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

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Yeu-Farn Lin¹, Gauthier Schang¹, Evan R. S. Buddle¹, Hailey Schultz², Thea L. Willis³,
Frederique Ruf-Zamojski⁴, Michel Zamojski⁴, Natalia Mendelev⁴, Ulrich Boehm⁵, Stuart C.
Sealfon⁴, Cynthia L. Andoniadou³, Daniel J. Bernard^{1,2}

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¹Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada

⁹ ²Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec, Canada

¹⁰ ³Centre for Craniofacial and Regenerative Biology, King's College London, London, UK

⁴Department of Neurology, Center for Advanced Research on Diagnostic Assays, Icahn School

12 of Medicine at Mount Sinai, New York, New York, USA

⁴Department of Experimental Pharmacology, Center for Molecular Signaling, Saarland
 University School of Medicine, Homburg, Germany

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Corresponding author: <u>daniel.bernard@mcgill.ca</u>; Tel. 514-554-6735; Department of
 Pharmacology and Therapeutics, 3655 Promenade Sir William Osler, McGill University,
 Montreal, Quebec, Canada; ORCID ID: 0000-0001-5365-5586

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1 Abstract

2 The inhibins control reproduction by suppressing follicle-stimulating hormone synthesis in pituitary gonadotrope cells. The newly discovered inhibin B co-receptor, TGFBR3L, is 3 4 selectively and highly expressed in gonadotropes in both mice and humans. Here, we describe Tafbr3I/TGFBR3L 5 our initial characterization of mechanisms controlling cell-specific 6 transcription. We identified two steroidogenic factor 1 (SF-1 or NR5A1) cis-elements in the proximal Tgfbr3I promoter in mice. SF-1 induction of murine Tgfbr3I promoter-reporter activity 7 was inhibited by mutations in one or both sites in heterologous cells. In homologous cells, 8 mutation of these *cis*-elements or depletion of endogenous SF-1 similarly decreased reporter 9 activity. We observed nearly identical results when using a human TGFBR3L promoter-reporter. 10 The *Tqfbr3I* gene was tightly compacted and *Tqfbr3I* mRNA expression was essentially absent 11 12 in gonadotropes of SF-1 (Nr5a1) conditional knockout mice. During murine embryonic development, Tgfbr3I precedes Nr5a1 expression, though the two transcripts are fully co-13 14 localized by embryonic day 18.5 and thereafter. Collectively, these data indicate that SF-1 directly regulates Tgfbr3I/TGFBR3L transcription and is required for post-natal expression of the 15 16 gene in gonadotropes.

1 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 (TGF β) ligands, such as the activins, which are currently thought to act in an autocrine/paracrine manner in the pituitary to stimulate transcription of the FSH β subunit (3).

9 FSH also stimulates the synthesis and secretion of activin-related TGF^β ligands, known as the inhibins, from the gonads. The ovaries produce inhibins A and B (4-6), whereas the adult 10 testes in most mammalian species produce inhibin B alone (7). Inhibins feedback to 11 12 gonadotropes to suppress FSH production by competitively binding to activin receptors (8). Inhibin binding to these receptors is enhanced by transmembrane co-receptors. The TGFB type 13 14 III receptor (TGFBR3), also known as betaglycan, can mediate the actions of both inhibins (8, 9); however, conditional deletion of the *Tqfbr3* gene in gonadotropes principally impairs inhibin 15 16 A, leaving inhibin B action intact (10). This observation led to the recent discovery of a 17 betaglycan-like protein, TGFBR3L, which functions as a specific inhibin B co-receptor in gonadotropes (11). 18

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.

