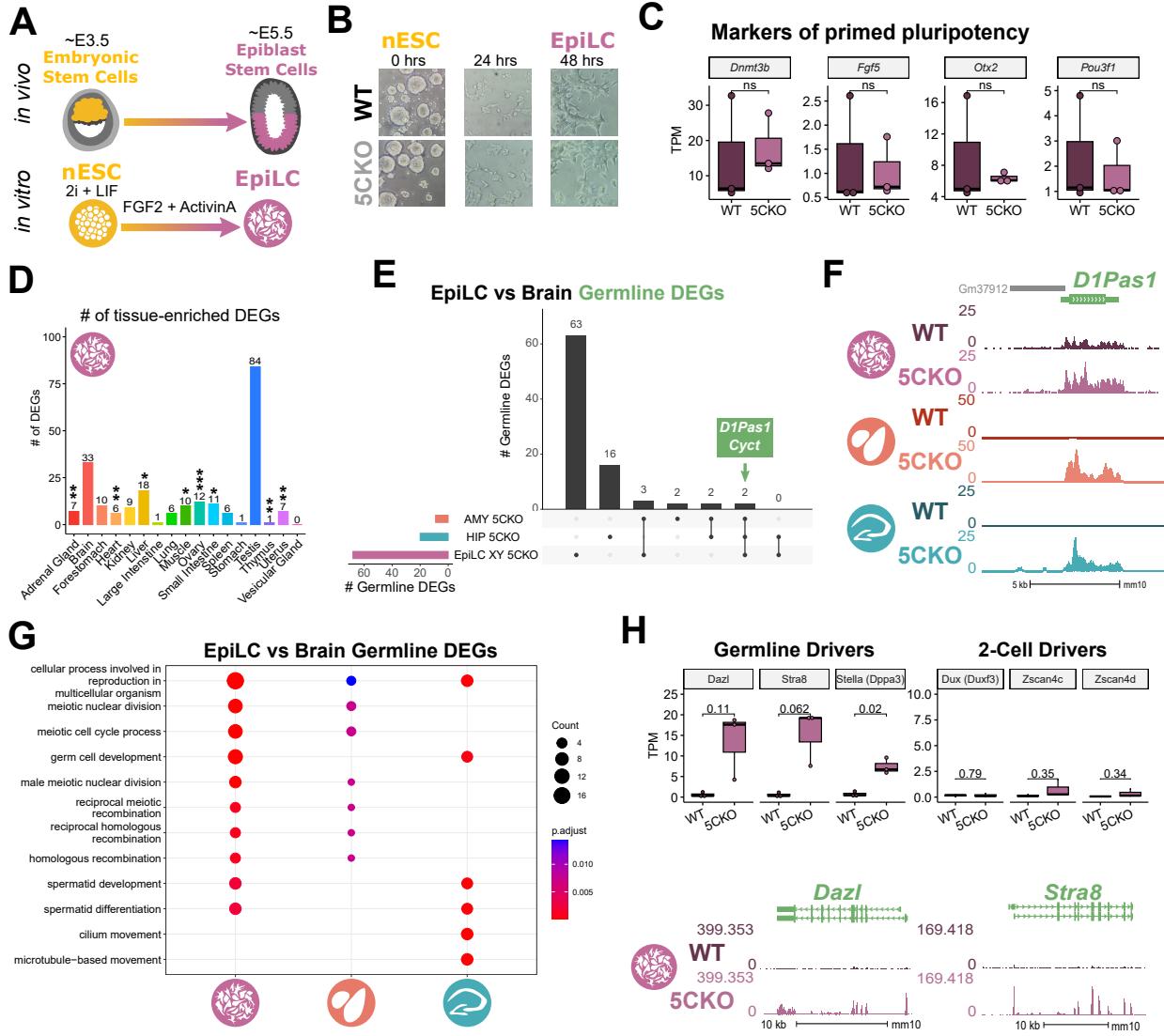


Figure 3: Kdm5c-KO epiblast-like cells express key drivers of germline identity **A.** Top - Diagram of *in vivo* differentiation of embryonic stem cells (ESCs) of the inner cell mass into epiblast stem cells. Bottom - *in vitro* differentiation of ESCs into epiblast-like cells (EpiLCs). **B.** Representative images of male wild-type (WT) and *Kdm5c*-KO (5CKO) cells during ESC to EpiLC differentiation. Brightfield images taken at 20X. **C.** 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). **D.** 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. **E.** Upset plot displaying the overlap of germline DEGs expressed in *Kdm5c*-KO EpiLCs, amygdala (AMY), and hippocampus (HIP) RNA-seq datasets. **F.** UCSC browser view of an example germline gene, *D1Pas1*, that is dysregulated *Kdm5c*-KO EpiLCs (top, purple. Average, $n = 3$), amygdala (middle, red. Average, $n = 4$), and hippocampus (bottom, blue. Average, $n = 4$). **G.** enrichPlot gene ontology analysis comparing enriched biological processes 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 - UCSC browser view of *Dazl* and *Stra8* expression in wild-type and *Kdm5c*-KO EpiLCs (Average, $n = 3$).

