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1 **Steroidogenic factor 1 regulates transcription of the inhibin B co-receptor in pituitary**  
2 **gonadotrope cells**

3  
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15  
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## 1 **Abstract**

2 The inhibins control reproduction by suppressing follicle-stimulating hormone synthesis in  
3 pituitary gonadotrope cells. The newly discovered inhibin B co-receptor, TGFBR3L, is  
4 selectively and highly expressed in gonadotropes in both mice and humans. Here, we describe  
5 our initial characterization of mechanisms controlling cell-specific *Tgfb3l/TGFBR3L*  
6 transcription. We identified two steroidogenic factor 1 (SF-1 or NR5A1) *cis*-elements in the  
7 proximal *Tgfb3l* promoter in mice. SF-1 induction of murine *Tgfb3l* promoter-reporter activity  
8 was inhibited by mutations in one or both sites in heterologous cells. In homologous cells,  
9 mutation of these *cis*-elements or depletion of endogenous SF-1 similarly decreased reporter  
10 activity. We observed nearly identical results when using a human *TGFBR3L* promoter-reporter.  
11 The *Tgfb3l* gene was tightly compacted and *Tgfb3l* mRNA expression was essentially absent  
12 in gonadotropes of SF-1 (*Nr5a1*) conditional knockout mice. During murine embryonic  
13 development, *Tgfb3l* precedes *Nr5a1* expression, though the two transcripts are fully co-  
14 localized by embryonic day 18.5 and thereafter. Collectively, these data indicate that SF-1  
15 directly regulates *Tgfb3l/TGFBR3L* transcription and is required for post-natal expression of the  
16 gene in gonadotropes.

## 1 Introduction

2           Reproduction is controlled by intricate endocrine feedforward/feedback loops between  
3 the brain, pituitary gland, and the gonads. Follicle-stimulating hormone (FSH) is a dimeric  
4 glycoprotein produced by pituitary gonadotrope cells that regulates ovarian follicle development  
5 and estrogen biosynthesis in females and spermatogenesis in males (1, 2). FSH production is  
6 stimulated by gonadotropin-releasing hormone (GnRH) from the brain and by transforming  
7 growth factor  $\beta$  (TGF $\beta$ ) ligands, such as the activins, which are currently thought to act in an  
8 autocrine/paracrine manner in the pituitary to stimulate transcription of the FSH $\beta$  subunit (3).

9           FSH also stimulates the synthesis and secretion of activin-related TGF $\beta$  ligands, known  
10 as the inhibins, from the gonads. The ovaries produce inhibins A and B (4-6), whereas the adult  
11 testes in most mammalian species produce inhibin B alone (7). Inhibins feedback to  
12 gonadotropes to suppress FSH production by competitively binding to activin receptors (8).  
13 Inhibin binding to these receptors is enhanced by transmembrane co-receptors. The TGF $\beta$  type  
14 III receptor (TGFB3), also known as betaglycan, can mediate the actions of both inhibins (8,  
15 9); however, conditional deletion of the *Tgfbr3* gene in gonadotropes principally impairs inhibin  
16 A, leaving inhibin B action intact (10). This observation led to the recent discovery of a  
17 betaglycan-like protein, TGFB3L, which functions as a specific inhibin B co-receptor in  
18 gonadotropes (11).

19           Whereas betaglycan is broadly expressed, including in the pituitary, gonads, and adrenal  
20 glands (12), *Tgfbr3/TGFB3L* expression appears to be restricted to gonadotropes in adult  
21 mice and humans (11, 13-15). The mechanisms conferring this cell-specific expression have not  
22 been elucidated. Here, we report that the nuclear receptor, steroidogenic factor 1 (SF-1, product  
23 of the *Nr5a1* gene), regulates *Tgfbr3/TGFB3L* transcription via conserved regulatory elements  
24 in the proximal promoter. *In vivo*, SF-1 is necessary for the maintenance, but not the initial  
25 expression of *Tgfbr3* in gonadotropes.

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## Materials and methods

### DNA constructs

The wild-type murine -999/+1 *Tgfb3l* and human -996/+1 *TGFBR3L* luciferase promoter-reporters were produced by PCR amplification of genomic DNA (see Table 1 for primers). The PCR products were ligated into pGL3-Basic (Promega, Madison, WI, USA) or pA3-luc (16). The murine SF-1 expression construct was described previously (17). The mutant promoter-reporters were constructed using the QuikChange protocol with primers described in Table 1. All constructs were confirmed by sequencing (G enome Qu ebec, Montreal, QC, CAN).

### 5' Rapid Amplification of cDNA Ends (RACE)

5' RACE was performed using the FirstChoice RLM-RACE kit (AM1700, Invitrogen, Waltham, MA, USA), following the manufacturer's protocol. Briefly, 10 µg of intact 5' capped mRNA from the pituitary of a male C57BL6 mouse was decapped and ligated to the 5' RACE adaptor. The ligated mRNA was reverse transcribed with MMLV-RT using a gene-specific primer located in exon 4 (see Table 1). Nested PCR was performed on the resulting cDNA using adaptor-specific forward primers and gene-specific reverse primers (see Table 1). PCR products were ligated into the pGEM-T Easy vector (A1360, Promega) and sequenced (G enome Qu ebec, Montreal, QC, CAN).

### Cell culture and promoter-reporter assays

All cells were cultured at 37°C in a humidified incubator with 5% CO<sub>2</sub>. Human embryonic kidney (HEK) 293T cells (ATCC CRL-3216; RRID: CVCL\_0063; provided by Dr. Terry H ebert, McGill University) were cultured in DMEM (319-005-CL, Wisent, St-Bruno, QC, CAN) supplemented with 5% (v/v) fetal bovine serum (FBS; 098150, Wisent). Immortalized murine

1 gonadotrope-like L $\beta$ T2 cells (18) (RRID: CVCL\_0398; provided by Dr. Pamela Mellon,  
2 University of California, San Diego, CA, USA) were cultured in DMEM supplemented with 10%  
3 (v/v) FBS.

4 Promoter-reporter assays were performed as previously described (16). Briefly,  
5 HEK293T and L $\beta$ T2 cells were seeded at densities of 50,000 and 150,000 cells per well,  
6 respectively, in 48-well plates. The following day, HEK293T cells were transfected using PEI at  
7 a ratio of 1:3 ( $\mu$ g DNA to  $\mu$ g PEI). L $\beta$ T2 cells were transfected using Lipofectamine 3000  
8 (L3000015, ThermoFisher Scientific, Burlington, ON, CAN) following the manufacturer's  
9 protocol. Control (D-001210-05) and *Nr5a1* (D-051262-01) short interfering RNAs (siRNAs)  
10 were purchased from Dharmacon (Lafayette, CO, USA). For assays where siRNAs and the  
11 murine promoter-reporter were co-transfected, the pA3-luc backbone was used. Twenty-four  
12 hours after transfection, cells were serum starved for an additional 24 h. After starvation, cells  
13 were lysed in 50  $\mu$ l/well passive lysis buffer [25 mM Tris-phosphate (pH 7.8), 10% (v/v) glycerol,  
14 1% (v/v) Triton X-100, 1 mg/mL bovine serum albumin (BSA), 2 mM EDTA] for 10 min at room  
15 temperature with agitation. Twenty  $\mu$ l of cell lysis supernatant was combined with 100  $\mu$ l assay  
16 buffer [15 mM potassium phosphate (pH 7.8), 25 mM glycylglycine, 15 mM MgSO<sub>4</sub>, 4 mM  
17 EDTA, 2 mM ATP, 1 mM DTT, 0.04 mM D-luciferin], and luciferase activity was measured on an  
18 Orion II microplate luminometer (Berthold Detection Systems, Oak Ridge, TN, USA). All  
19 experiments were performed in technical triplicates and the experiments repeated as indicated  
20 in the figures.

#### 21 DNA affinity purification assay and immunoblot

22 Thirty  $\mu$ l of Dynabeads® M-280 (11205D, Dynal, Invitrogen) were washed three times  
23 with 2x B&W buffer [10 mM Tris, pH 7.5, 1 mM EDTA, 2 M NaCl], then 10  $\mu$ M each of wild-type  
24 or mutant biotinylated double-stranded probe (see Table 2) were incubated with the beads in 1x  
25 B&W buffer at room temperature for 15 min. Beads were washed twice with 2x B&W buffer and  
26

1 once with 1x binding buffer [5% (v/v) glycerol, 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM  
2 dithiothreitol, 0.15% Triton X-100, 100 mM NaCl, 4 mM MgCl<sub>2</sub>], then blocked for 30 min at room  
3 temperature using 1% (w/v) BSA in binding buffer, and lastly resuspended in 50 µl of 1x binding  
4 buffer. LβT2 cells were grown until confluent in 10 cm plates and harvested using 1 mL of DNAP  
5 lysis buffer [300 mM NaCl, 20 mM Tris–HCl (pH 7.5), 1% (v/v) Triton X-100, 1 mM PMSF, 2  
6 µg/ml leupeptin and aprotinin]. One hundred µl of clarified lysate was combined in a 500 µl  
7 reaction with 100 µl of 5x binding buffer, 10 µl of 0.5 µg/µl salmon sperm DNA (Invitrogen,  
8 15632011), and 50 µl of DNA-bound streptavidin magnetic beads. The reaction was incubated  
9 at 4°C for 2 h with agitation, followed by 5 washes in 1x binding buffer. Bound proteins were  
10 eluted in 40 µl of 0.1% SDS at 100°C for 5 min.

11 Ten µl of 5x Laemmli buffer [250 mM Tris pH 6.8, 10% SDS, 50% glycerol, 0.02%  
12 bromophenol blue, and 10% β-mercaptoethanol] was added and eluted proteins were resolved  
13 by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) on a 10% resolving  
14 gel prepared using a 30% (w/w) acrylamide/bis-acrylamide (29:1) solution in running buffer (25  
15 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.3). Proteins were transferred to Protran  
16 nitrocellulose membranes (GE 10600001, Millipore Sigma, Oakville, Ontario, CA) in Towbin  
17 buffer (25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol), blocked with 5% milk (w/v) in Tris-  
18 buffered saline [TBS; 150 mM NaCl, 10 mM Tris (pH 8.0)] containing 0.05% (v/v) Tween 20  
19 (TBST) and incubated overnight at 4°C with agitation with an antibody against SF-1 diluted in  
20 blocking buffer (1:1000; D1Z2A; Cell Signaling Technology, Danvers, MA, USA;  
21 RRID:AB\_2798030). The next day, membranes were washed in TBST and incubated in  
22 horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5,000; AP182P; Millipore  
23 Sigma; RRID:AB\_92591) in blocking buffer for 1 h at room temperature with agitation.  
24 Membranes were once again washed in TBST, and bands were visualized using enhanced  
25 chemiluminescence substrate (NEL105001, PerkinElmer, Waltham, MA, USA) and an  
26 Amersham Imager 600 (GE Healthcare, Chicago, IL, USA).



