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Forkhead Protein, FoxL2, In Activin Regulation of FSH Gene Expression

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in

Biology

by

Patrick S. Corpuz

Committee in Charge:

Professor Pamela L. Mellon, Chair
Professor Paul Price, Co-Chair
Professor Chris Armour

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The thesis of Patrick S. Corpuz is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2009

This work is dedicated to my family and friends. Thank you for giving me the strength and support to carry on this journey and for bringing balance to my life.

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LIST OF ABBREVIATIONS

α-GSU	alpha glycoprotein subunit
ACTH	adrenocorticotropin hormones
ActR	activin receptor
ALK	activin receptor-like kinase
AP1	activating protein 1
ARE	androgen response element
BMP	bone morphogenetic protein
BPES	Blepharophimosis Ptosis Epicanthus Inversus Syndrome
CoSmad	common-mediator smad
DEBP	distal element-binding proteins
EMSA	electrophoretic mobility shift assay
FAST	forkhead transcription factor FoxH1
Fox	forkhead
FSH	follicle stimulating hormone
GH	growth hormone

GnRH	gonadotropin-releasing hormone
GnRHR	gonadotropin-releasing hormone receptor
GRAS	GnRH activating sequence
GRE	glucocorticoid response elements
HPG	hypothalamic-pituitary-gonadal axis
HRE	hormone response element
ISmad	inhibitory smad
LH	luteinizing hormone
Lhx3	LIM-homeodomain transcription factor gene
MAPK	mitogen-activated protein kinase
MH1-SBE	mad-homology 1 domain smad-binding element
MSH	melanocyte-stimulating hormone
NFY	nuclear factor Y
NLS	nuclear localization signals
oFSHβ	ovine FSHß subunit
Pbx1	pre B-cell leukemia transcription factor-1

Pitx2	paired-like homeodomain transcription factor-2
Prep1	Pbx regulating protein-1
PRL	prolactin
Prop1	Prophet of Pit1 homeodomain factor-1
RSmad	receptor-regulated Smad
RUNX	Runt-related transcription factor
SBE	smad binding element
SF1	steroidogenic factor-1
TAB1	TGFβ-activated kinase binding protein-1
TAK1	TGFβ-activated kinase-1
TALE	three-amino-acid loop extension
TGFβ	transforming growth factor-β tonight
TSH	thyroid stimulating hormone

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ABSTRACT OF THE THESIS

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In the anterior pituitary, activin is a potent inducer of the FSHβ gene. The gene is a critical subunit that regulates the synthesis of FSH and thus regulates reproduction. Smad3 is a transcription factor that conveys activin responsiveness, and we have identified a novel region of the FSHβ promoter as a major site of Smad3 action. In this region, we have identified a protein of the Forkhead (Fox) family of transcription factors that binds to a site. The activity for the FoxL2 transcription factor is located at -350 from the transcriptional start site. This site is critical for induction by activin, Smad3

and activin receptor, ALK7. FoxL2 is a novel player in pituitary gene expression that has been shown to interact with Smad3 and play a role in ovary development and therefore reproduction. Furthermore, we identified other FoxL2 binding sites at -200 and -153 that bind FoxL2 with varying affinity. These sites also play a role in activin and Smad3 induction. In the porcine promoter, the -153 site is a high affinity binding site for FoxL2, while in the human promoter, the -200 site confers higher affinity binding. Nevertheless, both sites confer a role in activin induction. Therefore, FoxL2 is a critical player for activin regulation of the FSHβ gene in multiple species, although the affinity of the sites may vary.

I

Introduction

The Hypothalamic-Pituitary-Gonadal Axis

The hypothalamic-pituitary-gonadal (HPG) axis is essential for mammalian sexual maturation and reproduction and controls several pivotal stages of the life cycle including sexual maturation, puberty, the menstrual cycle, pregnancy, and menopause. The hypothalamus is the main regulatory center that controls the release of trophic hormones from its neurons, including the decapeptide, gonadotropin-releasing hormone (GnRH), into the hypophyseal portal system, which is a set of blood vessels that directly delivers the hypothalamic peptide hormones to the anterior pituitary. GnRH is released in a pulsatile manner and traverses the portal system binding to and activating a seven-transmembrane GnRH receptor (GnRHR) located on the surface of gonadotrope cells within the anterior pituitary. This G-protein coupled receptor transfers the ligand-binding signal across the membrane and targets specific proteins in the transduction pathways that lead to the transcription and secretion of the gonadotropin hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH are heterodimeric glycoproteins that are released in a tightly regulated manner

into the circulation to target the gonads where they mediate folliculogenesis, steroidogenesis and gametogenesis. Specifically, FSH acts on the testes to regulate sperm production in the male and on the ovaries to regulate follicular growth and development in the female. LH increases testosterone production from the Leydig cells of males, while in females, LH regulates ovulation as well as steroidogenesis in maturing follicles (1). This steroidogenesis results in the production of sex steroids, such as androgens, estrogens and progestins. Furthermore, the peptide hormones, activin and inhibin, are produced by the gonads. Both steroids and peptide hormones are vital for sexual maturation and crucial for feedback regulation at the level of the hypothalamus and/or anterior pituitary. Activin and inhibin are secreted by the pituitary gonadotrope cells, as well, and act in both autocrine and paracrine manners (2). Feedback regulation occurs in an autocrine, paracrine, or endocrine manner resulting in the altered level of expression and release of GnRH, FSH and LH.

Anterior Pituitary

The pituitary gland is located inferior to the hypothalamus at the center base of the brain. It is divided into three sections: the anterior, intermediate and posterior pituitaries, each having significant anatomical differences. The posterior pituitary is derived from the ventral diencephalon from neural ectoderm, similar to the hypothalamus, while the anterior and

intermediate pituitary lobes are derived from an invagination in the oral ectoderm otherwise known as Rathke's pouch (3). The posterior pituitary stores and secretes oxytocin and vasopressin, which are synthesized by the hypothalamus. The intermediate lobe contains melanotropes, which produce and secrete melanocyte-stimulating hormone (MSH). Often termed the "master" gland of the body for its multicomponent endocrine control over peripheral tissues, the anterior pituitary is the location of five morphologically distinct endocrine cell types important for a vast number of bodily functions including growth, reproduction, blood pressure, thyroid functions, metabolism, and nervous system functions. Each endocrine cell type in the anterior pituitary is responsible for the production of specific hormones including the following: adrenocorticotropin hormones (ACTH) from corticotropes; thyroid-stimulating hormone (TSH) from thyrotropes; growth hormone (GH) from somatotropes; prolactin (PRL) from lactotropes; and FSH and LH from gonadotropes (3).

The human and rat anterior pituitary cell populations contain only about 10-15% gonadotropes, making studies of gonadotropin secretion and regulation difficult (3). Furthermore, studies were hampered by the lack of a highly differentiated gonadotrope cell line that adequately expressed FSH and LH. Substantial progress has been made in elucidating basal expression of LH in studies that used the heterologous cell line, CV1 cells, derived from African green monkey kidney fibroblast cells (4). CV1 cells are useful for their

relatively high transfection efficiency as well as low basal levels of expression of the cellular factors investigated in this study. However, these cells do not express endogenous steroid or gonadotrope peptide-hormone receptors such as GnRHR and activin receptors; and, thus, studies of hormonal regulation were impeded by the lack of a proper gonadotrope cell model.

In light of these limitations, our laboratory was able to create differentiated and immortalized cell lines that were derived from pituitary tumors. The tumors were induced by targeted oncogenesis utilizing a promoter sequence from the rat LHβ gene linked to the protein-coding sequences of the SV40 T-antigen oncogene (5). This process resulted in the creation of a useful mouse model for gonadotrope cells called LβT2. The LβT2 cell line represents a differentiated cell line capable of expressing mRNA for GnRHR and both the α-subunit glycoprotein (αGSU) and β-subunits of LH and FSH (5). In response to GnRH activation, LβT2 cells demonstrate the ability to increase intracellular calcium concentrations ($[Ca^{2+}]_i$) and exocytosis, indicating that LβT2 cells are useful models for the investigation of secretory mechanisms (6). Additional properties of this cell line include responsiveness to glucocorticoid treatment and pulsatile, concentration-dependent GnRH administration with both resulting in an increase of GnRHR mRNA, consistent with normal pituitary gonadotrope function (7). In a time dependent manner, starting at 48 hours after hormonal treatment with activin A, LβT2 cells dramatically increased FSH secretion in the mouse

model; this response was potentiated as early as 24 hours after treatment if activin was simultaneous with pulses of GnRH (8). LβT2 cells have been shown to express all four activin receptor RNA forms and the activin Bβ-subunit, which are appropriately responsive to activin signaling as demonstrated by transient transfection experiments with activin on the ovine FSHβ promoter (2). Regulation by GnRH of the FSHβ and LHβ promoters by the endogenous activin autocrine loop has been shown to be blocked by follistatin, indicating the presence of an activin/follistatin system that may interact with GnRH action (2). Therefore, studies of the LβT2 cells implicate use of these immortalized anterior pituitary gonadotropes as a fully differentiated cell line capable of expression a spectrum of markers including αGSU, FSHβ, LHβ, activin and activin receptors, inhibin, follistatin, and GnRHR. With the advent of LβT2 cells, there are now the tools to further dissect the regulatory components and molecular mechanisms governing gene expression in highly differentiated gonadotrope cells.

GnRH induction of LH and FSH is a major regulatory mechanism that controls gonadal function. GnRH action is applied either through direct targeting and binding to GnRHR on gonadotrope cells or indirectly through feedback loops that involve mediatory hormones such as activin. Activin, produced by the gonads and locally in the pituitary, is also a major regulator of gonadotrope function and gonadotropin expression. The direct control of gonadotropin release involves the intermittent hormonal input of GnRH from

the hypothalamus to stimulate the pituitary gonadotropes, which results in synthesis and release of LH and FSH in a close, temporal relationship (9). GnRH, indirectly and in conjunction with activin regulation of LH and FSH expression, promote pituitary cells to produce activins, follistatin and inhibins; these also influence the function of gonadotropes and are involved in the maintenance of normal reproductive activity. *In vitro* and *in vivo* animal studies are substantiating the importance of activins and inhibins as modulators in the reproductive axis, implicating that members of the transforming growth factor-β superfamily may control reproductive function in addition to orchestrating and modulating developmental growth programs, homeostasis of several cell types, and differentiation profiles (10).

Regulation of FSH

FSH is a heterodimer composed of α- and β-subunits, and hormonal activity is expressed only with strong and noncovalent interactions between these subunits (11). The α-subunit gene is highly conserved between species and is common and identical between FSH, LH, TSH and chorionic gonadotropin hormone. Because it is produced in excess, the α-subunit gene is not considered a major determinant to the biosynthetic rate of FSH or LH. Also, GnRH pulses regulate it less stringently (12). Thus, the hormones elicit their biological responses through the unique β-subunit, which provides

biological specificity. Because the α-subunit is usually found in excess to the intact FSH hormone and free β-subunits are found at very low basal levels, it has been suggested that FSH β-subunit synthesis is the rate-limiting step for the hormone (13). Changes in FSH expression and secretion result from the regulation of distinct transcriptional units sensitive to various hormone and steroidal activity.

The importance of FSH is underscored by studies implicating the lack of FSH hormone production in a number of abnormal reproductive phenotypes. Female FSH β-subunit null mice are infertile due to block in folliculogenesis preceding the formation of antral follicles (14). Frame-shift mutations in the FSH β-subunit gene, resulting in a truncated FSH β-subunit, result in amenorrhea and infertility in women, coinciding with undetectable serum FSH levels, low LH levels, and high estradiol levels. A point mutation in the human FSH receptor causes infertility in females due to a hereditary hypergonadotropic ovarian failure (14). Because FSH is necessary for spermatogenesis and Sertoli cell growth, male mice lacking FSH have impaired reproductive function and a reduction in testicle size, although fertile. In contrast, human males lacking FSH are infertile (14). To understand abnormalities in FSH expression, the underlying mechanism of FSHβ gene transcription must be elucidated.

Following the development of the LβT2 cell line, several contributions have been made in the elucidation of the promoter elements that regulate the

transcription of the FSHβ gene. Transcription factor pituitary homeobox 1 (Ptx1) can activate the rat FSHβ gene (rFSHβ) by binding to the proximal AAATCC site, located at -53/-48 of the rFSHβ gene promoter, a site that is functionally important for basal, but not effective in altering fold response to GnRH on the rFSHβ gene promoter (15). Reproductive disorders with loss of FSH have been attributed to a mutation in the site encoding LHX3, LIM-homeodomain transcription factor gene, demonstrating the importance of LHX3 transcription factors for the FSHβ basal promoter activity (16). Studies on the porcine FSHβ subunit gene in GH3 cells reported that Prophet of Pit1 homeodomain factor (Prop1) controlled gene expression and modulates synthesis of FSH. Defective Prop1 transcription factors result in combined pituitary hormone deficiency consequently leading to hypogonadism and dwarfism (17). Preceding the appearance of FSHβ in the developing mouse pituitary, the orphan nuclear receptor known as steroidogenic factor 1 (SF1), encoded by the *Ftz-F1* gene, mediates gonadal development (18). SF1 null mice are infertile since SF1 plays a crucial role in adrenal and gonadal development (19). Two conserved gonadotrope-specific elements, GSEs, that bind SF1 were identified at approximately -341 and -239 to the transcriptional start site of FSHβ (20). The heterotrimeric nuclear factor-Y (NFY) is ubiquitously expressed factor that can regulate the expression of genes implicated in the functions of major histocompatibility complex II genes and genes involved in cholesterol metabolism (20-22). An NF-Y binding

site with the sequence ATTGG was identified on the mouse FSHβ promoter at approximately -75 bp from the transcriptional start site. The A subunit of the transcription factor NFY was shown to interact physically to SF1 and confer direct activity on the FSHβ promoter, thereby contributing to LβT2 cell specificity (20).

Hormonal Regulation of FSHβ expression

Various types of hormones also play critical roles in the production of FSH. GnRH is a primary regulator of FSHβ gene expression *in vivo*. Investigations indicate that GnRH signaling involves the induction of activation protein 1 (AP1) through induction of the protooncogenes, c-Fos and c-Jun, in ovine (23) and mouse FSHβ promoters (24). GnRH also interacts with activin to synergistically induce FSHβ gene expression, augmenting phosphorylation of second messengers and elevating c-Fos expression through increased p38 activity (25). Putative AP1 binding sites at -120 and -83 were identified on the ovine FSHβ promoter gene through computer analysis. Analysis of the proximal promoter revealed highly stimulated c-Jun and c-Fos proteins involved in FSH production (26). In later studies using LβT2 cells with mouse FSHβ promoter, a putative site was determined to be a novel AP1 comprised of a CCAAT box and an AP1 half site juxtaposed to a binding site for NFY; the AP1 proteins are necessary and sufficient for FSHβ

transcription (24). Recent studies have shown that an AP1 site is conserved on the human FSHβ promoter (27). The bone morphogenetic proteins (BMPs), members of the transforming growth factor-β (TGFβ) family, are also thought to play pivotal role in the reproduction system and FSH production. BMPs have been shown to inhibit FSHβ mRNA expression in sheep primary pituitary cells while studies in the mouse LβT2 cells show BMP4 to be stimulatory activin-stimulated transcription of the FSHβ gene (28). BMP6 and BMP7 were shown to increase ovine FSHβ gene transcription and secretion of FSH in LβT2 cells (29). Additionally, basal expression of the mouse FSHβ gene is maintained by BMP7 (29). Most recently, BMP2 has been shown to regulate murine FSHβ subunit transcription through type I receptors, BMPR1A, and can do so independently or synergistically with activin (30). Additional hormones regulating FSH secretion include estrogen, which suppresses FSHβ expression (31); follistatins from the pituitary and inhibins from the gonads which feedback to modulate FSH secretion (32); and androgen, glucocorticoid, and progestin which induce expression of murine FSHβ subunit gene through binding of receptors to the hormone response element (HRE) at -381 of the proximal promoter (33). Further, androgen receptor binding to androgen response elements (AREs) within the FSHβ gene at -245/-231 and -153/139 induces androgen stimulated expression of an ovine FSHβ reporter gene (34) while glucocorticoid regulation of FSHβ

expression occurs through glucocorticoid response elements (GREs) located at -381, -197, and -139 of the FSHβ promoter (35), and progesterone induces expression of the FSHβ promoter gene through a progesterone receptor (PR) in the mouse promoter (36). Some of these hormones have been shown to synergize with activin. In addition to the synergistic effect of GnRH with activin (25), glucocorticoids (35), androgens and progesterone (36) also synergize with activin. There is, indeed a high complexity of factors that contribute to FSHβ gene expression, many of which have been shown to interact with activin. Activin is the most potent inducer of FSHβ transcription, however, relatively little is known about activin regulation of FSHβ gene transcription in the gonadotrope cells. Elucidation of the mechanisms involved in activin signaling will be essential in analyzing and understanding FSH secretion.

Activin Signaling Through Smads

Activin is a member of the TGFβ superfamily and is implicated as a potent regulator of FSH synthesis. The production and secretion of activin was originally found in the gonads with its target as the pituitary in regulation FSH secretion by inducing FSHβ gene expression in gonadotrope cells in a positive feedback manner (37). However, the gonadotrope cell itself

can express activin within the pituitary to act in an autocrine manner to induce FSH production as well (38, 39).

