

1 Erosion of somatic tissue identity with loss of the X-linked intellectual
2 disability factor KDM5C

3

4 **Abstract**

5 **Introduction**

6 A single genome holds the instructions to generate all the myriad of cell types found within the adult organism. This
7 is, in part, accomplished by chromatin regulators that can either promote or impede lineage-specific gene expression
8 through DNA and histone modifications^{1,2}. Although many chromatin regulators were initially identified for their roles in
9 shaping cellular and tissue identity^{3,4}, recent advancements in next generation sequencing unexpectedly revealed their
10 dysregulation underlies many neurodevelopmental disorders (NDDs)⁵. Many studies have suggested this connection between
11 neurodevelopment and chromatin regulators is due to their regulation of brain-specific genes, such as orchestrating the
12 transcriptional programs for synaptic maturation⁶ and the transition between neuronal and glial differentiation in neural
13 precursor cells⁷. However, loss of some chromatin regulators can also result in the ectopic transcription of tissue-specific
14 genes outside of their target environment^{3,4,8}. Very few studies have investigated the misexpression of tissue-specific genes
15 in chromatin-linked neurodevelopmental disorders^{9,10} and it is currently unclear if this partial loss of brain identity contributes
16 to neurodevelopmental impairments.

17 To elucidate the role of tissue identity in chromatin-linked neurodevelopmental disorders, it is essential to first characterize
18 the types of genes dysregulated and the molecular mechanisms underlying their de-repression. In this study, we characterized
19 the aberrant expression of tissue-enriched genes with loss of lysine demethylase 5C (KDM5C, also known as SMCX or
20 JARID1C). KDM5C is a chromatin regulator that can repress gene expression through erasure of histone 3 lysine 4
21 di- and trimethylation (H3K4me2/3)¹¹, marks enriched at active gene promoters. Pathogenic mutations in *KDM5C* cause
22 Intellectual Developmental Disorder, X-linked, Syndromic, Claes-Jensen Type (MRXSCJ, OMIM: 300534), whose predominant
23 features include intellectual disability, seizures, aberrant aggression, and autistic behaviors^{12–14}. *Kdm5c* knockout (-KO)
24 mice recapitulate key MRXSCJ patient phenotypes, including hyperaggression, increased seizure propensity, and learning
25 impairments^{10,15}. RNA sequencing (RNA-seq) of the *Kdm5c*-KO brain unexpectedly revealed ectopic expression of testis
26 genes within the hippocampus¹⁰. It is currently unclear if these testis genes impair *Kdm5c*-KO neurodevelopment and if this
27 dysregulation in *Kdm5c*-KO brain tissue identity is unique to testis genes.

28 Intriguingly, some of the ectopic testis transcripts expressed within the *Kdm5c*-KO brain have known functions in germ
29 cells¹⁰, suggesting KDM5C may play a role in demarcating somatic and germline identity. Germ cells produce the gametes
30 (e.g. sperm and eggs) that pass on their genetic material to the next generation while the somatic cells perform all the other
31 bodily functions. Distinguishing between soma and germline is a key feature of multicellularity and occurs in mammals soon
32 after the embryo is implanted into the uterine wall. Chromatin regulators play a key role in decommissioning germline genes
33 as the embryo transitions from naive to primed pluripotency by placing repressive histone H2A lysine 119 monoubiquitination

34 (H2AK119ub1)¹⁶, histone 3 lysine 9 trimethylation (H3K9me3)^{16,17}, and DNA CpG methylation¹⁷⁻¹⁹ at germline gene promoters.
35 KDM5C may also be involved in this early decommissioning of germline genes, as re-expression of KDM5C in neurons fails
36 to suppress their dysregulation¹⁰. In support of this, KDM5C was very recently shown to repress *Deleted in azoospermia*
37 *like (Dazl)*, a key regulator of germline development, in mouse embryonic stem cells (ESCs)^{20,21}. However, KDM5C binding
38 and *Kdm5c*-KO germline gene misexpression has yet to be globally characterized during early embryogenesis. Given that
39 *Dazl* and other germline genes can be expressed in ESCs and in the 2-cell stage, it is unclear if KDM5C has a direct role
40 in long-term germline gene silencing in post-implantation epiblast. Systematically characterizing KDM5C's role in germline
41 gene repression will unveil key mechanisms underlying of soma versus germline identity and provide molecular footholds for
42 testing the impact of ectopic germline gene expression on neurodevelopment.

43 To illuminate KDM5C's role in tissue identity, here we characterized the aberrant transcription of tissue-enriched genes
44 within the male *Kdm5c*-KO brain and epiblast-like stem cells (EpiLCs), an *in vitro* model of the post-implantation embryo. We
45 observed general dysregulation of tissue-enriched genes the *Kdm5c*-KO brain and EpiLCs, including misexpression of genes
46 typically highly enriched within the testis, liver, muscle, and ovary. Both the *Kdm5c*-KO amygdala and hippocampus had
47 significant enrichment of testis genes that are typically unique to germ cells. While the *Kdm5c*-KO brain primarily expressed
48 germline genes important for late spermatogenesis, *Kdm5c*-KO EpiLCs aberrantly expressed key drivers of germline identity
49 and meiosis, including *Dazl* and *Stra8*. KDM5C was highly enriched at germline gene promoters in EpiLCs but only bound to
50 a subset of germline genes expressed in *Kdm5c*-KO cells, indicating germline-enriched mRNAs can be aberrantly transcribed
51 through indirect mechanisms. Finally, we found KDM5C promotes the embryonic silencing of germline genes in somatic cells
52 by aiding the placement of DNA methylation in EpiLCs through H3K4me3 removal. Thus, we propose KDM5C plays a crucial
53 role in the development of tissue identity during early embryogenesis, including the establishment of the soma-germline
54 boundary.

55 Results

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

- 57 • note: should I compare amygdala and hippocampus? scandaglia only looked at hippocampus

58 Previous RNA sequencing (RNA-seq) in the adult hippocampus ectopic expression of some testis genes within the *Kdm5c*
59 knockout (-KO) brain¹⁰. Since tissue-enriched genes have not been systematically characterized in the *Kdm5c*-KO brain,
60 it is currently unclear if this erosion of brain tissue identity is a major consequence of *Kdm5c* loss and if it is unique to
61 testis-enriched genes. Therefore, we first globally assessed the expression of previously characterized mouse tissue-enriched
62 genes²² in our published mRNA-seq datasets of the amygdala and hippocampus in adult mice with constitutive knockout of
63 *Kdm5c*²³.