## 1 Chromatin Immunoprecipitation

2 L $\beta$ T2 cells were seeded at a density of 1.5 million cells/well in a 6-well plate. After three  
3 days, formaldehyde was added to a final concentration of 1%, and crosslinking was performed  
4 for 10 min at room temperature. The reaction was then quenched with 125 mM glycine for 5 min  
5 at room temperature. Cells were scraped and collected in 1 mL of ice-cold PBS and centrifuged  
6 at 800 x g for 10 minutes at 4°C. The cell pellet was then resuspended in 100  $\mu$ l of Nuc101 EZ  
7 lysis buffer (NUC101, Millipore Sigma) with protease inhibitor cocktail (04693116001, Millipore  
8 Sigma) and 1 mM PMSF for 5 minutes on ice, then centrifuged at 500 x g for 5 minutes at 4°C.  
9 Supernatant was discarded and the pellet washed in Nuc101 EZ lysis buffer as above. The  
10 nuclei were then divided into three tubes for digestion.

11 Nuclei (in 50  $\mu$ l of Nuc101 EZ lysis buffer) were mixed with 6  $\mu$ l of 10x MNase buffer [10  
12 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 50% glycerol], 0.44  $\mu$ l of 200 mM DTT, and 1  
13  $\mu$ l of diluted MNase (M0247S, New England Biolabs, Ipswich, MA, USA; diluted 1:10 in MNase  
14 reaction buffer [50 mM Tris-HCl (pH 7.9), 5 mM CaCl<sub>2</sub>]) in a total reaction volume of 60  $\mu$ l.  
15 Nuclei were digested for 10 minutes at 37°C and the digestion was stopped with 6.6  $\mu$ l of 100  
16 mM EDTA and 6.6  $\mu$ l of 1% Triton X-100/1% sodium deoxycholate. Chromatin was incubated  
17 on ice for 20 minutes, then 220  $\mu$ l of complete IP buffer [20 mM Tris-HCl (pH 8), 2 mM EDTA,  
18 150 mM NaCl, 0.1% Triton X-100, 1x protease inhibitor cocktail, 1 mM PMSF] was added. Ten  
19 percent of the chromatin was removed and kept as "input". NaCl was added to the input  
20 chromatin to a final concentration of 0.2 M and protein:DNA complexes were reverse-cross-  
21 linked overnight at 65°C. The next day, samples were incubated for 30 min at 37°C with 10  $\mu$ g  
22 RNase A in a total volume of 200  $\mu$ l followed by 1 hour at 55°C with 10  $\mu$ g proteinase K in a  
23 total volume of 200  $\mu$ l. DNA was extracted with phenol:chloroform:isoamyl alcohol and  
24 precipitated with sodium acetate and ethanol overnight at -20°C. The DNA was pelleted at  
25 15,000 rpm at 4°C for 20 min, washed with 70% ethanol, dried, dissolved in 30  $\mu$ l of 10 mM Tris-  
26 HCl (pH 8.0), and quantified using Nanodrop.

1 Ten  $\mu\text{g}$  of chromatin was precleared for 1 hour at  $4^{\circ}\text{C}$  with 10  $\mu\text{l}$  of Dynal protein G  
2 beads (10003D, Invitrogen) on an end-over-end rotator, then incubated with protein G beads  
3 conjugated to either rabbit IgG (2729, Cell Signaling Technology; RRID: AB\_1031062) or rabbit  
4 anti-SF-1 (12800, Cell Signaling Technology; RRID:AB\_2798030) overnight. To conjugate the  
5 antibodies to the beads, 10  $\mu\text{l}$  of beads (washed three times in complete IP buffer) were  
6 incubated with 1  $\mu\text{g}$  of antibody in a total volume of 200  $\mu\text{l}$  for 4 hours at  $4^{\circ}\text{C}$  on an end-over-  
7 end rotator. The next day, after overnight incubation with chromatin, beads were sequentially  
8 washed with complete IP buffer (1 x 5 minutes at  $4^{\circ}\text{C}$ ), low salt buffer [20 mM Tris-HCl (pH 8), 2  
9 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS] (2 x 5 minutes at  $4^{\circ}\text{C}$ ), and high salt  
10 buffer [20 mM Tris-HCl (pH 8), 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS] (1 x 5  
11 minutes followed by 1 x 10 minutes at  $4^{\circ}\text{C}$ ). Chromatin was eluted for 90 minutes at  $65^{\circ}\text{C}$  in 30  
12  $\mu\text{l}$  elution buffer (1 M  $\text{NaHCO}_3$ , 1% SDS), then reverse-cross-linked, digested with RNase A and  
13 proteinase K, and extracted as described above.

14 Input and immunoprecipitated chromatin were analysed using qPCR (described below)  
15 using primers listed in Table 1.

#### 17 Generation of *Nr5a1* conditional knockout mice

18 The *Nr5a1*<sup>fl/fl</sup> and *Gnrhr*<sup>IRES-Cre/IRES-Cre</sup> (GRIC) mice were previously described (19, 20).  
19 Cre-mediated recombination occurs in the germ line of male GRIC mice (21); thus, the GRIC  
20 allele was introduced via the female in all crosses. *Nr5a1*<sup>fl/fl</sup> males (Jackson Laboratory, 007041)  
21 (22) were crossed with GRIC females to produce *Nr5a1*<sup>fl/+</sup>; *Gnrhr*<sup>GRIC/+</sup> progeny. *Nr5a1*<sup>fl/fl</sup> males  
22 were then crossed with *Nr5a1*<sup>fl/+</sup>; *Gnrhr*<sup>GRIC/+</sup> females to produce *Nr5a1*<sup>fl/fl</sup>; *Gnrhr*<sup>+/+</sup> (control) and  
23 *Nr5a1*<sup>fl/fl</sup>; *Gnrhr*<sup>GRIC/+</sup> (conditional knockout; cKO) animals. Genotyping and assessment of  
24 genomic recombination were conducted as previously described (23) (primers listed in Table 1).  
25 All animals were housed on a 12 h light: 12 h dark cycle and given access to food and water *ad*  
26 *libitum*. All animal work was conducted in accordance with federal and institutional guidelines

1 and with the approval of the McGill University Facility Animal Care Committee-DOW-A (protocol  
2 5204).

3

#### 4 Organ collection and processing

5 Testes, seminal vesicles, ovaries, and uteri were dissected from control and cKO males  
6 at 8-10 weeks of age, and females at 9-10 weeks of age. Control females were collected at  
7 random points in the estrous cycle; cKO females were acyclic. All reproductive organs were  
8 weighed on an analytical balance. Pituitary glands were snap frozen in liquid nitrogen and  
9 stored at -80 °C until analysis.

10

#### 11 Blood collection and hormone analyses

12 Blood was collected by cardiac puncture and allowed to coagulate at room temperature  
13 for approximately 30 min. Whole blood was centrifuged at 3000 rpm for 10 min at room  
14 temperature. Serum was collected and stored at -20°C until hormone analyses were conducted.

15 Serum FSH was assessed using a Milliplex kit (Millipore, MPTMAG-49K, custom-made  
16 for FSH only) following the manufacturer's instructions (lower detection limit: 23.7 pg/mL;  
17 dynamic range: 61.0 pg/mL to 250 000 pg/mL; limit of quantification [LOQ]: 61.0 pg/mL; intra-  
18 assay coefficient of variation [CV] < 15%). Serum LH was measured using an in-house  
19 sandwich ELISA (24) (lower detection limit: 0.117 ng/mL; dynamic range: 0.117 ng/mL to 30  
20 ng/mL; LOQ: 0.516 ng/mL; intra-assay CV < 10%) (25).

21

#### 22 RNA extraction and reverse transcription quantitative PCR (RT-qPCR)

23 RNA was extracted from tissues and L $\beta$ T2 cells using TRIzol Reagent (15596018;  
24 Invitrogen, Waltham, MA, USA) following the manufacturer's protocol. Two hundred ng total  
25 RNA (concentration determined using Nanodrop spectrophotometer) were reverse transcribed

1 using random hexamers (C1181, Promega) and M-MLV reverse transcriptase (M1701,  
2 Promega).

3 qPCR analysis was performed using EvaGreen (ABMMmix, Diamed, Mississauga, ON,  
4 CAN) and primers listed in Table 1 on a Corbett Rotorgene 600 instrument (Corbett Life  
5 Science, Sydney, NSW, AUS). mRNA levels were determined using the  $2^{-\Delta\Delta CT}$  method. Gene  
6 expression was normalized to ribosomal protein L19 (*Rpl19*). All primers were validated for  
7 efficiency and specificity.

8

### 9 Immunofluorescence

10 Eleven-week-old mice were perfused with 4% paraformaldehyde (PFA) in PBS and  
11 dissected pituitaries were post-fixed in 4% PFA (P6148, Millipore Sigma) at room temperature  
12 for 2 hours. Samples were washed in PBS, cryoprotected with 30% sucrose overnight at 4°C,  
13 and embedded in OCT (95057-838, VWR International, Radnor, PA, USA). Frozen tissue was  
14 sectioned at a thickness of 10  $\mu$ m using a Leica CM3050S cryostat, mounted on Fisherbrand  
15 Superfrost Plus slides (22-037-246, ThermoFisher Scientific), and stored at -80°C until use.  
16 Antigen retrieval was performed by incubating tissue in 0.01M citric acid-sodium citrate buffer  
17 (pH 6.0) with 0.05% Triton X-100 at 90-95°C for 30 min and cooled gradually until buffer  
18 temperature was between 30-40°C. Sections were washed with phosphate-buffered saline with  
19 0.1% Tween (PBST), blocked in 5% BSA (in PBST) for 1 h at room temperature, then incubated  
20 overnight at 4°C with an antibody against SF-1 (described above) (1:100, diluted in blocking  
21 solution). Slides were washed in PBST, incubated at room temperature with Alexa Fluor 647  
22 goat anti-rabbit (1:600; A27040, Invitrogen) for 1 h, washed in PBST, and mounted in Prolong  
23 Diamond Antifade Mountant with DAPI (P36966, Invitrogen). Fluorescent images were captured  
24 using a Leica SP8 confocal laser scanning microscope with a Leica HC PL CS2 63X/1.4 NA oil  
25 objective.

26

## 1 Single-cell analyses

2 Pituitaries were collected from 10- to 12-week-old control and cKO males, immediately  
3 snap frozen in liquid nitrogen, and stored at -80°C until analysis. Nuclei were isolated from  
4 individual pituitaries and single nucleus assay for transposase-accessible chromatin (snATAC-  
5 seq) was performed using the Single Cell ATAC reagent kit V1 (10x Genomics) as described  
6 (13). snATAC-seq data were processed using the Cell Ranger-ATAC pipeline version 1.2.0  
7 (10X Genomics). Samples processed in multiple wells were combined using the *aggr* function.  
8 Clustering was performed in *Signac* (26) following standard procedures, and clusters were  
9 annotated based on chromatin accessibility at the promoter of known pituitary cell type markers  
10 (13). We used *igvtools* 2.3.32 (27) to generate chromatin accessibility tracks for the  
11 gonadotropes using a window size of 400 base-pairs per cut-site. Tracks were normalized to  
12 both the number of gonadotropes and the median number of fragments per gonadotrope in  
13 each sample. snATAC-seq datasets can be found in the GEO data repository under the  
14 accession number GSE198907.

