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# Hormonal Regulation of FSH Beta-Subunit Gene Expression

A Thesis submitted in partial satisfaction of the

requirements for the Degree Master of Science

in

Biology

by

Arpi Hambarchyan

Committee in Charge:

Pamela L. Mellon, Chair
William McGinnis, Co-Chair
Jayant B. Ghiara

2011

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Co-Chair

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Chair

University of California, San Diego

2011

This work is dedicated to my family and friends. I appreciate all the love and support you have given me throughout this journey and for always believing in me. Thank you for always bringing joy and laughter to my life.

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| | |
|---|---|
| α-GSU | alpha glycoprotein subunit |
| ACTH | adrenocorticotropin hormones |
| ActR | activin receptor |
| ALK | activin receptor-like kinase |
| ARE | androgen response element |
| AP1 | activating protein 1 |
| BMP | bone morphogenetic protein |
| BPES | Blepharophimosis Ptosis Epicanthus Inversus Syndrome |
| CoSmad | common-mediator smad |
| EMSA | electrophoretic mobility shift assay |
| FAST | forkhead transcription factor FoxH1 |
| Fox | forkhead transcription factor |
| FSH | follicle stimulating hormone |
| GH | growth hormone |
| GnRH | gonadotropin-releasing hormone |
| GnRHR | gonadotropin-releasing hormone receptor |
| hFSHβ | human FSHβ subunit |
| HPG | hypothalamic-pituitary-gonadal axis |
| LH | luteinizing hormone |
| MAPK | mitogen-activated protein kinase |
| mFSHβ | mouse FSHβ subunit |
| MH1-SBE | mad-homology 1 domain smad-binding element |

| | |
|---|---|
| MSH | melanocyte-stimulating hormone |
| NFY | nuclear factor Y |
| oFSHβ | ovine FSHβ subunit |
| Pbx1 | pre B-cell leukemia transcription factor-1 |
| pFSHβ | porcine FSHβ subunit |
| PRL | prolactin |
| Prop1 | Prophet of Pit1 homeodomain factor-1 |
| rFSHβ | rat FSHβ subunit |
| SBE | smad binding element |
| SF1 | steroidogenic factor-1 |
| TGF-β | transforming growth factor-β |
| TSH | thyroid stimulating hormone |

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# ABSTRACT OF THE THESIS

**Hormonal Regulation of FSH Beta-Subunit Gene Expression**

by

Arpi Hambarchyan

Master of Science in Biology

University of California, San Diego, 2011

Professor Pamela L. Mellon, Chair

Professor William McGinnis, Co-Chair

In the anterior pituitary, GnRH and activin are key regulators of reproduction. Furthermore, GnRH and activin synergize to induce FSHβ gene expression. We have identified a novel region of the FSHβ promoter located at -23 from the transcriptional start site that is important for GnRH responsiveness. In addition, activin has been shown to exert its effects on FSHβ gene transcription through Smad transcription factors as well as through the newly identified transcription factor FoxL2. In the mouse FSHβ promoter, we identified the FoxL2 sites at -350 and -120 to be most critical for activin induction, FoxL2 induction as well as for the synergistic induction by activin and GnRH. There seems to be controversy regarding the necessity of Smad binding sites adjacent to FoxL2 sites to convey activin responsiveness in different species. Therefore, we sought to

determine whether FoxL2 or Smad binding site is a critical player in human FSHβ promoter by focusing on the homologous site at -132 from the transcriptional start site.

# I

# Introduction

## The Hypothalamic-Pituitary-Gonadal Axis

The hypothalamic-pituitary-gonadal (HPG) axis is a finely controlled system essential for mammalian sexual maturation and reproduction. The intricate regulation of hormones between these three endocrine organs controls critical stages of the life cycle including puberty, sexual maturation, the menstrual cycle, pregnancy, and menopause. The hypothalamus, located ventrally to the brain, functions in a wide variety of physiological and behavioral activities including the regulation of body temperature, thirst, hunger, sleep, stress response, and aggressive and sexual behaviors [1]. The hypothalamus contains neurosecretory neurons whose axons either terminate in the posterior pituitary or deposit their contents to the hypophyseal portal blood system, which leads to the anterior pituitary. These hypothalamic neurohormones, released by the axons, may either increase or decrease the synthesis and secretion of hormones of the pituitary gland.

Neurosecretory neurons of the hypothalamus release gonadotropin-releasing hormone (GnRH), a hypothalamic decapeptide, into the hypophyseal portal system in a pulsatile manner [2]. The blood vessels of the hypophyseal portal system directly deliver

the hypothalamic peptide hormones to the anterior pituitary. GnRH travels through the portal system where it binds and activates its seven-transmembrane GnRH receptor (GnRHR). The GnRH receptor belongs to the rhodopsin family of G protein-coupled receptors and is specifically expressed on the surface of pituitary gonadotrope cells [3]. Activation of GnRHR initiates a variety of intracellular signaling across the membrane and leads to the transcription and secretion of the gonadotropin hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH).

The pituitary gland is a central endocrine organ that regulates physiological functions including growth and development, metabolism, stress response and reproduction [4]. Within the HPG axis, it functions as a relay between the hypothalamus and the gonads by integrating hormonal signals from the hypothalamus and feedback from the gonads. More specifically, the mature anterior pituitary gonadotropes synthesize and secrete both LH and FSH differentially depending on the frequency and amplitude of GnRH pulses [5], as well as due to the regulation of steroid hormones from the gonads and peptide hormones from the gonads and pituitary [3]. The steroid hormones include estrogen, progesterone and testosterone, and peptide hormones include inhibin from the gonads and activin from the gonads and pituitary itself. However, the most important regulators of gonadotropin synthesis and release are GnRH from the hypothalamus and activin from both the gonads and the pituitary.

FSH and LH, heterodimeric glycoproteins composed of a common α-subunit and a unique β-subunit released from the anterior pituitary into the circulation, target the gonads where they mediate steroidogenesis, folliculogenesis, and gametogenesis [6]. FSH acts on the testes to stimulate maturation of seminiferous tubules and regulate sperm

production in males and on the ovaries to stimulate the growth and recruitment of immature ovarian follicles in females [7]. LH acts upon the Leydig cells of the testes and increases testosterone production in males and in females, triggers ovulation, as well as regulates steroidogenesis in maturing follicles [7]. This steroidogenesis results in the production of sex steroids including androgens, estrogens and progestins, which feedback at the level of the hypothalamus and pituitary to control normal reproductive function [6]. The gonads also produce the peptide hormones, activin and inhibin. Inhibin is composed of α and β-subunits and acts directly on pituitary cells to selectively suppress the secretion of FSH. Activin is composed of two β-subunits and opposes the action of inhibin, thus stimulating the release of FSH. Activin and inhibin are secreted by the pituitary and gonads and may act in an autocrine, paracrine and/or endocrine manner [8]. Both gonadal steroid and peptide hormones are critical for feedback regulation by the hypothalamus and/or anterior pituitary. This feedback regulation occurring in an autocrine, paracrine, or endocrine manner alters the level of GnRH, FSH, and LH expression.

**Anterior Pituitary**

The hypophysis, also known as the pituitary gland, is an important component of the endocrine system and therefore referred to as a "master gland." It is divided into two sections: the anterior pituitary, which includes the intermediate pituitary and the posterior pituitary. The posterior pituitary develops from the ventral diencephalon from the neural ectoderm, while the anterior pituitary is derived from an invagination in the oral ectoderm, known as Rathke's pouch [9]. The posterior pituitary stores and secretes

oxytocin and antidiuretic hormone, which are synthesized by the hypothalamus [9]. The intermediate pituitary contains melanotropes, which produce and secrete melanocyte-stimulating hormone (MSH). The anterior pituitary contains five distinct endocrine cell types, which secrete six hormones important for growth, development, metabolism, and reproduction functions. Each of these endocrine cell types in the anterior pituitary is responsible for the production of a specific hormone including growth hormone from somatotropes, thyroid-stimulating hormone from thyrotropes, adrenocorticotropin hormones from corticotropes, prolactin from lactotropes, while gonadotropes produce both FSH and LH [9].

Gonadotropes account for only 10-15% of the pituitary cell population, which is part of the reason that studying gonadotropin regulation and secretion in vivo has been difficult. Another reason has been the lack of an appropriate gonadotrope cell model. Our laboratory was able to develop immortalized pituitary cell lines derived from pituitary tumors. The tumors were induced by targeted oncogenesis using the SV40 T-antigen driven by the rat LHβ gene promoter [10]. This process established a useful gonadotrope cell model called LβT2. The LβT2 cell line represents a differentiated cell line capable of expressing steroidogenic factor-1 (SF-1), functional activin receptors, Smad proteins, GnRHR, and both the α-glycoprotein subunit (αGSU) and β-subunits of LH and FSH [10]. With the creation of LβT2 cells, there is now a representative model system to study the regulatory components and molecular mechanisms regarding gene expression in highly differentiated gonadotrope cells.

**Regulation of FSH**

FSH belongs to the glycoprotein hormone family along with LH, TSH and chorionic gonadotropin hormone, hCG. These hormones are heterodimers and contain a common α-subunit with unique β-subunits [11]. Therefore, the β-subunit provides the biological specificity of these hormones. In gonadotropes, the α-subunit is synthesized in excess and synthesis of the β-subunit is a rate limiting step in the production of functional gonadotropins [12, 13] . Thus, the regulation of FSHβ gene transcription is critical per mature hormone synthesis. Synthesis of FSH is primarily regulated by GnRH from the hypothalamus and activin from the gonads [14, 15]. In the ovary, FSH stimulates follicular maturation, and, in the testes, it regulates Sertoli cell sperm production. Diseases at the hypothalamic and pituitary levels that impair synthesis and secretion of FSH highlight the importance of normal FSH regulation. Animal models for overexpression of gonadotropin genes and targeted disruption of gonadotropin subunit and receptor genes have helped understand the phenotypic findings in humans. Kumar, et al., produced mice deficient in FSH and found that FSHβ-deficient male mice had reduced testis size due to decreased seminiferous tubule total volume, but were fertile. However, FSHβ-deficient females were infertile due to a block in follicle maturation. Furthermore, pathologically increased levels of gonadotropins may lead to central precocious puberty and primary hypogonadism [16]. A 2 bp deletion of the FSHβ gene results in a frameshift and truncated FSHβ protein which cannot associate with the α-subunit and causes amenorrhea and infertility in women [17]. The same FSHβ gene mutations found in males resulted in azoospermia, but normal puberty [18]. Thus, to

understand these reproductive pathologies in FSH expression, it is important to investigate and appreciate the underlying mechanism of FSHβ gene transcription.

**Basal Regulation of FSHβ**

In light of the development of the LβT2 cell line, several advancements have been made in understanding the promoter elements that regulate FSHβ gene transcription. The -140/-50 region of the rat (rFSHβ) gene promoter is important for basal activity of the promoter in LβT2 cell line. The AAATCC site located at -54/-49 of the rFSHβ gene promoter is functionally important for basal expression and binds the pituitary homeobox 1 (Ptx1) protein [19]. Ptx1 is one of the earliest markers of pituitary organogenesis and acts as a DNA-binding protein that can recognize and activate the promoters of multiple pituitary hormones including FSHβ [4, 19]. Ptx1-knockout mice exhibit pituitary defects with decreased expression of FSHβ, LHβ and TSHβ [4].

