

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

2 intellectual disability factor KDM5C

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5 Abstract

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

30 Introduction

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

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

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

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

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

Results

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

Previous RNA sequencing (RNA-seq) in the adult hippocampus revealed ectopic expression of some testis genes within the male *Kdm5c* knockout (-KO) brain¹⁰. It is currently unclear if the testis is the only tissue type misexpressed in the *Kdm5c*-KO brain. We thus characterized dysregulation of *Kdm5c*-KO brain tissue identity by systematically assessing the expression of genes enriched in 17 mouse tissues²⁶, using our published mRNA-seq datasets of the adult amygdala and hippocampus for male mice with constitutive

98 knockout of *Kdm5c*²⁷.

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

108 Interestingly, we also observed significant enrichment of ovary-biased DEGs in both the amygdala and
109 hippocampus (Amygdala p = 0.00574, Odds Ratio = 18.7; Hippocampus p = 0.048, Odds Ratio = 5.88,
110 Fisher's Exact) (Figure 1D). Ovary-enriched DEGs included *Zygotic arrest 1* (*Zar1*), which sequesters mRNAs
111 in oocytes for meiotic maturation and early zygote development³¹ (Figure 1D). Given the *Kdm5c*-KO mice
112 we analyzed are male, this demonstrates ectopic expression of tissue-enriched genes is independent of
113 organismal sex.

114 Although not consistent across brain regions, we also found significant enrichment of DEGs biased
115 towards two non-gonadal tissues - the liver (Amygdala p = 0.0398, Odds Ratio = 6.58, Fisher's Exact Test)
116 and the muscle (Hippocampus p = 0.0104, Odds Ratio = 6.95, Fisher's Exact Test). A liver-biased DEG
117 dysregulated in both the hippocampus and amygdala is *Apolipoprotein C-I* (*Apoc1*), a lipoprotein metabolism
118 and transport gene³² that has been implicated in Alzheimer's disease³³ (Figure 1E). For all tissue-enriched
119 DEGs, aberrantly expressed mRNAs are polyadenylated and spliced into mature transcripts (Figure 1C-E).
120 Of note, we observed little to no dysregulation of brain-enriched genes (Amygdala p = 1; Hippocampus p =
121 0.74, Fisher's Exact), despite the fact these are brain samples and the brain has the second highest total
122 number of tissue-enriched genes (708 genes). Together, these results suggest the aberrant expression of
123 tissue-enriched genes within the brain is a major effect of KDM5C loss.

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

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

131 To further validate if these testis DEGs are truly germline genes, we then assessed their expression in
132 somatic versus germ cells within the testis. We first compared their expression in wild-type testes to those

133 without germ cells³⁴, which was accomplished by heterozygous *W* and *Wv* mutations in the enzymatic
134 domain of *c-Kit* (*Kit*^{W/Wv})³⁵. Almost all *Kdm5c*-KO testis-enriched DEGs lost expression with germ cell
135 depletion (Figure 2B). We then assessed testis-enriched DEG expression in a published single cell RNA-seq
136 dataset that identified cell type-specific markers within the testis³⁶. Some *Kdm5c*-KO testis-enriched DEGs
137 were classified as specific markers for different germ cell types (e.g. spermatogonia, spermatocytes, round
138 spermatids, and elongating spermatids), yet none marked somatic cells (Figure 2C). Together, these data
139 demonstrate that the *Kdm5c*-KO brain aberrantly expresses germline genes, but not somatic testicular genes,
140 reflecting an erosion between somatic versus germline identity.

141 We then wanted to characterize germline gene misexpression with *Kdm5c* loss more deeply, but lacked
142 a comprehensive list of mouse germline genes. We therefore generated a list of germline-enriched genes
143 using RNA-seq datasets of *Kit*^{W/Wv} mice that included males and females at embryonic day 12, 14, and 16³⁷
144 and adult male testes³⁴. We defined genes as germline-enriched if their expression met the following criteria:
145 1) their expression is greater than 1 FPKM in wild-type gonads 2) their expression in any non-gonadal tissue
146 of adult wild type mice²⁶ does not exceed 20% of their maximum expression in the wild-type germline, and
147 3) their expression in the germ cell-depleted gonads, for any sex or time point, does not exceed 20% of
148 their maximum expression in the wild-type germline. These criteria yielded 1,288 germline-enriched genes
149 (Figure 2D), which was hereafter used as a resource to globally assess germline gene misexpression with
150 *Kdm5c* loss (Supplementary table 1).

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

152 Germ cells are typically distinguished from somatic cells soon after the embryo implants into the uterine
153 wall^{38,39}. A subset of epiblast stem cells become the primordial germ cells (PGCs) while the remainder
154 differentiate into the ectoderm, mesoderm, and endoderm to form the somatic tissues⁴⁰. This developmental
155 time point can be modeled *in vitro* through differentiation of embryonic stem cells (ESCs) into epiblast-like
156 stem cells (EpiLCs) (Figure 3A, top)^{41,42}. While some germline-enriched genes are also expressed in
157 naïve embryonic stem cells (ESCs) and in the 2-cell stage^{43–45}, they are silenced as they differentiate into
158 EpiLCs^{19,46}. Therefore, we tested if KDM5C was necessary for silencing germline genes at this developmental
159 stage by evaluating the impact of *Kdm5c* loss in male EpiLCs.

160 We first identified *Kdm5c*-KO EpiLC DEGs in our previously published RNA-seq dataset⁴⁷ (DESeq2, log2
161 fold change > 0.5, q < 0.1) and then assessed tissue-enriched gene expression. Similar to the *Kdm5c*-KO
162 brain, we observed general dysregulation of tissue-enriched genes, with the largest number of genes
163 belonging to the brain and testis, although they were not significantly enriched (Figure 3B). Markers of primed
164 pluripotency, such as *Dnmt3b*, *Fgf5*, *Pou3f1*, and *Otx2*, were unaltered in *Kdm5c*-KO EpiLCs (Figure 3C).
165 Furthermore, *Kdm5c*-KO cell morphology during ESC to EpiLC differentiation (Figure 3D) appeared normal,
166 indicating KDM5C loss does not impair EpiLC formation.

167 To determine if germline DEGs are constitutively dysregulated or if they can change over the course of

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

Notably, DEGs unique to *Kdm5c*-KO EpiLCs included key drivers of germline identity, such as *Stimulated by retinoic acid 8 (Stra8)* and *Deleted in azoospermia like (Dazl)* (Figure 3H). These genes are typically expressed during embryonic germ cell development to commit PGCs to the germline fate, but are also expressed later in life to trigger meiotic gene expression programs^{48–50}. Of note, some germline genes, including *Dazl*, are also expressed in the two-cell embryo^{44,51}. However, we did not see misexpression of two-cell embryo-specific genes, like *Duxf3 (Dux)* ($q = 0.337$) and *Zscan4d* ($q = 0.381$), indicating *Kdm5c*-KO in EpiLCs do not revert back to a 2-cell-like state (Figure 3H). Altogether, these results demonstrate that while the *Kdm5c*-KO brain primarily expresses spermiogenesis genes, *Kdm5c*-KO EpiLCs express key drivers of germline identity and meiosis.

