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Gonadotropin-releasing hormone regulates transcription of the inhibin B co-receptor, TGFBR3L, *via* early growth response one

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Follicle-stimulating hormone (FSH), a product of pituitary gonadotrope cells, regulates gonadal function and fertility. FSH production is stimulated by gonadotropin-releasing hormone (GnRH) and activin-class ligands of the TGFβ family. Inhibin A and B are TGFβ proteins that suppress FSH synthesis by competitively binding activin type II receptors in concert with the co-receptors betaglycan (TGFBR3) and TGFBR3L. Betaglycan mediates the actions of both inhibins and is broadly expressed. In contrast, TGFBR3L is inhibin B-specific and selectively expressed in gonadotropes. This cell-restricted expression is driven, in part, by steroidogenic factor 1 (SF-1, NR5A1), which stimulates *Tgfbr3l/TGFBR3L* transcription *via* two conserved promoter elements. *Tgfbr3l* expression is lost in mice lacking SF-1 in gonadotropes. However, SF-1 alone is unlikely to fully explain gonadotrope-restricted *Tgfbr3l/TGFBR3L* expression. Here, we report that GnRH induces binding of the transcription factor, early growth response 1 (EGR1), to the murine *Tgfbr3l* and human *TGFBR3L* promoters at a conserved *cis*-element between the two SF-1 binding sites. In homologous LβT2 cells, GnRH stimulation of *Tgfbr3l/TGFBR3L* promoter-reporters depends on EGR1 binding to this *cis*-element. In heterologous cells, over-expressed EGR1 independently and synergistically with SF-1 activates *Tgfbr3l/TGFBR3L* promoter-reporter activities. *In vivo*, *Tgfbr3l* mRNA expression is reduced in the pituitaries of: 1) GnRH-deficient mice, 2) wild-type mice treated with a GnRH receptor antagonist, and 3) gonadotrope-specific *Egr1* knockout mice. Gonadectomy, which increases GnRH pulse frequency, enhances *Tgfbr3l* expression in control but not gonadotrope-specific *Egr1* knockouts. Collectively, these data indicate that GnRH stimulates *Tgfbr3l/TGFBR3L* transcription *via* EGR1, which acts with SF-1 through conserved promoter elements.

The gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are dimeric glycoproteins produced by gonadotrope cells of the anterior pituitary gland. They are composed of a common alpha subunit (CGA) bound to distinct beta subunits (LHβ and FSHβ) (1). In females, FSH stimulates ovarian follicle growth (2, 3), whereas LH promotes the growth and ovulation of mature follicles and, for a time, the function of the corpora lutea (4). In males, FSH and LH act on testicular Sertoli and Leydig cells to promote spermatogenesis (2, 5) and testosterone production (4), respectively.

In mammals, gonadotrope cells produce both LH and FSH, but the synthesis and secretion of the two hormones is differentially regulated. Gonadotropin-releasing hormone (GnRH) is released in a pulsatile manner from the hypothalamus and stimulates LH and FSH production, with higher pulse frequencies favoring LH relative to FSH secretion (6–13), as seen in polycystic ovary syndrome (14, 15). This preferential effect on LH is explained, at least in part, by GnRH's frequency-dependent regulation of the transcription factor, early growth response 1 (EGR1) (16). EGR1 acts in concert with steroidogenic factor 1 (SF-1 or NR5A1) and *paired*-like homeodomain transcription factor 1 (PITX1) on conserved *cis*-elements in the LHβ (*Lhb/LHB*) subunit promoter to drive its transcription (17, 18). EGR1 has little to no effect on FSHβ (*Fshb/FSHB*) expression, as seen in global *Egr1* knockout mice, which exhibit isolated LH deficiency (19, 20).

FSH production is selectively regulated by ligands in the TGFβ superfamily. The activin-class ligands, which include myostatin, growth differentiation factor 11, and the activins, stimulate *Fshb* expression by binding to complexes of activin type I and II receptors (21). The related gonadal hormones, inhibin A and B, antagonize activin-class ligand action by competitively binding and sequestering activin type II receptors, thereby decreasing FSH production (22–24). Inhibins help shape the dynamic changes in FSH across estrous and menstrual cycles (25). Circulating inhibin and FSH levels are negatively correlated (26–28). In rodents, the secondary FSH surge on the morning of estrus, which drives a new wave of follicle development, depends on post-ovulatory decreases in both inhibin A and B (29, 30).

Inhibins require co-receptors to avidly compete with activin-class ligands for binding to activin type II receptors

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(31). Gonadotrope cells express the inhibin co-receptors, betaglycan (32) and TGFBR3L (33). Betaglycan, which mediates both inhibin A and B actions (31, 34), is widely expressed and is required for normal heart and liver development in mice (35). In contrast, expression of the inhibin B-specific co-receptor, TGFBR3L, is restricted to gonadotropes (33). This is explained, in part, by SF-1, which regulates transcription through two binding sites in the murine *Tgfbr3l* and human *TGFBR3L* promoters. *Tgfbr3l* expression is lost in gonadotrope-specific SF-1 knockout mice (36). However, SF-1 is also present in the gonads, adrenal glands, and the ventromedial hypothalamus (37), indicating that it is necessary but not sufficient for gonadotrope-specific *Tgfbr3l/TGFBR3L* expression. Here, we report that GnRH stimulates *Tgfbr3l/TGFBR3L* transcription *via* EGR1, which acts in concert with SF-1 *via* a conserved promoter *cis*-element.

Results

Early growth response 1 can stimulate transcription through a cis*-element in the* Tgfbr3l/TGFBR3L *promoter in heterologous cells*

In our previous analysis of *Tgfbr3l/TGFBR3L* transcriptional regulation (36), we noted the presence of a candidate EGR1 binding site in the promoters of both mice and humans, between two conserved SF-1 *cis*-elements (Fig. 1*A*). As EGR1 and SF-1 synergistically stimulate *Lhb/LHB* transcription (17, 18), we asked whether EGR1 may similarly regulate *Tgfbr3l/TGFBR3L*. EGR1 overexpression in heterologous HEK293T cells stimulated both murine *Tgfbr3l* and human *TGFBR3L* promoter-reporter activities (Fig. 1, *B* and *C*). These effects were blocked by mutating the candidate EGR1 binding site (see base pairs in red in Fig. 1*A*) (Fig. 1, *B* and *C*). Overexpressed SF-1 similarly stimulated promoter activity in heterologous cells, as previously reported (36) (Fig. 1, *D* and *E*). When co-expressed, EGR1 and SF-1 synergistically activated the *Tgfbr3l/TGFBR3L* promoter-reporters (Fig. 1, *D* and *E*).