64 We found a large proportion of genes significantly upregulated within the *Kdm5c*-KO brain (DESeq2²⁴, log2 fold change >
65 0.5, q < 0.1) are typically enriched in non-brain tissues (Amygdala: 35%, Hippocampus: 24%) (Figure 1A-B). The majority
66 of tissue-enriched differentially expressed genes (tissue-enriched DEGs) were testis genes (Figure 1A-C). Testis-biased
67 DEGs were significantly enriched for both brain regions (Amygdala p = 1.83e-05; Hippocampus p = 4.26e-11, Fisher's Exact
68 Test), even though the testis has the largest total number of tissue-biased genes compared to any other tissue (2,496 genes).
69 Of note, we did not observe enrichment of brain-enriched genes (Amygdala p = 1; Hippocampus p = 0.74, Fisher's Exact),
70 despite the fact these are brain samples and the brain has the second highest total number of tissue-enriched genes (708

71 genes).

72 In addition to the high enrichment of testis genes, we also identified aberrant expression of other tissue-enriched genes
73 within the *Kdm5c*-KO brain. Although the *Kdm5c*-KO mice were male, we also observed significant enrichment of ovary-biased
74 DEGs in both the amygdala and hippocampus (Amygdala p = 0.00574; Hippocampus p = 0.048, Fisher's Exact) (Figure 1D).
75 Intriguingly, many ovary and testis-biased DEGs have functions specific to germ cells and have no known role in the brain.
76 For example, the testis-biased DEG *FK506 binding protein 6* (*Fkbp6*) is a known regulator of piRNA expression and meiosis
77 in germ cells^{25,26} (Figure 1C) while the ovary-enriched DEG *Zygotic arrest 1* (*Zar1*) was recently shown to sequester mRNAs
78 in oocytes for meiotic maturation and early zygote development²⁷ (Figure 1D). Although not consistent across brain regions,
79 we also found enrichment of two non-gonadal tissues - the liver (Amygdala p = 0.0398, Fisher's Exact Test) and the muscle
80 (Hippocampus p = 0.0104, Fisher's Exact Test). An example of a liver-biased DEG is *Apolipoprotein C-I* (*Apoc1*), which is
81 involved in lipoprotein metabolism (Figure 1E). Testis, ovary, and liver-enriched DEGs showed little to no expression in the
82 wild-type brain, yet our mRNA-seq data indicate they are polyadenylated and spliced into mature transcripts (Figure 1C-E).
83 Together, these results suggest misexpression of testis and other tissue-enriched genes within the brain is a major effect of
84 KDM5C loss.

85 Germline genes are aberrantly expressed in the male *Kdm5c*-KO brain

86 The testis contains both germ cells (meiotic cells, e.g. spermatogonia) and somatic cells (non-meiotic, e.g. Leydig cells)
87 that support hormone production and germline functions. Select testis-enriched DEGs that were characterized previously
88 had germline-specific functions¹⁰, suggesting *Kdm5c*-KO cells fail to demarcate between the soma and germline. To test if
89 this holds true for all *Kdm5c*-KO testis-biased DEGs, we first assed their function through gene ontology. We found high
90 enrichment of germline-relevant ontologies, including spermatid development (GO: 0007286, p.adjust = 6.2e-12) and sperm
91 axoneme assembly (GO: 0007288, p.adjust = 2.45e-14) (Figure 2A).

92 To further validate if these testis DEGs are truly germline genes, we then compared their expression in somatic versus
93 germ cells within the testis. We first compared their expression within wild-type versus germ cell-depleted testes²⁸. In this
94 study, germ cell depletion was accomplished by heterozygous *W* and *Wv* mutations in the enzymatic domain of *c-Kit* (*Kit*^{W/Wv}),
95 which prevents the maturation of germ cells and results in overall germline loss²⁹. Almost all *Kdm5c*-KO testis-enriched
96 DEGs lost expression with germ cell depletion (Figure 2B). The only testis-enriched DEG that did not show considerable
97 downregulation with germline depletion was *FK506 binding protein 6* (*Fkbp6*), the aforementioned testis gene that regulates
98 piRNA expression and meiosis in germ cells^{25,26}. We then assessed testis-enriched DEG expression in a published single
99 cell RNA-seq dataset that identified cell type-specific markers within the testis³⁰. We found that while some testis-enriched
100 DEGs were classified as specific markers for different germ cell types (e.g. spermatogonia, spermatocytes, round spermatids,
101 and elongating spermatids) none marked somatic cells (Figure 2C). Together, these data suggest the somatic *Kdm5c*-KO
102 brain aberrantly expresses germline genes.

103 We wanted to more deeply characterize germline gene misexpression with *Kdm5c* loss, but lacked a comprehensive list of
104 mouse germline-enriched genes. To facilitate downstream analyses, we generated a list of male and female germline-enriched
105 genes by evaluating gene expression in germline-depleted (*Kit*^{W/Wv}) mice. Current available *Kit*^{W/Wv} datasets included males
106 and females at embryonic day 12, 14, and 16³¹, as well as adult male testes²⁸.

107 We defined genes as germline-enriched if their expression met the following criteria: 1) their expression is greater than 1
108 FPKM in wild-type gonads 2) their expression in any wild-type, non-gonadal tissue²² does not exceed 20% of their maximum
109 expression in the wild-type germline, and 3) their expression in the germ cell-depleted germline, for any sex or time point,

110 does not exceed 20% of their maximum expression in the wild-type germline. These criteria yielded 1,288 germline-enriched
111 genes (Figure 2D), which was hereafter used as a resource for assessing misexpression of germline genes with loss of
112 *Kdm5c* (Supplementary table 1).

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

114 Misexpression of germline genes in the adult *Kdm5c*-KO brain suggests mutants fail to demarcate germline and somatic
115 cellular identity. Germ cells are typically distinguished from somatic cells soon after the embryo implants into the uterine
116 wall^{32,33} when a subset of epiblast stem cells become the primordial germ cells (PGCs) while the remainder differentiate into
117 the ectoderm, mesoderm, and endoderm to form the somatic tissues³⁴. This developmental time point can be modeled *in*
118 *vitro* through differentiation of embryonic stem cells (ESCs) into post-implantation epiblast-like stem cells (EpiLCs) (Figure 3A,
119 top). Previous studies have demonstrated that while some germline-enriched genes are also expressed in embryonic stem
120 cells (ESCs), they are silenced as they differentiate into EpiLCs¹⁷. Therefore, we assessed if KDM5C was necessary for
121 initial germline gene silencing by evaluating the impact of *Kdm5c* loss in male EpiLCs.