1

2 Materials and methods

3

4 DNA constructs

5 The wild-type murine -999/+1 *Tgfbr3I* 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 <u>5' Rapid Amplification of cDNA Ends (RACE)</u>

5' RACE was performed using the FirstChoice RLM-RACE kit (AM1700, Invitrogen, 13 Waltham, MA, USA), following the manufacturer's protocol. Briefly, 10 µg of intact 5' capped 14 mRNA from the pituitary of a male C57BL6 mouse was decapped and ligated to the 5' RACE 15 16 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 17 adaptor-specific forward primers and gene-specific reverse primers (see Table 1). PCR 18 19 products were ligated into the pGEM-T Easy vector (A1360, Promega) and sequenced (Génome Québec, Montreal, QC, CAN). 20

21

22 Cell culture and promoter-reporter assays

All cells were cultured at 37°C in a humidified incubator with 5% CO₂. Human embryonic kidney (HEK) 293T cells (ATCC CRL-3216; RRID: CVCL_0063; provided by Dr. Terry Hébert, 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

gonadotrope-like LβT2 cells (18) (RRID: CVCL 0398; provided by Dr. Pamela Mellon, 1 University of California, San Diego, CA, USA) were cultured in DMEM supplemented with 10% 2 (v/v) FBS. 3

4 Promoter-reporter assays were performed as previously described (16). Briefly, 5 HEK293T and LBT2 cells were seeded at densities of 50,000 and 150,000 cells per well. respectively, in 48-well plates. The following day, HEK293T cells were transfected using PEI at 6 a ratio of 1:3 (µg DNA to µg PEI). LBT2 cells were transfected using Lipofectamine 3000 7 (L3000015, ThermoFisher Scientific, Burlington, ON, CAN) following the manufacturer's 8 protocol. Control (D-001210-05) and Nr5a1 (D-051262-01) short interfering RNAs (siRNAs) 9 were purchased from Dharmacon (Lafayette, CO, USA). For assays where siRNAs and the 10 murine promoter-reporter were co-transfected, the pA3-luc backbone was used. Twenty-four 11 12 hours after transfection, cells were serum starved for an additional 24 h. After starvation, cells were lysed in 50 µl/well passive lysis buffer [25 mM Tris-phosphate (pH 7.8), 10% (v/v) glycerol, 13 1% (v/v) Triton X-100, 1 mg/mL bovine serum albumin (BSA), 2 mM EDTA] for 10 min at room 14 temperature with agitation. Twenty µl of cell lysis supernatant was combined with 100 µl assay 15 16 buffer [15 mM potassium phosphate (pH 7.8), 25 mM glycylglycine, 15 mM MgSO₄, 4 mM EDTA, 2 mM ATP, 1 mM DTT, 0.04 mM D-luciferin], and luciferase activity was measured on an 17 Orion II microplate luminometer (Berthold Detection Systems, Oak Ridge, TN, USA). All 18 19 experiments were performed in technical triplicates and the experiments repeated as indicated in the figures. 20

- 21

DNA affinity purification assay and immunoblot 22

Thirty µl of Dynabeads® M-280 (11205D, Dynal, Invitrogen) were washed three times 23 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 B&W buffer at room temperature for 15 min. Beads were washed twice with 2x B&W buffer and 26

once with 1x binding buffer [5% (v/v) glycerol, 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM 1 dithiothreitol, 0.15% Triton X-100, 100 mM NaCl, 4 mM MgCl₂], then blocked for 30 min at room 2 temperature using 1% (w/v) BSA in binding buffer, and lastly resuspended in 50 µl of 1x binding 3 4 buffer. LBT2 cells were grown until confluent in 10 cm plates and harvested using 1 mL of DNAP lysis buffer [300 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1% (v/v) Triton X-100, 1 mM PMSF, 2 5 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, 15632011), and 50 µl of DNA-bound streptavidin magnetic beads. The reaction was incubated 8 9 at 4°C for 2 h with agitation, followed by 5 washes in 1x binding buffer. Bound proteins were eluted in 40 µl of 0.1% SDS at 100°C for 5 min. 10

