

1 ***Srd5a1* is differentially regulated and methylated during pre-pubertal development in the ovary and**
2 **hypothalamus**

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1 Abstract

2 5 α -reductase-1 catalyzes production of various steroids, including neurosteroids. We reported
3 previously that expression of its encoding gene, *Srd5a1*, drops in murine ovaries and hypothalamic pre-
4 optic area (POA) after early-life immune stress, seemingly contributing to delayed puberty and ovarian
5 follicle depletion, while in their ovaries the first intron was more methylated at two CpGs. Here we
6 hypothesized that this CpG-containing locus comprises a methylation-sensitive transcriptional enhancer
7 for *Srd5a1*. We found that ovarian *Srd5a1* mRNA increased 8-fold and methylation of the same two
8 CpGs decreased upto 75% between post-natal days (PND) 10-30. Estradiol (E₂) levels rise during this pre-
9 pubertal stage, and exposure of ovarian cells to E₂ increased *Srd5a1* expression. Chromatin
10 immunoprecipitation (ChIP) in an ovarian cell line confirmed ESR1 binding to this differentially-
11 methylated genomic region, and also enrichment of the enhancer modification, H3K4me1. Targeting
12 dCas9-DNMT3 to this locus increased CpG2 methylation 2.5-fold and abolished the *Srd5a1* response to
13 E₂. In the POA, *Srd5a1* mRNA levels decreased 70% between PND 7-10 and then remained constant
14 without correlation to CpG methylation levels. *Srd5a1* mRNA levels did not respond to E₂ in
15 hypothalamic GT1-7 cells, even after dCas9-TET1 reduced CpG1 methylation by 50%. The neonatal drop
16 in POA *Srd5a1* expression occurs at a time of increasing glucocorticoids, and treatment of GT1-7 cells
17 with dexamethasone reduced *Srd5a1* mRNA levels, while ChIP confirmed GR binding at the enhancer.
18 Our findings on the tissue-specific regulation of *Srd5a1* and its methylation-sensitive control by E₂ in the
19 ovaries illuminate epigenetic mechanisms underlying reproductive phenotypic variation which impact
20 life-long health.

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1 Introduction

2 Early-life stress has numerous well-recognized adverse effects on life-long health. Reproductive function
3 is particularly sensitive, and changes in pubertal timing, hormone levels, menstrual/estrous cycles and
4 even the size of the ovarian reserve are often evident following stressful events experienced in
5 childhood[1–6]. Such outcomes impact not only fertility, but also reproductive longevity with
6 consequences for post-menopausal health and susceptibility to non-communicable disease[7,8]. We
7 have previously reported that pre-pubertal immunological stress in a mouse model delayed puberty and
8 led to faster depletion of the ovarian follicle pool, seemingly due to reduced expression of the *Srd5a1*
9 gene in the preoptic area (POA) of the hypothalamus and in the ovaries[9]. We also demonstrated
10 causality for the role of reduced *Srd5a1* expression in the altered reproductive phenotype, through
11 inhibition of 5 α -reductase-1 which not only reduced GnRH release and mRNA levels in cultured cells, but
12 also delayed pubertal onset in mice[9].

13 The *Srd5a1* gene encodes the steroidogenic enzyme, 5 α -reductase-1, which is responsible for
14 conversion of testosterone to dihydrotestosterone (DHT) required for healthy ovarian follicle
15 growth[10]. In the brain, this enzyme catalyzes the production of neurosteroids, converting
16 deoxycorticosterone (DOC) and progesterone to their 5 α -reduced forms which are subsequently
17 converted to tetrahydrodeoxycorticosterone (THDOC; 3 α ,21-dihydroxy-5 α -pregnan-20-one) and
18 allopregnanolone (ALLO; 5 α -pregnane-3 α -ol-20-one). These neurosteroids act on multiple cell types to
19 regulate the hypothalamic-pituitary-adrenal (HPA) and gonadal (HPG) axes, playing important roles in
20 the stress response and likely also in early life programming of these two endocrine axes[1,9,11–14].

21 Our previous study reported that the promoter of *Srd5a1*, which is encompassed by a CpG island, is
22 completely unmethylated in mouse ovaries, but its “shore” in the 5' end of the *Srd5a1* first intron was
23 more methylated in the ovaries of mice after early-life immune stress than in controls, correlating with

1 its reduced expression. Such shores, at the margins of CpG islands, are regions of lower CpG density, the
2 methylation of which is usually conserved across species, tissue or cell-type, and is closely associated
3 with transcriptional repression [15,16]. Moreover, most changes in methylation during development and
4 reprogramming occur specifically in these regions of lower CpG density, strongly pointing to a functional
5 modification. Strikingly, in our previous work, we also saw increased methylation at the orthologous
6 genomic region in buccal DNA of Bangladeshi women who had grown up in Bangladesh where
7 immunological challenges in early-life are relatively high, compared to Bangladeshi migrant women who
8 had grown up in the UK, with better health care and fewer disease exposures in childhood [9]. Notably,
9 this orthologous region harbors several SNPs associated with altered reproductive function in
10 women [17]. Moreover, the women who had spent the first years of their lives in Bangladesh had a
11 shorter reproductive lifespan and lower age-matched ovarian reserve, which were also associated with
12 the higher childhood disease loads in Bangladesh [18–21].

13 An additional study on the same populations of Bangladeshi women indicated faster rates of epigenetic
14 ageing among those who had grown up in Bangladesh compared to migrant Bangladeshi women who
15 grew up in the UK. Furthermore, based on concordant DNA methylation at the *LHCGR/LHR* locus, the
16 methylation appeared to be more stable [22]. DNA methylation is responsive to multiple external and
17 internal signals and stressors, and altered methylation signatures at key regulatory regions of the
18 genome can mediate long-term effects on gene expression, profoundly affecting physiological
19 function [23–28]. We thus hypothesized that the region of the *Srd5a1* first intron where we found
20 differential methylation acts as a transcriptional enhancer that is regulated by DNA methylation to
21 control *Srd5a1* expression levels. We observed dynamic but distinct changes in *Srd5a1* expression levels
22 and methylation at this intronic enhancer in the ovaries and hypothalamic POA across the lifespan, and
23 describe cell-specific regulatory mechanisms of glucocorticoid-activated repression of *Srd5a1* in the
24 POA, and its methylation-sensitive stimulation by estradiol (E_2) in the ovary.

1 **Materials and Methods**

2 *Mice*

3 All mice (inbred C57BL/6) were held and handled humanely, after protocol approval by the Technion
4 Committee for the Supervision of Animal Experimentation (IACUC) and in accordance with their
5 guidelines. Following euthanasia, brains of these mice were removed and, as previously described [9],
6 placed ventral side up into a matrix (RWD-800-00149-00, model 68713: RWD Life Sciences) for coronal
7 sectioning of the tissue (i.e. parallel to the central line of the brain) using a razor blade. The sections
8 located between 5-6 mm (or 4.5-5.5 mm for the 7-10 d old mice) contain the POA (Allen Brain Atlas:
9 <https://mouse.brain-map.org/>) and these were collected for further processing. Two 1 mm holes were
10 punched in these sections, centering on the co-ordinates -0.465 or 0.465; 1.536; -2.37 mm from the
11 bregma; this sectioning should include harvest of the vascular organ of the lamina terminus, but not the
12 paraventricular hypothalamic nucleus. These two punched tissue specimens from the same mouse were
13 then combined into a single tube to which 1 ml TRIzol was added for DNA and/or RNA extractions. Brain
14 and ovarian tissues from sexually mature females (ages noted in figure legends) were collected in estrus,
15 verified by vaginal smears.

