Supplementary Materials for

TGFBR3L is an inhibin B co-receptor that regulates female fertility

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Published 15 December 2021, Sci. Adv. 7, eabl4391 (2021)
DOI: 10.1126/sciadv.abl4391

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Fig. S1. Tgfbr3l/TGFBR3L is specifically expressed in pituitary gonadotrope cells. (A and C) tSNE representations of scRNAseq of male murine and snRNAseq of male human pituitaries. Different colored clusters represent different pituitary cell types, which are labeled above the clusters: B cells (B), corticotropes (Cor), endothelial cells (En), gonadotropes (Gt), lactotropes (Lac), macrophages (Mac), melanotropes (Mel), pericytes (Per), pituicytes (Pit), somatotropes (Som), stem cells (Stem), T cells (T) and thyrotropes (Thy). (B and D) Feature plots of Tgfbr3l/TGFBR3L expression. (E) Tgfbr3l expression in additional male (blue) and female (red) tissues. Pituitary expression data are the same as in Fig. 1E. All expression was normalized to male pituitary.
Fig. S2. The chromatin is open at the promoter region of Tgfbr3l uniquely in gonadotropes. Chromatin accessibility plots from snATACseq of male murine pituitary. Some data from Fig. 1F are reproduced here, but this figure includes all cell types identified in the dissociated pituitaries. The dotted box indicates the promoter region of Tgfbr3l.
**Fig. S3.** The ZP domain of TGFBR3L shares 33% amino acid sequence identity with the ZP-C domain of betaglycan. Amino acid sequence alignments between mouse (A) and human (B) TGFBR3L ZP domain and TGFBR3 (betaglycan) ZP-C domain. UniProt accession numbers: D3YZZ2 (mouse TGFBR3L), H3BV60 (human TGFBR3L), O88393 (mouse TGFBR3), Q03167 (human TGFBR3).

|    | Mouse TGFBR3L | Human TGFBR3L | Amino Acids | Mouse TGFBR3 | Human TGFBR3 | Amino Acids |
|----|---------------|---------------|-------------|--------------|--------------|-------------|
| A  | GPLEVPADSHVFQALAARFSPRWGLALHCRSVPSSRTPALVLLRGCC  | GPLEVPADSHVFQALAARFSPRWGLALHCRSVPSSRTPALVLLRGCP  | 108         | GPLEVPADSHVFQALAARFSPRWGLALHCRSVPSSRTPALVLLRGCP  | GPLEVPADSHVFQALAARFSPRWGLALHCRSVPSSRTPALVLLRGCP  | 108         |
|    | G V + HV+V+ ++ + G A+ C ++ P S P ++ CP            | G V + HV+V+ ++ + G A+ C ++ P S P ++ CP            |             | G V + HV+V+ ++ + G A+ C ++ P S P ++ CP            | G V + HV+V+ ++ + G A+ C ++ P S P ++ CP            |             |
| B  | ADDSVSFSSPPR-----FAD-AVSRFSLFRLFVPNRSVQQFLHCQI  | ADDSVSFSSPPR-----FAD-AVSRFSLFRLFVPNRSVQQFLHCQI  | 148         | ADDSVSFSSPPR-----FAD-AVSRFSLFRLFVPNRSVQQFLHCQI  | ADDSVSFSSPPR-----FAD-AVSRFSLFRLFVPNRSVQQFLHCQI  | 148         |
|    | DDSV F R P A RFSF + VPN S+ FLHC+                | DDSV F R P A RFSF + VPN S+ FLHC+                |             | DDSV F R P A RFSF + VPN S+ FLHC+                | DDSV F R P A RFSF + VPN S+ FLHC+                |             |
|    | KDDSVKFEYSKRFVHFPFIEAVDCKKRFSFVFKSVFNTSLLLHCEL  | KDDSVKFEYSKRFVHFPFIEAVDCKKRFSFVFKSVFNTSLLLHCEL  | 706         | KDDSVKFEYSKRFVHFPFIEAVDCKKRFSFVFKSVFNTSLLLHCEL  | KDDSVKFEYSKRFVHFPFIEAVDCKKRFSFVFKSVFNTSLLLHCEL  | 706         |

