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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

16 Short title: SF-1 regulates *Tgfbr3l* transcription

17

18 Key words: Transcription, inhibin, receptor, pituitary, knockout mouse, cell line

19

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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 *Tgfbr3l/TGFBR3L*
6 transcription. We identified two steroidogenic factor 1 (SF-1 or NR5A1) *cis*-elements in the
7 proximal *Tgfbr3l* promoter in mice. SF-1 induction of murine *Tgfbr3l* 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 *Tgfbr3l* gene was tightly compacted and *Tgfbr3l* mRNA expression was essentially absent
12 in gonadotropes of SF-1 (*Nr5a1*) conditional knockout mice. During murine embryonic
13 development, *Tgfbr3l* 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 *Tgfbr3l/TGFBR3L* transcription and is required for post-natal expression of the
16 gene in gonadotropes.

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Introduction

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

FSH also stimulates the synthesis and secretion of activin-related TGFβ ligands, known as the inhibins, from the gonads. The ovaries produce inhibins A and B (4-6), whereas the adult testes in most mammalian species produce inhibin B alone (7). Inhibins feedback to gonadotropes to suppress FSH production by competitively binding to activin receptors (8). Inhibin binding to these receptors is enhanced by transmembrane co-receptors. The TGFβ type III receptor (TGFBR3), also known as betaglycan, can mediate the actions of both inhibins (8, 9); however, conditional deletion of the *Tgfbr3* gene in gonadotropes principally impairs inhibin A, leaving inhibin B action intact (10). This observation led to the recent discovery of a betaglycan-like protein, TGFBR3L, which functions as a specific inhibin B co-receptor in gonadotropes (11).

Whereas betaglycan is broadly expressed, including in the pituitary, gonads, and adrenal glands (12), *Tgfbr3l/TGFBR3L* expression appears to be restricted to gonadotropes in adult mice and humans (11, 13-15). The mechanisms conferring this cell-specific expression have not been elucidated. Here, we report that the nuclear receptor, steroidogenic factor 1 (SF-1, product of the *Nr5a1* gene), regulates *Tgfbr3l/TGFBR3L* transcription via conserved regulatory elements in the proximal promoter. *In vivo*, SF-1 is necessary for the maintenance, but not the initial expression of *Tgfbr3l* in gonadotropes.

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1

2 **Materials and methods**

3

4 DNA constructs

5 The wild-type murine -999/+1 *Tgfbr3l* and human -996/+1 *TGFBR3L* luciferase

6 promoter-reporters were produced by PCR amplification of genomic DNA (see Table 1 for

7 primers). The PCR products were ligated into pGL3-Basic (Promega, Madison, WI, USA) or

8 pA3-luc (16). The murine SF-1 expression construct was described previously (17). The mutant

9 promoter-reporters were constructed using the QuikChange protocol with primers described in

10 Table 1. All constructs were confirmed by sequencing (Génome Québec, Montreal, QC, CAN).

11

12 5' Rapid Amplification of cDNA Ends (RACE)

13 5' RACE was performed using the FirstChoice RLM-RACE kit (AM1700, Invitrogen,

14 Waltham, MA, USA), following the manufacturer's protocol. Briefly, 10 µg of intact 5' capped

15 mRNA from the pituitary of a male C57BL6 mouse was decapped and ligated to the 5' RACE

16 adaptor. The ligated mRNA was reverse transcribed with MMLV-RT using a gene-specific

17 primer located in exon 4 (see Table 1). Nested PCR was performed on the resulting cDNA using

18 adaptor-specific forward primers and gene-specific reverse primers (see Table 1). PCR

19 products were ligated into the pGEM-T Easy vector (A1360, Promega) and sequenced

20 (Génome Québec, Montreal, QC, CAN).

21

22 Cell culture and promoter-reporter assays

23 All cells were cultured at 37°C in a humidified incubator with 5% CO_2. Human embryonic

24 kidney (HEK) 293T cells (ATCC CRL-3216; RRID: CVCL_0063; provided by Dr. Terry Hébert,

25 McGill University) were cultured in DMEM (319-005-CL, Wisent, St-Bruno, QC, CAN)

26 supplemented with 5% (v/v) fetal bovine serum (FBS; 098150, Wisent). Immortalized murine

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1 gonadotrope-like LβT2 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 LβT2 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 (µg DNA to µg PEI). LβT2 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 µl/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 µl of cell lysis supernatant was combined with 100 µl assay 16 buffer [15 mM potassium phosphate (pH 7.8), 25 mM glycylglycine, 15 mM $MgSO_4$, 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

22 DNA affinity purification assay and immunoblot

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

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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 $MgCl_2$], 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).

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Chromatin Immunoprecipitation

LβT2 cells were seeded at a density of 1.5 million cells/well in a 6-well plate. After three days, formaldehyde was added to a final concentration of 1%, and crosslinking was performed for 10 min at room temperature. The reaction was then quenched with 125 mM glycine for 5 min at room temperature. Cells were scraped and collected in 1 mL of ice-cold PBS and centrifuged at 800 x g for 10 minutes at 4°C. The cell pellet was then resuspended in 100 µl of Nuc101 EZ lysis buffer (NUC101, Millipore Sigma) with protease inhibitor cocktail (04693116001, Millipore Sigma) and 1 mM PMSF for 5 minutes on ice, then centrifuged at 500 x g for 5 minutes at 4°C. Supernatant was discarded and the pellet washed in Nuc101 EZ lysis buffer as above. The nuclei were then divided into three tubes for digestion.