GnRH drives Tgfbr3l/TGFBR3L *transcription through EGR1 in homologous cells*

In gonadotropes, GnRH stimulates EGR1 expression (16, 17, 38). We therefore asked whether *Tgfbr3l/TGFBR3L* transcription is regulated by GnRH. First, using DNA affinity pulldown (DNAP) assays in homologous gonadotrope-like LβT2 cells, we showed that GnRH-induced EGR1 levels (Fig. 2*A*, lanes 4 and 7 to lane 1) and binding of the protein to 25-base-pair-long double-stranded DNA probes containing the putative EGR1 *cis*-elements in the murine *Tgfbr3l* (Fig. 2*A*, lane 5) and human *TGFBR3L* promoters (Fig. 2*A*, lane 8). This binding was not observed with probes containing mutations in the putative EGR1 binding sites (as described in Fig. 1*A*)

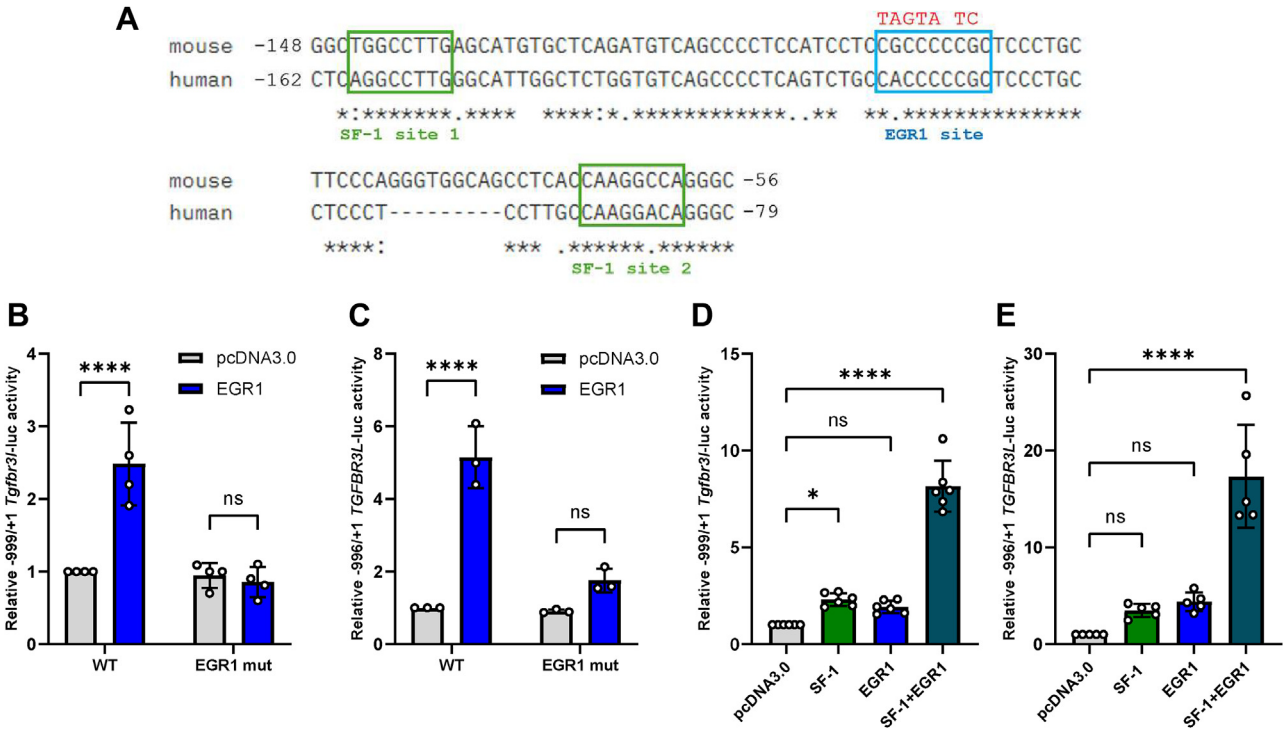

Figure 1. EGR1 stimulates murine and human *Tgfbr3l/TGFBR3L* transcription *via* a conserved *cis*-element in the promoter. *A*, alignment of the murine *Tgfbr3l* and human *TGFBR3L* promoters. The conserved SF-1 and EGR1 binding sites are boxed in *green* and *blue*, respectively. Mutated base pairs (in Figs. 1*B* and *C*, 2, *A*, *C* and *D*) are indicated above in *red*. HEK293T cells were transfected with 225 ng/well of the indicated murine −999/+1 *Tgfbr3l*-luc (*B*) or human −996/+1 *TGFBR3L*-luc (*C*) reporters, as well as 6.25 ng/well of either pcDNA3.0 (empty expression vector) or EGR1 expression vector. HEK293T cells were transfected with 225 ng/well of the wild-type murine (*D*) or human (*E*) reporter, as well as 25 ng/well of pcDNA3.0, SF-1, and/or EGR1 expression vectors. In *B*–*E*, protein lysates were collected, and reporter activity was measured by luciferase assay. Each dot on the graphs represents an independent experiment. Results are plotted as mean (bar heights) ± SD. Data were analyzed by two-way ANOVA followed by Sidak's multiple comparisons test in (*B* and *C*) or two-way ANOVA followed by Dunnett's multiple comparisons test in (*D* and *E*). *$p < 0.05$; ****$p < 0.0001$. EGR1 mut, mutated EGR1 site; ns, not significant; WT, wild-type.