|    | Mouse TGFBR3L | Human TGFBR3L | Amino Acids | Mouse TGFBR3 | Human TGFBR3 | Amino Acids |
|----|---------------|---------------|-------------|--------------|--------------|-------------|
| A  | GPLEVPADSRVFQALAARFSPRWGLALHCRSVPSSRPAPGPAALLREGCP | GPLEVPADSRVFQALAARFSPRWGLALHCRSVPSSRPAPGPAALLREGCP | 107         | GPLEVPADSRVFQALAARFSPRWGLALHCRSVPSSRPAPGPAALLREGCP | GPLEVPADSRVFQALAARFSPRWGLALHCRSVPSSRPAPGPAALLREGCP | 107         |
|    | G V + V+V+ ++ + G A+ C ++ P S P ++ CP            | G V + V+V+ ++ + G A+ C ++ P S P ++ CP            |             | G V + V+V+ ++ + G A+ C ++ P S P ++ CP            | G V + V+V+ ++ + G A+ C ++ P S P ++ CP            |             |
| B  | ADDSVAFPPPP-----PPSPGAARPARFSFSLFRLFVPNRSVQQFLHCQ | ADDSVAFPPPP-----PPSPGAARPARFSFSLFRLFVPNRSVQQFLHCQ | 149         | ADDSVAFPPPP-----PPSPGAARPARFSFSLFRLFVPNRSVQQFLHCQ | ADDSVAFPPPP-----PPSPGAARPARFSFSLFRLFVPNRSVQQFLHCQ | 149         |
|    | DSV F P P P + RFSF + VPN S+ FL C+L               | DSV F P P P + RFSF + VPN S+ FL C+L               |             | DSV F P P P + RFSF + VPN S+ FL C+L               | DSV F P P P + RFSF + VPN S+ FL C+L               |             |
|    | KDESVKYFSKPRVHFIPQADMK-RKRFSFVFKPVFNSTSFLLQCEL  | KDESVKYFSKPRVHFIPQADMK-RKRFSFVFKPVFNSTSFLLQCEL  | 707         | KDESVKYFSKPRVHFIPQADMK-RKRFSFVFKPVFNSTSFLLQCEL  | KDESVKYFSKPRVHFIPQADMK-RKRFSFVFKPVFNSTSFLLQCEL  | 707         |
Fig. S4. Experimentally determined nucleotide and amino acid sequence of human TGFBR3L. The nucleotide sequence of TGFBR3L was determined using a combination of 5’ and 3’ RACE and RT-PCR from 65–80-year-old human pituitary RNA. The amino acids of the predicted signal peptide are boxed, and those of the predicted transmembrane domain are underlined. Nucleotides are numbered at the left and amino acids at the right.
Fig. S5. TGFBR3L mediates inhibin B actions in vitro. (A and B) Inhibin sensitivity of CHO cell lines generated with stable integration of pCMV6 (grey) or stable expression of revised human TGFBR3L (red). Cells were transfected with the CAGA-luc reporter plasmid and treated with 1 nM activin A and the indicated concentrations of inhibin B (AnshLabs) or inhibin A (R&D). (C-D) COV434 cells were transfected with the A3-luc reporter and FAST2 plasmids and either pCMV6 (grey), rat betaglycan (blue), or murine TGFBR3L (red). Cells were either left untreated or treated with 200 pM activin A and the indicated concentrations of inhibin B (Monash U; C) or inhibin A (Monash U; D). (E) HEK293 cells were transfected with pCMV6 or murine TGFBR3L-Myc-DDK along with 2 nM of either control, Tgfbr3l, or Tgfbr3 (betaglycan) siRNA. Protein lysates were immunoblotted against FLAG. b-actin was used as a loading control. (F) LβT2 cells were transfected with the CAGA-luc reporter plasmid and either 4 nM control (grey), or 2 nM of each betaglycan and Tgfbr3l (yellow) siRNA. Cells were either left untreated or treated with 1 nM activin A and/or 5 nM inhibin B (AnshLabs). Data represent
mean values (± SEM) from 3-4 independent experiments. Data were analyzed by two-way ANOVA, followed by Tukey (A-D) or Sidak’s (F) multiple comparisons tests. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. 
Fig. S6. ActRII binds activin A. SPR sensorgrams of ActRII binding to immobilized activin A. Concentrations of receptor used are indicated in the top left corner of the graph. Kinetic fits are shown in orange over experimental curves in black.