16 *Quantitative PCR*

17 RNA was isolated with TRIzol, treated with DNase I, and digested and cleaned using R1014 RNA Clean &
18 Concentrator-5 kit (Zymo Research). The cDNA was synthesized using qScript Flex cDNA kit (95049
19 Quanta) with oligo dT, and real-time quantitative PCR (qPCR) carried out using PerfeCTa SYBR Green
20 FastMix (Quanta). Amplicon levels were quantified using standard curves and normalized to levels of
21 *Rplp0*, all as previously reported [29]. Primers are listed in Table 1.

22

1 *Methylation analysis*

2 DNA was extracted from tissues using TRIzol, after taking the upper phase for RNA extractions. The DNA
3 was then cleaned using the Quick-DNA Miniprep Plus Kit (D4068; Zymo), before bisulfite conversion
4 using the EZ-DNA Methylation-Gold Kit (D5005 Zymo), and two rounds of PCR-amplification (nested,
5 with outer and inner primers: Table 1) using Red Load Taq Master (Larova). For analysis of untreated cell
6 lines, where there is less variation in methylation levels between samples, the amplicons were purified
7 (DNA Clean and Concentrator kit D4004; Zymo) and cloned into pGEM-T-easy, before inserts from 7-20
8 randomly selected clones were sequenced and analyzed as previously described [30]. For tissue samples
9 from the mice, deep sequencing was performed: after bisulfite conversion, the region of interest was
10 cleaned and amplified with the listed primers (Table 1). An additional 8-12 cycles of PCR (30 sec each, at
11 65°C) were then performed using KAPA HiFi HotStart Ready mix X2 (Roche), with a different
12 combination of Illumina Nextera XT indexes (10 µM) for each sample. Samples were cleaned with PCR
13 purification kit (Zymo) between each PCR round. After addition of 50% Phi-X, these libraries were deep-
14 sequenced by 150 bp paired-end sequencing on Mi-seq (Illumina), at the Technion Genome Center. The
15 % methylation levels represent the relative number of cytosines found methylated out of the total
16 number sequenced at the same site.

17 *Cell culture*

18 The GT1-7 mouse hypothalamic GnRH neuronal cell line was cultured with high glucose DMEM
19 containing 10 % FBS, 1 % penicillin-streptomycin, sodium pyruvate and sodium bicarbonate (all from
20 Biological Industries, Beit Haemek), maintained at 37 °C with 5 % CO₂ at 50-90 % confluency, passaging
21 1-2 times a week. The media was replaced with the same media but containing charcoal-stripped FBS,
22 24 h before and during treatments with E₂ or Dex (Sigma), as described. The murine KK-1 granulosa cell
23 line (a gift from Ilpo Huhtaniemi, Imperial College, UK) was cultured as reported [31], maintained at 37 °C

1 with 5 % CO₂ at 30-80 % confluency, passaging 2-3 times a week. Steroid treatments were performed in
2 each cell line across several doses of the steroid (in this or our previous study [9]) which were chosen
3 initially based on those commonly used in similar cell culture gene expression analysis and specifically in
4 these cell types (e.g. [32–34]). Subsequently, after the dose-response analysis indicated which doses
5 elicited effects on gene expression, the optimal dose (lowest dose showing most significant effect) was
6 chosen for future experiments, and control genes or loci were included to confirm responses to these
7 treatments. Cells are tested regularly for mycoplasma and identity authenticated through hormone
8 responsiveness.

9 *Primary culture*

10 Upon harvest, ovaries were transferred immediately into Hanks' Balanced Salt Solution (HBSS) that
11 lacked Mg and Ca (Biological Industries). The HBSS was then replaced with papain solution [35] for 20
12 min at 37 °C, to loosen cell-cell interactions. Subsequently, the papain solution was replaced with
13 growth media containing charcoal-stripped FBS, as for KK-1 cells, and the tissue was pipetted several
14 times to separate the cells (mostly granulosa), which were then seeded into a 96-well plate.
15 Approximately 24 h later, fresh media with E₂, or ethanol as a vehicle control, was added for 24 h before
16 RNA extraction.

17 *Site-directed manipulation of the DNA methylation*

18 KK-1 cells were transfected with pCMV-dCas9-D3A plasmid (Addgene #78256 [36]) to stably express
19 catalytically dead Cas9 (dCas9) fused with the catalytic domain of DNMT3A and FLAG tag peptide. The
20 plasmid was first linearized (*NotI* HF [R3189S BioLabs] for 1 h at 37 °C), and 1 µg/ml plasmid transfected
21 using Lipofectamine 3000 (L3000015, Invitrogen) to cells at 70-80 % confluency in a 35 mm plate. The
22 transfected cells were selected with 600 µg/ml Bleomycin (Zeocin™ ant-zn-1, InvivoGen) for 2-3 weeks,

1 and clones tested for DNMT3A mRNA by qPCR, and protein by western blot with FLAG peptide
2 monoclonal antibody (Sigma F3165, RRID:AB_259529).

3 These cells were transiently transfected with gRNA (planned with *benchling.com*) for recruitment of the
4 dCas9-DNMT3A to the 5' and 3' ends of *Srd5a1* enhancer. Each annealed gRNA was ligated into
5 linearized (*Esp3I* [R0734S, BioLabs] for 2 h at 37 °C) pSB700 plasmid (#64046 Addgene) modified to
6 express mCherry fluorescent protein, with T4 DNA ligase (M180B, Promega) in T4 Rapid Ligation Buffer
7 X2 (C6711, Promega), for 60 min at room temperature. Cells were transfected with this plasmid as
8 above, and after ~48 h, FACS Aria-IIIu cell sorter separated the mCherry-positive and negative cells.
9 These cells were seeded on a 24-well plate with phenol red-free DMEM/F12 medium containing
10 charcoal-stripped FBS. After 24 h, the cells were exposed to 10 nM E₂ for 24 h, followed by RNA and DNA
11 extractions.

12 For the site-directed demethylation, GT1-7 cells were co-transfected with TETv4 plasmid (Addgene
13 #167983) and pSB700 containing the same gRNAs as above, or empty pSB700 as control. After ~48 h,
14 the cells that were successfully transfected for both plasmids (expressing mCherry and BFP) were
15 collected using the FACS Aria-IIIu cell sorter. These cells were seeded on a 96-well plate with charcoal
16 stripped serum-containing medium and harvested 24 h later.

17 *Chromatin immunoprecipitation (ChIP)*

18 ChIP was carried out as described [37,38] after formaldehyde cross-linking, sonicated to an average of
19 200 bp fragments and with the following antibodies: ESR1 (Abcam 32063, RRID:AB_732249), GR (Abcam
20 3671, RRID:AB_2236351), H3K4me1 (Abcam 8895, RRID:AB_306847), FLAG peptide (Sigma
21 F3165, RRID:AB_259529). The DNA was purified and regions amplified by qPCR (as detailed earlier;
22 primers in Table 1) from IP samples and from the input to which the IP amplicon levels were normalized.

23

1 *Statistical analysis*

2 All data are from multiple biological repeats (n-value) which were assayed individually. Results are
3 shown as mean \pm SEM. Parametric data were analyzed by two-tailed Student's *t*-test or one-way analysis
4 of variance (ANOVA) followed by Tukey-Kramer or Bonferroni *t*-test for multiple comparisons.
5 Methylation analysis (% methylation) utilized Mann-Whitney or Kruskal Wallis, Dunn's non-parametric *t*-
6 test. Significance was defined as $p < 0.05$.