15 Human scRNAseq datasets were previously published (28). The processed dataset was  
16 downloaded from the GEO data repository under the accession number GSE142653. Cells  
17 were filtered and the dataset was scaled as previously described (28) and count thresholds  
18 were adjusted where necessary to account for background. The filtered and scaled gene  
19 expression dataset was analyzed using Seurat v.4.0.4 and standard procedures (29, 30). Cell  
20 types were assigned as previously published, and clustering was performed on the subset of  
21 endocrine cells (28).

## 22 RNAscope mRNA *in situ* hybridisation

23 Wild type CD1 mice (MGI: 5649524) were purchased from Charles River Laboratories.  
24 To analyse embryonic stages of pituitary gland development, females and males were time-  
25 mated for the generation of embryos. Midday of the day of vaginal plug was considered  
26

1 embryonic day 0.5 (E0.5). Adult pituitary glands were collected from 8-week-old CD-1 males. All  
2 animals were housed on a 12 h light: 12 h dark cycle and given access to food and water *ad*  
3 *libitum* under compliance of the Animals (Scientific Procedures) Act 1986 and KCL ethical  
4 review.

5 Dissected embryos and adult pituitaries were fixed in 10% neutral buffered formalin  
6 (NBF) at room temperature for 16-24 h. Samples were then washed in PBS and dehydrated  
7 through graded ethanol series before paraffin embedding as previously described (31). Embryo  
8 samples were sectioned along the sagittal plane for ages E9.5-16.5 and frontal plane for older  
9 embryos and adult pituitaries, at a thickness of 5  $\mu\text{m}$ .

10 The RNAscope 2.5 HD Duplex assay was used according to the manufacturer's  
11 recommendations, with a combination of the following probes; Mm-Tgfr3l-C1 (Cat# 1040221-  
12 C1), Mm-Nr5a1-C2 (Cat# 445731-C2), Mm-Fshb-C2 (Cat# 445351-C2), Mm-Lhb-C2 (Cat#  
13 478401-C2). All reagents were from Advanced Cell Diagnostics (Newark, CA, USA). All sections  
14 were counterstained with Mayer's hematoxylin (Vector H-3404) and mounted with Vectamount  
15 Permanent Mounting Medium (Vector H-5000).

16 For brightfield images, slides were scanned using an Olympus BX34F Brightfield  
17 microscope.

18

### 19 Statistical analysis

20 Luciferase assays in HEK93T and L $\beta$ T2 cells were analyzed by one-way or two-way  
21 ANOVA, followed by *post-hoc* Holm-Sidak multiple comparisons tests. Where indicated, effects  
22 of genotype between two groups were assessed by unpaired *t*-tests with Welch's correction.  
23 Statistical analyses were performed using Prism 9, GraphPad software. Alpha was set at  $p <$   
24 0.05.

25

## 1 Results

### 2 Two SF-1 binding sites are located in the proximal *Tgfb $\beta$ 3I* promoter

3 We mapped the *Tgfb $\beta$ 3I* transcription start site (TSS, +1) in murine pituitary RNA using 5'  
4 rapid amplification of cDNA ends (5' RACE). Sequencing of several clones indicated that  
5 transcription was initiated at one of two sites: either 29 base pairs (at +1) or 52 base pairs (at -  
6 23) upstream of the start codon (Fig. 1A). The 29 base pair untranslated region (UTR) was more  
7 common (8 clones out of 11) and was therefore used to define the TSS. Both experimentally  
8 determined UTRs differed from the computationally predicted TSS (at -40) in GenBank  
9 (NM\_001195258.1). We then analysed the proximal promoter region for potential transcription  
10 factor binding sites and identified two candidate SF-1 *cis*-elements at -146 to -138  
11 (CTGGCCTTG, site 1) and -67 to -59 (CAAGGCCAG, site 2). These two elements were reverse  
12 complements of each other and differed from the consensus SF-1 binding motif by one base  
13 pair (32, 33).

14 To assess SF-1 binding to these sequences, we performed DNA affinity purification  
15 assays (DNAP) using biotinylated double-stranded DNA probes corresponding to each  
16 candidate *cis*-element (Fig. 1B). To ascertain specific binding, we also used DNA probes with 2-  
17 bp mutations in the putative SF-1 *cis*-elements. Endogenous SF-1 protein from L $\beta$ T2 cells  
18 bound to wild-type (Fig. 1B, lanes 2 and 4) but not mutant probes (lanes 3 and 5) corresponding  
19 to both *cis*-elements. Though the two *cis*-elements were identical (reverse complements of each  
20 other), site 1 appeared to bind SF-1 more strongly than site 2. Finally, we performed chromatin  
21 immunoprecipitation in L $\beta$ T2 cells to assess SF-1 binding in the context of native chromatin.  
22 Compared to IgG, we observed enrichment of SF-1 in a region of the *Tgfb $\beta$ 3I* promoter  
23 containing the two *cis*-elements but not in a negative (gene desert) control (34) (Fig. 1C).

24

### 1 SF-1 activates murine *Tgfr3l* promoter-reporter activity

2 To examine *Tgfr3l* transcription, we ligated ~1 kb of the murine *Tgfr3l* 5' flanking  
3 sequence (-999/+1) upstream of luciferase in the pGL3-basic reporter plasmid. In heterologous  
4 HEK293T cells, ectopically expressed SF-1 activated *Tgfr3l* reporter activity (Fig. 1D).  
5 Mutations in either or both SF-1 sites blunted or blocked this stimulatory effect. In homologous  
6 murine L $\beta$ T2 gonadotrope-like cells, mutations in the SF-1 binding sites significantly decreased  
7 basal reporter activity relative to wild-type (Fig. 1E). Reporter activity was also significantly  
8 attenuated following SF-1 knockdown in L $\beta$ T2 cells using a previously validated siRNA against  
9 *Nr5a1* (Fig. 1F) (17).

10

### 11 SF-1 also regulates human *TGFBR3L* promoter-reporter activity

12 The TSS of human *TGFBR3L* was previously determined (11). Alignment of the murine  
13 *Tgfr3l* and human *TGFBR3L* promoters demonstrated a high degree of sequence  
14 conservation, including in the two SF-1 *cis*-elements (Fig. 1A). Using DNAP, we demonstrated  
15 SF-1 binding to both *cis*-elements in the human promoter (Fig. 2A, lanes 2 and 4). Mutations in  
16 critical bp in these sites blocked SF-1 binding (lanes 3 and 5). In HEK293T cells, SF-1  
17 overexpression induced human *TGFBR3L* promoter-reporter activity (Fig. 2B). The mutations  
18 that blocked SF-1 binding (Fig. 2A) also abrogated SF-1 induction of reporter activity (Fig. 2B).  
19 In L $\beta$ T2 cells, mutations in these *cis*-element also decreased basal promoter-reporter activity  
20 (Fig. 2C), as did siRNA-mediated knockdown of SF-1 (Fig. 2D).

21

### 22 *Tgfr3l* expression is SF-1-dependent *in vivo*

23 To determine whether SF-1 regulates *Tgfr3l* expression *in vivo*, we generated  
24 gonadotrope-specific *Nr5a1* knockout mice by crossing *Gnrhr*<sup>GRIC</sup> and *Nr5a1*<sup>fl/fl</sup> animals. Cre is  
25 expressed in gonadotropes and in the male germline with the GRIC Cre-driver line (19). We  
26 observed recombination of the floxed *Nr5a1* allele in pituitaries (both sexes, lanes 2 and 12) and



1 in testis (lane 14, Fig. 3A) (21). There was no evidence of recombination in control mice, which  
2 harbored floxed alleles but no Cre (Fig. 3A, lanes 6-9 and 16-19). Ablation of SF-1 protein (Fig.  
3 3B) and *Nr5a1* mRNA expression (Fig. 3C) was demonstrated by immunofluorescence and RT-  
4 qPCR on pituitaries of conditional knockouts (*Nr5a1<sup>fl/fl</sup>;Gnrhr<sup>GRIIC/+</sup>*, cKO) compared to controls  
5 (*Nr5a1<sup>fl/fl</sup>*).

6 SF-1 is required for gonadotropin synthesis and fertility *in vivo* (20, 35). Here, we  
7 observed profound impairments in gonadotropin subunits (*Lhb* and *Fshb*) and *Gnrhr*, but not  
8 *Cga*, mRNA levels in pituitaries of female and male cKOs (Fig. 3C). Serum LH and FSH levels  
9 were correspondingly reduced in cKO mice (Fig. 3D). Both female and male cKOs were infertile,  
10 with severely hypoplastic gonads and accessory sex organs (ovaries and uteri in females;  
11 testes and seminal vesicles in males; Fig. 3E-F). Collectively, the data demonstrate that loss of  
12 SF-1 expression and function was complete in our model.

13 Consistent with the *in vitro* reporter data (Fig. 1), pituitary *Tgfb3l* expression was  
14 abrogated in both female and male cKO mice (Fig. 4A). Next, we performed single-nucleus  
15 ATAC-sequencing on pituitaries of male control and cKO mice. In the controls, we identified  
16 open chromatin around the *Tgfb3l* promoter in gonadotropes (upper track in Fig. 4B). In  
17 contrast, the corresponding region was closed in cKO mice (lower track). The *Cga* promoter  
18 was open in gonadotropes of both genotypes (Fig. 4C), consistent with their equivalent  
19 expression of the gene (Fig. 3C).

20

#### 21 *Tgfb3l* precedes *Nr5a1* expression in the embryonic murine pituitary

22 Next, we examined *Tgfb3l* expression during murine pituitary development. Using  
23 mRNA *in situ* hybridization (RNAscope) on CD1 mouse embryos, we observed a low level of  
24 *Tgfb3l* expression as early as E13.5 (Fig. 5A). In contrast, *Nr5a1* mRNA was first detected at  
25 E15.5 (Fig. 5A). *Tgfb3l* co-localized with *Nr5a1* (Fig. 5A) and *Lhb* by E18.5 (Fig. 5B). *Tgfb3l*

1 co-localization with *Fshb* was not complete at E18.5 (Fig. 5C). In adult (8-week-old) males,  
2 *Tgfb3l* co-localized with *Nr5a1* (Fig. 5D), *Fshb* (Fig. 5E), and *Lhb* (Fig. 5F).

3 Finally, to gain insight into TGFBR3L regulation in human development, we analyzed a  
4 human embryonic pituitary single-cell RNA sequencing database (28) (Fig. 6A). *TGFBR3L* and  
5 *NR5A1* expression were enriched in gonadotropes 7-25 weeks post-fertilization (Fig. 6B and C).

## 7 Discussion

8 The novel inhibin B co-receptor, TGFBR3L, appears to be uniquely or principally  
9 expressed in pituitary gonadotropes (11, 13-15). Here, we demonstrate that the gonadotrope-  
10 specific transcription factor SF-1 regulates murine *Tgfb3l* expression, at least in part, via two  
11 cis-elements in the proximal promoter. The data also suggest that this mechanism is conserved  
12 in humans. Beyond binding to these elements, how SF-1 regulates *Tgfb3l* is not yet clear.  
13 However, the closed chromatin state of the *Tgfb3l* locus in gonadotropes of conditional  
14 knockout (cKO) mice suggests that SF-1 promotes chromatin accessibility through the  
15 recruitment of histone modifying enzymes (36-38).