Nuclei (in 50 µl of Nuc101 EZ lysis buffer) were mixed with 6 µl of 10x MNase buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 50% glycerol], 0.44 µl of 200 mM DTT, and 1 µl of diluted MNase (M0247S, New England Biolabs, Ipswich, MA, USA; diluted 1:10 in MNase reaction buffer [50 mM Tris-HCl (pH 7.9], 5 mM $CaCl_2$]) in a total reaction volume of 60 µl. Nuclei were digested for 10 minutes at 37°C and the digestion was stopped with 6.6 µl of 100 mM EDTA and 6.6 µL of 1% Triton X-100/1% sodium deoxycholate. Chromatin was incubated on ice for 20 minutes, then 220 µL of complete IP buffer [20 mM Tris-HCl (pH 8), 2 mM EDTA, 150 mM NaCl, 0.1% Triton X-100, 1x protease inhibitor cocktail, 1 mM PMSF] was added. Ten percent of the chromatin was removed and kept as "input". NaCl was added to the input chromatin to a final concentration of 0.2 M and protein:DNA complexes were reverse-cross-linked overnight at 65°C. The next day, samples were incubated for 30 min at 37°C with 10 µg RNase A in a total volume of 200 µL followed by 1 hour at 55°C with 10 µg proteinase K in a total volume of 200 µL. DNA was extracted with phenol:chloroform:isoamyl alcohol and precipitated with sodium acetate and ethanol overnight at -20°C. The DNA was pelleted at 15,000 rpm at 4°C for 20 min, washed with 70% ethanol, dried, dissolved in 30 µl of 10 mM Tris-HCl (pH 8.0), and quantified using Nanodrop.

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1 Ten µg of chromatin was precleared for 1 hour at 4°C with 10 µl 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 µl of beads (washed three times in complete IP buffer) were
6 incubated with 1 µg of antibody in a total volume of 200 µl for 4 hours at 4°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°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°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°C). Chromatin was eluted for 90 minutes at 65°C in 30
12 µl elution buffer (1 M $NaHCO_3$, 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.
16
17 <u>Generation of *Nr5a1* conditional knockout mice</u>
18 The *Nr5a1*$^{fl/fl}$ and *Gnrhr*$^{IRES\text{-}Cre/IRES\text{-}Cre}$ (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*$^{fl/fl}$ males (Jackson Laboratory, 007041)
21 (22) were crossed with GRIC females to produce *Nr5a1*$^{fl/+}$;*Gnrhr*$^{GRIC/+}$ progeny. *Nr5a1*$^{fl/fl}$ males
22 were then crossed with *Nr5a1*$^{fl/+}$;*Gnrhr*$^{GRIC/+}$ females to produce *Nr5a1*$^{fl/fl}$;*Gnrhr*$^{+/+}$ (control) and
23 *Nr5a1*$^{fl/fl}$;*Gnrhr*$^{GRIC/+}$ (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

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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 LβT2 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

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

3 qPCR analysis was performed using EvaGreen (ABMMmix, Diamed, Missisauga, 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 μm 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.

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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

23 RNAscope mRNA *in situ* hybridisation

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

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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 μm.

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-Tgfbr3l-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 LβT2 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.

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1 **Results**

2 Two SF-1 binding sites are located in the proximal *Tgfbr3l* promoter

3 We mapped the *Tgfbr3l* 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 LβT2 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 LβT2 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 *Tgfbr3l* promoter
23 containing the two cis-elements but not in a negative (gene desert) control (34) (Fig. 1C).

24

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1 SF-1 activates murine *Tgfbr3l* promoter-reporter activity

2 To examine *Tgfbr3l* transcription, we ligated ~1 kb of the murine *Tgfbr3l* 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 *Tgfbr3l* reporter activity (Fig. 1D).
5 Mutations in either or both SF-1 sites blunted or blocked this stimulatory effect. In homologous
6 murine LβT2 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 LβT2 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 *Tgfbr3l* 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 LβT2 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 *Tgfbr3l* expression is SF-1-dependent *in vivo*

23 To determine whether SF-1 regulates *Tgfbr3l* expression *in vivo*, we generated
24 gonadotrope-specific *Nr5a1* knockout mice by crossing $Gnrhr^{GRIC}$ and $Nr5a1^{fl/fl}$ 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

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

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

Consistent with the *in vitro* reporter data (Fig. 1), pituitary *Tgfbr3l* expression was abrogated in both female and male cKO mice (Fig. 4A). Next, we performed single-nucleus ATAC-sequencing on pituitaries of male control and cKO mice. In the controls, we identified open chromatin around the *Tgfbr3l* promoter in gonadotropes (upper track in Fig. 4B). In contrast, the corresponding region was closed in cKO mice (lower track). The *Cga* promoter was open in gonadotropes of both genotypes (Fig. 4C), consistent with their equivalent expression of the gene (Fig. 3C).

Tgfbr3l precedes *Nr5a1* expression in the embryonic murine pituitary

Next, we examined *Tgfbr3l* expression during murine pituitary development. Using mRNA *in situ* hybridization (RNAscope) on CD1 mouse embryos, we observed a low level of *Tgfbr3l* expression as early as E13.5 (Fig. 5A). In contrast, *Nr5a1* mRNA was first detected at E15.5 (Fig. 5A). *Tgfbr3l* co-localized with *Nr5a1* (Fig. 5A) and *Lhb* by E18.5 (Fig. 5B). *Tgfbr3l*

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co-localization with *Fshb* was not complete at E18.5 (Fig. 5C). In adult (8-week-old) males, *Tgfbr3l* co-localized with *Nr5a1* (Fig. 5D), *Fshb* (Fig. 5E), and *Lhb* (Fig. 5F).