8 **Results**

9 ***Srd5a1* is differentially regulated in the ovaries and hypothalamus across the lifespan**

10 Having found previously that *Srd5a1* expression is reduced in the POA and ovaries following early-life
11 immunological challenge, and that this was associated in the ovaries with increased methylation at two
12 CpGs in the first intron[9], we looked first at how *Srd5a1* mRNA and methylation levels at these *loci* vary
13 across early development under normal conditions. We found that *Srd5a1* expression levels changed
14 across the pre-pubertal period, in a manner that differed markedly in the two tissues. Ovarian *Srd5a1*
15 mRNA levels increased dramatically between mice at postnatal day (PND) 10 and 30, but were much
16 lower in post-pubertal mice examined at PND 45 (Fig. 1A). However in the POA, they decreased sharply
17 in mice aged between PND 7 and 10 and appeared relatively consistent in the mice older than this (Fig.
18 1B).

19 Levels of methylation at these two intronic CpGs (CpG1 and CpG2) were measured by bisulfite
20 conversion and high throughput sequencing (MiSeq). In the ovaries, a significant drop in DNA
21 methylation (between PND 7-20) was seen prior to the increased in expression of *Srd5a1*: at the second
22 CpG (CpG2) the levels dropped more dramatically from 24 to 7%, and at both CpGs they appeared to

1 remain consistent thereafter (Fig. 1C,E). In the POA, there appeared to be some drop in methylation
2 between PND 7 to 10, though this was not statistically significant, perhaps due to the small sample size.
3 It is clear, however, that the drop in *Srd5a1* expression in the POA of the mice at this early neonatal
4 stage was not negatively correlated with change in the methylation levels. The methylation was
5 generally higher in the POA than in the ovaries, and from PND 10, levels at both CpGs appeared to
6 increase with aging (Fig 1D,F). *Srd5a1* is thus clearly regulated differently in these two tissues.

7 8 **E₂ increases *Srd5a1* expression in ovarian granulosa cells, and estrogen receptor-1 (ESR1) binds the** 9 **locus of the differentially methylated CpGs at a transcriptional enhancer**

10 The dramatic increase in ovarian *Srd5a1* expression between PND 10 to 30 suggested that the gene
11 might be regulated by gonadal steroids, supported by the fact that E₂ activity has been shown already by
12 PND 15[39,40], and our previous observations that E₂ induced an increase in *Srd5a1* mRNA levels in the
13 KK-1 ovarian granulosa cell line [9]. Granulosa cells are the most abundant cell type in the ovary and the
14 main cell type that expresses *Srd5a1* ([www.proteinatlas.org/ENSG00000145545-](http://www.proteinatlas.org/ENSG00000145545-SRD5A1/single+cell+type/ovary)
15 [SRD5A1/single+cell+type/ovary](http://www.proteinatlas.org/ENSG00000145545-SRD5A1/single+cell+type/ovary)[41,42]), and we confirmed the *Srd5a1* response to 24 h E₂ in primary
16 ovarian cells from 30 d-old mice (Fig 2A). To examine further the mechanisms through which E₂
17 regulates *Srd5a1*, we performed CHIP for ESR1 in the same KK-1 ovarian granulosa cells. In E₂-treated
18 cells, ESR1 was enriched in the region of the first exon-intron boundary in accordance with the presence
19 of two half estrogen response elements (EREs: consensus TGACC and non-consensus GGGCA[43]; Fig
20 2B,C). ESR1 was also enriched further downstream in the intron, where there are two more consensus
21 half-sites, one of which encompasses CpG1, while the other is located closer to CpG2 (Fig 2B,C). The
22 orthologous region in the human genome, which includes the differentially methylated CpGs and early

1 menopause-associated SNPs identified previously[9], contains two sequences predicted (by JASPAR) to
2 function as EREs.

3 In the human genome, this orthologous region is indicated (GeneHancer[44], visualized in the UCSC
4 genome browser: <http://genome.ucsc.edu>) to act as a transcriptional enhancer, and ENCODE data show
5 it is highly enriched with H3K4 monomethylation (H3K4me1)[9], a typical histone modification of
6 transcriptional enhancers[45]. In the mouse genome, this locus is enriched with ENCODE candidate *cis*-
7 Regulatory Elements (cCREs) comprising a proximal enhancer-like signature (pELS; Fig 2C). We thus
8 performed ChIP for this histone modification in the mouse ovarian granulosa cell line, which revealed
9 that H3K4me1 is strongly enriched at this locus (Fig 2D), indicating that it indeed likely functions as a
10 transcriptional enhancer in these cells.

11
12 **In ovarian KK-1 granulosa cells, methylation of the intronic enhancer CpG2 prevents E₂ stimulation of**
13 ***Srd5a1* expression**

14 Given the drop in methylation levels at the two intronic enhancer CpGs in the ovaries prior to the pre-
15 pubertal increase in *Srd5a1* expression (Fig 1), we went on to investigate a possible regulatory role for
16 this methylation. To ascertain the utility of the KK-1 ovarian cell line for this purpose, we first examined
17 the *Srd5a1* proximal promoter (-123 to +62 bp from the transcriptional start site [TSS]) and enhancer
18 (+870 to +1081 bp from the TSS), both of which we found to be virtually unmethylated in these cells (Fig
19 3A). We next investigated whether inducing DNA methylation at this enhancer in the ovarian granulosa
20 cell line would be sufficient to inhibit *Srd5a1* expression. For this, we used KK-1 cells stably expressing a
21 FLAG-tagged dCas9-DNMT3A catalytic domain, targeted to the enhancer by two site-specific gRNAs (Fig
22 3B). Binding of the chimeric protein to this locus was confirmed by ChIP, and was seen to be enriched at
23 the enhancer only in the presence of the gRNAs (Fig 3C). Methylation levels were assessed (by bisulfite

1 conversion and MiSeq), and were increased 2.5-fold at CpG2, while CpG1 was not affected (Fig 3D).
2 Strikingly, this treatment abolished the response of *Srd5a1* to E₂, while that of the control E₂-activated
3 gene, *Greb1* was unaltered (Fig 3E). We have thus established a facilitating role for the demethylation at
4 this site in E₂ up-regulation of *Srd5a1*.

5
6 **In immortalized GnRH neuronal GT1-7 cells, *Srd5a1* mRNA levels are not affected by E₂ even after**
7 **reduction in methylation at the intronic enhancer CpG1**

8 Given the differential expression of *Srd5a1* in the POA and ovaries, we looked for the underlying
9 mechanisms, and first asked whether this enhancer might be methylated at additional sites other than
10 CpG1 and CpG2 in the hypothalamic POA. However, the other four CpGs in the H3K4me1-enriched
11 region (+605 to +847) had very low levels of DNA methylation (<10 %) in the POA of both young (7 d)
12 and adult (60 d) mice (Fig 4A), suggesting an unlikely regulatory role in this context.

13 Although methylation at the intronic enhancer CpGs in the POA did not appear to be correlated with
14 *Srd5a1* expression across early development (Fig 1), in light of our findings in the ovaries, we wanted to
15 determine whether methylation of these CPGs had any effect on *Srd5a1* expression in neuronal cells.