122 We first identified *Kdm5c*-KO EpiLC DEGs through DESeq2 analysis of our previously published RNA-seq dataset³⁵
123 (log2 fold change > 0.5, q < 0.1). Similar to the *Kdm5c*-KO brain, we observed general dysregulation of tissue-enriched
124 genes, with the largest number of genes belonging to the brain and testis, although they were not significantly enriched
125 (Figure 3B). Using our curated list, we found *Kdm5c*-KO EpiLCs aberrantly expressed 54 germline-enriched genes, including
126 the previously characterized hippocampal DEG¹⁰ *Cytochrome C, testis-specific (Cyct)* (Figure 3C). Although we observed
127 aberrant expression of many tissue-enriched genes, we did not observe any significant difference in primed pluripotency
128 genes (Figure 3D) or gross changes in *Kdm5c*-KO cell morphology during differentiation (Figure 3E), indicating KDM5C loss
129 does not impair EpiLC formation.

130 To evaluate if all germline DEGs are constitutively dysregulated or change over the course of development, we then
131 compared EpiLC germline DEGs to those expressed in the *Kdm5c*-KO brain. The majority of EpiLC germline DEGs were
132 unique to EpiLCs, with only *Cyct* shared across all sequencing datasets (Figure 3F). We then compared the function of
133 EpiLC and brain germline DEGs and found particularly high enrichment of meiosis-related gene ontologies in EpiLCs (Figure
134 3G), such as meiotic cell cycle (GO: 0051321, p.adjust = 4.44e-07). While there was modest enrichment of meiotic gene
135 ontologies in both brain regions, the *Kdm5c*-KO hippocampus showed strong enrichment of late-stage sperm genes, such
136 as those involved in the sperm axoneme. This shift from meiotic genes to later spermatogenesis genes in the hippocampus
137 suggests the germline developmental program could occur ectopically as *Kdm5c*-KO cells progresses through somatic
138 tissue development. **note: this is strengthened by the ChIP-seq data since KDM5C is not directly bound to many**
139 **brain/flagellar DEGs. This point might be stronger in the ChIPseq figure**

140 Interestingly, DEGs unique to *Kdm5c*-KO EpiLCs included key drivers of germline identity, such as *Stimulated by retinoic*
141 *acid 8 (Stra8)* and *Deleted in azoospermia like (Dazl)* (Figure 3H). These genes are typically expressed during embryonic
142 germ cell development to commit PGCs to the germline fate, but are also expressed later in life to trigger meiotic gene
143 expression programs^{36–38}. Of note, some germline genes, including *Dazl*, are also expressed in the two-cell embryo. However,
144 we did not see misexpression of two-cell embryo-specific genes, like *Zscan4d* (q = 0.381), indicating *Kdm5c*-KO in EpiLCs do
145 not revert back to a 2-cell state (Figure 3H).

146 *Dazl* is an RNA-binding protein that regulates the translation of germline mRNAs and is essential for germ cell function³⁹.
147 A significant portion of germline transcripts misexpressed in *Kdm5c*-KO EpiLCs are known binding targets of DAZL, including
148 *Stra8*⁴⁰ (p = 1.698e-07, Fisher's Exact Test). This suggests expression of DAZL protein could promote the translation of other

149 aberrant germline transcripts, influencing their ability to impact *Kdm5c*-KO cellular function. We thus tested DAZL protein
150 expression in *Kdm5c*-KO EpiLCs through immunocytochemistry (Figure 3H). We observed about 25% of *Kdm5c*-KO EpiLCs
151 expressed DAZL protein and it was localized to the cytoplasm ($p = 0.0015$, Welch's t-test), consistent with the pattern of DAZL
152 expression in spermatogonia⁴⁰. Altogether these results suggest tissue-specific genes are misexpressed during *Kdm5c*-KO
153 embryogenesis, including key drivers of germline identity that can be translated into protein.

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

- 155 • **note: do Direct vs indirect DEGs motif analysis**
156 • However, it is currently unclear if KDM5C binds to all germline DEGs and if its binding is maintained at any germline
157 genes in neurons.

158 Previous work suggests KDM5C represses germline genes during early development, as re-expression of KDM5C in
159 knockout neuronal cultures fails to suppress hippocampal germline DEGs and KDM5C is not bound to their promoters
160 in neurons¹⁰. There is some evidence KDM5C binds to select germline gene promoters in ESCs¹⁰, including two recent
161 independent screens that found KDM5C binds to Dazl's promoter^{20,21}. As KDM5C's binding at germline gene promoters has
162 not been systematically characterized, it is currently unclear what types of germline genes KDM5C regulates and if its binding
163 is maintained at any germline genes in neurons.

164 To further characterize the mechanism behind *Kdm5c*-KO germline gene misexpression, we analyzed KDM5C chromatin
165 immunoprecipitation followed by DNA sequencing (ChIP-seq) datasets in EpiLCs³⁵ and primary neuron cultures (PNCs) from
166 the cortex and hippocampus¹⁵. EpiLCs had a higher total number of KDM5C peaks than PNCs (EpiLCs: 5,808, PNCs: 1,276,
167 MACS2 q < 0.1 and fold enrichment > 1) and KDM5C was primarily localized to gene promoters in both cell types (EpiLCs:
168 4,190, PNCs: 745 +/- 500kb from TSS), although PNCs showed increased localization to non-promoter regions (Figure 4A).

169 The majority of promoters bound by KDM5C in PNCs were also bound in EpiLCs (513 shared promoters), however a
170 large portion of gene promoters were only bound by KDM5C in EpiLCs (3677 EpiLC only promoters) (Figure 4B). We then
171 performed gene ontology analysis to compare the functions of genes bound by KDM5C at the promoter in different cell
172 types. While there were no significant ontologies for genes with KDM5C only bound in PNCs, gene ontologies for peaks
173 shared between PNCs and EpiLCs were enriched for functions involving nucleic acid turnover, such as deoxyribonucleotide
174 metabolic process (GO:0009262, p.adjust = 8.28e-05) (Figure 4C). Germline-specific ontologies were only enriched in
175 peaks unique to EpiLCs, such as meiotic nuclear division (GO: 0007127 p.adjust = 6.77e-16) and meiotic cell cycle process
176 (GO:1903046, p.adjust = 5.05e-16) (Figure 3C). When comparing KDM5C binding at all germline gene promoters, KDM5C
177 was only bound to a subset of germline gene promoters in EpiLCs and was not bound to any in PNCs (Figure 4D). Together,
178 this suggests KDM5C is recruited to a subset of germline gene promoters in EpiLCs, including meiotic genes, but does not
179 regulate germline genes in neurons.