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

It is currently unknown if the misexpression of germline genes is influenced by chromosomal sex, as previous studies on germline gene repressors have focused on their regulation in males^{18–20,52,53}. We explored the impact of sex upon germline gene misexpression by comparing their dysregulation in male *Kdm5c* hemizygous knockout (XY *Kdm5c*-KO), female homozygous knockout (XX *Kdm5c*-KO) and female heterozygous (XX *Kdm5c*-HET) EpiLCs.⁴⁷ We first identified differentially expressed genes (DEGs) compared to sex-matched wild-type controls (DESeq2²⁸, log2 fold change > 0.5, $q < 0.1$) and then filtered for germline-enriched genes.

Homozygous and heterozygous females expressed over double the number of germline-enriched genes than hemizygous males (Figure 4A). While the majority of germline DEGs in *Kdm5c*-KO males were also dysregulated in females (74%), there were also many male-specific and female-specific germline DEGs (Figure 4B). We the compared the known functions of germline genes dysregulated only in females (XX only - XX *Kdm5c*-KO, XX *Kdm5c*-HET, or both), only in males (XY only), or in all samples (shared). Female-specific germline DEGs were enriched for meiotic (GO:XXX meiotic cell cycle) and flagellar (GO:XXX cilium movement) functions, male-specific DEGs had mitochondrial and cell signaling gene functions (GO:XXX protein localization to mitochondrion). Germline transcripts expressed in both sexes were enriched for meiotic

202 (meiotic nuclear division) and egg-specific functions (female gamete generation).
203 The majority of germline genes expressed in both sexes had a greater log2 fold change from wild-type
204 in females compared to males (Figure 4D). This increased degree of dysregulation in females, along with
205 the increased total number of germline genes, indicates females are more sensitive to the loss of KDM5C-
206 mediated suppression of germline genes. Female sensitivity could be due to improper X chromosome
207 inactivation (XCI) in *Kdm5c* mutants, as many spermatogenesis genes lie on the X chromosome^{54,55}.
208 However, both shared and female-specific germline DEGs were not biased towards the X chromosome, with
209 the majority of genes instead lying on autosomes (Figure 4G). Thus, while female EpiLCs are more prone to
210 germline gene misexpression with KDM5C loss, it is likely independent of potential defects in XCI.

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

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

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

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

233 To address these questions, we analyzed KDM5C chromatin immunoprecipitation followed by DNA
234 sequencing (ChIP-seq) datasets in EpiLCs⁴⁷ and primary forebrain neuron cultures (PNCs)¹⁵. EpiLCs had a

235 higher total number of KDM5C peaks than PNCs (EpiLCs: 5,808, PNCs: 1,276, MACS2 q < 0.1 and fold
236 enrichment > 1, removal of *Kdm5c*-KO false positive peaks) and KDM5C was primarily localized to gene
237 promoters in both cell types (EpiLCs: 4,190, PNCs: 745 +/- 500bp from the TSS), although PNCs showed
238 increased localization to non-promoter regions (Figure 5A).

239 The majority of promoters bound by KDM5C in PNCs were also bound in EpiLCs (513 shared promoters),
240 however a large portion of gene promoters were only bound by KDM5C in EpiLCs (3677 EpiLC only
241 promoters) (Figure 5B). Genes bound by KDM5C in both PNCs and EpiLCs were enriched for functions
242 involving nucleic acid turnover, such as deoxyribonucleotide metabolic process (GO:0009262, p.adjust =
243 8.28e-05) (Figure 5C). Germline-specific ontologies were only enriched in promoters unique to EpiLCs, such
244 as meiotic nuclear division (GO: 0007127 p.adjust = 6.77e-16) and meiotic cell cycle process (GO:1903046,
245 p.adjust = 5.05e-16) (Figure 5C). There were no ontologies significantly enriched for genes only bound
246 by KDM5C in PNCs. We next evaluated KDM5C binding around the transcription start site (TSS) of all
247 germline-enriched genes. In EpiLCs, We observed modest KDM5C signal at about half of all germline genes
248 (Figure 5D). Based on our ChIP-seq peak cut-off criteria, KDM5C was strongly bound to about 10% of
249 germline gene promoters in EpiLCs (Figure 5E). In condordance with our gene ontology results, we did not
250 observe KDM5C accumulation at any germline gene promoters in PNCs (Figure 5D). Together, these results
251 demonstrate KDM5C is significantly enriched at a subset of germline gene promoters in EpiLCs, including
252 meiotic genes, but does not directly regulate germline genes in neurons.

253 Many germline-specific genes are suppressed by the transcription factor heterodimers E2F/DP1 and
254 MGA/MAX, which respectively bind E2F and E-box motifs^{20,52,53,56,57}. To elucidate if KDM5C is recruited to
255 germline gene promoters by a similar mechanism, we used HOMER to identify transcription factor motifs
256 enriched at KDM5C-bound or unbound germline gene promoters⁵⁸ (TSS +/- 500 bp, q-value < 0.1). MAX
257 and E2F6 binding sites were significantly enriched at germline genes bound by KDM5C in EpiLCs, but
258 not at germline genes unbound by KDM5C (MAX q-value: 0.0068, E2F6 q-value: 0.0673, E2F q-value:
259 0.0917) (Figure 5F). One third of KDM5C-bound promoters contained the consensus sequence for either
260 E2F6 (E2F, 5'-TCCCGC-3'), MGA (E-box, 5'-CACGTG-3'), or both, but only 17% of KDM5C-unbound genes
261 contained these motifs (Figure 5G). KDM5C-unbound germline genes were instead enriched for multiple
262 RFX transcription factor binding sites (RFX q-value < 0.0001, Rfx2 q-value < 0.0001, Rfx5 q-value < 0.0001)
263 (Figure 5H). RFX transcription factors bind X-box motifs⁵⁹ to promote ciliogenesis^{60,61}. Enriched RFX
264 transcription factors included RFX2, a central regulator of post-meiotic spermatogenesis^{62,63}. Interestingly,
265 RFX2 mRNA is upregulated in *Kdm5c*-KO EpiLCs (Figure 5I), but is not a direct target of KDM5C. Thus,
266 distinct transcription factor programs regulate the expression of KDM5C-bound and unbound germline genes.