16 The GT1-7 cell line is derived from GnRH-producing hypothalamic POA neurons, and *Srd5a1* is expressed
17 in primary GnRH neurons[46]), so we first examined methylation at the *Srd5a1* proximal promoter and
18 enhancer in this cell line. As in the ovarian cell line (Fig 3A), the *Srd5a1* promoter was practically
19 unmethylated in these neuronal cells, but both enhancer CpGs (CpG1 and CpG2) were highly methylated
20 (Fig 4B) in line with the findings in the primary cells (Fig 1). This differential enhancer methylation also
21 correlated with very different *Srd5a1* expression levels which were more than 12-fold higher in the
22 ovarian KK1 cell line than in the neuronal GT1-7 cells (Fig 4C).

1 Following our findings in the ovarian cell lines, we next examined whether in this estrogen-responsive
2 GT1-7 neuronal cell line, *Srd5a1* mRNA levels might be increased by E₂. However, no effect on *Srd5a1*
3 was seen, despite a clear increase in expression of the positive control gene, *Greb1* (Fig 4D). To
4 determine whether a reduction in the enhancer DNA methylation in these cells might be sufficient to
5 allow increased *Srd5a1* expression, we targeted the demethylating dCas9-TET1 catalytic domain[47] to
6 the region with two site-specific gRNAs (Fig 4E). After 3 d, the methylation levels were reduced by >50 %
7 at CpG1, but were unaltered at CpG2 (Fig 4F). Despite the significant reduction in methylation at CpG1,
8 there was no apparent effect on *Srd5a1* expression, and *Srd5a1* mRNA levels were still not responsive to
9 E₂ exposure (Fig 4G).

10

11 **The glucocorticoid, dexamethasone, represses *Srd5a1* expression in GT1-7 neuronal cells, and the**
12 **glucocorticoid receptor is found at the intronic enhancer.**

13 The levels of both glucocorticoids and the glucocorticoid receptor (GR) are particularly low in the
14 immediate neonatal hyposensitive period, increasing after about PND 8-10[48,49], with circulating
15 corticosterone levels rising by as much as 300-fold between PND 5-15[50]. This timing corresponds with
16 the dramatic drop in *Srd5a1* levels (Fig 1B). Moreover, our previous studies found that early-life immune
17 stress (PND 23-30) caused a reduction in POA *Srd5a1* expression[9], indicating a possible role for
18 glucocorticoids in regulating *Srd5a1* in this region of the brain. The glucocorticoid receptor (GR or Nr3c1)
19 is expressed in primary GnRH neurons[46], so we treated GT1-7 cells with the synthetic glucocorticoid
20 dexamethasone (Dex) for 24-72 h to assess its effects on *Srd5a1* expression. Dex significantly reduced
21 *Srd5a1* mRNA levels (Fig 5A, B), although the effect was not dose- dependent over 1-100 nM (Fig 5A).

22 To explore further the effect of glucocorticoids on *Srd5a1*, we also performed CHIP for GR in these
23 neuronal cells to identify the GR binding sites in this region of the gene. GR was enriched at the *Srd5a1*

1 intronic enhancer, although not detected at various putative GR response elements (GREs), including
2 those indicated in other tissues (Chip-atlas.org). Despite Dex treatment strongly increasing GR binding at
3 the control, *Fkbp5* locus, it did not seem to affect GR binding at the *Srd5a1* enhancer (Fig 5C), suggesting
4 glucocorticoid modification of receptor activity rather than DNA binding at this site. Notably, Dex was
5 seen previously to have no effect on *Srd5a1* expression in KK-1 ovarian cells[9]. Together, these findings
6 support the existence of a cell-specific regulatory mechanism of glucocorticoid-activated repression in
7 the POA, in contrast to the cell-specific stimulatory effects of E₂ in the ovary.

8

9 Discussion

10 5 α reductase-1 plays a central role in endocrine systems regulating brain function, the stress response
11 and reproduction. This, together with the discovery that the encoding gene, *SRD5A1*, is sensitive to
12 epigenetic modifications, emphasizes the need to understand the molecular mechanisms regulating its
13 expression. Based on our previous findings of altered reproductive function following early-life immune
14 stress[9], we hypothesized here that reduced *Srd5a1* expression in the ovaries and hypothalamus is due
15 to increased methylation in the first intron that was evident in the mouse ovarian and women's buccal
16 DNA in the earlier study. We have now identified this locus as a transcriptional enhancer, the activity of
17 which is affected by methylation. Surprisingly, however, we saw very different patterns of *Srd5a1*
18 expression in the ovaries and POA across murine postnatal development.

19 The finding that *Srd5a1* mRNA levels in the ovaries peaked at PND 30, is similar to reports in male mice
20 in which *Srd5a1* levels in the testes were seen to peak at PND 25[51]. Although the very low levels of E₂
21 in prepubertal mice make accurate measurements difficult, its levels and/or activity are reportedly
22 elevated and play a role in reproductive development already by PND 15[39,40]. This, together with the
23 stimulatory effect of E₂ on *Srd5a1* expression in cultured ovarian cells, suggested that E₂ might be

1 responsible for the increase in ovarian *Srd5a1* levels at this stage of development. A stimulatory effect
2 of E_2 on *Srd5a1* expression has been reported in human breast cancer cell lines, mediated through ESR1
3 binding to an upstream distal enhancer, tethered via additional proteins [52]. We found ESR1 at the
4 novel intronic enhancer, providing an additional locus of activation via a half ERE suggesting also a
5 tethering mechanism, which has been reported for ESR1 binding in many cellular and genomic
6 contexts [53–55]. The exact mechanisms of ESR1 binding to this *Srd5a1* enhancer, as well as its co-
7 activators and regulatory mechanisms which are often diverse and complex [53,56–59], have yet to be
8 identified.

9 DNA methylation has been shown to affect ESR1 binding at numerous genomic loci [60–63] and the fact
10 that the increase in ovarian *Srd5a1* expression in prepubertal mice followed a drop in DNA methylation
11 suggested a possible connection. This connection was confirmed when increased methylation at one of
12 the CpGs abolished the *Srd5a1* response to E_2 . Targeting of DNMT3A to this region increased
13 methylation levels only at the more distal CpG (CpG2), while the ERE sites are located a little further
14 upstream and overlap CpG1. However, DNA methylation affects transcription factor (TF) binding
15 through various mechanisms [64]. At the TF binding site, methylation can alter TF binding kinetics,
16 stability and/or its dissociation, which may be position-dependent within the motif [65–68]. Methylated
17 DNA is also recognized by specific methylated DNA-binding proteins which recruit additional chromatin
18 modifying proteins that alter the chromatin landscape. Such changes, including nucleosome positioning
19 and histone variants, would certainly impact binding site accessibility and dynamics [69]. Moreover,
20 methylation alters the mechanical characteristics of DNA, such as its shape, flexibility and hydration [70–
21 72], any of which would likely affect TF binding in the vicinity.

22 Epigenetic modifications play various roles in the central control of puberty [73,74], and methylation
23 levels change across sexual development [75–77], particularly at *loci* enriched with high affinity EREs [76].
24 In breast cancer patients, the negative correlation between methylation and ESR1 induction of gene

1 expression involved mostly CpGs located at enhancers >1 kbp downstream of the transcription start
2 site[78,79], corresponding with the location of the intronic enhancer of *Srd5a1*. Our findings suggest
3 that the drop in methylation during early postnatal development is required for E₂ induction of *Srd5a1*
4 expression in the ovary. This drop in methylation is, however, unlikely sufficient for gene activation
5 which would depend also on circulating E₂ levels and activity (e.g. as regulated by α -fetoprotein[39]), as
6 well as the expression of ESR1 in granulosa cells of the developing follicles[80].