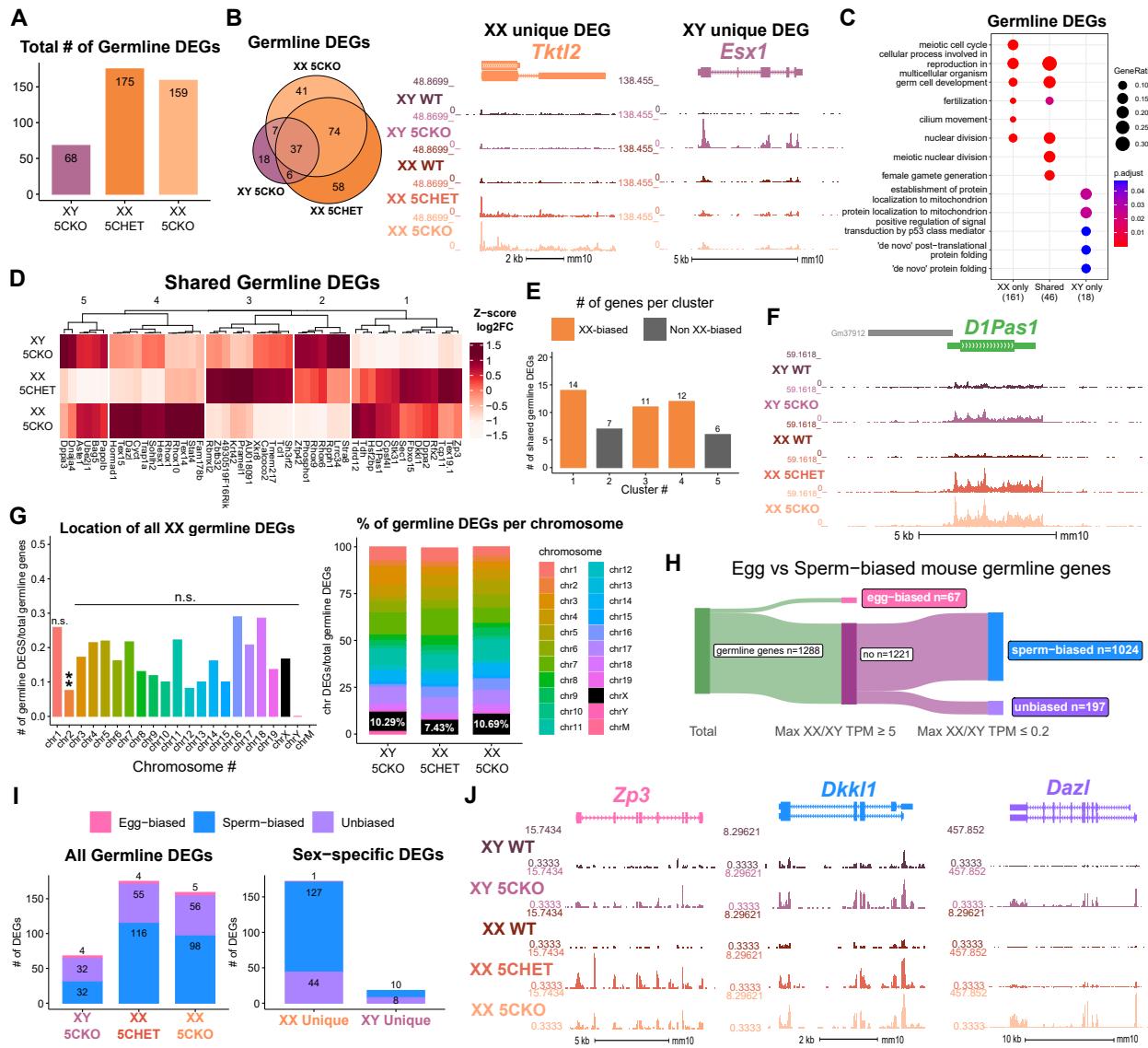


Figure 4: Chromosomal sex influences *Kdm5c*-KO germline gene misexpression. **A.** Total number of germline-enriched RNA-seq DEGs for male hemizygous *Kdm5c* knockout EpiLCs (XY 5CKO, purple), female heterozygous *Kdm5c* knockout (XX 5CHET, orange), female homozygous *Kdm5c* knockout (XX 5CKO, light orange) EpiLCs. **B.** Left - Eulerr overlap of *Kdm5c* mutant male and female EpiLC germline DEGs. Right - Example of germline DEGs unique to females or males, *Tktl2* and *Esx1*. **C.** enrichPlot gene ontology analysis comparing enriched biological processes for germline DEGs shared between *Kdm5c* mutant males and females (Shared), or unique to one sex (XX only or XY only). **D.** Heatmap of germline DEGs shared between male and female mutants. Color is the average log 2 fold change from sex-matched wild-type, z-scored across rows. **E.** Number of genes within each cluster from D. Clusters with higher expression in females compared to males (XX-biased) highlighted in orange. **F.** UCSC browser view of a male and female shared germline DEG *D1Pas1* that is more highly expressed in female mutants (Average, n = 3). **G.** Left - Number of all female germline DEGs located on each chromosome over the total number of germline-enriched genes on that chromosome. P-values for Fisher Exact Test, ** p < 0.01, n.s. non-significant. Germline DEGs were only significant for chromosome 2, in which they were significantly depleted. Right - Percentage of germline DEGs that lie on each chromosome for each *Kdm5c* mutant. X chromosome highlighted in black. **H.