Another of the DNA-binding proteins that regulates the porcine FSHβ (pFSHβ) is a pituitary-specific transcription factor, Prophet of Pit-1 (Prop-1), which is important for the development of Pit-1 lineage hormone-producing cells and other lineages such as the gonadotrope cells, and therefore production of FSH [20, 21]. Prop-1 indirectly regulates αGSU and FSHβ gene expression and defects in murine Prop-1 protein cause a deficiency in GH, PRL, and TSH and lead to Ames dwarfism with decreased levels of FSH and LH [4, 22]. Human PROP1 has similar functions in pituitary development and mutations within it are the leading cause of combined pituitary hormone deficiency in humans [23].

The binding site for ubiquitous transcription factor, nuclear factor Y (NF-Y), was identified on the mouse FSHβ promoter at the -76 position. NF-Y binds the CCAAT element and is an important basal regulator of many genes. The orphan nuclear receptor, steroidogenic factor 1 (SF-1), is an essential regulator for adrenal and gonadal steroidogenesis [24]. SF-1 knockout mice have impaired expression of gene products important in reproduction including LHβ, FSHβ, αGSU, and GnRHR, and die shortly after birth [24]. SF-1 binding sites were identified at -341 bp and -239 bp of the mouse FSHβ promoter. Mutation of SF-1, combined with a mutation in the NF-Y element, significantly decreased FSHβ promoter activity, indicating that SF-1 may functionally interact with NF-Y in LβT2 cells [25].

**GnRH Regulation of FSHβ Expression**

Various hormones play a critical role in the production of FSH, but GnRH and activin are the most important regulators. GnRH, released from the hypothalamus, regulates FSHβ gene expression and synthesis and secretion of the mature hormone. GnRH regulation occurs through direct targeting and binding to GnRHR on gonadotrope cells that elicit signaling cascades that induce transcription of early response genes, c-Fos, c-Jun, and egr-1.

GnRH induces FSHβ through induction of AP1 in multiple species. Two functional activating protein-1 (AP1) sites have been identified in the promoter region of the ovine FSHβ (oFSH) [26, 27] that are important for GnRH activation of the oFSHβ gene promoter in primary pituitary cultures [28]. In the murine FSHβ promoter, AP1

binds its consensus half site GTCA and an adjacent CCAAT box, a NF-Y site involved in basal expression [29]. In this study, it was determined that AP1 proteins are necessary and sufficient for FSHβ gene expression by GnRH [29].

**Activin Signaling**

Activin is the most potent inducer of FSHβ gene promoter, illustrated by targeted disruption of activin signaling in the mouse which suppresses FSH secretion and causes defects in spermatogenesis in males and impaired ovarian follicle development in females [30]. It is a member of the transforming growth factor beta (TGF-β) superfamily of proteins. Activin secreted from the gonads regulates FSH in an endocrine manner, while activin from the gonadotrope cells in the pituitary regulates FSH in an autocrine manner [31, 32]. Frozen porcine follicular fluid was used to purify this FSH-releasing substance [15], and it was determined that there are three forms of activin: activin A composed of two β-subunits of inhibin A (βAβA), activin B composed of two β-subunits of inhibin B (βBβB), or activin AB, composed of one β-subunit of inhibin A and B each (βAβB) [15]. Attisano, et al., determined that activin receptors belong to the TGF-β family receptors and activate the same family of signaling molecules and transcription factors [33]. Activin signals through a heteromeric receptor complex and intracellular signaling molecules known as Smad proteins [34]. Ligands of the TGF-β superfamily initiate downstream signaling by interacting with two heterodimeric receptors that belong to single-transmembrane serine-threonine kinase proteins, classified as type I and type II [35-37]. Upon ligand binding, Activin type II receptors, ActII or ActRIIB, bind and

phosphorylate Activin type I receptors, activin receptor-like kinase (ALK) [38, 39]. Activated type I receptors phosphorylate transcription factors in the cytoplasm known as Smads 2 and 3 [40, 41]. Bone morphogenetic proteins (BMP), which are members of the TGF-β family and similar to activin, have recently been shown to stimulate FSHβ transcription. BMPs are expressed in LβT2 cells, in adult murine pituitary and in sheep primary pituitary cells [42]. BMP2 and BMP4 signal through the interaction of ligands with BMP type I receptors (BMPR1A and BMPR1B) which recruit type II receptors (BMPR2) to phosphorylate the type I receptors. Activated type I receptors then phosphorylate intracellular signaling proteins, Smads 1 and 5, which form heteromeric complexes with common Smad 4 and accumulate in the nucleus to alter gene expression. Recently, it has been shown that BMP2 regulates murine FSHβ transcription through BMPR1A receptors [43, 44], but to a much lesser degree than activin.

Smads in the nucleus coordinate with a set of proteins that are specific to a particular cell type, and these set of proteins determine the DNA sequences that the Smad complex will bind and the co-activators or co-repressors that it will recruit to regulate target genes [36, 45]. More specifically, Smad2 and 3 are activated by TGF-β and activin receptors, Smad 1, 5, and 8 by BMP receptors, while Smad 6 and 7 are inhibitors of Smad-receptor interactions, and Smad 4 is a co-Smad that acts in conjunction with all the Smads and is important in DNA binding and recruitment of transcriptional cofactors [36, 46]. The target DNA sequence for Smad binding is a palindrome 5' GTCTAGAC 3,' or either half-site [47, 48] called the Smad-binding element (SBE). DNA binding is mediated by a β-hairpin structure of Smad3 Mad-homology 1 domain (MH1) [47]. Smad MH1 domain affinity for SBE sites is very weak and lacks selectivity. Therefore, Smads

interact with other DNA-binding transcription factors for specific, high-affinity binding of a Smad complex to a target gene. For example, in early Xenopus development, activin regulates a homeobox gene element called ARE which binds the DNA-binding protein of the forkhead family FAST-1. FAST-1 is expressed only in the oocyte and early development and is a transducer of activin signaling and also binds Smad2 [49].

Studies of the molecular mechanism by which activin induces FSHβ gene expression show that activin responsiveness maps to multiple sites throughout the mouse FSHβ promoter [50, 51]. Activin-responsive elements in the FSHβ promoter have been identified at -267, -153, -120 and more recently, at -350 and -208 [38]. The activin responsive element identified at -267 is a classical Smad-binding element. The -120 site, identified as a Smad half site, binds Pbx/Prep homeodomain proteins that have been shown to interact with homeotic Hox proteins to regulate cell-specific gene transcription and developmental pathways, and more recently to be involved in activin regulation of FSHβ expression by recruiting Smad 3 to the promoter [41]. A novel player, FoxL2 was shown to be involved in activin induction of FSHβ promoter even though there is a lack of Smad binding elements within some of these sites. The mouse -350 site plays a role in activin induction of the FSHβ gene and was determined to contain a functional FoxL2 binding site, however, the homologous human site did not appear to have a significant role in activin induction. Nonetheless, human FSHβ sites homologous to the mouse -208 and -153 sites, bind FoxL2, and are necessary for full activin response [38]. Additionally, the -120 site in the mouse promoter was identified as a FoxL2 element in addition to the Pbx element [52].

FoxL2 is a forkhead transcription factor expressed in the eye, ovary and pituitary gland [53]. FoxL2 loss of function mutations cause Blepharophimosis-Ptosis-Epicanthus Inversus Syndrome (BPES), an autosomal dominant disorder characterized by eyelid malformations comorbid with premature ovarian failure (POF). POF results from excessive loss of ovarian follicles leading to a shortened reproductive period. FoxL2 is excluded from developing male gonads and appears to be specifically required for female sex determination [54]. The importance of FoxL2 is underscored in mouse knockout models where most homozygous mutant mice are not viable [55]. Furthermore, removal of FoxL2 from the adult mouse ovary induces the SOX9 transcription factor important for male sex determination, leading to ovary-to-testis transformation, the replacement of granulose cells by Sertoli cells, and formation of seminiferious tubules instead of follicles [56].

**Synergy between GnRH and Activin Signaling**

Synergy is an interaction of two hormones, which results in a combined effect greater than the sum of their individual effects. Although GnRH responsive sites and activin responsive sites on the mouse FSHβ promoter are distinct, molecular studies have shown that GnRH and activin can synergistically induce FSHβ gene expression [50, 57]. Follistatin, a potent activin-binding protein, is secreted by gonadotrope cells within the pituitary [58] and inhibits the synthesis and secretion of FSH by inhibiting activin binding to its receptor. Follistatin treatment of LβT2 cells has been shown to block GnRH stimulation of the regulatory region of β-subunit genes of the ovine and mouse promoters [50] suggesting that GnRH stimulation of FSHβ is dependent on the activity of

the activin autocrine loop [8]. However, the ovine and rat [59] LHβ promoters were not affected by pretreatment with follistatin, suggesting that endogenous activin is not sufficient for LHβ transcription [8]. Thus, the synergy observed on the FSHβ promoter is specific and not replicated on the LHβ promoter, which may be a mechanism involved in the differential regulation of FSH and LH. Studies in LβT2 cells investigating the synergistic effects of GnRH and activin have mapped Smad3 activation of the rat FSHβ gene to the -266/-259 Smad-binding site. With activin treatment, four protein-DNA complexes were identified to bind this Smad-binding site, and an increase in intensity of these complexes were identified after co-treatment with activin and GnRH agonist, indicating the functioning of cross-talk between activin and GnRH-signaling pathways [57]. Norwitz, et al., have also shown that GnRH stimulates activin-signaling pathways in the gonadotrope and induces nuclear translocation of Smad proteins, further supporting the involvement of Smad proteins in both GnRH and activin signaling [60]. Zhang, et al., investigated how activin exposure modulates the transcriptional response of LβT2 pituitary gonadotropes to GnRH [61] and suggested that although activin has little effect on gonadotrope gene expression alone, it dramatically alters the cellular response to GnRH. For instance, GnRH-regulated genes in the absence of activin are involved in cytoskeletal and organelle reorganization, while in the presence of activin, they are involved in protein processing and differentiation. Thus, the presence of activin may be modulating gonadotropin transcriptional response to GnRH, indicating a potential role for both activin-dependent and GnRH-dependent transcription factors interacting to induce the synergistic effects. This synergy has recently been shown to be dependent on Smad

and AP1 binding sites in the mouse FSHβ promoter. It has been suggested that Smad and AP1 may physically interact after DNA binding to promote higher affinity binding and recruitment of co-activators involved in the synergistic induction of FSHβ gene expression [50].

**Summary**

Proper regulation of the hypothalamic-pituitary-gonadal axis is critical for normal reproductive function including growth and sexual maturation, puberty, menstruation, pregnancy, and menopause. GnRH from the hypothalamus, activin from the pituitary and gonads, and inhibin and follistatin from the gonads stringently regulate the transcription and therefore the synthesis and secretion of FSH and LH. Hormonal regulation and expression of the glycoproteins FSH and LH is accomplished by the interaction of hypothalamic GnRH and feedback loops by gonadal and pituitary steroidal and peptide hormones. The β-subunits of these heterodimeric glycoproteins are rate-limiting in their synthesis and secretion, and provide the biological specificity of these hormones. The importance of the proper regulation of FSHβ subunit is underscored by the resulting reproductive pathologies that cause infertility including premature ovarian failure and polycystic ovarian syndrome in females and reduced sperm production in males.

Previous studies have identified a number of transcription factors and DNA-binding sequences that regulate transcription of the FSHβ gene promoter. Both GnRH and activin are potent regulators of FSHβ gene expression, and they are critical players in this regulation on their own as well as in synergy. However, the mechanism for the

synergistic regulation of FSHβ by activin and GnRH remains unclear. The focus of this study is to further investigate regulatory elements involved in GnRH and activin activation as well as their synergistic regulation of the FSHβ gene.

