

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

3  
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 **Germline genes are aberrantly expressed in the male *Kdm5c*-KO brain**

64 The testis contains both germ cells (e.g. spermatogonia) and somatic cells (e.g. Leydig cells) that support hormone production  
65 and germline functions. We then wondered if the high enrichment of testis genes in the *Kdm5c*-KO brain reflected a failure  
66 to demarcate between the soma and germline. Gene ontology analysis of testis-biased DEGs revealed enrichment of  
67 germline-relevant ontologies, including spermatid development (GO:0007286, p.adjust =  $6.2 \times 10^{-12}$ ) and sperm axoneme  
68 assembly (GO:0007288, p.adjust =  $2.45 \times 10^{-14}$ ) (Figure 2A).

69 To further validate if these testis DEGs are truly germline genes, we then compared their expression in a published  
70 RNA-seq 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-  
71 enriched DEGs lose their expression with germ cell depletion (Figure 2B). The only testis DEG that did not show considerable  
72 downregulation with germline depletion was *FK506 binding protein 6* (*Fkbp6*), a known regulator of piRNA expression and

73 meiosis in germ cells<sup>9,10</sup>. We additionally assessed testis DEG expression in a published testis single cell RNA-seq dataset  
74 that identified cell type-specific markers<sup>11</sup>. We found that while some testis-enriched DEGs were classified as specific  
75 markers for different germ cell types (e.g. spermatogonia, spermatocytes, round spermatids, and elongating spermatids)  
76 none marked somatic cells (Figure 2C). Together, these data suggest the *Kdm5c*-KO brain expresses germline genes.

77 We then aimed to globally characterize germline gene misexpression in *Kdm5c*-KO mice, but lacked a comprehensive list  
78 of mouse germline-enriched genes. To facilitate downstream analyses, we generated a list of male and female germline-  
79 enriched genes by evaluating gene expression in wild-type and germline-depleted (*Kit<sup>W/Wv</sup>*) mice. We assessed gene  
80 expression in male and female mice at embryonic day 12, 14, and 16 embryos, as well as male postnatal day 6 and adult  
81 testes. We defined genes as germline-enriched if their expression met the following criteria: 1) their expression is greater than  
82 1 FPKM in wild-type germline 2) their expression in any wild-type, non-gonadal tissue<sup>2</sup> does not exceed 20% of maximum  
83 expression in wild-type germline, and 3) their expression in the germ cell-depleted germline, for any sex or time point, does  
84 not exceed 20% of their maximum expression in the wild-type germline. These criteria yielded 1287 germline-enriched genes.

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

86 Misexpression of germline genes in the *Kdm5c*-KO brain suggests mutants fail to demarcate germline and somatic cellular  
87 identity. Germ cells are typically distinguished from somatic cells soon after the embryo implants into the uterine wall<sup>12,13</sup>  
88 when a subset of epiblast stem cells become the primordial germ cells (PGCs) while the remainder differentiate into the  
89 ectoderm, mesoderm, and endoderm to form the body's somatic tissues<sup>14</sup>. This developmental time point can be modeled *in*  
90 *vitro* through differentiation of embryonic stem cells (ESCs) into post-implantation epiblast-like stem cells (EpiLCs) (Figure  
91 3A, top). Previous studies have demonstrated that while some germline-enriched genes are also expressed in embryonic  
92 stem cells (ESCs), they are silenced as they differentiate into EpiLCs<sup>15</sup>. Therefore, we assessed if KDM5C was necessary  
93 for initial germline gene silencing by assessing their expression in male *Kdm5c*-KO EpiLCs using our previously generated  
94 RNA-seq dataset<sup>16</sup>.

95 We did not observe any apparent morphology changes between wild-type and *Kdm5c*-KO cells during ESC to EpiLC  
96 differentiation (Figure 3A, bottom) and found no significant difference in primed pluripotency genes that indicate proper EpiLC  
97 differentiation (Figure 3B). We then identified *Kdm5c*-KO EpiLC DEGs through DESeq2<sup>4</sup> (log2 fold change > 0.5, q < 0.1) and  
98 again observed widespread dysregulation of tissue-enriched genes, with the largest number of genes belonging to the brain  
99 and testis, although they were not significantly enriched (Figure 3C).