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

Discussion

The novel inhibin B co-receptor, TGFBR3L, appears to be uniquely or principally expressed in pituitary gonadotropes (11, 13-15). Here, we demonstrate that the gonadotrope-specific transcription factor SF-1 regulates murine *Tgfbr3l* expression, at least in part, via two cis-elements in the proximal promoter. The data also suggest that this mechanism is conserved in humans. Beyond binding to these elements, how SF-1 regulates *Tgfbr3l* is not yet clear. However, the closed chromatin state of the *Tgfbr3l* locus in gonadotropes of conditional knockout (cKO) mice suggests that SF-1 promotes chromatin accessibility through the recruitment of histone modifying enzymes (36-38).

Whereas SF-1 is necessary for *Tgfbr3l* expression, it is likely not sufficient. Though restricted to gonadotropes among pituitary cell types, SF-1 is expressed in other tissues that do not express *Tgfbr3l* (11), including the ventromedial hypothalamus, adrenal glands, and gonads (39). Within gonadotropes, SF-1 physically and functionally interacts with early growth response 1 (EGR1) and *paired*-like homeodomain transcription factors to regulate gonadotrope-specific expression of *Lhb* (17). In contrast, SF-1 cooperates with LIM homeodomain proteins to regulate *Gnrhr* promoter activity (40). It is possible that SF-1 might interact with similar or distinct transcription factors to confer gonadotrope-specific *Tgfbr3l* expression. Notably, there is a candidate EGR1 binding site between the two SF-1 *cis*-elements in the *Tgfbr3l* promoter, which is the subject of our ongoing investigations.

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Importantly, the dependence of *Tgfbr3l* expression on SF-1 may be an emergent property. *Nr5a1* mRNA is reliably detected in developing murine pituitary on embryonic day 14.5, but not at E13.5 (35). Using RNAscope, *Tgfbr3l* mRNA was first detected at E13.5, when *Nr5a1* mRNA was absent. These data indicate that the initial expression of *Tgfbr3l* is SF-1 independent. Nevertheless, *Nr5a1* and *Tgfbr3l* are co-expressed at least as early as E15.5 and continue to be thereafter. The loss of *Tgfbr3l* mRNA in *Nr5a1* cKO mice demonstrates that, at least postnatally, the gene is uniquely/preferentially expressed in gonadotropes in an SF-1 dependent manner. Though we have not yet established what initially drives *Tgfbr3l* expression, this mechanism does not compensate for the loss of SF-1 in adulthood.

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

Gnrhr and the gonadotropin β subunits (*Lhb* and *Fshb*) are canonical markers of the gonadotrope lineage. The expression of all three depends on SF-1 (35). Recent single-cell and single-nucleus RNA-sequencing analyses of murine, rat, and human pituitaries similarly establish *Tgfbr3l/TGFBR3L* as another gonadotrope-specific gene (13, 41, 42)}. It is therefore notable that its expression is also SF-1 dependent. These data demonstrate SF-1's role as a master regulator of gonadotrope identity. Nevertheless, as the expression of two of these genes, *Gnrhr* (21) and *Tgfbr3l*, precedes *Nr5a1* developmentally, gonadotrope-lineage specification appears to be SF-1 independent. Indeed, treatment of *Nr5a1* knockout mice with exogenous GnRH is sufficient to increase gonadotropin production (43), indicating that gonadotropes are present even in the absence of SF-1. It will be interesting to determine whether GnRH treatment similarly induces *Tgfbr3l*. Collectively, the data indicate that SF-1 is required for the full expression of the gonadotrope-specific transcriptome, but is not required for gonadotrope specification per se.

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1 Finally, we should note that deciphering mechanisms controlling *Tgfbr3l/TGFBR3L*
2 expression may have translational relevance. Female *Tgfbr3l* 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 *Tgfbr3l/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.
10
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
21 secured the grant funding for the research. All authors approved the final version of the
22 manuscript.
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1 **Figure Legends**

2

3 **Figure 1. SF-1 activates murine *Tgfbr3l* transcription via two *cis*-elements in the proximal**
4 **promoter.** (A) Alignment of the murine and human *Tgfbr3l/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 LβT2 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 LβT2 cells (n=4).
12 (D) HEK293T cells were transfected with 225 ng/well of the indicated murine -999/+1 *Tgfbr3l*-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) LβT2 cells were transfected with 225 ng/well of the
16 indicated promoter-reporters. (F) LβT2 cells were transfected with 225 ng/well of the -999/+1
17 *Tgfbfr3l*-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

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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 LβT2 cells were transfected with 225 ng/well of the indicated promoter-reporters. (D) LβT2 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 $Nr5a1^{fl/fl}$;$Gnrhr^{GRIC/+}$ (cKO; left panels) and $Nr5a1^{fl/fl}$ (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 µm. (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

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1 **Figure 4. SF-1 is required for *Tgfbr3l* 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 *Tgfbr3l* 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) *Tgfbr3l* and (C) *Cga* genes in gonadotropes of 10-12-week-old control
6 (orange) and cKO (grey) males. Exon 1 and the promoter of *Tgfbr3l* 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 *Tgfbr3l* expression in fetal and adult murine pituitaries.** (A) mRNA *in*
10 *situ* hybridization (RNAscope) for *Tgfbr3l* (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 µm. Duplex RNAscope for *Tgfbr3l* (blue) and (B) *Lhb* or (C) *Fshb* (red)
13 on heads of CD1 embryos at E18.5. Scale bars: 200 µm (top) and 50 µm (bottom).. Duplex
14 RNAscope for *Tgfbr3l* (blue) and (D) *Nr5a1*, (E) *Fshb*, or (F) *Lhb* (red) on pituitaries of 8-week-
15 old CD1 males. Scale bars: 50 µm.
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