Fig. S7. Male *Tgfbr3l* knockout mice have no overt reproductive phenotypes. Body (A), testicular (B), seminal vesicle (C), and epidydimal (D) weights. Serum FSH (E) and LH (F). Representative testicular histology with H&E staining from WT (G) and KO (H) males. Scale bars: 200 µm. Data were collected from 9- to 13-wk-old males. Data were analyzed by two-tailed unpaired *t* test with Welch’s correlation.
Fig. S8. Many reproductive phenotypes appear to be normal in Tgfbr3l knockout mice. (A) Age of vaginal opening (days) and (B) estrous cyclicity (% time spent in each cycle stage) over the course of 21 days. M: metestrus, D: diestrus, P: proestrus, E: estrus. (C) Body weight, (D) litter frequency per 30 days, and (E) ovarian and (F) uterine weights. (G) Ovarian mRNA expression of Cyp19a1, Inha, Inhba, and Inhbb. (H) Number of oocytes ovulated following superovulation. Serum inhibin A and B (I), P4 (J) and LH (K). (L) Pituitary mRNA expression of Lhb, Cga, and Gnrhr. (M and N) Inhibin sensitivity in pituitary cells cultured from WT (grey) and KO (red) males. Pituitaries were isolated from 9- to 13-wk-old males. Cells were treated with the indicated concentrations of inhibit B (AnshLabs; M) and inhibit A (N) Twenty-four hours after treatment, Fshb mRNA expression was measured by RT-qPCR. Data represent mean values (± SEM) from 4-5 independent experiments. (O) Serum FSH levels in WT (grey) and KO (red) on the morning of diestrus before (pre) and 11 h after (post) treatment with anti-inhibin serum (AIS). Data in panels C, E-G, and I-L were collected from 9-wk-old females on the
morning of diestrus. Pituitary culture and AIS data were analyzed by two-way ANOVA, followed by Sidak’s multiple comparisons test. All other data were analyzed by two-tailed unpaired \( t \) tests with Welch’s correlation. n.s., no significant difference. ** \( P<0.01 \), *** \( P<0.001 \).
Fig. S9. Schematic representation of the genetic crosses used to generate double knockout animals. Four genetic crosses were required to obtain double knockout (Gnrhr\textsuperscript{Cre/+};Tgfbr3\textsuperscript{fl/fl};Tgfbr3l\textsuperscript{-/-}; dKO) animals with a global deletion of Tgfbr3l and gonadotrope-specific deletion of betaglycan (Tgfbr3). The final genetic cross used to generate the dKO animals gave three other control genotypes (enumerated at the bottom of the figure). Gnrhr\textsuperscript{Cre/+};Tgfbr3\textsuperscript{fl/fl};Tgfbr3l\textsuperscript{+/-} (referred to as Tgfbr3l\textsuperscript{+/-}) harbored two floxed betaglycan (Tgfbr3) alleles and one null Tgfbr3l allele, but lacked Cre recombinase. Gnrhr\textsuperscript{Cre/+};Tgfbr3\textsuperscript{fl/fl};Tgfbr3l\textsuperscript{-/-} (referred to as Tgfbr3l\textsuperscript{-/-}) were null for Tgfbr3l, with two floxed betaglycan alleles and no Cre. Gnrhr\textsuperscript{Cre/+};Tgfbr3\textsuperscript{fl/fl};Tgfbr3l\textsuperscript{+/-} lacked betaglycan in gonadotropes and one Tgfbr3l allele globally. All four genotypes from this final cross were obtained at the expected Mendelian ratios; the frequency of each, from a sample of 275 animals, are shown in the figure.
Fig. S10. Normal FSH synthesis in female Tgfbr3l heterozygous knockout mice. (A) Serum FSH and (B) pituitary Fshb mRNA expression in Tgfbr3l wildtype (WT) and heterozygous (HET) knockout females. Rpl19 was used as a housekeeping gene. WT data here are the same as in Fig. 5J-K. Data were collected from 9-wk-old females on the morning of diestrus. Each dot in the graphs represents an individual female. Data were analyzed by a two-tailed unpaired t test with Welch’s correlation.