100 Using our curated list, we found 54 germline-enriched genes were misexpressed in *Kdm5c*-KO EpiLCs, including *Cyct*  
101 (Figure 3D). To evaluate whether germline mRNAs are constitutively dysregulated or change over the course of development,  
102 we then compared EpiLC germline DEGs to those expressed in the *Kdm5c*-KO brain. The majority of EpiLC germline DEGs  
103 were unique to EpiLCs, with only *Cyct* shared across sequencing datasets (Figure 3E).

104 We then compared the function of EpiLC and brain germline DEGs through gene ontology and found particularly high  
105 enrichment of meiosis-related gene ontologies in EpiLCs (Figure 3F), such as meiotic cell cycle (GO:0051321, p.adjust =  
106  $4.44 \times 10^{-7}$ ). While a few meiosis-related genes are misexpressed in the *Kdm5c*-KO brain, DEGs unique to *Kdm5c*-KO EpiLCs  
107 included master regulators of germline identity, such as *Stimulated by retinoic acid 8 (Stra8)* and *Deleted in azoospermia*  
108 *like (Dazl)* (Figure 3G). These genes are typically expressed during embryonic germ cell development to commit PGCs  
109 to the germline fate, but are also expressed later in life to trigger meiotic gene expression programs<sup>17-19</sup>. Of note, some  
110 germline genes, including *Dazl*, are also expressed in the two-cell embryo. However, we did not see misexpression of two-cell  
111 embryo-specific genes, like *Zscan4d* (q = 0.381).

112 *Dazl* is an RNA-binding protein that regulates the translation of germline mRNAs and is essential for germ cell function<sup>20</sup>.  
113 Interestingly, a significant portion of germline transcripts expressed in *Kdm5c*-KO EpiLCs are known binding targets of DAZL,  
114 including *Stra8*<sup>21</sup> ( $p = 1.698e-07$ , Fisher's Exact Test). This suggests expression of DAZL protein would enable the translation  
115 of other aberrant germline transcripts, influencing their ability to impact on *Kdm5c* mutant cellular function. We therefore  
116 stained *Kdm5c*-KO EpiLCs for DAZL protein expression through immunocytochemistry (Figure 3H). We found about 25% of  
117 *Kdm5c*-KO EpiLCs expressed DAZL protein in their cytoplasm ( $p = 0.0015$ , Welch's t-test), consistent with the subcellular  
118 localization observed when DAZL is stained in spermatogonia<sup>21</sup>. Altogether these results suggest *Kdm5c*-KO EpiLCs fail  
119 to decommission germline genes during early embryogenesis, including master regulators of germline identity that can be  
120 translated into protein.