267 Finally, we compared KDM5C binding at the promoters of RNA-seq DEGs to determine if the germline
268 genes transcribed in *Kdm5c*-KO cells are direct targets of KDM5C (Figure 5J-L). In EpiLCs, KDM5C was
269 bound to about one third of EpiLC-specific and brain-specific germline DEG promoters (EpiLC only: 36%,
270 Brain only: 33.3%). Again, we did not observe any KDM5C binding at germline gene promoters in PNCs,

even for brain-specific DEGs. Some notable differences in KDM5C binding for EpiLC-specific DEGs included *Dazl* and *Stra8*, two key drivers of germline identity discussed above. Although both mRNAs are expressed in *Kdm5c*-KO EpiLCs, KDM5C is only bound to *Dazl*'s promoter and not *Stra8*'s in EpiLCs (Figure 5K). In contrast to the unique DEGs, 3 out of the 4 genes dysregulated in both the brain and EpiLCs are bound by KDM5C around the TSS. Altogether, this suggests KDM5C decommissions a subset of germline genes in EpiLCs but is no longer recruited to germline gene promoters in neurons. Furthermore, the majority of germline mRNAs expressed in *Kdm5c*-KO cells are dysregulated independent of direct KDM5C recruitment to their promoters.

KDM5C promotes *de novo* DNA methylation at germline genes (if there are differences, say at germline gene CpG islands)

In the early embryo, germline gene promoters are initially decorated with repressive histone modifications and are then silenced long-term via DNA CpG methylation (CpGme)^{18,19,46,64}. Our results above indicate KDM5C also acts at germline gene promoters during this time period. However, how KDM5C interacts with other germline gene silencing mechanisms is currently unclear. KDM5C is generally thought to suppress transcription through erasure of histone 3 lysine 4 di- and trimethylation (H3K4me2/3)¹¹, yet KDM5C's catalytic activity was recently shown to be dispensable for suppressing *Dazl* in undifferentiated ESCs⁵¹. Since H3K4me3 impedes *de novo* CpGme placement^{65,66}, KDM5C's catalytic activity may instead be required later in development for long-term silencing of germline genes. In support of this, CpGme is markedly reduced at two germline gene promoters in the *Kdm5c*-KO adult hippocampus¹⁰.

Based on the above observations, we hypothesized KDM5C erases H3K4me3 to promote the initial placement of CpGme at germline gene promoters in EpiLCs. To test this hypothesis, we first characterized KDM5C's substrates, histone 3 lysine 4 di- and trimethylation (H3K4me2/3) in our previously published ChIP-seq datasets of the wild type and *Kdm5c*-KO amygdala²⁷ and EpiLCs⁴⁷. In congruence with previous work in the *Kdm5c*-KO hippocampus¹⁰, we observed aberrant accumulation of H3K4me3 around the transcription start site (TSS) of germline genes in the *Kdm5c*-KO amygdala (Figure 6A). We additionally found a marked increase in H3K4me2 around the TSS of germline genes in *Kdm5c*-KO EpiLCs (Figure 6B).

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

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

- 305 • Germline genes are known to accumulate CpGme at (CGIs) during the transition from naive to primed
306 pluripotency.
- 307 • We first identified the number of germline genes with CGIs neighboring their promoter.
308 – Found XXX% of germline genes had CGIs, XXX% of which were direct KDM5C targets in EpiLCs
309 (Eulerr).
- 310 • We then curated a list of germline genes that significantly gained CpGme in wild-type exEpiLCs
311 compared to ESCs.
312 – Example gene bedgraph
313 – Majority of CGI germline genes?
- 314 • Out of the CGI genes, which had significantly reduced CpGme in 5CKO (heatmap of % methylation)
315 – Highlight interesting genes affected vs unaffected by KDM5C (especially if same/different from
316 E2F6, PRC1.6, Setdb1 targets)
317 – CGIs that never gain DNAme in WT
318 – Although wild-type cells accumulated high levels of DNA methylation at germline gene promoters
319 over the course of ESCs to exEpiLC differentiation, DNA methylation was markedly reduced in
320 *Kdm5c*-KO exEpiLCs (Figure 6F).
- 321 • Non-CGI germline genes, any significant changes at their promoter? (Heatmap again?)
322 • Altogether, this suggests KDM5C is dynamically regulated during ESC to EpiLC differentiation and
323 promotes germline gene silencing via DNA methylation during early embryogenesis.

324 Discussion

325 In the above study, we demonstrate KDM5C's pivotal role in the development of tissue identity, resulting in
326 substantial misexpression of tissue-enriched genes within the adult *Kdm5c*-KO hippocampus and amygdala.
327 In addition to testis genes identified previously¹⁰, we found significant enrichment of muscle, liver, and even
328 ovary-biased genes aberrantly expressed within the male *Kdm5c*-KO brain. Deeper characterization of
329 testis-enriched DEGs revealed they were not somatic testis genes, but germline genes, thus demonstrating
330 KDM5C's crucial role in establishing the soma-germline boundary. Given the majority of tissue-enriched
331 DEGs are testis and ovary genes with no known brain functions, it is currently unknown if they impair
332 *Kdm5c*-KO brain function and contribute to MRXSCJ-related phenotypes like intellectual disability and
333 aberrant aggression. However, select liver and muscle-biased DEGs do have known roles within the brain,
334 such as the liver-enriched lipid metabolism gene *Apolipoprotein C-I* (*Apoc1*)³² that is highly expressed in the

335 *Kdm5c*-KO amygdala and hippocampus. Intriguingly, *Apoc1* overexpression in the mouse brain can impair
336 learning and memory⁶⁷ and is implicated in Alzheimer's disease in humans³³.

337 We found a substantial portion of genes upregulated in *Kdm5c*-KO brain are germline genes with no
338 known fucntions within the brain. Distinguishing the germline from the soma is a key feature of multicellularity
339 and sexual reproduction¹⁶. Previous work characterizing chromatin regualtors that silence germ cell-specific
340 transcription has predominatly focused on their repression of key marker genes in embryonic stem cells
341 (ESCs), such as *Dazl* and *Ddx4*^{18,19,53}. To characterize KDM5C's role in germline gene repression at a
342 genome-wide level throughout life, we curated a list of mouse germline-enriched genes using publically
343 available germ cell-depleted RNA-seq datasets from *Kit^{WWv}* mice^{34,37}. This resource enabled us to identify 1)
344 the extent of germline gene dysregulation in *Kdm5c*-KO cells, 2) the types of germline genes misexpressed
345 at different developmental time points, and 3) which groups of germline genes are directly and indirectly
346 regulated by KDM5C. This list of germline-enriched genes serves as a resource for future studies to
347 systematically assess soma-germline dysregulation.