Fig. S11. Male Tgfbr3l/betaglycan double knockout mice have no overt reproductive phenotypes. Body (A), testicular (B), seminal vesicle (C), and epidydimal (D) weights. Serum levels of FSH (E) and LH (F). Representative testicular histology with H&E staining from Tgfbr3l/− (G) and dKO (H) males. Scale bars: 200 μm. Data were collected from 9- to 13-wk-old males. Data were analyzed by two-tailed unpaired t tests with Welch’s correlation.
Fig. S12. Many reproductive phenotypes appear to be normal in Tgfbr3l/betaglycan double knockout females. (A) Age of vaginal opening (days) and (B) estrous cyclicity (% time spent in each cycle stage) over the course of 21 days. M: metestrus, D: diestrus, P: proestrus, E: estrus. (C) Body weight, (D) serum LH, and (E) pituitary gene expression of Lhb, Cga, Gnrhr, and Nr5a1. Data in panels C-E were collected from 9-wk-old females on the morning of diestrus. Rpl19 was used as a housekeeping gene for all gene expression analyses. Data were analyzed by two-tailed unpaired t tests with Welch’s correlation. *** P < 0.001.
Fig. S13. Homology model of inhibin A with presumptive betaglycan interacting residues highlighted. (A) Ribbon/stick diagram (inhibin α subunit in cyan and inhibin βA subunit in gray) and (B) surface representation of a homology model of inhibin A. Neither the structure of inhibin A nor inhibin B has been reported. Note that we modeled inhibin A rather inhibin B because the structure of activin A (homodimer of inhibin βA subunits) has been reported. The residues highlighted in the α (red) and βA (yellow) subunits are homologous to those identified by either NMR or mutagenesis, as mediating the binding of TGFβ2 to the ZP-C domain of betaglycan. It is therefore likely that one or more of the (red) residues in the α subunit also contribute to the binding of inhibin A and B to betaglycan. Residues (yellow) in the wrist helix of inhibin βA subunit may contribute to differences in inhibin A and B affinity for betaglycan. In particular, Thr61 (T61') and His65 (H65') in the inhibin βA subunit differ at the corresponding positions in the inhibin βB subunit, Ala61 and Gln65, respectively. It is possible that these, and perhaps other, differences in the wrist helix of the two inhibin β subunits could explain affinity differences between inhibin B and inhibin A for TGFBR3L.
**Fig. S14. Activin B actions are unchanged TGFBR3L expressing cells.** CHO cells with stable integration of pCMV6 (grey) or murine TGFBR3L-Myc-DDK (red) were transiently transfected with the CAGA-luc reporter plasmid and treated with the indicated concentrations of activin B. Data represent mean values (± SEM) from 3 independent experiments.
Fig. S15. Relative purity of commercially sourced inhibin B. Equal masses of inhibin B from either R&D (left lane) and AnshLabs (right lane) were resolved by SDS-PAGE under non-reducing conditions and immunoblotted against the inhibin βB subunit. As shown, Ansh inhibin B was not as pure as R&D inhibin B. Therefore, we could not accurately estimate IC$_{50}$s in Figs. 3A, 7B, S5B, and S8M. Inhibin B from R&D was limiting and therefore reserved for siRNA knockdown (Fig. 3C and E) and SPR experiments (Fig. 4).
Table S1. SPR binding constants for ActRII, BGZP-C, and TGFBR3L to TGFβ2 E84A, inhibin A, and inhibin B and ActRII to activin A.