There are three possible molecular species of activin composed of homo- or heterodimers designated as activin A (βAβA), activin B (βBβB), and activin AB (βAβB) (40). As a TGFβ superfamily protein, it signals through heterodimeric receptors composed of two single membrane spanning serine-threonine kinases designated type I and type II (41). Upon ligand binding, type II serine-threonine kinases receptors, ActRII and ActRIIB, interact and phosphorylate the type I receptor, activin receptor-like kinase (ALK). Type RII receptors are constitutively phosphorylated, but activation of downstream signaling does not require phosphorylation of RII receptors (41). ALK4 preferentially binds Activin A, while ALK7 binds activin B. Single amino acid substitutions in either ALK4 or ALK7 allow these receptors to generate activity through autophosphorylation (42). Activated type I receptors phosphorylate transcription factors located in the cytoplasm known as Smads. There are eight isoforms encoded in the human and mouse genomes. Receptor-associated Smads, specific for activin, are Smad2 and Smad3 (43, 44). Phosphorylation and activation of Smad2 and/or 3 leads to the association with a common Smad mediator, Smad4, which causes translocation into the nucleus where Smads may act alone in the regulation of target genes or complex with binding proteins to induce coactivation or corepression (45, 46). Smad4, the co-Smad, serves as a common partner to all

the receptor-regulated Smads (RSmads), which include Smad1, 2, 3, 5, and 8. Smad1, 5, and 8, are substrates for the bone morphogenic proteins (BMP) and anti-Mullerian receptors, while inhibitory Smads (ISmads), Smad6 and 7, act as inhibitors of Smad-receptor or Smad-Smad interactions (47). Transient transfections of Smad3, but not Smad1, 2, 4, 5, or 8 stimulate endogenous FSHβ mRNA levels. However, results from Smad2 transfection studies remain inconclusive due to the inability to overexpress the full Smad2 protein in LβT2 cells (43). It has been shown that Smad2 activity on gene transcription is dependent on interactions with other factors while Smad3 (as a heterodimer with the co-Smad4) acts independently for DNA-binding. This difference is attributed to their structural differences, where Smad2 contains surface-exposed amino acid residues not present in Smad3 (48). The importance of Smad3 is demonstrated in studies where decreased expression of Smad3 abrogates stimulation of FSHβ gene transcription, since normally, Smad3 is sufficient to stimulate FSHβ promoter activity in LβT2 cells (48). Also, the limiting factor for activin induction of FSHβ seems to be Smad3 (49, 50). Mice deficient in Smad3 have lower levels of FSHβ mRNA (24).

Smads are constantly undergoing shuttling between the nucleus and cytoplasm in a process that is mediated by the phosphorylation of receptors. Receptor activity leads to a decrease in RSmad affinity for cytoplasmic anchors and increases affinity for nuclear factors (46). The nuclear import and export of Smads 2, 3, and 4 can occur without the intervention of nuclear

transport factors such as nuclear localization signals (NLS) (51). The target sequence and binding specificity of Smad proteins has been identified as 5'-GTCTAGAC-3' (52), called the Smad-binding element (SBE). The Smad-responsive region may contain multiple SBEs or half sites, in some instances with the addition of an extra base as in 5'-CAGAC-3' (51). Smad3 Mad-homology 1 domain recognizes Smad-binding element (MH1-SBE) through a β-hairpin that can recognize the sequence, 5'-GTCT-3', which is the minimal binding sequence for Smad3 (53). Consensus SBE sites that confer activin responsiveness have been characterized at multiple locations on the FSHβ promoter including the -134 site (44), -153 site (35, 44), and the -267 site (25). Furthermore, some SBE sites are necessary factors for synergistic expression of the FSHβ gene via cross-talk of steroid hormones and activin signaling pathways (36). In addition to Smad proteins, SBE sites may require the contribution of other transcription factors in order to elicit activin responsiveness.

Due to their low DNA-binding activity, Smads interact with other DNA-binding transcription factors on the promoters of target genes. In frog embryos, activin responses are mediated through Smad2 and 4 interaction with a winged-helix forkhead transcription factor FoxH1 (FAST1) (54). Distal element-binding proteins (DEBPs) in *Xenopus* embryos are shown to interact and recruit Smad2 and Smad4 to induce the expression of the Mixer gene in *Xenopus*, which is a signaling target of activin/TGFβ activity (55). The BMP

and TGFβ pathways involved in skeletal development and bone formation contain Smads as substrates for signaling, which are dependent on interaction with Runt-related transcription factor-2 (RUNX2) to execute signaling in osteoblasts (56). Smad interaction with transcription factors is highly represented throughout several signaling systems in various model organisms. Thus, it is intuitive to approach the questions concerning the mechanisms of FSHβ gene expression with investigations exploring and identifying possible key players in the pathway for activin-mediated Smad activation of FSHβ genes. The activation of both GnRH and activin pathways result in synergistic induction of FSHβ, indicating that Smad and AP1 may physically interact following DNA binding to recruit coactivators that can facilitate the observed synergy (25). Members of the three-amino-acid loop extension (TALE) superclass of homeodomain proteins, Pre B-cell leukemia transcription factor (Pbx1) and Pbx regulating protein-1 (Prep1), have been identified as Smad partners in mediating activin action capable of interacting with Smad4 and binding onto an SBE located at -134 from the transcriptional start site in the ovine promoter, which corresponds to -120 in the mouse gene (44). Pbx and Prep1 complexes with Smad may possibly bind Smad2 and/or Smad3, in addition to Smad4, as indicated in glutathione-S-transferase interaction assays (44). An additional pituitary-expressed transcription factor pertinent to activin-dependent induction of FSHβ, known as Paired-like

homeodomain transcription factor-2 (Pitx2), also complexes with Smad proteins, specifically Smad3, to induce the rat FSHβ promoter (57).

Summary

The regulation of the hypothalamic-pituitary-gonadal axis is essential for reproductive growth and maintenance. Proper maturation at pivotal check points in mammalian reproductive life depend on the proper functioning of the HPG-axis, including sexual development, puberty, menstrual cycles, pregnancy and menopause. GnRH, activin, inhibin, and follistatin help to regulate hormonal levels by stringently controlling the transcriptional mechanisms involved in the production of FSH and LH. Proper regulation of LH and FSH is accomplished by tight regulation and interaction of GnRH and feedback control by gonadal and pituitary activin and steroid hormones. Levels of FSH synthesis are determined by the expression of the unique β-subunit gene, which confers hormone biospecificity and acts as a limiting component. The importance of the FSH β-subunit is demonstrated by the infertility of individuals that lack its expression.

Previous studies have elucidated a number of transcription factors and target sequences that play a role in modulating FSHβ expression. However, the mechanism for activin control of FSH synthesis remains relatively unclear. Activin seems to be a critical player both alone and in synergy with

other hormones. Truncation analysis of the FSHβ promoter shows a step-wise decrease in activin induction with every progressing truncation, as previously reported by our laboratory. This indicates the presence of multiple activin regulatory elements in the FSHβ promoter. It is the focus of this study to reveal the regulatory elements that confer control of the expression of the FSHβ gene.

II

Materials and Methods

Plasmid Constructs

The expression vector, Smad3 in pcDNA3, was kindly provided by Dr. Joan Massague. The Smad4 expression vector in pcDNA3 was kindly provided by Dr. Theresa A. Guise. Constitutively active ALK4 and ALK7 plasmids in pcDNA3 were kindly provided by Dr. Carl-Henrik Heldin and Dr. Peter T. Dijke. TAB2 and TAB3 plasmids linked to an HA-tag in the pCMV backbone were generously provided by Dr. Jun Tsuji and Tsuji laboratory members. The goat FoxL2 expression vector in a pcDNA3.1 backbone was kindly provided by Dr. Reiner Veitia. The -1028 bp human FSHβ promoter linked to a luciferase-reporter in a pGL3-basic vector was kindly provided by Dr. Daniel J. Bernard.

Cell Culture and Transient Transfections

LβT2 immortalized cell lines were cultured at 37° C in 10 cm plates in DMEM (Cellgro, Mediatech, Inc., Herndon, VA) containing 10% fetal bovine serum (Omega Scientific Inc., Tarzana, CA) and penicillin/Streptomycin until approximately 80% confluent. Cells were split into 12-well plates 1 day prior

to transfection and transfected using Fugene 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) in accordance to the manufacturer's protocol. Wells were transfected with 500 ng of either the mouse or human FSHβ-luciferase reporter plasmids, 100 ng of the β-galactosidase reporter plasmid driven by the Herpes virus thymidine kinase promoter to serve as an internal control for transfection efficiency, 200 ng of either one or a combination of the following expression vectors: FoxL2, Smad2, Smad3, Smad4, or pcDNA3.1 control, or 200 ng of a constitutively active activin receptor, ALK7QD, as indicated in the figure legends. Prior to harvesting, cells were incubated in serum-free DMEM containing 0.1% BSA and antibiotics overnight. Cells transfected with mouse FSHβ promoters were treated (unless otherwise stated) with 10 ng/mL activin (Calbiochem, La Jolla, CA) in serum-free DMEM 5 hrs prior to lysing, and human constructs were treated with the same dose 24 hrs prior to lysis.

Luciferase and β-galactosidase Assays

Subsequent to activin treatment, cells were washed with 1X phosphate buffer saline (PBS) and lysed with 60 μL of a 0.1 M K-phosphate buffer at pH 7.8 containing 0.2% Triton X-100. A 96-well luminometer plate was loaded with 20 μL of each of the lysates and luciferase activity was measured after injection of a buffer containing 100 mM Tris-HCl with pH 7.8, 15 mM $MgSO_4$,

10 mM ATP and 65 μM luciferin per well, using a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA). The Galacto-Light Assay (Tropix, Bedford, MA) was performed according to the manufacturer's protocol and used to measure galactosidase activity.

Mutagenesis

Mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. All mutagenesis in the mouse FSHβ-luciferase promoter was conducted using the 1 kb promoter and/or the 1028 bp promoter for the human FSHβ-luciferase. Mutations were confirmed via dideoxyribonucleotide sequencing performed by DNA Sequencing Shared Resources at the University of California, San Diego, Cancer Center. Wild-type promoters were mutated with the oligonucleotides described in Tables 2 and 3. PCR conditions for mutagenesis were conducted as follows: 95°C for 30 sec., 95°C for 30 sec., 55°C for 1 min., 68°C for 14 min., repeat steps 2-4 for an addition 18 cycles, 1 hr of Dpn treatment. PCR products (1.5 μl) were transformed into 50 μl XL1 Blue Supercompetent Cells (Stratgene, La Jolla, CA) via heat shock with the following temperatures and incubation times: on ice for 30 min, 42°C for 45 sec, on ice for 2 min. Cells were recovered with 450 μl SOC

at 37°C for 1 hr. Seventy-five μl and 350 μl of bacteria were plated on LB-amp plates and cultivated at 37°C overnight.

Hormones

Activin was purchased from Calbiochem, La Jolla, CA and diluted in 0.1% bovine serum albumin suspended in phosphate buffer solution to a concentration of 10 μg/mL.

Multimer Cloning

The oligonucleotides (oligos) presented in Table 6 were synthesized by Integrated DNA Technologies. Single stranded oligos were diluted with Tris-EDTA (TE) to a concentration of approximately 100 μM. Complimentary single stranded oligos were annealed using 10 μl of each strand from the original 100 μM stock. Ten μl of each strand was placed in a solution containing 10% of a 0.5 M NaCl solution and brought to a boil for three minutes. The solution was allowed to slowly cool to room temperature, producing approximately 10 μM annealed oligos. Annealed oligos were phosphorylated using a T4 kinase and adenosine triphosphate. Following phosphorylation, annealed oligos were ligated to a pGL3 vector (driven by a thymidine-kinase luciferase promoter) previously digested with restriction enzymes – Nhe, Xho and/or KpnI – and treated with calf intestinal

phosphatase. The ligation reaction was performed using Ready-To-Go T4 DNA Ligase (Amersham Biosciences/GE Healthcare) according to manufacturer's protocol. Ligated samples were transformed into DH5α Max Efficiency Competent Cells (Invitrogen, Carlsbad, CA) and plated on LB-amp plates. DNA was extracted from the colonies using the Qiagen Miniprep Kit (Qiagen Sciences, Maryland) then analyzed for an insert by PCR with the following conditions: 95°C for 4 min., 95°C for 30 sec., 56°C for 30 sec., 72°C for 1 min., repeat step 2-4 for an additional 40 cycles, 72°C for 10 min. PCR products were run on a 2% agarose gel for analysis and sequences were confirmed by sequencing through DNA Sequencing Shared Resources at the University of California, San Diego, Cancer Center. Sequence alignment analyses were performed using Accelrys DS Gene.

EMSA

LβT2 cells or Cos-1 cells were cultured at 37°C in 10 cm plates in DMEM (Cellgro, Mediatech, Inc., Herndon, VA) containing 10% fetal bovine serum (Omega Scientific Inc., Tarzana, CA) and penicillin until approximately 60% confluent. Cells were transfected using Fugene 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN) in accordance to the manufacturer's protocol. Cells were transfected 5 μg FoxL2, Smad3 or Smad4 expression vectors. After 24 hours, nuclear extracts were obtained by swelling the cells with hypotonic buffer containing the following: 20 mM Tris pH 7.4,

10 mM NaCl, 1 mM $MgCl_2$, 1 mM PMSF, protease inhibitor cocktail from Sigma (Sigma-Aldrich), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA. Cells were broken by passing through a $25^5/_8$ G needle 3 times. Samples were centrifuged at 4000 rpm for 4 min and the nuclear pellets were resuspended in hypertonic buffer containing: 20 mM Hepes pH 7.8, 20% glycerol, 420 mM KCl, 1.5 mM $MgCl_2$, 1 mM PMSF, protease inhibitor cocktail (Sigma-Aldrich), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA. After incubation on ice for 20 min., samples were centrifuged at 10,000 rpm for 10 min. and the supernatants were aliquoted and frozen until use. Protein determination was performed using the Bradford reagent (Bio-Rad, Hercules, CA).

Single stranded oligonucleotides (Table 4) were obtained from Integrated DNA Technologies and diluted with Tris-EDTA (TE) to 100 μM. The oligos were annealed and labeled with γ^{32}P ATP using T4 Polynucleotide Kinase (New England Biolabs, Inc., Beverly, MA) and column purified using Micro Bio-Spin Chromatography Columns (Bio-Rad Laboratories, Inc., Hercules, CA) according to manufacturers' protocols. Binding reactions contained 2 μg of nuclear proteins in a total volume of 20 μl containing the following: 10 mM Hepes pH 7.8, 50 mM KCl, 0.5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 0.25 μg didC, 5 mM DTT, 5 fmol of labeled probe. Oligos for competition assays are described in Table 5. Competition and antibody shift assays were performed using 200-fold excess of unlabeled oligonucleotide or 1 μg antibody respectively. The following antibodies were used in EMSAs:

Smad4 (Santa Cruz Biotechnology, Santa Cruz, CA), Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA), FoxL2 (Santa Cruz Biotechnology, Santa Cruz, CA), IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Reactions were loaded onto a 5% nondenaturing polyacrylamide gel and ran in 0.25X Tris-borate-EDTA buffer. Gels were run at 250 V/cm^2 constant voltage and dried. Autoradiography was performed to identify complexes.

Immunoprecipitation

LβT2 cells or Cos-1 cells were cultured at 37°C in 10 cm plates in DMEM containing 10% fetal bovine and penicillin until approximately 60% confluent. Cells were transfected using Fugene 6 reagent in accordance to the manufacturer's protocol and transfected with 5 μg expression vectors. After 24 hours, cells were rinsed with 1X PBS and lysed with buffer containing 20 mM Tris pH 7.4, 140 mM NaCl, protease inhibitors, 1 mM PMSF, 10 mM NaF, 1% NP-40, 0.5 mM EDTA, and 1mM EGTA. Approximately 1 mg of protein extracts were thawed on ice and 5 μl of antibodies were added to each sample. Prior to use and after thawing, 5 μl of protease inhibitors and 10 mM of NaF were added. Samples were incubated at 4°C for 1 hour and 20 μl of Protein A or Protein G Magnetic Beads (New England Biolabs) were added and further incubated at 4°C for approximately 45 min. The immunoprecipitates were cleared from the beads using a magnetic rack and washed 3 times in the lysis buffer. The immunoprecipitated protein mixture

was resuspended in 25 µl of a 2 X sample buffer containing 125 mM Tris pH 6.8, 20% glycerol, 4% SDS (10% stock), 10 mM ß-mercaptoethanol, and 0.02% bromophenol blue.

Western Blot

LβT2 cells or Cos-1 cells were cultured at 37°C in 10 cm plates and transfected with pcDNA3.1, FoxL2, TAK1, TAB1, Smad 2, Smad 3, or ALK4-HA or not transfected if treated with hormones. After overnight starvation in serum-free DMEM supplemented with 0.1% BSA cells were treated with 10 nM activin for 1 hr or 3 hr. Whole cell extracts were harvested after hormone treatment. The cells were rinsed with 1X PBS and lysed with lysis buffer containing the following: 20 mM Tris pH 7.4, 140 mM NaCl, protease inhibitors (Sigma-Aldrich), 1 mM PMSF, 10 mM NaF, 1% NP-40, 0.5 mM EDTA, and 1 mM EGTA. Protein concentration was determined using the Bradford reagent (Bio-Rad, Hercules, CA).

Equal amounts of proteins from whole cell extracts were loaded with 2 X or 4 X sample buffer into an SDS-PAGE gel containing a 4% stacking gel and a 10% or 12.5% separating gel. After the proteins were resolved by electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane, they were blocked with 10% milk in wash buffer containing 20 mM Tris pH 7.4, 0.1% Tween, 150 mM NaCl, and 0.5% BSA. Following overnight treatment in blocking buffer at 4°C, the membranes were blotted

with specific antibodies. The following antibodies were used: HA.11 monoclonal antibody made in mouse (Covance, Emeryville, California), rabbit polyclonal anti-HA tag (Abcam, Cambridge, MA), mouse monoclonal anti-Flag (Sigma, St. Louis, MO), rabbit polyclonal anti-Smad 3 (Abcam, Cambridge, MA), rabbit polyclonal anti-Smad 4 (Upstate Cell Signaling Solutions, Lake Placid, NY). Bands were detected with a secondary antibody to rabbit or mouse IgG linked to horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Santa Cruz, CA) and Enhanced Chemiluminescence (ECL) reagent (GE Healthcare).

Statistical Analysis

All experiments were performed a minimum of three times. Transfections were performed in triplicates within each experiment. Luciferase over β-galactosidase ratio serves to normalize transfection efficiency. Between trials of experiments, pGL3 luciferase reporter activity was measured in parallel to assess variability between the experiments. Mean from the triplicate within one experiment of normalized luciferase over β–galactosidase values were averaged from three of the experiments and statistical analysis of variance (ANOVA) was performed using the JMP7 program. Significance was set at $p \leq 0.05$. An asterisk was used to represent statistically significant data as analyzed by one-way ANOVA.

Table 1: Primers for mouse 10 bp internal deletions
Only forward primers are listed.