7 In contrast with its expression and up-regulation in the ovaries, *Srd5a1* expression was not increased by
8 E₂ in GnRH neuronal cells, and we did not detect any major changes in its expression in the POA towards
9 puberty when circulating and brain E₂ levels are elevated [39,40]. The only dramatic change evident in
10 POA *Srd5a1* expression levels along the life course was a drop in the neonate, following the stress-
11 hyporesponsive period when circulating glucocorticoid levels start to increase [49,81,82]. Taken together
12 with the fact that Dex inhibited *Srd5a1* expression in GnRH neuronal cells, and 5 α reductase affects
13 GnRH synthesis, secretion and pubertal timing [9], the glucocorticoid repression of *Srd5a1* that impacts
14 reproductive function likely occurs in this part of the brain, although this gene is expressed other
15 hypothalamic glial and neuronal cell types (data in[83]). Given that corticosterone is at a nadir already
16 during late embryonic through perinatal development, *Srd5a1* levels in the POA are presumably high
17 during this time which is a period of exquisite POA sensitivity to gonadal steroids[84,85] and when
18 sexual dimorphism of the POA is established[85,86]. Thus, even subtle changes in *Srd5a1* expression
19 levels at this time might have important implications for early life development and later sexual
20 maturation and reproductive function.

21 The effects of the glucocorticoid on *Srd5a1* transcription are likely mediated directly, given that GR is
22 associated with the intronic enhancer in GnRH neuronal cells, though Dex treatment did not appear to
23 increase GR binding at the enhancer, suggesting that the repression is mediated via GR interacting
24 proteins and co-repressors. Although a consensus palindromic GRE is not found in this genomic locus, a

1 half site (GGGACA) reported previously to mediate monomeric GR repression of gene expression [87] is
2 located at the region of enriched binding. GR actions are highly context-specific not only in terms of its
3 DNA binding, but also in its mechanisms of activation, co-regulatory proteins, and the resulting
4 outcomes[88,89]. GR represses transcription of numerous genes, and at the *Crh* promoter,
5 glucocorticoid-induced GR recruits DNMT3b to induce DNA methylation as well as recruitment of other
6 proteins such as MeCP2 that are involved in the repression [81,90]. However, DNA methylation at the
7 intronic enhancer in the POA did not increase with (or prior to) the drop in *Srd5a1* levels, indicating that
8 methylation at this site is not responsible for the drop in *Srd5a1* expression in the neonate.

9 In the ovary, the peri-pubertal stimulatory effect of E_2 on *Srd5a1* appears to be dependent on low
10 enhancer DNA methylation which we found elevated following pre-pubertal immune challenge [9].
11 However in the POA, the dominant regulation appears to be mediated by the HPA axis and GR acting
12 directly at the *Srd5a1* intronic enhancer. Although we did not detect reduced *Srd5a1* expression in the
13 POA in the older mice, an age-related drop in 5α -reduced neurosteroids was reported recently [91]
14 which might perhaps be due to the increase in glucocorticoid levels that occurs over the lifespan [92].
15 We have thus shown that this gene is regulated through cell-specific mechanisms some of which help
16 explain the long-term effects and particular sensitivity to stress experienced early in life [1,9].

17 Guided by our earlier work, our study is limited in that it has focused on this one element of the early-
18 life stress-response which is certainly complex and involves multiple cell types in both the POA and
19 other tissues, encompassing 5α reductase-1-dependent and independent mechanisms. However, 5α
20 reductase-1 is widely expressed, and its epigenetic regulation in other regions of the brain [93–96], as
21 well as its role in catalyzing production of additional neurosteroids [11,12,14] have been reported,
22 indicating multiple and diverse downstream effects on various endocrine systems. The epigenetic
23 regulation of 5α reductase-1, together with recent indications of its role in pathologies such as
24 polycystic ovarian syndrome (PCOS) and metabolic syndrome [14,97] emphasize the need for further

1 research to uncover more fully these and additional mechanisms underlying variation in reproductive
2 phenotypes as influenced by early-life experiences, and also how such responses affect health across the
3 life course.

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8 **Data Availability**

9 Some or all datasets generated during and/or analyzed during the current study are not publicly
10 available but are available from the corresponding author on reasonable request.

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16 Legends

17 **Fig 1. *Srd5a1* is differentially regulated in ovaries and hypothalamus across the lifespan.** (A,B) *Srd5a1*
 18 mRNA levels in (A) ovaries and (B) the hypothalamic preoptic area (POA) of mice at various ages (from 1-
 19 4 litters at each time point; for > 50 d old, mice in each group were not identical ages and the average
 20 age is shown). The mRNA levels were normalized to those of *Rplp0* and are shown relative to levels at
 21 the first time point; mean \pm SEM (some of the ovarian data is from [9]). (C-F) The % DNA methylation (% of
 22 cytosines methylated out of the total number sequenced at the same site) measured by bisulfite

1 conversion followed by MiSeq deep sequencing at the (C, D) first and (E, F) second CpG in (C, E) ovaries
2 and (D, F) POA. In all graphs, n-values at each point are shown; $p > 0.05$ (ANOVA, Tukey-Kramer t-test) for
3 groups sharing the same letter. Shaded boxes mark periods of significant change.

4 **Fig 2. E₂ increases Srd5a1 expression in ovarian granulosa cells, and estrogen receptor (ESR1) binds**
5 **the locus of the differentially methylated CpGs at a transcriptional enhancer.** (A) *Srd5a1* mRNA levels
6 in mouse ovarian primary cell culture (mice were 30 d old) after exposure to E₂ (100 nM) for 24 h;
7 mean ± SEM, n=3, 4; **: $P < 0.01$. (B) Chromatin immunoprecipitation (ChIP) for ESR1 in murine ovarian
8 granulosa KK-1 cells with or without 2 h exposure to E₂ (10 nM) followed by qPCR for loci across the 5'
9 end of the gene and first intron, with *Pgr* as positive control. IP/input levels are mean ± SEM (n=7, except
10 at 223-371, 676-828 and *Pgr* where n=4). Student's *t*-test compared treated and non-treated groups, *:
11 $P < 0.05$. (C) Schematic (adapted from UCSC genome browser; <http://genome.ucsc.edu>) showing the
12 locus of the 5' end of the *Srd5a1* gene (in dark blue: UTR (thin bar), first exon (thick bar) and part of first
13 intron [thin line]) in the mouse genome, with the location of CpG1 and CpG2, and ½ ERE motif sites
14 marked (arrows). The CpG island (green bar) and several regions identified by ENCODE as proximal
15 enhancer like sequences (pELS; orange bars) are shown, as well as the regions we found enriched for
16 ESR1 or H3K4me1 in KK-1 cells (long pale blue and grey diamonds, respectively, centered on the
17 amplicon center and in accordance with the resolution determined by sonication). (D) ChIP assay for
18 H3K4me1, performed and presented as in Fig 2B (n=4); ###: $P < 0.001$ (ANOVA, Tukey-Kramer t-test)
19 compared to all other means.