348 RNA-seq of epiblast-like cells (EpiLCs) revealed dysregulation of *Kdm5c*-KO tissue identity begins during
349 early emrbyogenesis. *In vivo*, germline genes are typically decommissioned in epiblast stem cells and remain
350 silenced as the epiblast differentiates into somatic tissues⁴⁰. However, a small subset of epiblast stem cells
351 will receive signals to reactivate germline gene expression to become the primordial germ cells (PGCs) that
352 will ultimately form the mature germline^{38,39}. This process can be mimicked *in vitro* by differentiating EpiLCs
353 into primordial germ cell-like cells (PGCLCs)⁴¹. Therefore, misexpression of germline genes in EpiLCs might
354 suggest *Kdm5c*-KO ESCs are progressing beyond EpiLC differentiation and instead becoming PGCLCs. Yet,
355 *Kdm5c*-KO EpiLCs properly express primed pluripotency genes, including *Otx2* - an epiblast stem cell marker
356 that is known to repress differentiation into PGCs/PGCLCs⁶⁸. Furthermore, we observed no difference in
357 cellular morphology during *Kdm5c*-KO ESC to EpiLC differentiation. Proper EpiLC differentiation, together
358 with *Kdm5c*-KO mice being viable, suggests germline gene expression is occuring ectopically in conjunction
359 with typical developmental programs, rather than a complete shift to towards germline identity.

- 360 • XX vs XY
- 361 • While many germline genes have roles in both the male and female germline, some display sex-biased
362 expression or have functions unique to eggs and sperm. Many of the germline genes characterized in
363 the male *Kdm5c*-KO brain have sperm-specific functions. It is currently unclear if sperm genes are also
364 dysregulated in female *Kdm5c* mutants, or if they would instead misexpress egg-biased genes.
- 365 • Motif analysis
- 366 – only 1/3 had max/e2f - Unlikely to be the major recruitment mechanism for KDM5C
- 367 – KDM5C does not contain motif-specific binding
- 368 – controls cilia in mutiple cell types, initially identified in neurons. Shared axis that could contribute
369 to NDDs

- 370 * RFX Cilia in NDDs - <https://pubmed.ncbi.nlm.nih.gov/33658631/>
- 371 – Enriched RFX transcription factors included the spermiogenesis regulator RFX2, whose mRNA is
372 also significantly upregulated in *Kdm5c*-KO EpiLCs (Figure 4).
- 373 – <https://www.nature.com/articles/srep20435>
- 374 – <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4498915/>
- 375 – <https://pubmed.ncbi.nlm.nih.gov/32725242/>
- 376 • However, CPG islands greatly determine KDM5C recruitment - KDM5C is previously known to be
377 enriched at CGIs. CGIs not typically methylated, germline genes exception
- 378 We then globally characterized KDM5C binding at germline-enriched gene promoters through KDM5C
379 ChIP-seq in EpiLCs and primary hippocampus and cortex neuron cultures (PNCs).
- 380 Our analysis of KDM5C ChIP-seq data provided novel insights into direct and indirect mechanisms by
381 which germline genes can be misexpressed in *Kdm5c*-KO cells.
- 382 While we observed high enrichment of KDM5C peaks at germline genes in EpiLCs, KDM5C was not
383 bound to germline gene promoters in PNCs. This suggests dysregulation of germline genes in the mature
384 *Kdm5c*-KO brain is due to loss of repression during embryogenesis, which is consistent with previous
385 work that found introducing human KDM5C into *Kdm5c*-KO PNCs does not repress two aberrant germline
386 transcripts¹⁰. Although enriched at germline gene promoters in EpiLCs, KDM5C was only bound to about a
387 third of the germline genes aberrantly transcribed in *Kdm5c*-KO EpiLCs. One notable EpiLC DEG not bound
388 by KDM5C is *Stra8*, a transcription factor activated by retinoic acid signaling in germ cells to promote meiotic
389 initiation^{69,70}. However, retinoic acid can only activate *Stra8* expression when DAZL is present. Unlike *Stra8*,
390 *Dazl* is a direct target of KDM5C in EpiCs and is both transcribed and translated in *Kdm5c*-KO EpiLCs.
391 Expression of indirect DEGs like *Stra8* indicates germline genes can be aberrantly expressed in *Kdm5c*-KO
392 cells through downstream activation by other ectopic germline programs. These ectopic germline programs
393 appear to loosely mimic the trajectory of typical germline development, as germline genes important for early
394 germ cell development and meiosis are expressed in *Kdm5c*-KO EpiLC while late spermatogenesis genes
395 are expressed in the mature *Kdm5c*-KO brain. Altogether, this indicates once a subset of germline genes
396 are activated due to loss of direct KDM5C repression in the early embryo, these ectopic germline programs
397 can continue to progress in the background of *Kdm5c*-KO somatic development.
- 398 It is important to note that while we focused on KDM5C's regulation of germline genes, some germline-
399 enriched genes are also expressed in naïve ESCs and at the 2-cell stage due to their roles in pluripotency and
400 self-renewal. For example, *Dazl* is primarily known for committing PGCs to the germline fate and regulating
401 the translation of germline-specific RNAs, but is also expressed at the 2-cell stage⁷¹, in naïve ESCs⁴³, and in
402 the inner cell mass⁴³. Very recently, two screens of *Dazl*-repressors identified KDM5C as a direct regulator in
403 ESCs^{51,72}. Interestingly, one study found *Kdm5c*-KO ESCs also aberrantly expressed 2-cell-specific genes,
404 indicating KDM5C negatively regulates totipotency⁵¹. However, out of the four regulators characterized,

405 KDM5C was the only factor whose repression of *Dazl* was independent of the 2-cell specific transcription
406 factor *Dux*⁵¹. Although expressed at the 2-cell stage and in ESCs, *Dazl* is typically silenced when ESCs
407 differentiate into EpiLCs¹⁹. We found KDM5C also represses *Dazl* in EpiLCs, but *Kdm5c*-KO EpiLCs did
408 not express 2-cell specific genes like *Dux* and *Zscan4c*. Together, this suggests the 2-cell-like state in
409 *Kdm5c*-KO ESCs is an indirect consequence of KDM5C's primary role in germline gene repression.

410 *Dazl* and other germline-enriched genes are initially decommissioned in ESCs by repressive histone
411 modifications and then *de novo* DNA CpG methylation (CpGme) in EpiLCs^{19–21,64,73,74}. Unlike the previously
412 characterized germline gene suppressors, KDM5C removes an active histone mark, histone 3 lysine 4 di-
413 and trimethylation (H3K4me2/3)¹¹. In this study, we observed a global increase in H3K4me2/3 in *Kdm5c*-KO
414 EpiLCs and amygdala around the transcription start site (TSS) of germline-enriched genes. However,
415 KDM5C's catalytic activity may not be required for germline gene silencing, as it was recently found to be
416 dispensible for repressing *Dazl* in ESCs⁵¹. Although not necessary in ESCs, KDM5C's catalytic activity be
417 required for CpGme in EpiLCs, as H3K4me3 impedes *de novo* CpGme placement^{65,66}. This is supported
418 by previous work in the *Kdm5c*-KO adult hippocampus, which found CpGme is significantly eroded at at
419 least two germline promoters¹⁰. To elucidate the mechanism behind KDM5C-mediated silencing of germline
420 genes, we characterized KDM5C in ESCs and EpiLCs and found its expression is dynamically regulated
421 during differentiation. Furthermore, KDM5C acts during the transition from naïve to primed pluripotency to
422 promote the initial placement of CpGme at CpG islands for the long-term silencing of germline genes.