180 To determine if the germline mRNAs expressed in the *Kdm5c*-KO brain and EpiLCs are direct targets of KDM5C, we then
181 compared KDM5C binding at RNA-seq DEG promoters (Figure 4E). About one third of EpiLC specific and brain-specific
182 (hippocampus, amygdala, or both) germline DEGs were bound by KDM5C in EpiLCs (EpiLC only: 36%, Brain only: 33.3%).
183 Some notable differences in KDM5C binding for EpiLC-specific DEGs included *Dazl* and *Stra8*, two key drivers of germline
184 identity discussed above. Although both mRNAs are expressed in *Kdm5c*-KO EpiLCs, KDM5C is only bound to *Dazl*'s
185 promoter and not *Stra8*'s in EpiLCs (Figure 4F). In contrast to the unique DEGs, 3 out of the 4 genes dysregulated in both the
186 brain and EpiLCs bound by KDM5C (Figure 4G). Again, we did not observe any KDM5C binding at germline gene promoters

187 in PNCs, even for brain-specific DEGs (Figure 4H). Altogether, this suggests the majority of germline mRNAs expressed in
188 *Kdm5c*-KO cells are dysregulated independent of direct KDM5C recruitment to their promoters during embryogenesis.

189 **KDM5C erases H3K4me3 to promote long-term repression of germline genes via DNA methylation**

190 Although KDM5C is generally thought to suppress transcription through erasure of histone 3 lysine 4 di- and trimethylation
191 (H3K4me2/3)¹¹, recent studies in ESCs have suggested KDM5C's repression *Dazl* is independent of its catalytic activity²⁰.
192 Somatic repression of germline genes is typically established during the transition between naive and primed pluripotency,
193 which modeled in vitro as ESCs to EpiLC differentiation. In ESCs, chromatin regulators place repressive histone modifications
194 at germline gene promoters, including histone 2A 119 monoubiquitination (H2AK119ub1) and histone 3 lysine 9 trimethylation
195 (H3K9me3)^{16,17,41}. Germline genes are then silenced long-term in EpiLCs by *de novo* placement of DNA CpG methylation¹⁷.
196 It has been proposed KDM5C may promote germline gene silencing via H3K4me3 removal since DNA methylation is lost at
197 select germline gene promoters in the hippocampus¹⁰ and H3K4me3 can impair DNA methylation placement^{42,43}. However,
198 KDM5C was recently shown to repress *Dazl* in ESCs independent of its catalytic activity²⁰. Because KDM5C's role in germline
199 gene repression has only been characterized in ESCs and in the mature brain, it is currently unclear to what extent KDM5C is
200 involved during transition between ESCs and EpiLCs and if its catalytic activity is required for long-term silencing.

201 To elucidate KDM5C's role in germline gene silencing, we first characterized KDM5C's substrates, histone 3 lysine 4
202 di- and trimethylation (H3K4me2/3) in our previously published ChIP-seq datasets in wild type and *Kdm5c*-KO amygdala²³
203 and EpiLCs³⁵. In congruence with previous work in the *Kdm5c*-KO hippocampus¹⁰, we observed aberrant accumulation of
204 H3K4me3 around the transcription start site (TSS) of germline genes in the *Kdm5c*-KO amygdala (Figure 5A). We additionally
205 found a marked increase in H3K4me2 germline gene TSSs in *Kdm5c*-KO EpiLCs (Figure 5B). Increase in H3K4me2 and
206 H3K4me3 was highest in *Kdm5c*-KO cells that were highest genes that are bound by KDM5C at their promoter in EpiLCs
207 (**note: do analysis to check if true**).

208 To assess KDM5C's embryonic role in germline gene silencing, we first characterized KDM5C's expression in ESCs and
209 EpiLCs by harvesting RNA and protein at 0 hours (ESCs), 24 hours, and 48 hours (EpiLCs). While *Kdm5c* mRNA steadily
210 decreased from 0 to 48 hours of differentiation, KDM5C protein initially increased from 0 to 24 hours but then decreased to
211 near knockout levels by 48 hours (Figure 5C).

212 We then determined the role of KDM5C in the initial placement of DNA methylation at germline gene promoters by
213 performing whole genome bisulfite sequencing in wild-type and *Kdm5c*-KO ESCs and 96 hour extended EpiLCs (exEpiLCs)
214 (**note: check**). - While wild-type cells accumulate high levels of DNA methylation over the course of ESCs to exEpiLC
215 differentiation, DNA methylation is markedly reduced in *Kdm5c*-KO

216 **Discussion**

217 Random thoughts - The demarcation of the germ vs soma is a key feature of multicellularity - Anything known about tissue-
218 biased gene expression in other H3K4me regulators? - Our data suggests the germline developmental program is occurring
219 ectopically as *Kdm5c*-KOs progresses through somatic tissue development - tissue-biased gene expression: - However unlike
220 the gonadal-biased DEGs, many liver and muscle-biased DEGs have a known involvement in brain function. For example, the
221 liver-biased DEG is *Apolipoprotein C-I (Apoc1)*, is important for lipoprotein metabolism but has also been shown to influence
222 learning and memory. - Otx2 is expressed in EpiLCs and is known to repress PGC identity. - It's properly expressed in *Kdm5c*-

223 KO EpiLCs, further supporting they aren't just becoming PGCs <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6485399/>

224 • Altogether, these data indicate that while some germline genes are misexpressed due to direct loss of KDM5C binding
225 during emryogenesis, secondary downstream mechanisms can also promote their aberrant transcription.

226 • KDM5C is dynamically regulated during the window of embryonic germline gene silencing, our EpiLC ChIP-seq is likely
227 catching the tail end of KDM5C's main involvement.

228 • Papers to read/reference:

229 – Reconstitution of the Mouse Germ Cell Specification Pathway in Culture by Pluripotent Stem Cells: [https://www.cell.com/fulltext/S0092-8674\(11\)00771-9](https://www.cell.com/fulltext/S0092-8674(11)00771-9)

