

1 Misexpression of germline programs with loss of the X-linked intellectual  
2 disability factor KDM5C

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

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

- 19 • Loss of KDM5C can result in the misexpression of genes typically only found in the testis  
20     – Misexpression of tissue-enriched genes hasn't been systematically characterized - Unclear if these genes are  
21         exceptions or if other tissue-specific genes are dysregulated  
22     – Interestingly, these genes (Cyct, D1pas1) typically function in the germline  
23     – Germ cells (meiotic cells) are typically distinguished from somatic cells very early on in embryogenesis and is a  
24         key feature of multicellularity  
25     – Chromatin regulators are very important for decommissioning germline genes and act successively the embryo  
26         implants into the uterine wall  
27         \* Most studies have focused on ESCs, which have a similar transcriptome to germ cells / 2-cells  
28         \* recently, KDM5C was shown to repress DAZL in ESCs, independent of its catalytic activity  
29         \* However, DNA methylation is lost in the mature 5CKO brain, DNA methylation is placed later and it's unclear if  
30             it's required for long-term repression (maybe too specific, just trying to go into the fact that the mechanism is  
31             partially understood but unclear)  
32     – Systematic characterization of ectopic germline genes hasn't been done  
33         \* unknown if other germline-enriched genes are dysregulated, including oocyte-specific genes  
34         \* Crucially, it's unknown if misexpression of the germline program leads to functional consequences in 5CKO  
35         cells.

36 **Results**

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

38 RNA sequencing (RNA-seq) studies surprisingly revealed the aberrant expression of testis genes within the adult *Kdm5c*  
39 knockout (-KO) hippocampus<sup>1</sup>. Given the high abundance of testis-enriched genes within the mouse transcriptome, it is  
40 currently unclear if their misexpression in the *Kdm5c*-KO brain is indicative of random de-repression throughout the genome  
41 or instead reflects an impairment in tissue identity. To distinguish these possibilities, we globally assessed the expression of  
42 previously characterized tissue-enriched genes<sup>2</sup> in our previously published mRNA-seq datasets of the *Kdm5c*-KO amygdala  
43 and hippocampus<sup>3</sup>

44 We found many genes significantly upregulated within the *Kdm5c*-KO brain (DESeq2<sup>4</sup>, log2 fold change > 0.5, q < 0.1) are  
45 typically enriched in non-brain tissues, with the majority of differentially expressed genes (DEGs) displaying biased expression  
46 towards the testis (Figure 1A-C). Even though the testis has the largest number of tissue-biased genes compared to any  
47 other tissue (2496 genes), testis-biased DEGs were significantly enriched for both brain regions (Amygdala p =  $1.83 \times 10^{-5}$ ;  
48 Hippocampus p =  $4.26 \times 10^{-11}$ , Fisher's Exact).

49 Even though the *Kdm5c*-KO mice are male, we also observed significant enrichment of ovary-biased DEGs within both  
50 brain regions (Amygdala p = 0.00574; Hippocampus p = 0.048, Fisher's Exact) (Figure 1D). Intriguingly, many ovary and  
51 testis-biased DEGs have functions specific to germ cells and have no known role in the brain. For example, the testis-biased  
52 DEG *Cytochrome C, testis* (*Cyct*) is a testis-specific cytochrome c isoform found in spermatozoa mitochondria and is important  
53 for flagellar beating<sup>5,6</sup> (Figure 1C). The ovary-enriched DEG *Zygotic arrest 1* (*Zar1*) was recently shown to sequester mRNAs  
54 in oocytes for meiotic maturation and early zygote development<sup>7</sup> (Figure 1D). Although not consistent across brain regions,  
55 we also found enrichment of two non-gonadal tissues - the liver (Amygdala p = 0.0398, Fisher's Exact) and the muscle  
56 (Hippocampus p = 0.0104, Fisher's Exact). One example of a liver-biased DEG is *Apolipoprotein C-I* (*Apoc1*), which is  
57 involved in lipoprotein metabolism (Figure 1E). Overall, these tissue-enriched genes show little to no expression in the  
58 wild-type brain, yet our mRNA-seq data indicate they are polyadenylated and spliced into mature transcripts (Figure 1C-E).