16 Whereas SF-1 is necessary for *Tgfb3l* expression, it is likely not sufficient. Though  
17 restricted to gonadotropes among pituitary cell types, SF-1 is expressed in other tissues that do  
18 not express *Tgfb3l* (11), including the ventromedial hypothalamus, adrenal glands, and gonads  
19 (39). Within gonadotropes, SF-1 physically and functionally interacts with early growth response  
20 1 (EGR1) and *paired*-like homeodomain transcription factors to regulate gonadotrope-specific  
21 expression of *Lhb* (17). In contrast, SF-1 cooperates with LIM homeodomain proteins to  
22 regulate *Gnrhr* promoter activity (40). It is possible that SF-1 might interact with similar or  
23 distinct transcription factors to confer gonadotrope-specific *Tgfb3l* expression. Notably, there is  
24 a candidate EGR1 binding site between the two SF-1 *cis*-elements in the *Tgfb3l* promoter,  
25 which is the subject of our ongoing investigations.

1           Importantly, the dependence of *Tgfb3l* expression on SF-1 may be an emergent  
2 property. *Nr5a1* mRNA is reliably detected in developing murine pituitary on embryonic day  
3 14.5, but not at E13.5 (35). Using RNAscope, *Tgfb3l* mRNA was first detected at E13.5, when  
4 *Nr5a1* mRNA was absent. These data indicate that the initial expression of *Tgfb3l* is SF-1  
5 independent. Nevertheless, *Nr5a1* and *Tgfb3l* are co-expressed at least as early as E15.5 and  
6 continue to be thereafter. The loss of *Tgfb3l* mRNA in *Nr5a1* cKO mice demonstrates that, at  
7 least postnatally, the gene is uniquely/preferentially expressed in gonadotropes in an SF-1  
8 dependent manner. Though we have not yet established what initially drives *Tgfb3l* expression,  
9 this mechanism does not compensate for the loss of SF-1 in adulthood.

10           *NR5A1* and *TGFBR3L* are also co-expressed in human embryonic pituitary  
11 development. However, based on the available data, we cannot determine precisely when these  
12 transcripts first emerge relative to one another. Regardless, as in mouse, SF-1 and TGFBR3L  
13 are co-expressed in adult human gonadotropes, as well as in gonadotrope tumors (14, 28).

14           *Gnrhr* and the gonadotropin  $\beta$  subunits (*Lhb* and *Fshb*) are canonical markers of the  
15 gonadotrope lineage. The expression of all three depends on SF-1 (35). Recent single-cell and  
16 single-nucleus RNA-sequencing analyses of murine, rat, and human pituitaries similarly  
17 establish *Tgfb3l/TGFBR3L* as another gonadotrope-specific gene (13, 41, 42)}. It is therefore  
18 notable that its expression is also SF-1 dependent. These data demonstrate SF-1's role as a  
19 master regulator of gonadotrope identity. Nevertheless, as the expression of two of these  
20 genes, *Gnrhr* (21) and *Tgfb3l*, precedes *Nr5a1* developmentally, gonadotrope-lineage  
21 specification appears to be SF-1 independent. Indeed, treatment of *Nr5a1* knockout mice with  
22 exogenous GnRH is sufficient to increase gonadotropin production (43), indicating that  
23 gonadotropes are present even in the absence of SF-1. It will be interesting to determine  
24 whether GnRH treatment similarly induces *Tgfb3l*. Collectively, the data indicate that SF-1 is  
25 required for the full expression of the gonadotrope-specific transcriptome, but is not required for  
26 gonadotrope specification per se.

1 Finally, we should note that deciphering mechanisms controlling *Tgfb3l/TGFBR3L*  
2 expression may have translational relevance. Female *Tgfb3l* knockout mice have elevated FSH  
3 levels and enhanced fertility due to impaired inhibin B negative feedback (11). Therefore, either  
4 blocking inhibin B binding to TGFBR3L or reducing TGFBR3L expression would provide means  
5 to increase endogenous FSH levels. Such an outcome could be favorable in the context of  
6 assisted reproduction. Though SF-1 itself may not be an ideal therapeutic target, delineation of  
7 the *Tgfb3l/TGFBR3L* transcriptional regulatory machinery may uncover a more suitable and  
8 selective approach to decrease gene expression. The results reported here provide an  
9 important first step in this direction.

#### 11 **Data Availability Statement**

12 Most data are available in the manuscript. ATAC-seq data were deposited in GEO: GSE198907  
13

#### 14 **Author Contributions**

15 YFL and DJB were responsible for the experimental design, data analyses, and manuscript  
16 preparation. YFL and ERSB conducted the *in vitro* experiments. GS, HS, and YFL were  
17 responsible for tissue collection, mouse colony management, and analyses from the SF-1  
18 strain. TLW performed the RNAscope. UB provided the GRIC strain. NM isolated nuclei from  
19 individual pituitaries. FRZ performed the snATAC-seq experiments and MZ analyzed the  
20 snATAC-seq datasets. HS analyzed the human scRNA-seq datasets. DJB, CLA, and SCS  
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6

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## 1 References

- 2 1. Edson, M. A., Nagaraja, A. K., and Matzuk, M. M. (2009) The mammalian ovary from  
3 genesis to revelation. *Endocr Rev* **30**, 624-712
- 4 2. Simoni, M., Weinbauer, G. F., Gromoll, J., and Nieschlag, E. (1999) Role of FSH in male  
5 gonadal function. *Ann Endocrinol (Paris)* **60**, 102-106
- 6 3. Fortin, J., Ongaro, L., Li, Y., Tran, S., Lamba, P., Wang, Y., Zhou, X., and Bernard, D. J.  
7 (2015) Minireview: Activin Signaling in Gonadotropes: What Does the FOX say... to the  
8 SMAD? *Molecular Endocrinology* **29**, 963-977
- 9 4. Woodruff, T. K., D'Agostino, J., Schwartz, N. B., and Mayo, K. E. (1988) Dynamic  
10 changes in inhibin messenger RNAs in rat ovarian follicles during the reproductive cycle.  
11 *Science* **239**, 1296-1299
- 12 5. Woodruff, T. K., Meunier, H., Jones, P. B., Hsueh, A. J., and Mayo, K. E. (1987) Rat  
13 inhibin: molecular cloning of alpha- and beta-subunit complementary deoxyribonucleic  
14 acids and expression in the ovary. *Mol Endocrinol* **1**, 561-568
- 15 6. Davis, S. R., Krozowski, Z., McLachlan, R. I., and Burger, H. G. (1987) Inhibin gene  
16 expression in the human corpus luteum. *J Endocrinol* **115**, R21-23
- 17 7. Anawalt, B. D., Bebb, R. A., Matsumoto, A. M., Groome, N. P., Illingworth, P. J.,  
18 McNeilly, A. S., and Bremner, W. J. (1996) Serum inhibin B levels reflect Sertoli cell  
19 function in normal men and men with testicular dysfunction. *J Clin Endocrinol Metab* **81**,  
20 3341-3345
- 21 8. Lewis, K. A., Gray, P. C., Blount, A. L., MacConell, L. A., Wiater, E., Bilezikjian, L. M.,  
22 and Vale, W. (2000) Betaglycan binds inhibin and can mediate functional antagonism of  
23 activin signalling. *Nature* **404**, 411-414
- 24 9. Makanji, Y., Temple-Smith, P. D., Walton, K. L., Harrison, C. A., and Robertson, D. M.  
25 (2009) Inhibin B Is a More Potent Suppressor of Rat Follicle-Stimulating Hormone  
26 Release than Inhibin A in Vitro and in Vivo. *Endocrinology* **150**, 4784-4793

- 1 10. Li, Y., Fortin, J., Ongaro, L., Zhou, X., Boehm, U., Schneyer, A., Bernard, D. J., and Lin,  
2 H. Y. (2018) Betaglycan (TGFBR3) Functions as an Inhibin A, but Not Inhibin B,  
3 Coreceptor in Pituitary Gonadotrope Cells in Mice. *Endocrinology* **159**, 4077-4091
- 4 11. Brule, E., Wang, Y., Li, Y., Lin, Y. F., Zhou, X., Ongaro, L., Alonso, C. A. I., Buddle, E. R.  
5 S., Schneyer, A. L., Byeon, C. H., Hinck, C. S., Mendeleev, N., Russell, J. P., Cowan, M.,  
6 Boehm, U., Ruf-Zamojski, F., Zamojski, M., Andoniadou, C. L., Sealfon, S. C., Harrison,  
7 C. A., Walton, K. L., Hinck, A. P., and Bernard, D. J. (2021) TGFBR3L is an inhibin B co-  
8 receptor that regulates female fertility. *Sci Adv* **7**, eabl4391
- 9 12. Bilandzic, M., and Stenvers, K. L. (2011) Betaglycan: a multifunctional accessory. *Mol*  
10 *Cell Endocrinol* **339**, 180-189
- 11 13. Ruf-Zamojski, F., Zhang, Z., Zamojski, M., Smith, G. R., Mendeleev, N., Liu, H.,  
12 Nudelman, G., Moriwaki, M., Pincas, H., Castanon, R. G., Nair, V. D., Seenarine, N.,  
13 Amper, M. A. S., Zhou, X., Ongaro, L., Toufaily, C., Schang, G., Nery, J. R., Bartlett, A.,  
14 Aldridge, A., Jain, N., Childs, G. V., Troyanskaya, O. G., Ecker, J. R., Turgeon, J. L.,  
15 Welt, C. K., Bernard, D. J., and Sealfon, S. C. (2021) Single nucleus multi-omics  
16 regulatory landscape of the murine pituitary. *Nat Commun* **12**, 2677
- 17 14. Sjostedt, E., Kolnes, A. J., Olarescu, N. C., Mitsios, N., Hikmet, F., Sivertsson, A.,  
18 Lindskog, C., Oystese, K. A. B., Jorgensen, A. P., Bollerslev, J., and Casar-Borota, O.  
19 (2020) TGFBR3L-An Uncharacterised Pituitary Specific Membrane Protein Detected in  
20 the Gonadotroph Cells in Non-Neoplastic and Tumour Tissue. *Cancers (Basel)* **13**
- 21 15. The Human Protein Atlas.
- 22 16. Wang, Y., Fortin, J., Lamba, P., Bonomi, M., Persani, L., Roberson, M. S., and Bernard,  
23 D. J. (2008) Activator protein-1 and smad proteins synergistically regulate human follicle-  
24 stimulating hormone beta-promoter activity. *Endocrinology* **149**, 5577-5591