Ten µl of 5x Laemmli buffer [250 mM Tris pH 6.8, 10% SDS, 50% glycerol, 0.02% 11 12 bromophenol blue, and 10% β-mercaptoethanol] was added and eluted proteins were resolved by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) on a 10% resolving 13 gel prepared using a 30% (w/w) acrylamide/bis-acrylamide (29:1) solution in running buffer (25 14 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.3). Proteins were transferred to Protran 15 nitrocellulose membranes (GE 10600001, Millipore Sigma, Oakville, Ontario, CA) in Towbin 16 buffer (25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol), blocked with 5% milk (w/v) in Tris-17 buffered saline [TBS: 150 mM NaCl, 10 mM Tris (pH 8.0)] containing 0.05% (v/v) Tween 20 18 19 (TBST) and incubated overnight at 4°C with agitation with an antibody against SF-1 diluted in blocking buffer (1:1000; D1Z2A; Cell Signaling Technology, Danvers, 20 MA, USA: RRID:AB_2798030). The next day, membranes were washed in TBST and incubated in 21 22 horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5,000; AP182P; Millipore Sigma; RRID:AB 92591] in blocking buffer for 1 h at room temperature with agitation. 23 24 Membranes were once again washed in TBST, and bands were visualized using enhanced chemiluminescence substrate (NEL105001, PerkinElmer, Waltham, MA, USA) and an 25 Amersham Imager 600 (GE Healthcare, Chicago, IL, USA). 26

1 Chromatin Immunoprecipitation

2 L β 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 guenched 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 at 800 x g for 10 minutes at 4°C. The cell pellet was then resuspended in 100 µl of Nuc101 EZ 6 lysis buffer (NUC101, Millipore Sigma) with protease inhibitor cocktail (04693116001, Millipore 7 Sigma) and 1 mM PMSF for 5 minutes on ice, then centrifuged at 500 x g for 5 minutes at 4°C. 8 Supernatant was discarded and the pellet washed in Nuc101 EZ lysis buffer as above. The 9 nuclei were then divided into three tubes for digestion. 10

Nuclei (in 50 µl of Nuc101 EZ lysis buffer) were mixed with 6 µl of 10x MNase buffer [10 11 12 mM Tris-HCI (pH 7.5), 50 mM NaCI, 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 13 reaction buffer [50 mM Tris-HCI (pH 7.9], 5 mM CaCl₂]) in a total reaction volume of 60 µl. 14 Nuclei were digested for 10 minutes at 37°C and the digestion was stopped with 6.6 µl of 100 15 16 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-HCI (pH 8), 2 mM EDTA, 17 150 mM NaCl, 0.1% Triton X-100, 1x protease inhibitor cocktail, 1 mM PMSF] was added. Ten 18 19 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-20 linked overnight at 65°C. The next day, samples were incubated for 30 min at 37°C with 10 µg 21 22 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 23 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 µl of 10 mM Tris-HCI (pH 8.0), and quantified using Nanodrop. 26

Ten µg of chromatin was precleared for 1 hour at 4°C with 10 µl of Dynal protein G 1 2 beads (10003D, Invitrogen) on an end-over-end rotator, then incubated with protein G beads conjugated to either rabbit IgG (2729, Cell Signaling Technology; RRID: AB 1031062) or rabbit 3 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 incubated with 1 µg of antibody in a total volume of 200 µl for 4 hours at 4°C on an end-over-6 7 end rotator. The next day, after overnight incubation with chromatin, beads were sequentially washed with complete IP buffer (1 x 5 minutes at 4°C), low salt buffer [20 mM Tris-HCl (pH 8), 2 8 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS] (2 x 5 minutes at 4°C), and high salt 9 buffer [20 mM Tris-HCI (pH 8), 2 mM EDTA, 500 mM NaCI, 1% Triton X-100, 0.1% SDS] (1 x 5 10 minutes followed by 1 x 10 minutes at 4°C). Chromatin was eluted for 90 minutes at 65°C in 30 11 12 µl elution buffer (1 M NaHCO₃, 1% SDS), then reverse-cross-linked, digested with RNase A and proteinase K, and extracted as described above. 13