59 Of note, we did not observe enrichment of brain-enriched genes (Amygdala p = 1; Hippocampus p = 0.74, Fisher's  
60 Exact), even though the brain has the second highest total number of tissue-enriched genes (708 genes). Altogether, these  
61 results suggest misexpression of testis and other tissue-enriched genes within the *Kdm5c*-KO brain is not due to random  
62 de-repression of all genes, but rather due to a dysregulation of tissue identity.

63 **Male and female germline genes are aberrantly expressed in the male *Kdm5c*-KO brain**

64 Intriguingly, many *Kdm5c*-KO testis-enriched DEGs have functions specific to male germ cells, such as *Cytochrome C, testis*  
65 (*Cyct*), a testis-specific cytochrome c isoform found in spermatozoa mitochondria and is important for flagellar  
66 beating<sup>5,6</sup>. We therefore wondered if the high enrichment of testis genes within the *Kdm5c*-KO brain reflected a failure to  
67 demarcate the soma and germline, as the testis contains both germ cells (e.g. spermatogonia) as well as somatic cells  
68 (e.g. Leydig cells) that support hormone production and germline functions. Gene ontology analysis of testis DEGs revealed  
69 enrichment of germline-relevant ontologies in the *Kdm5c*-KO amygdala and hippocampus, including spermatid development  
70 (GO:0007286) and sperm axoneme assembly (GO:0007288) (Figure 1A).

71 To validate if these testis DEGs are truly germline genes, we then compared their expression in a published RNA-seq  
72 dataset of wild-type (WT) and germ cell-depleted (*Kit<sup>W/Wv</sup>*) mouse testes<sup>8</sup>. We found almost all *Kdm5c*-KO testis-enriched

73 DEGs lose their expression with germ cell depletion (Figure 1B). The only testis DEG that did not show considerable  
74 downregulation with germline depletion was *FK506 binding protein 6* (*Fkbp6*), a known regulator of piRNA expression and  
75 meiosis in germ cells<sup>9,10</sup>. We additionally assessed testis DEG expression in a published testis single cell RNA-seq dataset  
76 that identified cell type-specific markers<sup>11</sup>. We found that while some testis-enriched DEGs were classified as specific  
77 markers for multiple stages of germ cell development - including spermatogonia, spermatocytes, round spermatids, and  
78 elongating spermatids, none marked somatic cells (Figure 1C). Altogether, these data suggest testis genes expressed within  
79 the *Kdm5c*-KO brain are actually male germ cell genes.

80 Interestingly, we also observed significant enrichment of ovary genes within the male *Kdm5c*-KO brain (Amygdala p =  
81 0.00574; Hippocampus p = 0.048, Fisher's Exact). Although there are relatively few ovary-enriched DEGs compared to  
82 testis-DEGs (Amygdala: 12 testis DEGs, 2 ovary DEGs; Hippocampus: 33 testis DEGs, 2 ovary DEGs), they also have  
83 known germline-specific functions. For example, *Zygotic arrest 1* (*Zar1*) was recently shown to regulate oocyte translation by  
84 sequestering mRNAs<sup>7</sup> (Figure 1D).

85 • To assess if ovary-enriched DEGs were also germline specific, we assessed their expression in wild-type and germline-  
86 depleted (*Kit<sup>W/Wv</sup>*) female embryos, when female germ cells undergo the initial stages of development and meiosis.

87 – We found XYZ.

88 – Altogether, these results indicate male *Kdm5c*-KO mice express both male and female germline genes within the  
89 amygdala and hippocampus.

90 • One difficulty in globally characterizing the misexpression of germline genes is a comprehensive list of mouse germline-  
91 enriched genes is currently lacking.

92 • To facilitate downstream analyses, we generated a list of male and female germline-enriched genes by evaluating  
93 expression in wild-type and germline-depleted (*Kit<sup>W/Wv</sup>*) male and female mouse embryos from embryonic day 12, 14,  
94 and 16.