230 – 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/>

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320 **Figures and Tables**

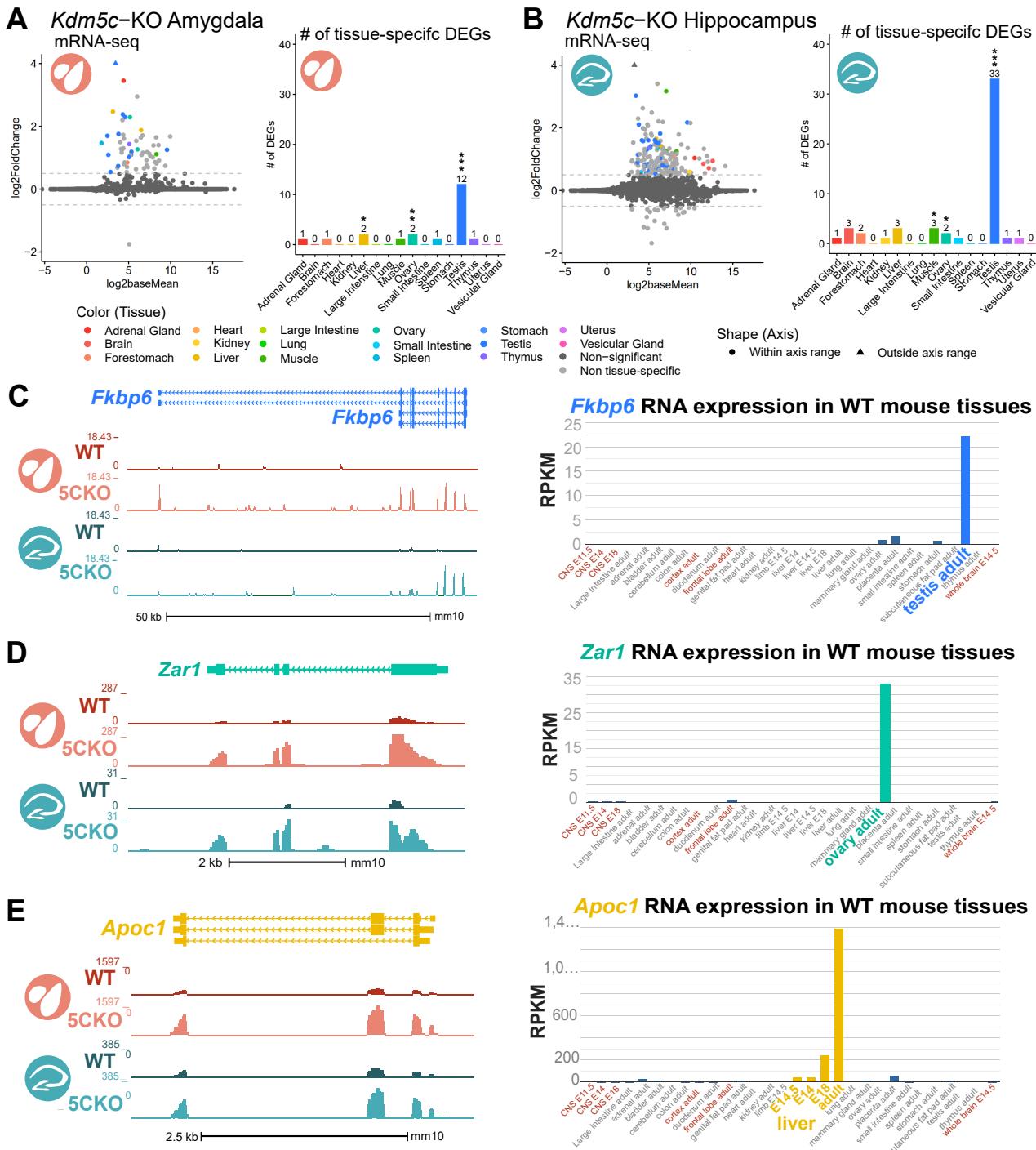


Figure 1: Tissue-enriched genes are misexpressed in the *Kdm5c-KO* brain. **A.** Expression of tissue-enriched genes in the male *Kdm5c-KO* amygdala. Left - MA plot of mRNA-seq. Right - Number of tissue-enriched differentially expressed genes (DEGs). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Fisher's exact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

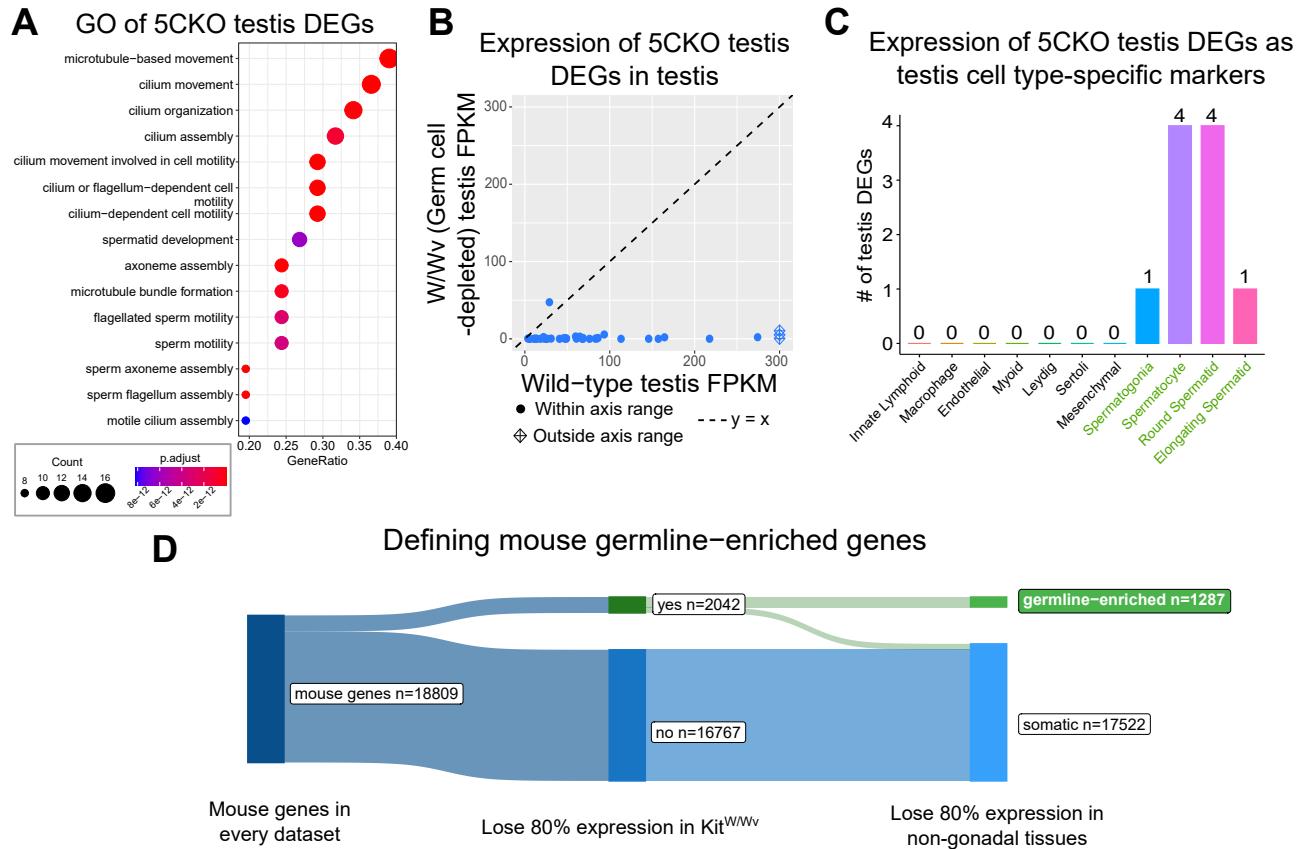


Figure 2: Aberrant transcription of germline genes in the *Kdm5c*-KO in the brain. **A.** enrichPlot gene ontology (GO) of *Kdm5c*-KO amygdala and hippocampus testis-enriched DEGs **B.** Expression of testis DEGs in wild-type (WT) testis versus germ cell-depleted (W/Wv) testis. Expression is in Fragments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