20 **Fig 3. In ovarian KK-1 granulosa cells, methylation of the intronic enhancer CpG2 prevents E₂**
21 **stimulation of Srd5a1 expression.** (A) DNA methylation (bisulfite conversion and sequencing) at the
22 *Srd5a1* promoter (-123 to +62 bp) and intronic enhancer (+870 to +1081, including CpG1 and CpG2) in
23 ovarian KK-1 cells: each column represents a single CpG site, and each row a repeat; black circles
24 represent CpGs that are methylated and white circles those that are not. (B) Targeted DNA methylation

1 was performed by stable expression of a FLAG-tagged dCas9-DNMT3A catalytic domain, recruited to the
2 enhancer of *Srd5a1* by two site-specific gRNAs (thick green lines). (C) CHIP assay for FLAG peptide in KK-1
3 cells stably expressing dCas9-DNMT3A-FLAG, after transfection with the gRNAs or empty vector,
4 followed by qPCR for the *Srd5a1* intronic enhancer, an upstream region or *Gapdh* as controls; IP/input
5 levels presented as in Fig 2B (n=3). Student's t-test compared levels in cells with and without
6 transfection of the gRNAs; *: <0.05. (D) Levels of DNA methylation (measured by bisulfite conversion
7 and MiSeq) in these cells at CpG1 and CpG2 of the *Srd5a1* intronic enhancer, shown relative to those in
8 control cells (no gRNAs); mean±SEM (n=3). (E) *Srd5a1* and *Greb1* mRNA levels in these cells with or
9 without E₂ (10 nM, 24 h). The mRNA levels were analyzed and presented as before (n=3); **: p<0.01,
10 ***: p<0.001 compared to untreated controls.

11 **Fig 4. In GnRH neuronal GT1-7 cells, *Srd5a1* mRNA levels are not affected by E₂ even after reduction in**
12 **intronic enhancer CpG1 methylation.** (A) DNA methylation at the intronic enhancer and ~300 bp
13 adjacent upstream region in the POA of 7 and 60 d old female mice was performed and is presented as
14 % methylation, mean±SEM (n=4); small or capital letters designate statistical tests for each age group
15 separately (Kruskal Wallis, Dunn's test); CpG (0) had no detectable methylation. (B) DNA methylation
16 was assessed at the *Srd5a1* promoter (-124 to +62 bp) and intronic enhancer (CpG1 and CpG2) in GT1-7
17 cells, as in Fig 3A and is presented similarly. (C,D) *Srd5a1* mRNA levels in (C) KK-1 and GT1-7 cells, or (D)
18 GT1-7 cells after 10-100 nM E₂ exposure (*Greb1* serves as positive control), measured and presented as
19 before, (n=4); *: p<0.05, ***: p<0.001. (E) Targeted demethylation was performed by over-expression of
20 dCas9-TET1 catalytic domain, recruited to the *Srd5a1* enhancer by the same site-specific gRNAs as in Fig
21 3B (thick green lines). (F) DNA methylation (bisulfite conversion and MiSeq) at CpG1 and CpG2 of the
22 *Srd5a1* intronic enhancer in the GT1-7 cells expressing the dCas9-TET1. Levels are presented relative to
23 those in control cells (no gRNAs); mean±SEM (n=3); *: P<0.05. (G) *Srd5a1* mRNA levels in similarly

1 transfected cells, with or without exposure to E₂ (10 nM), presented as before (n=3); P>0.05 in *t*-test for
2 all comparisons.

3 **Fig 5. The glucocorticoid, dexamethasone, represses *Srd5a1* expression in GT1-7 neuronal cells, and**
4 **the glucocorticoid receptor is found at the intronic enhancer.** (A,B) *Srd5a1* mRNA levels in GT1-7 cells
5 after (A) 1-100 nM dexamethasone (Dex; n=6-7) for 24 h, with *Gnrh* and *Fkbp5* as controls, or (B) 10 nM
6 Dex for 24-72 h (n=3-4); data analyzed and presented as before. (C) ChIP assay for GR in GT1-7 cells after
7 Dex exposure (10 nM ,24 h), and qPCR for identification of binding at the *Srd5a1* promoter, enhancer
8 and additional putative sites (from chip-atlas.org), with *Fkbp5* as positive control, performed and
9 presented as in Fig 2B (n=3). Student's *t*-test compared treated and non-treated groups; ***: P<0.001,
10 otherwise p>0.05.

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1 **Table 1: Primers**

Primer number	Gene	position relative to TSS	Sequence
qPCR			
#184	mRPLP0	140 F	GCGACCTGGAAGTCCAATA
#185	hRPLP0	240 R	ATCTGCTTGGAGCCCACAT
#1493	Srd5a1	755 F	GAATATGTATCTTCAGCCAAC
#1494	Srd5a1	925 R	GGTAATCTTCAAACCTTCTCG
#1891	Gnrh	67F	GATCCTCAAACCTGATGGCCG
#1892	Gnrh	271R	CTCCTCGCAGATCCCTGAG
#1931	Fkbp5	1479F	GAGTCCAAAGCCTCAGAGTC
#1932	Fkbp5	1696R	GCCAACACCTTCTCGAAGTC
#1672	Greb1	591F	GCCGAGCAGACAATGAGGAA
#1673	Greb1	806R	CAGGCTGGGAGACTTAGCAC
qPCR following ChIP			
#2656	Srd5a1	-200F	GTGCTCCGCTGTGGCGCTGA
#2657	Srd5a1	-65R	AGGGCGCCTTAGTCTCGAGC
#2560	Srd5a1	-384F	TGGACGACCTGATCGTAGC
#2561	Srd5a1	-228R	GCCTACACAGCAAAGACCC
#2297	Srd5a1	12F	GTATCTTCTGGTGGTGCTAG
#2367	Srd5a1	150R	GCCACATATAAGCTCAGGAG
#2673	Srd5a1	223F	GATGCGCTAGTCTACCTGG
#2674	Srd5a1	371R	GAAGGCAGCTCCTGTAGGA
#2272	Srd5a1	483F	GTCTCCCTCCTGCGCTTG
#2273	Srd5a1	636R	GAAATCCGGACCACTGTGC
#2664	Srd5a1	676F	GCGATGCCATCCAAGCTGC
#2665	Srd5a1	828R	CTCTGAAATTGCTCCAGTCC

#2287	Srd5a1	862F	CTTTCCCAGGAGGTGTTATG
#2652	Srd5a1	970R	GGGTCAGTTAAAGATAAGACC
#2288	Srd5a1	1095R	GACTTTCCCATGTCCCAAATG
#2233	Srd5a1	1152F	GTTGTGTTAATAGCCTCTGC
#2234	Srd5a1	1322R	GCTGTTACACAGAGAACTCG
#2438	Srd5a1	3413F	GTAAACCCTCCGAGATAGAC
#2439	Srd5a1	3565R	CCCACTCTGTGCTACTAAGTG
#2554	Srd5a1	8465F	CTCAAAGTCCCCACTCTAG
#2555	Srd5a1	8679R	CTTTCATGGATGGATCAC
#2496	Srd5a1	12621F	GGCAAGTAACAGAGGAAGAG
#2497	Srd5a1	12773R	CCCTTCACTCTGCTTTACA
#2440	Srd5a1	18043F	GCGTGGTAGGGGACAAGAG
#2441	Srd5a1	18197R	CCACATCTGGAATCAGGTAC
#2498	Srd5a1	21898F	GAGGTTTCCATAAGGGAGCA
#2499	Srd5a1	22054R	ATGAAGTGGCAACGCCTTTC
#2233	Fkbp5	1479F	GAGTCCAAAGCCTCAGAGTC
#2234	Fkbp5	1696R	TGGACGACCTGATCGTAGC
#1404	Pgr	459F	AGGACAGGAGCTGACCAAGA
#1405	Pgr	640R	AGTCATGACGACCCAAGCTC
PCR on bisulfite converted DNA			
#1628	Srd5a1 BS	-123F	AAGGAGTTTTTAGTTAATGTGTGTAG
#1629	Srd5a1 BS	62R	AAACACAACTAACACCACCAAAA
#1822	Srd5a1 BS	-301F	GGGTTAGATTGTGGAGGGG
#1823	Srd5a1 BS	123R	CAAACAACCCACAAAAACCAAC
#1824	Srd5a1 BS	845F	GTGTGAGATGGTATGAATTTTTTTT
#1825	Srd5a1 BS	871F	GGAGGTGTTATGTGAAAAATGTTT