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1 **Table 1: Primers**

2

Murine *Tgfbr3l* promoter amplification	
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	AAAACTCGAGGTAGCTGATGCAACCATACGTAG
-999/+1 promoter amplification R	AAAAAAGCTTGACCGGCAGCGAGCACCT
Human *Tgfbr3l* promoter amplification	
-996/+1 promoter amplification F	AAAACTCGAGTAGGCGTAGCATCCCTCTC
-996/+1 promoter amplification R	TTTTAAGCTTGGACCAGCTGAGGTCGGA
***Tgfbr3l/TGFBR3L* mutagenesis**	
Tgfbr3l SF-1 site 1 mut F	ATCTGAGCACATGCTCAATTCCAGCCATGGATAAGGGC
Tgfbr3l SF-1 site 1 mut R	GCCCTTATCCATGGCTGGAATTGAGCATGTGCTCAGAT
Tgfbr3l SF-1 site 2 mut F	GGTGGCAGCCTCACCAATTCCAGGGCTACC
Tgfbr3l SF-1 site 2 mut R	GGTAGCCCTGGAATTGGTGAGGCTGCCACC
TGFBR3L SF-1 site 1 mut F	CCAGAGCCAATGCCCAATTCCTGAGGGGATTAAGGG
TGFBR3L SF-1 site 1 mut R	CCCTTAATCCCCTCAGGAATTGGGCATTGGCTCTGG
TGFBR3L SF-1 site 2 mut F	GGCGGCCCTGTAATTGGCAAGGAGGGAGGCA
TGFBR3L SF-1 site 2 mut R	TGCCTCCCTCCTTGCCAATTACAGGGCCGCC
qPCR primers	
Fshb F	GTGCGGGCTACTGCTACACT
Fshb R	CAGGCAATCTTACGGTCTCG
Gnrhr F	TTCGCTACCTCCTTTGTCGT
Gnrhr R	CACGGGTTTAGGAAAGCAAA
Lhb F	ACTGTGCCGGCCTGTCAACG
Lhb R	AGCAGCCGGCAGTACTCGGA
Nr5a1 F	AGGAGTTCGTCTGTCTCAAGTTCCT
Nr5a1 R	ACAAGGTGTAATCCAACAGGGCAG
Rpl19 F	CGGGAATCCAAGAAGATTGA
Rpl19 R	TTCAGCTTGTGGATGTGCTC
Tgfbr3l F	CCTGACACCAGTGCCTTTGA
Tgfbr3l R	CTAGGGGACGGACGAGGTAT
Tgfbr3l SF-1 ChIP qPCR F	TCAGTACATCAAGAAAGCCC
Tgfbr3l SF-1 ChIP qPCR R	GTACCCAGCCCTCTAGGT
Gene desert qPCR F	GTCACAGAAACGCAAAGGTTTA
Gene desert qPCR R	CCCAAAGTCATGTTGTACTTGATAG
Genotyping primers	
GRIC F	GGACATGTTCAGGGATCGCCAGGC
GRIC R	GCATAACCAGTGAAACAGCATTGCTG
Nr5a1 F	AGGAGTTCGTCTGTCTCAAGTTCC
Nr5a1 R	ACAAGGTGTAATCCAACAGGGCAG
Nr5a1 R (recombinant)	TGCGTGCAATCCATCTTGTTCAAT

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Table 2: DNAP probes

Tgfbr3l SF-1 site 1 WT sense	TATCCATGGCTGGCCTTGAGCATGT
Tgfbr3l SF-1 site 1 WT antisense	ACATGCTCAAGGCCAGCCATGGATA
Tgfbr3l SF-1 site 1 Mut sense	TATCCATGGCTGGAATTGAGCATGT
Tgfbr3l SF-1 site 1 Mut antisense	ACATGCTCAATTCCAGCCATGGATA
Tgfbr3l SF-1 site 2 WT sense	AGCCTCACCAAGGCCAGGGCTACCT
Tgfbr3l SF-1 site 2 WT antisense	AGGTAGCCCTGGCCTTGGTGAGGCT
Tgfbr3l SF-1 site 2 Mut sense	AGCCTCACCAATTCCAGGGCTACCT
Tgfbr3l SF-1 site 2 Mut antisense	AGGTAGCCCTGGAATTGGTGAGGCT
TGFBR3L SF-1 site 1 WT sense	TAATCCCCTCAGGCCTTGGGCATTG
TGFBR3L SF-1 site 1 WT antisense	CAATGCCCAAGGCCTGAGGGGATTA
TGFBR3L SF-1 site 1 Mut sense	TAATCCCCTCAGGAATTGGGCATTG
TGFBR3L SF-1 site 1 Mut antisense	CAATGCCCAATTCCTGAGGGGATTA
TGFBR3L SF-1 site 2 WT sense	CTCCTTGCCAAGGACAGGGCCGCCT
TGFBR3L SF-1 site 2 WT antisense	AGGCGGCCCTGTCCTTGGCAAGGAG
TGFBR3L SF-1 site 2 Mut sense	CTCCTTGCCAATTACAGGGCCGCCT
TGFBR3L SF-1 site 2 Mut antisense	AGGCGGCCCTGTAATTGGCAAGGAG