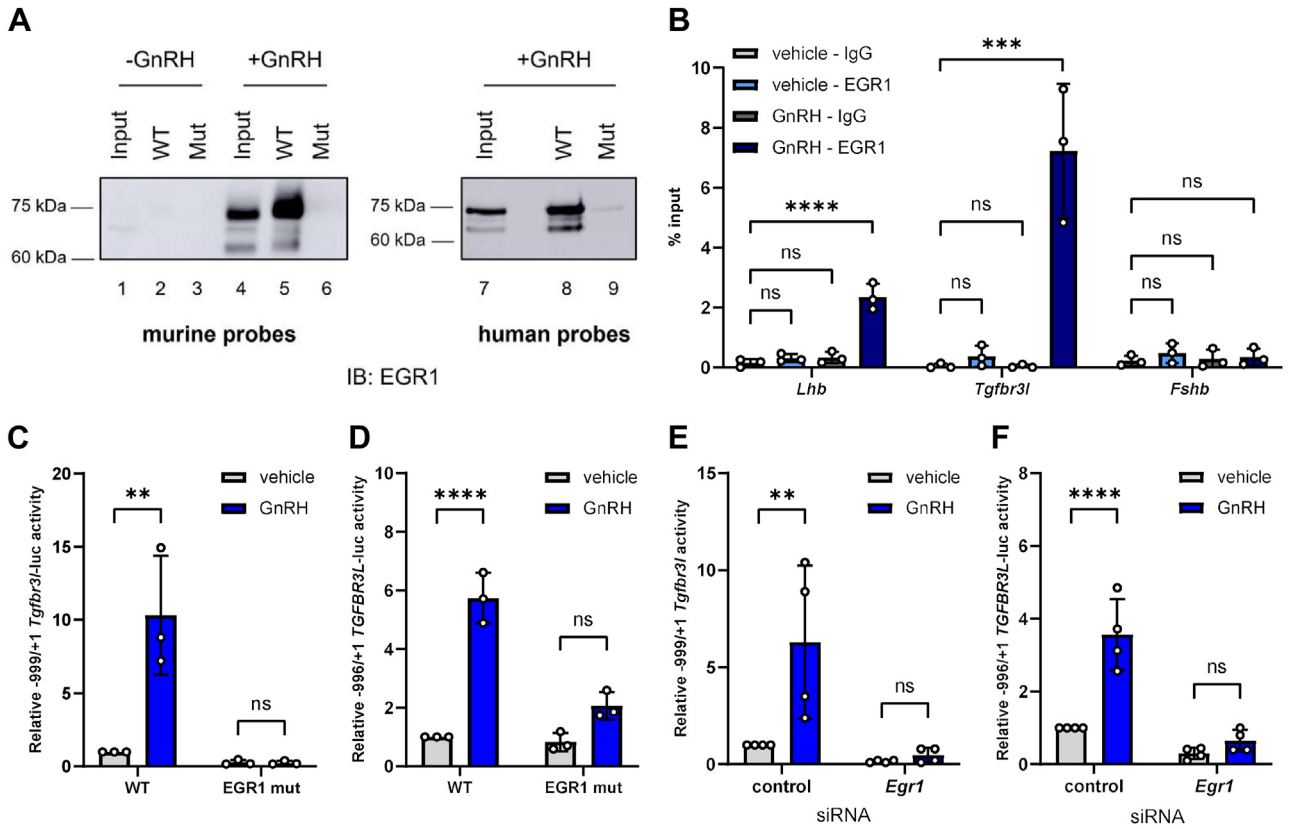

Figure 2. GnRH stimulates the murine *Tgfbr3l* and human *TGFBR3L* promoters through EGR1. *A*, DNAP using double-stranded DNA probes containing the wild-type and mutant EGR1 *cis*-elements in the murine *Tgfbr3l* and human *TGFBR3L* promoters. LβT2 cells were treated (+GnRH) or not (−GnRH) with 100 nM GnRH for 2 h and lysates were incubated with the indicated wild-type (WT) or mutant (Mut) probes. Whole-cell protein lysates from LβT2 cells (input) and proteins interacting with the probes were analyzed *via* immunoblot (IB) using an EGR1 antibody. *B*, chromatin immunoprecipitation analysis of EGR1 binding to the indicated promoter regions in LβT2 cells treated or not (vehicle) with 100 nM GnRH for 2 h. LβT2 cells were transfected with 225 ng/well of the indicated murine (*C*) or human (*D*) reporters. Cells were treated or not with 100 nM GnRH for 6 h prior to collection of lysates for luciferase assay. LβT2 cells were co-transfected with 225 ng/well of the wild-type murine (*E*) or human (*F*) reporters and 10 nM of control or *Egr1* siRNA. Cells were treated or not with 100 nM GnRH for 6 h prior to collection of lysates for luciferase assays. Dots, bar heights, and error bars are as defined in Figure 1. Data were analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test in (*B*) or two-way ANOVA followed by Sidak's multiple comparisons test in (*C–F*). $^{**}p < 0.01$; $^{****}p < 0.0001$. EGR1 mut, mutated EGR1 site; ns, not significant; WT, wild-type.

(Fig. 2*A*, lanes 6 and 9). Second, in chromatin immunoprecipitation assays performed in LβT2 cells, GnRH stimulated EGR1 recruitment to the murine *Tgfbr3l* and *Lhb* promoters, but not to the *Fshb* promoter (Fig. 2*B*). Finally, GnRH stimulated murine *Tgfbr3l* and human *TGFBR3L* promoter-reporter activities in LβT2 cells (Fig. 2, *C* and *D*). These effects were blocked by mutations in the putative EGR1 sites (as described in Fig. 1*A*) (Fig. 2, *C* and *D*) or by siRNA-mediated knockdown of endogenous *Egr1* (Fig. 2, *E* and *F*).

EGR1 regulates Tgfbr3l *expression* in vivo

To determine whether *Tgfbr3l* expression depends on EGR1 *in vivo*, we generated gonadotrope-specific *Egr1* knockout mice by crossing $Gnrhr^{GRIC}$ (39) to $Egr1^{fx/fx}$ mice. Recombination was observed in gonadotropes, a small number of non-GnRH neurons in the hypothalamus, and the male germline in GRIC mice, as previously described (40, 41) (Fig. 3*A*).

EGR1 is required for LH, but not FSH, production *in vivo*, as observed in global *Egr1* knockout mice (19, 20). Here, male and female conditional knockout mice ($Egr1^{fx/fx}$;$Gnrhr^{GRIC/+}$, hereafter cKO) displayed dramatic reductions in serum LH and pituitary *Lhb* mRNA relative to controls ($Egr1^{fx/fx}$) (Fig. 3, *B–D*). Serum FSH was modestly decreased in male but not female cKOs, but *Fshb* mRNA was unchanged in both sexes (Fig. 3, *B–D*). Expression of *Cga* and the GnRH receptor (*Gnrhr*) was higher in cKO mice, while *Nr5a1* levels did not differ between genotypes (Fig. 3, *C* and *D*). Of particular note in the current context, pituitary *Tgfbr3l* expression was decreased in both male and female cKOs (Fig. 3, *C* and *D*).

cKO males had smaller testes and seminal vesicles (Fig. 3, *E–G*) and decreased intratesticular testosterone relative to controls (Fig. 3*H*). cKO females had threadlike uteri and smaller ovaries than controls (Fig. 3, *I* and *J*). Their ovaries possessed antral follicles but lacked corpora lutea (Fig. 3*K*). cKO females had inhibin B levels equivalent to those of controls (Fig. 3*L*), suggesting that the number of growing follicles was not different between genotypes (42), consistent with the unchanged serum FSH levels.

Gonadotropin-releasing hormone regulates Tgfbr3l *expression* in vivo

The *in vitro* data indicated that GnRH stimulates *Tgfbr3l/TGFBR3L* transcription *via* EGR1. To determine whether

