

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) hippocampus¹. 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 genes² in our previously published mRNA-seq datasets of the *Kdm5c*-KO amygdala
43 and hippocampus³

44 We found many genes significantly upregulated within the *Kdm5c*-KO brain (DESeq2⁴, 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 = 1.83×10^{-5} ;
48 Hippocampus p = 4.26×10^{-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^{5,6} (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 development⁷ (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, q = 6.2×10^{-12}) and sperm axoneme assembly
68 (GO:0007288, q = 2.45×10^{-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^{W/Wv}*) mouse testes⁸. 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^{9,10}. We additionally assessed testis DEG expression in a published testis single cell RNA-seq dataset
74 that identified cell type-specific markers¹¹. 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*^{W/Wv}) 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.

82 We defined genes as germline-enriched if their expression met the following criteria: 1) their expression is greater than 1
83 FPKM in wild-type germline 2) their expression in any wild-type, non-gonadal tissue² does not exceed 20% of maximum
84 expression in wild-type germline, and 3) their expression in the germ cell-depleted germline, for any sex or time point, does
85 not exceed 20% of their maximum expression in the wild-type germline. These criteria yielded 1287 germline-enriched
86 genes. To more comprehensively assess the impact of sex on germline gene misexpression, we additionally categorized
87 if germline-enriched genes displayed biased expression in the wild-type female or male germline. We defined sex-biased
88 genes as those whose expression in the opposite sex, at any time point, is no greater than 20% of the gene's maximum
89 expression in a given sex. This yielded 65 egg-biased, 1023 sperm-biased, and 199 unbiased germline-enriched genes,
90 which is consistent with the testis overall having a more unique transcriptome than the ovary².

91 ***Kdm5c*-KO cells express master regulators of germline identity in an *in vitro* model of early 92 embryogenesis**

93 Misexpression of germline genes in the *Kdm5c*-KO brain suggests mutants fail to demarcate germline and somatic cellular
94 identity. Germ cells are typically distinguished from somatic cells soon after the embryo implants into the uterine wall^{12,13}
95 when a subset of epiblast stem cells become the primordial germ cells (PGCs) while the remainder differentiate into the
96 ectoderm, mesoderm, and endoderm to form the body's somatic tissues¹⁴. This developmental time point can be modeled *in*
97 *vitro* through differentiation of embryonic stem cells (ESCs) into post-implantation epiblast-like stem cells (EpiLCs) (Figure 3A,
98 top). Previous studies have demonstrated that while some germline-enriched genes are also expressed in embryonic stem
99 cells (ESCs), they are silenced as they differentiate into EpiLCs¹⁵. Therefore, to elucidate the mechanism underlying aberrant
100 germline genes expression in the mature *Kdm5c*-KO brain, we first assessed their expression in male *Kdm5c*-KO EpiLCs
101 using our previously generated RNA-seq dataset¹⁶.

- 102 • We did not observe any apparent morphology changes between wild-type and *Kdm5c*-KO cells during ESC to EpiLC
103 differentiation (Figure 3A, bottom). Additionally, *Kdm5c*-KO EpiLCs downregulated ESC pluripotency genes and
104 upregulated EpiLC differentiation genes similar to wild-type (Figure 3B).
- 105 • We then identified *Kdm5c*-KO EpiLC DEGs through DESeq2⁴ (log2 fold change > 0.5, q < 0.1) and observed widespread
106 dysregulation of tissue-enriched genes, with the largest number of genes belonging to the brain and testis (Figure 3C).
- 107 • Of these testis genes, we also observed misexpression of germline-enriched genes, including *Cyct* (Figure 3D)
- 108 • To evaluate whether germline mRNAs are constitutively dysregulated or change over the course of development, we
109 compared EpiLC germline DEGs to those expressed in the *Kdm5c*-KO brain. The majority of EpiLC germline DEGs

were unique to EpiLCs, with only a few shared across brain sequencing datasets (Figure 3E). Only two germline genes were consistently misexpressed in all datasets, *DNA segment, Chr 1, Pasteur Institute 1 (D1Pas1)* and *Testis expressed gene 14 (Tex14)*.

