

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-specific 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-specific
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-specific 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-specific genes, including testis genes, are enriched within the *Kdm5c*-KO brain**

38 note: the 5CKO DEGs table we should make a column for if the gene is tissue-specific and which tissue it's in
39 see if any liver or muscle genes are dysregulated in both tissues, could add as a bigwig

40 Previous RNA sequencing studies performed in the adult male *Kdm5c* knockout (-KO) hippocampus identified aberrant
41 transcription of genes that are typically only expressed in the testis¹. Given the high abundance of testis-enriched genes
42 within the mouse transcriptome, it is currently unclear if their misexpression in the *Kdm5c*-KO brain is indicative of random
43 de-repression throughout the genome or instead reflect widespread impairment of tissue identity. To distinguish these
44 possibilities, we globally assessed if tissue-specific genes were significantly enriched in our previous mRNA-seq dataset of
45 the *Kdm5c*-KO amygdala and hippocampus². We classified differentially expressed genes (DEGs) as tissue-specific if they
46 were significantly upregulated in the *Kdm5c*-KO brain compared to wild-type controls (DESeq2³, log2 fold change > 0.5, q <
47 0.1) and were previously identified as uniquely enriched within a given mouse tissue⁴.

- 48 • Based on these criteria, we observed widespread misexpression of tissue-enriched genes within the *Kdm5c*-KO
49 amygdala and hippocampus, with the majority of genes belonging to the testis (Figure 1A-C).
- 50 • Even though the testis has the largest number of tissue-specific genes compared to any other tissue (2496 genes),
51 testis genes were significantly enriched for both brain regions (Amygdala p = 1.83×10^{-5} ; Hippocampus p = 4.26×10^{-11} ,
52 Fisher's Exact).
- 53 • This dysregulation of tissue identity is not unique to the testis, as we also observed significant misexpression of ovary,
54 liver, and muscle genes within the *Kdm5c*-KO brain (Figure 1A-E).

55 Importantly, these tissue-specific genes show little to no expression in the wild-type brain and have no known brain
56 functions, yet our mRNA-seq data indicates they are polyadenylated and spliced into mature transcripts (Figure 1C,G).

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

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

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

69 To validate if these testis DEGs are truly germline genes, we then compared their expression in a published RNA-seq
70 dataset of wild-type (WT) and germ cell-depleted (Kit^{W/Wv}) mouse testes⁷. We found almost all *Kdm5c*-KO testis-enriched

71 DEGs lose their expression with germ cell depletion (Figure 1B). 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^{8,9}. We additionally assessed testis DEG expression in a published testis single cell RNA-seq dataset
74 that identified cell type-specific markers¹⁰. We found that while some testis-enriched DEGs were classified as specific
75 markers for multiple stages of germ cell development - including spermatogonia, spermatocytes, round spermatids, and
76 elongating spermatids, none marked somatic cells (Figure 1C). Altogether, these data suggest testis genes expressed within
77 the *Kdm5c*-KO brain are actually male germ cell genes.

78 Interestingly, we also observed significant enrichment of ovary genes within the male *Kdm5c*-KO brain (Amygdala p =
79 0.00574; Hippocampus p = 0.048, Fisher's Exact). Although there are relatively few ovary-enriched DEGs compared to
80 testis-DEGs (Amygdala: 12 testis DEGs, 2 ovary DEGs; Hippocampus: 33 testis DEGs, 2 ovary DEGs), they also have
81 known germline-specific functions. For example, *Zygotic arrest 1* (*Zar1*) was recently shown to regulate oocyte translation by
82 sequestering mRNAs¹¹ (Figure 1D).

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

85 – We found XYZ.

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

88 • One difficulty in globally characterizing the misexpression of germline genes is a comprehensive list of mouse germline-
89 specific genes is currently lacking.

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

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

95 We defined genes as germline-enriched if their expression met the following criteria: 1) their expression is greater than 1
96 FPKM in wild-type germline 2) their expression in any wild-type, non-gonadal tissue⁴ does not exceed 20% of maximum
97 expression in wild-type germline, and 3) their expression in the germ cell-depleted germline, for any sex or time point, does
98 not exceed 20% of their maximum expression in the wild-type germline. These criteria yielded 1287 germline-enriched
99 genes. To more comprehensively assess the impact of sex on germline gene misexpression, we additionally categorized if
100 germline-enriched genes displayed biased expression in the wild-type female (XX) or male germline. We defined sex-biased
101 genes as those whose expression in the opposite sex, at any time point, is no greater than 20% of the gene's maximum
102 expression in a given sex. This yielded 65 XX-biased, 1023 XY-biased, and 199 unbiased germline-enriched genes, which is
103 consistent with the testis overall having a more unique transcriptome than the ovary⁴.