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

16

17 Generation of Nr5a1 conditional knockout mice

The Nr5a1^{fl/fl} and Gnrhr^{JRES-Cre/IRES-Cre} (GRIC) mice were previously described (19, 20). 18 Cre-mediated recombination occurs in the germ line of male GRIC mice (21); thus, the GRIC 19 allele was introduced via the female in all crosses. *Nr5a1*^{fl/fl} males (Jackson Laboratory, 007041) 20 (22) were crossed with GRIC females to produce Nr5a1^{fl/+}: Gnrhr^{GRIC/+} progeny. Nr5a1^{fl/fl} males 21 were then crossed with Nr5a1^{fl/+}; Gnrhr^{GRIC/+} females to produce Nr5a1^{fl/fl}; Gnrhr^{+/+} (control) and 22 Nr5a1^{fl/fl}: Gnrhr^{GRIC/+} (conditional knockout; cKO) animals. Genotyping and assessment of 23 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 libitum. All animal work was conducted in accordance with federal and institutional guidelines 26

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

3

4 Organ collection and processing

Testes, seminal vesicles, ovaries, and uteri were dissected from control and cKO males at 8-10 weeks of age, and females at 9-10 weeks of age. Control females were collected at random points in the estrous cycle; cKO females were acyclic. All reproductive organs were weighed on an analytical balance. Pituitary glands were snap frozen in liquid nitrogen and 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 for approximately 30 min. Whole blood was centrifuged at 3000 rpm for 10 min at room 13 temperature. Serum was collected and stored at -20°C until hormone analyses were conducted. 14 Serum FSH was assessed using a Milliplex kit (Millipore, MPTMAG-49K, custom-made 15 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; intraassay coefficient of variation [CV] < 15%). Serum LH was measured using an in-house 18 19 sandwich ELISA (24) (lower detection limit: 0.117 ng/mL; dynamic range: 0.117 ng/mL to 30 ng/mL; LOQ: 0.516 ng/mL; intra-assay CV < 10%) (25). 20

- 21
- 22

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

RNA was extracted from tissues and LβT2 cells using TRIzol Reagent (15596018;
 Invitrogen, Waltham, MA, USA) following the manufacturer's protocol. Two hundred ng total
 RNA (concentration determined using Nanodrop spectrophotometer) were reverse transcribed

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

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

8

9 <u>Immunofluorescence</u>

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

1 <u>Single-cell analyses</u>

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 (13). snATAC-seg data were processed using the Cell Ranger-ATAC pipeline version 1.2.0 6 7 (10X Genomics). Samples processed in multiple wells were combined using the aggr function. Clustering was performed in Signac (26) following standard procedures, and clusters were 8 annotated based on chromatin accessibility at the promoter of known pituitary cell type markers 9 We used igvtools 2.3.32 (27) to generate chromatin accessibility tracks for the 10 (13). gonadotropes using a window size of 400 base-pairs per cut-site. Tracks were normalized to 11 12 both the number of gonadotropes and the median number of fragments per gonadotrope in each sample. snATAC-seq datasets can be found in the GEO data repository under the 13 14 accession number GSE198907.

Human scRNAseq datasets were previously published (28). The processed dataset was downloaded from the GEO data repository under the accession number GSE142653. Cells were filtered and the dataset was scaled as previously described (28) and count thresholds were adjusted where necessary to account for background. The filtered and scaled gene expression dataset was analyzed using Seurat v.4.0.4 and standard procedures (29, 30). Cell types were assigned as previously published, and clustering was performed on the subset of 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 embryonic day 0.5 (E0.5). Adult pituitary glands were collected from 8-week-old CD-1 males. All
animals were housed on a 12 h light: 12 h dark cycle and given access to food and water *ad libitum* under compliance of the Animals (Scientific Procedures) Act 1986 and KCL ethical
review.

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

The RNAscope 2.5 HD Duplex assay was used according to the manufacturer's recommendations, with a combination of the following probes; Mm-Tgfbr3I-C1 (Cat# 1040221-C1), Mm-Nr5a1-C2 (Cat# 445731-C2), Mm-Fshb-C2 (Cat# 445351-C2), Mm-Lhb-C2 (Cat# 478401-C2). All reagents were from Advanced Cell Diagnostics (Newark, CA, USA). All sections were counterstained with Mayer's hematoxylin (Vector H-3404) and mounted with Vectamount Permanent Mounting Medium (Vector H-5000).

For brightfield images, slides were scanned using an Olympus BX34F Brightfieldmicroscope.