423 The above work provides the mechanistic foundation for KDM5C's role in germline gene repression
424 and offers novel insight into how dysregulation in *Kdm5c*-KO tissue identity changes over the course of
425 development. Although their impact upon neurodevelopment is currently unclear, ectopic germline transcripts
426 are also found in models of other related neurodevelopmental disorders⁷⁵, including Immunodeficiency,
427 Centromere instability, Facial anomalies syndrome 1 (ICF1 OMIM: #242860)^{76,77}, Kleefstra syndrome
428 1 (OMIM: #610253)⁹, and MeCP2 duplication syndrome (MDS, OMIM: #300260)⁷⁸. Like KDM5C, the
429 chromatin regulators underlying these conditions, DNA methyltransferase 3b (DNMT3B), H3K9me1/2
430 methyltransferases G9A/GLP, methyl-CpG -binding protein 2 (MECP2), primarily silence gene expression.
431 Thus, KDM5C is among a growing cohort of chromatin-linked neurodevelopmental disorders that may have a
432 similar underlying cause of germline versus soma dysregulation. However, further research is required to
433 determine the ultimate impact of these germline genes and the extent to which this phenomenon occurs in
434 humans.

435 • Last paragraph

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

438 **Materials and Methods**

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

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

444 We curated a list of germline-enriched genes using an RNA-seq dataset from wild-type and germline-
445 depleted (Kit^{W/W^v}) male and female mouse embryos from embryonic day 12, 14, and 16³⁷, as well as adult
446 male testes³⁴. Germline-enriched genes met the following criteria: 1) their expression is greater than 1
447 FPKM in wild-type germline 2) their expression in any wild-type somatic tissues²⁶ does not exceed 20%
448 of maximum expression in wild-type germline, and 3) their expression in the germ cell-depleted (Kit^{W/W^v})
449 germline, for any sex or time point, does not exceed 20% of maximum expression in wild-type germline.

450 **Cell culture**

451 We utilized our previously established cultures of male wild-type and *Kdm5c* knockout (-KO) embryonic
452 stem cells⁴⁷. Sex was confirmed by genotyping *Uba1/Uba1y* on the X and Y chromosomes with the following
453 primers: 5'-TGGATGGTGTGCCAATG-3', 5'-CACCTGCACGTTGCCCTT-3'. Deletion of *Kdm5c* was
454 confirmed through the primers 5'-ATGCCCATATTAAGAGTCCTG-3', 5'-TCTGCCTTGATGGACTGTT-3',
455 and 5'-GGTTCTAACACTCACATAGTG-3'.

456 Embryonic stem cells (ESCs) and epiblast-like cells were cultured using previously established
457 methods⁴². Briefly, ESCs were initially cultured in primed ESC (pESC) media consisting of KnockOut
458 DMEM (Gibco#10829–018), fetal bovine serum (Gibco#A5209501), KnockOut serum replacement
459 (Invitrogen#10828–028), Glutamax (Gibco#35050-061), Anti-Anti (Gibco#15240-062), MEM Non-essential
460 amino acids (Gibco#11140-050), and beta-mercaptoethanol (Sigma#M7522). They were then transitioned
461 into ground-state, “naïve” ESCs (nESCs) by culturing for four passages in N2B27 media containing
462 DMEM/F12 (Gibco#11330–032), Neurobasal media (Gibco#21103–049), Gluamax, Anti-Anti, N2 sup-
463 plement (Invitrogen#17502048), and B27 supplement without vitamin A (Invitrogen#12587-010), and
464 beta-mercaptoethanol. Both pESC and nESC media were supplemented with 3 µM GSK3 inhibitor
465 CHIR99021 (Sigma #SML1046-5MG), 1 µM MEK inhibitor PD0325901 (Sigma #PZ0162-5MG), and 1,000
466 units/mL leukemia inhibitory factor (LIF, Millipore#ESG1107).

467 nESCs were differentiated into epiblast-like cells (EpiLCs, 48 hours) and extendend EpiLCs (exEpiLCs,
468 96 hours) by culturing in N2B27 media containing DMEM/F12, Neurobasal media, Gluamax, Anti-Anti,
469 N2 supplement, B27 supplement (Invitrogen#17504044), beta-mercaptoethanol, fibroblast growth factor 2
470 (FGF2, R&D Biotechne 233-FB), and activin A (R&D Biotechne 338AC050CF), as previously described⁴².

471 **Immunocytochemistry (ICC)**

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

481 **RNA sequencing (RNA-seq)**

482 After ensuring read quality via FastQC (v0.11.8), reads were then mapped to the mm10 *Mus musculus*
483 genome (Gencode) using STAR (v2.5.3a), during which we removed duplicates and kept only uniquely
484 mapped reads. Count files were generated by FeatureCounts (Subread v1.5.0), and BAM files were
485 converted to bigwigs using deeptools (v3.1.3) and visualized by the UCSC genome browser. RStudio (v3.6.0)
486 was then used to analyze counts files by DESeq2 (v1.26.0)²⁸ to identify differentially expressed genes
487 (DEGs) with a q-value (p-adjusted via FDR/Benjamini–Hochberg correction) less than 0.1 and a log2 fold
488 change greater than 0.5. For all DESeq2 analyses, log2 fold changes were calculated with IfcShrink using
489 the ashR package⁷⁹. MA-plots were generated by ggpibr (v0.6.0), and Eulerr diagrams were generated by
490 eulerr (v6.1.1). Boxplots and scatterplots were generated by ggpibr (v0.6.0) and ggplot2 (v3.3.2). The Upset
491 plot was generated via the package UpSetR (v1.4.0)⁸⁰. Gene ontology (GO) analyses were performed by
492 the R package enrichPlot (v1.16.2) using the biological processes setting and compareCluster.

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

494 ChIP-seq reads were aligned to mm10 using Bowtie1 (v1.1.2) allowing up to two mismatches. Only
495 uniquely mapped reads were used for analysis. Peaks were called using MACS2 software (v2.2.9.1) using
496 input BAM files for normalization, with filters for a q-value < 0.1 and a fold enrichment > 1. We removed
497 “black-listed” genomic regions that often give aberrant signals. Common peak sets were obtained in R via
498 DiffBind (v3.6.5). In the case of KDM5C ChIP-seq, *Kdm5c*-KO peaks were then subtracted from wild-type
499 samples using bedtools (v2.25.0). Peak proximity to genome annotations was determined by ChIPSeeker
500 (v1.32.1). Gene ontology (GO) analyses were performed by the R package enrichPlot (v1.16.2) using the
501 biological processes setting and compareCluster. Enriched motifs were identified using HOMER⁵⁸. Average
502 binding across the genome was visualized using deeptools (v3.1.3). Bigwigs were visualized using the
503 UCSC genome browser.