Name	Sequence
Δ400-391	5′-AGATGCAGAAGTACTTCCTACACTTGGAGTGTTCAGTCTGTTC-3′
Δ390-381	5′-GTACTTCCTATTTGTTCATAGTTCAGTCTGTTCTTGGATCAAT-3′
Δ380-371	5′-TTTGTTCATACACTTGGAGTTTCTTGGATCAATTAAGACATAT-3′
Δ370-361	5′-TAAACCAAAATATGTCTTAATTCAGACTGAACACTCCAAGTG-3′
Δ360-351	5′-CATTGCGAAGGTAAACCAAAATATGATCCAAGAACAGACTGAAC-3′
Δ350-341	5′-GCTTTGGCTCCATTGCGAAGGTATGTCTTAATTGATCCAAGAA-3′
Δ340-331	5′-CTGAACATTGCTTTGGCTCCATAACCAAAATATGTCTTAATT-3′
Δ330-321	5′-AGAATCCTTTCTGAACATTGCTTGCGAAGGTAAACCAAAATA-3′
Δ320-310	5′-TGCGAACTCAGAATCCTTTCTTTGGCTCCATTGCGAAGGTA-3′
Δ310-301	5′-TCTTTAACTTGGCGAACTCAGAGAACATTGCTTTGGCTCCAT-3′

Table 2: Mutated mouse FSHβ promoter sequence
Bold and underlined base-pairs represent mutations from wild-type.

Name	Sequence
-350wt	5′-TTCTTGGATCAATTAAGACATATTTTGGTTTACCTTCGCAATGGAGCCAAAG-3′
-348mut	5′-TTCTTGGATCAATTAAGACAT**GGG**TTGGTTTACCTTCGCAATGG-3′
-345mut	5′-TTCTTGGATCAATTAAGACATATT**AAA**GTTTACCTTCGCAATGG-3′
-342mut	5′-ATCAATTAAGACATATTTTG**AAA**TACCTTCGCAATGGAGCCAAAG-3′
-330wt	5′-TGGTTTACCTTCGCAATGGAGCCAAAGCAATGTTCAGAAAGGATTCTGAGTTCGC-3′
-328mut	5′-TGGTTTACCTTCGCAAT**TTT**GCCAAAGCAATGTTCAGAAAGGATT-3′
-325mut	5′-TGGTTTACCTTCGCAATGGA**TTT**AAAGCAATGTTCAGAAAGGATT-3′
-322mut	5′-TGGTTTACCTTCGCAATGGATTT**CCC**GCAATGTTCAGAAAGGATT-3′
-319mut	5′-TCGCAATGGAGCCAAA**TTT**ATGTTCAGAAAGGATTCTGAGTTCGC-3′
-200wt	5′-GCTGCCATATCAGATTCGGTTTGTACAGAAACCATCATCATCACTGAT-3′
-200mut	5′-GCTGCCATATCAGA**CC**CGG**CCC**GTACAG**GG**ACCATCATCATCACTGAT-3′
-153wt	5′-TTCTGCTCTGTGGCATTTAGACTGCTTTGGCGAGGCTTGATCTCC-3′
-153mut	5′-TTCTGCTCTGTGGCA**GGG**AGACTGC**GGG**GGCGAGGCTTGATCTCC-3′

Table 3: Mutated human FSHβ promoter sequence
Bold and underlined base-pairs represent mutations from wild-type.

Name	Sequence
-350wt	5′-AGCAATTTATTAACCATATTTTTTAATGCATCTCCTGAACAGAGT-3′
-350mut	5′-AGCAATTTATTAACCATAT**AAAAA**AATGCATCTCCTGAACAGAGT-3′
-200wt	5′-GCTACTGTATCAAATTTAATTTGTACAAAATCATCATCTCTAGTA-3′
-200mut	5′-GCTACTGTATCAAA**GGG**AA**GGG**GTACA**GG**ATCATCATCTCTAGTA-3′
-153wt	5′-TTCTAATCTACTGCGTTTAGACTACTTTAGTAAAGCTTGATCTCC-3′
-153mut	5′-TTCTAATCTACTGCG**GGG**AGACTAC**GGG**AGTAAAGCTTGATCTCC-3′

Table 4: Oligonucleotides used as radiolabeled EMSA probes

Probe	Sequence
Mus -360/-330	5′-AATTTAGACATATTTTGGTTTACCTTCGCA-3′
Mus -335/-305	5′-TCGCAATGGAGCCAAAGCAATGTTCAGAAA-3′
Mus -267/-249	5′-CAGAAAGAATAGTCTAGACTCTAGAGTCAC-3′
Mus -200	5′-CATATCAGATTCGGTTTGTACAGAAACCATCATCA-3′
Mus -153	5′-GCTCTGTGGCATTTAGACTGCTTTGGCGAGGCTTG-3′
Homo -360/-330	5′-AATTTATTAACCATATTTTTTAATGCATCT-3′
Homo -200	5′-TGTATCAAATTTAATTTGTACAAAATCATCATCTC-3′
Homo -153	5′-AATCTACTGCGTTTAGACTACTTTAGTAAAGCTTG-3′

Table 5: Oligonucleotides used as unlabeled competitors in EMSAs

Bold and underlined base-pairs represent mutations from the wild-type probe.

Competitor	Sequence
WT	5′-AATTTAGACATATTTTGGTTTACCTTCGCA-3′
A	5′-AAT**CCC**GACATATTTTGGTTTACCTTCGCA-3′
B	5′-AATTTA**TTT**ATATTTTGGTTTACCTTCGCA-3′
C	5′-AATTTAGAC**GGG**TTTTGGTTTACCTTCGCA-3′
D	5′-AATTTAGACATA**GGG**TGGTTTACCTTCGCA-3′
E	5′-AATTTAGACATATTT**CCC**TTTACCTTCGCA-3′
F	5′-AATTTAGACATATTTTGG**GGG**ACCTTCGCA-3′
G	5′-AATTTAGACATATTTTGGTTT**GGG**TTCGCA-3′
H	5′-AATTTAGACATATTTTGGTTTACC**GGG**GCA-3′
I	5′-AATTTAGACATATTTTGGTTTACCTTC**TTT**-3′

Table 6: Multimer sequences.

Bold and underlined base-pairs represent the repeated sequence.

Primer	Sequence
SBE+FoxL2 (first half)	5′-CTAG**CTAGTCTAGACTCATATTTTGGTTTAC**CTAGTCTAGACTCATATTTTGGTTTACCC-3′
SBE+FoxL2 (second half)	5′-CTAGT**CTAGACTCATATTTTGGTTTAC**CTAGTCTAGACTCATATTTTGGTTTACCG-3′
FoxL2	5′-CTAG**CATATTTTGGTTTA**CATATTTTGGTTTACATATTTTGGTTTACATATTTTGGTTTACC-3′

III

Results

Constitutively Active Activin Receptor Induces FSHβ Transcription

Activin, a member of the TGFβ superfamily of proteins, is a major inducer of FSHβ gene transcription and is paramount in the overall expression of FSH. It signals through two heterodimeric receptors containing serine-threonine kinases, classified as type I and type II (41). Activated type II receptors, ActRII or ActRIIB, bind and phosphorylate type I receptors, activin-like kinase, (ALK). ALK4 has been shown to have a higher affinity for activin A, while ALK7 selectively binds activin B. Therefore, to analyze the molecular mechanism for activin signaling, we first investigated whether activin receptors are sufficient to induce FSHβ. Single amino acid substitutions in either ALK4 or ALK7 allow these receptors to generate intracellular signals in the absence of their ligands due to autophosphoryation (42). To test the hypothesis that these constitutively active forms of the receptors ALK4 and ALK7 (caALK4 and caALK7) can induce FSHβ in the absence of activin, we overexpressed caALK4 and

caALK7 in LβT2 cells. Transient transfections of caALK4 and caALK7 resulted in 26.5% and 61.2% fold increases in induction of the 1.5 kb mouse FSHβ promoter, respectively (Fig. 1a), demonstrating that activin receptors can potently induce the promoter and are sufficient to mimic the effects of activin.

To identify which promoter elements convey caALK response, truncation analysis was performed on the 1.5 kb FSHβ promoter, where different lengths of the promoter were transiently transfected into LβT2 cells together with caALK4 or caALK7. To determine fold induction, and eliminate the influence of basal expression, luciferase levels from caALK transfected cells were normalized to the luciferase levels from cells transfected with empty vector control for each truncation. Significant decreases in fold induction by both ALK4 and ALK7 occurs between all truncations in the distal region upstream from -800. More interestingly, there are more significant decreases in activin induction when the regions between -398 bp and -304 bp and -304 and -230 are truncated (Fig. 1b-c). The fact that the ALK4 and ALK7 effects map to multiple regions corresponds to our previously published data that activin maps to many regions in the mouse FSHβ promoter (25). The decrease in fold induction when the region from -304 to -230 is removed is not surprising, since the previously identified full consensus Smad binding element (SBE) at -267 is present in this region of the promoter. However, the largest decrease of 66.1% of the induction occurs

when the region between -398 and -304 is truncated. This area represents a novel activin-responsive region in the FSHβ promoter and was the focus of our investigation.

TGFβ-activated Kinase 1 (TAK1) Activity Does Not Map to the Activin-Responsive Region

Studies analyzing FSHβ expression suggest that Smad-independent pathways involving TGFβ-activated kinase 1 (TAK1), a member of the MAPK kinase kinase (MAPKKK) family, is a modulator for ovine FSHβ gene transcription (58). Expression of TAK1 with its binding protein, TAB1, induce autophosphorylation of TAK1 and mimic the effects of kinase-activated TAK1, independently of receptor activity (59). To assess whether TAK1 mediates activin induction of the mouse FSHβ gene, we cotransfected TAK1 and TAB1 into LβT2 cells with the 1.5 kb FSHβ promoter. TAK1 confers small induction of the mouse FSHβ promoter when cotransfected with its binding protein, TAB1 (Fig. 2a). The fold induction by TAK1 alone is not significant (Fig. 2a), but with the addition of TAB1, the induction increased to significant levels over pGL3. To determine whether TAK1 activity on the 1.5 kb FSHβ promoter maps to one of the activin receptors' responsive regions, truncation analysis was performed and compared to mapping of caALK. The truncation analysis reveals a significant decrease in FSHβ gene

expression, induced by TAK1 and TAB1 overexpression, in the region between -500 bp and -398 bp (Fig. 2b). While with further truncation, luciferase activity decreases to a level as low as the empty backbone, pGL3 (Fig. 2b). In comparison to the region of TAK1/TAB1 activity in Figure 2b, there is no overlap of TAK1/TAB1 responsive regions to that of either caALK4 or caALK7. This indicates that the signaling cascade involving ALK4 and ALK7 does not depend on the TAK1/TAB1 proteins for induction of the mouse FSHβ promoter. Therefore, we further investigated other potential factors that may be involved with the ALK receptors' signaling to the FSHβ promoter.

Smad3 Responsiveness Correlates with the Activity of caALK

The activin signaling pathway involves Smad3-dependent mechanisms that have been implicated in the stimulation of FSHβ transcription. Furthermore, a Smad3-responsive site has already been identified on the FSHβ promoter at -267 (51, 52). Therefore, to identify additional Smad3 responsive regions, truncation analysis was performed with the overexpression of Smad3 and the same FSHβ promoter constructs used in the analysis of caALK4 and caALK7.

Significant decreases in fold induction by Smad3 overexpression were identified between -1.5 kb and -1 kb, -398 bp and -304 bp, and -304 bp and -230 bp, all of which overlap with sites of induction by caALK4 and caALK7 (Fig. 3). The largest decrease in induction is observed when the region between -398 bp and -304 bp is truncated (Fig. 3), from 9.1 fold with the -398 truncation to 2.5 fold with the -304 truncation. There is also a significant attenuation of induction between -304 bp and -230 bp that contains the SBE site at -267. Surprisingly, Smad3 induction is significantly diminished with truncation in the -398 and -304 region despite the fact that there appears to be no full consensus SBE site at this location. The same region also confers the largest induction by caALK4 and 7. Since the region between -398 and -304 is a novel Smad3 responsive region, we focused on that area to identify elements critical for this induction.

To further narrow down the region between -398 bp and -304 bp, we constructed 10 bp internal deletions in this area and used these mutations in transient tranfection assays with the overexpression of ALK7, Smad3 and activin treatment. Since both ALK4 and ALK7 map to the same regions, and the fold induction by caALK7 is higher than the fold induction of caALK4 and therefore the effects of the mutations easier to assess, we utilized ALK7 in subsequent experiments. Deletions on -350/-341 and -330/-310 from the transcriptional start site reduced fold induction by activin. Although the -360/-351 and -340/-331 regions also played a role in Smad3 and ALK7

induction, there was no significant decrease observed due to activin, which is possibly due to their proximity and interaction with the -350/-340 deletion. The 10 base-pair internal deletions demonstrate the presence of activin-responsive elements between -350 bp and -340 bp and between -330 bp and -310 bp, as indicated by significant decreases in FSHβ activity (Fig. 4c) which overlap with activity by caALK7 or Smad3 as demonstrated by a significant decrease by the two treatments at these regions (Fig. 4a-b). Therefore, these elements are strongly implicated in the induction of the FSHβ promoter by activin and activin signaling components.

Analysis of the -350 bp Region on the Mouse FSHβ Promoter Reveals a FoxL2 Site

Thus far, the localization of activin-induced activity on the FSHβ promoter has identified two novel elements, 10 bp and 20 bp in length, at -350 and -330. To further investigate these regions, EMSAs (Electrophoretic Mobility Shift Assays) were used to determine whether cell nuclear extracts contained nuclear proteins capable of binding to these regions of the FSHβ promoter. Since these sites confer responsiveness to Smad3, in order to assess whether Smad proteins can bind these regions, radiolabeled probes encompassing these sites were incubated with nuclear extracts from Cos-1 cells cotransfected with Smad3 and/or Smad4 expression vectors. In addition,

a radiolabeled probe encompassing the SBE site at -267 (SBE probe) was also incubated with Cos-1 cells to serve as a positive control for Smad binding. Smad3 and Smad4, in combination or singly as indicated above each lane, do not bind to either probe encompassing regions of interest (Fig. 5a). Smad3 and/or Smad4 cotransfected Cos-1 cells (lanes 2-4) have similar banding patterns to that of pcDNA3.1-transfected cells of lane 1. In contrast, incubation of the SBE probe with nuclear extracts from Cos-1 cells transfected with Smad4 expression vector elicits a very strong signal, indicated by arrow 1 (Fig. 5a-b). Therefore, neither probe is capable of binding to Smad proteins in Cos-1 cell extracts.

The probes were then used to assess whether activin treatment of LβT2 cells induces complexes to bind these regions by incubating the probes with nuclear extracts from control or activin-treated cells. Extracts from the activin-treated LβT2 cells, either one hour or three hours treatment, do not show different binding compared to those from the untreated cells (lane 10-11). Also, activin treatments result in banding patterns similar to control versus activin. The SBE probe detects the presence of an activin-induced band previously characterized as a Smad-containing complex, indicated by arrow 2 in lanes 13 and 14 of Figure 5a-b. Therefore, we could not detect Smad binding to either one of these regions, nor did activin treatment cause binding complexes in LβT2 cells to change.

We then performed *in silico* analysis of -350/-340 bp and -330/-310 bp sequences using several on-line-based sequence analyzing programs for putative transcription factor-binding sites; including CONSITE (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/), Jaspar (http://jaspar.cgb.ki.se/), and Transfac (http://www.genome.jp/dbget-bin/www_bfind?trasnfac), and identified several putative transcription factors binding sites for the upstream region. The upstream -350/-341 region contains sites for binding of several proteins, including Runt-related transcription factors RUNX1, RUNX2, RUNX3; and forkhead transcription factor genes FoxF1, FoxA1, and FoxL2. However, the -330/-310 probe does not contain any putative elements, since the results of the on-line analysis by Transfac, Jaspar, Consite, etc identified no candidates. Therefore, we focused our study on the -350/-340 region. EMSAs were conducted utilizing nuclear extracts of LβT2 cells treated with control and activin. The probe was incubated with nuclear extracts and antibodies for the putative transcription factors identified by on-line analysis. Inclusion of the FoxL2 antibody resulted in a supershift of a specific band (Fig. 6). The supershift (arrow 1) of the complex indicated by arrow 2 is achieved in lane 3 and 4 by the FoxL2 antibody (αFoxL2) (Fig. 6). The nonspecific antibody, IgG, serves as control. The supershift by anti-FoxL2 (αFoxL2) is detected in both the control or activin-treated nuclear extracts from LβT2 cells as indicated above the lanes. This demonstrates that the FoxL2 protein can bind to the -350/-340 region (Fig. 6).

To determine which nucleotides are necessary for FoxL2 binding, EMSAs were conducted using 200-fold excess of unlabeled oligonucleotides containing three base-pair scanning mutations as competitors (Fig. 7b). As outlined in Figure 7b, the scanning mutations (underlined and bold) were introduced into the wild-type oligonucleotide sequence that served as a probe. The unlabeled scanning mutations were incubated with nuclear extracts from activin-treated LβT2 cells in addition to the radiolabeled wild-type probe. The oligonucleotides that cannot compete indicate that the mutated base-pairs are needed for protein binding. This is evident in mutations C-F, indicated by arrow 2. The mutants that are able to compete for FoxL2 binding are A, B, and G-I, indicated by the weaker bands marked by arrow 2. This denotes that mutants A, B, and G-I, as with the unlabeled wild type in lane 2, can effectively outcompete the labeled wild-type probe to bind the FoxL2 protein, leading to a decreased intensity of the band (Fig. 7a, arrow 2). The addition of αFoxL2 in lane 12 induces a supershift as indicated by arrow 1 (Fig. 7a). Results of the competition assay indicate that the sequence necessary for FoxL2 binding is 5'-ATATTTTGGTTTA-3' (Fig. 7b).