# II

# Methods and Materials

## Plasmid Constructs

The -1000 bp of the mouse FSHβ promoter and -398 bp of the mouse FSHβ promoter linked to a luciferase reporter in pGL3 vector (FSHβ-luc) were created in the Mellon laboratory. The mouse FoxL2 and mouse FoxL2 mutant expression vectors were kindly provided by Dr. Louise Bilezikjan from the Salk Institute, San Diego. The -1000 human FSHβ promoter linked to a luciferase-reporter in pGL3 vector was generously provided by Dr. Daniel J. Bernard from McGill University, Canada. The human FoxL2 and human FoxL2 mutant expression vectors were kindly provided by Dr. Jeehyeon H. Bae from CHA University, Korea.

## Mutagenesis

Point mutations and internal deletions of the mouse FSHβ-luc plasmid were performed using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Wild-type promoters were mutated with the oligonucleotides described in (TABLE 2, 4, 6, and 8). PCR conditions for mutagenesis were 95$^{o}$C for 30 seconds, 55$^{o}$C for 1 minute, 68$^{o}$C for 14 min; repeated for 18 cycles, followed by incubation with Dpn for one hour. A 1.5 µl sample of each PCR

product was transformed into 50 µl XL1 Blue Supercompetent Cells (Stratgene, La Jolla, CA). The samples were incubated on ice for 30 minutes, heat shocked at 42°C for 40 seconds, then incubated on ice for 2 minutes. Bacterial cells were incubated with 450 µl SOC at 37°C for 1 hour. Following incubation, 50 µl and 300 µl of bacteria were plated on LB-amp plates and cultivated at 37°C for 16 hours. Mutations were confirmed by dideoxynucleotide sequencing performed by the DNA sequencing Shared Resource, University of California, San Diego Cancer Center.

**Cell Culture and Transient Transfection**

LβT2 cells were cultured at 37°C in 10 cm plates in Dulbecco's modified Eagle's medium (DMEM, Cellgro, Mediatech, Inc., Herndon, VA) with 10% fetal bovine serum (Omega Scientific Inc., Tarzana, CA) and 1% penicillin/Streptomycin. LβT2 cells were split into 12-well plates 1 day prior to transfection. Following the manufacturer's instructions, transfection was performed using Fugene 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN). Each well was transfected with 0.5 µg of the -1000 mouse, -398 mouse or -1000 human FSHβ promoter linked to a luciferase-reporter. An expression plasmid containing β-galactosidase reporter driven by the Herpes virus thymidine kinase promoter at a concentration of 0.1 ug was also transfected as an internal control for transfection efficiency. Expression vectors were transfected at 0.2 µg/well at the same time as indicated in figure legends.

The day after transfection, the cells were switched to serum-free DMEM supplemented with 0.1% bovine serum albumin and antibiotics overnight. Cells transfected with mouse FSHβ promoters (unless otherwise stated) were treated with 100

nM GnRH (Sigma) or 10 ng/ml activin (Calbiochem, CA) in serum-free DMEM 5 hours prior to lysing. Human FSHβ promoters were treated with 10 nM GnRH 5 hours prior to lysis or with 50ng/ml of activin 24 hours prior to lysis.

**Luciferase and β-galactosidase Assays**

Following hormone treatment, cells were washed with 1X phosphate buffer saline (PBS) and lysed with 60 µl of a 0.1M potassium phosphate buffer (pH 7.8) with 0.2% Triton X-100. Equal volumes of each lysate were placed in 96-well luminometer plates. Luciferase activity was measured using a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) after injecting 100 µl of a buffer containing 100 mM Tris-HCL (pH 7.8), 15 mM $MgSO_4$, 10 mM ATP, and 65 µM luciferin per well. Galactosidase activity was measured using the Galacto-light assay (Tropix, Bedford, MA) following the manufacturer's instructions.

**Hormones**

GnRH was purchased from Sigma-Aldrich, St. Louis, Missouri and was diluted in 0.1% bovine serum albumin suspended in phosphate buffer solution to 10 µM concentration for mouse FSHβ promoter and human FSHβ promoter experiments. Activin was purchased from Calbiochem, La Jolla, CA and diluted in 0.1% bovine serum albumin suspended in phosphate buffer solution to a 10 µg/ml concentration for mouse FSHβ promoter experiments and to a 50 µg/ml concentration for human FSHβ promoter experiments.

**TBP promoter Sub-Cloning**

Wild-type and mutant TATA-Binding Protein (TBP) promoters in pGL2 vector were digested using 15 µg of plasmid vector DNA, 1 µl SacI restriction enzyme, 2 µl NEB1 buffer, 0.2 µl BSA, and brought to a final volume of 20 µl with $H_2O$. pGL3 vector was digested using 15 µg vector DNA, 1 µl SacI restriction enzyme, 2 µl NEB1 buffer, 0.2 µl BSA, and brought to a final volume of 20 µl with $H_2O$. The digests were incubated at 37$^{o}$C. After two hours of incubation, the pGL3 vector digests was prevented from reclosure by adding 1 µl calf intestine alkaline phosphatase, 0.5 µl NEB buffer 3, and 3.5 µl $H_2O$. The digests were incubated at 37$^{o}$C for an additional hour placed in 95$^{o}$C heat block for 5 minutes to stop the enzymatic reactions, and run on a 1% agarose gel. The digest bands were cut and extracted following the manufacturer's instructions for the QIAquick Gel Extraction Kit and quantified by gel analysis. 5:1 molar ratio of TBP promoter and pGL3 vector were ligated using Ready-To-Go T4 DNA ligase (Amersham Bioschiences) according to manufacturer's instructions. Ligation samples were transformed into DH5α max Efficiency Competent Cells (Invitrogen, Carlsbad, CA) and plated on LB-amp plates. Using the Qiagen Miniprep Kit (Qiagen Sciences, Maryland), DNA from the colonies was extracted. Extracted DNA was digested with restriction enzymes and run on 1% agarose gel using the same conditions as previously described to verify the presence of TBP promoter inserts. Samples with successful ligation of TBP promoter and pGL3 vector were sequenced by the DNA sequencing Shared Resource, University of California, San Diego Cancer Center. Sequence alignment was confirmed by Accelrys DS Gene.

**Electrophoretic Mobility Shift Assay (EMSA)**

LβT2 cells were cultured at 37$^{o}$C in 10 cm plates in DMEM containing 10% fetal bovine serum and penicillin. After 2 hours of vehicle or GnRH treatment, nuclear extracts were obtained by swelling the cells on ice using a hypotonic buffer containing 20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM PMSF protease inhibitor (Sigma-Aldrich), 10 mM NaF, 0.5 mM EDTA, and 0.1 mM EGTA. The cells were lysed by passing through a 25-gauge needle 3 times and centrifuged at 4000 rpm for 4 minutes. The nuclear pellet was extracted in hypertonic buffer containing 20 mM Hepes, pH 7.8, 20% glycerol, 420 mM KCl, 1.5 mM $MgCl_2$, 1 mM PMSF, 10 mM NaF, 0.5 mM EDTA, and 0.1 mM EGTA. The samples were incubated on ice for 20 minutes then centrifuged at 10,000 rpm for 10 minutes. The supernatants were aliquoted (10 µl) and frozen until use. Protein determination was performed using the Bradford reagent (Bio-Rad, Hercules, CA) and standard curve run in each determination. Single stranded oligonucleotides were obtained from Integrated DNA technologies and diluted to 100 µM with Tris-EDTA (TE). The oligonucleotides were labeled with [$_{\gamma}$-$^{32}$P]ATP using T4 kinase and purified using Micro Bio-spin Chromatography Columns (Bio-Rad Laboratories, Inc., Hercules, CA) following the manufacturer's instructions. The binding reaction used 2 µg of nuclear proteins per sample in a total volume of 20 µl containing 10 mM Hepes, pH 7.8, 50 mM KCl, 5 mM MgCl2, 5 mM DTT, 10% glycerol, 0.1% NP-40, 0.25 µg didC, and 5 fmol of labeled probe. In the competition assays, competitor oligonucleotides were used at a 200-fold excess of unlabeled probe, and in the antibody shift assays 1 µg antibody was used. The antibodies used in supershift assays included GATA2, GATA3, GATA4 and IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Reactions were loaded on a 5% acrylamide

gel in 0.25X Tris-borate-EDTA buffer and electrophoresed at 1-V/$cm^2$ constant voltage. After drying, the gels were exposed to autoradiography to identify complexes.

**Statistical Analysis**

All experiments were performed a minimum of three times unless otherwise noted. 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 one-way or two-way statistical analysis of variance (ANOVA) followed by the Tukey's posthoc test was performed using the JMP program. Significance was set at $p \leq 0.05$. An asterisk was used to represent statistically significant data as analyzed by ANOVA.

**Table 1: Primers for 10 bp internal deletions in mouse FSHβ promoter**

| Name | Sequence |
|---|---|
| mFSHβ -70/-60 forward | 5′- CAGGCTTTATGTTGGTATTGGTCCCAGTAAATCCACAGGGTTTT -3′ |
| mFSHβ -70/-60 reverse | 5′- AAAACCCTGTGGATTTACTGGGACCAATACCAACATAAAGCCTG -3′ |
| mFSHβ -60/-50 forward | 5′- GTTGGTATTGGTCATGTTAACACCACAGGGTTTTAAGTTTGTAT -3′ |
| mFSHβ -60/-50 reverse | 5′- ATACAAACTTAAAACCCTGTGGTGTTAACATGACCAATACCAAC -3′ |
| mFSHβ -50/-40 forward | 5′- GTCATGTTAACACCCAGTAAATTTAAGTTTGTATAAAAGATGAG -3′ |
| mFSHβ -50/-40 reverse | 5′- CTCATCTTTTATACAAACTTAAATTTACTGGGTGTTAACATGAC -3′ |
| mFSHβ -40/-30 forward | 5′- CACCCAGTAAATCCACAGGGTTATAAAAGATGAGGTGTAACTTG -3′ |
| mFSHβ -40/-30 reverse | 5′- CAAGTTACACCTCATCTTTTATAACCCTGTGGATTTACTGGGTG -3′ |
| mFSHβ -30/-20 forward | 5′- ATCCACAGGGTTTTAAGTTTGTAGGTGTAACTTGACTCAGTGTT -3′ |
| mFSHβ -30/-20 reverse | 5′- AACACTGAGTCAAGTTACACCTACAAACTTAAAACCCTGTGGAT -3′ |
| mFSHβ -20/-10 forward | 5′- TTTTAAGTTTGTATAAAAGATGTGACTCAGTGTTCAGCTTTCCC -3′ |
| mFSHβ -20/-10 reverse | 5′- GGGAAAGCTGAACACTGAGTCACATCTTTTATACAAACTTAAAA -3′ |
| mFSHβ -10/-1 forward | 5′- GTATAAAAGATGAGGTGTAACTTTCAGCTTTCCCCAGAAGAGAA -3′ |
| mFSHβ -10/-1 reverse | 5′- TTCTCTTCTGGGGAAAGCTGAAGTTACACCTCATCTTTTATAC -3′ |