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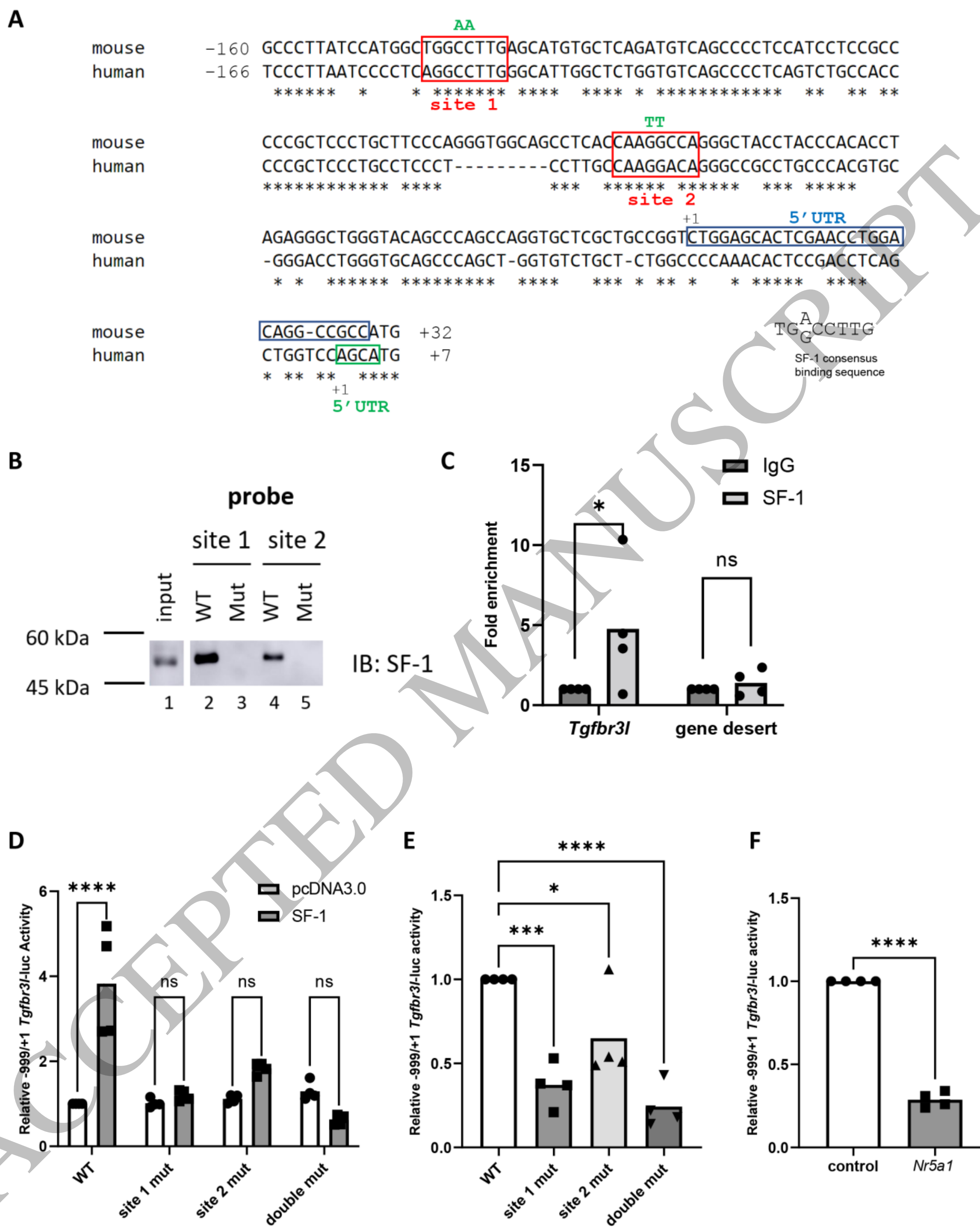

Figure 1

1

2 165x220 mm (.42 x DPI)

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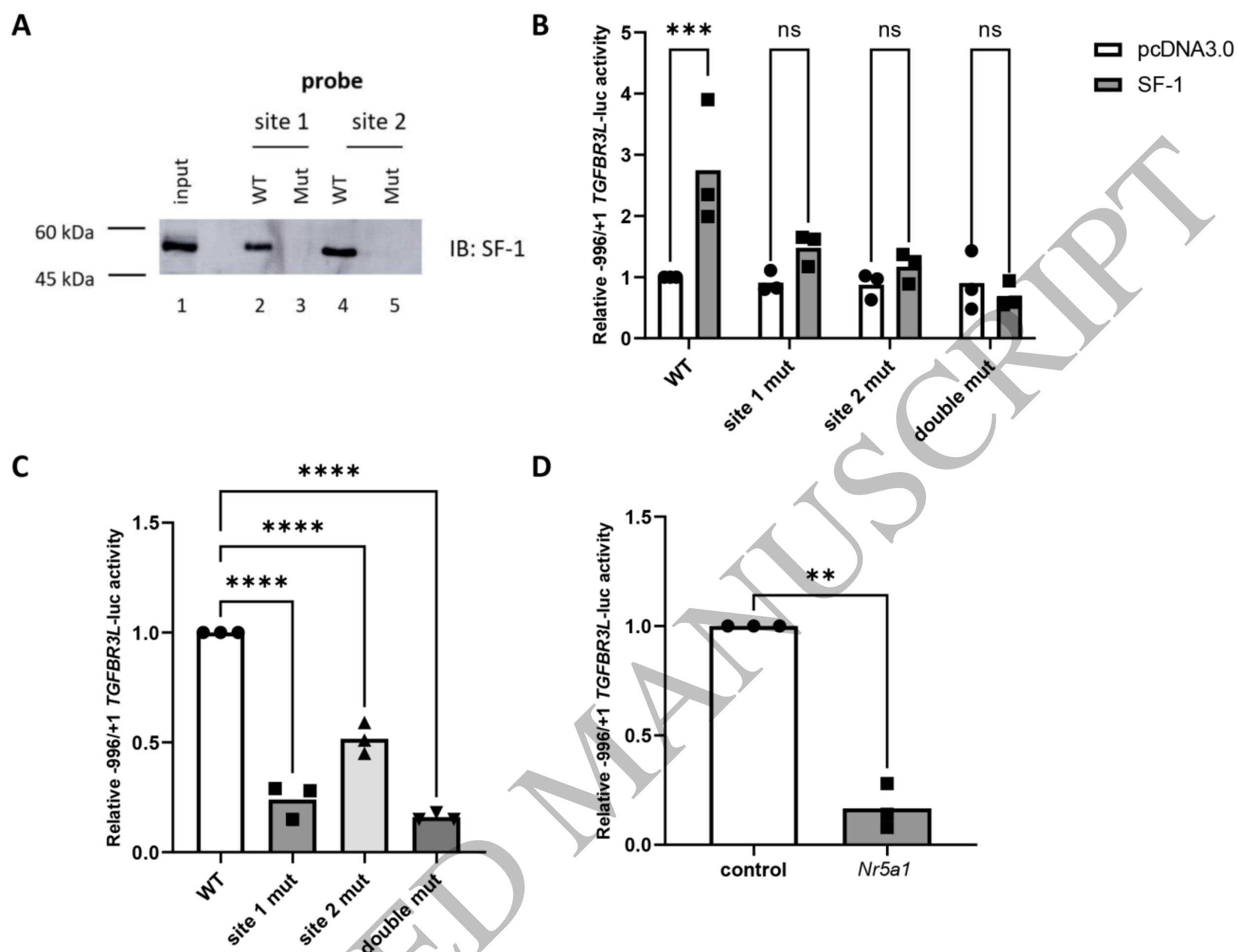