Sequence analysis of the human FSHβ promoter shows a strong homology to the mouse FSHβ promoter at the FoxL2 site located approximately -350 bp from the transcriptional start site (Fig. 8c). Therefore, we conducted EMSAs to analyze whether FoxL2 proteins are capable of binding to the human FSHβ probe of the same region. FoxL2 does not bind

the homologous human element (Fig. 8a, lanes 5-8). Furthermore, Cos-1 cells transfected with FoxL2 expression vectors do not demonstrate any FoxL2 bands (Fig. 8b, lanes 3-4). Therefore, the human FSHβ promoter does not bind the FoxL2 at the -350 bp element. Further analysis of the mouse sequence indicated the presence of a SBE half site, 5'-AGAC-3', 5' of the FoxL2 binding site (Fig. 7b). When comparing the human FSHβ promoter sequence to that of the mouse, the human FSHβ promoter does not contain the Smad-half site 5' of the putative FoxL2 site (Fig. 8c). Therefore, the human FSHβ promoter was mutated to include the Smad-half site (+Smad-half) to further assess whether the Smad-half site is needed for FoxL2 binding. The mouse probe was also mutated to eliminate the Smad-half site to asses whether it is necessary to FoxL2 binding (Fig. 8c). The addition of the Smad-half site did not contribute to FoxL2 binding to the human promoter (Fig. 8a, lanes 13-16), nor did the mutation of the Smad-half site eliminate binding to the mouse promoter (Fig. 8a, lanes 9-12). With the addition of αFoxL2 in LβT2 cell extracts, we observe a supershift indicated by arrow 1 of the complex that forms at arrow 2 (Fig. 8a, lanes 3, 4, 11 and 12). FoxL2 binding occurs in Cos-1 cells transiently transfected with FoxL2 expression vector for both the wild type and mutated mouse probes but not in either the wild type or mutated human probes (Fig. 8b). We conclude that Smad-half site is not necessary for FoxL2 binding to the -350 bp site on the mouse FSHβ promoter.

Since the base pairs necessary for FoxL2 binding have been established through EMSAs and competition assays, we performed mutagenesis of these base pairs to assess their functionality for induction of the FSHβ promoter. We created three base-pair mutations throughout this region. The mutant, "-348", in Figure 9a-c, indicates that the three base pairs from -347 to -349 have been mutated, while "-345" indicates that the -344 to -346 base-pairs are mutated, and so on. Also included in this figure are mutations for the -335/-305 region in which the identities of the proteins that bind this region remains inconclusive. We determine that all mutations cause a decrease in caALK7, Smad3, and activin induction (Fig. 9a-c). Induction by constitutively active ALK7 decreases 50-80% with any three base-pair mutations between -350/-340 and -330/-310 (Fig. 9a), while induction by Smad3 decreases 50-70% (Fig. 9b), and fold induction by activin decreases 30-50% (Fig. 9c). Though the key transcription factor(s) binding in the region from -335/-305 bp is still unidentified, this region plays a role in activin induction of the FSHβ gene. On the other hand, the -350/-340 region contains a functional FoxL2 binding site, and all nine base pairs that are critical for FoxL2 binding are important for activin responsiveness.

FoxL2 Potentiates Induction of the FSHβ Promoter

To assess whether the -350 FoxL2 site is interacting with Smads at the -267 SBE site, mutations of the -350 FoxL2 site and the -267 SBE site were

created in the 1 kb FSHβ promoter and cotransfected into cells with overexpressed caALK7, Smad3, or with activin treatment. As a result, the drop in induction by the mutation in the SBE site is not further increased by the addition of the mutation of the -350 FoxL2 site (Fig. 10a-c). Figure 10c demonstrates that activin treatment is attenuated by 48.3% with the -267 SBE mutant and 25.8% by the -350 FoxL2 mutant, while the double mutation containing both mutated sites is still maintained at 51.6%, indicating no interaction between these two sites.

To assess the sufficiency of the -350 FoxL2 site for FSHβ promoter activity and to further test whether the -350 FoxL2 site can interact with the -267 SBE site, multimers containing four consecutive repeats of the -350 FoxL2 alone or -350 FoxL2 and -267 SBE sites in combination were constructed on the minimal Tk-Luciferase pGL3 reporter gene (Tk-Luc). Multimers are utilized to assess the sufficiency of the sites to promote Tk-Luc activity. The -350 FoxL2 site is not sufficient to induce a response by caALK7, Smad3, or activin treatment since luciferase activity for these multimers is not induced as compared empty vector control (Fig. 11). In contrast, a multimer of the consensus -267 SBE site was sufficient to induce activity by all three treatments. By linking the -350 FoxL2 site to the -267 SBE site and creating a multimer, a small increase in caALK7 and Smad3 activity was induced in comparison to the -267 SBE site alone (Fig. 11). However, when evaluating the effect of activin, the FoxL2 with the SBE site is not induced

higher than the SBE site alone, indicating as above that these two sites do not interact (Fig. 11).

We have shown that FoxL2 regulates the FSHβ promoter through binding to a site at -350 bp from the transcriptional start site. To investigate how FoxL2 proteins regulate the expression of the FSHβ promoter, a FoxL2 expression vector was transiently transfected into LβT2 cells with or without activin treatment. Transient transfection of FoxL2 expression vectors in LβT2 cells does not significantly increase FSHβ promoter activity as compared to control (Fig. 12a) since LβT2 cells endogenously express high levels of FoxL2. However, transfection of FoxL2 in activin-treated cells increases induction from 3.6 by activin alone to 6.6 fold with the combination (Fig. 12a) representing a significant increase. Thus, FoxL2 potentiates activin treatment in LβT2 cells.

The CV1 cell was used in subsequent experiments because it is a heterologous cell line that may be expressing less FoxL2, and CV1 cells have relatively higher transfection efficiency to LβT2 cells. Overexpression of caALK7 or Smad proteins in CV1 cells did not induce the FSHβ promoter (Fig. 12b), while transfection of FoxL2 induces 4.5 fold when comparing to empty-vector control. In addition, cotransfection of FoxL2 with caALK7, Smad3, and/or Smad3/4 elicits potentiated induction as compared to empty-vector or FoxL2 alone (Fig. 12b). FSHβ promoter activity increased from 4.5 to 5.7, 7.4, or 15.1 fold when cotransfected with caALK7, Smad3, or Smad3/4

respectively (Fig. 12b). Therefore, FoxL2 expression can potentiate the effects of activin receptor or Smad proteins on the FSHβ promoter.

To further investigate the effects of the -350 FoxL2 site, the site was mutated and the promoter transfected into CV1 cells to compare with the wild-type promoter. Mutation of the -350 FoxL2 site reduced fold induction by 33.8% (Fig. 13a). Furthermore, the potentiated effect of Smad3/4 response by the FoxL2 expression vector (seen in Figure 12b) is attenuated by 38.9% (Fig. 13b). The decrease in response due to the mutated FoxL2 site further indicates that FoxL2 and its binding site at -350 bp confers responsiveness on the FSHβ promoter.

Together, these results indicate that Smad3 is indirectly acting on the -350 bp site of the promoter through interactions with FoxL2, which can then bind directly to the site. While there is no interaction between the -350 FoxL2 and -267 SBE sites, the Smad3 effect both maps to the FoxL2 site and is potentiated by FoxL2 overexpression in the heterologous cell line. Thus, although there is no interaction between the sites and since -350 and -267 function independently to convey activin responsiveness, there is likely Smad3 and FoxL2 protein-protein interaction at the FoxL2 sites on the mouse FSHβ promoter, as was recently shown for activin induction of the follistatin promoter.

FoxL2 Mapping in CV1 Cells Reveals Additional Putative FoxL2-Responsive Regions

To identify whether there are additional FoxL2 sites, truncation analysis was performed in CV1 cells with overexpressed FoxL2. A significant decrease in fold induction occurs between the -230 bp and -127 bp region (Fig. 14a). The truncation of -230 bp and -127 bp causes a decrease in induction by 51.6% (Fig. 14a). Sequence analysis of this region reveals two putative FoxL2 binding sites (Fig. 14b). These regions are highly conserved in the mouse and human FSHβ promoters. To test whether this region contains a FoxL2-regulated site, EMSAs were performed with both radiolabeled human and mouse probes for the -200 bp region and the -153 bp region. Radiolabeled probes were incubated with LβT2 nuclear extracts with or without activin treatment (Fig. 15a). The mouse probe encompassing the -153 bp site and the human probes for both the -200 and -153 FoxL2 putative sites exhibit a band that is supershifted upon the addition of the antibody to FoxL2 as indicated by the arrow (Fig. 15a). We were unable to identify whether FoxL2 binds the -200 site in the mouse due to a comigrating band. The same radiolabeled probes were incubated with Cos1 nuclear extracts containing overexpressed FoxL2 and the arrow in Figure 15b indicates the FoxL2 protein band. Each probe detects the FoxL2 consistent with the findings in LβT2 cells displayed in Figure 15a. To test whether the -200 and the -153 site are essential for

FSHβ promoter response, mutations were made in the putative FoxL2 sites. The mutations in the newly identified sites at -200 and -153 both reduce induction of the mouse FSHβ promoter by caALK7, Smad3, and activin treatment (Fig. 16a-c).

Since the homologous sites for -200 and -153 in the human promoters bind the FoxL2 protein in EMSAs, we tested whether the newly discovered FoxL2 sites can convey activin responsiveness of the human FSHβ promoter. Thus, we mutated the -350, -200, and -153 sites in the human FSHβ promoter and tested the effects of caALK7 and activin treatment on activity (Fig. 17a-b). Mutation of the -350 FoxL2 site does not appear to have significant effects on human FSHβ promoter activity since it does not decrease activin induction, consistent with a lack of FoxL2 binding to the site. However, the -153 and -200 mutations completely eliminate activin and ALK7 responsiveness of the human FSHβ promoter (Fig. 17a-b). The FoxL2 sites at -153 and -200 are the only activin-responsive sites identified on the human FSHβ promoter.

Therefore, the FoxL2 factor plays a critical role in activin induction of both the human and mouse FSHβ gene. Although the exact position of the FoxL2 binding sites may differ among species, FoxL2 exerts its effect through binding to multiple FoxL2 elements within the FSHβ promoter.

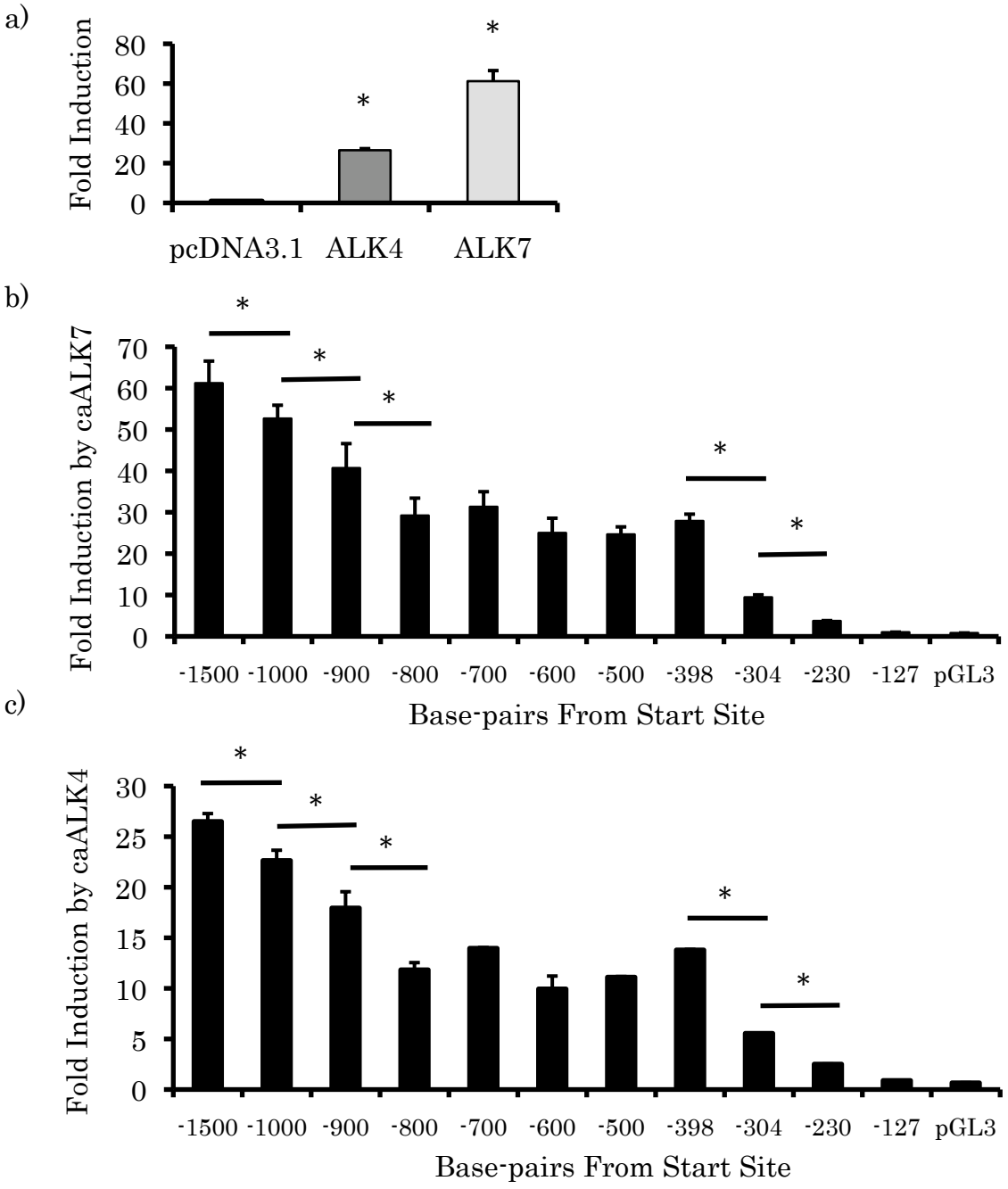


Figure 1. Constitutively active ALK activity maps to multiple regions on the FSHβ promoter.

LβT2 cells were cotransfected with the specified mouse FSHβ promoter truncations, fused with a luciferase reporter gene, and caALK4 or caALK7. a) caALK4 and 7 significantly induces promoter activity. b-c) ALK sites are located in distal regions and between -398/-230. Luciferase β-gal ratio was normalized to pGL3 and values are represented by three experiments done in triplicates. (*) represents a significant difference in induction with $p<0.05$.

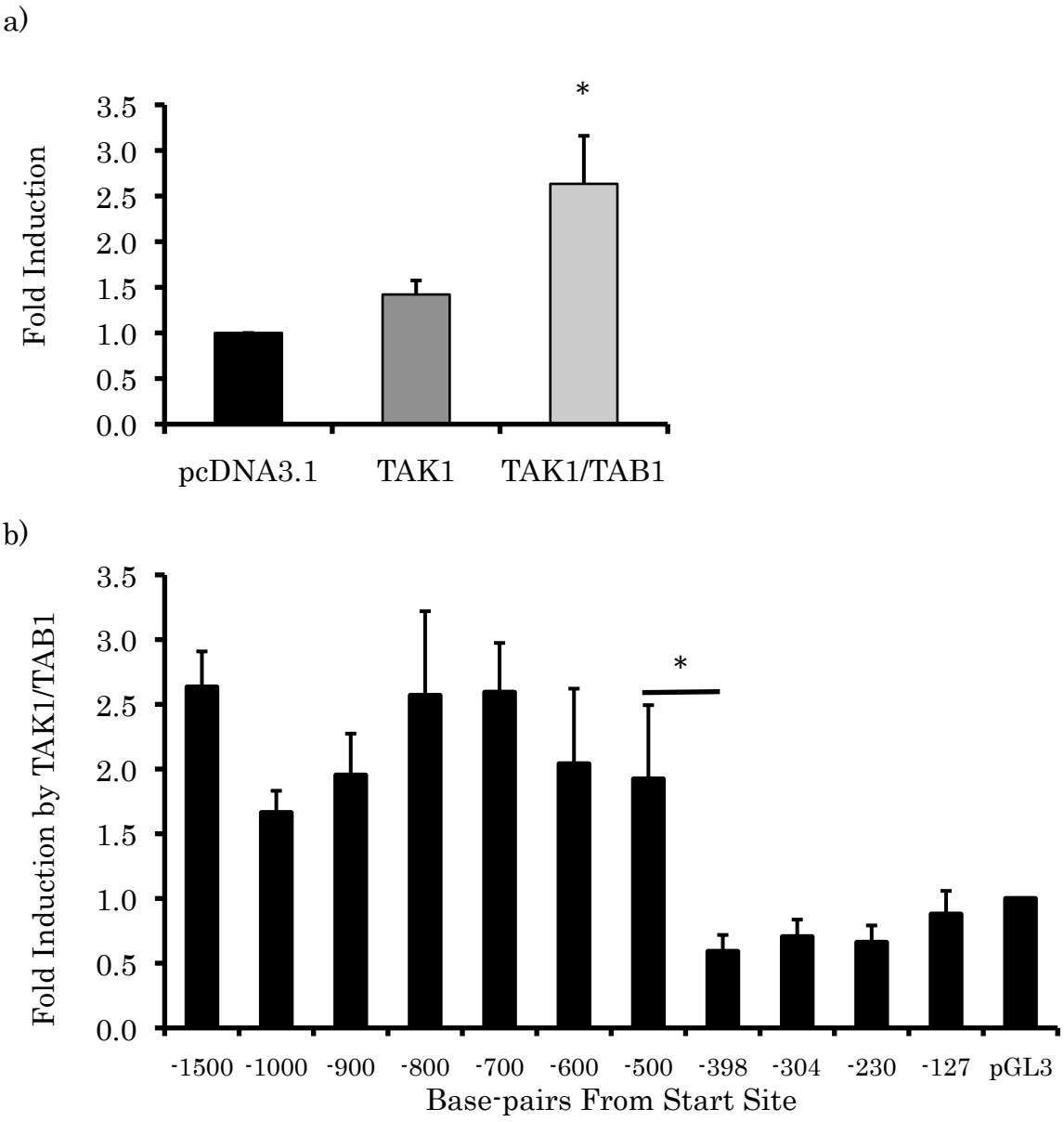


Figure 2: TAK1/TAB1 maps to -500/-398 bp from the transcriptional start site.

LβT2 cells were cotransfected with the specified mouse FSHβ promoter truncations, fused with a luciferase reporter gene, and TAK1 and/or TAB1. Luciferase β-gal ratio was normalized to pGL3 and values are represented by three experiments done in triplicates. a) TAK1/TAB1 significantly induces promoter activity. b) There is a 64.7% reduction in induction due to the -500/-398 truncation. (*) represents a significant difference in fold induction with $p<0.05$.