**Table 2: Deletions in mouse FSHβ promoter**
Bold and underlined base-pairs represent deletions from wild-type.

| Name | Sequence |
|---|---|
| mFSHβ WT | 5′- CATGTTAACACCCAGTAAATCCACAGGGTTTTAAGTTTGTATAAAAGATGAGGTGTAACTTGACTCAGTGTTCAGCTTTCCCCAGAAGAGAA -3′ |
| mFSHβ-70 | 5′- **CATGTTAACA**CCCAGTAAATCCACAGGGTTTTAAGTTTGTATAAAAGATGAGGTGTAACTTGACTCAGTGTTCAGCTTTCCCCAGAAGAGAA -3′ |
| mFSHβ -60 | 5′- CATGTTAACA**CCCAGTAAAT**CCACAGGGTTTTAAGTTTGTATAAAAGATGAGGTGTAACTTGACTCAGTGTTCAGCTTTCCCCAGAAGAGAA -3′ |
| mFSHβ -50 | 5′- CATGTTAACACCCAGTAAAT**CCACAGGGTT**TTAAGTTTGTATAAAAGATGAGGTGTAACTTGACTCAGTGTTCAGCTTTCCCCAGAAGAGAA -3′ |
| mFSHβ -40 | 5′- CATGTTAACACCCAGTAAATCCACAGGGTT**TTAAGTTTGT**ATAAAAGATGAGGTGTAACTTGACTCAGTGTTCAGCTTTCCCCAGAAGAGAA -3′ |
| mFSHβ -30 | 5′- CATGTTAACACCCAGTAAATCCACAGGGTTTTAAGTTTGT**ATAAAAGATG**AGGTGTAACTTGACTCAGTGTTCAGCTTTCCCCAGAAGAGAA -3′ |
| mFSHβ -20 | 5′- CATGTTAACACCCAGTAAATCCACAGGGTTTTAAGTTTGTATAAAAGATG**AGGTGTAACT**TGACTCAGTGTTCAGCTTTCCCCAGAAGAGAA -3′ |
| mFSHβ -10 | 5′- CATGTTAACACCCAGTAAATCCACAGGGTTTTAAGTTTGTATAAAAGATGAGGTGTAACT**TGACTCAGTG**TTCAGCTTTCCCCAGAAGAGAA -3′ |

**Table 3: Primers for -208 FoxL2 site mutations in mouse FSHβ promoter**

| Name | Sequence |
|---|---|
| -208 FoxL2 Mut1 forward | 5′- GCTGCCATATCAGACCCGGCCCGTACAGGGACCATCATCACTGAT -3′ |
| -208 FoxL2 Mut1 reverse | 5′- ATCAGTGATGATGGTCCCTGTACGGGCCGGGTCTGATATGGCAGC -3′ |
| -208 FoxL2 Mut2 forward | 5′- GCTGCCATATCAGACCCGGTTTGTACAGAAACCATCATCACTGAT -3′ |
| -208 FoxL2 Mut2 reverse | 5′- ATCAGTGATGATGGTTTCTGTACAAACCGGGTCTGATATGGCAGC -3′ |
| -208 FoxL2 Mut3 forward | 5′- GCTGCCATATCAGATTCGGCCCGTACAGAAACCATCATCACTGAT -3′ |
| -208 FoxL2 Mut3 reverse | 5′- ATCAGTGATGATGGTTTCTGTACGGGCCGAATCTGATATGGCAGC -3′ |
| -208 FoxL2 Mut4 forward | 5′- GCTGCCATATCAGATTCGGTTTGTACAGGGGCCATCATCACTGAT -3′ |
| -208 FoxL2 Mut4 reverse | 5′- ATCAGTGATGATGGCCCCTGTACAAACCGAATCTGATATGGCAGC -3′ |

**Table 4: Mutated -208 FoxL2 sites in mouse FSHβ promoter**
Bold and underlined base-pairs represent mutations from wild-type.

| Name | Sequence |
|---|---|
| -208 FoxL2 WT | 5′- GCTGCCATATCAGATTCGGTTTGTACAGAAACCATCATCACTGAT -3′ |
| -208 FoxL2 Mut1 | 5′- GCTGCCATATCAGA**CC**CGG**CCC**GTACAG**GG**ACCATCATCACTGAT -3′ |
| -208 FoxL2 Mut2 | 5′- GCTGCCATATCAGA**CC**CGGTTTGTACAGAAACCATCATCACTGAT -3′ |
| -208 FoxL2 Mut3 | 5′- GCTGCCATATCAGATTCGG**CCC**GTACAGAAACCATCATCACTGAT -3′ |
| -208 FoxL2 Mut4 | 5′- GCTGCCATATCAGATTCGGTTTGTACAG**AAA**CCATCATCACTGAT -3′ |

**Table 5: Primers for TATA site mutations in mouse FSHβ promoter and LHβ promoter**

| Name | Sequence |
|---|---|
| LHβ TATA Mut forward | 5′- CACCCCCACAACCCGCAGGTCCCCAGCCAGGTGCCCAAGGTAGGG -3′ |
| LHβ TATA Mut reverse | 5′- CCCTACCTTGGGCACCTGGCTGGGGACCTGCGGGTTGTGGGGGTG -3′ |
| FSHβ TATA Mut2 forward | 5′- CAGGGTTTTAAGTTTGCCCCAAAGATGAGGTGTAACTTGACTC -3′ |
| FSHβ TATA Mut2 reverse | 5′- GAGTCAAGTTACACCTCATCTTTGGGGCAAACTTAAAACCCTG -3′ |
| FSHβ TATA Mut3 forward | 5′- CAGGGTTTTAAGTTTGTATAAAAGCCCAGGTGTAACTTGACTC -3′ |
| FSHβ TATA Mut3 reverse | 5′- GAGTCAAGTTACACCTGGGCTTTTATACAAACTTAAAACCCTG -3′ |

**Table 6: Mutated TATA sites in mouse FSHβ promoter and LHβ promoter**
Bold and underlined base-pairs represent mutations from wildtype.

| Name | Sequence |
|---|---|
| LHβ WT | 5′- GTGGCCTTGCCACCCCCACAACCCGCAGGTATAAAGCCAGGTGCCCAAGGTAGGGAAGGT -3′ |
| LHβ TATA Mut | 5′- GTGGCCTTGCCACCCCCACAACCCGCAGGT**CCCC**AGCCAGGTGCCCAAGGTAGGGAAGGT -3′ |
| FSHβ WT | 5′- CAGGGTTTTAAGTTTGTATAAAAGATGAGGTGTAACTTGACTC -3′ |
| FSHβTATA Mut2 | 5′- CAGGGTTTTAAGTTTG**CCCC**AAAGATGAGGTGTAACTTGACTC -3′ |
| FSHβ TATA Mut3 | 5′- CAGGGTTTTAAGTTTGTATAAAAG**CCC**AGGTGTAACTTGACTC -3′ |

**Table 7: Primers for -120 site mutations in human FSHβ promoter**

| Name | Sequence |
|---|---|
| -132 FoxL2 forward | 5′- TAAAGCTTGATCTCCCTGTCTATAGAAACACTGATTCACTTACAG -3′ |
| -132 FoxL2 reverse | 5′- CTGTAAGTGAATCAGTGTTTCTATAGACAGGGAGATCAAGCTTTA -3′ |
| -132 Smad forward | 5′- TAAAGCTTGATCTCCCTGTAGATCTAAACACTGATTCACTTACAG -3′ |
| -132 Smad reverse | 5′- CTGTAAGTGAATCAGTGTTTAGATCTACAGGGAGATCAAGCTTTA -3′ |

**Table 8: Mutated -120 sites in human FSHβ promoter**
Bold and underlined base-pairs represent mutations from wildtype.

| Name | Sequence |
|---|---|
| -132 WT | 5′- TACTTTAGTAAAGCTTGATCTCCCTGTCTATCTAAACACTGA -3′ |
| -132 FoxL2 mut | 5′- TACTTTAGTAAAGCTTGATCTCCCTGTCTAT**AG**AAACACTGA -3′ |
| -132 Smad mut | 5′- TACTTTAGTAAAGCTTGATCTCCCTGT**AG**ATCTAAACACTGA -3′ |

**Table 9: Oligonucleotides used as radiolabeled EMSA probes**

| Name | Sequence |
|---|---|
| FSHβ wt forward | 5′- TTTAAGTTTGTATAAAAGATGAGGTGTAAC -3′ |
| FSHβ wt reverse | 5′- GTTACACCTCATCTTTTATACAAACTTAAA -3′ |
| FSHβ GATA Consensus forward | 5′- AGCTCAAGATAGAGGACAGACATAAGACTT -3′ |
| FSHβ GATA Consensus reverse | 5′- AAGTCTTATCTCTGTCCTCTATCTTGAGCT -3′ |

**Table 10: Oligonucleotdies used as unlabeled EMSA competitors**

| Name | Sequence |
|---|---|
| FSHβ wt competitor forward | 5′- TTTAAGTTTGTATAAAAGCCCAGGTGTAAC -3′ |
| FSHβ wt competitor reverse | 5′- GTTACACCTGGGCTTTTATACAAACTTAAA -3′ |
| FSHβ mut competitor forward | 5′- TTTAAGTTTGTATAAAAGCCCAGGTGTAAC -3′ |
| FSHβ mut competitor reverse | 5′- GTTACACCTGGGCTTTTATACAAACTTAAA -3′ |

# III

# Results

## A Novel Element in the FSHβ Promoter that is Required for GnRH Responsiveness

GnRH, a tropic peptide hormone synthesized and released from neurons within the hypothalamus, is a major regulator of FSHβ gene transcription. Studies of the molecular mechanism by which GnRH induces FSHβ gene expression show that GnRH responsiveness is retained in the 95 bp proximal region of the mouse FSHβ promoter [29]. The 95 bp proximal region of the mouse FSHβ promoter contains an AP1 half site, which, when mutated, significantly reduces GnRH responsiveness [29]. However, significant GnRH responsiveness is maintained even with the mutation of the -76 AP1 site. To identify additional elements that contribute to GnRH responsiveness of the FSHβ promoter, we constructed a series of 10 bp internal deletions throughout the 95 bp proximal region in the -398 base pair luciferase reporter and used them in transient transfection assays to compare their induction to the induction of the wild-type -398 promoter. To normalize for transfection efficiency, cells were transfected with an internal control reporter thymidine kinase β-galactosidase and to normalize 3 repeats of the experiment and to eliminate the potential effect of the empty vector, the luciferase to β-galactosidase ratio for each truncation was normalized to luciferase/β-gal ratio of the empty vector (pGL3). To more easily observe the GnRH effect without influence of the

changes in basal expression, luciferase levels from GnRH-treated cells were normalized to the luciferase levels from cells treated with vehicle control for each mutation or deletion and results were represented as fold induction. We used the previously described point mutation in the AP1 site of the -70/-69 residues, as our control [29].

Herein, we show that a -70/-61 deletion containing the AP1 site and the AP1 point mutation both reduced fold induction of the mouse FSHβ promoter following GnRH treatment. A mutation or a deletion of the AP1 site [29] significantly reduced GnRH responsiveness of the mouse FSHβ promoter by about 50% (Fig. 1A), demonstrating that the AP1 site is necessary for maximal GnRH induction of FSHβ gene expression. LβT2 cells were transiently transfected with the series of 10 bp internal deletions of the mouse FSHβ gene downstream of the -95 bp promoter region and treated with GnRH. The decrease in GnRH fold induction observed between the -70 and -61 internally deleted region is not surprising, since the previously identified AP1 half site is present in this region of the promoter. An increase in GnRH fold induction was observed when the -60/-51 and -50/-41 region of the FSHβ promoter was internally deleted (Fig. 1B), due to the dramatic decrease in basal expression. When data are not normalized to fold induction, the luciferase/β-galactosidase ratio for GnRH-treated samples are lower in these deletions than in WT, except that basal expression in vehicle-treated samples is proportionally even lower, resulting in apparent increase in fold induction (data not shown). Decrease in basal expression in these deletions is likely due to the presence of Ptx1 sites [19]. Ptx1 is a pituitary-specific transcription factor which is expressed at highest levels in gonadotrope-derived cells and an early marker for pituitary

organogenesis [19]. Thus, the observed apparent increase in fold induction is likely due to the decreased basal expression since these internal deletions removed homologous Ptx1 binding sites within the mouse FSHβ gene promoter. However, a significant decrease in fold induction, without a change in basal expression, (data not shown) was observed with the deletion in -30/-21 region. This area represents a novel GnRH-responsive region in the mouse FSHβ promoter and became the focus of our investigation.