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

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

- 115 • We did not observe any apparent morphology changes between wild-type and *Kdm5c*-KO cells during ESC to EpiLC
116 differentiation (Figure 3A, bottom). Additionally, *Kdm5c*-KO EpiLCs downregulated ESC pluripotency genes and
117 upregulated EpiLC differentiation genes similar to wild-type (Figure 3B).
- 118 • We then identified *Kdm5c*-KO EpiLC DEGs through DESeq2³ (log2 fold change > 0.5, q < 0.1) and observed widespread
119 dysregulation of tissue-enriched genes, with the largest number of genes belonging to the brain and testis (Figure 3C).
- 120 • Of these testis genes, we also observed misexpression of germline-enriched genes, including *Cyct* (Figure 3D)
- 121 • To evaluate whether germline mRNAs are constitutively dysregulated or change over the course of development, we
122 compared EpiLC germline DEGs to those expressed in the *Kdm5c*-KO brain. The majority of EpiLC germline DEGs
123 were unique to EpiLCs, with only a few shared across brain sequencing datasets (Figure 3E). Only two germline genes
124 were consistently misexpressed in all datasets, *DNA segment, Chr 1, Pasteur Institute 1 (D1Pas1)* and *Testis expressed*
125 *gene 14 (Tex14)*.
- 126 • We then compared the typical function of brain and EpiLC germline DEGs and found EpiLC germline DEGs displayed
127 strong enrichment of meiosis-related gene ontologies, including XXXXX (Figure 3F).
- 128 • Surprisingly, germline DEGs unique to *Kdm5c*-KO EpiLCs included master regulators of germline identity, such as
129 *Stimulated by retinoic acid 8 (Stra8)* and *Deleted in azoospermia like (Dazl)* (Figure 3G). These genes are typically
130 expressed during embryonic germ cell development to commit PGCs to the germline fate, but they are also expressed
131 later in life to trigger meiotic gene expression programs¹⁷⁻¹⁹.
- 132 • *Dazl* is a translational regulator essential for germ cell function²⁰. Interestingly, a significant portion of germline
133 transcripts expressed in *Kdm5c*-KO EpiLCs are known binding targets of DAZL, including *Stra8*²¹ (XY *Kdm5c*-KO
134 EpiLCs p = 1.698e-07, Fisher's Exact Test). This suggests expression of DAZL protein would enable the translation of
135 other aberrant germline transcripts, influencing their ability to impact on *Kdm5c* mutant cellular function.
- 136 • Therefore, we then stained *Kdm5c*-KO EpiLCs for DAZL protein expression through immunocytochemistry (Figure 3H).
137 We found about 25% of *Kdm5c*-KO EpiLCs expressed DAZL protein in their cytoplasm (p = 0.0015, Welch's t-test, XY
138 KO versus WT), consistent with the subcellular localization observed when DAZL is stained in spermatogonia²¹.

139 **Discussion**

- 140 • Papers to read/reference:
- 141 – 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)
- 142 – 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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145 **References**