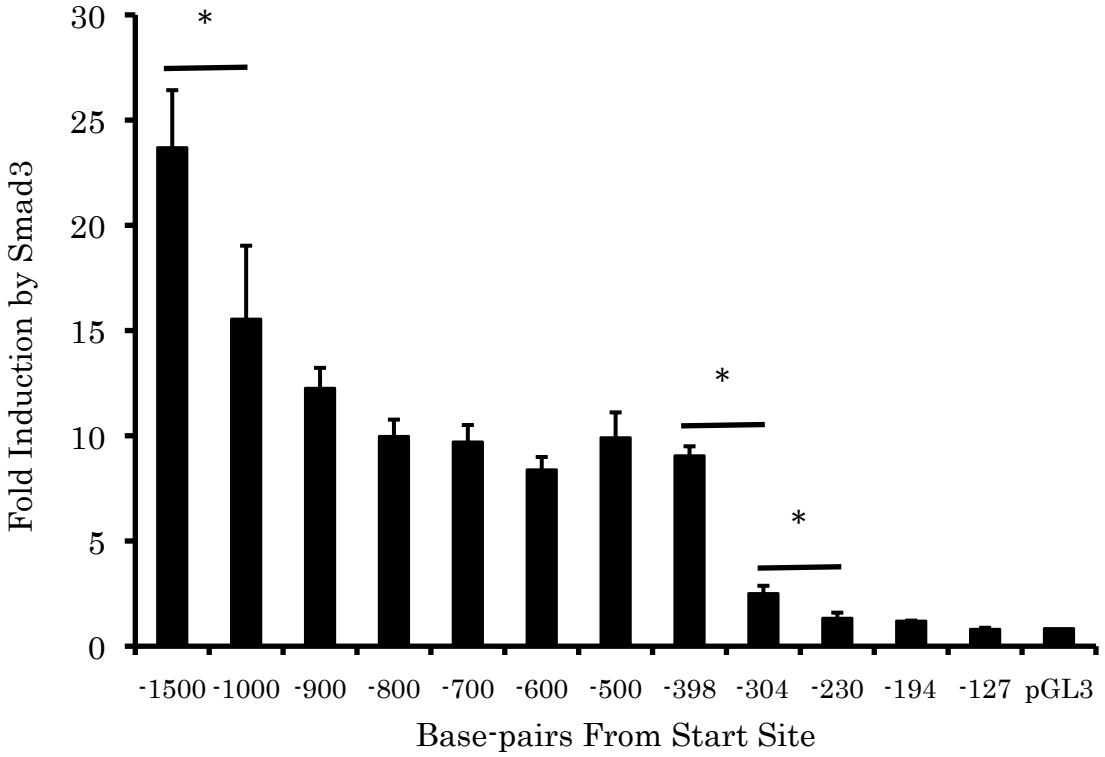


Figure 3: Smad3 activity maps to distinct regions overlapping response sites of caALK.

LβT2 cells were cotransfected with the specified mouse FSHβ promoter truncations, fused with a luciferase reporter gene, and Smad3. Luciferase β-gal ratio was normalized to pGL3 and values are represented by three experiments done in triplicates. There is a 34.4%, 72.3%, and 46.7% reduction in induction due to the -1500/-1000, -398/-304, and -304/-230 truncations respectively. (*) represents a significant decrease in fold induction with $p<0.05$.

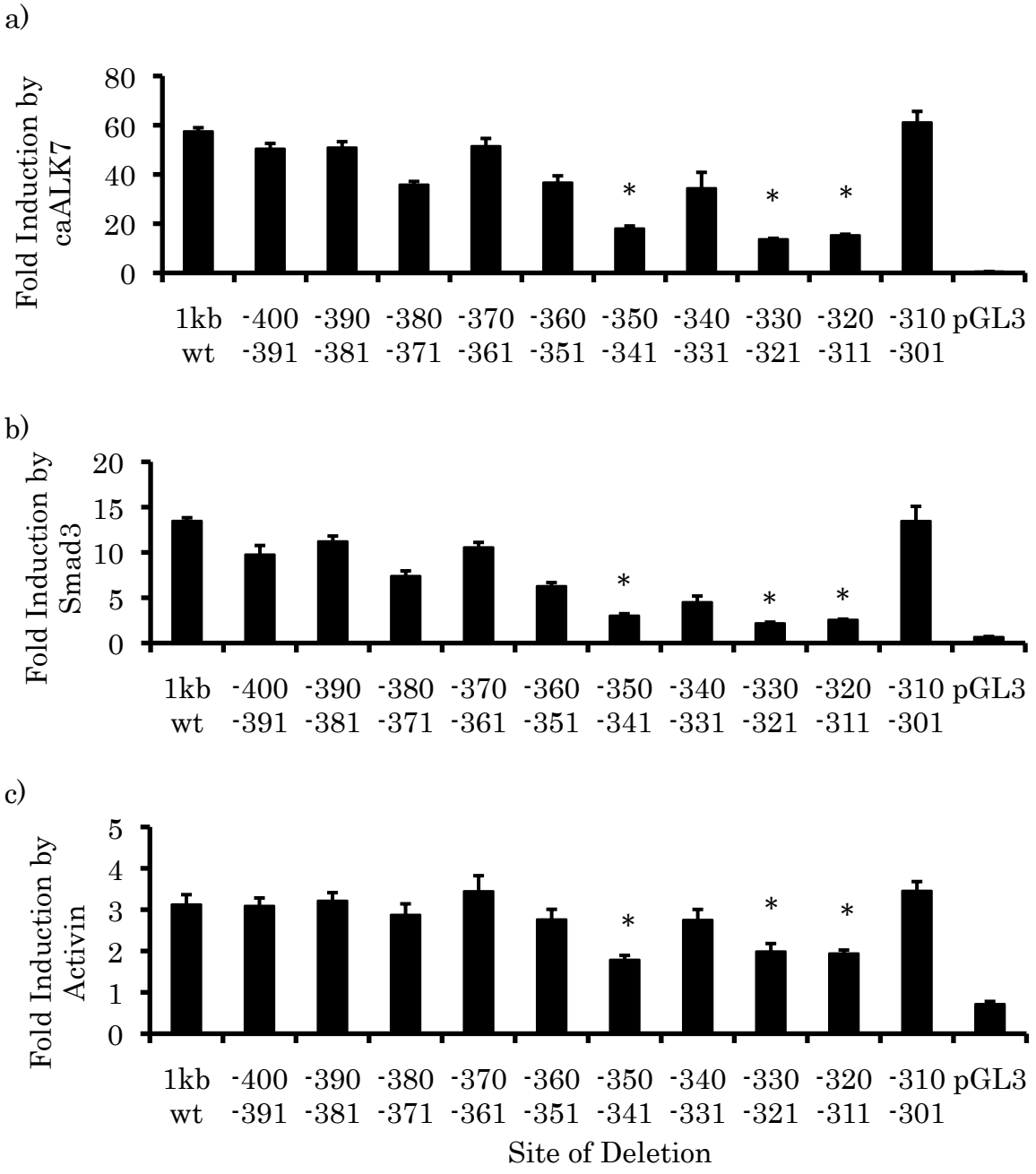
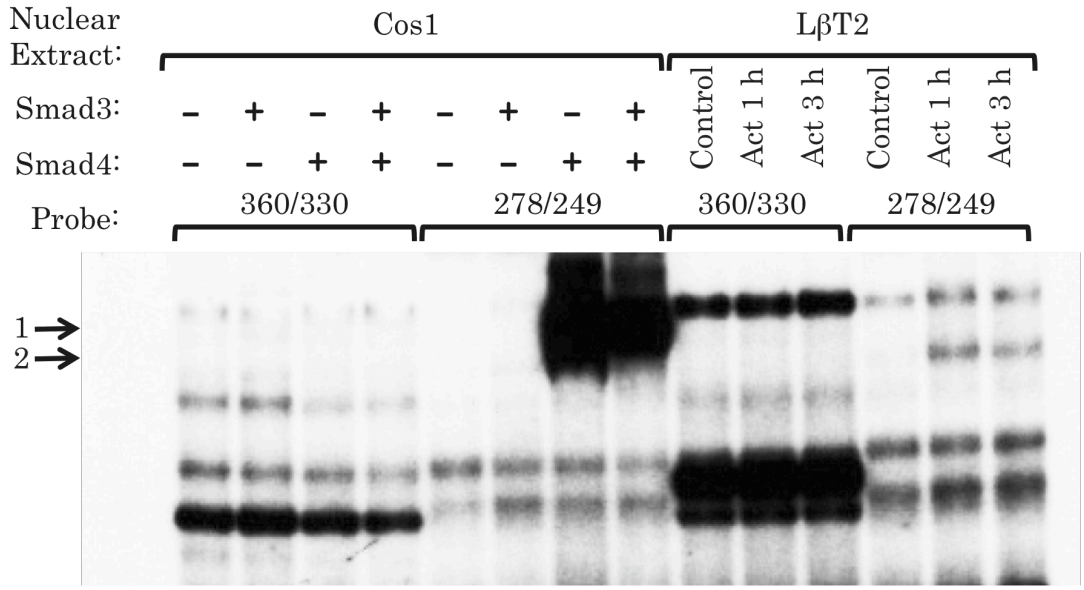


Figure 4: Constitutively active ALK, Smad3, and activin activity overlap at -350/-341 and -330/311 regions.

LβT2 cells were transfected with the specified 10 bp internal deletion of the mouse FSHβ promoter fused with a luciferase reporter gene. Cells were also transfected with caALK7 (a), Smad3 (b), or treated with activin (c). Luciferase β-gal ratio was normalized to pGL3 and values are represented by three experiments done in triplicates. (*) represent significant reduction from wild-type at sites that overlap among all three treatments, $p<0.05$.

a)



b)

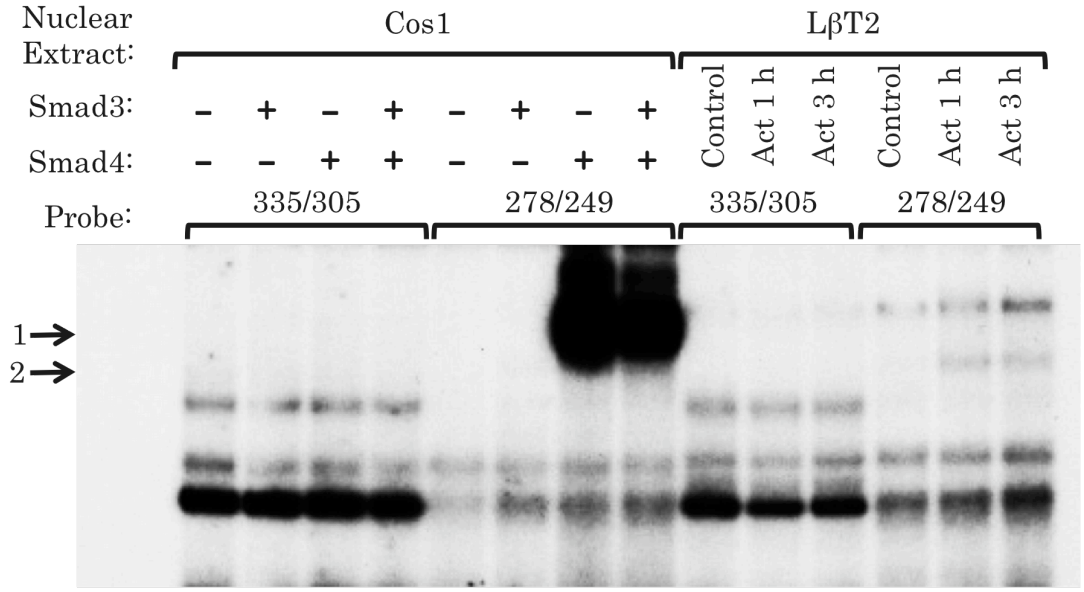


Figure 5: The -350/-340 and -330/-310 probes cannot detect Smad protein binding.

Nuclear extracts of Cos1 cells transfected with Smad3 and LβT2 cells treated with activin were incubated with radiolabeled probes, indicated in Table 4. a) -360/-330 and -267/-249 probes. b) -335/-305 and -267/-249 probes. Arrow 1 represents Smad protein bound to the SBE -267/-249 probe. Arrow 2 represents an activin induced Smad band bound to the SBE -267/-249 probe.

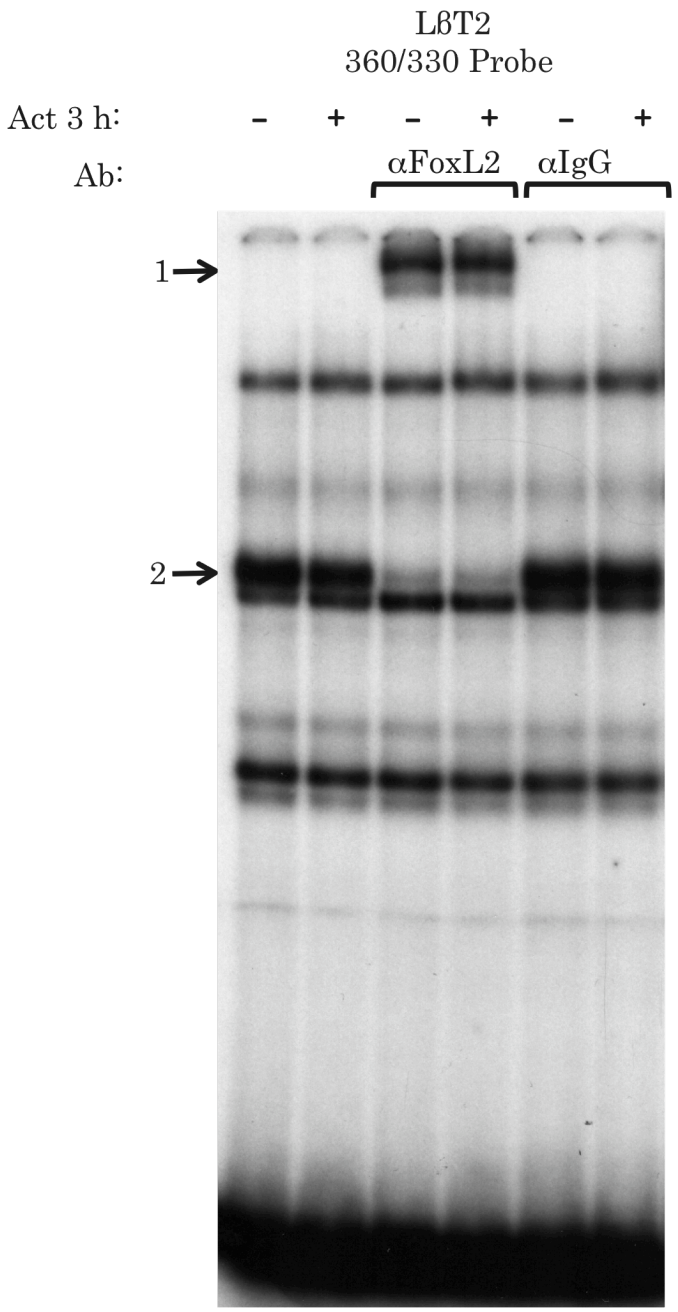
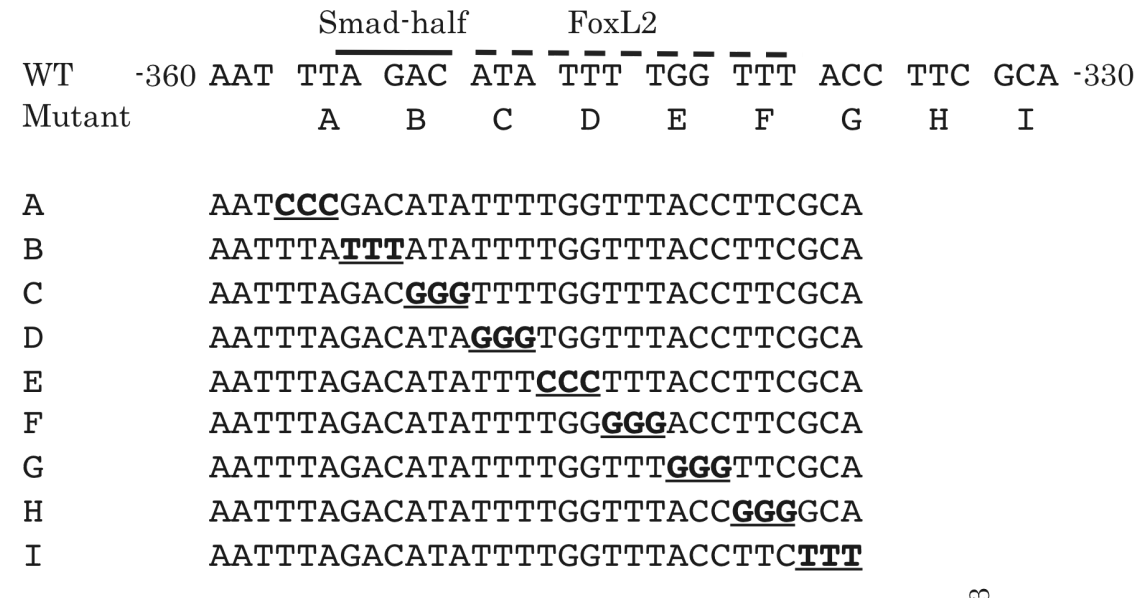


Figure 6: FoxL2 proteins bind to the -360/-330 region in the FSHβ promoter.
Nuclear extracts of LβT2 cells treated with three hours of activin or control were incubated with the -360/-330 probe and antibodies for FoxL2 and IgG. Arrow 1 represents the supershift of the FoxL2 complex by the addition of αFoxL2. Arrow 2 represents the FoxL2 complex prior to the addition of its antibody. IgG represents control for nonspecific antibody binding.

a)



b)