| Surface       | Analyte   | Concentration Range of Analyte (µM) | $k_{on}$ (M$^{-1}$ s$^{-1}$) | $k_{off}$ (s$^{-1}$) | $K_d$ (nM) | $R_{max}$ (RU) |
|---------------|-----------|-------------------------------------|------------------------------|----------------------|------------|----------------|
| TGFβ2 E84A   | ActRII    | 1 - 0.0078                           | ND*                          | ND*                  | ND*        | ND*            |
| TGFβ2 E84A   | BGZP-C    | 0.5 - 0.0078                         | $3.7 \pm 0.1 \times 10^2$   | $0.073 \pm 0.002$   | $195 \pm 2$| $328 \pm 1$   |
| TGFβ2 E84A   | TGFBR3L   | 4 - 0.03125                          | ND*                          | ND*                  | ND*        | ND*            |
| Inhibin A    | ActRII    | 0.125 - 0.0078                       | $8.8 \pm 0.2 \times 10^2$   | $0.0310 \pm 0.0005$ | $35.3 \pm 0.3$| $227.2 \pm 0.7$|
| Inhibin A    | BGZP-C    | 8 - 0.125                            | $2 \pm 1 \times 10^4$       | $0.4 \pm 0.2$       | $1.72 \pm 0.08 \times 10^4$ | $247 \pm 8$ |
| Inhibin A    | TGFBR3L   | 0.5 - 0.250                          | ND*                          | ND*                  | ND*        | ND*            |
| Inhibin B    | ActRII    | 0.125 - 0.0078                       | $6.87 \pm 0.02 \times 10^5$ | $1.217 \pm 0.003 \times 10^{-2}$ | $17.71 \pm 0.006$ | $67.4 \pm 0.1$ |
| Inhibin B    | BGZP-C    | 4 - 0.0313                           | ND*                          | ND*                  | ND*        | ND*            |
| Inhibin B    | TGFBR3L   | 0.25 - 0.0078                        | $6.0 \pm 0.1 \times 10^5$   | $1.40 \pm 0.01 \times 10^{-2}$ | $52.0 \pm 0.6$ | $17.49 \pm 0.08$ |
| Activin A    | ActRII    | 0.25 - 0.0078                        | $9.8 \pm 0.1 \times 10^5$   | $0.058 \pm 0.001$   | $59 \pm 2$  | $740 \pm 1$   |