## 121 Discussion

- 122 • tissue-biased gene expression:
  - 123 – However unlike the gonadal-biased DEGs, many liver and muscle-biased DEGs have a known involvement in brain  
124 function. For example, the liver-biased DEG is *Apolipoprotein C-I* (*Apoc1*), is important for lipoprotein metabolism  
125 but has also been shown to influence learning and memory (Figure 1E).
- 126 • Papers to read/reference:
  - 127 – 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)
  - 128 – 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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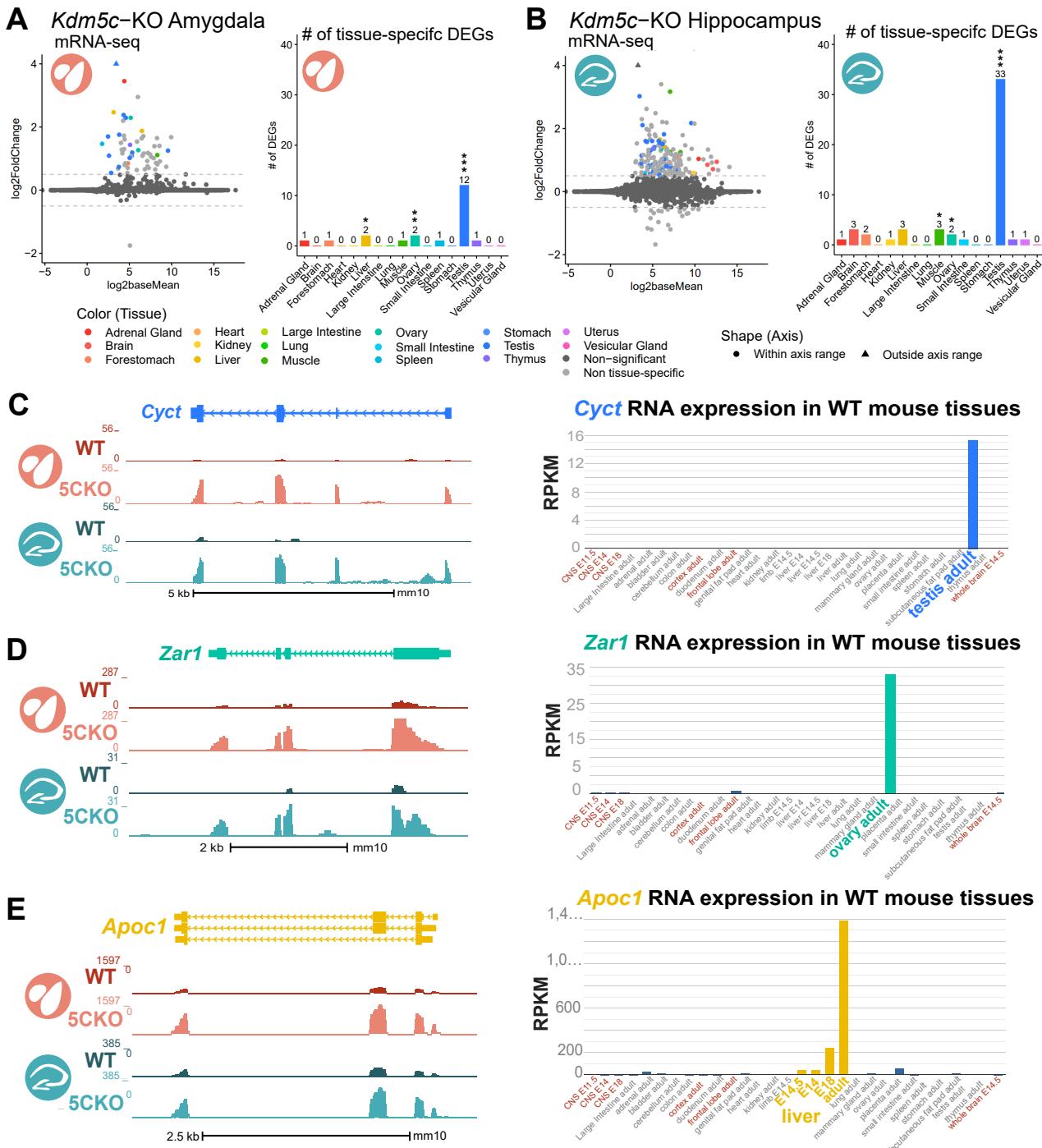
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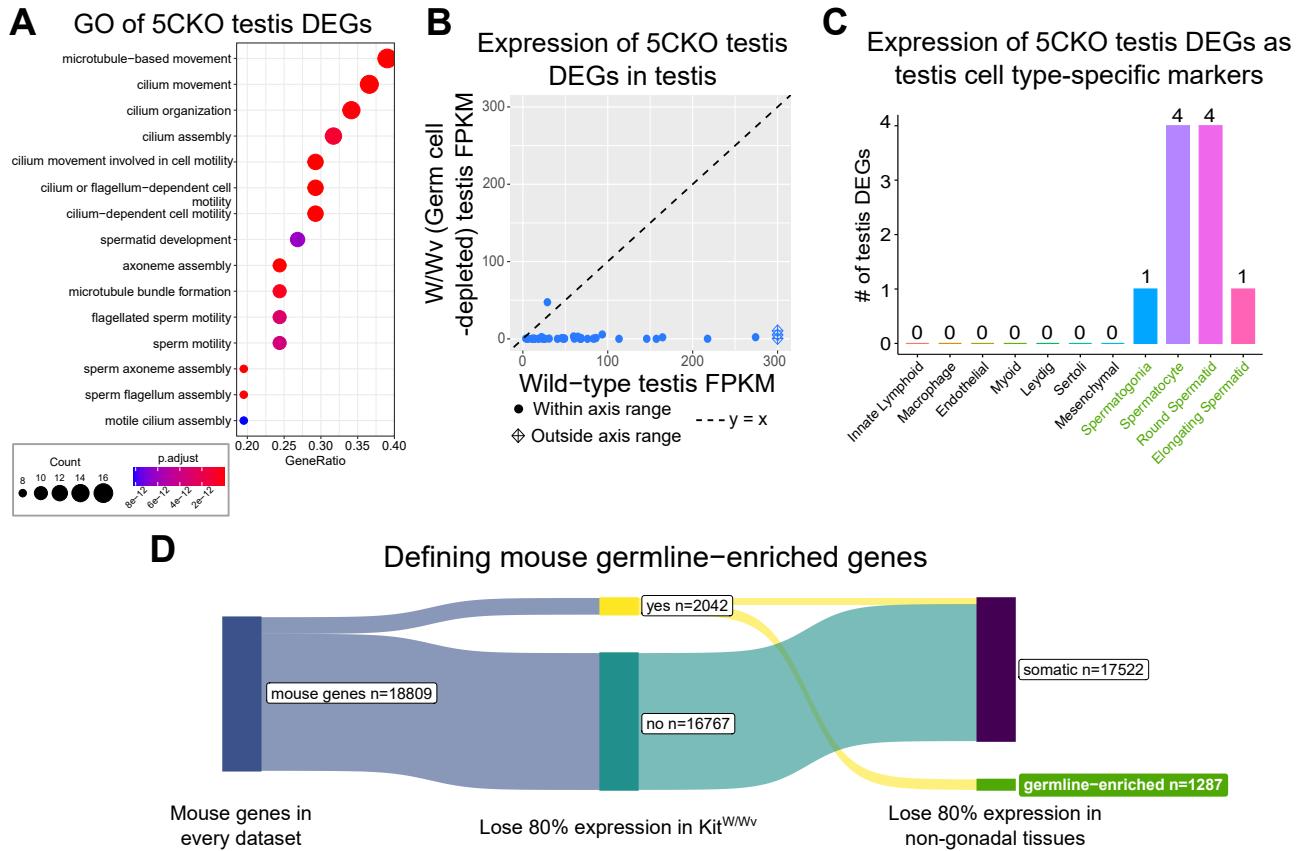
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**174 Figures and Tables**