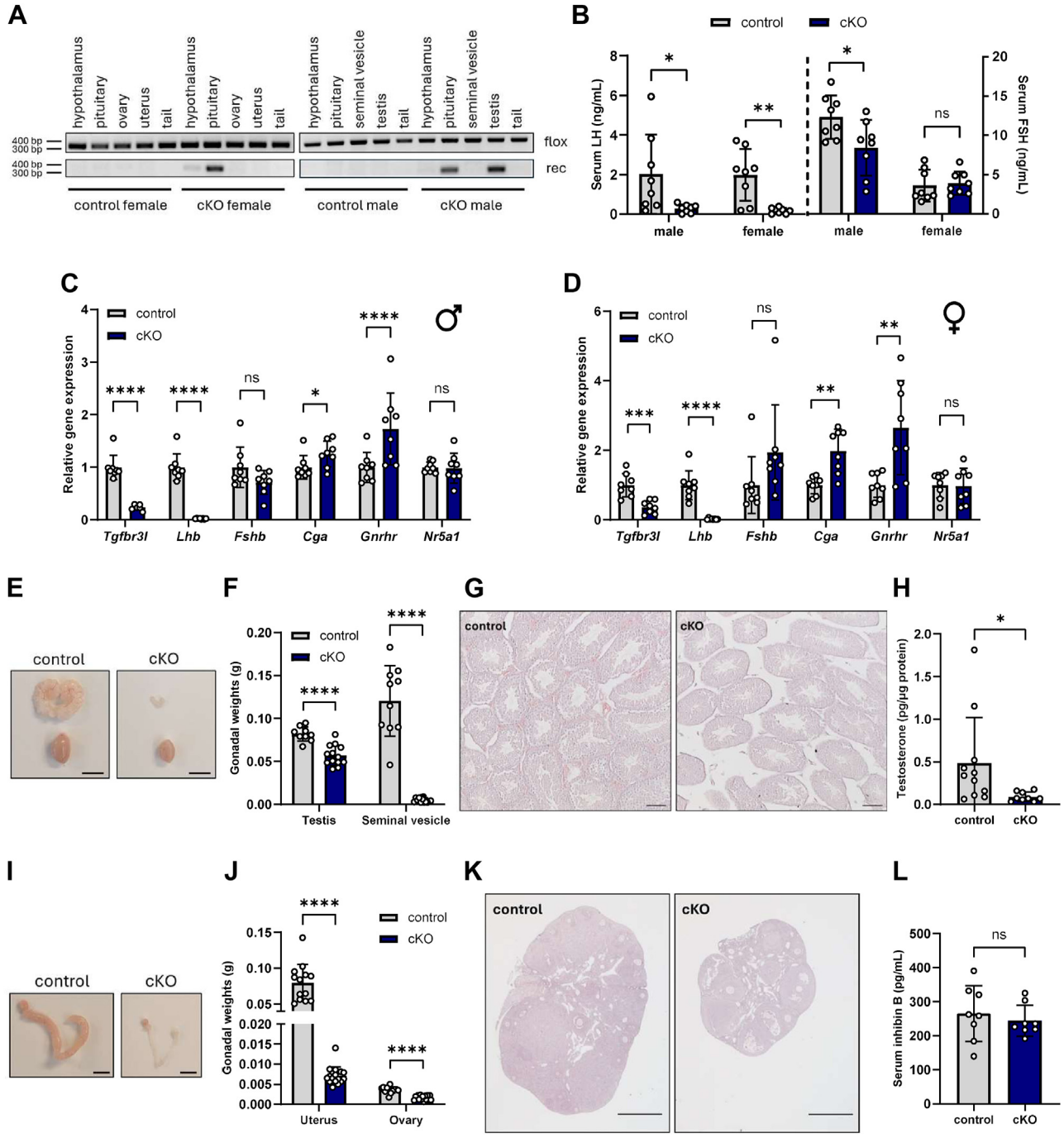

Figure 3. Gonadotrope-specific *Egr1* knockout mice exhibit decreased pituitary *Tgfbr3l* expression and LH deficiency. *A*, genomic DNA was extracted from the indicated tissues of *Egr1*$^{fx/fx}$ (control) and *Egr1*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$ (cKO) mice and analyzed by PCR for the presence of the floxed (flox) or recombined (rec) *Egr1* alleles. *B*, serum LH and FSH levels in male and female control and cKO mice. Pituitary expression of *Tgfbr3l*, *Lhb*, *Fshb*, *Cga*, *Gnrhr*, and *Nr5a1* in 8- to 10-week-old control and cKO male (*C*) and female (*D*) mice. Representative images (*E*) and weights (*F*) of testes and seminal vesicles from control and cKO males. Scale bars: 5 mm. *G*, testicular histology of sections stained with H&E. Scale bars: 100 μm. *H*, intratesticular testosterone levels normalized to total testicular protein. Representative images (*I*) and weights (*J*) of uteri and ovaries from control and cKO females. Scale bars: 5 mm. *K*, ovarian histology sections stained with H&E. Scale bars: 500 μm. *L*, serum inhibin B levels in female control and cKO mice. Each dot on the graphs represents an individual animal. Results are plotted as mean (bar heights) ± SD. Female data here and in all subsequent figures are from females at random stages of the estrous cycle (control) or acyclic females (mutant). Data were analyzed by two-tailed unpaired t-tests. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. ns, not significant.

GnRH similarly regulates *Tgfbr3l* expression *in vivo*, we first treated adult wild-type C57BL/6 male and female mice daily with vehicle or 1 mg/kg of the GnRH receptor antagonist, Cetrorelix, for 1 week. To compare responses in males and females, we first performed two-way ANOVA followed by Sidak's multiple comparisons test. Using this approach, Cetrorelix treatment significantly decreased pituitary *Tgfbr3l*, *Lhb*, *Fshb*, and *Gnrhr* mRNA levels in males but not females (Fig. 4, *A*–*D*). As expression of all these genes was higher in males than females, we suspected that the effect of sex might

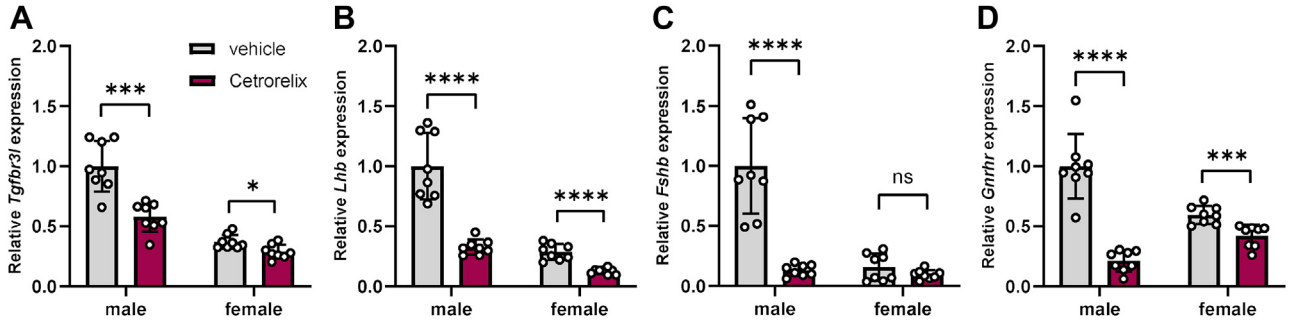

Figure 4. GnRH receptor antagonism decreases *Tgfbr3l* expression *in vivo*. Eight-week-old C57BL/6 male and female mice were injected daily with 1 mg/kg of Cetrorelix or vehicle for 1 week. Pituitaries were collected 24 h after the last injection. Pituitary expression of *Tgfbr3l* (*A*), *Lhb* (*B*), *Fshb* (*C*), and *Gnrhr* (*D*). Expression was normalized to vehicle-treated males. Dots, bar heights, and error bars are defined in Figure 3. Data from each sex were analyzed separately by two-tailed unpaired t-tests. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$. ns, not significant.

mask the treatment effect in females. Therefore, we then analyzed each sex separately using unpaired t-tests. Transcript levels of *Tgfbr3l*, *Lhb*, and *Gnrhr* were significantly decreased in both males and females following Cetrorelix treatment. The decrease in *Fshb* mRNA levels was significant in Cetrorelix-treated males but not females (Fig. 4, *A*–*D*).

Next, we measured the pituitary expression of the above genes in *hpg* mice, which carry an inactivating mutation in the *Gnrh1* gene (43, 44). Both male and female *hpg/hpg* mice had significantly lower pituitary *Tgfbr3l* expression compared to wild-type (+/+) males and females (Fig. 5*A*). *Fshb*, *Lhb*, and *Gnrhr* expression was similarly decreased, or trended downwards, in *hpg*/*hpg* mice (Fig. 5, *B*–*D*). Of note, wild-type males had higher *Tgfbr3l*, *Lhb*, and *Fshb* mRNA levels than wild-type females, but these sex differences were not present in *hpg/hpg* mice (Fig. 5, *A*–*C*).

Post-gonadectomy increases in Tgfbr3l *expression are EGR1-dependent*

Finally, we asked whether increased GnRH signaling would alter *Tgfbr3l* expression in an EGR1-dependent manner. Gonadectomy increases GnRH pulse frequency by removing steroid negative feedback on the GnRH pulse generator (45–50). Pituitary *Tgfbr3l* expression was elevated in gonadectomized control ($Egr1^{fx/fx}$) but not cKO animals (Fig. 6*A*). As expected, in controls, gonadectomy increased pituitary *Lhb* expression in both sexes; these effects were blocked in cKO mice (Fig. 6*B*). Similarly, serum LH was significantly elevated or trended upwards in control, but not cKO animals post-gonadectomy (Fig. 6*C*). Both control and cKO females exhibited post-ovariectomy increases in pituitary *Fshb* mRNA and serum FSH (Fig. 6, *D* and *E*). *Fshb* expression and serum FSH were increased post-castration in cKO, but not control males (Fig. 6, *D* and *E*). *Gnrhr* expression was unaltered by gonadectomy in control and cKO animals of both sexes, though we again observed elevated levels in cKO females (Fig. 6*F*).

Discussion

Here, we show that GnRH stimulates *Tgfbr3l/TGFBR3L* transcription by inducing the expression of EGR1, which binds in concert with SF-1 to conserved *cis*-elements in the murine and human promoters. This bears a remarkable resemblance to the mechanism through which GnRH regulates *Lhb/LHB* transcription in multiple species (17, 18). Thus, the cooperative actions of EGR1 and SF-1 underlie the cell-specific expression of two gonadotrope-restricted genes and also explain, at least in part, their regulation by GnRH. Interestingly, this is not the case for two other gonadotrope-specific and GnRH-regulated genes, *Fshb/FSHB* and *Gnrhr/GNRHR*, which rely on SF-1, but not EGR1 for their expression (19, 20, 36, 51, 52). These data suggest that there may be a physiological need for the coordinated regulation of LH and TGFBR3L synthesis by GnRH.