95 – **note: need to figure out why these time points. We have P6 and adult for males but not females so it would  
96 be biased towards males if we included the later ages**

97 We defined genes as germline-enriched if their expression met the following criteria: 1) their expression is greater than 1  
98 FPKM in wild-type germline 2) their expression in any wild-type, non-gonadal tissue<sup>2</sup> does not exceed 20% of maximum  
99 expression in wild-type germline, and 3) their expression in the germ cell-depleted germline, for any sex or time point, does  
100 not exceed 20% of their maximum expression in the wild-type germline. These criteria yielded 1287 germline-enriched  
101 genes. To more comprehensively assess the impact of sex on germline gene misexpression, we additionally categorized if  
102 germline-enriched genes displayed biased expression in the wild-type female (XX) or male germline. We defined sex-biased  
103 genes as those whose expression in the opposite sex, at any time point, is no greater than 20% of the gene's maximum  
104 expression in a given sex. This yielded 65 XX-biased, 1023 XY-biased, and 199 unbiased germline-enriched genes, which is  
105 consistent with the testis overall having a more unique transcriptome than the ovary<sup>2</sup>.

106 **Kdm5c-KO cells express master regulators of germline identity in an *in vitro* model of early  
107 embryogenesis**

108 Misexpression of germline genes in the *Kdm5c*-KO brain suggests mutants fail to demarcate germline and somatic cellular  
109 identity. Germ cells are typically distinguished from somatic cells soon after the embryo implants into the uterine wall<sup>12,13</sup>  
110 when a subset of epiblast stem cells become the primordial germ cells (PGCs) while the remainder differentiate into the  
111 ectoderm, mesoderm, and endoderm to form the body's somatic tissues<sup>14</sup>. This developmental time point can be modeled *in*  
112 *vitro* through differentiation of embryonic stem cells (ESCs) into post-implantation epiblast-like stem cells (EpiLCs) (Figure 3A,  
113 top). Previous studies have demonstrated that while some germline-enriched genes are also expressed in embryonic stem  
114 cells (ESCs), they are silenced as they differentiate into EpiLCs<sup>15</sup>. Therefore, to elucidate the mechanism underlying aberrant  
115 germline genes expression in the mature *Kdm5c*-KO brain, we first assessed their expression in male *Kdm5c*-KO EpiLCs  
116 using our previously generated RNA-seq dataset<sup>16</sup>.

- 117 • We did not observe any apparent morphology changes between wild-type and *Kdm5c*-KO cells during ESC to EpiLC  
118 differentiation (Figure 3A, bottom). Additionally, *Kdm5c*-KO EpiLCs downregulated ESC pluripotency genes and  
119 upregulated EpiLC differentiation genes similar to wild-type (Figure 3B).
- 120 • We then identified *Kdm5c*-KO EpiLC DEGs through DESeq2<sup>4</sup> (log2 fold change > 0.5, q < 0.1) and observed widespread  
121 dysregulation of tissue-enriched genes, with the largest number of genes belonging to the brain and testis (Figure 3C).
- 122 • Of these testis genes, we also observed misexpression of germline-enriched genes, including *Cyct* (Figure 3D)
- 123 • To evaluate whether germline mRNAs are constitutively dysregulated or change over the course of development, we  
124 compared EpiLC germline DEGs to those expressed in the *Kdm5c*-KO brain. The majority of EpiLC germline DEGs  
125 were unique to EpiLCs, with only a few shared across brain sequencing datasets (Figure 3E). Only two germline genes  
126 were consistently misexpressed in all datasets, *DNA segment, Chr 1, Pasteur Institute 1 (D1Pas1)* and *Testis expressed*  
127 *gene 14 (Tex14)*.
- 128 • We then compared the typical function of brain and EpiLC germline DEGs and found EpiLC germline DEGs displayed  
129 strong enrichment of meiosis-related gene ontologies, including XXXXX (Figure 3F).
- 130 • Surprisingly, germline DEGs unique to *Kdm5c*-KO EpiLCs included master regulators of germline identity, such as  
131 *Stimulated by retinoic acid 8 (Stra8)* and *Deleted in azoospermia like (Dazl)* (Figure 3G). These genes are typically  
132 expressed during embryonic germ cell development to commit PGCs to the germline fate, but they are also expressed  
133 later in life to trigger meiotic gene expression programs<sup>17-19</sup>.
- 134 • *Dazl* is a translational regulator essential for germ cell function<sup>20</sup>. Interestingly, a significant portion of germline  
135 transcripts expressed in *Kdm5c*-KO EpiLCs are known binding targets of DAZL, including *Stra8*<sup>21</sup> (XY *Kdm5c*-KO  
136 EpiLCs p = 1.698e-07, Fisher's Exact Test). This suggests expression of DAZL protein would enable the translation of  
137 other aberrant germline transcripts, influencing their ability to impact on *Kdm5c* mutant cellular function.
- 138 • Therefore, we then stained *Kdm5c*-KO EpiLCs for DAZL protein expression through immunocytochemistry (Figure 3H).  
139 We found about 25% of *Kdm5c*-KO EpiLCs expressed DAZL protein in their cytoplasm (p = 0.0015, Welch's t-test, XY  
140 KO versus WT), consistent with the subcellular localization observed when DAZL is stained in spermatogonia<sup>21</sup>.