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188 **Figures and Tables**

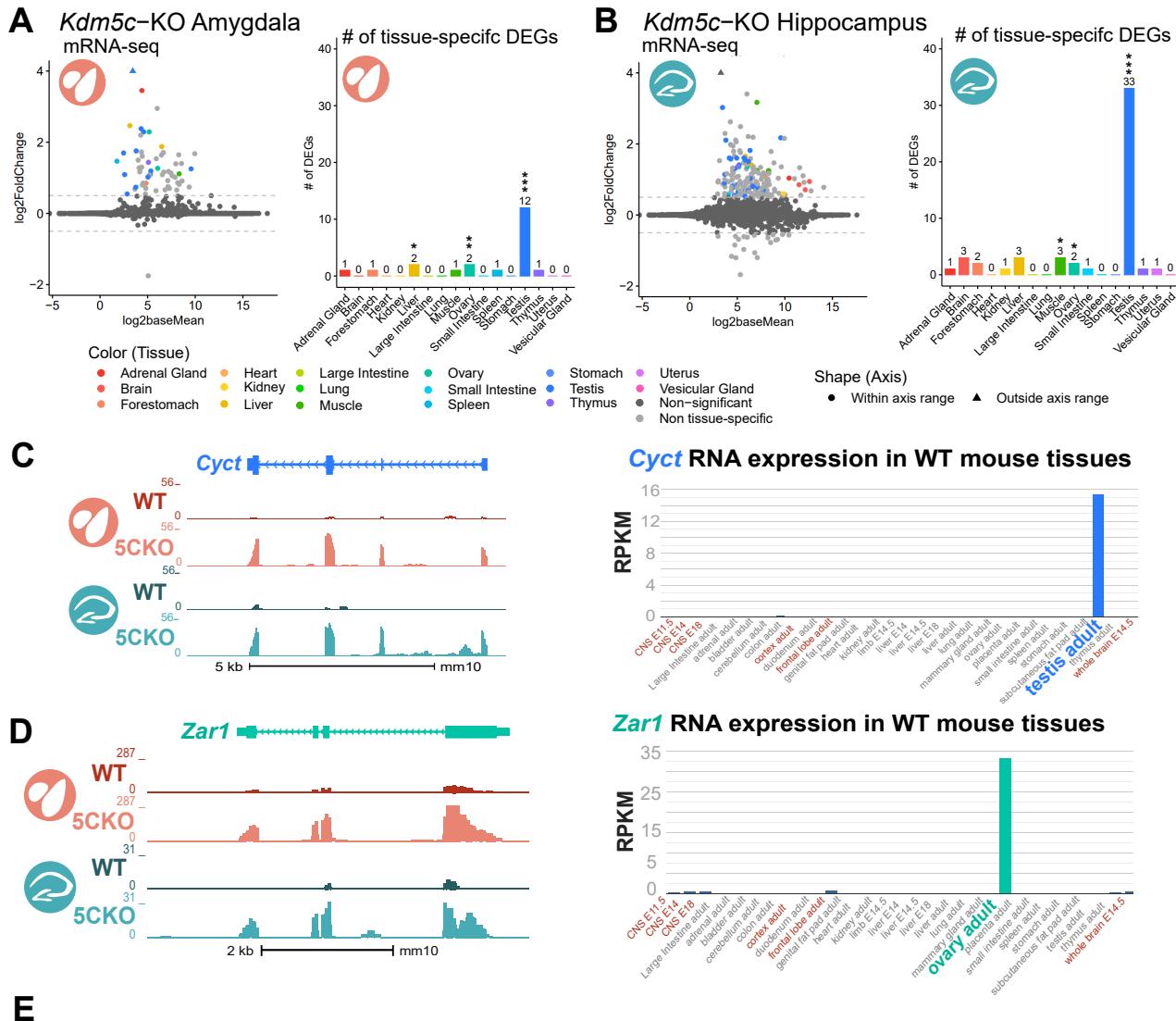


Figure 1: Misexpression of tissue-specific genes in the *Kdm5c*-KO in the brain **A.** Expression of tissue-enriched genes in the male *Kdm5c*-KO amygdala. Left - MA plot of mRNA-sequencing. Right - The number of tissue-enriched differentially expressed genes (DEGs). * p<0.05, ** p<0.01, *** p<0.001,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