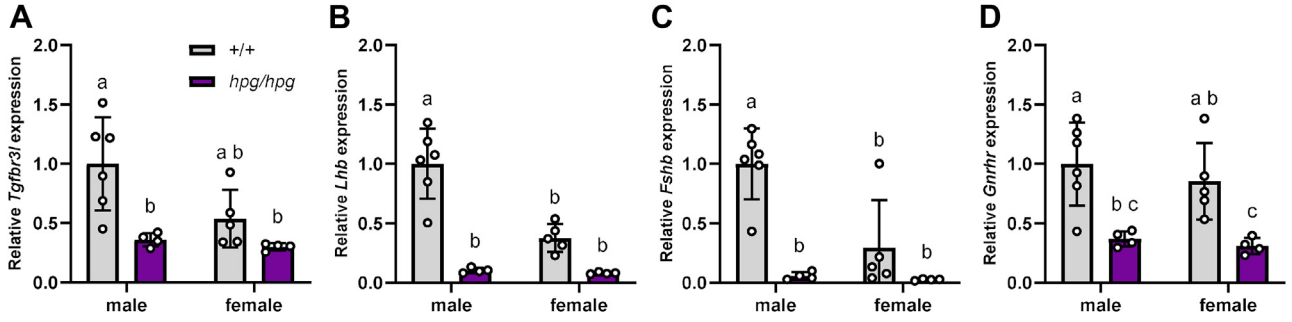

Figure 5. GnRH-deficient mice have decreased *Tgfbr3l* expression. Pituitary expression of *Tgfbr3l* (*A*), *Lhb* (*B*), *Fshb* (*C*), and *Gnrhr* (*D*) in 9- to 12-week-old +/+ (wild-type) and *hpg/hpg* males and females. Expression was normalized to wild-type males. Dots, bar heights, and error bars are defined in Figure 3. Data were analyzed by two-way ANOVA followed by Sidak's multiple comparisons test. Bars with different letters differ significantly.

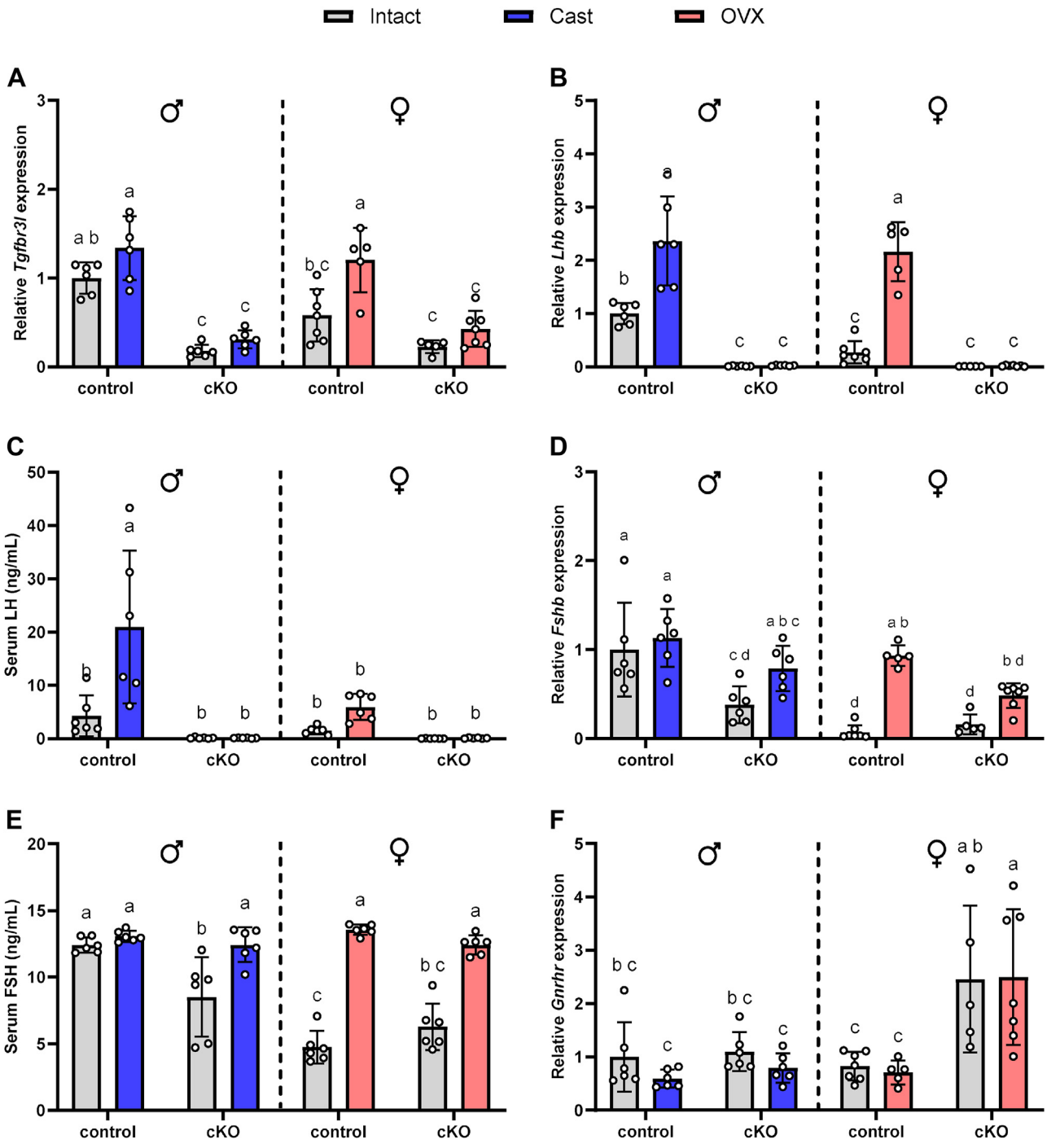

Figure 6. Gonadectomy increases *Tgfbr3l* expression in an EGR1-dependent manner. Nine- to 10-week-old *Egr1*$^{fx/fx}$ (control) and *Egr1*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$ (cKO) mice underwent bilateral gonadectomy (Cast or OVX) or remained intact. Pituitaries and serum were collected 2 weeks post-surgery. *A*, pituitary expression of *Tgfbr3l*. *B*, pituitary expression of *Lhb*. *C*, serum LH levels. *D*, pituitary expression of *Fshb*. *E*, serum FSH levels. *F*, pituitary *Gnrhr* expression. Gene expression was normalized to the intact control males. Dots, bar heights, and error bars are as defined in Figure 3. Data were analyzed by three-way ANOVA followed by Sidak's multiple comparisons test. Bars with different letters differ significantly.