**The TATA Box is Not Involved in FSHβ Promoter Responsiveness to GnRH**

Closer scrutiny of the DNA sequence of the mouse FSHβ promoter revealed the presence of a TATA box within the -30/-21 region that conveys GnRH responsiveness. The -31/-21 sequence is TATAAAAGATG, and the TATA box is found at -31/-25. Genomic studies have shown that TATA-promoters belong to the class of focused core promoters, which control tissue-specific genes [62]. Since the TATA box is commonly found in the proximal promoter and is considered a major regulatory element, we tested the hypothesis that the TATA box mediates GnRH induction of the mouse FSHβ gene expression. The TATA box is recognized by TATA-binding protein (TBP) that directs accurate transcription initiation by RNA polymerase II [62]. There is evidence that TBP can be transcriptionally regulated in certain situations, [63], [64], although TBP is mostly a house keeping gene with high basal expression. To assess the involvement of TATA box and TBP, we introduced a 4 bp mutation in the -31 to -28 region within the FSHβ promoter and transfected it into the LβT2 cells. The results demonstrate that the presence

of a TATA box does not affect GnRH fold induction since mutation within the 5' TATA DNA sequence does not significantly decrease GnRH responsiveness of the mouse FSHβ promoter compared to the wildtype mouse FSHβ promoter (Fig. 2A). Not surprisingly, the TATA box mutation does reduce basal expression of the FSHβ promoter, indicating that we created functional mutation that prevents TBP binding (Fig. 2B). To assess whether GnRH may regulate FSHβ gene transcription through TBP, we obtained a TBP promoter/luciferase reporter and transfected LβT2 cells. The results demonstrate that GnRH does not induce the TBP-promoter and therefore TBP is not a likely transcriptional intermediary of GnRH induction of FSHβ (Fig. 2C).

**The TATA Box is involved in LHβ Promoter Responsiveness to GnRH**

Since the β subunits of LH and FSH confer the biological specificity of these gonadotrope hormones and are the rate-limiting steps for production of the mature hormones [3], we also investigated whether LHβ gene expression involves regulation via the TATA box that is also found within the LHβ promoter. We mutated the TATA box within the LHβ promoter and used the mutation in transient transfection assays with GnRH treatment. Contrary to the lack of effect on fold induction observed in the FSHβ promoter, the mutation within the TATA box of the LHβ promoter significantly reduced GnRH induction, as well as basal expression of LHβ gene, by 79% and 56%, respectively (Fig. 3A, 3B). Therefore, the presence of a functional TATA box plays a role in maximal GnRH induction of LHβ promoter, while it does not play a crucial role in GnRH induction of FSHβ promoter.

**GnRH Responsiveness Maps to the 3' End of the -30/-21 FSHβ Gene Promoter Region**

Thus far, mouse FSHβ gene induction by GnRH has been mapped to the -30/-21 gene promoter region, but the TATA box DNA sequence containing nucleotides TATA (-31/-28) has shown to not play a role. To further map the critical DNA sequence housing the DNA binding elements responsible for GnRH responsiveness, we constructed an internal 4 bp TAAA (-29/-26) mutation within the TATA box DNA sequence as well as a 3 bp ATG (-23/-21) mutation in the 3' end of the -30/-21 FSHβ gene promoter**.** These mutations were used in transient transfection assays with GnRH treatment and analyzed for fold induction (Fig. 4). Similar to the TATA (-31/-28) sequence mutation, the internal TAAA (-29/-26) mutation also did not significantly affect GnRH responsiveness. However, the mutation of the promoter region -23/-21 showed a significant decrease in GnRH responsiveness, similar to the 30% reduction observed with this 10 bp internal deletion. Thus, the novel element in the FSHβ promoter required for GnRH responsiveness mapped to the ATG sequence in the 3' end of the -30/-21 region. We focused on the -23/-21 region of the FSHβ promoter to identify proteins that bind this region and are critical for this induction.

**Analysis of the -30/-21 Region on the FSHβ Promoter Reveals GATA Proteins do not Bind This Region of Interest**

The localization of GnRH-induced activity on the FSHβ promoter has identified the novel element at -23 GATG. Searching through the literature, we hypothesized that

GATA proteins, which have DNA binding sequences most similar to the GATG sequence found within our novel element, may play a role in the induction of FSHβ promoter by GnRH. To further investigate this region, EMSAs (Electrophoretic Mobility Shift Assays) were used to determine whether nuclear proteins were capable of binding to this region of the FSHβ promoter. To assess whether GATA proteins can bind this region, radiolabeled FSHβ probe encompassing the region -41 to -12 was incubated with nuclear extracts from LβT2 cells treated with vehicle control or GnRH treatment. In addition, unlabeled wildtype and mutant oligonucleotides, with the same -23/-21 mutations as those used in transfections, were used in 200-fold excess in the EMSA to compete with the FSHβ radiolabeled probe. Wildtype competitor was used to determine specificity of protein complexes and mutant competitor was used to identify the complex that binds the base pairs that have functional significance in transfection assays. GnRH treatment causes several complexes to change their binding to the probe (Fig. 5A arrows 1 and 2). Arrow 1 represents a specific complex induced by GnRH treatment, but is not our complex of interest since it was competed with the mutant oligonucleotide, indicated by a weaker band. Arrow 2 represents our complex of interest because it is induced by GnRH treatment but not competed with the mutant oligonucleotide. Although several complexes were induced by GnRH, they did not contain GATA 2, 3, or 4 proteins since the addition of antibodies did not supershift any of the complexes (Fig. 5A). The wildtype FSHβ competitor was able to compete with the radiolabeled probe, indicated by weaker bands. Mutant FSHβ competitor was able to compete with the radiolabeled probe for the protein indicated by arrow 1, but it was not able to compete with the radiolabeled probe

for the protein indicated by arrow 2. This indicates that our complex of interest is the specific band induced by GnRH treatment (arrow 2). However, this specific band, in addition to other complexes induced by GnRH treatment, also did not result in a supershift upon inclusion of the various GATA antibodies (Fig. 5A). Therefore, this FSHβ probe is not capable of binding to GATA proteins 2, 3, or 4 in LβT2 cell extracts in the -41/-12 region of interest.

To confirm that GATA proteins are expressed in LβT2 cells and that our antibodies are specific, we performed EMSAs incubating nuclear extracts from LβT2 control or GnRH-treated cells with a radiolabeled GATA consensus probe (Fig. 5B). We observed that inclusion of the GATA2 antibody resulted in a supershift of a specific band, indicated with arrow 1. The supershift (arrow ss 1) of the complex indicated by arrow 1 is achieved in lane 10 and 11 by the GATA2 antibody. The inclusion of GATA4 antibody resulted in a supershift (arrow ss 2) of the complex indicated by arrow 2 in lanes 14 and 15 (Fig. 5B). These resulting supershifts indicate that GATA2 and GATA4 proteins are present in LβT2 cell nuclear extracts. Unlabeled wildtype FSHβ and mutant FSHβ nucleotides were also used in 200-fold excess in the EMSA to compete with the GATA consensus-radiolabeled probe. However, FSHβ oligonucleotides do not compete with the GATA consensus probe for any of the complexes, which further indicates that GATA proteins do not bind to the FSHβ promoter under our conditions. Thus, although GATA2 and 4 proteins are expressed in the LβT2 cells, they do not bind FSHβ promoter within the -41/-12 region. Because we could not identify the proteins involved in the GnRH induction of the FSHβ promoter that bind -23/-21 sequence, we shifted our focus

to study the synergistic affects of GnRH and activin on this novel region of FSHβ promoter.

**GnRH and Activin Synergism in the -30/-21 Region of the FSHβ Promoter**

Given that activin is also a major physiological regulator of FSHβ gene transcription by itself and it synergizes with GnRH to bring forth a much higher level of induction than either hormone alone, we set out to determine whether the newly identified GnRH responsive site on the mouse FSHβ promoter was also important for activin or synergistic induction of FSHβ gene expression. Thus, to analyze the role in synergy, we used the -30/-21 internal deletion FSHβ promoter in transfections with GnRH, activin and co-treatment. Although, the -30/-21 region of the mouse FSHβ promoter was not found to be critical for activin induction of FSHβ gene expression, deletion of this region significantly reduced induction by GnRH, as previously discussed, and also reduced the synergistic affects of GnRH and activin on FSHβ gene expression (Fig. 6A).

Given that the -23/-21 region on the mouse FSHβ promoter was responsible for the significant decrease in GnRH induction of FSHβ gene expression, we focused on this same region for synergistic induction of FSHβ gene expression by GnRH and activin. We used the same -23/-21 mutation in the FSHβ promoter as in Figure 4A, the mutated AP1 site FSHβ promoter, and the -30/-21 internal deletion (Fig. 6B), to compare to the wildtype FSHβ promoter. The -23/-21 mutation of the mouse FSHβ promoter was also found to be important for the synergistic effect of activin and GnRH on FSHβ gene

expression as the mutations of these 3 bps reduced induction by co-treatment. Therefore, the -23/-21 DNA sequence containing ATG nucleotides plays a critical role in GnRH induction of FSHβ promoter and is necessary for maximal synergistic affects of GnRH and activin hormones on FSHβ gene expression.

**Synergistic Regulation of Mouse FSHβ Promoter by GnRH and Activin**

Although the -23/-21 region of the FSHβ promoter plays a critical role in GnRH induction of the FSHβ gene expression as well as in the synergistic affects of GnRH and activin, we could not identify the proteins that bind this element. Therefore, we decided to study the synergistic regulation of the mouse FSHβ promoter by GnRH and activin. Previously in our lab, it had been identified that the -350, -267, -208, -153, and -120 sites on the mouse FSHβ promoter are important for activin responsiveness [38, 41, 51]. A consensus Smad-binding element (SBE) resides at -267 of the mouse FSHβ promoter and has previously been identified as a Smad-responsive site important for activin induction of FSHβ gene expression [39, 48]. Our laboratory previously identified -153 and -120 sites as Smad half sites important for activin induction [41]. While proteins that bind -153 had not been initially identified, later it was determined to bind FoxL2 [38], and -120 binds Pbx/Prep heterodimers and/or FoxL2 [41], [52]. The -350 and -208 sites were newly identified FoxL2 sites important for activin induction [38]. FoxL2 is a forkhead transcription factor expressed in the eye, ovary and pituitary gland [53] and plays an essential role in embryogenesis, cell differentiation, and tumorigenesis [65]. Mutations of FoxL2 have been shown to be responsible for the Blepharophimosis-Ptosis-Epicanthus-

inversus Syndrome (BPES), which is an autosomal dominant disorder resulting in eyelid dysplasia that may be comorbid with premature ovarian failure [52] .

In our pursuit of understanding the synergistic regulation of FSHβ by GnRH and activin, we first investigated which of these newly identified activin-responsive sites are critical for the synergistic regulation. To assess which of these sites are involved in synergy, we transiently transfected LβT2 cells with the FSHβ promoter mutated at the -350, -208, -153, or -120 site with GnRH, activin or co-treatment and analyzed for fold induction. None of these sites resulted in a significant change in GnRH regulation of FSHβ promoter (Fig. 7A). Not surprisingly, all of the sites were observed to be important for activin responsiveness of FSHβ promoter as their mutations significantly reduced activin induction compared to the wildtype promoter (Fig. 7B). Although all of the sites were observed to play a role in the synergistic effects of GnRH and activin, the -120 site was most critical as it resulted in the largest reduction in fold induction by co-treatment (Fig. 7C).