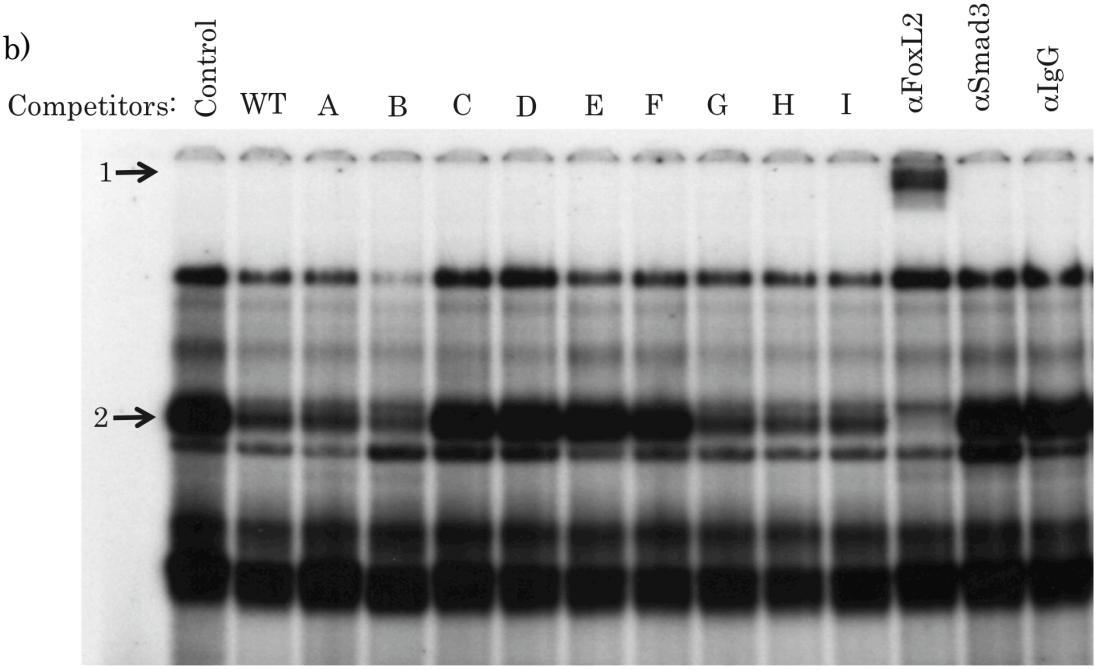
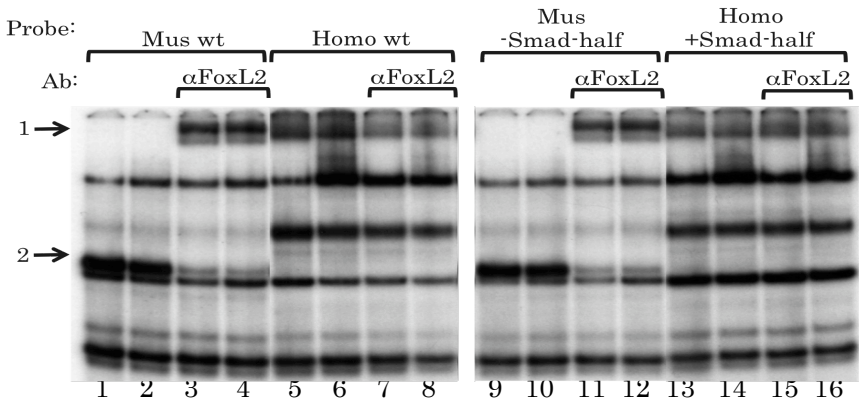
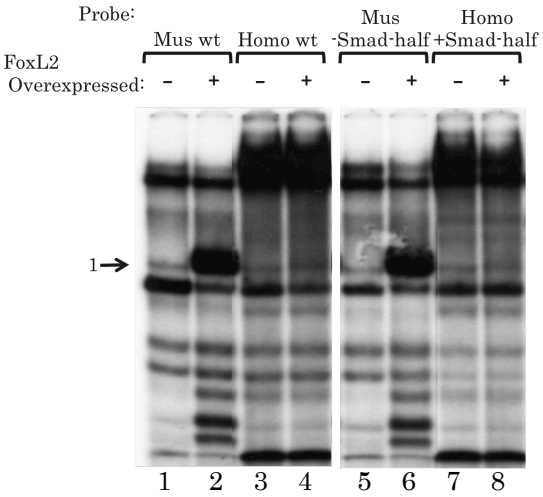


Figure 7: FoxL2 binds to the sequence 5'-ATATTTTGGTTT-3'.
a) An alignment of the wild-type sequence of the -360/-330 region and the oligonucleotide competitors with scanning mutations as shown. b) Unlabeled nucleotides used in 200-fold excess in the EMSA compete with the radiolabeled probe. Nuclear extracts of LβT2 cells treated with activin bind to the radiolabeled probe. The FoxL2 complex supershifts with the addition of αFoxL2 as shown with arrow 1. αSmad3 and αIgG are negative controls. Mutants C-F cannot compete for FoxL2 binding as indicated by arrow 2.

a)



b)



c)

```
Mus WT     AATTAAGACATATTTTGGTTTACCTTCGCAATGGA
Homo WT    AATTTATTAACCATATTTTTTAATGCATCTCCTGA

Mus
—Smadhalf AATTAAACCATATTTTGGTTTACCTTCGCAATGGA
Hum
+Smadhalf AATTTATTAGACATATTTTTTAATGCATCTCCTGA
```

Figure 8: Human FSHβ homology to mouse not sufficient for FoxL2 binding in human.

a) EMSA utilizing LβT2 nuclear extracts. b) EMSA utilizing Cos1 nuclear extracts transfected with FoxL2. c) Homology of mouse wt and human wt probe with mutations in putative Smad sites indicated in bold and underlined. a) Arrow 1 represents the supershift of the complex indicated by arrow 2 due to the addition of αFoxL2. b) Arrow 1 represents a FoxL2 induced band. Human probe contains considerable homology to the mouse but does not bind FoxL2 protein. A putative Smad-half site, AGAC, found in the human is not necessary or sufficient for FoxL2 binding.

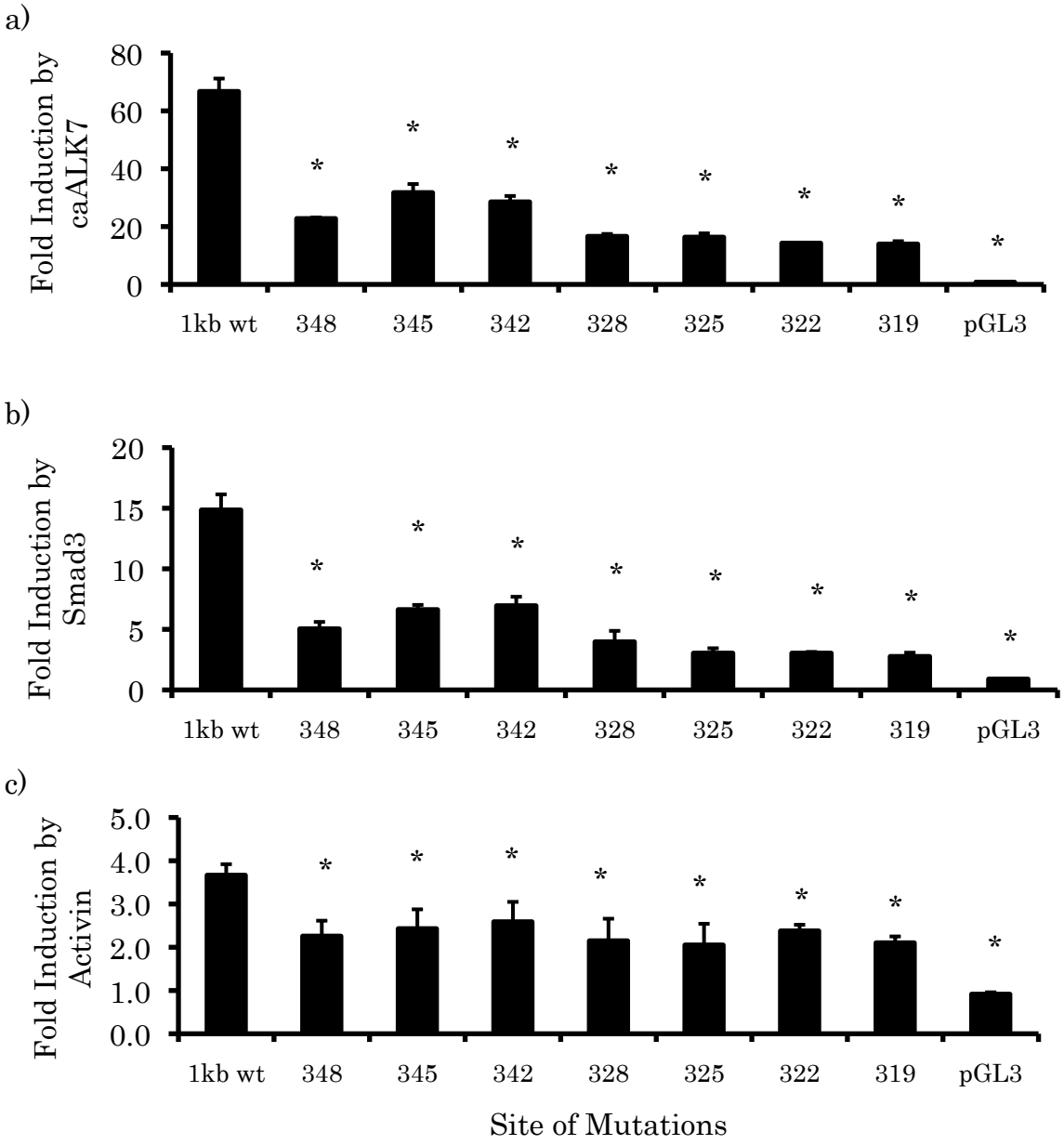


Figure 9: The -350/340 and -330/-310 regions are necessary for induction of the FSHβ gene by caALK, Smad3, and activin.

Three base-pair mutations of the mouse FSHβ promoter, as indicated in Table 2, were transfected into LβT2 cells with caALK7 (a), Smad3 (b), or activin treatment (c). Every mutation elicits a significant reduction in response from wild-type where $p<0.05$. This is observed in all three treatments (a-c). Luciferase β-gal ratio was normalized to pGL3 and values are represented by three experiments done in triplicates.

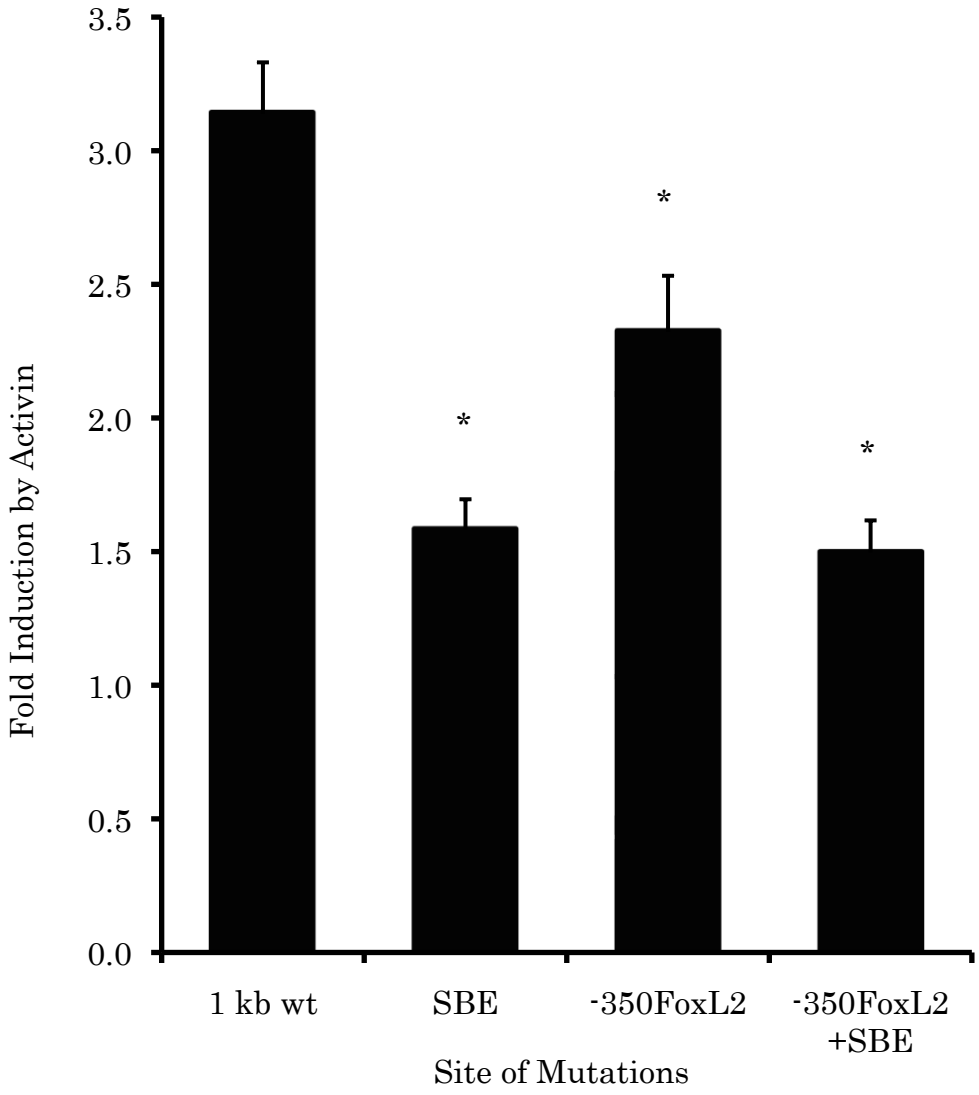


Figure 10: The -350 FoxL2 and SBE sites do not interact to induce FSHβ promoter activity.

Both single and double mutations were created at the -350 FoxL2 and SBE sites on the mouse FSHβ promoter, fused to a luciferace reporter gene, and transfected into LβT2 cells. Luciferase β-gal ratio was normalized to pGL3 and values are represented by three experiments done in triplicates. Cells were treated with activin for five hours prior to lysis. Single mutations decreased significantly from wild-type as did the double mutation. However the magnitude of reduction for the double mutation was similar to that of the single mutations. (*) represent significant difference in fold induction from the 1 kb wild-type promoter with $p<0.05$.

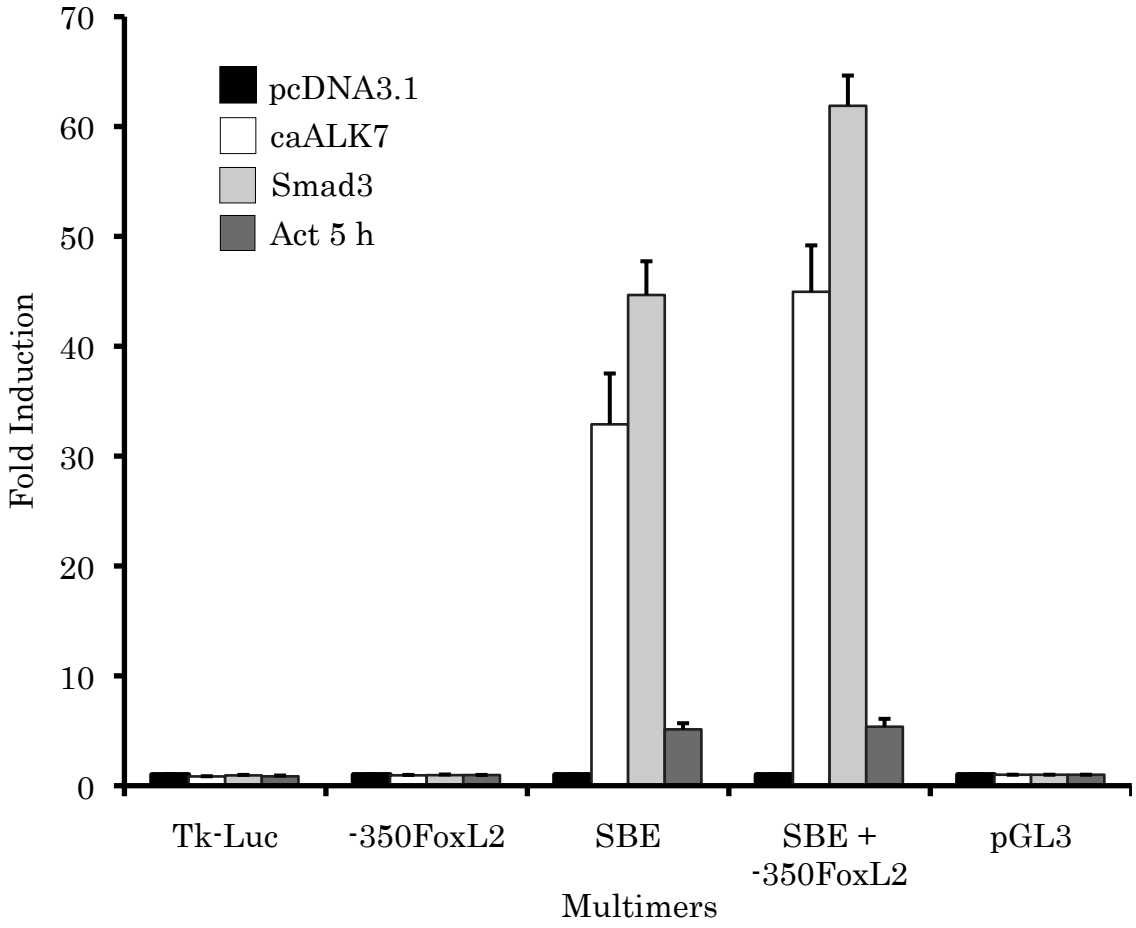


Figure 11: FoxL2 mutlimer is not sufficient to induce FSHβ promoter activity.
Multimers containing four repeats of either -350 FoxL2, SBE or both sites were created as indicated in Table 6 and transfected into LβT2 cells in combination with either pcDNA, caALK7, Smad3, or five hour activin treatment. The multimers were fused with the Tk-Luc minimal promoter. There is no induction of activity from the -350 FoxL2 multimer and a very strong induction of activity with the SBE multimer. In combination, there is a slight increase of activity by caALK7 and Smad3 from that of the SBE multimer, but there is no increase in response by activin treatment. Luciferase β-gal ratio was normalized to pGL3 and values are represented by three experiments done in triplicates.