Though the mechanisms underlying *Tgfbr3l* and *Lhb* transcription are similar, there are also clear differences. For example, there are two EGR1 binding sites in the *Lhb/LHB* promoter (17, 18), while we only identified a single EGR1 site in the murine *Tgfbr3l* and human *TGFBR3L* promoters. We nevertheless observed robust enrichment of EGR1 on the murine *Tgfbr3l* promoter following GnRH treatment in LβT2 cells. Another difference is that gonadotrope-specific inactivation of *Egr1* led to the almost complete loss of *Lhb*, while *Tgfbr3l* was reduced but still expressed in the pituitaries of these mice. Thus, EGR1 is required for *Lhb* but not *Tgfbr3l* transcription *in vivo*. In contrast, the inactivation of *Nr5a1* (SF-1) in gonadotropes led to the absence of both *Lhb* and *Tgfbr3l* (36). In the latter case, SF-1 deficiency is associated

with compaction of the *Tgfbr3l* promoter (36). This suggests that SF-1 might possess pioneer activity on this gene and that EGR1 binds to the promoter and stimulates transcription only after it is rendered accessible by SF-1. Pioneer activity often occurs in enhancers (53–55). Using single-nucleus ATAC-sequencing data from murine and human pituitaries (56, 57), we searched for potential enhancers by co-accessibility in gonadotropes. Though the results of the analysis were inconclusive, we cannot rule out a role for enhancers (or SF-1 and EGR1 actions therein) in *Tgfbr3l/TGFBR3L* transcription.

Another difference between *Lhb* and *Tgfbr3l* regulation may relate to a third transcription factor, PITX1, which partners with SF-1 and EGR1 to stimulate *Lhb/LHB* transcription (17, 18). We were unable to locate a PITX1 binding site in the *Tgfbr3l/TGFBR3L* promoters, though this does not rule out a role for the protein (or the related PITX2) in the expression of the genes. PITX1 could, for example, regulate transcription through a tethering mechanism (*i.e.*, *via* association with SF-1 or EGR1) (17, 58) or by binding a non-canonical *cis*-element. This will be important to explore moving forward, as the combination of SF-1 and EGR1 is likely insufficient to fully account for the gonadotrope-specific expression of *Tgfbr3l/TGFBR3L*. That is, SF-1 and EGR1 are co-expressed in other cell types, including somatic cells in the gonads (59, 60). To our knowledge, there are no gonadotrope-specific knockout mouse models of PITX1 or PITX2. Therefore, we currently lack insight into the potential roles of these proteins in *Tgfbr3l* expression *in vivo*.

In our initial characterization of TGFBR3L (33), and again here, we observed sex differences in pituitary *Tgfbr3l* expression, with higher levels in males. Though we currently lack a mechanistic explanation, it is tempting to propose a role for sex differences in GnRH signaling. That is, it is possible that GnRH induces greater increases in EGR1 expression in male relative to female gonadotropes, which in turn would be expected to drive greater induction of *Tgfbr3l* transcription in males. This will be difficult to assess experimentally, given the transient nature of *Egr1* expression and the low abundance of gonadotropes in the pituitary. Nevertheless, the extant data are consistent with our hypothesis. First, both *Tgfbr3l* and *Lhb* mRNA levels are higher in males than females. Second, *Tgfbr3l* levels are comparable in pituitaries of male and female *hpg* (GnRH-deficient) mice. It is also possible that gonadal hormones may contribute to the observed sex difference. Indeed, gonadectomized control males and females have similar *Tgfbr3l* mRNA levels. In both sexes, post-gonadectomy increases in *Tgfbr3l* expression were EGR1-dependent, suggesting that any gonadal hormone (steroid) effects are, at least partly, mediated *via* feedback on GnRH secretion.

Finally, as mentioned earlier, the apparent co-regulation of LH and TGFBR3L synthesis may be physiologically relevant. GnRH differentially regulates LH and FSH synthesis and secretion in a frequency-dependent manner. Enhanced stimulation of LH at higher GnRH pulse frequencies is almost certainly explained by the greater induction of EGR1 under these conditions (16). Why FSH production is not similarly increased at high GnRH pulse rates is less clear. This stems, in part, from the lack of a clearly defined mechanism through which GnRH regulates *Fshb* transcription (61, 62). Different models of pulse frequency decoding have been proposed based mainly on work in cultured cells (8, 12, 13, 63). None, to our knowledge, have been validated *in vivo* and others (*e.g.*, differential Gα protein activation) have been refuted (62, 64, 65). Based on the data presented here, we propose that increases in GnRH pulse frequency, as occur at the luteal to follicular phase transition during the menstrual cycle, will stimulate increases in *TGFBR3L* expression, enhancing gonadotrope sensitivity to inhibin B. This, in turn, may contribute to suppression of FSH later in the follicular phase, as inhibin B levels increase (25). Similarly, in women with polycystic ovary syndrome, LH levels and pulse frequency are increased (14), whereas FSH levels are reduced or unchanged (15). Concomitant increases in TGFBR3L would render their gonadotropes more sensitive to the FSH suppressing/restraining effects of inhibin B, which may or may not be elevated in this condition (66–68).

In conclusion, the gonadotrope-restricted expression of the inhibin B co-receptor, TGFBR3L, is explained, at least in part, by the cooperative actions of the transcription factors SF-1 and EGR1. EGR1 expression in gonadotropes is GnRH-dependent, linking *Tgfbr3l* transcription and inhibin B regulation of FSH to GnRH signaling. Whether and how other hormones, including gonadal steroids, regulate TGFBR3L has yet to be determined. It is also unclear whether additional transcription factors, such as PITX1 or PITX2, contribute to the gonadotrope-specific expression of TGFBR3L.

Experimental procedures

DNA constructs

The wild-type murine −999/+1 *Tgfbr3l*- and human −996/+1 *TGFBR3L*-luciferase promoter–reporters (36) and the murine SF-1 and EGR1 expression constructs (18) were previously described. The mutant promoter–reporters were constructed using a modified QuikChange protocol with PfuTurbo DNA Polymerase (200,250, Agilent Technologies) and primers described in Table 1. All constructs were confirmed by sequencing (Génome Québec).

Cell culture and promoter-reporter assays

Human embryonic kidney (HEK) 293T cells (ATCC CRL-3216; RRID:CVCL_0063; provided by Dr Terry Hébert, McGill University) were cultured in Dulbecco's modified Eagle's medium (DMEM; 319–005-CL, Wisent) supplemented with 5% (v/v) fetal bovine serum (FBS; 098150, Wisent). Immortalized murine gonadotrope-like LβT2 cells (69) (RRID:CVCL_0398; provided by Dr Pamela Mellon, University of California) were cultured in DMEM supplemented with 10% (v/v) FBS. All cells were cultured at 37 °C in a humidified incubator with 5% CO_2 and certified negative for *mycoplasma*. The authenticity of the LβT2 cells was confirmed as previously described (70).

HEK293T and LβT2 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

Table 1
Primers

	Sequence
Tgfbr3l/TGFBR3L mutagenesis	
Tgfbr3l EGR1 site mut F	GATGTCAGCCCCTCCATCCTCTAGTACTCCTCCCTGCTTCCCAG GGTGGC
Tgfbr3l EGR1 site mut R	GCCACCCTGGGAAGCAGGGAGGAGTACTAGAGGATG GAGGGGCTGACATC
TGFBR3L EGR1 site mut F	GTGTCAGCCCCTCAGTCTGCTAGTACTCCTCCCTGCCTCCCTCCTTGC
TGFBR3L EGR1 site mut R	GCAAGGAGGGAGGCAGGGAGGAGTACTAGCAGACTGAGGGGCTGACAC
qPCR primers	
Cga F	TCCCTCAAAAAGTCCAQGAGC
Cga R	GAAGAGAATGAAGAATATGCAG
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
Fshb EGR1 ChIP qPCR F	GGAGTGTTCAGTCTGTTCTTGG
Fshb EGR1 ChIP qPCR R	CCCACTCCCTCACCTTGTAA
Lhb EGR1 ChIP qPCR F	CAATTCACTGGGACACTGGA
Lhb EGR1 ChIP qPCR R	TTGGGCACCTGGCTTTATAC
Tgfbr3l EGR1 ChIP qPCR F	TCAGTACATCAAGAAAGCCC
Tgfbr3l EGR1 ChIP qPCR R	GTACCCAGCCCTCTAGGT
Genotyping primers	
Egr1 geno F	TCAAGGTGTTTTTCAGCCTGAGT
Egr1 geno R	GATTGGATTGGCCACCACCTCTGGC
Egr1 rec F	TTTGATGAGCGGGGTTGCCC
GRIC F	CCTGGAAAATGCTTCTGTCCG
GRIC R	CAGGGTGTTATAAGCAATCCC
hpg geno F	TGAGATGCAAACACACCACTCT
hpg geno R1	AGGCTTGGAGAGCTGTAAGG
hpg geno R2	GTTTCAGTGCATCCTCTCAGG