141 **Discussion**

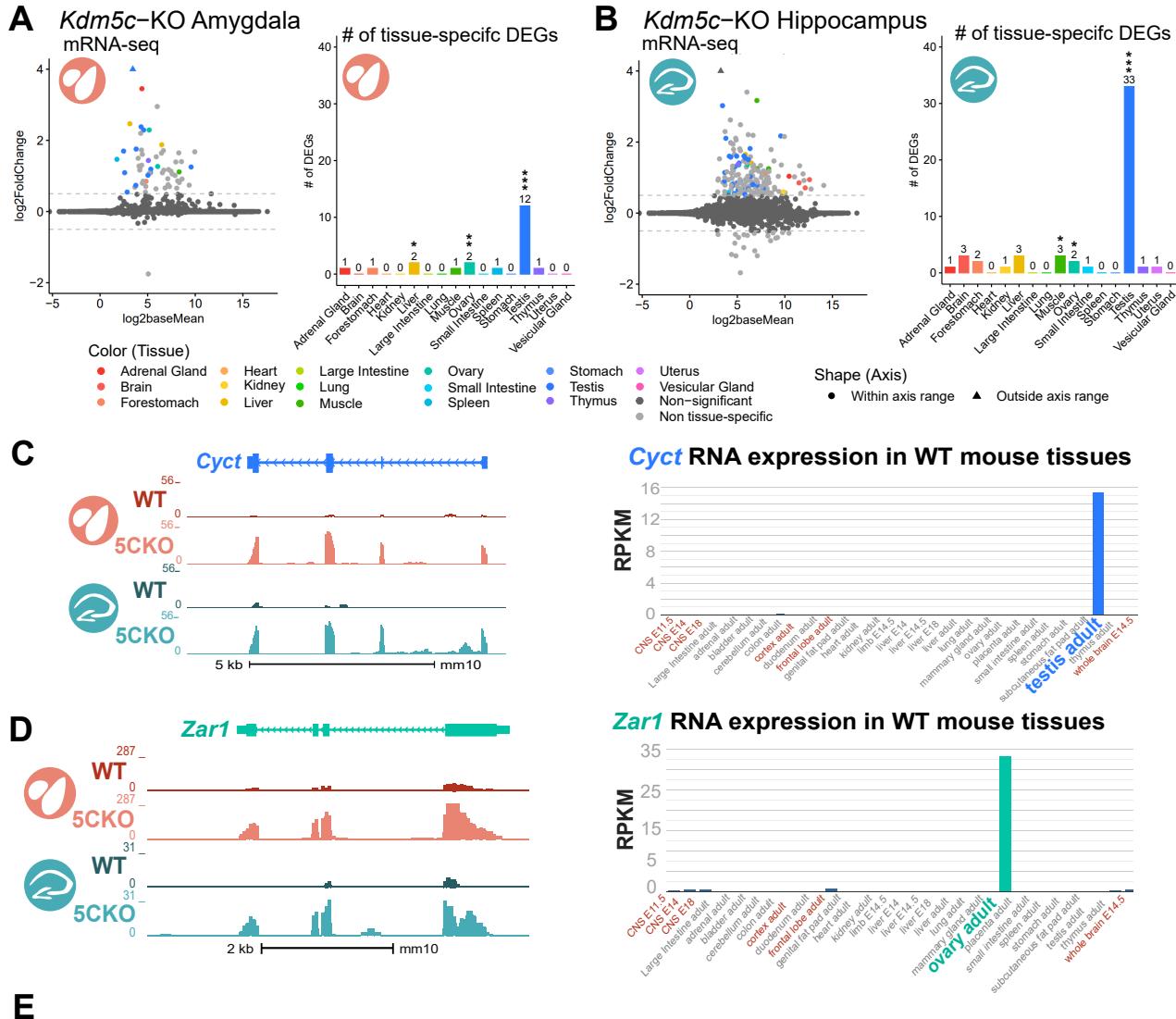
- 142 • tissue-biased gene expression:
- 143 – However unlike the gonadal-biased DEGs, many liver and muscle-biased DEGs have a known involvement in brain  
144 function. For example, the liver-biased DEG is *Apolipoprotein C-I (Apoc1)*, is important for lipoprotein metabolism  
145 but has also been shown to influence learning and memory (Figure 1E).
- 146 • Papers to read/reference:
- 147 – 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)
- 148 – 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/>