** Sankey diagram classifying egg-biased (pink) and sperm-biased (blue) and unbiased (purple) mouse germline-enriched genes. **I.** Number of egg, sperm, or unbiased germline DEGs for male and female *Kdm5c* mutants. **J.** UCSC browser view of egg-biased (*Zp3*), sperm-biased (*Dkk1*), and unbiased (*Dazl*) germline genes dysregulated in both male and female *Kdm5c* mutants (Average of n = 3).

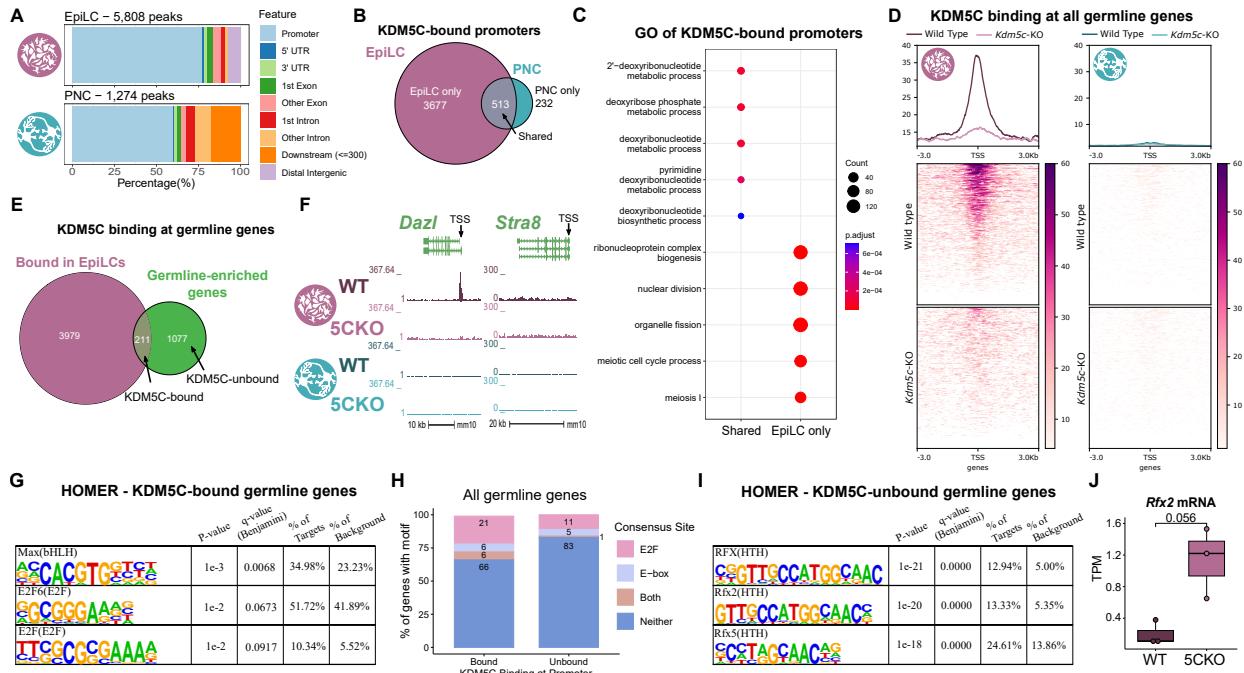


Figure 5: KDM5C binds to a subset of germline gene promoters during early embryogenesis. **A.** ChIPseeker localization of KDM5C peaks at different genomic regions in EpiLCs (top) and hippocampal and cortex primary neuron cultures (PNCs, bottom). **B.** Overlap of genes with KDM5C bound to their promoters ($TSS \pm 500$) 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.** Eulerr overlap of germline-enriched genes (green) with significant KDM5C binding at their promoter in EpiLCs (purple). **F.** Example KDM5C ChIP-seq signal around the *Dazl* TSS but not *Stra8* in EpiLCs. **G.** HOMER motif analysis of all KDM5C-bound germline gene promoters, highlighting significant enrichment of MAX, E2F6, and E2F motifs. **H.** Number of all gene promoters bound or unbound by KDM5C with instances of the E2F or E-box consensus sequence. **I.** HOMER motif analysis of all KDM5C-unbound germline gene promoters, highlighting significant enrichment of RFX family transcription factor motifs. **J.** Expression of RNA-seq DEG *Rfx2* in wild-type and *Kdm5c*-KO EpiLCs. P-value of Welch's t-test, expression in transcripts per million (TPM).

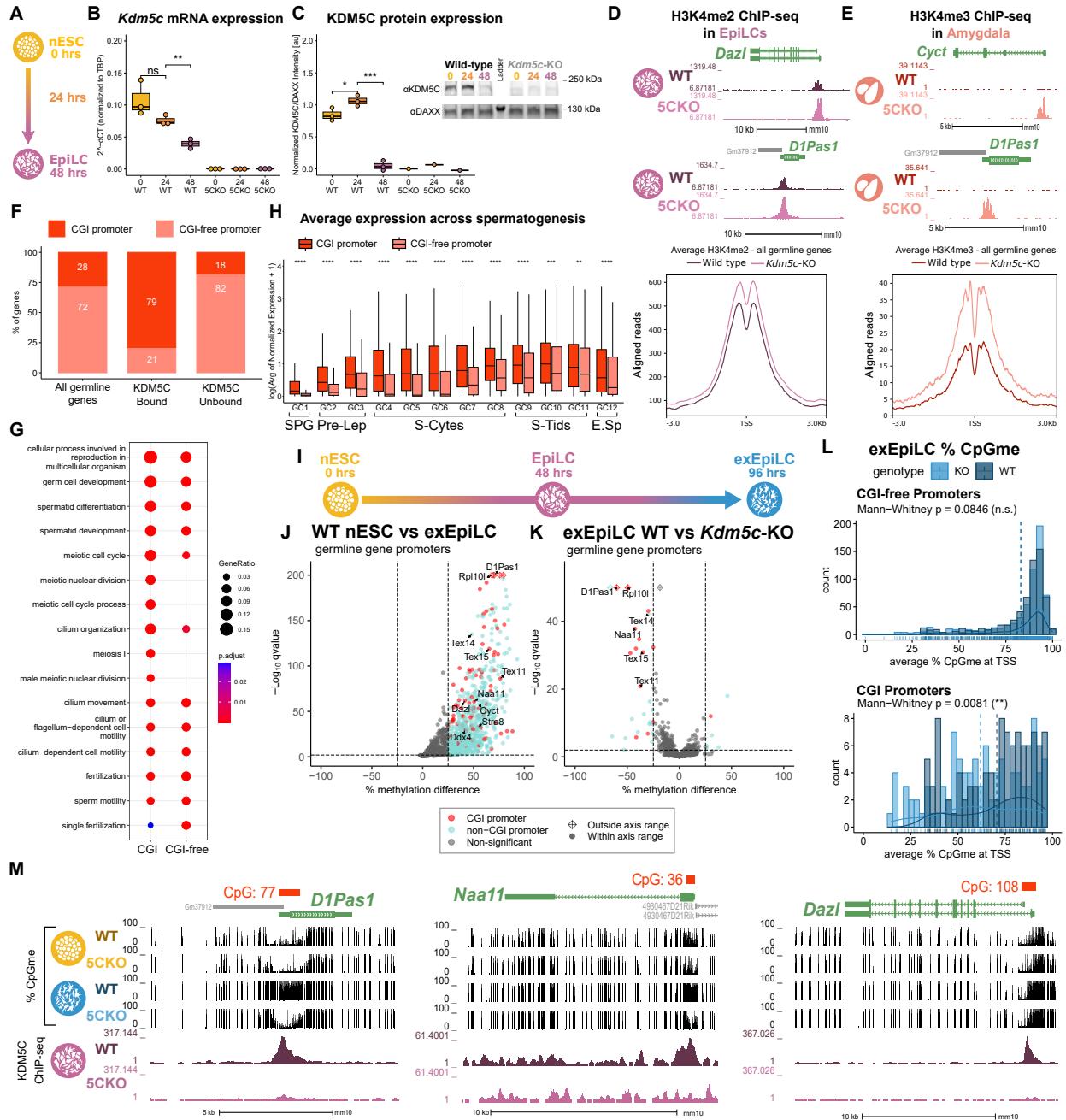