polyethylenimine (PEI) at a ratio of 1:3 (μg DNA to μg PEI). LβT2 cells were transfected using Lipofectamine 3000 (L3000015, ThermoFisher Scientific) following the manufacturer's protocol. Control (D-001210–05) and *Egr1* (D-040286–01) short interfering RNAs (siRNAs) were purchased from Dharmacon and previously validated (18). For assays where siRNAs and the promoter-reporter were co-transfected, the pA3-luc (rather than pGL3-Basic) backbone was used and the p3000 reagent was excluded, as per the manufacturer's protocol. Twenty-four hours after transfection, cells were serum starved for an additional 24 h. Following serum starvation, HEK293T cells were lysed and LβT2 cells were treated for 6 h with vehicle or GnRH (100 nM) (L7134, Sigma-Aldrich) and then lysed. Cells were lysed in 50 μl/well passive lysis buffer (25 mM Tris-phosphate [pH 7.8], 10% [v/v] glycerol, 1% [v/v] Triton X-100, 1 mg/ml bovine serum albumin (BSA), 2 mM ethylenediaminetetraacetic acid [EDTA]) for 10 min at room temperature with agitation. Twenty microliters of cell lysis supernatant were combined with 100 μl of assay buffer (15 mM potassium phosphate [pH 7.8], 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EDTA, 2 mM adenosine triphosphate, 1 mM dithiothreitol, 0.04 mM D-luciferin), and luciferase activity was measured on an Orion II microplate luminometer (Berthold Detection Systems). All experiments were performed in technical triplicates, and the experiments were repeated as indicated in the figures.

DNA affinity pull-down

The wild-type and mutant biotinylated double-stranded DNA probes (see Table 2) were immobilized to Dynabeads M-280 (11205D, Dynal, Invitrogen). For this, 30 μl of Dyanabeads were washed three times with 2x B&W buffer (2x; 10 mM Tris, pH 7.5, 1 mM EDTA, 2 M NaCl), and then

Table 2
DNAP probes

	Sequence
Tgfbr3l EGR1 site WT sense	ATCCTCCGCCCCCGCTCCCTGCTTC
Tgfbr3l EGR1 site WT antisense	GAAGCAGGGAGCGGGGGCGGAGGAT
Tgfbr3l EGR1 site mut sense	ATCCTCTAGTACTCCTCCCTGCTTC
Tgfbr3l EGR1 site mut antisense	GAAGCAGGGAGGAGTACTAGAGGAT
TGFBR3L EGR1 site WT sense	GTCTGCCACCCCCGCTCCCTGCCTC
TGFBR3L EGR1 site WT antisense	GAGGCAGGGAGCGGGGGTGGCAGAC
TGFBR3L EGR1 site mut sense	GTCTGCTAGTACTCCTCCCTGCCTC
TGFBR3L EGR1 site mut antisense	GAGGCAGGGAGGAGTACTAGCAGAC

incubated with 10 μM of wild-type or mutant probes in 1x B&W buffer at room temperature for 15 min. Beads were washed twice with 2x B&W buffer and once with 1x binding buffer (5% [v/v] glycerol, 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.15% Triton X-100, 100 mM NaCl, 4 mM $MgCl_2$), blocked for 30 min at room temperature using 1% (w/v) BSA in binding buffer, and lastly resuspended in 50 μl of 1x binding buffer. LβT2 cells were grown until confluent in 10-cm plates, treated with vehicle or 100 nM GnRH for 1 h, 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 phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin and aprotinin). One hundred μL of clarified lysates were combined in a 500-μL reaction with 100 μl of 5x binding buffer, 10 μl of 0.5 μg/μl salmon sperm DNA (15632011, Invitrogen), and 50 μl of DNA-bound streptavidin magnetic beads. The reactions were incubated at 4 °C for 2 h with agitation, followed by five washes in 1x binding buffer. Bound proteins were eluted in 40 μl of 0.1% sodium dodecyl sulfate (SDS) [w/v] at 100 °C for 5 min and resolved by SDS-PAGE on an 8% resolving gel using a 30% (w/w) acrylamide/bis-acrylamide (29:1) solution. Immunoblotting of the eluted proteins was performed as described previously (36), using an antibody against EGR1 (1:500, C-19, sc-189, Santa Cruz Biotechnology; RRID:AB_2231020) and a horseradish peroxidase–conjugated anti-rabbit secondary antibody (1:5000, 1706515, Bio-Rad Laboratories; RRID:AB_11125142). Bands were visualized with enhanced chemiluminescence substrate (NEL105001, PerkinElmer) and an Amersham Imager 600 (GE Healthcare). EGR1 antibody specificity was demonstrated both by the protein size in western blot and the dependency of its expression on GnRH treatment in these cells (17).

Chromatin immunoprecipitation (ChIP)

LβT2 cells were grown until confluent in 10-cm plates, treated with vehicle or 100 nM GnRH for 2 h, and then crosslinked with 1% formaldehyde in DMEM for 10 min at room temperature. The reaction was quenched with 125 mM glycine for 5 min at room temperature. Cells were removed from the plates with cell scrapers and collected in 1 ml of ice-cold phosphate-buffered saline (PBS) and centrifuged at 800*g* for 10 min at 4 °C. The cell pellets were resuspended in 1 ml of cell lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 0.5 mM ethylene-bis(oxyethylenenitrilo)tetraacetic acid [EGTA], 0.25% [v/v] Triton X-100, 1 mM PMSF, 1 × protease inhibitor cocktail [4693116001, Sigma-Aldrich]) and incubated for 10 min on ice, and then centrifuged at 800*g* for 10 min at 4 °C. The pelleted nuclei were resuspended in 100 μl of nuclei lysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% SDS [w/v], 1 mM PMSF, 1 × protease inhibitor cocktail), incubated for 15 min on ice, and sonicated for 15 min (30 s on/30 s off) using a Bioruptor Standard (UCD-200, Diagenode). Sheared chromatin was pelleted at 15,000*g* for 10 min at 4 °C. Ten percent of the chromatin was removed and kept as "input." Twenty μg of proteinase K and NaCl to a final concentration of 0.3 M were combined with the input sample in a final volume of 200 μl, and the protein:DNA complexes were reverse crosslinked overnight at 65 °C. The next day, samples were incubated with 10 μg of RNase A in a final volume of 200 μl for 30 min at 37 °C. 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 a Nanodrop spectrophotometer.