#1826	Srd5a1 BS	1081R	CCAAATATCACAAAACCTCAACTTC
#1827	Srd5a1 BS	1148R	CATTCTCCCAACCTCTCTAAAAA
#2238	Srd5a1 BS	597F	GTGTTTGGTTAGGGATAGTGGT
#2217	Srd5a1 BS	875R	CCTCCTAAAAAAAATTCATACCAT
#2215	Srd5a1 BS	605F	TTAGGGATAGTGGTATAGTGGTT
#2216	Srd5a1 BS	847R	CACAAAAAAAACAAAACATCTCTAAA
#1881	Srd5a1 BS+ adapter - MiSeq	871F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGAGG TGTTATGTGAAAAATGTTT
#1882	Srd5a1 BS+ adapter - MiSeq	1081R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCAA ATATCACAAAACCTCAACTTC
#2258	Srd5a1 BS+ adapter - MiSeq	605F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTAGG GATAGTGGTATAGTGGTT
#2259	Srd5a1 BS+ adapter - MiSeq	847R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCACA AAAAAAAACAAAACATCTCTAAA
sgRNAs			
#2460	Srd5a1 sgRNA5	849F	<u>CACCGAGATGGTATGAATCTTTCCC</u>
#2461	Srd5a1 sgRNA5	869R	AAACGGGAAAGATTCATACCATCTC
#2462	Srd5a1 sgRNA6	1105F	CACCGCTCTGATCCTAAAGTATTCA
#2463	Srd5a1 sgRNA6	1125R	<u>AAACTGAATACTTTAGGATCAGAGC</u>

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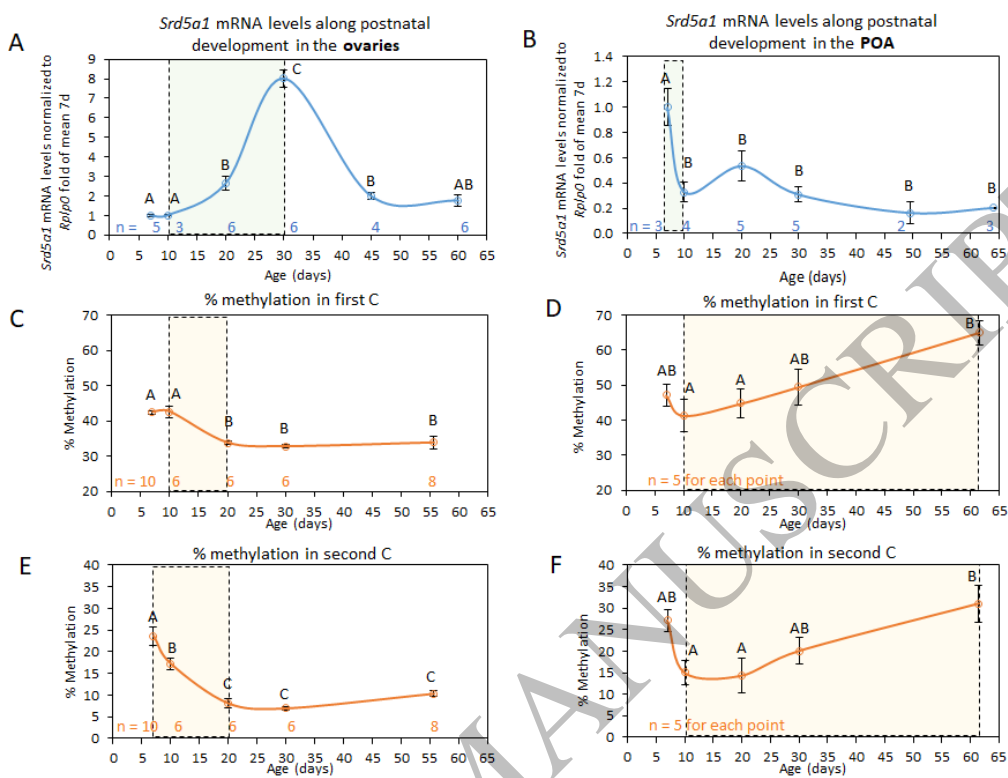
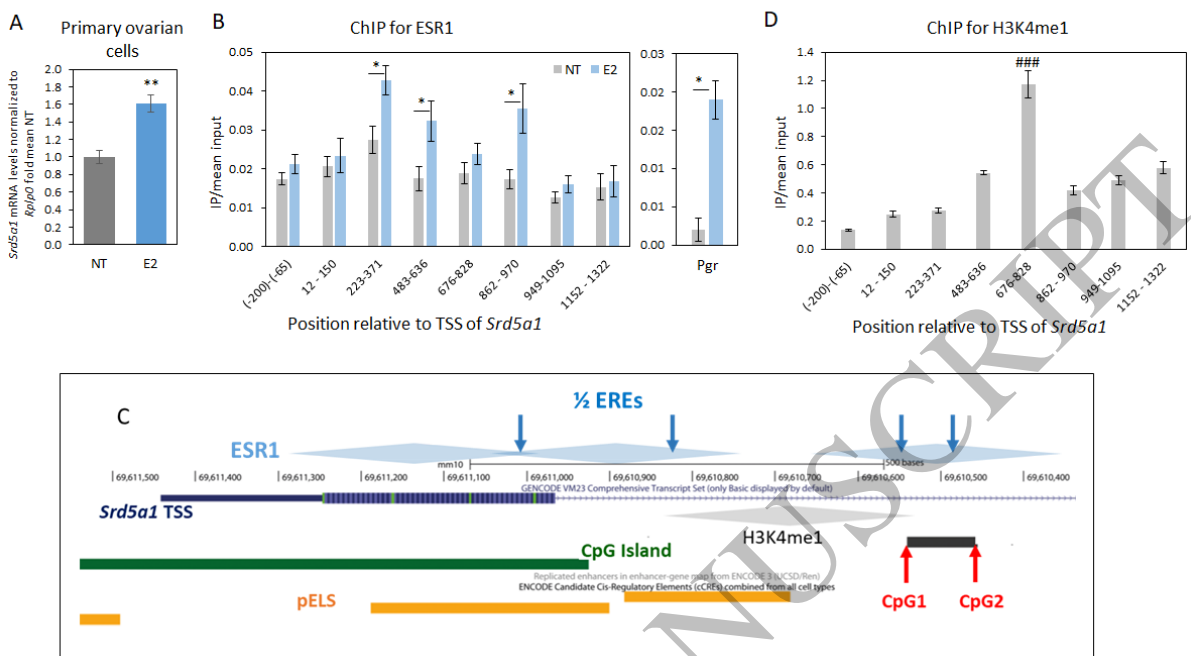


Figure 1
159x110 mm (x DPI)

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Figure 2
159x96 mm (x DPI)