**Activin-responsive Sites -350 and -120 Confer FoxL2 Induction of FSHβ Promoter**

The sites, -350, -208, -153, and -120, were all identified to bind FoxL2 in EMSA and play a role in activin induction. However, which of these sites can confer induction by FoxL2 was not determined. To determine this, we overexpressed FoxL2 with FSHβ wildtype and FoxL2 site mutants. We determined that mutation of the -350 site reduced induction by FoxL2 overexpression by 37%, while mutation of the -120 site reduced induction by FoxL2 overexpression by 63% (Fig. 8). Although in EMSA, the -208 and

-153 sites bind FoxL2 with much lower affinity than -350 and -120, their mutations did not reduce induction by FoxL2 overexpression. Thus, these sites may interact with the -350 and -120 sites and contribute to activin responsiveness, but are not strong FoxL2 sites. However, FoxL2 conveys activin responsiveness in LβT2 cells at -350 and -120 sites.

**The Homologous Mouse -120 Site in the Human FSHβ Promoter is Important for FoxL2 Induction**

The human FSHβ promoter also responds to activin but to a much smaller degree than the mouse FSHβ promoter. Previously in our lab, two sites on the human FSHβ promoter at -223 and -164, which are equivalent to the -208 and -153 sites of the mouse FSHβ promoter, were identified. FoxL2 was shown to bind these sites and mutation of these sites eliminates activin response [38]. Furthermore, in the ovine FSHβ gene promoter, an activin-responsive element at -134 was identified, which is highly conserved across species, and was shown to bind complexes containing Smad4 and the TALE homeodomain proteins Pbx1 and Prep1 [41]. Later, this site was identified as a FoxL2 site in the mouse promoter [52]. This site corresponds to the mouse -120 site consisting of the nucleotide sequence 5’ GTCTAAACAA 3’, which contains both a Smad half-site (5’ GTCT 3’) and a FoxL2 site (5’ CTAAACAA 3’), which are overlapping. Therefore, it could not be distinguished whether the FSHβ promoter responsiveness to activin mapped to this region is through Smad or FoxL2. To investigate whether Smad or FoxL2 play a more critical role in the activin induction of the FSHβ

promoter, we concentrated on the human FSHβ promoter, which has a nucleotide sequence of 5' GTCTATCTAAACAA 3' and is homologous to the mouse -120 site and the ovine -134 site. We constructed a human FSHβ promoter with a 2 bp mutation within the Smad half-site (-132 Smad mut) and a human FSHβ promoter with a 2 bp mutation within the FoxL2 site (-132 FoxL2 mut). We used our mutated human FSHβ promoters compared to wildtype 1 kb human FSHβ promoter in transient transfection with overexpression of FoxL2. Our results show that the -132 FoxL2 site is necessary for full FoxL2 induction of FSHβ gene expression, since mutation of the FoxL2 site, but not the Smad site, reduced induction by FoxL2 overexpression (Fig. 9B). We used our mutated human FSHβ promoters compared to the wildtype 1 kb human FSHβ promoter in transient transfections with activin treatment (Fig. 9B). Our results show that neither mutation decreased activin responsiveness of the human promoter. Although the homologous site in the mouse promoter has the most profound effect on both activin and FoxL2 responsiveness, in the human promoter this site doesn't play a role in activin responsiveness (Fig. 9A). Thus, this site represents species-specific regulation of FSHβ expression.

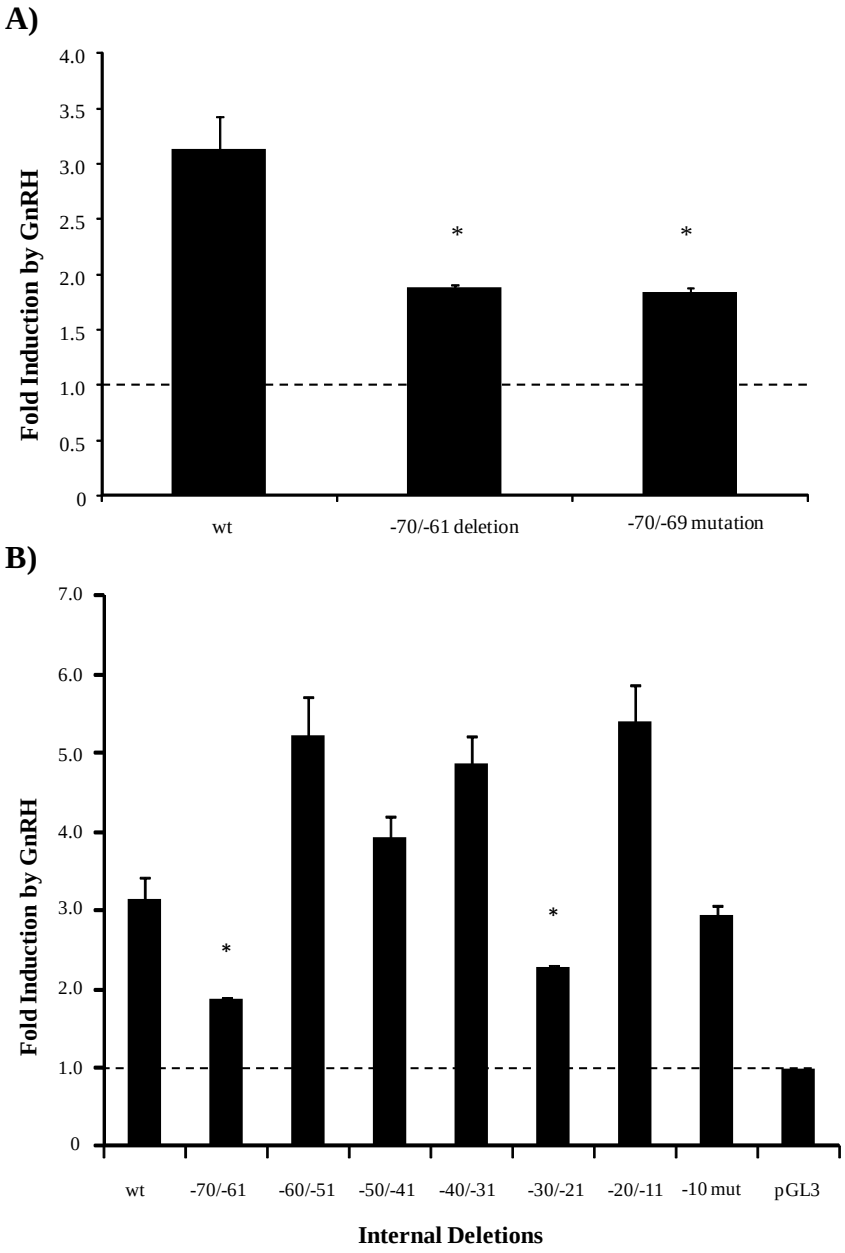



**Figure 1: GnRH responsiveness of the mouse FSHβ promoter maps to -70/-61 region and -30/-21 region**

LβT2 cells were transfected with the specified -398 mouse FSHβ promoter, fused to a luciferase reporter gene. A) A 10 bp internal deletion containing the AP1 site (-70/-61 deletion) and mutation of the AP1 site (-70/-69 mutation), within the -398 base pair luciferase construct, significantly decrease GnRH responsiveness of FSHβ promoter compared to the wildtype (wt) promoter. B) Mouse FSHβ promoter with the specified 10 bp internal deletions were transfected into LβT2 cells, and results show significant decrease in GnRH responsiveness at the -30/-21 region, in addition to the -70/-61 region. Luciferase/β-galactosidase (luciferase/β-gal) ratio was represented as fold induction, normalized to pGL3 and values represent a mean of three experiments done in triplicate. (*) represents a significant difference in induction with $p<0.05$.

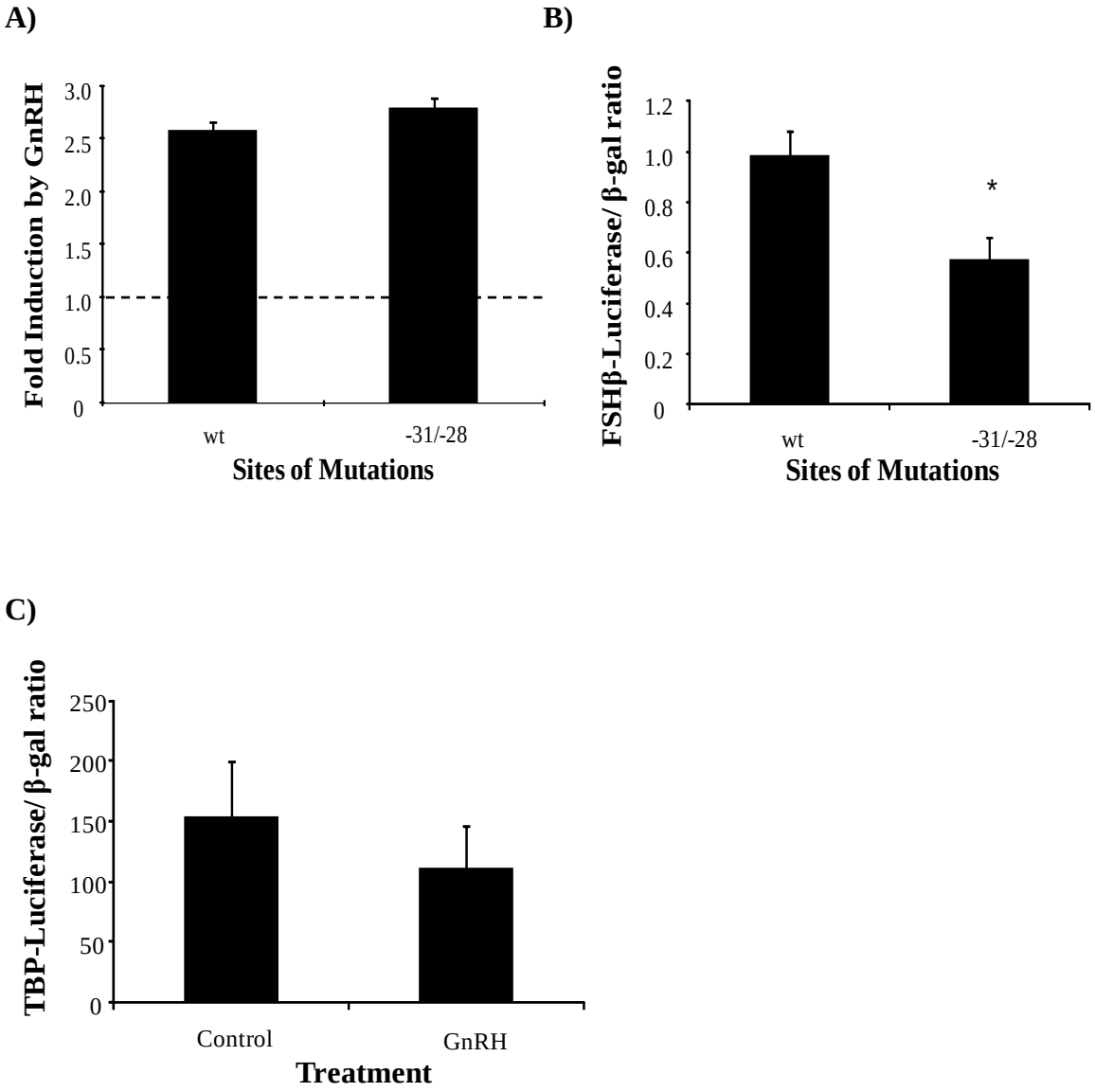



**Figure 2:TATA box does not play a role in FSHβ induction by GnRH**
Wildtype (wt) and a mutation within the 5' end of the TATA box DNA (-31/-28) sequence were transiently transfected in LβT2 cells. A mutation within the TATA box does not significantly affect GnRH fold induction. B) A mutation within the TATA box does decrease basal expression of the FSHβ promoter. Luciferase/β-gal ratio is represented as fold induction over basal expression, normalized to pGL3. C) TBP promoter-luciferase was transfected in LβT2 cells and treated with vehicle or GnRH. (*) represents a significant difference in fold induction from the wild type reporter with $p<0.05$.