18

19 Statistical analysis

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

1 Results

2 <u>Two SF-1 binding sites are located in the proximal *Tgfbr3l* promoter</u>

We mapped the Tafbr3I transcription start site (TSS, +1) in murine pituitary RNA using 5' 3 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 determined UTRs differed from the computationally predicted TSS (at -40) in GenBank 8 9 (NM_001195258.1). We then analysed the proximal promoter region for potential transcription factor binding sites and identified two candidate SF-1 cis-elements at -146 to -138 10 (CTGGCCTTG, site 1) and -67 to -59 (CAAGGCCAG, site 2). These two elements were reverse 11 12 complements of each other and differed from the consensus SF-1 binding motif by one base 13 pair (32, 33).

To assess SF-1 binding to these sequences, we performed DNA affinity purification 14 assays (DNAP) using biotinylated double-stranded DNA probes corresponding to each 15 16 candidate cis-element (Fig. 1B). To ascertain specific binding, we also used DNA probes with 2bp mutations in the putative SF-1 *cis*-elements. Endogenous SF-1 protein from LBT2 cells 17 bound to wild-type (Fig. 1B, lanes 2 and 4) but not mutant probes (lanes 3 and 5) corresponding 18 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 immunoprecipitation in LBT2 cells to assess SF-1 binding in the context of native chromatin. 21 22 Compared to IgG, we observed enrichment of SF-1 in a region of the Tgfbr3I promoter containing the two cis-elements but not in a negative (gene desert) control (34) (Fig. 1C). 23

1 <u>SF-1 activates murine *Tgfbr3I* promoter-reporter activity</u>

2 To examine Tgfbr3l transcription, we ligated ~1 kb of the murine Tgfbr3l 5' flanking sequence (-999/+1) upstream of luciferase in the pGL3-basic reporter plasmid. In heterologous 3 4 HEK293T cells, ectopically expressed SF-1 activated Tgfbr3I reporter activity (Fig. 1D). 5 Mutations in either or both SF-1 sites blunted or blocked this stimulatory effect. In homologous murine LBT2 gonadotrope-like cells, mutations in the SF-1 binding sites significantly decreased 6 basal reporter activity relative to wild-type (Fig. 1E). Reporter activity was also significantly 7 attenuated following SF-1 knockdown in LBT2 cells using a previously validated siRNA against 8 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 Tafbr3I and human TGFBR3L promoters demonstrated a high degree of sequence 13 conservation, including in the two SF-1 cis-elements (Fig. 1A). Using DNAP, we demonstrated 14 SF-1 binding to both *cis*-elements in the human promoter (Fig. 2A, lanes 2 and 4). Mutations in 15 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 that blocked SF-1 binding (Fig. 2A) also abrogated SF-1 induction of reporter activity (Fig. 2B). 18 19 In LBT2 cells, mutations in these *cis*-element also decreased basal promoter-reporter activity (Fig. 2C), as did siRNA-mediated knockdown of SF-1 (Fig. 2D). 20

21

22

Tgfbr3l expression is SF-1-dependent in vivo

To determine whether SF-1 regulates *Tgfbr3l* expression *in vivo*, we generated gonadotrope-specific *Nr5a1* knockout mice by crossing *Gnrhr*^{GRIC} and *Nr5a1*^{fl/fl} animals. Cre is expressed in gonadotropes and in the male germline with the GRIC Cre-driver line (19). We observed recombination of the floxed *Nr5a1* allele in pituitaries (both sexes, lanes 2 and 12) and 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 RTqPCR on pituitaries of conditional knockouts (*Nr5a1*^{fl/fl}; *Gnrhr*^{GRIC/+}, cKO) compared to controls
(*Nr5a1*^{fl/fl}).

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 *Tgfbr3I* 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 *Tgfbr3I* 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 <u>Tgfbr3I precedes Nr5a1 expression in the embryonic murine pituitary</u>

Next, we examined *Tgfbr3I* expression during murine pituitary development. Using mRNA *in situ* hybridization (RNAscope) on CD1 mouse embryos, we observed a low level of *Tgfbr3I* expression as early as E13.5 (Fig. 5A). In contrast, *Nr5a1* mRNA was first detected at E15.5 (Fig. 5A). *Tgfbr3I* co-localized with *Nr5a1* (Fig. 5A) and *Lhb* by E18.5 (Fig. 5B). *Tgfbr3I*

co-localization with Fshb was not complete at E18.5 (Fig. 5C). In adult (8-week-old) males, 1 2 Tgfbr3l 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).