151 **References**

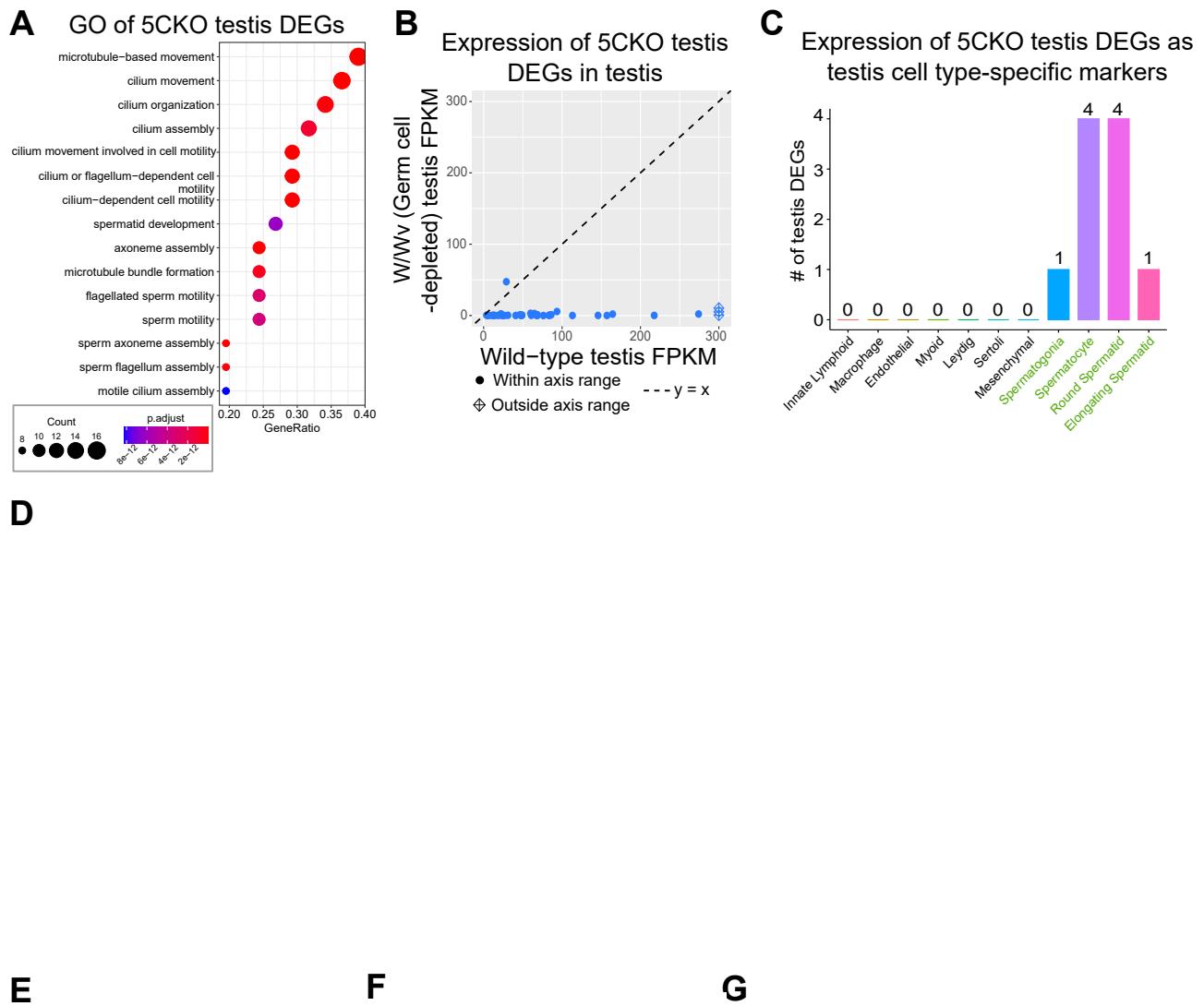
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