2 **Figure 2**

3 **165x191 mm (.42 x DPI)**

4

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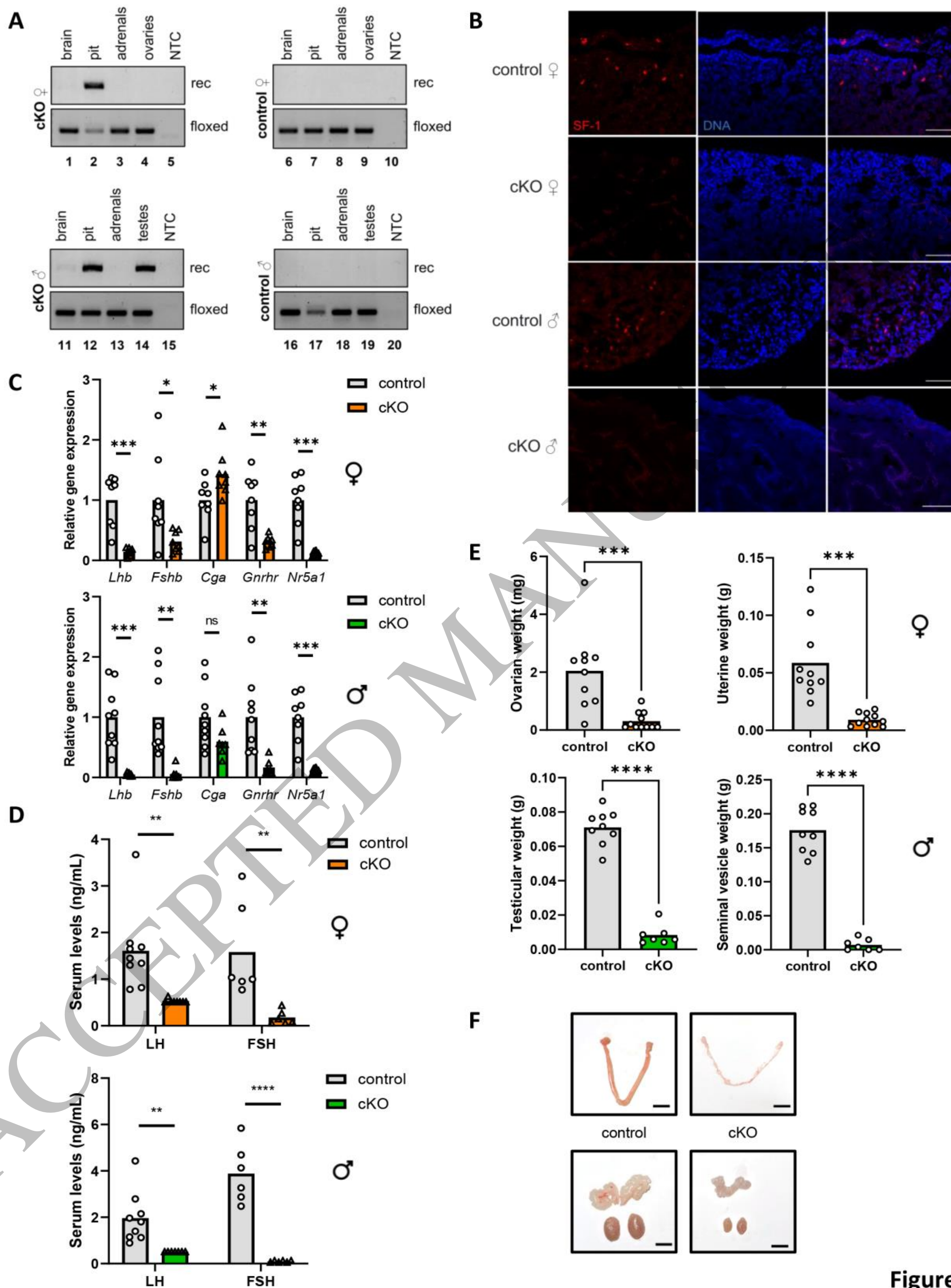

Figure 3

1

2 165x220 mm (.42 x DPI)