6

Discussion 7

The novel inhibin B co-receptor, TGFBR3L, appears to be uniquely or principally 8 9 expressed in pituitary gonadotropes (11, 13-15). Here, we demonstrate that the gonadotropespecific transcription factor SF-1 regulates murine Tgfbr3I expression, at least in part, via two 10 cis-elements in the proximal promoter. The data also suggest that this mechanism is conserved 11 in humans. Beyond binding to these elements, how SF-1 regulates Tgfbr3I is not yet clear. 12 However, the closed chromatin state of the Tgfbr3l locus in gonadotropes of conditional 13 knockout (cKO) mice suggests that SF-1 promotes chromatin accessibility through the 14 recruitment of histone modifying enzymes (36-38). 15

16 Whereas SF-1 is necessary for Tgfbr3I expression, it is likely not sufficient. Though 17 restricted to gonadotropes among pituitary cell types, SF-1 is expressed in other tissues that do not express Tafbr31 (11), including the ventromedial hypothalamus, adrenal glands, and gonads 18 19 (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 20 expression of Lhb (17). In contrast, SF-1 cooperates with LIM homeodomain proteins to 21 22 regulate Gnrhr promoter activity (40). It is possible that SF-1 might interact with similar or distinct transcription factors to confer gonadotrope-specific Tgfbr3I expression. Notably, there is 23 24 a candidate EGR1 binding site between the two SF-1 *cis*-elements in the *Tgfbr31* promoter, 25 which is the subject of our ongoing investigations.

Importantly, the dependence of Tafbr3I expression on SF-1 may be an emergent 1 2 property. Nr5a1 mRNA is reliably detected in developing murine pituitary on embryonic day 14.5, but not at E13.5 (35). Using RNAscope, Tafbr3/ mRNA was first detected at E13.5, when 3 4 Nr5a1 mRNA was absent. These data indicate that the initial expression of Tgfbr31 is SF-1 5 independent. Nevertheless, Nr5a1 and Tgfbr3l are co-expressed at least as early as E15.5 and 6 continue to be thereafter. The loss of Tafbr3/ mRNA in Nr5a1 cKO mice demonstrates that, at least postnatally, the gene is uniquely/preferentially expressed in gonadotropes in an SF-1 7 dependent manner. Though we have not yet established what initially drives Tafbr3I expression, 8 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 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 15 16 single-nucleus RNA-sequencing analyses of murine, rat, and human pituitaries similarly establish Tgfbr3I/TGFBR3L as another gonadotrope-specific gene (13, 41, 42)}. It is therefore 17 notable that its expression is also SF-1 dependent. These data demonstrate SF-1's role as a 18 master regulator of gonadotrope identity. Nevertheless, as the expression of two of these 19 genes, Gnrhr (21) and Tgfbr3l, precedes Nr5a1 developmentally, gonadotrope-lineage 20 specification appears to be SF-1 independent. Indeed, treatment of Nr5a1 knockout mice with 21 22 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 23 24 whether GnRH treatment similarly induces Tgfbr3I. Collectively, the data indicate that SF-1 is 25 required for the full expression of the gonadotrope-specific transcriptome, but is not required for gonadotrope specification per se. 26

Finally, we should note that deciphering mechanisms controlling Tafbr3I/TGFBR3L 1 2 expression may have translational relevance. Female Tgfbr3I 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 assisted reproduction. Though SF-1 itself may not be an ideal therapeutic target, delineation of 6 the Tgfbr3I/TGFBR3L transcriptional regulatory machinery may uncover a more suitable and 7 selective approach to decrease gene expression. The results reported here provide an 8 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

YFL and DJB were responsible for the experimental design, data analyses, and manuscript 15 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 strain. TLW performed the RNAscope. UB provided the GRIC strain. NM isolated nuclei from 18 individual pituitaries. FRZ performed the snATAC-seq experiments and MZ analyzed the 19 snATAC-seq datasets. HS analyzed the human scRNA-seq datasets. DJB, CLA, and SCS 20 secured the grant funding for the research. All authors approved the final version of the 21 22 manuscript.