- 1 17. Fortin, J., Lamba, P., Wang, Y., and Bernard, D. J. (2009) Conservation of mechanisms  
2 mediating gonadotrophin-releasing hormone 1 stimulation of human luteinizing hormone  
3 beta subunit transcription. *Mol Hum Reprod* **15**, 77-87
- 4 18. Alarid, E. T., Windle, J. J., Whyte, D. B., and Mellon, P. L. (1996) Immortalization of  
5 pituitary cells at discrete stages of development by directed oncogenesis in transgenic  
6 mice. *Development* **122**, 3319-3329
- 7 19. Wen, S., Schwarz, J. R., Niculescu, D., Dinu, C., Bauer, C. K., Hirdes, W., and Boehm,  
8 U. (2008) Functional characterization of genetically labeled gonadotropes.  
9 *Endocrinology* **149**, 2701-2711
- 10 20. Zhao, L., Bakke, M., Krimkevich, Y., Cushman, L. J., Parlow, A. F., Camper, S. A., and  
11 Parker, K. L. (2001) Steroidogenic factor 1 (SF1) is essential for pituitary gonadotrope  
12 function. *Development* **128**, 147-154
- 13 21. Wen, S., Ai, W., Alim, Z., and Boehm, U. (2010) Embryonic gonadotropin-releasing  
14 hormone signaling is necessary for maturation of the male reproductive axis. *Proc Natl*  
15 *Acad Sci U S A* **107**, 16372-16377
- 16 22. Luo, X., Ikeda, Y., and Parker, K. L. (1994) A cell-specific nuclear receptor is essential  
17 for adrenal and gonadal development and sexual differentiation. *Cell* **77**, 481-490
- 18 23. Zhou, X., Wang, Y., Ongaro, L., Boehm, U., Kaartinen, V., Mishina, Y., and Bernard, D.  
19 J. (2016) Normal gonadotropin production and fertility in gonadotrope-specific Bmpr1a  
20 knockout mice. *J Endocrinol* **229**, 331-341
- 21 24. Steyn, F. J., Wan, Y., Clarkson, J., Veldhuis, J. D., Herbison, A. E., and Chen, C. (2013)  
22 Development of a methodology for and assessment of pulsatile luteinizing hormone  
23 secretion in juvenile and adult male mice. *Endocrinology* **154**, 4939-4945
- 24 25. Schang, G., Ongaro, L., Schultz, H., Wang, Y., Zhou, X., Brule, E., Boehm, U., Lee, S.  
25 J., and Bernard, D. J. (2020) Murine FSH Production Depends on the Activin Type II  
26 Receptors ACVR2A and ACVR2B. *Endocrinology* **161**



- 1 26. Stuart, T., Srivastava, A., Madad, S., Lareau, C. A., and Satija, R. (2021) Single-cell  
2 chromatin state analysis with Signac. *Nat Methods* **18**, 1333-1341
- 3 27. Thorvaldsdottir, H., Robinson, J. T., and Mesirov, J. P. (2013) Integrative Genomics  
4 Viewer (IGV): high-performance genomics data visualization and exploration. *Brief*  
5 *Bioinform* **14**, 178-192
- 6 28. Zhang, S., Cui, Y., Ma, X., Yong, J., Yan, L., Yang, M., Ren, J., Tang, F., Wen, L., and  
7 Qiao, J. (2020) Single-cell transcriptomics identifies divergent developmental lineage  
8 trajectories during human pituitary development. *Nat Commun* **11**, 5275
- 9 29. Butler, A., Hoffman, P., Smibert, P., Papalexi, E., and Satija, R. (2018) Integrating  
10 single-cell transcriptomic data across different conditions, technologies, and species. *Nat*  
11 *Biotechnol* **36**, 411-420
- 12 30. Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W. M., 3rd,  
13 Hao, Y., Stoeckius, M., Smibert, P., and Satija, R. (2019) Comprehensive Integration of  
14 Single-Cell Data. *Cell* **177**, 1888-1902 e1821
- 15 31. Russell, J. P., Lim, X., Santambrogio, A., Yianni, V., Kemkem, Y., Wang, B., Fish, M.,  
16 Haston, S., Grabek, A., Hallang, S., Lodge, E. J., Patist, A. L., Schedl, A., Mollard, P.,  
17 Nusse, R., and Andoniadou, C. L. (2021) Pituitary stem cells produce paracrine WNT  
18 signals to control the expansion of their descendant progenitor cells. *Elife* **10**
- 19 32. Doghman, M., Figueiredo, B. C., Volante, M., Papotti, M., and Lalli, E. (2013) Integrative  
20 analysis of SF-1 transcription factor dosage impact on genome-wide binding and gene  
21 expression regulation. *Nucleic Acids Res* **41**, 8896-8907
- 22 33. Keri, R. A., and Nilson, J. H. (1996) A steroidogenic factor-1 binding site is required for  
23 activity of the luteinizing hormone beta subunit promoter in gonadotropes of transgenic  
24 mice. *J Biol Chem* **271**, 10782-10785

- 1 34. Bohaczuk, S. C., Thackray, V. G., Shen, J., Skowronska-Krawczyk, D., and Mellon, P. L.  
2 (2021) FSHB Transcription is Regulated by a Novel 5' Distal Enhancer With a Fertility-  
3 Associated Single Nucleotide Polymorphism. *Endocrinology* **162**
- 4 35. Ingraham, H. A., Lala, D. S., Ikeda, Y., Luo, X., Shen, W. H., Nachtigal, M. W., Abbud,  
5 R., Nilson, J. H., and Parker, K. L. (1994) The nuclear receptor steroidogenic factor 1  
6 acts at multiple levels of the reproductive axis. *Genes Dev* **8**, 2302-2312
- 7 36. Harris, J., Gouhier, A., and Drouin, J. (2021) MECHANISMS IN ENDOCRINOLOGY:  
8 Pioneer transcription factors in pituitary development and tumorigenesis. *Eur J*  
9 *Endocrinol* **184**, R1-R15
- 10 37. Mayran, A., Sochodolsky, K., Khetchoumian, K., Harris, J., Gauthier, Y., Bemmo, A.,  
11 Balsalobre, A., and Drouin, J. (2019) Pioneer and nonpioneer factor cooperation drives  
12 lineage specific chromatin opening. *Nat Commun* **10**, 3807
- 13 38. Zaret, K. S., and Carroll, J. S. (2011) Pioneer transcription factors: establishing  
14 competence for gene expression. *Genes Dev* **25**, 2227-2241
- 15 39. Schimmer, B. P., and White, P. C. (2010) Minireview: steroidogenic factor 1: its roles in  
16 differentiation, development, and disease. *Mol Endocrinol* **24**, 1322-1337
- 17 40. Granger, A., Bleux, C., Kottler, M. L., Rhodes, S. J., Counis, R., and Laverriere, J. N.  
18 (2006) The LIM-homeodomain proteins Isl-1 and Lhx3 act with steroidogenic factor 1 to  
19 enhance gonadotrope-specific activity of the gonadotropin-releasing hormone receptor  
20 gene promoter. *Mol Endocrinol* **20**, 2093-2108
- 21 41. Fletcher, P. A., Smiljanic, K., Maso Prévise, R., Iben, J. R., Li, T., Rokic, M. B.,  
22 Sherman, A., Coon, S. L., and Stojilkovic, S. S. (2019) Cell Type- and Sex-Dependent  
23 Transcriptome Profiles of Rat Anterior Pituitary Cells. *Frontiers in Endocrinology* **10**
- 24 42. Zhang, Z., Zamojski, M., Smith, G. R., Willis, T. L., Yianni, V., Mendeleev, N., Pincas, H.,  
25 Seenarine, N., Amper, M. A. S., Vasoya, M., Cheng, W. S., Zaslavsky, E., Nair, V. D.,  
26 Turgeon, J. L., Bernard, D. J., Troyanskaya, O. G., Andoniadou, C. L., Sealfon, S. C.,

1 and Ruf-Zamojski, F. (2022) Single nucleus transcriptome and chromatin accessibility of  
2 postmortem human pituitaries reveal diverse stem cell regulatory mechanisms. *Cell Rep*  
3 **38**, 110467

4 43. Ikeda, Y., Luo, X., Abbud, R., Nilson, J. H., and Parker, K. L. (1995) The nuclear  
5 receptor steroidogenic factor 1 is essential for the formation of the ventromedial  
6 hypothalamic nucleus. *Mol Endocrinol* **9**, 478-486  
7

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## 1 **Figure Legends**

2

3 **Figure 1. SF-1 activates murine *Tgfb3l* transcription via two *cis*-elements in the proximal**  
 4 **promoter.** (A) Alignment of the murine and human *Tgfb3l/TGFBR3L* promoters. In both cases,  
 5 +1 refers to the transcription start site. The most common murine 5' untranslated region (5'  
 6 UTR, +1/+29) is boxed in blue and the human 5' UTR is boxed in green. The conserved SF-1  
 7 binding sites are boxed in red and labeled as site 1 and site 2. Mutated base pairs (in B, C, and  
 8 D) are indicated above in green. (B) DNAP using probes corresponding to the wild-type and  
 9 mutant murine SF-1 *cis*-elements. Whole cell protein lysates from L $\beta$ T2 cells (input) or proteins  
 10 interacting with the probes were analyzed via immunoblot (IB) using an SF-1 antibody. (C)  
 11 Chromatin immunoprecipitation for SF-1 of the indicated genomic regions in L $\beta$ T2 cells (n=4).  
 12 (D) HEK293T cells were transfected with 225 ng/well of the indicated murine -999/+1 *Tgfb3l-luc*  
 13 reporters as well as 3.125 ng/well of either pcDNA3.0 (empty expression vector) or SF-1  
 14 expression vector. WT, wild-type; site 1 mut, mutated SF-1 site 1; site 2 mut, mutated SF-1 site  
 15 2; double mut, both SF-1 sites mutated. (E) L $\beta$ T2 cells were transfected with 225 ng/well of the  
 16 indicated promoter-reporters. (F) L $\beta$ T2 cells were transfected with 225 ng/well of the -999/+1  
 17 *Tgfb3l-luc* reporter and 10 nM of control or *Nr5a1* siRNA. In D-F, lysates were collected and  
 18 reporter activity measured by luciferase assay. Data represent the mean of three or more  
 19 independent experiments performed in triplicate. Data were analyzed by two-way ANOVA  
 20 followed by Holm-Sidak multiple comparisons test in (D), one-way ANOVA followed by  
 21 Dunnett's multiple comparisons test in (E), and two-tailed unpaired t test with Welch's correction  
 22 in (C and F). ns, not significant; \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001; \*\*\*\*, p<0.0001.

23

24 **Figure 2. Mechanisms of human *TGFBR3L* transcriptional regulation by SF-1 are**  
 25 **conserved.** (A) DNAP was performed as in Fig. 1B, but with probes corresponding to the wild-  
 26 type and mutant human SF-1 *cis*-elements. (B) HEK293T cells were transfected with 225

1 ng/well of the indicated human -996/+1 *TGFBR3L*-luc reporters as well as 3.125 ng/ well of  
 2 either pcDNA3.0 (empty expression vector) or SF-1 expression vector. WT, wild-type; site 1  
 3 mut, mutated SF-1 site 1; site 2 mut, mutated SF-1 site 2; double mut, both sites mutated. (C)  
 4 L $\beta$ T2 cells were transfected with 225 ng/well of the indicated promoter-reporters. (D) L $\beta$ T2 cells  
 5 were transfected with 225 ng/well of the -996/+1 *TGFBR3L*-luc reporter and 10 nM of control or  
 6 *Nr5a1* siRNA. In B-D, luciferase assays were performed as in Fig. 1. Data represent the mean  
 7 of three independent experiments performed in triplicate and were analyzed by two-way ANOVA  
 8 followed by Holm-Sidak multiple comparisons test (B), one-way ANOVA followed by Dunnett's  
 9 multiple comparisons test (C), or by a two-tailed unpaired t test with Welch's correction (D). ns,  
 10 not significant; \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001; \*\*\*\*, p<0.0001.