3

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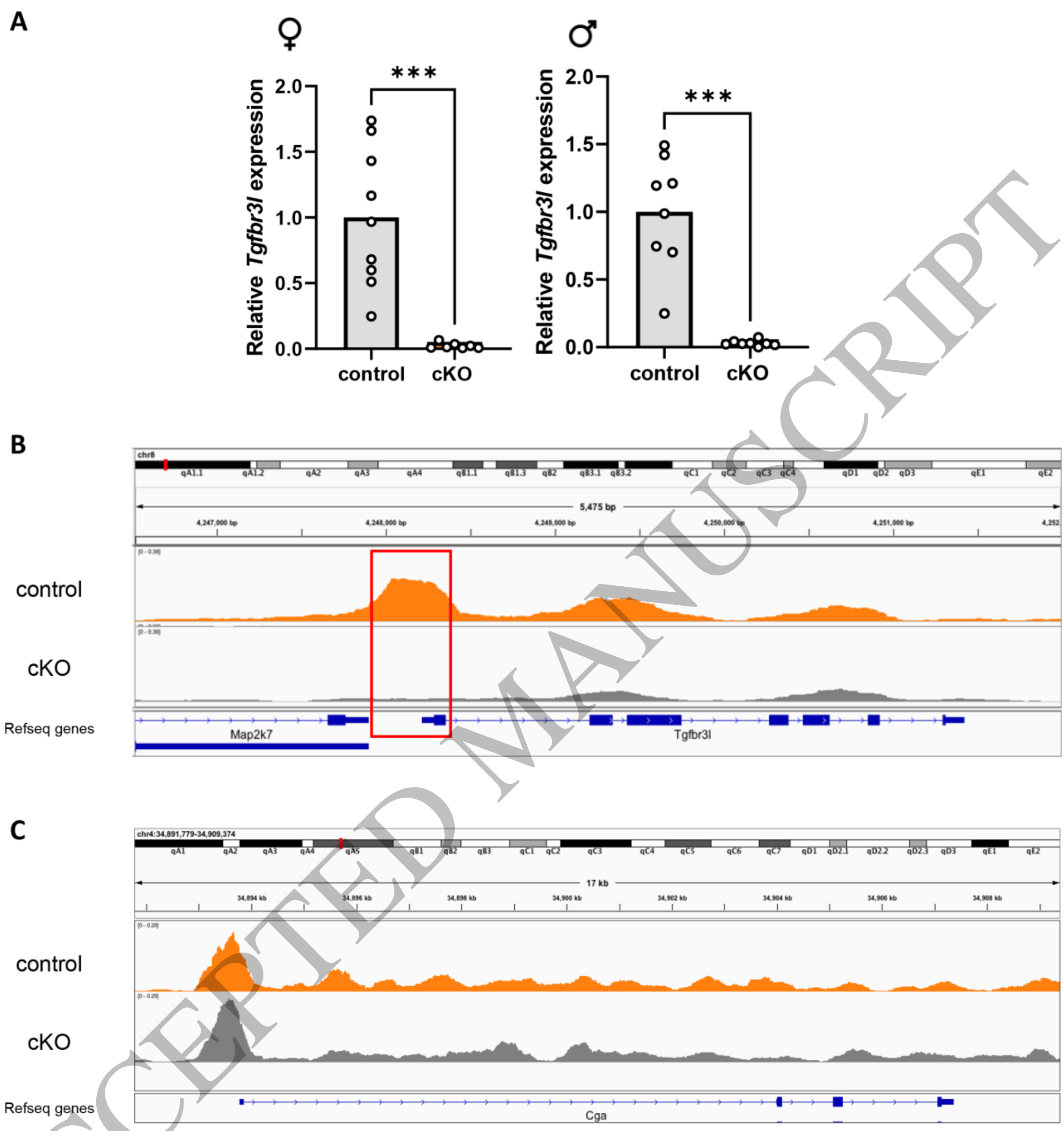

1 Figure 4

2 165x206 mm (.42 x DPI)

3

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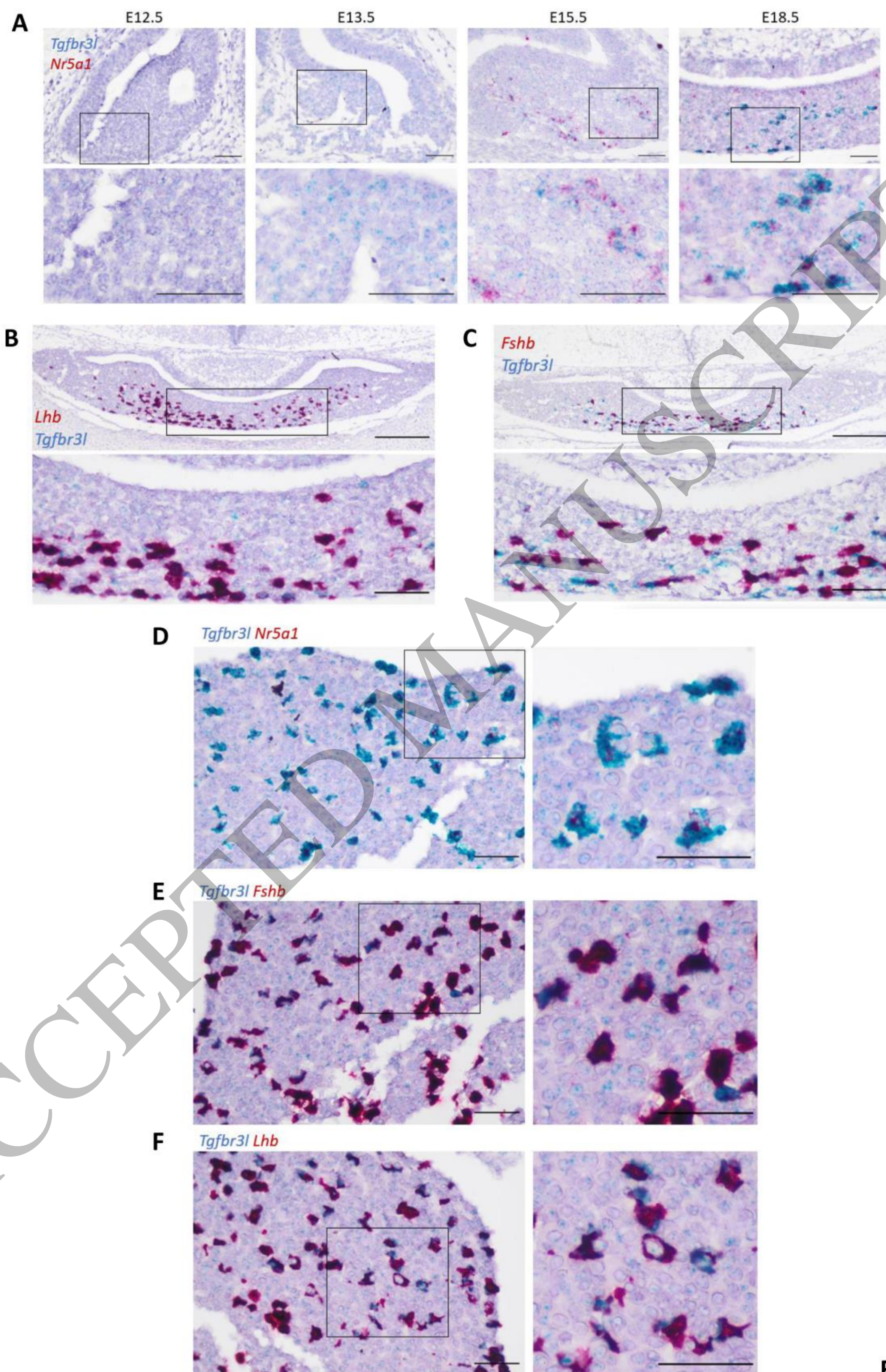