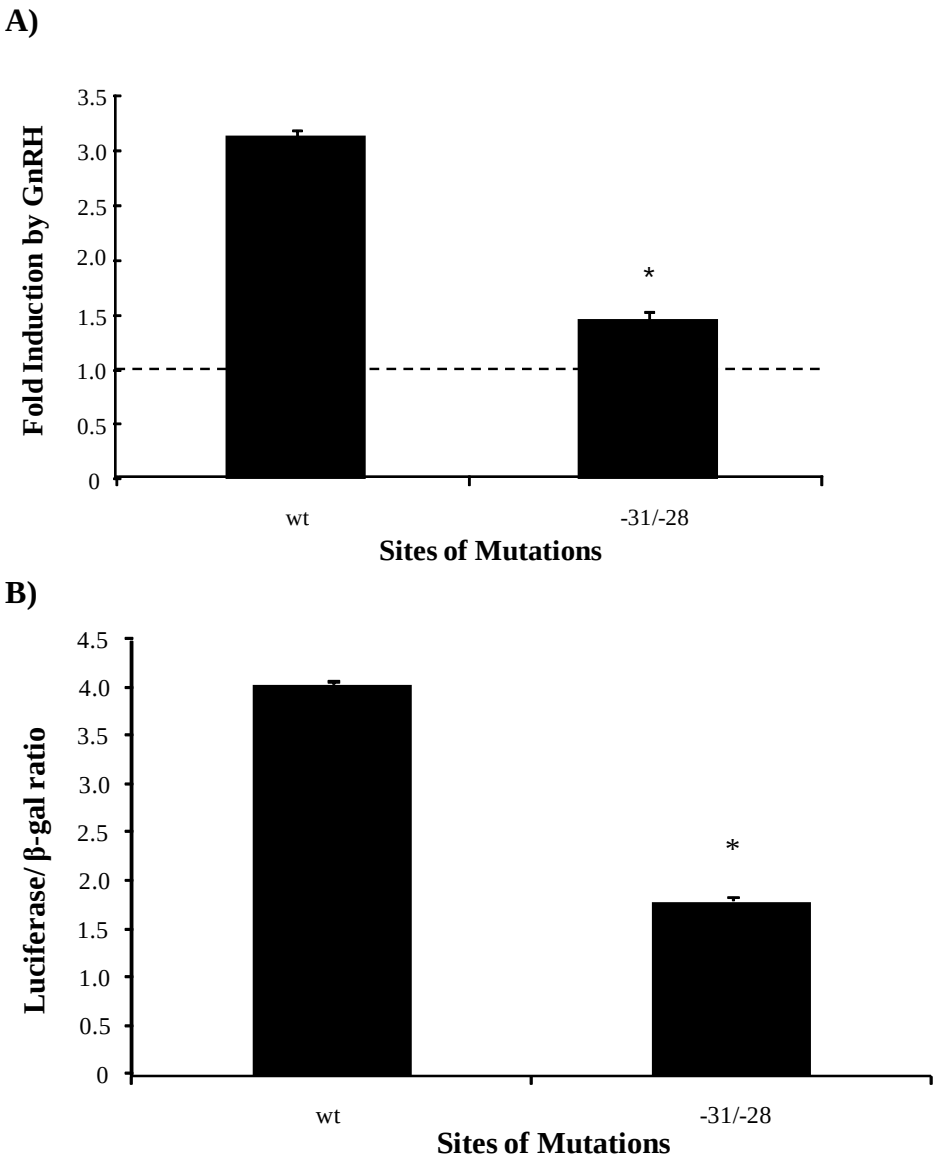



**Figure 3: TATA box plays a role in LHβ induction by GnRH**
LβT2 cells were transfected with the wildtype (wt) 1.8 kb LHβ promoter or a mutation in the TATA box, fused with a luciferase reporter gene and treated with vehicle or GnRH. A) GnRH fold induction is significantly reduced by 79% due to the mutation of the TATA box. B) There is a 56% reduction in basal expression due to the mutation of the TATA box. (*) represents a significant difference in fold induction from wild type with $p<0.05$.

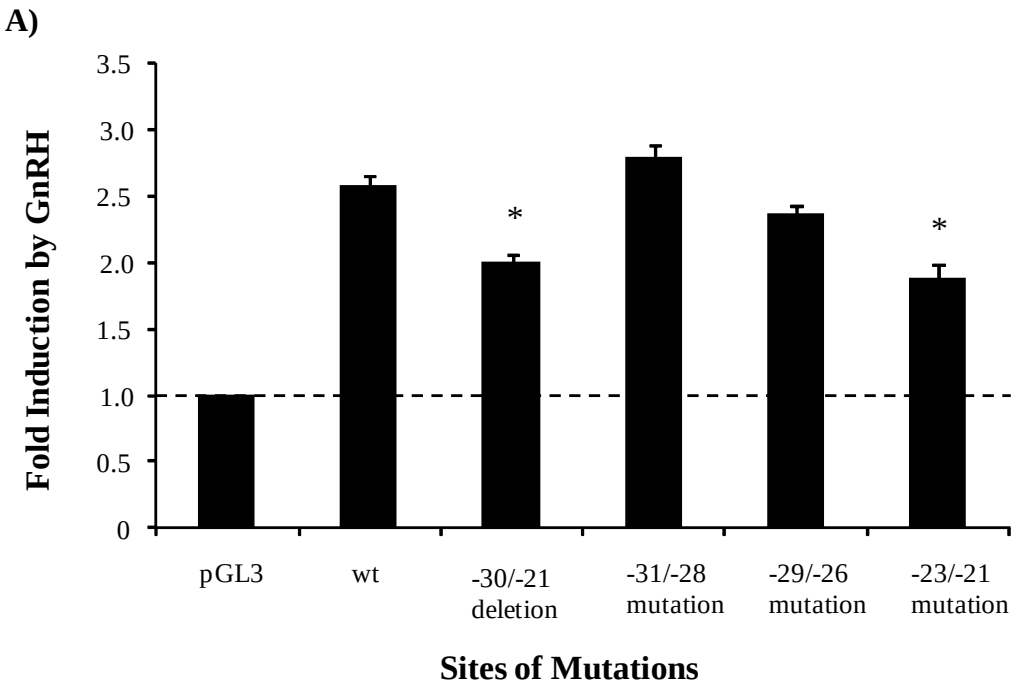



**Figure 4: Novel element maps to -23/-21 region of FSHβ promoter**
A) LβT2 cells were transfected with wildtype (wt) FSHβ promoter, the -30/-21 internal deletion, or mutations as indicated below the bar, fused with a luciferase reporter gene and treated with GnRH. Significant reduction in GnRH responsiveness by 30% corresponds to the mutation of -23/-21 base pairs in the FSHβ promoter, similar to the reduction observed with the 10 bp internal deletion of the -30/-21 region. Values represent a mean of three experiments done in triplicate. (*) represents a significant difference in fold induction with $p<0.05$.

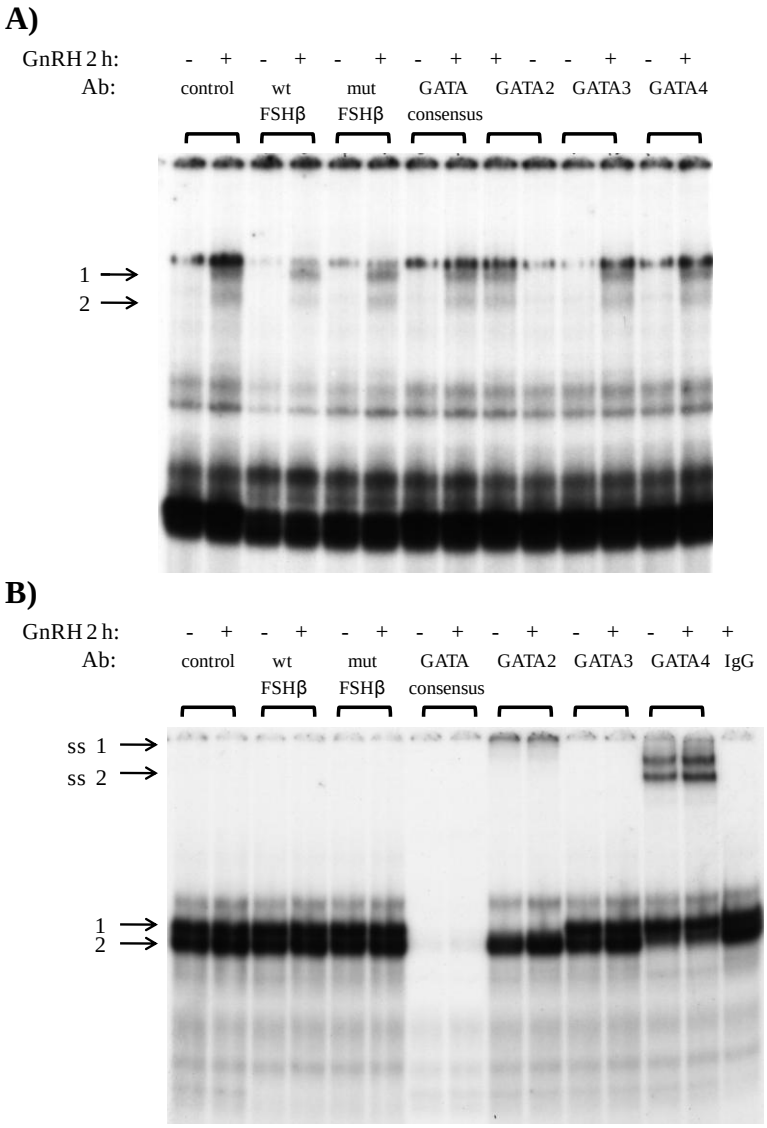



**Figure 5: GATA proteins do not bind FSHβ promoter within our region of interest**
A) Nuclear extracts from LβT2 cells treated with GnRH or vehicle were incubated with radiolabeled wildtype FSHβ probe (-41/-12) and antibodies for GATA proteins. Unlabeled wildtype FSHβ sequence and mutant FSHβ oligonucleotides with mutation in the ATG residues of interest, as well as GATA consensus, were used in 200-fold excess to compete with the radiolabeled probe. Arrows indicate complexes that change following GnRH treatment. Arrow 2 represents our complex of interest induced by GnRH treatment, since it was not competed with the mutant oligonucleotide. B) Nuclear extracts of LβT2 cells treated with GnRH or vehicle were incubated with radiolabeled GATA consensus probe and antibodies for GATA proteins and non-specific IgG control antibody. Unlabeled wildtype FSHβ and mutant FSHβ nucleotides were used in 200-fold excess in the EMSA to compete with the radiolabeled probe. Arrow 1 represents the complex containing GATA 2 protein since the complex was supershifted with antibodies to GATA 2 (arrow ss 1) Arrow 2 indicates GATA 4 containing complex, which was supershifted upon the addition of GATA 4 antibody indicated by arrow ss 2. IgG represents control for nonspecific antibody binding.