- We then compared the typical function of brain and EpiLC germline DEGs and found EpiLC germline DEGs displayed strong enrichment of meiosis-related gene ontologies, including XXXXX (Figure 3F).
- Surprisingly, germline DEGs unique to *Kdm5c*-KO EpiLCs included master regulators of germline identity, such as *Stimulated by retinoic acid 8 (Stra8)* and *Deleted in azoospermia like (Dazl)* (Figure 3G). These genes are typically expressed during embryonic germ cell development to commit PGCs to the germline fate, but they are also expressed later in life to trigger meiotic gene expression programs^{17–19}.
- *Dazl* is a translational regulator essential for germ cell function²⁰. Interestingly, a significant portion of germline transcripts expressed in *Kdm5c*-KO EpiLCs are known binding targets of DAZL, including *Stra8*²¹ (XY *Kdm5c*-KO EpiLCs p = 1.698e-07, Fisher's Exact Test). This suggests expression of DAZL protein would enable the translation of other aberrant germline transcripts, influencing their ability to impact on *Kdm5c* mutant cellular function.
- Therefore, we then stained *Kdm5c*-KO EpiLCs for DAZL protein expression through immunocytochemistry (Figure 3H). We found about 25% of *Kdm5c*-KO EpiLCs expressed DAZL protein in their cytoplasm (p = 0.0015, Welch's t-test, XY KO versus WT), consistent with the subcellular localization observed when DAZL is stained in spermatogonia²¹.

126 Discussion

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

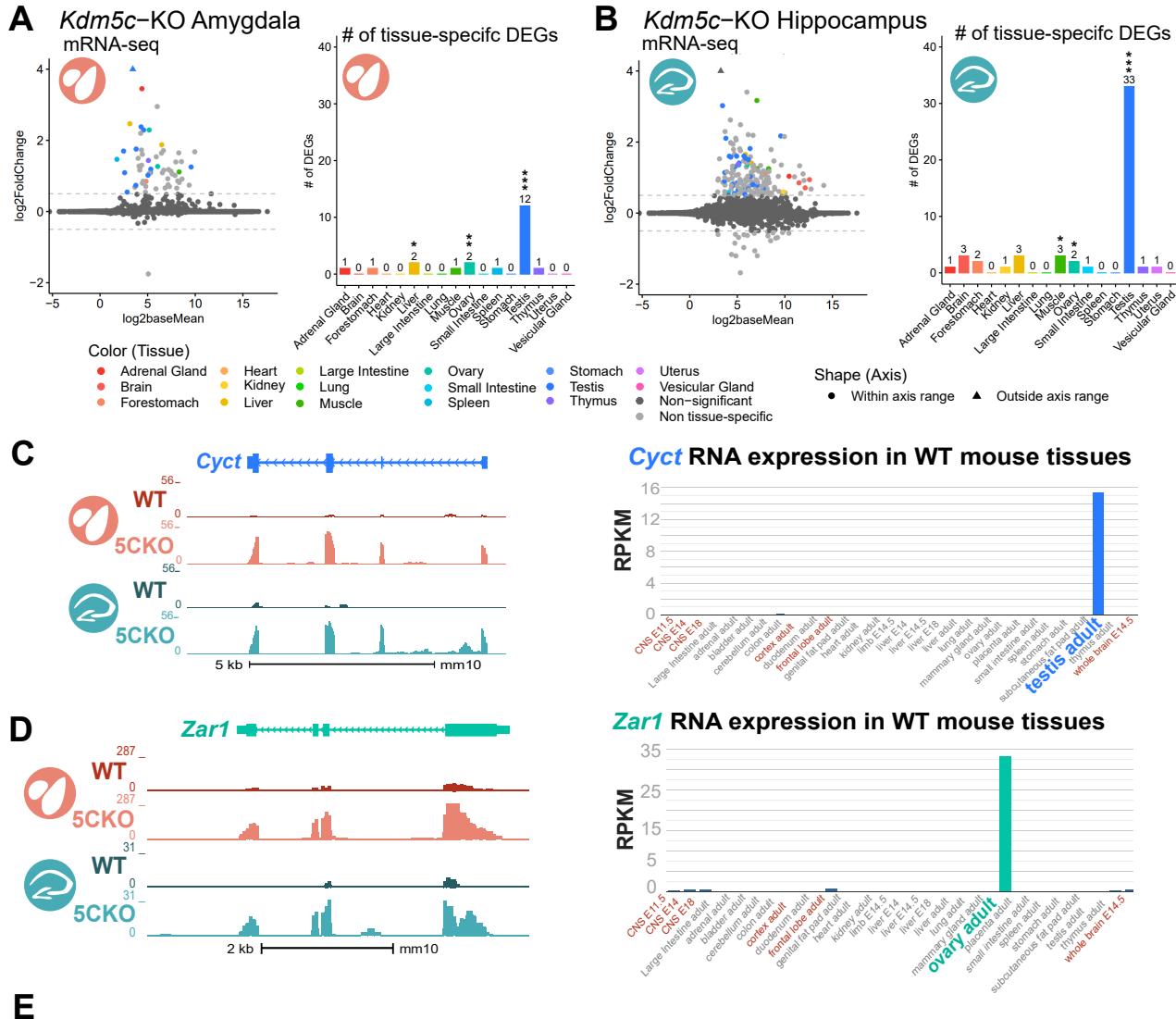


Figure 1: Misexpression of tissue-enriched genes in the *Kdm5c*-KO in the brain **A.** Expression of tissue-enriched genes in the male *Kdm5c*-KO amygdala. Left - MA plot of mRNA-sequencing. Right - The number of tissue-enriched differentially expressed genes (DEGs). * p<0.05, ** p<0.01, *** p<0.001, 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.