11  
 12 **Figure 3. Gonadotrope-specific SF-1 knockout mice exhibit hypogonadotropic**  
 13 **hypogonadism.** (A) Genomic DNA was extracted from the indicated tissues  
 14 of *Nr5a1<sup>fl/fl</sup>;Gnrhr<sup>GRIC/+</sup>* (cKO; left panels) and *Nr5a1<sup>fl/fl</sup>* (control; right panels) mice and analyzed  
 15 by PCR for the presence of the floxed or recombined (rec) *Nr5a1* alleles. (B) Pituitary sections  
 16 from 11-week-old control and cKO mice were analyzed for SF-1 by immunofluorescence (red);  
 17 DAPI (blue) was used to stain nuclei. Scale bars: 50  $\mu$ m. (C) cDNA was prepared from total  
 18 RNA isolated from individual pituitary glands of 8-10-week-old control and cKO female (top) and  
 19 male (bottom) mice and analyzed by RT-qPCR for expression of *Lhb*, *Fshb*, *Cga*, *Gnrhr*, and  
 20 *Nr5a1*. (D) Serum LH and FSH levels in female (top) and male (bottom) control and cKO mice.  
 21 (E) Ovarian, uterine, testicular, and seminal vesicle weights and (F) representative images of  
 22 gonads and accessory sex organs from control and cKO females (top) and males (bottom).  
 23 Scale bars: 5 mm. Female data in all panels represent randomly cycling females. Data were  
 24 analyzed by two-tailed unpaired t tests with Welch's correlation. ns, not significant; \*, p<0.05; \*\*,  
 25 p<0.01; \*\*\*, p<0.001; \*\*\*\*, p<0.0001.

26

1 **Figure 4. SF-1 is required for *Tgfb3l* expression in adult murine pituitary glands.** (A)  
2 Pituitary cDNA from control and cKO female and male mice (described in Fig. 3) were analyzed  
3 for *Tgfb3l* expression by RT-qPCR. Data were analyzed by two-tailed unpaired t tests with  
4 Welch's correlation. \*\*\*,  $p < 0.001$ . Chromatin accessibility, as measured with single-nucleus  
5 ATAC-seq, over the (B) *Tgfb3l* and (C) *Cga* genes in gonadotropes of 10-12-week-old control  
6 (orange) and cKO (grey) males. Exon 1 and the promoter of *Tgfb3l* are boxed in red in panel B.  
7 Shown are representative tracks from a control and a cKO animal.

8  
9 **Figure 5. *Nr5a1* and *Tgfb3l* expression in fetal and adult murine pituitaries.** (A) mRNA *in*  
10 *situ* hybridization (RNAscope) for *Tgfb3l* (blue) and *Nr5a1* (red) on heads of wild-type CD1  
11 embryos at the indicated ages. Boxed regions in the top panels are magnified in the bottom  
12 panels. Scale bars: 50  $\mu\text{m}$ . Duplex RNAscope for *Tgfb3l* (blue) and (B) *Lhb* or (C) *Fshb* (red)  
13 on heads of CD1 embryos at E18.5. Scale bars: 200  $\mu\text{m}$  (top) and 50  $\mu\text{m}$  (bottom).. Duplex  
14 RNAscope for *Tgfb3l* (blue) and (D) *Nr5a1*, (E) *Fshb*, or (F) *Lhb* (red) on pituitaries of 8-week-  
15 old CD1 males. Scale bars: 50  $\mu\text{m}$ .

16  
17 **Figure 6. *TGFBR3L* and *NR5A1* expression is enriched in gonadotropes of human fetal**  
18 **pituitaries.** (A) UMAP plots of scRNAseq of 21 human embryonic pituitaries collected 7-25  
19 weeks post-fertilization. Different colored clusters represent different pituitary cell types. (B)  
20 Feature plots of *TGFBR3L* and *NR5A1* expression. (C) Violin plots of *TGFBR3L* and *NR5A1*  
21 expression in the defined cell lineages.

22

1 **Table 1: Primers**

2

<b>Murine <i>Tgfr3l</i> promoter amplification</b>	
5' RACE gene-specific RT primer	GGACGGACGAGGTATTGTGA
5' RACE gene-specific outer R	CCTGCGTCCGGTATTCAATG
5' RACE gene-specific inner R	GGGCGTGAAGAAGGTGTTAC
-999/+1 promoter amplification F	AAAACCTCGAGGTAGCTGATGCAACCATACGTAG
-999/+1 promoter amplification R	AAAAAAGCTTGACCGGCAGCGAGCACCT
<b>Human <i>Tgfr3l</i> promoter amplification</b>	
-996/+1 promoter amplification F	AAAACCTCGAGTAGGCGTAGCATCCCTCTC
-996/+1 promoter amplification R	TTTTAAGCTTGGACCAGCTGAGGTCGGA
<b><i>Tgfr3l</i>/<i>TGFR3L</i> mutagenesis</b>	
<i>Tgfr3l</i> SF-1 site 1 mut F	ATCTGAGCACATGCTCAATTCCAGCCATGGATAAGGGC
<i>Tgfr3l</i> SF-1 site 1 mut R	GCCCTTATCCATGGCTGGAATTGAGCATGTGCTCAGAT
<i>Tgfr3l</i> SF-1 site 2 mut F	GGTGGCAGCCTCACCAATTCCAGGGCTACC
<i>Tgfr3l</i> SF-1 site 2 mut R	GGTAGCCCTGGAATTGGTGAGGCTGCCACC
<i>TGFR3L</i> SF-1 site 1 mut F	CCAGAGCCAATGCCCAATTCTGAGGGGATTAAGGG
<i>TGFR3L</i> SF-1 site 1 mut R	CCCTTAATCCCTCAGGAATTGGGCATTGGCTCTGG
<i>TGFR3L</i> SF-1 site 2 mut F	GGCGGCCCTGTAATTGGCAAGGAGGGAGGCA
<i>TGFR3L</i> SF-1 site 2 mut R	TGCCTCCCTCCTTGCCAATTACAGGGCCGCC
<b>qPCR primers</b>	
<i>Fshb</i> F	GTGCGGGCTACTGCTACACT
<i>Fshb</i> R	CAGGCAATCTTACGGTCTCG
<i>Gnrhr</i> F	TTCGCTACCTCCTTTGTCGT
<i>Gnrhr</i> R	CACGGGTTTAGGAAAGCAA
<i>Lhb</i> F	ACTGTGCCGGCCTGTCAACG
<i>Lhb</i> R	AGCAGCCGGCAGTACTCGGA
<i>Nr5a1</i> F	AGGAGTTCGTCTGTCTCAAGTTCCT
<i>Nr5a1</i> R	ACAAGGTGTAATCCAACAGGGCAG
<i>Rpl19</i> F	CGGGAATCCAAGAAGATTGA
<i>Rpl19</i> R	TTCAGCTTGTGGATGTGCTC
<i>Tgfr3l</i> F	CCTGACACCAGTGCCTTTGA
<i>Tgfr3l</i> R	CTAGGGGACGGACGAGGTAT
<i>Tgfr3l</i> SF-1 ChIP qPCR F	TCAGTACATCAAGAAAGCCC
<i>Tgfr3l</i> SF-1 ChIP qPCR R	GTACCCAGCCCTCTAGGT
Gene desert qPCR F	GTCACAGAAACGCAAAGGTTTA
Gene desert qPCR R	CCCAAAGTCATGTTGACTTGATAG
<b>Genotyping primers</b>	
GRIC F	GGACATGTTTCAGGGATCGCCAGGC
GRIC R	GCATAACCAGTGAAACAGCATTGCTG
<i>Nr5a1</i> F	AGGAGTTCGTCTGTCTCAAGTTC
<i>Nr5a1</i> R	ACAAGGTGTAATCCAACAGGGCAG
<i>Nr5a1</i> R (recombinant)	TGCGTGCAATCCATCTTGTTCAAT

3

1

2 **Table 2: DNAP probes**

3

<i>Tgfr3l</i> SF-1 site 1 WT sense	TATCCATGGCTGGCCTTGAGCATGT
<i>Tgfr3l</i> SF-1 site 1 WT antisense	ACATGCTCAAGGCCAGCCATGGATA
<i>Tgfr3l</i> SF-1 site 1 Mut sense	TATCCATGGCTGGAATTGAGCATGT
<i>Tgfr3l</i> SF-1 site 1 Mut antisense	ACATGCTCAATTCCAGCCATGGATA
<i>Tgfr3l</i> SF-1 site 2 WT sense	AGCTCACCAAGGCCAGGGCTACCT
<i>Tgfr3l</i> SF-1 site 2 WT antisense	AGGTAGCCCTGGCCTTGGTGAGGCT
<i>Tgfr3l</i> SF-1 site 2 Mut sense	AGCTCACCAATTCCAGGGCTACCT
<i>Tgfr3l</i> SF-1 site 2 Mut antisense	AGGTAGCCCTGGAATTGGTGAGGCT
<i>TGFR3L</i> SF-1 site 1 WT sense	TAATCCCCTCAGGCCTTGGGCATTG
<i>TGFR3L</i> SF-1 site 1 WT antisense	CAATGCCCAAGGCCTGAGGGGATTA
<i>TGFR3L</i> SF-1 site 1 Mut sense	TAATCCCCTCAGGAATTGGGCATTG
<i>TGFR3L</i> SF-1 site 1 Mut antisense	CAATGCCCAATTCCTGAGGGGATTA
<i>TGFR3L</i> SF-1 site 2 WT sense	CTCCTTGCCAAGGACAGGGCCGCCT
<i>TGFR3L</i> SF-1 site 2 WT antisense	AGGCGGCCCTGTCCTTGGCAAGGAG
<i>TGFR3L</i> SF-1 site 2 Mut sense	CTCCTTGCCAATTACAGGGCCGCCT
<i>TGFR3L</i> SF-1 site 2 Mut antisense	AGGCGGCCCTGTAATTGGCAAGGAG