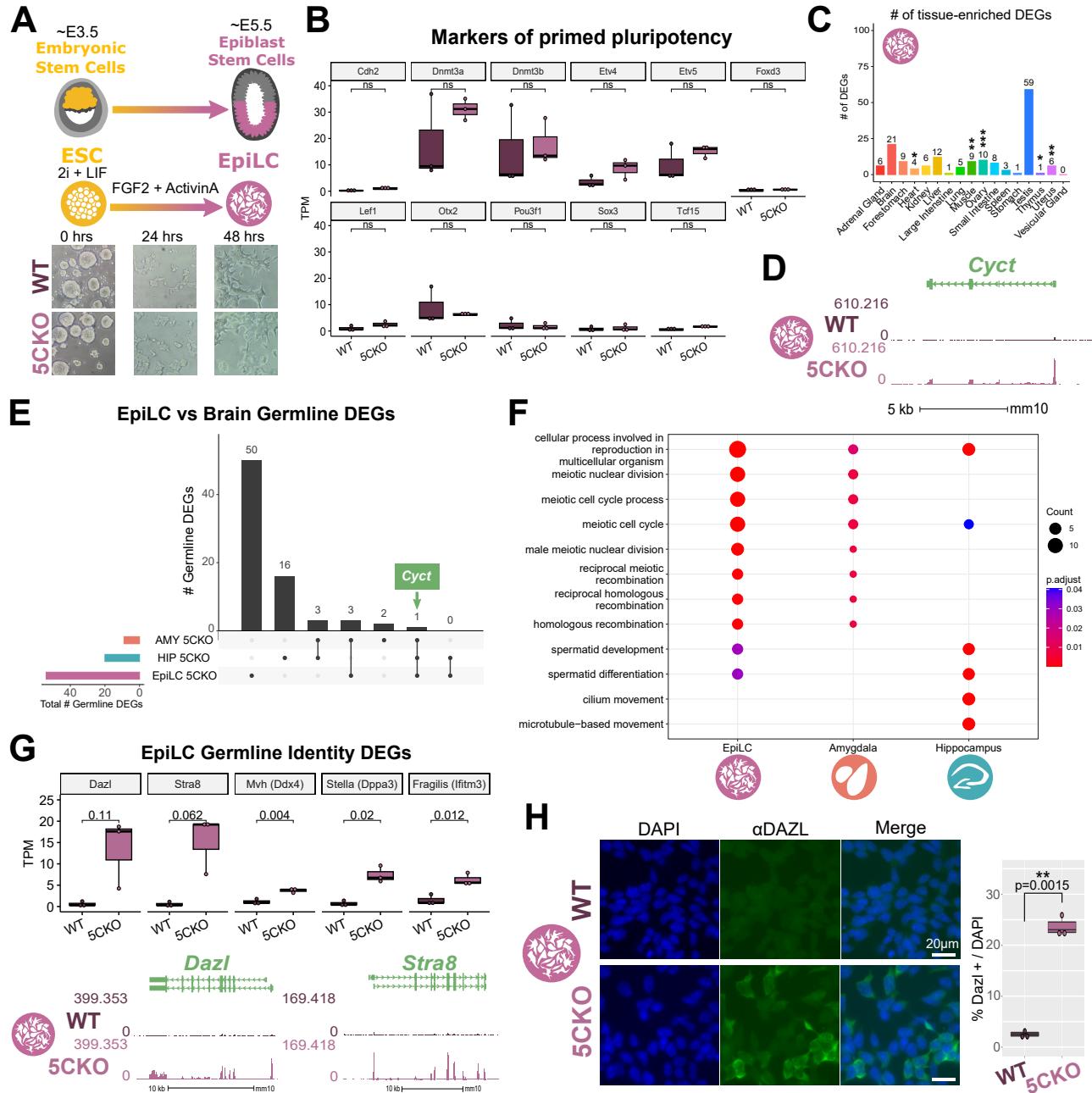
**175** —



**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 - 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* (*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 - 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 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. 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 mutant EpiLC, amygdala (AMY), and hippocampus (HIP) RNA-seq datasets. **F**. enrichPlot comparing enriched gene ontologies for *Kdm5c*-KO EpiLCs, 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

176 **Figure outline:**

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

180 **Figure 2: The male *Kdm5c*-KO brain expresses male and female germline-enriched genes** \* Gene ontology of  
181 testis DEGs in the amygdala and hippocampus - ontologies are germline ontologies \* Expression of testis DEGs in germline-  
182 depleted testis (this is adult testis data) \* scRNAseq of testis - # of testis DEGs that are germline-specific markers \* Although  
183 far fewer, 5CKO brain also expresses ovary-enriched genes(NCBI and bigwigs of Zar1) \* These ovary enriched genes are  
184 also germline specific (NCBI/Li tissues are in adult ovary, so it would be best to show they're oocyte-specific in adult ovary.  
185 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  
186 W/Wv data since oocytes are developed at this point? Or both?) \* Defining what is/isn't a germline gene, and which are  
187 male/female biased using embryonic W/Wv data

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

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

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

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