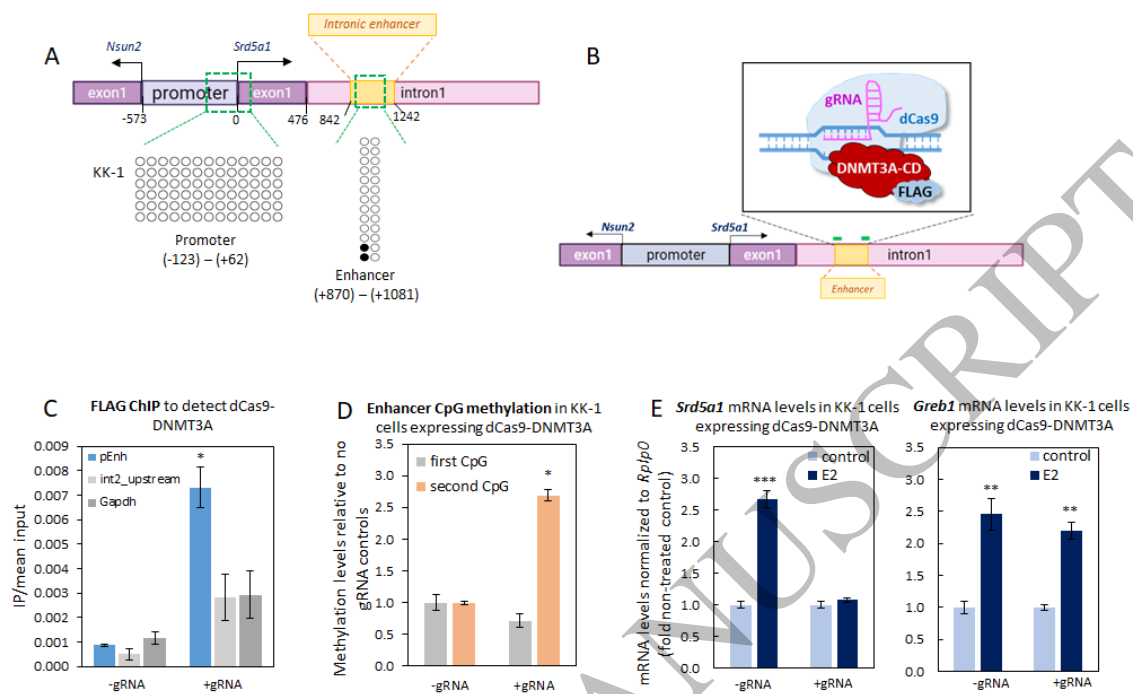


Figure 3
159x107 mm (x DPI)

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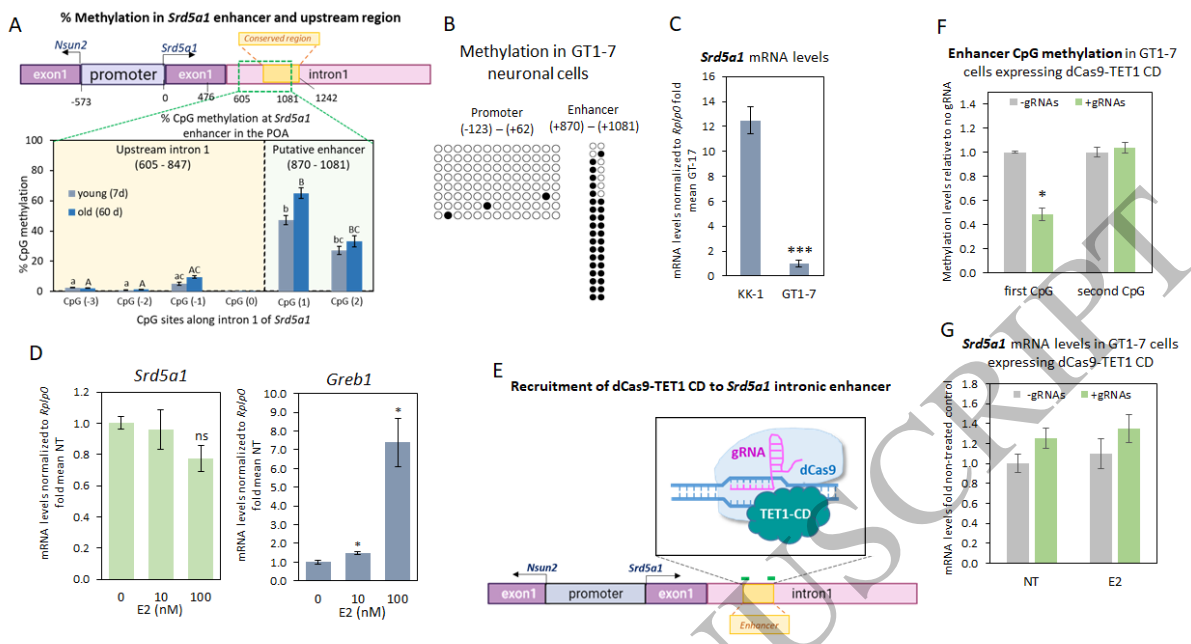


Figure 4
159x84 mm (x DPI)

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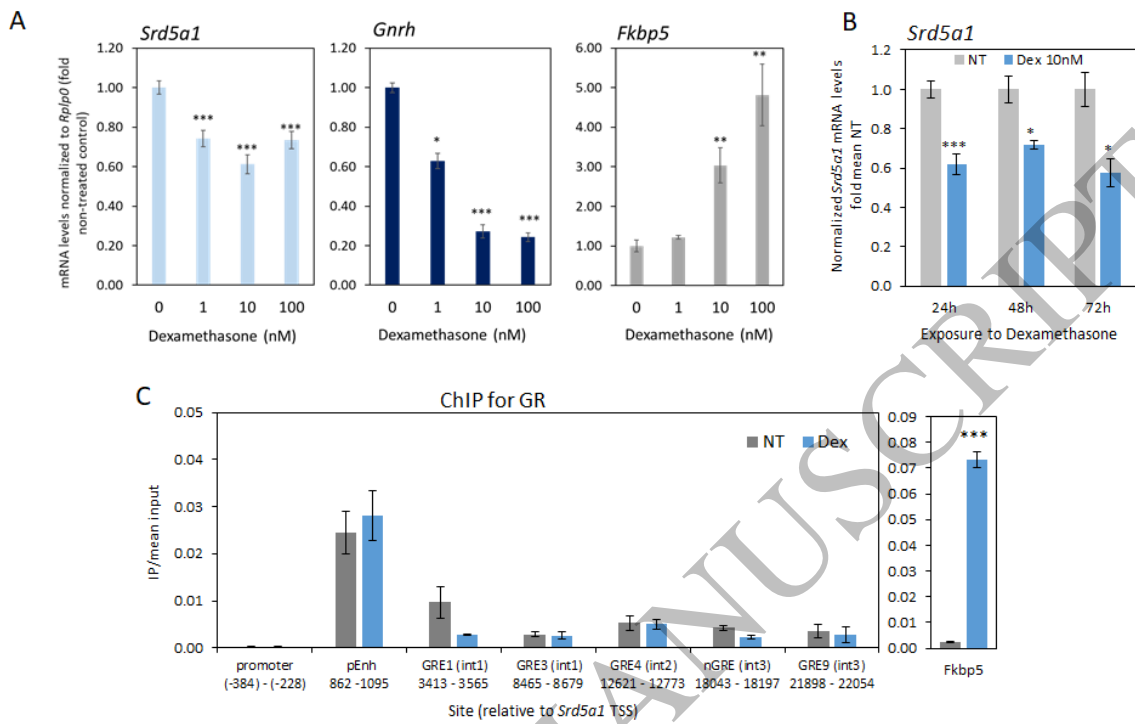


Figure 5
159x106 mm (x DPI)

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