4

5



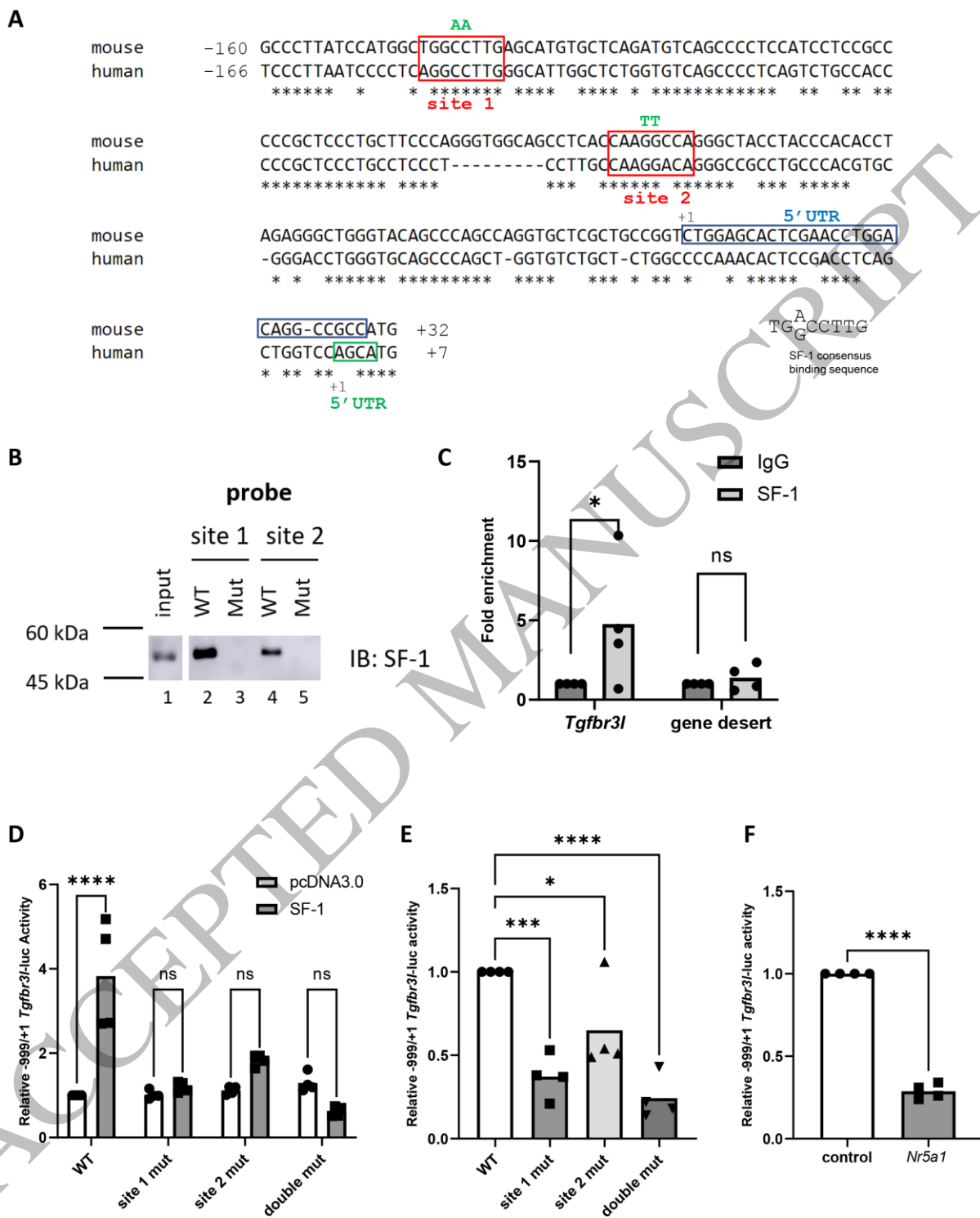
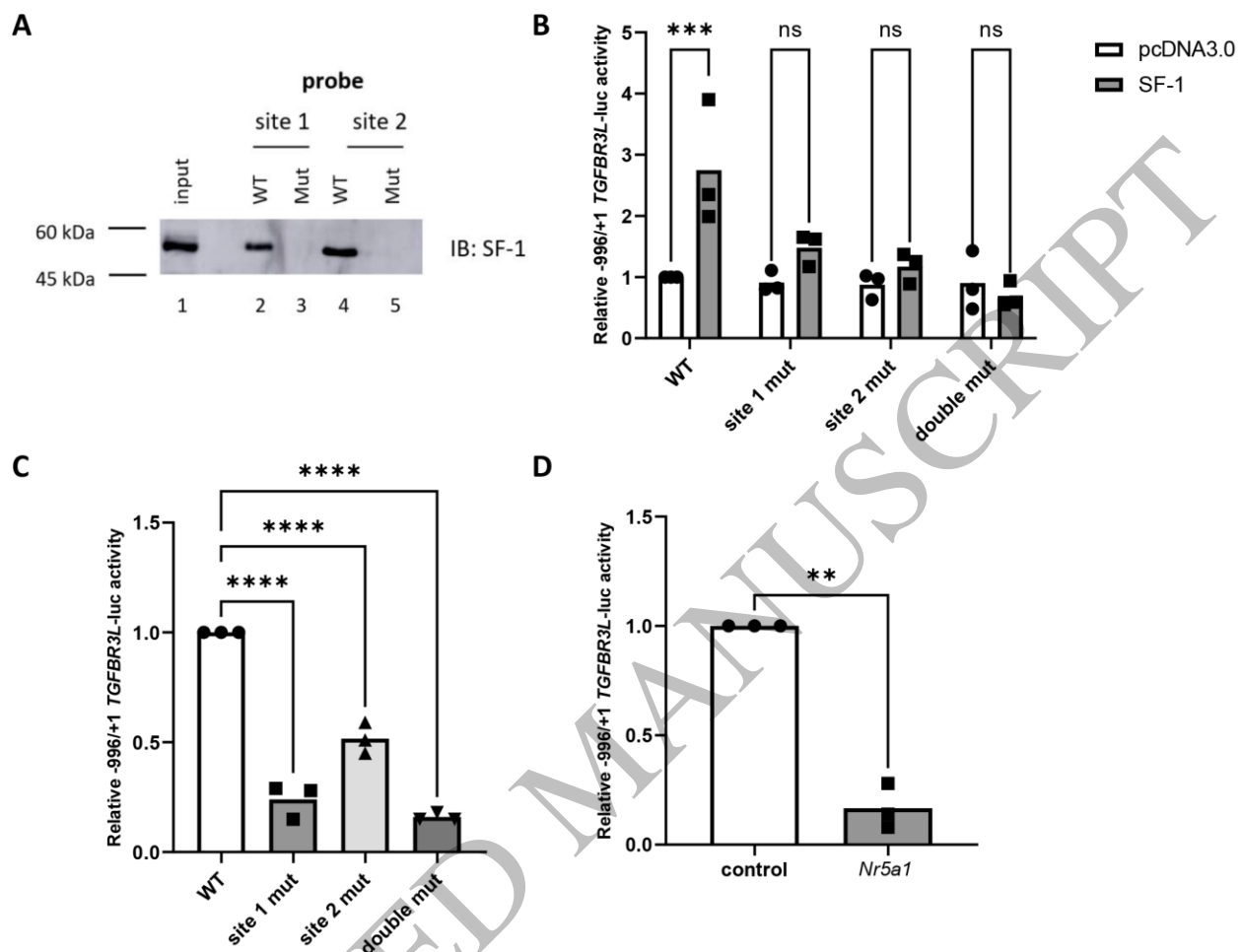


Figure 1

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2

165x220 mm (.42 x DPI)

1



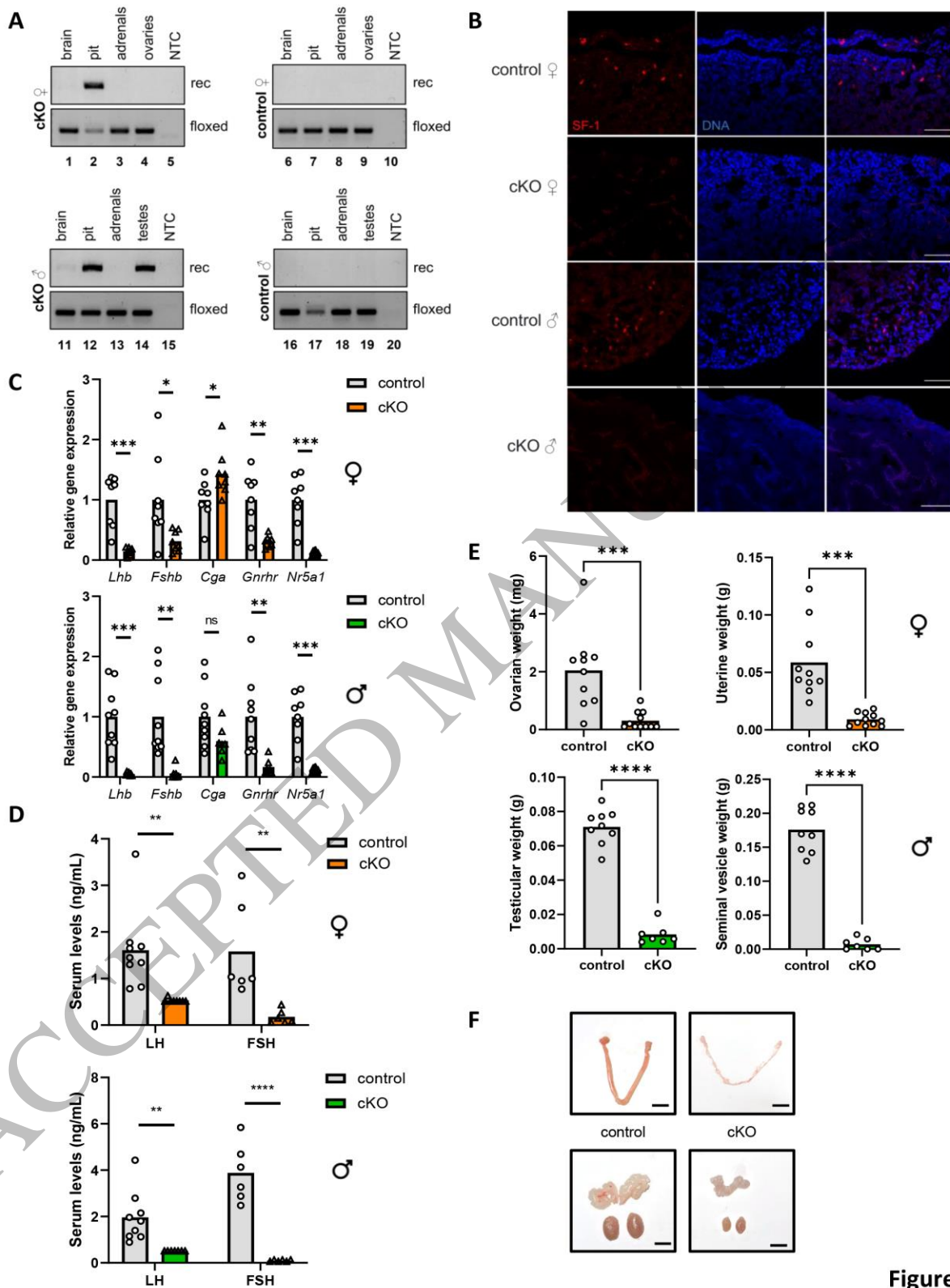
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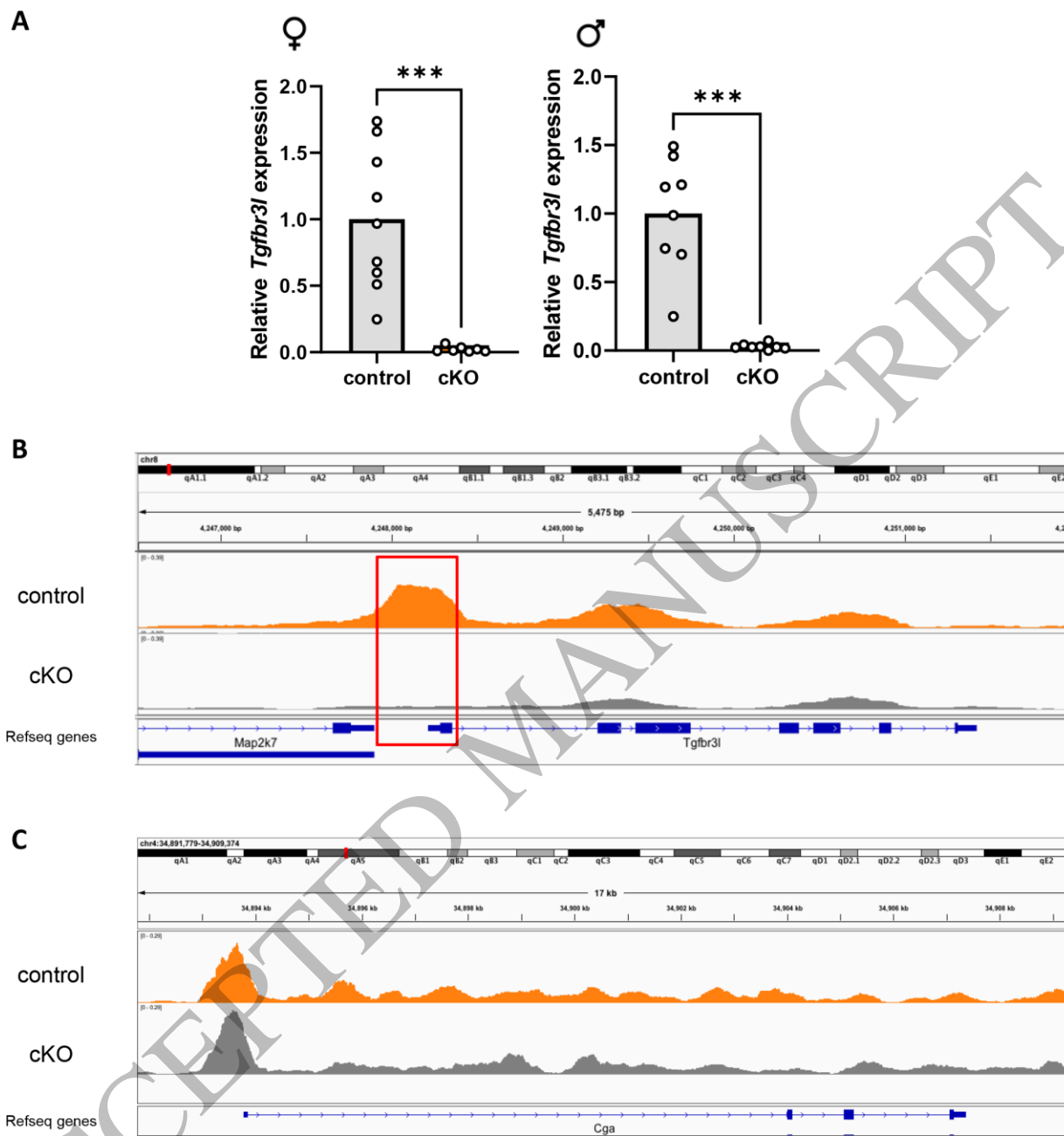
Figure 2