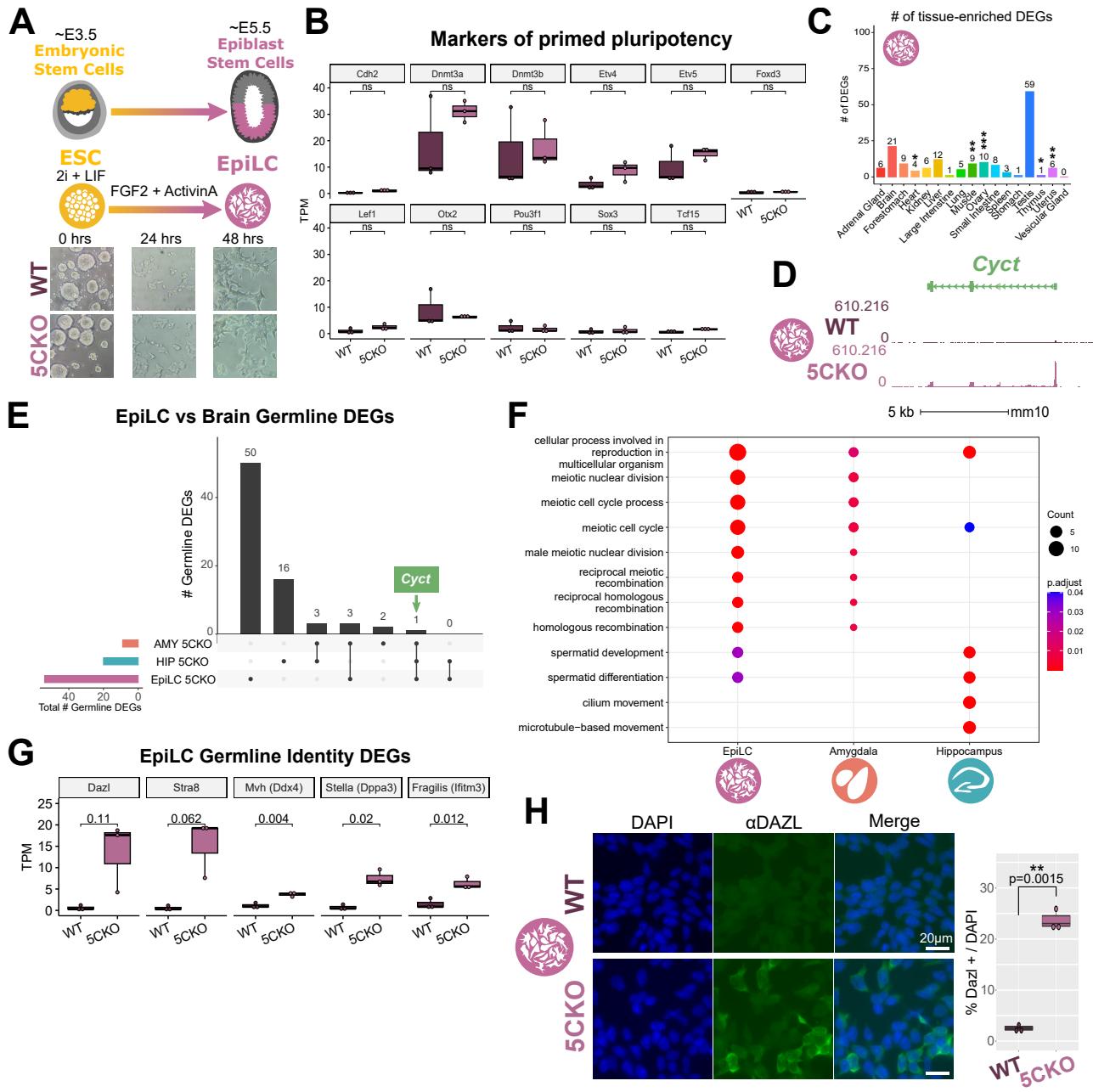
194 **Figures and Tables**