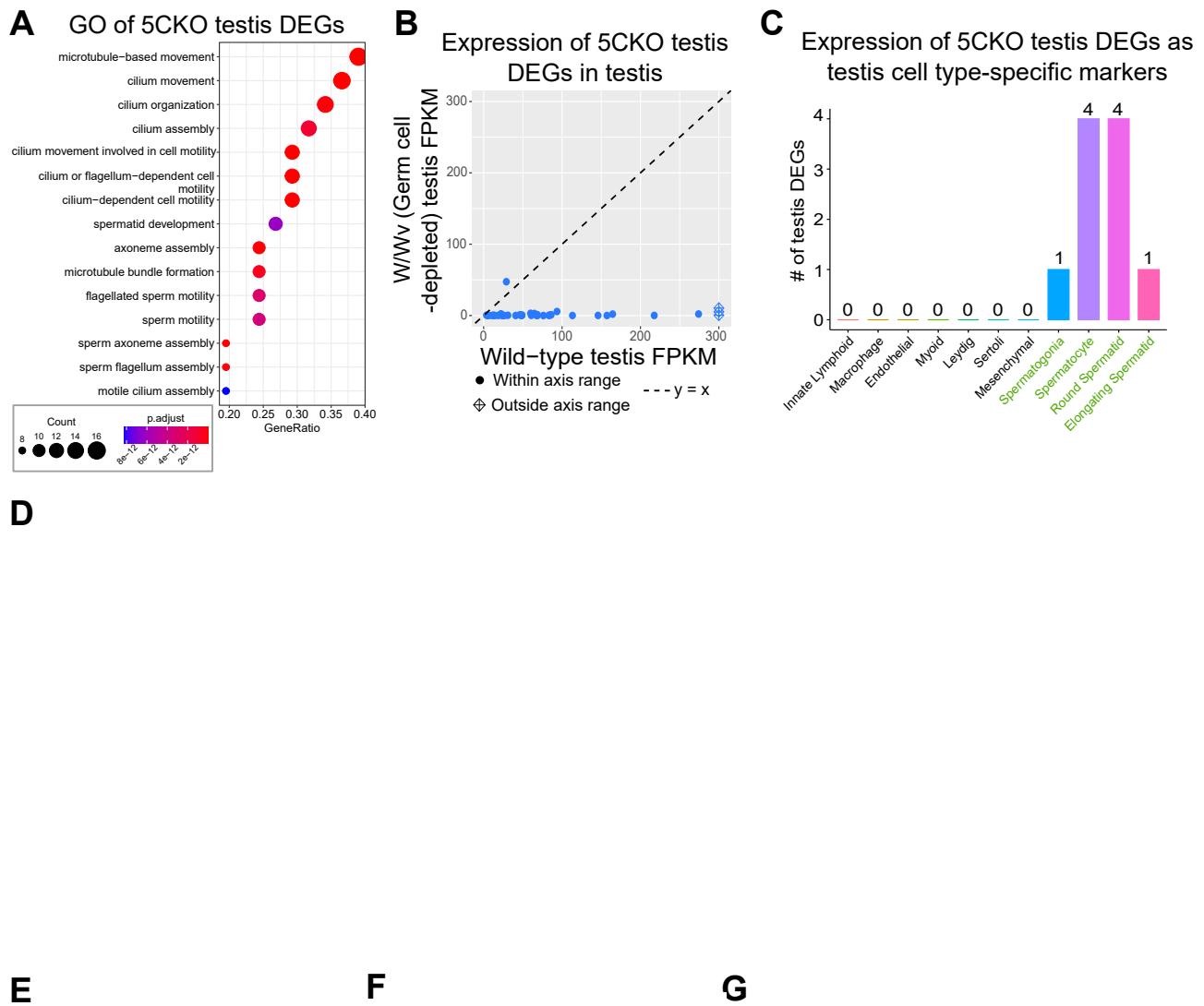


Figure 2: Aberrant transcription of germline genes in the *Kdm5c*-KO in the brain. **A.** enrichPlot gene ontology (GO) of *Kdm5c*-KO amygdala and hippocampus testis-enriched DEGs **B.** Expression of testis DEGs in wild-type (WT) testis versus germ cell-depleted (W/Wv) testis. Expression is in Fragments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