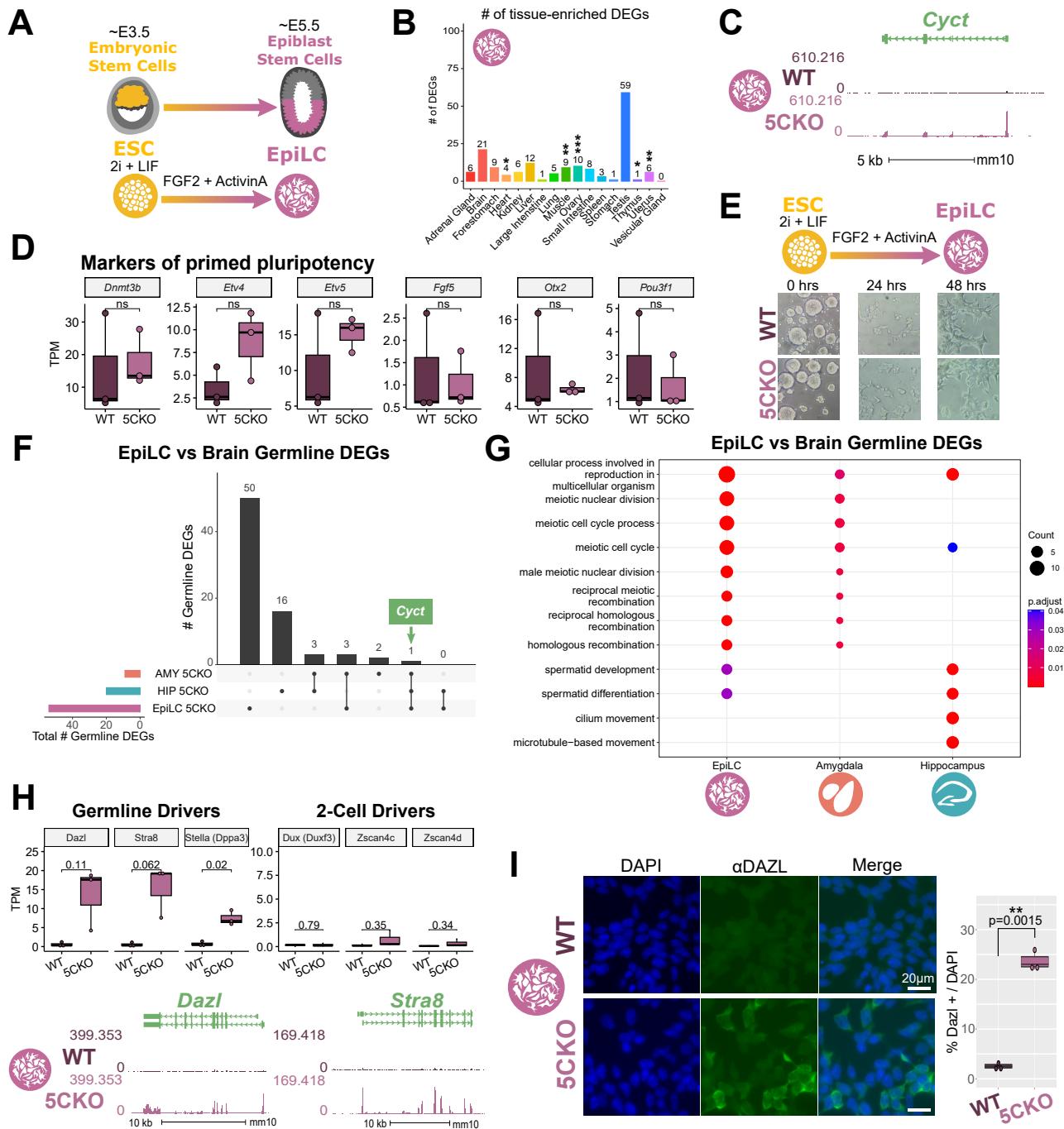


Figure 3: Kdm5c-KO epiblast-like cells express key drivers regulators of germline identity **A.** Top - Diagram of *in vivo* differentiation of embryonic stem cells (ESCs) of the inner cell mass into epiblast stem cells. Middle - *in vitro* differentiation of ESCs into epiblast-like cells (EpiLCs). Bottom - representative images of wild-type (WT) and *Kdm5c*-KO ESC to EpiLC differentiation. Brightfield images taken at 20X. **B.** 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). **C.** Number of tissue-enriched differentially expressed genes (DEGs). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Fisher's exact test. **D.** Average bigwigs of an example germline gene, *Cyct*, that is dysregulated *Kdm5c*-KO EpiLCs. **E.** Upset plot displaying the overlap of germline DEGs expressed in *Kdm5c*-KO EpiLCs, amygdala (AMY), and hippocampus (HIP) RNA-seq datasets. **F.** enrichPlot comparing enriched gene ontologies for *Kdm5c*-KO EpiLC, amygdala, and hippocampus germline DEGs. **G.** Top - Example germline identity DEGs unique to EpiLCs, p-values for Welch's t-test. Bottom - Average bigwigs of *Dazl* and *Stra8* expression in wild-type and *Kdm5c*-KO EpiLCs **H.** Immunocytochemistry of DAZL in male wild-type (WT) and *Kdm5c*-KO (5CKO) EpiLCs. Percentage of DAZL-positive cells normalized to DAPI, p-value for Welch's t-test.