**Figure 1: Misexpression of tissue-enriched genes in the *Kdm5c*-KO in the brain** **A.** Expression of tissue-enriched genes in the male *Kdm5c*-KO amygdala. Left - MA plot of mRNA-sequencing. Right - The 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 - Bigwigs of an example aberrantly expressed testis-enriched gene, *Cytochrome C, testis-specific* (*Cyct*) 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 - Bigwigs of an example ovary-enriched germline 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.



**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. Germline cell types are highlighted in green, somatic cell types in black. **D.** Expression of ovary-enriched DEGs in a wild-type versus germ cell-depleted testis



**Figure 3: Male and female *Kdm5c* mutant epiblast-like cells express master 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 and primordial germ cells. Bottom - *in vitro* differentiation of ESCs into epiblast-like cells (EpiLCs). **B.** Left - Bigwigs of an example germline gene, *Cyct* that is dysregulated in male and female *Kdm5c* mutant EpiLCs. Right - Overlap of all germline DEGs between male and female EpiLCs. **C.** Chromosome location of germline genes misexpressed in male and female EpiLCs. **D.** Number of EpiLC germline DEGs that show sex-biased expression in the wild-type gonads. **E.** Upset plot displaying the overlap of germline DEGs expressed in male and female *Kdm5c* mutant EpiLC and brain RNA-seq datasets. **F.** Expression of genes key for early germ cell development in *Kdm5c* mutant EpiLCs in transcripts per million (TPM) with p-values for Welch's t-test compared to wild type of the same sex. **G.** Immunocytochemistry of DAZL in male wild-type (WT) and *Kdm5c*-KO (5CKO) EpiLCs.

195 **Figure outline:**

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

199 **Figure 2: The male *Kdm5c*-KO brain expresses male and female germline-enriched genes** \* Gene ontology of  
200 testis DEGs in the amygdala and hippocampus - ontologies are germline ontologies \* Expression of testis DEGs in germline-  
201 depleted testis (this is adult testis data) \* scRNASeq of testis - # of testis DEGs that are germline-specific markers \* Although  
202 far fewer, 5CKO brain also expresses ovary-enriched genes(NCBI and bigwigs of Zar1) \* These ovary enriched genes are  
203 also germline specific (NCBI/Li tissues are in adult ovary, so it would be best to show they're oocyte-specific in adult ovary.  
204 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  
205 W/Wv data since oocytes are developed at this point? Or both?) \* Defining what is/isn't a germline gene, and which are  
206 male/female biased using embryonic W/Wv data

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

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

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

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