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

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

509 **Data availability**

510 **Published datasets**

511 All published datasets are available at the Gene Expression Omnibus (GEO) <https://www.ncbi.nlm.nih>
512 .gov/geo. Published RNA-seq datasets analyzed in this study included the male wild-type and *Kdm5c*-KO
513 adult amygdala and hippocampus²⁷ (available at GEO: GSE127722) and male wild-type and *Kdm5c*-KO
514 EpiLCs⁴⁷ (available at GEO: GSE96797).

515 Previously published ChIP-seq experiments included KDM5C in wild-type and *Kdm5c*-KO EpiLCs⁴⁷ (avail-
516 able at GEO: GSE96797) and mouse primary neuron cultures (PNCs) from the cortex and hippocampus¹⁵
517 (available at GEO: GSE61036). ChIP-seq of histone 3 lysine 4 dimethylation in male wild-type and *Kdm5c*-KO
518 EpiLCs⁴⁷ is also available at GEO: GSE96797. ChIP-seq of histone 3 lysine 4 trimethylation in wild-type and
519 *Kdm5c*-KO male amygdala²⁷ are available at GEO: GSE127817.

520 **Data analysis**

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

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693 **Figures and Tables**

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



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



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

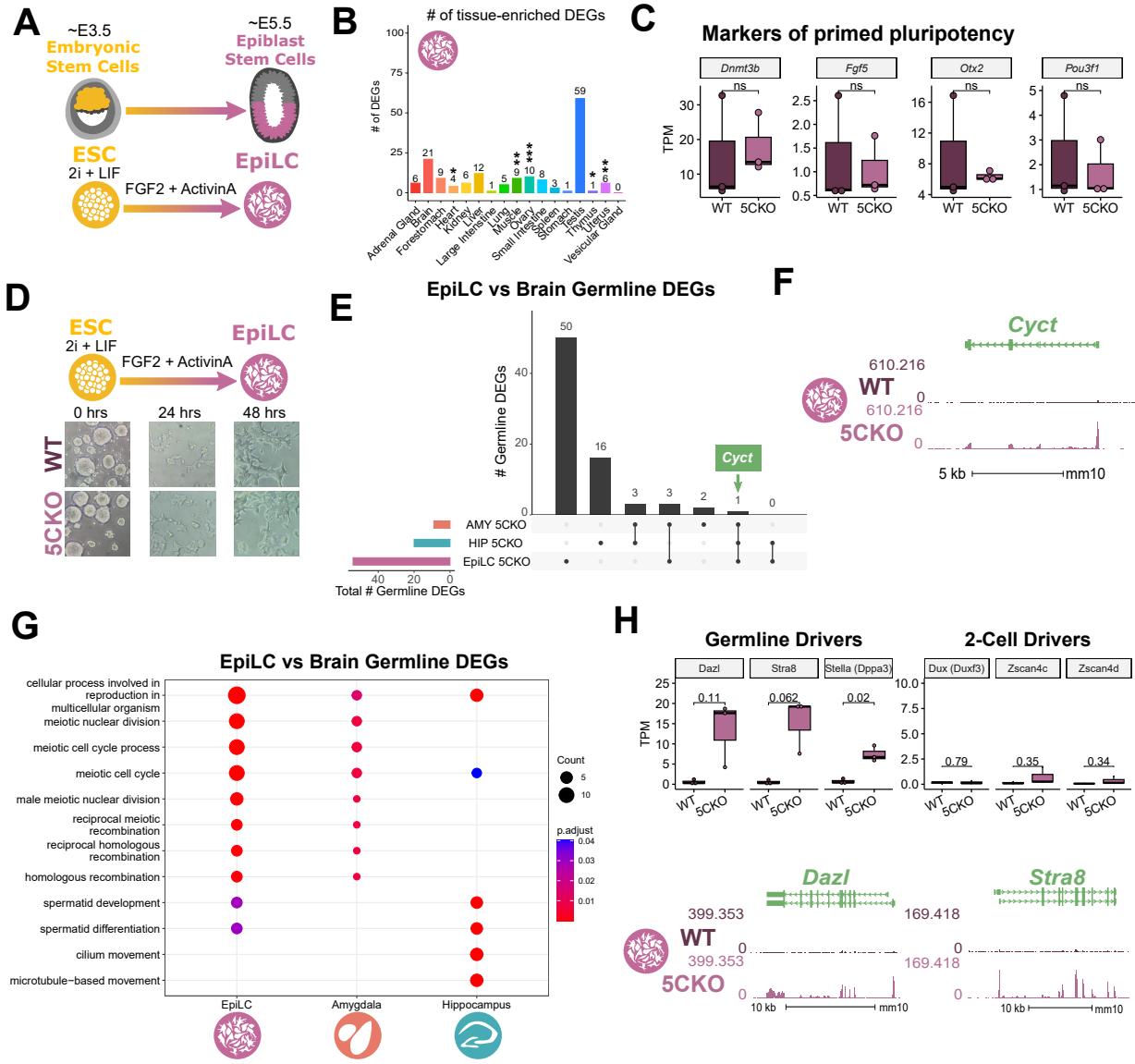


Figure 3: *Kdm5c*-KO epiblast-like cells express key drivers of germline identity