Figure 6: KDM5C promotes long-term silencing of germline genes via DNA methylation at CpG islands. **A.** Diagram of embryonic stem cell (ESC) to epiblast-like cell (EpiLC) differentiation and collection time points. **B.** Real time quantitative PCR (RT-qPCR) of *Kdm5c* mRNA expression in wild-type (WT) and *Kdm5c*-KO (5CKO) ESCs at 0, 24, and 48 hours of differentiation into EpiLCs. Expression calculated in comparison to TBP mRNA expression ($2^{-\Delta\Delta CT}$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Welch's t-test. **C.** 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. **D.** Top - Representative UCSC browser view of histone 3 lysine 4 dimethylation (H3K4me2) ChIP-seq signal at two germline genes in wild-type and *Kdm5c*-KO EpiLCs. Bottom - Average H3K4me2 signal at the TSS of all germline-enriched genes in wild-type (dark purple) and *Kdm5c*-KO (light purple) EpiLCs. **E.** Top - Representative UCSC browser view of histone 3 lysine 4 trimethylation (H3K4me3) ChIP-seq signal at two germline genes in the wild-type (WT) and *Kdm5c*-KO (5CKO) adult amygdala. Bottom - Average H3K4me3 signal at the transcription start site (TSS) of all germline-enriched genes in wild-type (dark red) and *Kdm5c*-KO (light red) amygdala. **F.** Percentage of germline genes that harbor CpG islands (CGIs) in their promoters (CGI promoter, red), based on UCSC annotation. Left - percentage of all germline-enriched genes, middle - KDM5C-bound germline genes, and right - KDM5C-unbound germline genes. (Legend continued on next page.)