*Not determined due to weak binding
### Table S2. Primers

| **Single guide RNAs** |                                                                             |
|-----------------------|-----------------------------------------------------------------------------|
| sgRNA-1               | TTTGGGATGCGGCTGAGCAT                                                         |
| sgRNA-2               | TACAACCATGTACCAGCTTAG                                                       |
| sgRNA-3               | GCCGCCTAAACGAGGACGAGG                                                       |
| sgRNA-4               | GCGGGCGCCTTAACGACGGA                                                        |

| **Genotyping Primers** |                                                                             |
|-------------------------|-----------------------------------------------------------------------------|
| GRIC Fw                 | CCTGGAAAAATGCTTCTGTCCG                                                       |
| GRIC Rv                 | CAGGGTGTATATAAGCAATTC                                                        |
| Tgfbr3 floxed Fw        | TTGACTCTCAGTGCAATTG                                                         |
| Tgfbr3 floxed Rv        | CTCAGCATAGACAGGAATGTAC                                                       |
| Tgfbr3l Fw              | CTGGGACCTCAGTAAGCAC                                                         |
| Tgfbr3l Rv              | TGAAGGGACCCAGACC                                                           |

| **Human TGFBR3L amplification** |                                                                             |
|-------------------------------|-----------------------------------------------------------------------------|
| 5’ RACE gene specific RT      | GTGGCGGGGAAGGCGACAG                                                         |
| 5’ RACE gene specific outer Rv| ACGAACACCGCCGCTGG                                                          |
| 5’ RACE gene specific inner Rv| CTCTGTGTCCGAGCCGCT                                                        |
| 3’ RACE gene specific outer Fw| GTGTGGCACCTCCCTGAG                                                         |
| 3’ RACE gene specific inner Fw| GTCTCGTCTGTCGCA                                                           |
| Amplification exon* 2-3 Fw    | AGACCCCTCTCTCAGCTG                                                         |
| Amplification exon* 2-3 Rv    | AAATGCACCGAGGGTTGG                                                         |
| Amplification exon* 3-4 Fw    | ACACCTCTGTCGCTTCC                                                         |
| Amplification exon* 3-4 Rv    | TGACACGATAGGCCGTG                                                          |
| Amplification exon* 4-5 Fw    | CCGACACTGTCGAGTGG                                                         |
| Amplification exon* 4-5 Rv    | AGACGAGGGCTGGAAGA                                                         |
| Amplification exon* 5-6 Fw    | TGCACTGCACGCCCCTGA                                                        |
| Amplification exon* 5-6 Rv    | TACAGGGACCTCCTCGG                                                         |

| **Cloning Primers** |                                                                             |
|---------------------|-----------------------------------------------------------------------------|
| murine Tgfbr3l ECD amplification Fw | ATAGCTTTGGCGGCCGCAAGAAG                                                     |
| murine Tgfbr3l ECD amplification Rv | CCTCGGTTAAGACTGAGTTGTTCCAATGG                                                  |
| human ActRII ECD amplification Fw     | TTAGGGTTAAGGCGCCGCA                                                         |
| human ActRII ECD amplification Rv      | TGCCTCCCTGGTCTGGAGTCA                                                       |
| human INHBA amplification Fw           | ATAAACATATTGCGGCTGAGTGTGAG                                                  |
| human INHBA amplification Rv           | TATTAAGGCTTGTATGAGCACCACCACTC                                               |

| **qPCR primers** |                                                                             |
|------------------|-----------------------------------------------------------------------------|
| Cga Fw           | TCCCTCAAAAAGTCACAGAG                                                        |
| Cga Rv           | GAAGAGAATGAAGAATATGC                                                        |
| Cyp19a1 Fw       | GACAGGACCCTTGTTGGA                                                          |
| Cyp19a1 Rv       | GAGGTTACGGCCACCTCA                                                         |
| Fshb Fw          | GTGCAGGGCTACGTGCTA                                                        |
| Fshb Rv          | CAGGCAATCTTACGCTGCT                                                         |
| Gnrhr Fw         | TTGCCTACCTCGTTGCTG                                                         |
| Gnrhr Rv         | CACGGGTGTTAGGAAAGCA                                                        |
| Inha Fw          | CTCCCAAGGCTATCCTTCC                                                         |
| Primer   | Sequence                  |
|----------|---------------------------|
| *Inha*  Rv | TGGCCGGAATACATAAGTGA       |
| *Inhba* Fw | GAGGGGCCGAAATGAAATGAA     |
| *Inhba* Rv | CACTGCCTTCTTTGGAAATCT     |
| *Inhbb* Fw | AGATCATCAGCTTTTCAGACAGACA |
| *Inhbb* Rv | TCTCCAGGACATAGGGGAGC      |
| *Lhb*  Fw | ACTGTGCCGGCCTGTCAACG      |
| *Lhb*  Rv | AGCAGCCGGCAGTACTCGGA      |
| *Nr5a1* Fw | AGGAGTTCGTCTGTCTCAAGTTTCT |
| *Nr5a1* Rv | ACAAGGTGTAATCACAACAGCGCAG |
| *Rpl19* Fw | CGGGATCTCAAGAAGATTGA      |
| *Rpl19* Rv | TTCAGCTTGTGGATGTGCTC      |
| *Tgfbr3l* exon 1-2 Fw | CTCCAGTAAGCAGGTACAC      |
| *Tgfbr3l* exon 1-2 Rv | CGTCCCTCAGCATGTGATAACTC  |
| *Tgfbr3l* exon 3-4 Fw | CCTGACACACAGGTTCCTTGA    |
| *Tgfbr3l* exon 3-4 Rv | CTAGGGACGGGACGGTGTAT     |

*exon numbering refers to the exons in the revised sequence (MW464126).