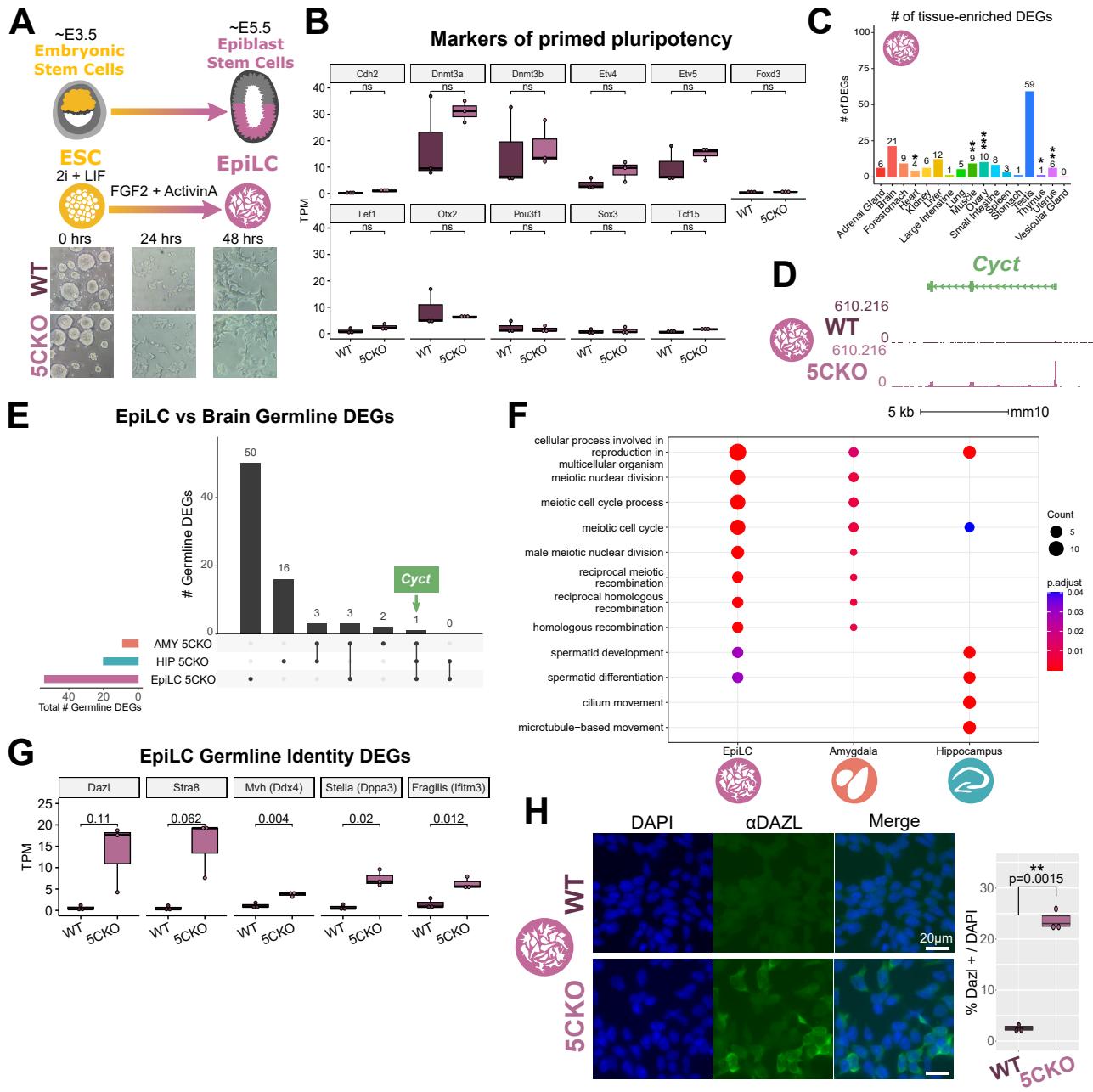


Figure 3: Male and female *Kdm5c* mutant epiblast-like cells express master regulators of germline identity **A.** Top - Diagram of *in vivo* differentiation of embryonic stem cells (ESCs) of the inner cell mass into epiblast stem cells and primordial germ cells. Bottom - *in vitro* differentiation of 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.

180 **Figure outline:**

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

184 **Figure 2: The male *Kdm5c*-KO brain expresses male and female germline-enriched genes** * Gene ontology of
185 testis DEGs in the amygdala and hippocampus - ontologies are germline ontologies * Expression of testis DEGs in germline-
186 depleted testis (this is adult testis data) * scRNASeq of testis - # of testis DEGs that are germline-specific markers * Although
187 far fewer, 5CKO brain also expresses ovary-enriched genes(NCBI and bigwigs of Zar1) * These ovary enriched genes are
188 also germline specific (NCBI/Li tissues are in adult ovary, so it would be best to show they're oocyte-specific in adult ovary.
189 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
190 W/Wv data since oocytes are developed at this point? Or both?) * Defining what is/isn't a germline gene, and which are
191 male/female biased using embryonic W/Wv data

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

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

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

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