23

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Figure 1. SF-1 activates murine *Tgfbr31* transcription via two *cis*-elements in the proximal 3 4 promoter. (A) Alignment of the murine and human Tgfbr3l/TGFBR3L promoters. In both cases, +1 refers to the transcription start site. The most common murine 5' untranslated region (5' 5 UTR, +1/+29) is boxed in blue and the human 5' UTR is boxed in green. The conserved SF-1 6 7 binding sites are boxed in red and labeled as site 1 and site 2. Mutated base pairs (in B, C, and D) are indicated above in green. (B) DNAP using probes corresponding to the wild-type and 8 9 mutant murine SF-1 *cis*-elements. Whole cell protein lysates from LBT2 cells (input) or proteins interacting with the probes were analyzed via immunoblot (IB) using an SF-1 antibody. (C) 10 Chromatin immunoprecipitation for SF-1 of the indicated genomic regions in L β T2 cells (n=4). 11 12 (D) HEK293T cells were transfected with 225 ng/well of the indicated murine -999/+1 Tgfbr3I-luc reporters as well as 3.125 ng/well of either pcDNA3.0 (empty expression vector) or SF-1 13 14 expression vector. WT, wild-type; site 1 mut, mutated SF-1 site 1; site 2 mut, mutated SF-1 site 2; double mut, both SF-1 sites mutated. (E) LBT2 cells were transfected with 225 ng/well of the 15 16 indicated promoter-reporters. (F) LBT2 cells were transfected with 225 ng/well of the -999/+1 Tafbfr3I-luc reporter and 10 nM of control or Nr5a1 siRNA. In D-F, lysates were collected and 17 reporter activity measured by luciferase assay. Data represent the mean of three or more 18 19 independent experiments performed in triplicate. Data were analyzed by two-way ANOVA followed by Holm-Sidak multiple comparisons test in (D), one-way ANOVA followed by 20 Dunnett's multiple comparisons test in (E), and two-tailed unpaired t test with Welch's correction 21 22 in (C and F). ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

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Figure 2. Mechanisms of human *TGFBR3L* transcriptional regulation by SF-1 are conserved. (A) DNAP was performed as in Fig. 1B, but with probes corresponding to the wildtype 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 mut, mutated SF-1 site 1; site 2 mut, mutated SF-1 site 2; double mut, both sites mutated. (C) 3 4 LBT2 cells were transfected with 225 ng/well of the indicated promoter-reporters. (D) LBT2 cells 5 were transfected with 225 ng/well of the -996/+1 TGFBR3L-luc reporter and 10 nM of control or Nr5a1 siRNA. In B-D, luciferase assays were performed as in Fig. 1. Data represent the mean 6 7 of three independent experiments performed in triplicate and were analyzed by two-way ANOVA followed by Holm-Sidak multiple comparisons test (B), one-way ANOVA followed by Dunnett's 8 9 multiple comparisons test (C), or by a two-tailed unpaired t test with Welch's correction (D). ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.001. 10

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12 Figure 3. Gonadotrope-specific SF-1 knockout mice exhibit hypogonadotropic was extracted from 13 hypogonadism. (A) Genomic DNA the indicated tissues of Nr5a1^{fl/fl}; Gnrhr^{GRIC/+} (cKO; left panels) and Nr5a1^{fl/fl} (control; right panels) mice and analyzed 14 by PCR for the presence of the floxed or recombined (rec) Nr5a1 alleles. (B) Pituitary sections 15 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 RNA isolated from individual pituitary glands of 8-10-week-old control and cKO female (top) and 18 19 male (bottom) mice and analyzed by RT-qPCR for expression of Lhb, Fshb, Cga, Gnrhr, and Nr5a1. (D) Serum LH and FSH levels in female (top) and male (bottom) control and cKO mice. 20 (E) Ovarian, uterine, testicular, and seminal vesicle weights and (F) representative images of 21 22 gonads and accessory sex organs from control and cKO females (top) and males (bottom). Scale bars: 5 mm. Female data in all panels represent randomly cycling females. Data were 23 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.