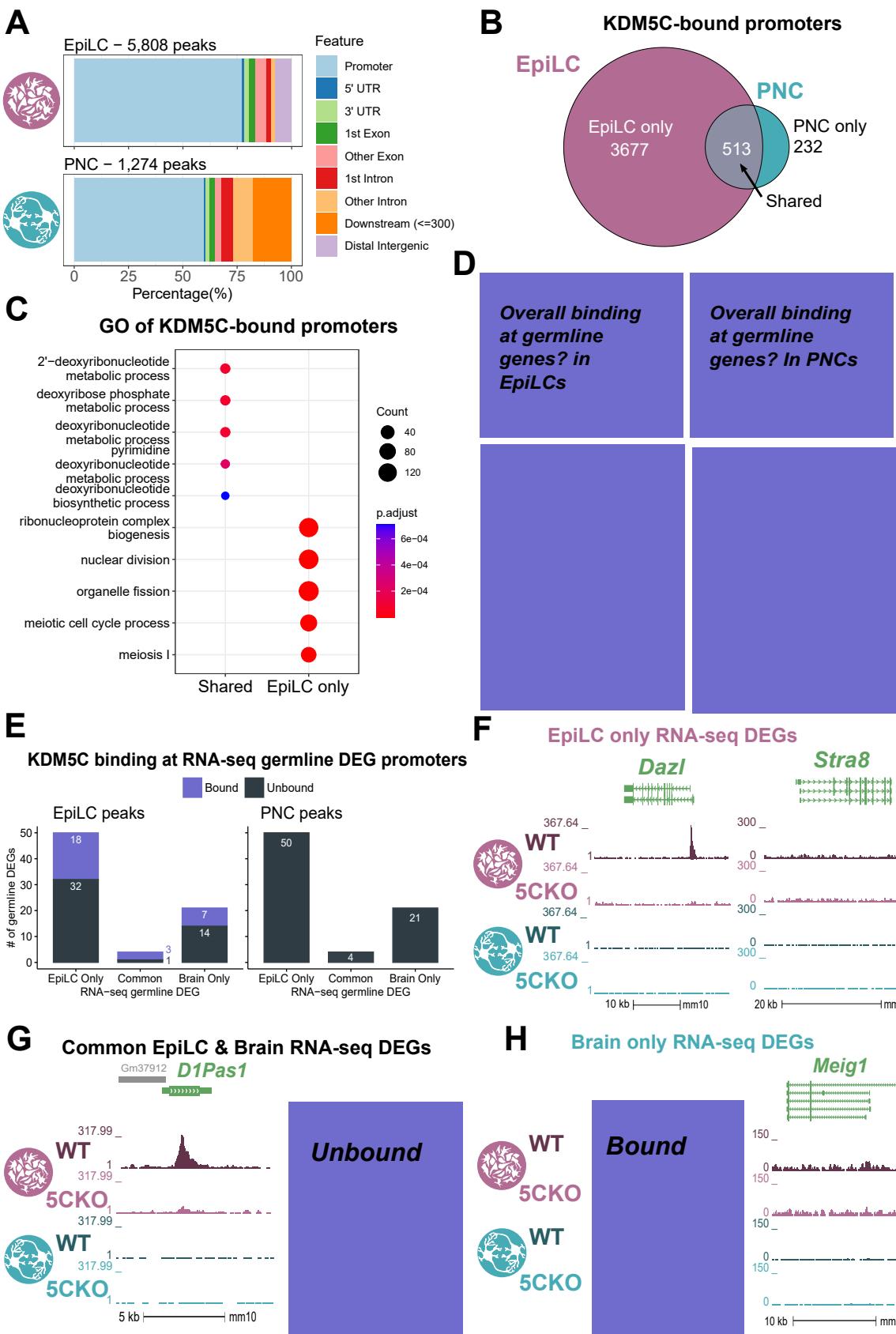


Figure 4: KDM5C binds to a subset of germline gene promoters during early embryogenesis **A.** ChIPseeker localization of KDM5C peaks at different genomic regions in EpiLCs (top) and hippocampal and cortex primary neuron cultures (PNCs, bottom). **B.** Overlap of genes with KDM5C bound to their promoters in EpiLCs (purple) and PNCs (blue). **C.** Gene ontology (GO) comparison of genes with KDM5C bound to their promoter. 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 at 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 4: KDM5C binds to a subset of germline gene promoters during early embryogenesis. (Legend continued.) **F.** Example ChIP-seq bigwigs of DEGs unique to EpiLCs. Although both are expressed in EpiLCs, KDM5C is bound to the *Dazl* promoter but not the *Stra8* promoter in EpiLCs. **F.** Bigwigs of the upregulated imprinted gene *Dlk1* that shows rescue in the double mutant brain. **G.** Example ChIP-seq bigwigs of DEGs common between brain and EpiLCs. KDM5C is bound to the *D1Pas1* promoter but not the *XXX* promoter in EpiLCs. **H.** Example ChIP-seq bigwigs of DEGs unique to the brain. KDM5C is bound to the *XXX* promoter but not the *Meig1* promoter