**Figure 3**

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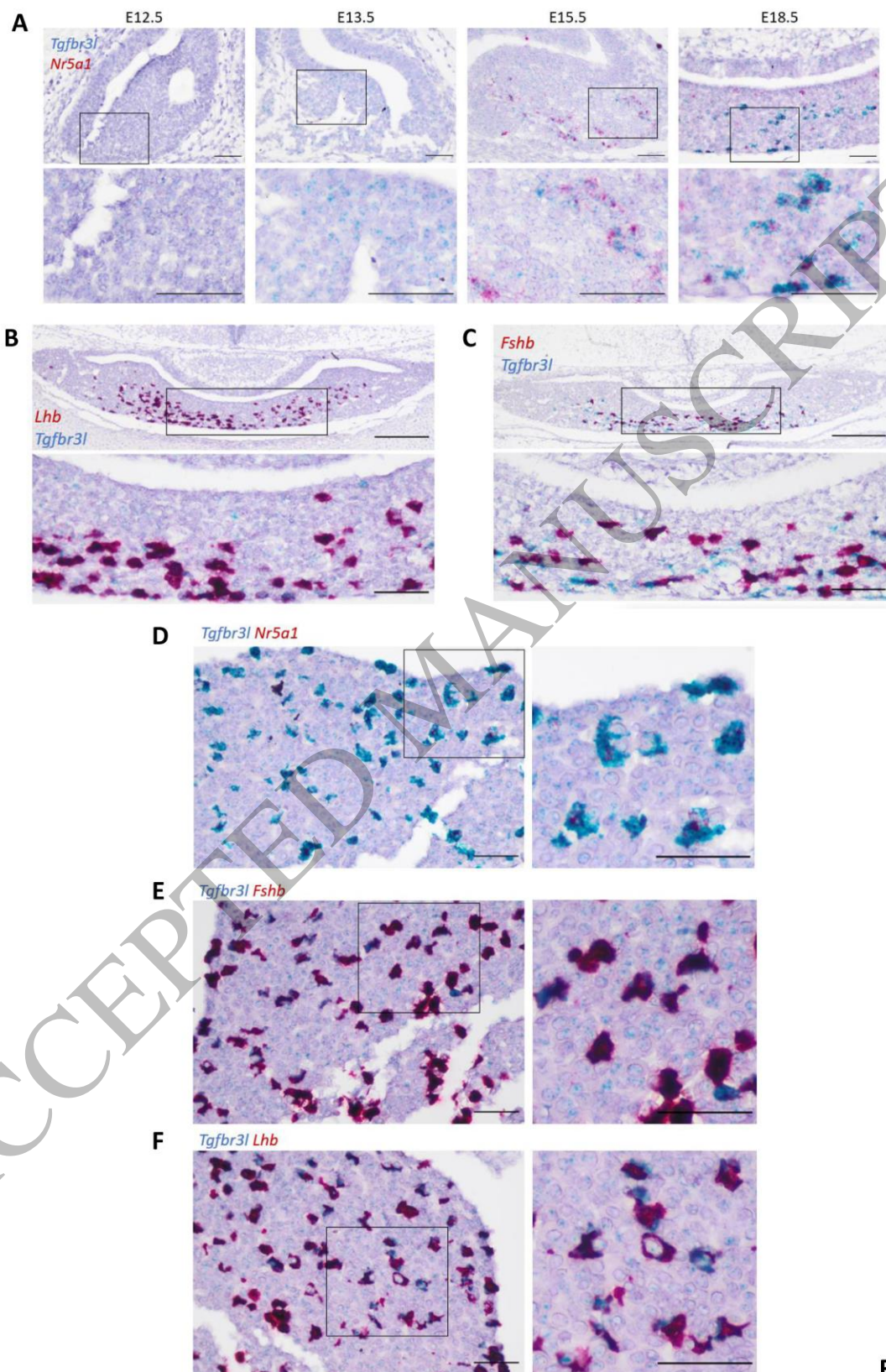
165x220 mm (.42 x DPI)



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**Figure 4**

165x206 mm (.42 x DPI)



**Figure 5**

1  
2

165x220 mm (.42 x DPI)

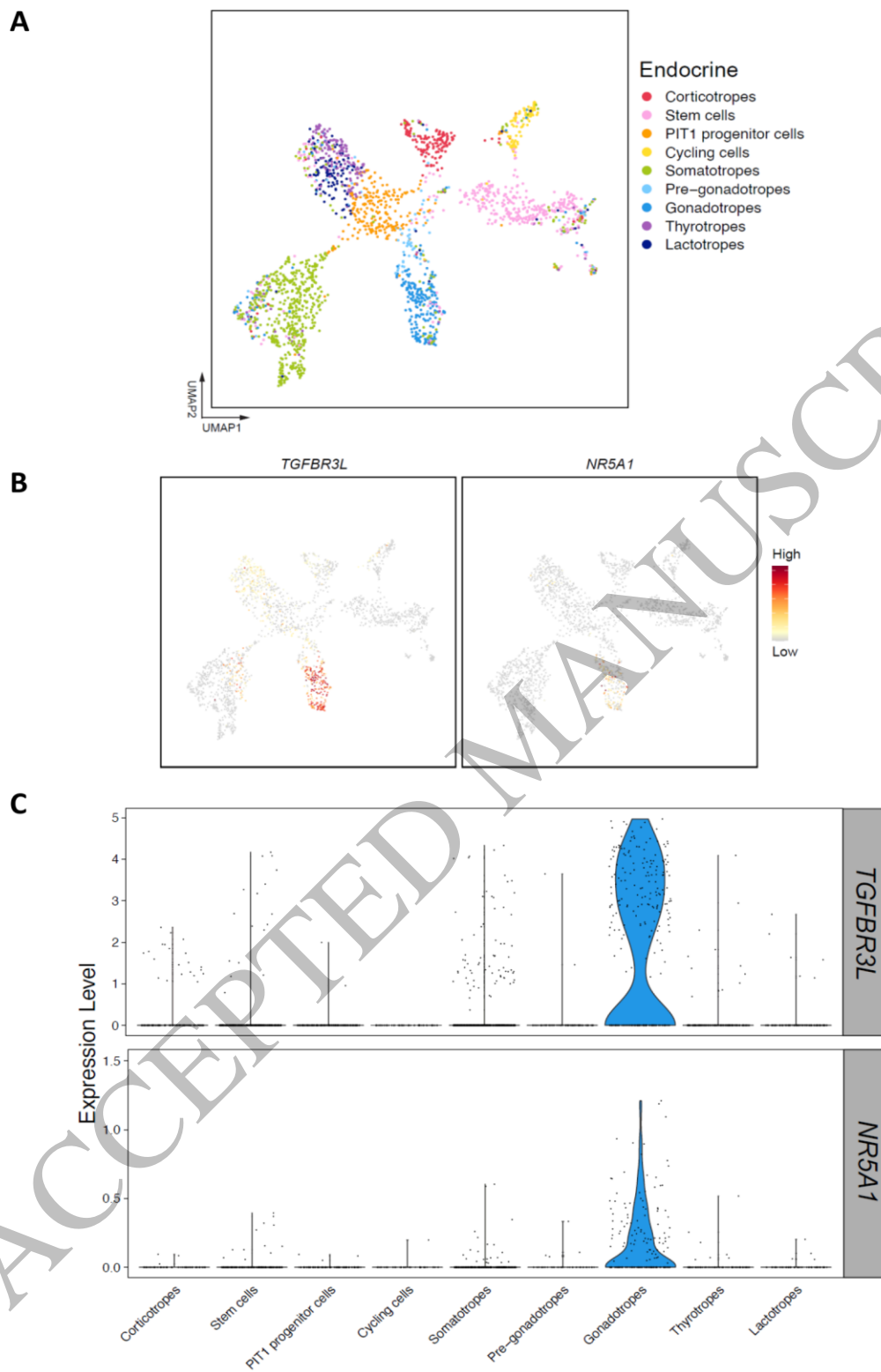


Figure 6

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162x229 mm (.42 x DPI)