Figure 4. SF-1 is required for *Tgfbr3l* expression in adult murine pituitary glands. (A) Pituitary cDNA from control and cKO female and male mice (described in Fig. 3) were analyzed for *Tgfbr3l* expression by RT-qPCR. Data were analyzed by two-tailed unpaired t tests with Welch's correlation. ***, p<0.001. Chromatin accessibility, as measured with single-nucleus ATAC-seq, over the (B) *Tgfbr3l* and (C) *Cga* genes in gonadotropes of 10-12-week-old control (orange) and cKO (grey) males. Exon 1 and the promoter of *Tgfbr3l* are boxed in red in panel B. Shown are representative tracks from a control and a cKO animal.

8

Figure 5. *Nr5a1* and *Tgfbr3I* expression in fetal and adult murine pituitaries. (A) mRNA *in situ* hybridization (RNAscope) for *Tgfbr3I* (blue) and *Nr5a1* (red) on heads of wild-type CD1
embryos at the indicated ages. Boxed regions in the top panels are magnified in the bottom
panels. Scale bars: 50 µm. Duplex RNAscope for *Tgfbr3I* (blue) and (B) *Lhb* or (C) *Fshb* (red)
on heads of CD1 embryos at E18.5. Scale bars: 200 µm (top) and 50 µm (bottom).. Duplex
RNAscope for *Tgfbr3I* (blue) and (D) *Nr5a1*, (E) *Fshb*, or (F) *Lhb* (red) on pituitaries of 8-weekold CD1 males. Scale bars: 50 µm.

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

1 Table 1: Primers

| Murine Tgfbr3I promoter amplification | | |
|---------------------------------------|--|--|
| 5' RACE gene-specific RT | GGACGGACGAGGTATTGTGA | |
| primer | | |
| 5' RACE gene-specific outer R | CCTGCGTCCGGTATTCAATG | |
| 5' RACE gene-specific inner R | GGGCGTGAAGAAGGTGTTAC | |
| -999/+1 promoter amplification | AAAACTCGAGGTAGCTGATGCAACCATACGTAG | |
| F | | |
| -999/+1 promoter amplification | AAAAAAGCTTGACCGGCAGCGAGCACCT | |
| R | | |
| Human Tgfbr3l promoter ampl | ification | |
| -996/+1 promoter amplification | AAAACTCGAGTAGGCGTAGCATCCCTCTC | |
| F | | |
| -996/+1 promoter amplification | TTTTAAGCTTGGACCAGCTGAGGTCGGA | |
| R | | |
| Tgfbr3I/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 | TGCCTCCTCCTTGCCAATTACAGGGCCGCC | |
| 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 | |
| N/rEad D (recembinent) | TGCGTGCAATCCATCTTGTTCAAT | |

2 Table 2: DNAP probes

| Tgfbr3l SF-1 site 1 WT sense | TATCCATGGCTGGCCTTGAGCATGT |
|--|---------------------------|
| <i>Tgfbr3I</i> SF-1 site 1 WT antisense | ACATGCTCAAGGCCAGCCATGGATA |
| <i>Tgfbr3I</i> SF-1 site 1 Mut sense | TATCCATGGCTGGAATTGAGCATGT |
| <i>Tgfbr3I</i> SF-1 site 1 Mut antisense | ACATGCTCAATTCCAGCCATGGATA |
| Tgfbr3l SF-1 site 2 WT sense | AGCCTCACCAAGGCCAGGGCTACCT |
| <i>Tgfbr3I</i> SF-1 site 2 WT antisense | AGGTAGCCCTGGCCTTGGTGAGGCT |
| <i>Tgfbr3I</i> 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

165x220 mm (.42 x DPI)

Α









165x220 mm (.42 x DPI)



Figure 6

162x229 mm (.42 x DPI)