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

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

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

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

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

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

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

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

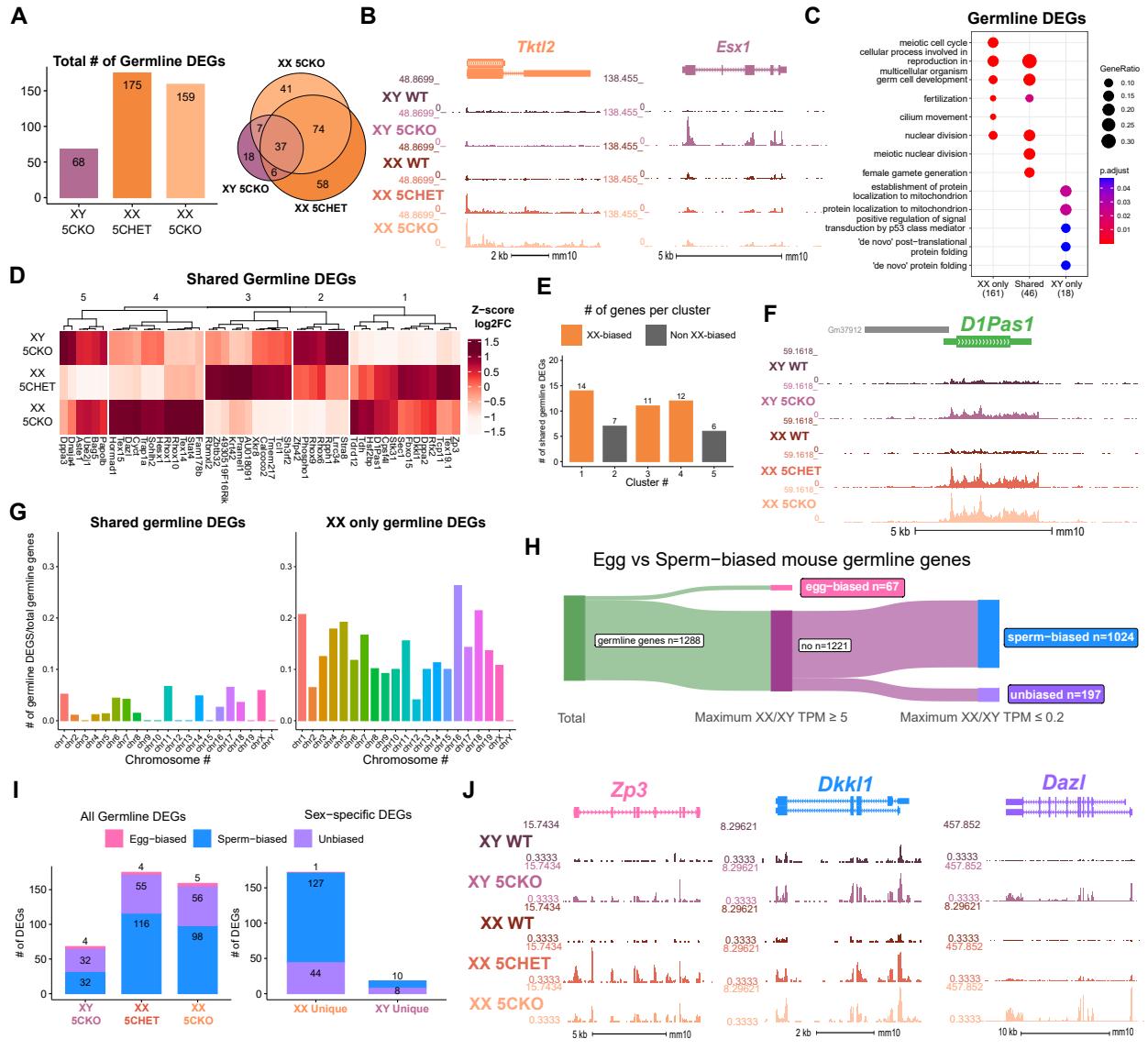


Figure 4: Chromosomal sex influences *Kdm5c*-KO germline gene misexpression. **A-B.** Expression of tissue-enriched genes (Li et al 2017) in the male *Kdm5c*-KO amygdala (A) and hippocampus (B). Left - MA plot of mRNA-seq. Right - Number of tissue-enriched differentially expressed genes (DEGs). * $p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

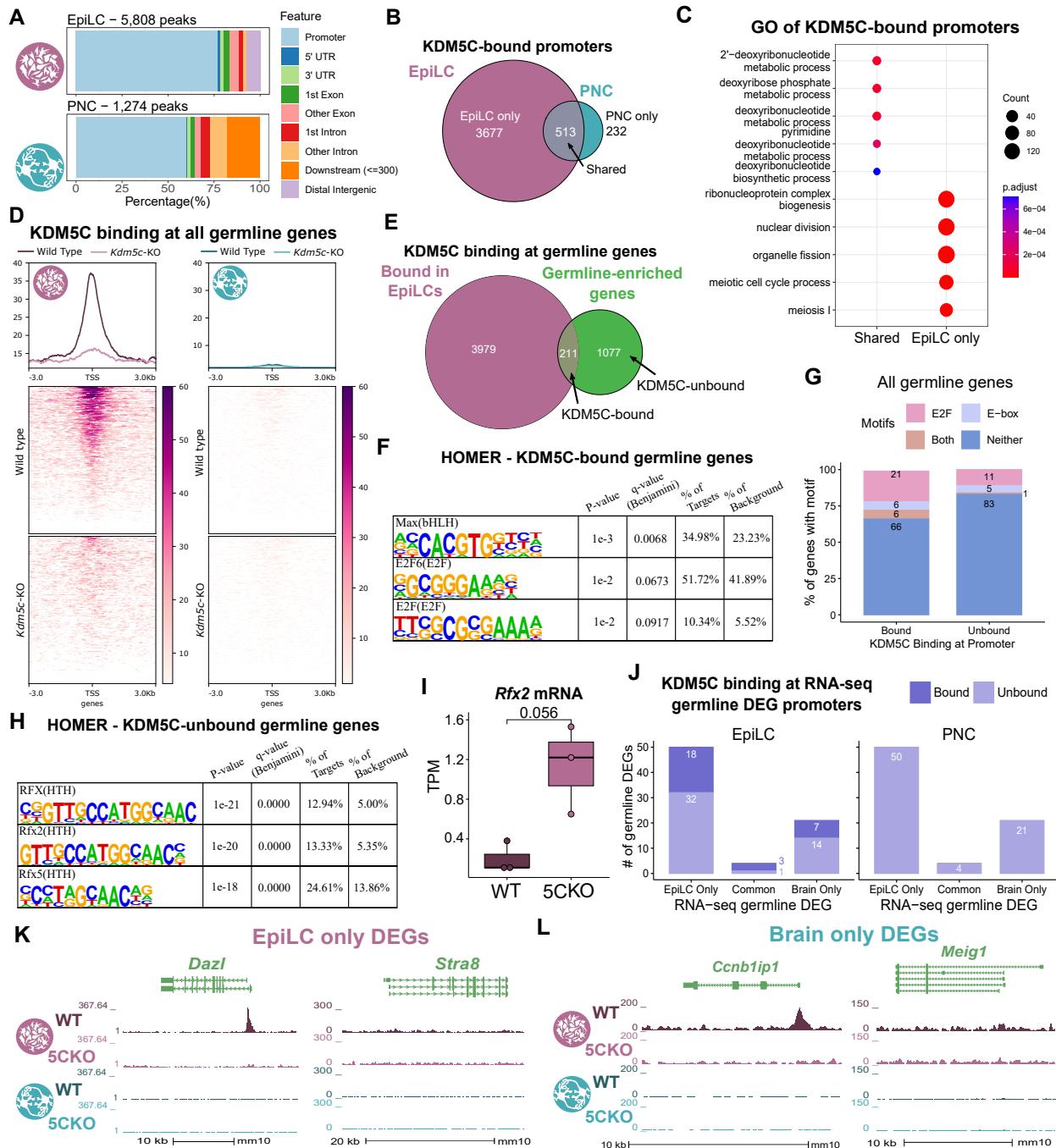
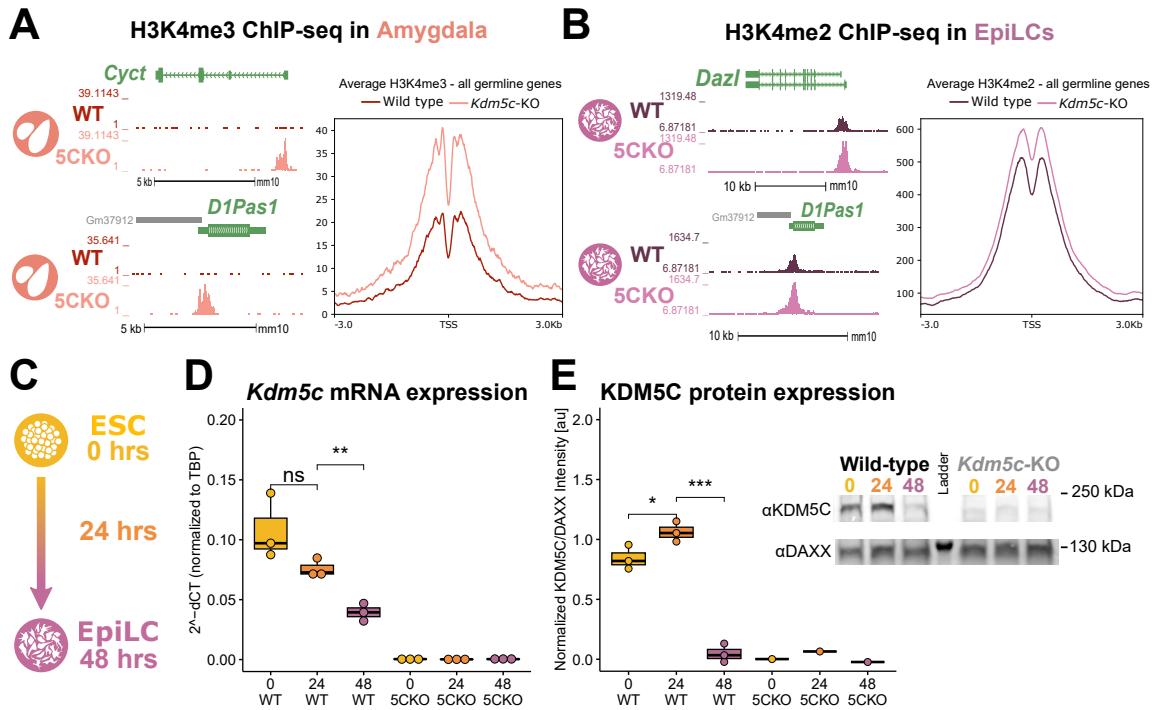