Dynal protein G beads (10003D, Invitrogen) were blocked with salmon sperm DNA (7 ng salmon sperm DNA/μl of beads) and BSA (0.1 μg BSA/μl of beads) for 30 min at room temperature. Ten μg of chromatin (determined by concentration of "input" sample) were diluted 1:10 in dilution buffer (11.7 mM Tris-HCl [pH 8.0], 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS [w/v], 1.1% [v/v] Triton X-100) and precleared for 30 min at 4 °C with 10 μl of blocked protein G beads on an end-over-end rotator, and then incubated at 4 °C with 1 μg of either rabbit IgG (2729, Cell Signaling Technology; RRID:AB_1031062) or rabbit anti-EGR1 (44D5, Cell Signaling Technology; RRID:AB_2097035) overnight. The next day, the antigen:antibody complexes were incubated with 15 μl of blocked protein G beads for 2 h at 4 °C on an end-over-end rotator. Beads were then sequentially washed with agitation in dilution buffer (1 × 5 min at 4 °C), low salt buffer (20 mM Tris-HCl [pH 8.0], 2 mM EDTA, 150 mM NaCl, 0.1% SDS [w/v], 1% [v/v] Triton X-100) (1 × 5 min at 4 °C), high salt buffer (20 mM Tris-HCl [pH 8.0], 2 mM EDTA, 500 mM NaCl, 0.1% SDS [w/v], 1% [v/v] Triton X-100) (3 × 5 min at 4 °C), LiCl buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 250 mM LiCl, 1.43% NP-40 [v/v], 1% [v/v] sodium deoxycholate) (1 × 5 min at 4 °C), and TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) (1 × 5 min at 4 °C). Chromatin was eluted for 30 min at 65 °C in 200 μl of elution buffer (100 mM $NaHCO_3$, 1% SDS [w/v]), reverse crosslinked, and digested with RNase A and proteinase K as above. 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, and dissolved in 30 μl of 10 mM Tris-HCl (pH 8.0). Input and immunoprecipitated chromatin were analyzed using qPCR as described below, using primers in Table 1.

Animals

All animal work was conducted in accordance with federal and institutional guidelines and with the approval of the McGill University Animal Care Committee DOW-A (protocol 5204). All animals were housed on a 12 h light:12 h dark cycle and given access to food and water *ad libitum*.

Egr1$^{fx/fx}$ males (MGI:6316290) were crossed with *Gnrhr*$^{GRIC/GRIC}$ (39, 40) females to produce *Egr1*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ progeny. *Egr1*$^{fx/fx}$ males were then crossed with *Egr1*$^{fx/+}$;*Gnrhr*$^{GRIC/+}$ females to produce *Egr1*$^{fx/fx}$;*Gnrhr*$^{+/+}$ (control) and *Egr1*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$ (conditional knockout; cKO) animals. *hpg/hpg* animals (43) were produced by intercrossing *hpg/+* males and females to obtain +/+ (wild-type) and *hpg/hpg*

littermates. Mice were genotyped by PCR using primers in Table 1.

Organ collection

Pituitaries, testes, seminal vesicles, ovaries, and uteri were dissected from 8- to 9-week-old control (*Egr1*$^{fx/fx}$) and cKO (*Egr1*$^{fx/fx}$;*Gnrhr*$^{GRIC/+}$) animals. Control females were collected at random points in the estrous cycle; cKO females were acyclic. All reproductive organs were weighed on an analytical balance and either snap frozen in liquid nitrogen for storage at −80 °C, or fixed in 10% neutral buffered formalin (HT501128, Sigma-Aldrich) overnight at 4 °C and stored in 70% ethanol. Pituitary glands were snap frozen in liquid nitrogen and stored at −80 °C until analysis.

Pituitaries were dissected from 9- to 12-week-old wild-type and *hpg/hpg* males and females, snap-frozen in liquid nitrogen, and stored at −80 °C until further analysis. Wild-type females were collected at random points in the estrous cycle; *hpg/hpg* females were acyclic.

Histochemical staining

Fixed ovaries and testes were dehydrated in a series of graded ethanol baths (80%, 1 × 1 h; 95%, 1 × 1 h; 100%, 2 × 1 h), cleared with Histoclear (NDIHS-200, Diamed) for 2 × 30 min, and then embedded in paraffin (18–604–991, ThermoFisher Scientific). Sections were cut at a thickness of 7 µm using a Shandon Finesse 325 microtome.

For hematoxylin and eosin (H&E) staining, tissue sections were cleared with Histoclear (2 × 5 min) and rehydrated in graded ethanol baths (100% and 70%, 5 min each). Slides were stained with hematoxylin (Gill No.3, GHS332, Sigma-Aldrich) and eosin (AC611815000, ThermoFisher Scientific), dehydrated in graded ethanol baths (70% and 100%, 5 min each), cleared in Histoclear (2 × 5 min), and mounted with Permount (SP15–100, ThermoFisher Scientific). H&E images were acquired with a Zeiss AxioImager M2 Imaging microscope equipped with a Zeiss Axiocam 506 Color camera and Zeiss ZenPro software v3.11 (Zeiss Canada Ltd).

Cetrorelix treatment

Eight-week-old C57BL/6 males and females (Charles River Laboratories) were injected once daily intraperitoneally (i.p.) with 1 mg/kg Cetrorelix (C5249, Sigma-Aldrich) or saline for a total of 7 days. Cardiac blood and pituitaries were collected 24 h after the last injection, and pituitaries were snap-frozen in liquid nitrogen and stored at −80 °C until further analysis.

Gonadectomy

Ovariectomies and castrations were performed on 9- to 10-week-old mice under isoflurane general anesthesia with standard aseptic techniques following McGill standard operating procedures #206 and #207. The same procedures were applied to sham-operated females except that the gonads were not removed. Intact males did not undergo sham surgery. Pituitaries and cardiac blood were collected 2 weeks post-gonadectomy. Pituitaries were snap-frozen in liquid nitrogen and stored at −80 °C until further analysis.

Blood collection and hormone analyses

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 temperature. Serum was collected and stored at −20 °C until further analysis.

Serum LH and FSH were measured using in-house sandwich enzyme-linked immunosorbent assays as previously described (detection ranges, 0.117–30 ng/ml and 0.03125–0.5 ng/ml, respectively) (71, 72). Serum inhibin B was measured by ELISA (AL-163, AnshLabs; detection range 6–1143 pg/ml).

Testes were manually homogenized in PBS and testosterone was extracted according to the manufacturer's protocol. Intratesticular testosterone was measured by ELISA (582,701, Cayman Chemical Company; detection range 3.9–500 pg/ml) and normalized to total testicular protein (measured using a Pierce BCA protein assay kit [23,227, ThermoFisher Scientific]).

RNA extraction and reverse transcription quantitative PCR

RNA was extracted from tissues using TRIzol Reagent (15596018; Invitrogen) following the manufacturer's protocol. Pituitaries were homogenized in 500 µl TRIzol using a Polytron PT10-35 homogenizer. Two hundred nanograms of total RNA (concentration determined using Nanodrop spectrophotometer) were reverse transcribed using random hexamers (C1181, Promega) and MMLV reverse transcriptase (M1701, Promega).

qPCR analysis was performed using BlasTaq (G891, Applied Biological Materials Inc) and primers listed in Table 1 on a Corbett Rotorgene 600 instrument (Corbett Life Science). Relative 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.

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 10 software. Luciferase assays and ChIP assays were analyzed by one-way or two-way analysis of variance (ANOVA), followed by *post hoc* multiple comparisons tests. For *in vivo* studies, unpaired t-tests or two-way ANOVA followed by Sidak's multiple comparisons test were used to assess statistical significance among experimental groups. Alpha was set at $p < 0.05$. F values, p values, and degrees of freedom from two- and three-way ANOVAs are reported in Table S1. In histograms, bar heights are group means (±SD). Individual data points reflect experimental replicates or individual animals.

Data availability

All data are available in the manuscript.

Supporting information—This article contains supporting information.

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Abbreviations—The abbreviations used are: CGA, chorionic gonadotropin alpha subunit; DNAP, DNA affinity pulldown; EGR1, early growth response 1; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; PITX1, paired-like homeodomain transcription factor 1.

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