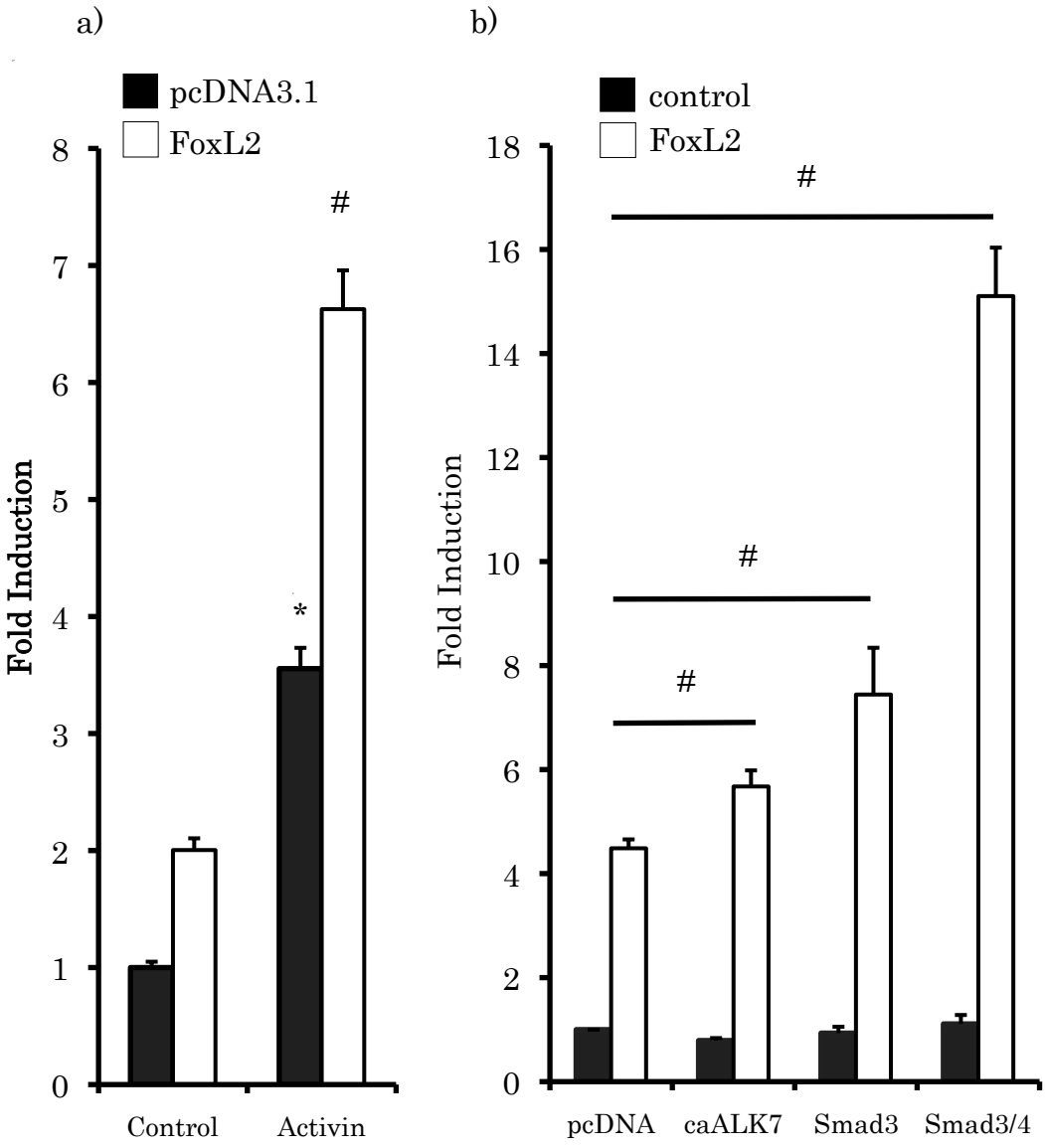


Figure 12: Overexpressed FoxL2 potentiates effects of activin in LβT2 cells and caALK7, Smad3, and Smad3/4 in Cos1 cells.
LβT2 cells were transfected with 1 kb mouse FSHβ promoter-luc reporter construct in either LβT2 cells treated with activin or Cos1 cells transfected with pcDNA control, caALK7, Smad3, or Smad3/4. Cells were also cotransfected with FoxL2 expression vector. a) Responsiveness of LβT2 cells with activin treatment and overexpressed FoxL2 increased significantly from activin treatment alone. Overexpressed FoxL2 significantly increased effects by control. b) All transfection treatments in Cos1 cells were potentiated by the addition of FoxL2 expression vector. Luciferase β-gal ratio was normalized to pGL3 and values are represented by three experiments done in triplicates. (#) and (*) represent significant changes in induction with $p<0.05$.

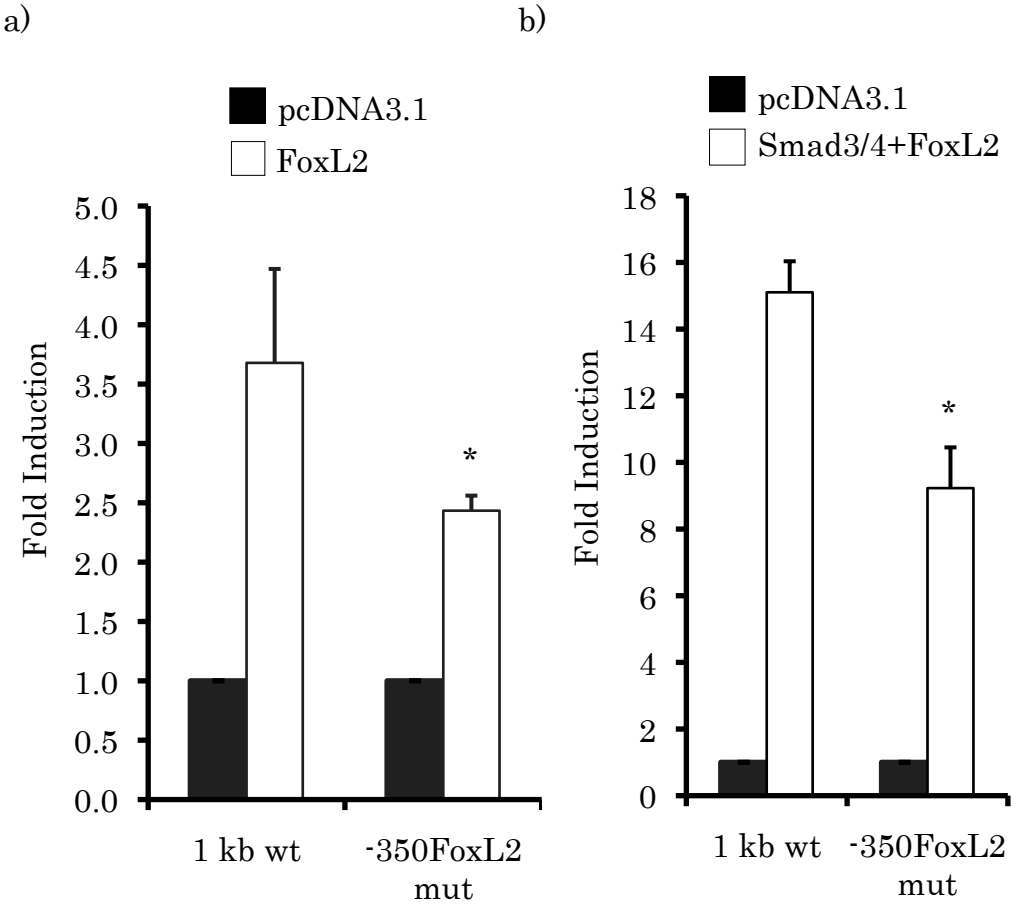
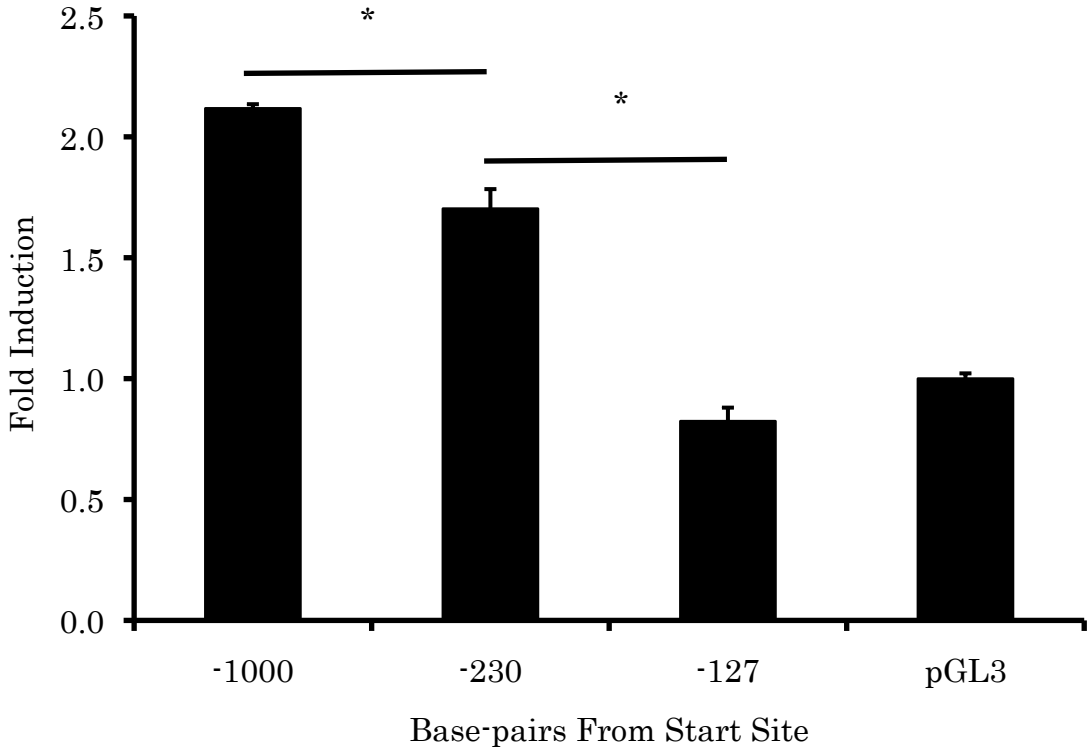


Figure 13: FoxL2 site at -350 is necessary for full FSHβ promoter response.

The -350 FoxL2 site was mutated on the mouse FSHβ promoter, fused to a luciferase reporter gene, and transfected into CV1 cells. a) Induction by FoxL2 expression vector decreased by 33.8% due to the mutation in the FoxL2 site. b) Induction by cotransfected Smad3/4 and FoxL2 expression vectors decreased by 38.9%. Luciferase β-gal ratio was normalized to empty vector and values are represented by three experiments done in triplicates. (*) represent significant changes in induction from wild-type with $p<0.05$.

a)



b)

```
Mus   -200: 213-CATATCAGATTCGGTTTGTACAGAAACCATCATCA-179
Homo -200: 213-TGTATCAAATTTAATTTGTACA AAATCATCATCTC-179

Mus   -153: 163-GCTCTGTGGCATTTAGACTGCTTTGGCGAGGCTTG-129
Hum   -153: 163-AATCTACTGCGTTTAGACTACTTTAGTAAAGCTTG-129
```

Figure 14: Putative FoxL2 binding sites in the -230/-127 region of the FSHβ promoter.

a) CV1 cells were transfected with truncations of the mouse FSHβ promoter, fused with a luciferase reporter gene, and overexpressed FoxL2 expression vectors. Luciferase β-gal ratio was normalized to pGL3 and values are represented by three experiments done in triplicates. There is a 51.6% reduction in induction due to the -230/-127 truncation. (*) represents a significant difference in fold induction with $p<0.05$. b) There is a strong homology of the mouse FSHβ promoter to that of the human in this region. Bold and underline base-pairs represent putative FoxL2 binding sites.

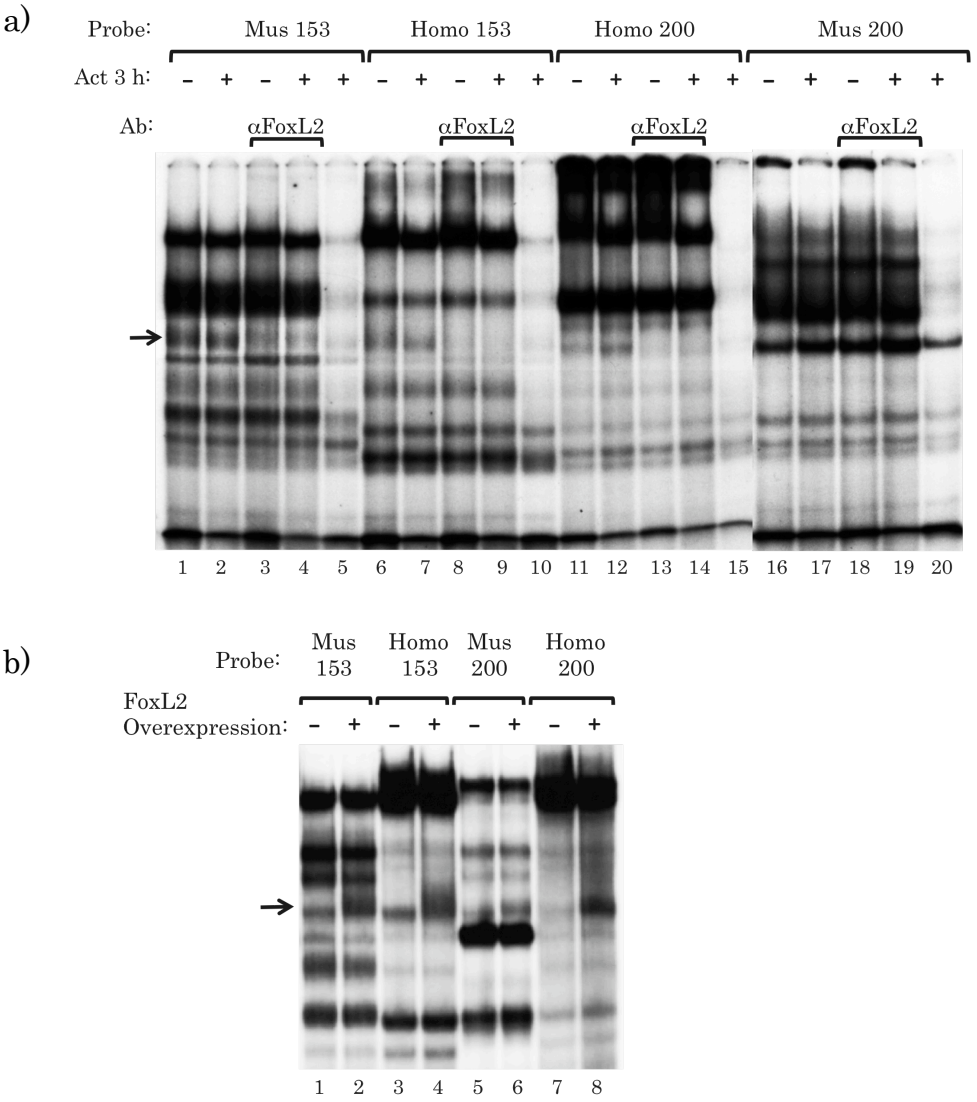


Figure 15: Putative FoxL2 binding sites in the -153 bp mouse region and the -200 and -153 bp human regions.

Nuclear extracts of LβT2 cells, treated with activin, and Cos1 cells, transfected with FoxL2, were incubated with radiolabeled human and mouse probes encompassing the -200 and -153 bp regions on the FSHβ promoter, as indicated in Table 4. a) LβT2 nuclear extracts contain FoxL2 protein that are able to bind to the -153 and -200 probes in both the mouse and human sequences, as indicated by the arrow. The complex is supershifted with the addition of αFoxL2. b) Cos1 cells transfected with FoxL2 demonstrate detection of FoxL2 by the -153 and -200 mouse and human probes, as indicated by the arrow. Identification of the FoxL2 band in the -200 mouse probe is indeterminate due to a comigrating band.

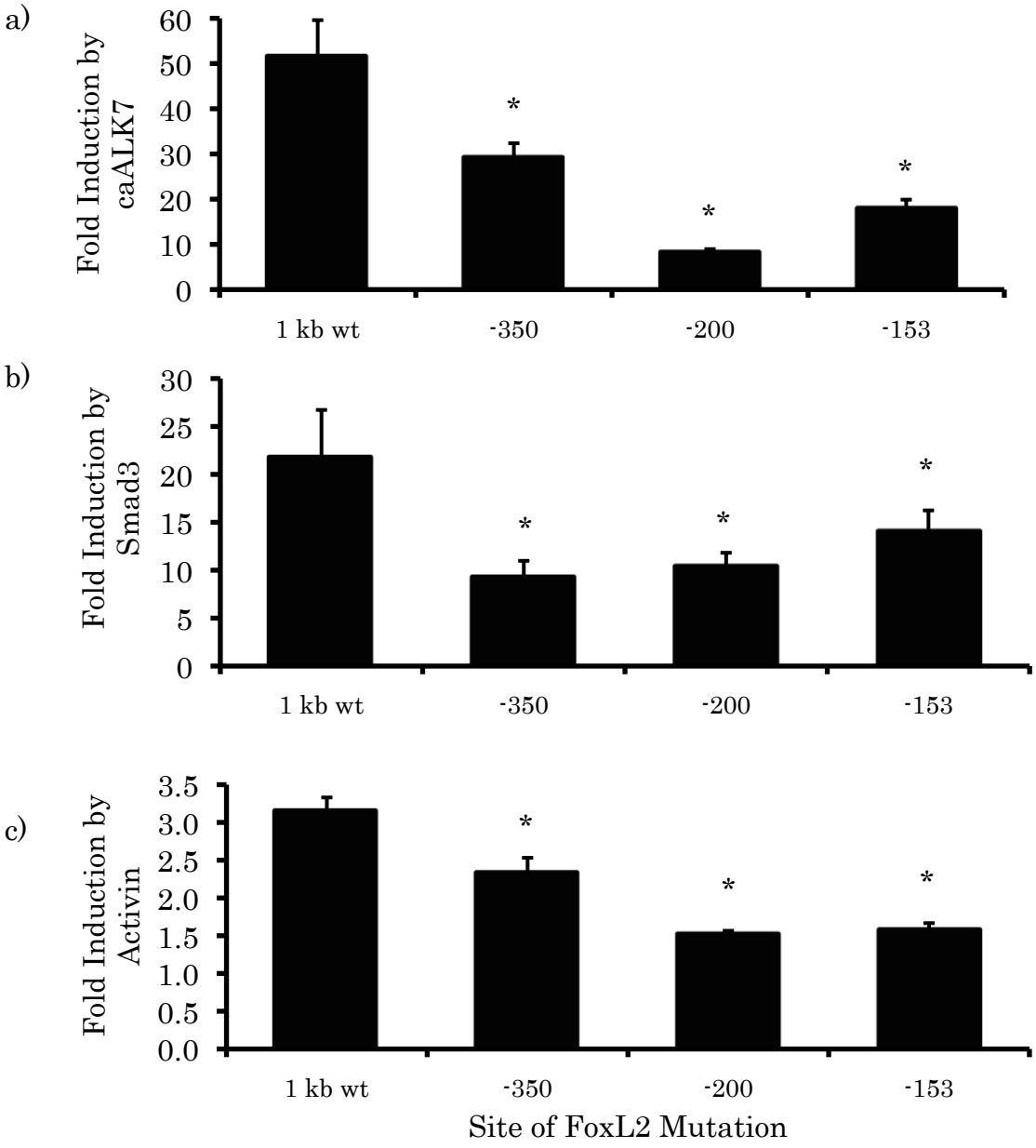


Figure 16: Downstream putative FoxL2 sites at -200 bp and -153 bp play a role in mouse FSHβ promoter activity.