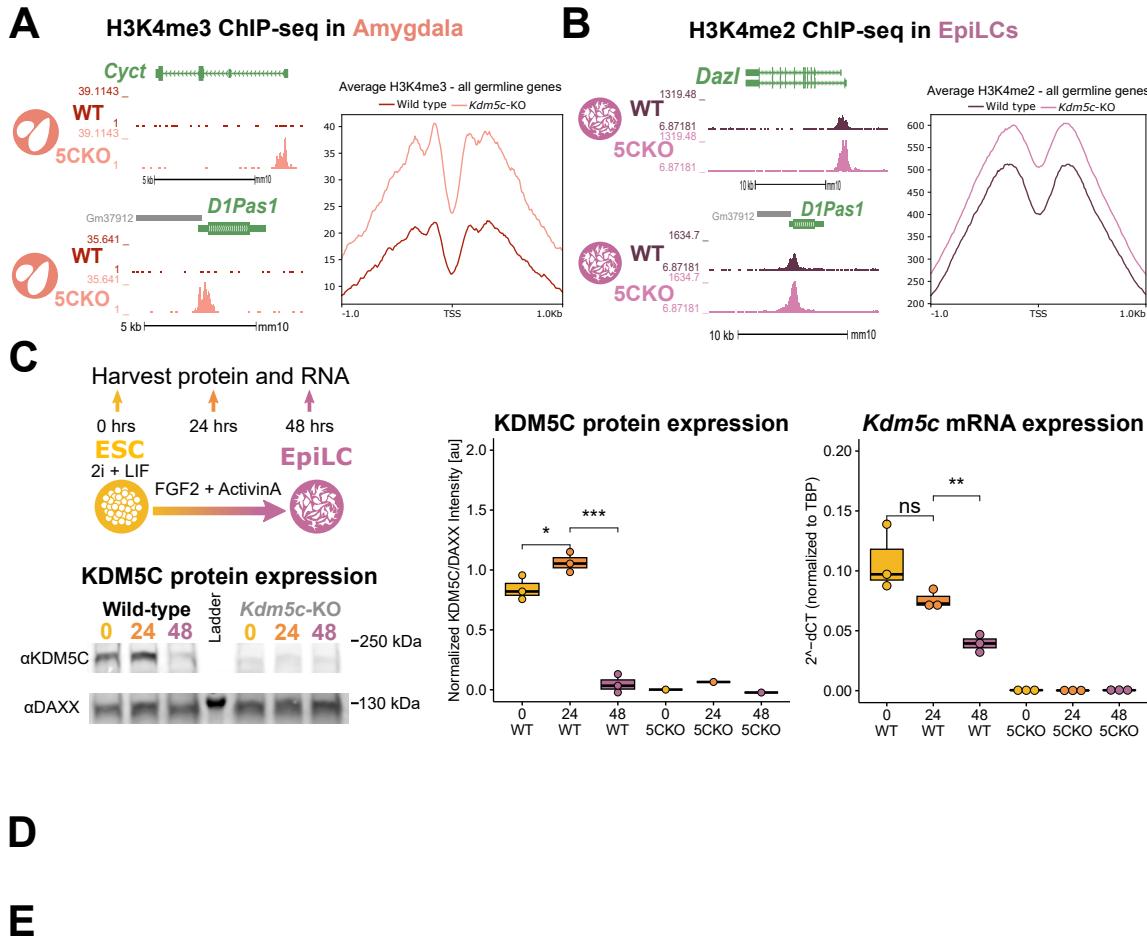


Figure 5: KDM5C's catalytic activity promotes long-term silencing of germline genes via DNA methylation. **A.** Left - Bigwigs of representative histone 3 lysine 4 trimethylation (H3K4me3) ChIP-seq peaks at two germline genes in the wild-type (WT) and *Kdm5c*-KO (5CKO) adult amygdala. Right - Average H3K4me3 at the transcription start site (TSS) of all germline-enriched genes in wild-type (dark red) and *Kdm5c*-KO 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. **C.** RNA and protein expression of KDM5C across ESC to EpiLC differentiation. Top left - diagram of differentiation protocol and collection time points. Bottom left - representative lanes of Western blot for KDM5C and DAXX. Middle - KDM5C protein expression normalized to DAXX. Quantified intensity using ImageJ (artificial units - au). Right - RT-qPCR of *Kdm5c* RNA expression, calculated in comparison to TBP expression ($2^{-\Delta\Delta CT}$). **D.** XXX. **E.** XXX.

321 **Notes**

322 **Figure outline:**

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

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

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

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

342 **Figure 4: Loss of KDM5C's catalytic activity impairs DNAme placement and long-term silencing of germline
343 genes** * Increase in H3K4me3 in *Kdm5c*-KO amygdala at germline genes * Increase in H3K4me2 in EpiLCs at germline
344 genes * *Kdm5c* binding in EpiLCs vs PNCs to show that germline repression is happening in early embryo * Previous studies
345 only looked at ESCs, unknown if catalytic activity is required for long-term repression, especially since DNA methylation is
346 placed later). KDM5C RNA and protein ESC → EpiLC (increasing then decreasing) * RNA expression of germline genes with
347 catalytic dead rescue (Ilakkiya) * DNA methylation in WT and 5CKO EpiLCs (Ilakkiya)

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

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

358 Introduction: * Chromatin regulators are important for cellular identity * H3K4me1-3 linked to active gene promoters and
359 enhancers * Surprisingly, mutations in Chromatin regulators lead to many NDDs (including many H3K4 regulators) * Recent

360 studies have shown some chromatin regulators are important for regulating neuron-specific gene expression/chromatin
361 stat_compare_means * However, loss of some chromatin regulators can also lead to ectopic expression of tissue-enriched
362 genes * Very few studies have looked at these genes and it's unclear if these genes contribute to NDD impairments. *
363 Necessary to first characterize the mechanism behind their derepression to identify molecular footholds into testing their
364 contribution to neuronal impairments and potential for therapeutic intervention

- 365 • Loss of KDM5C can result in the misexpression of genes typically only found in the testis
- 366 – Misexpression of tissue-enriched genes hasn't been systematically characterized - Unclear if these genes are
367 exceptions or if other tissue-specific genes are dysregulated
- 368 – Interestingly, these genes (Cyct, D1pas1) typically function in the germline
- 369 – Germ cells (meiotic cells) are typically distinguished from somatic cells very early on in embryogenesis and is a
370 key feature of multicellularity
- 371 – Chromatin regulators are very important for decommissioning germline genes and act successively the embryo
372 implants into the uterine wall
- 373 * Most studies have focused on ESCs, which have a similar transcriptome to germ cells / 2-cells
- 374 * recently, KDM5C was shown to repress DAZL in ESCs, independent of its catalytic activity
- 375 * However, DNA methylation is lost in the mature 5CKO brain, DNA methylation is placed later and it's unclear if
376 it's required for long-term repression (maybe too specific, just trying to go into the fact that the mechanism is
377 partially understood but unclear)
- 378 – Systematic characterization of ectopic germline genes hasn't been done
- 379 * unknown if other germline-enriched genes are dysregulated, including oocyte-specific genes
- 380 * Crucially, it's unknown if misexpression of the germline program leads to functional consequences in 5CKO
381 cells.

382 **Germline gene repression background:**

383 Interestingly, some of the ectopic testis transcripts identified in the *Kdm5c*-KO brain are typically expressed in germ cells¹⁰.
384 Unlike somatic cells, germ cells (e.g. sperm and eggs) undergo meiosis and pass on their genetic material to the next genera-
385 tion. The germline and the soma are typically distinguished during early embryogenesis, when germline genes are silenced
386 in epiblast stem cells soon after implantation and only reactivated in a subset to form the germline. Chromatin regulators
387 play a key role in decommissioning germline genes as the embryo transitions from naive to primed pluripotency by placing
388 repressive histone H2A lysine 119 monoubiquitination (H2AK119ub1)¹⁶, histone 3 lysine 9 trimethylation (H3K9me3)^{16,17},
389 and DNA CpG methylation¹⁷⁻¹⁹ at germline gene promoters. KDM5C may also be involved in this early decommissioning of
390 germline genes, as re-expression of KDM5C in neurons fails to suppress their dysregulation¹⁰. In support of this, KDM5C
391 was very recently shown to repress *Deleted in azoospermia like* (*Dazl*), a key regulator of germline development, in mouse
392 embryonic stem cells (ESCs)^{20,21}. In support of this, two independent screens in mouse embryonic stem cells (ESCs) recently
393 identified KDM5C as a repressor of *Deleted in azoospermia like* (*Dazl*), a key regulator of germline development. However,
394 KDM5C's role in embryonic germline gene repression is currently unclear, given that *Dazl* is also expressed in ESCs and in
395 the 2-cell stage and germline gene misexpression has yet to be globally characterized during *Kdm5c*-KO embryogenesis.