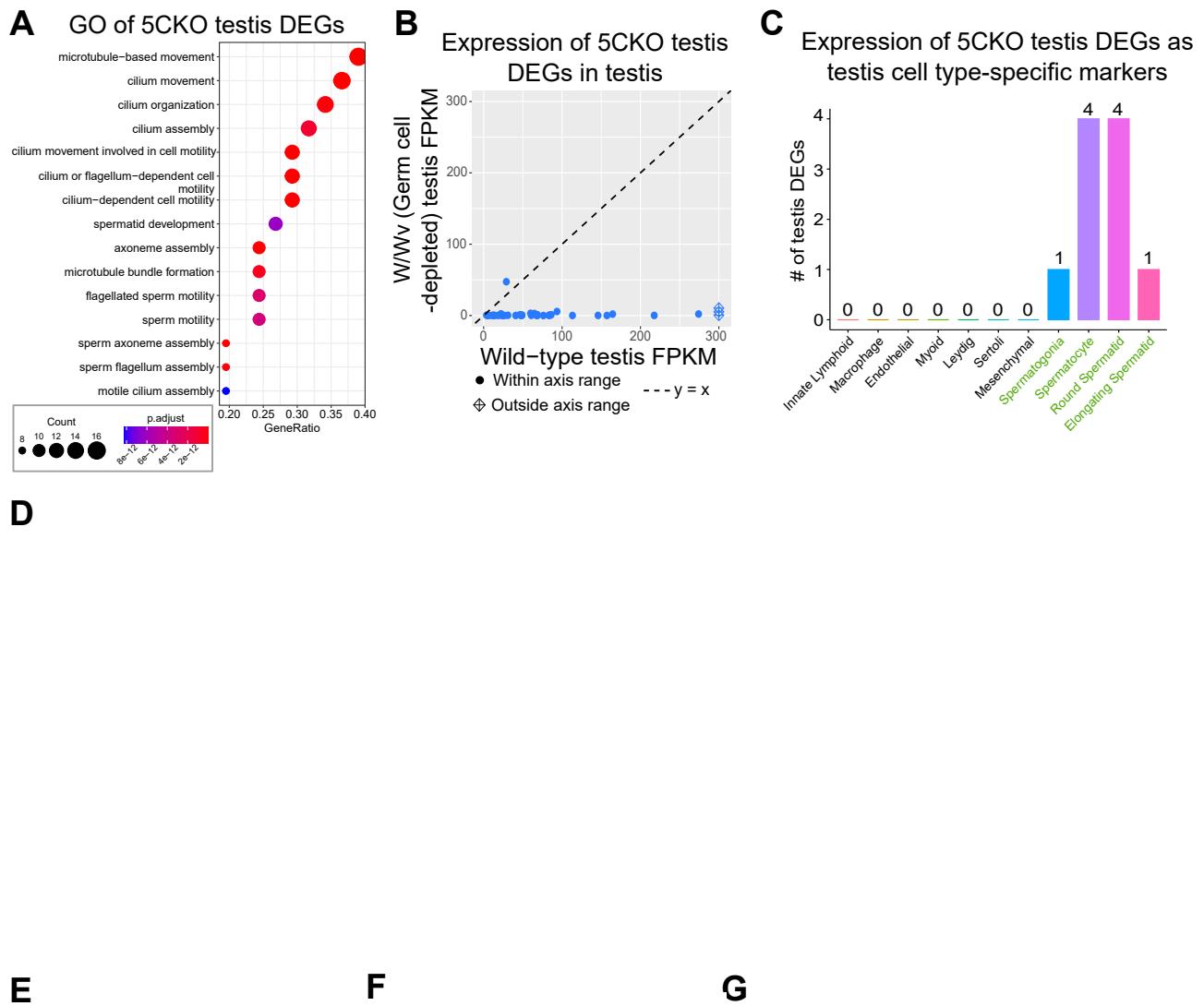


Figure 2: Ovary and testis germline genes are expressed in the *Kdm5c*-KO in the brain. **A.** enrichPlot gene ontology (GO) of *Kdm5c*-KO amygdala and hippocampus testis-enriched DEGs **B.** Expression of testis DEGs in wild-type (WT) testis versus germ cell-depleted (W/Wv) testis. Expression is in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