Mutations at the -350 FoxL2, -200, and -153 bp sites were created on the mouse FSHβ promoter fused with a luciferase reporter gene as outlined in Table 2 and 3. These constructs were transfected into LβT2 cells in addition to caALK7 (a), Smad3 (b), or activin treatment (c). Each mutation significantly reduces induction by each treatment. Luciferase β-gal ratio was normalized to pGL3 and values are represented by three experiments done in triplicates. (*) represents a significant difference in induction with $p<0.05$.

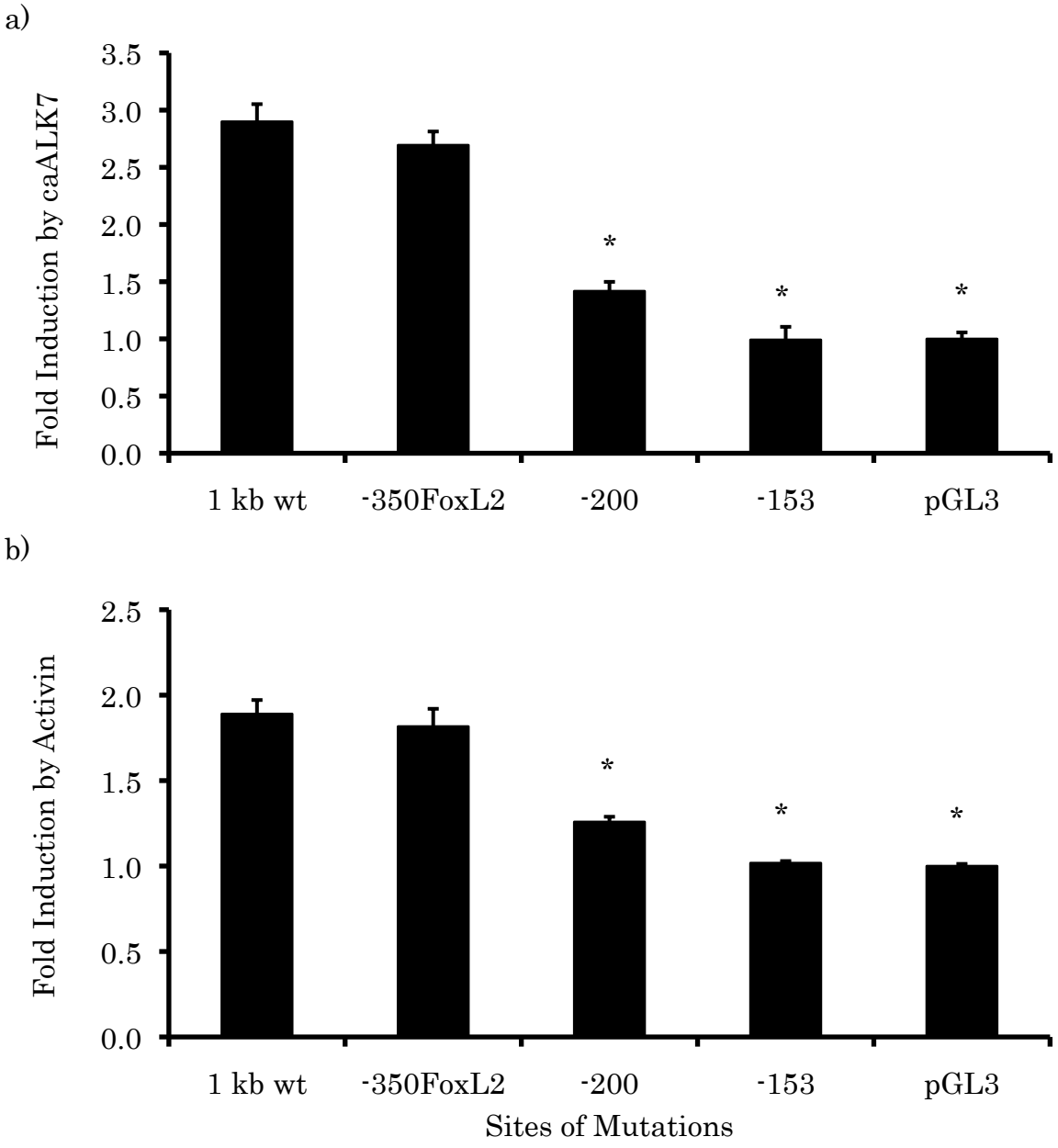


Figure 17: Downstream putative FoxL2 sites at -200 bp and -153 bp play a role in human FSHβ promoter activity.
Mutations at the -350 FoxL2, -200, and -153 bp sites were created on the human FSHβ promoter fused with a luciferase reporter gene as outlined in Table 2 and 3. These constructs were transfected into LβT2 cells in addition to caALK7 or activin treatment. The -200 and -153 mutations significantly reduce induction by each treatment. Luciferase β-gal ratio was normalized to pGL3 and values are represented by three experiments done in triplicates. (*) represent a significant difference in fold induction with $p<0.05$.

IV

Discussion

In this study, we identify a critical player for activin induction of the FSHβ gene. Follicle-stimulating hormone is essential for regulation of reproductive function and is responsible for proper ovulation and steroidogenesis in the hypothalamic-pituitary-gonadal axis. Activin plays a critical role in FSH synthesis, since activin is a very potent inducer of FSHβ gene expression. Truncations of the mouse 1.5 kb FSHβ promoter result in a significant decrease in activin induction in a step-wise manner, indicating that there are multiple activin-responsive sites (25). The activin–responsive elements previously identified in the promoter, do not account for complete response of the FSHβ gene. Thus, we sought to understand the mechanisms underlying the full response to activin and determine the molecular basis of control of FSHβ transcription.

We determined that caALK4 and 7 are sufficient to induce FSHβ activity in the absence of the activin ligand (Fig. 1a). The effects of caALK4 and 7 map to identical regions on the promoter (Fig. 1b-c), suggesting that activin A and B have similar responsive sites and implying that all of the effects of activin occur through one of these receptors. Because caALK7 is the

more potent inducer of FSHβ transcription, we utilized it in further studies. We sought out possible regulators of activin activity and because activin is a member of the TGFβ superfamily, we assessed the effects of the TGFβ-activated kinase (TAK1) on FSHβ expression. TAK1 was incapable of inducing a significant response on the FSHβ promoter alone; however, in combination with its binding protein, TAB1, there is a slight increase of response (Fig. 2a). Mapping of this activity through truncation analysis indicates that its putative target gene on the promoter is between -500 and -398 bp (Fig. 2b). This region does not overlap with activin responsive elements when compared to Figure 1b-c, therefore it is unlikely that TAK1/TAB1 convey activin responsiveness to the FSHβ promoter, contrary to previous conclusions that implicate TAK1 in activin signaling (58). Though a decrease in FSHβ induction is seen when a dominant-negative TAK1 (DN-TAK1) is used (data not shown), these effects may be due to the role of TAK1 in other signaling pathways rather than that of activin.

Another protein that has been implicated in regulating FSHβ gene transcription is Smad3. Activin-responsive elements in the FSHβ promoter have been identified at -267, which is a classical Smad-biding element (SBE), -153, which binds unknown proteins, and -120, which binds Pbx/Prep homeodomain proteins that recruit Smads (35, 44). Mutations in these sites result in dramatic effects on both induction by activin and by Smad3 overexpression, diminishing the ability of Smad3 to activate FSHβ (35, 44).

Our experiments with truncations of the promoter reveal another putative Smad responsive region, indicated by a larger decrease in induction that occurs when the region between -398 and -304 bp is deleted. Further resolution of this region reveals responsive sites at -350/-340 and -330/-310 (Fig. 4a-c). Since Smads do not bind these sites (Fig. 5a-c), but some of the Smad3 effect maps to this region (Fig. 3), it is likely that Smads interact with other factors to induce activin responses in this region.

Further investigation is required to determine the mechanism of regulation in the -330/-310 region, since we were not yet able to identify the factors that bind to this region, although the internal deletions and mutations clearly demonstrate its importance (Fig. 4a-c and Fig. 9a-c). In contrast, analysis of the -350/-340 bp sequence and subsequent gel shift experiments indicate a binding site for FoxL2. FoxL2 is a member of the forkhead family of transcription factors that share a conserved DNA-binding domain (60). Underscoring the importance of FoxL2 in reproductive function are various loss of functions from mutations and genetic disorders involving FoxL2 such as BPES, which causes premature ovarian failure in patients (61), and disruptions in granulosa cell differentiation and failure of oocyte growth (62). The pituitary and the gonadotropes also expresses FoxL2 (63), though the role of this protein in the pituitary needs further investigation.

FoxL2 binding to the FSHβ promoter does not change with activin treatment as compared to control cells. Additionally, the amount of FoxL2 in

LβT2 cells does not change following activin treatment, as indicated by western blotting (data not shown). Mutations of the FoxL2 site, though they do not affect the basal expression of the FSHβ gene (data not shown), have a profound effect on fold induction by activin. Therefore, we conclude that following Smad3 activation by activin treatment, Smad3 is recruited to the promoter by interaction with FoxL2. Indeed, protein-protein interaction between Smad3 and FoxL2 has already been demonstrated to play a role in follistatin gene induction by activin (64). This was confirmed by our experiments with overexpression of FoxL2 and Smad3 in the heterologous cells line, CV1, as discussed below.

In LβT2 cells, activin induction is potentiated by overexpression of FoxL2 (Fig. 10a), further demonstrating the role of FoxL2 in activin signaling. In CV1 cells, a heterologous cell line that does not contain the molecules required for gonadotropin synthesis, ALK7, Smad3, and Smad3/4 overexpression alone do not significantly induce the FSHβ gene (Fig. 10b), though each of them induces FSHβ in LβT2 cells (Fig. 4a-c). However, upon the addition of FoxL2, there is a substantial increase in FSHβ expression, as well as a potentiation of ALK7, Smad3, and Smad3/4 in CV1 cells (Fig. 10b). The results in CV1 cells demonstrate that FoxL2 plays an important role in inducing FSHβ activity (Fig. 10b), since lack of FoxL2 in non-gonadotrope cells causes the FSHβ promoter to be non-responsive to ALK7 and Smad induction. Furthermore, mutation of the -350 site reduces the effect of FoxL2

overexpression, indicating functional significance of this site (Fig. 11a-b). However, the effect of FoxL2 overexpression was not completely abrogated with this mutation, indicating the presence of other FoxL2 sites in the promoter that we have subsequently identified. Recently, studies have shown that FoxL2 participates in activin-dependent transcription of the follistatin gene through recruitment of Smad3. This study conferred expression of follistatin in another heterologous cell line, HEK293T, following the overexpression of Smad3 and FoxL2, similar to what we observe in FSHβ expression (64). FoxL2 also plays a role in activin induction of the GnRH receptor gene, through a site that comprises the GnRH activating sequence (GRAS), which recruits Smad3, 4, and AP1, again involving Smad3 and FoxL2 (65). It was also suggested that FoxL2 could be functionally or physically engaged with Smad3 and that a trimeric complex involving Smad3, 4, and FoxL2 is a possibility (64).

Although there is sequence homology between the mouse and human promoters in the -350/-340 sequence, FoxL2 does not bind the human promoter, while it binds the mouse with relatively high affinity. Our experiments, both with competition analysis (Fig. 7a-b) and mutations of the "AGAC" (Fig. 8a-c), indicate that the "AGAC" Smad half-site is not necessary for FoxL2 binding, nor does its mutation significantly affect activin responsiveness. Therefore, the differences between the mouse and human promoters in FoxL2 binding are conferred by other base pairs. Upon further

analysis of the -350/-340 FoxL2 site and identification of the -153 and -200 sites, we concluded that the repetitive stretch of three thymidines (TTT) is important, since FoxL2 binds as a homodimer (66).

To further demonstrate the role of FoxL2 sites in signaling, we created mutations of the -350/-340 FoxL2 site and observed decreases in fold induction by FoxL2 overexpression in LβT2 cells, as well as an attenuation of Smad3/4/FoxL2 overexpression in CV1 cells (Fig. 11a-b). A multimer of the -350 FoxL2 site is not sufficient to induce transcriptional activity by activin (Fig. 12), nor does it increase responsiveness of an SBE multimer, indicating that these sites do not interact. Double mutations of both the -267 SBE site and the -345 FoxL2 site did not cause the additional decrease in activin activity that is seen when the sites are mutated singly (Fig. 13), indicating that the interaction of FoxL2 and Smad sites is not facilitated by their cognate sequences (Fig. 13). Since the -267 and -350 sites do not interact, but Smad3 activity maps to the FoxL2 site, we hypothesize that FoxL2 and Smad proteins interact only on the FoxL2 sites.

Other FoxL2 sites are likely to be present in the FSHβ gene since the mutation of the -350 FoxL2 site did not completely eliminate the induction in LβT2 cells nor with overexpression of FoxL2 in the CV1 cells (Fig. 11a-b). Overexpression of FoxL2 in CV1 cells and truncation analysis indicates that additional putative FoxL2 sites are located between the -230 and -127 bp region (Fig. 14a-b). Analysis of this region reveals putative FoxL2 binding

sites at -200 and -153 bp (Fig. 14b). Previously, our lab had investigated an activin-responsive element at the -153 site in which the associated proteins were unknown (44). The site was critical for activin and Smad3 activation of FSHβ since a substantially significant drop of induction occurs upon its mutation (35, 44). However, the -200 site is a novel site in activin induction of the FSHβ gene. We were unable to demonstrate FoxL2 binding to the -200 region of the mouse promoter due to a co-migrating complex (Fig. 15a-b). Mutations of the mouse promoter at both -200 and -153 sites result in a significant decrease of activity (Fig. 16a-c), indicating that these sites are important for maintaining a full activin response. Because Smad3 activity maps to the FoxL2 binding sites, although Smad3 cannot bind to these sites, it is likely that protein-protein interactions are essential for Smad3 interactions with these sites through FoxL2, similar to the Smad3 and FoxL2 interaction we described earlier on the -350 FoxL2 site.

Due to the sequence conservation in the proximal -400 bp of the mouse and human promoters, we were interested to determine whether these sites play a role in the induction of the human FHSβ gene. The human FSHβ promoter was shown to respond to activin, albeit to a smaller degree than the mouse and with longer treatment. The difference in the level of the response to activin was attributed to the presence of the high-affinity SBE in the mouse promoter at -267 that is absent in the human sequence. Despite the absence of the SBE in the human promoter, the human gene is induced by

activin, though to a smaller degree. Therefore, we examined a role of the FoxL2 sites in the human promoter in response to activin. Consistent with the lack of binding to the -350 site, mutation of this region does not reduce the response to activin or to induction by ALK7 overexpression which mimics activin (Fig 17a-b). However, EMSAs utilizing the human probes encompassing the -153 and -200 regions reveal that FoxL2 can bind to both regions, confirmed by supershifts induced by the FoxL2 antibody (Fig. 15a-b). Mutations of the -153 and -200 bp sites in human completely abrogate ALK7 and activin activity down to levels of control (Fig. 17a-b). The decreased activity with mutations indicate that these sites are necessary for the human gene to respond to activin and that FoxL2 binding mediates this activity since binding is apparent in EMSA.

Therefore, multiple FoxL2 responsive elements are mediating the full response of FSHβ induction. In human, FoxL2 binds the -200 site with high affinity and the -153 site with lower affinity. In the mouse promoter, FoxL2 binds the -350 site with high affinity and the -153 site with lower affinity, while we were not able to demonstrate binding to the -200 site. All of these sites contribute to activin responsiveness of the FSHβ gene in both species. In human, the -200 and -153 FoxL2 sites are necessary for full activin response, whereas the -350, -200 and -153 FoxL2 sites are necessary for full activin response in the mouse. These sites are the first identified activin-responsive

elements of the human FSHβ promoter, and in the mouse promoter, FoxL2 is a novel player in activin induction of the FSHβ gene.

This study demonstrates that a novel target of activin-responsiveness is the forkhead transcription factor, FoxL2, which participates in activin induction through interactions with Smad proteins. Furthermore, the FSHβ proximal promoter contains multiple FoxL2 sites, which also confer activin responsiveness. A potent Smad response maps to -350, -200 and -153 bp, though no Smad binding elements exist in these regions. The forkhead transcription factor, FoxL2, binds to and activates through these sites, and is likely to interact with Smad3 to induce FSHβ gene transcription. This report has served to lay a foundation for further studies of FoxL2 and Smad3 interaction and the role of these proteins in activin signaling by elucidating functional FoxL2 sites on the FSHβ promoter. Future studies can now look into the mechanism by which the multiple FoxL2 sites at -350, -200 and -153 may interact to facilitate Smad3 interaction and full activin responsiveness.

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