Figure 5: KDM5C binds to a subset of germline gene promoters during early embryogenesis **A.** ChIPseeker localization of KDM5C peaks at different genomic regions in EpiLCs (top) and hippocampal and cortex primary neuron cultures (PNCs, bottom). **B.** Overlap of genes with KDM5C bound to their promoters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

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



F

G

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

698 Notes

699 Things to do

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

704 Dazl

705 We were particularly interested in the aberrant transcription of *Dazl*, since it is essential for germ cell
706 development and promotes the translation of germline mRNAs⁸¹. A significant portion of germline transcripts
707 misexpressed in *Kdm5c*-KO EpiLCs are known binding targets of DAZL, including *Stra8*⁸² ($p = 1.698e-07$,
708 Fisher's Exact Test). This suggests expression of DAZL protein could promote the translation of other
709 aberrant germline transcripts, influencing their ability to impact *Kdm5c*-KO cellular function. We thus tested
710 DAZL protein expression in *Kdm5c*-KO EpiLCs through immunocytochemistry (Figure 3I). We observed
711 about 25% of *Kdm5c*-KO EpiLCs expressed DAZL protein and it was localized to the cytoplasm ($p = 0.0015$,
712 Welch's t-test), consistent with the pattern of DAZL expression in spermatogonia⁸². Altogether these results
713 suggest tissue-specific genes are misexpressed during *Kdm5c*-KO embryogenesis, including key drivers of
714 germline identity that can be translated into protein.

- 715 • We additionally found *Kdm5c*-KO EpiLCs ectopically express DAZL protein that is localized to the
716 cytoplasm, similar to its morphology in spermatogonia⁸². **note: maybe just put in results.** Could
717 move around depending upon if I get pheno working.

718 Discussion notes

- 719 • For other paper:
 - 720 – for methods: Heatmaps of gene expression were generated using the base R functions scale and
721 hclust and visualized using the R package ComplexHeatmap (v2.12.1).
 - 722 – * 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>
- 724 • Talk about different motifs if we do see differences there and if it explains direct vs indirect dysregulation
 - 725 – Could also mention PRC1.6/KDM5C binds pcgf6 in ESCs
- 726 • Maybe talk about other regulators/if anything is known about them long term? Or save for 2nd paper.

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

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

763 **Figure outline:**

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

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

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

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

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

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

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

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

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

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

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

828 **Germline gene repression background:**

829 Interestingly, some of the ectopic testis transcripts identified in the *Kdm5c*-KO brain are typically ex-
830 pressed in germ cells¹⁰. Unlike somatic cells, germ cells (e.g. sperm and eggs) undergo meiosis and pass
831 on their genetic material to the next generation. The germline and the soma are typically distinguished during
832 early embryogenesis, when germline genes are silenced in epiblast stem cells soon after implantation and
833 only reactivated in a subset to form the germline. Chromatin regulators play a key role in decommissioning
834 germline genes as the embryo transitions from naïve to primed pluripotency by placing repressive histone
835 H2A lysine 119 monoubiquitination (H2AK119ub1)¹⁸, histone 3 lysine 9 trimethylation (H3K9me3)^{18,19}, and
836 DNA CpG methylation^{19–21} at germline gene promoters. KDM5C may also be involved in this early decom-
837 missioning of germline genes, as re-expression of KDM5C in neurons fails to suppress their dysregulation¹⁰.
838 In support of this, KDM5C was very recently shown to repress *Deleted in azoospermia like (Dazl)*, a key
839 regulator of germline development, in mouse embryonic stem cells (ESCs)^{51,72}. In support of this, two
840 independent screens in mouse embryonic stem cells (ESCs) recently identified KDM5C as a repressor of
841 *Deleted in azoospermia like (Dazl)*, a key regulator of germline development. However, KDM5C's role in
842 embryonic germline gene repression is currently unclear, given that Dazl is also expressed in ESCs and in
843 the 2-cell stage and germline gene misexpression has yet to be globally characterized during *Kdm5c*-KO
844 embryogenesis.