Figure 6: KDM5C promotes long-term silencing of germline genes via DNA methylation at CpG islands. (Legend continued.) **G.** enrichPlot gene ontology analysis of germline genes with (CGI-promoter) or without (CGI-free) CGIs in their promoter. **H.** Expression of germline genes with (CGI-promoter, red) or without (CGI-free, salmon) CGIs in their promoter across stages of spermatogenesis from Green et al 2018. SPG - spermatogonia, Pre-Lep - preleptotene spermatocytes, S-Cytes - meiotic spermatocytes, S-Tids - post-meiotic haploid round spermatids, E.Sp - elongating spermatids. Wilcoxon test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. **I.** Diagram of ESC to extended EpiLC (exEpiLC) differentiation. **J.** Volcano plot of whole genome bisulfite sequencing (WGBS) comparing CpG methylation (CpGme) at germline gene promoters ($TSS \pm 500$) in wild-type (WT) ESCs versus exEpiLCs. Significantly differentially methylated promoters ($q < 0.01$, $|methylated difference| > 25\%$) with CGIs in red, CGI-free promoters in light blue **K.** Volcano plot of WGBS of wild-type (WT) versus *Kdm5c*-KO exEpiLCs for germline gene promoters. Promoters with CGIs in red, CGI-free promoters in light blue. **L.** Histogram of average percent CpGme at the promoter of germline genes with or without CGIs. Wild-type in navy and *Kdm5c*-KO (KO) in light blue. Dashed lines are average methylation for each genotype, p-values for Mann-Whitney U test. **M.** UCSC browser view of germline genes, showing UCSC annotated CGI with number of CpGs, representative CpGme in wild-type (WT) and *Kdm5c*-KO (5CKO) ESCs and exEpiLCs, and KDM5C ChIP-seq signal in EpiLCs.