1

2 **165x220 mm (.42 x DPI)**

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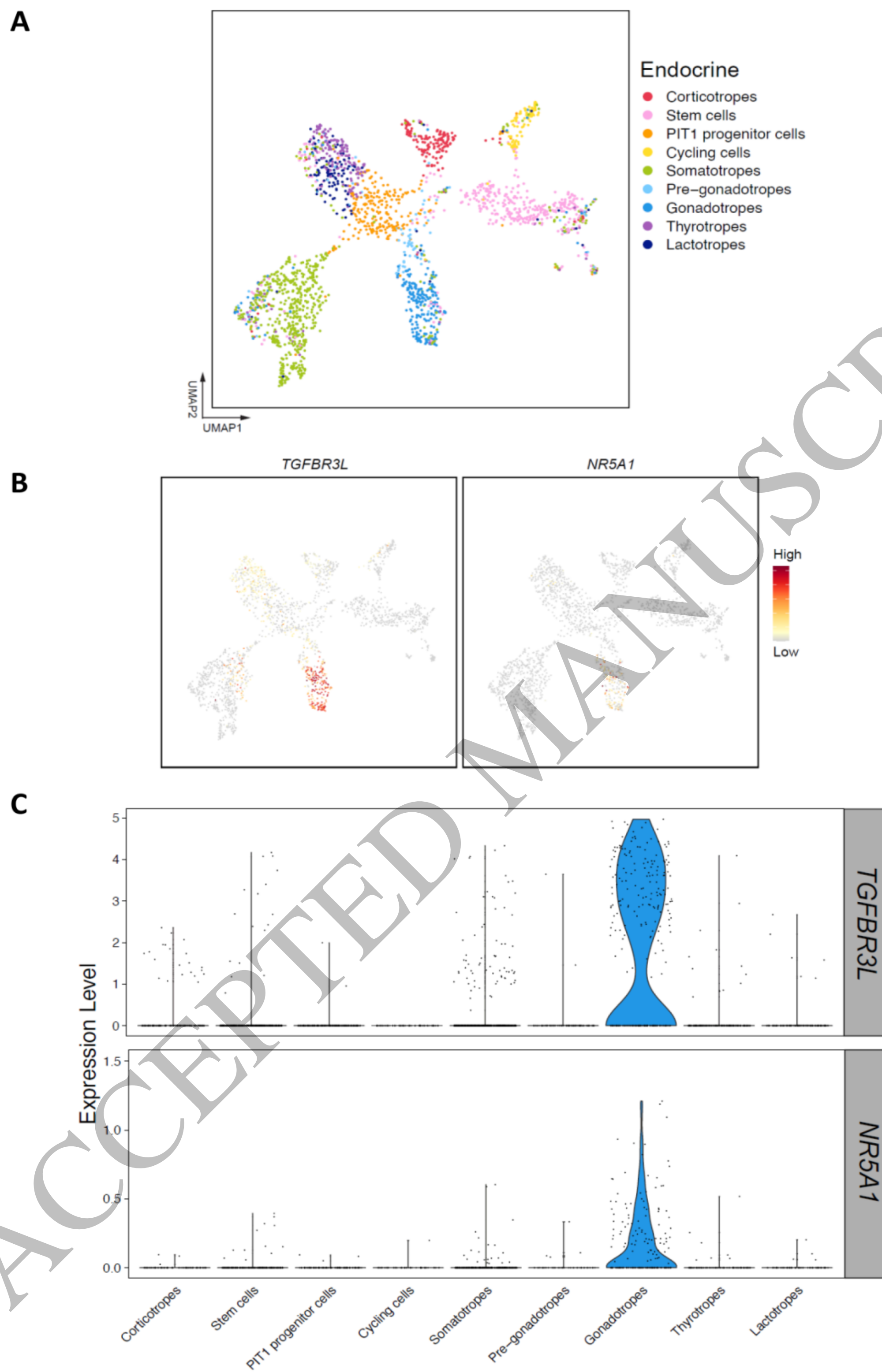

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1 **Figure 6**

2 **162x229 mm (.42 x DPI)**