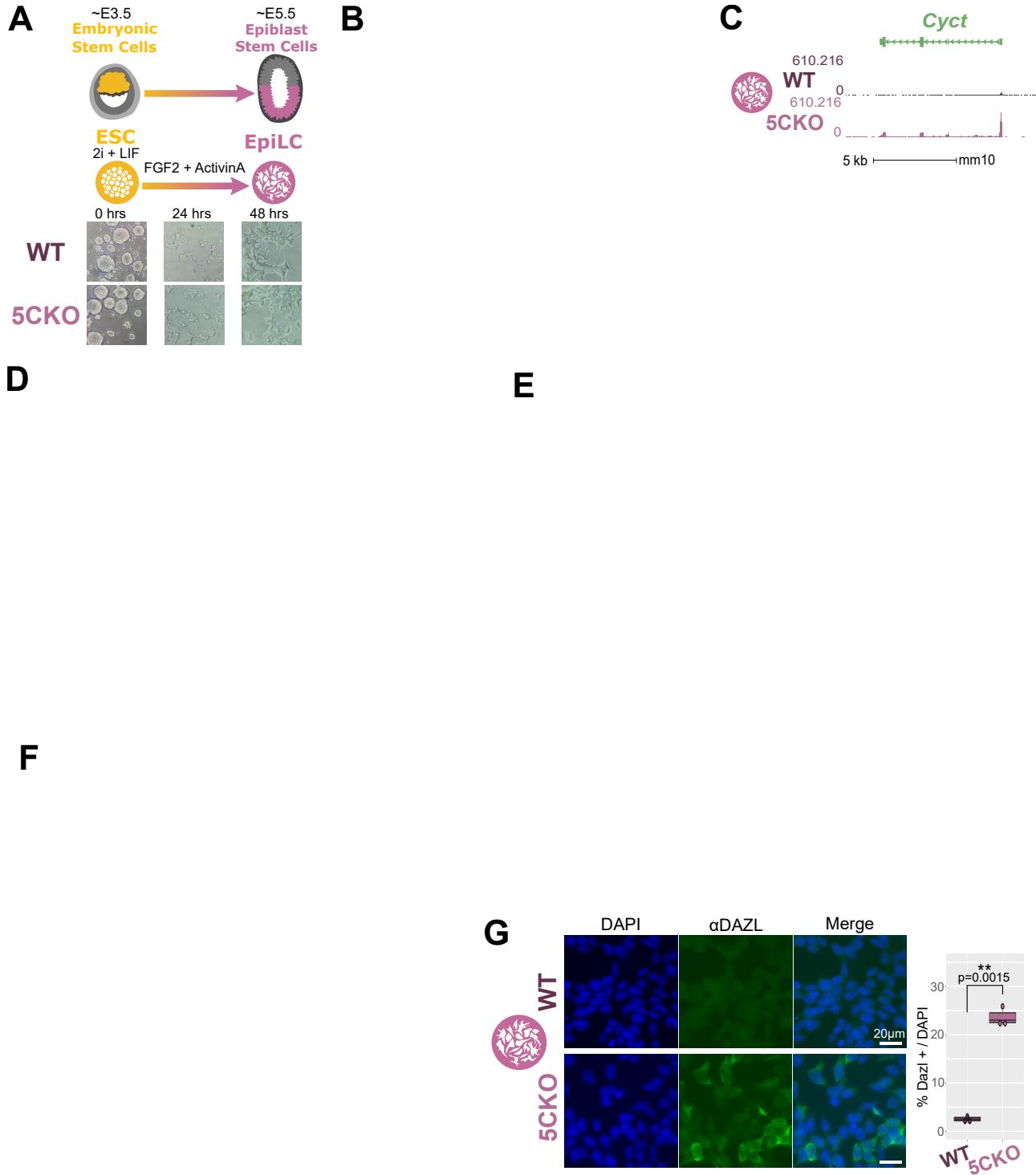


Figure 3: Male and female *Kdm5c* mutant epiblast-like cells express master regulators of germline identity **A.** Top - Diagram of *in vivo* differentiation of emrbyonic stem cells (ESCs) of the inner cell mass into epiblast stem cells and primordial germ cells. Bottom - *in vitro* differentiation of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

189 **Figure outline:**

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

193 **Figure 2: The male *Kdm5c*-KO brain expresses male and female germline-enriched genes** * Gene ontology of
194 testis DEGs in the amygdala and hippocampus - ontologies are germline ontologies * Expression of testis DEGs in germline-
195 depleted testis (this is adult testis data) * scRNAseq of testis - # of testis DEGs that are germline-specific markers * Although
196 far fewer, 5CKO brain also expresses ovary-enriched genes(NCBI and bigwigs of Zar1) * These ovary enriched genes are
197 also germline specific (NCBI/Li tissues are in adult ovary, so it would be best to show they're oocyte-specific in adult ovary.
198 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
199 W/Wv data since oocytes are developed at this point? Or both?) * Defining what is/isn't a germline gene, and which are
200 male/female biased using embryonic W/Wv data

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

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

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

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