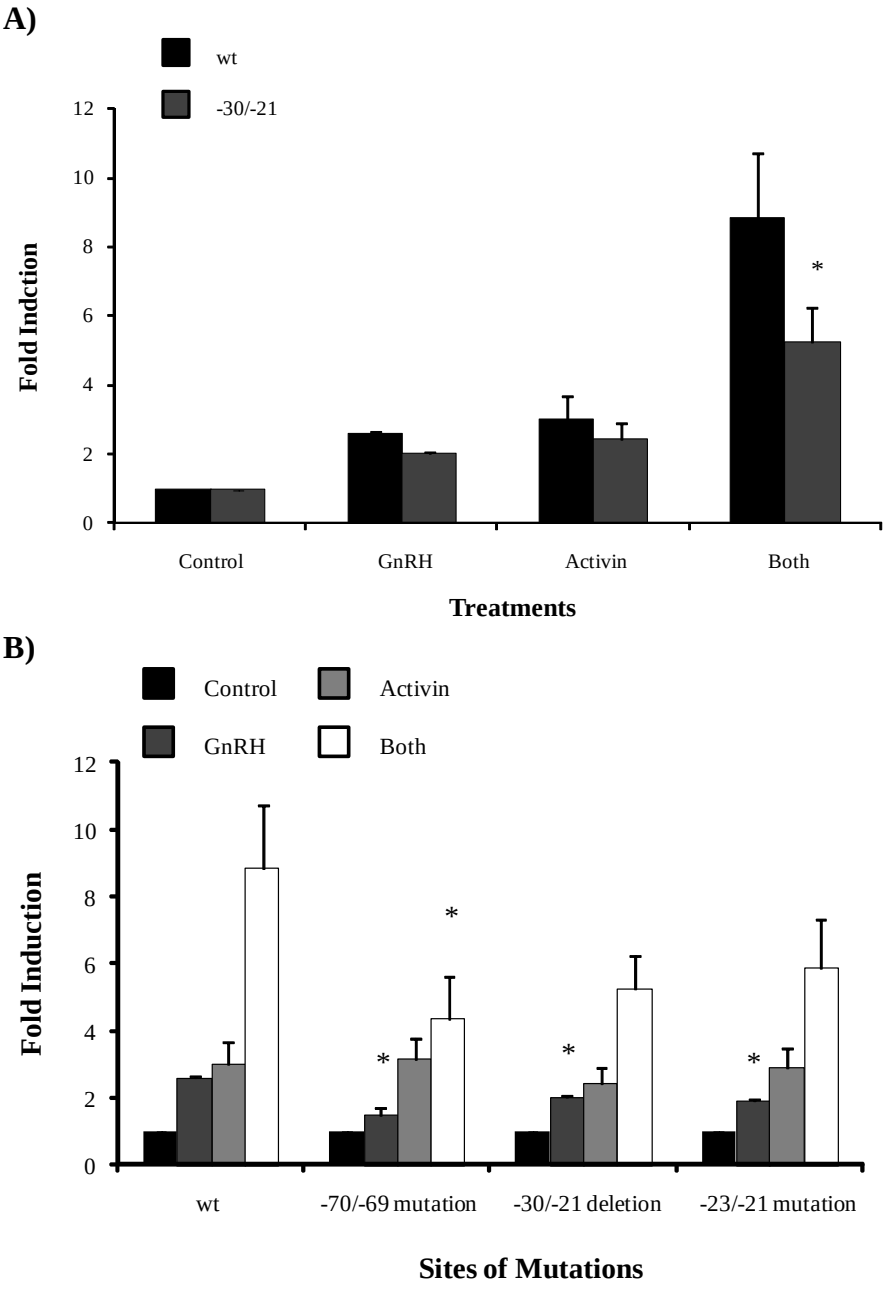



**Figure 6: -30/-21 region is involved in synergistic induction of FSHβ by GnRH and activin**

LβT2 cells were transfected with wildtype (wt) FSHβ promoter or -30/-21 internal deletion fused with a luciferase reporter gene and treated with GnRH, activin or co-treated with both hormones. Deletion reduced not only GnRH responsiveness but induction by co-treatment also. B) LβT2 cells were transfected with the FSHβ wild type (wt), -76 AP1 site mutant, -30/-21 internal deletion, or -23/-21 mutant and treated with vehicle, GnRH, activin, or co-treated with both hormones. Luciferase/β-gal ratio was normalized to pGL3 and values represent a mean of three experiments done in triplicate. (*) represents a significant difference in fold induction from the wild type with $p<0.05$.

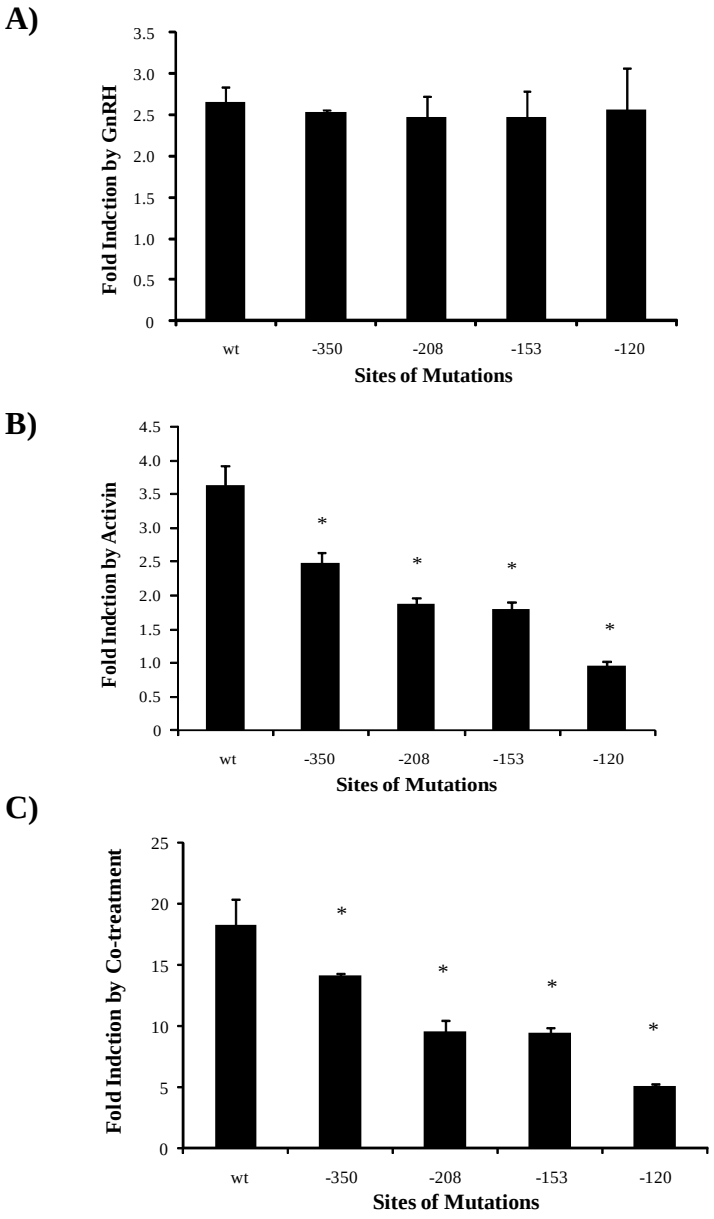



**Figure 7: Analysis of activin-responsive sites for GnRH, activin and synergy responsiveness of FSHβ promoter**

LβT2 cells were transiently transfected with the FSHβ promoter mutated at the -350 site, -208 site, -153 site or -120 site and treated with GnRH, activin or co-treatment and analyzed for fold induction. A) None of the activin-responsive sites were found to be critical for GnRH induction of FSHβ promoter. B) All of the sites were important for activin responsiveness of FSHβ promoter. C) -120 site was most critical for the synergistic effects of GnRH and activin. Luciferase/β-gal ratio was represented as fold induction, normalized to pGL3 and values represent a mean of three experiments done in triplicate. (*) represents a significant difference in induction with $p<0.05$.

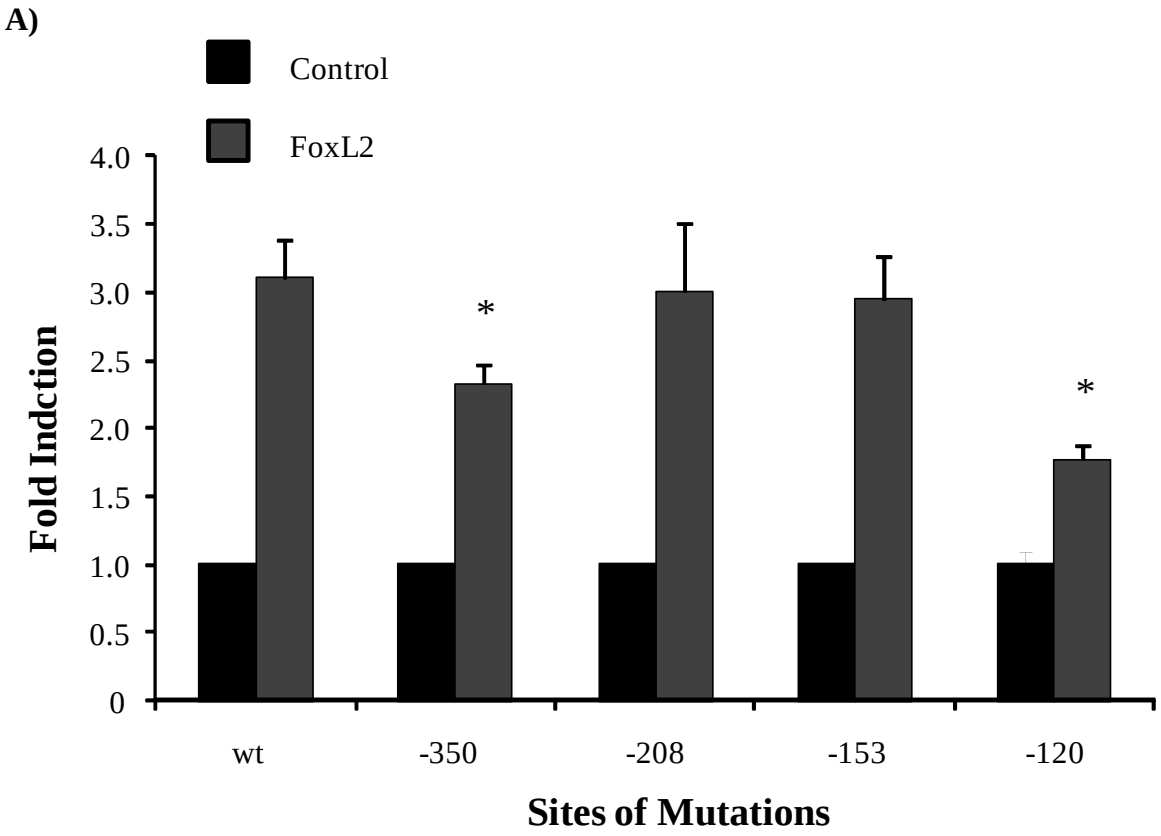



**Figure 8: -350 and -120 sites of FSHβ promoter are necessary for FoxL2 induction**
A) LβT2 cells co-transfected with FSHβ wildtype or the indicated FoxL2 site mutants, and overexpression vector containing FoxL2 cDNA. Mutations at activin-responsive sites -350 and -120 reduce the induction of the FSHb promoter by FoxL2 overexpression by 37% and 63%, respectively. Luciferase/β-gal ratio was represented as fold induction, normalized to pGL3 and values represent a mean of three experiments done in triplicate. (*) represents a significant difference in induction with $p<0.05$.

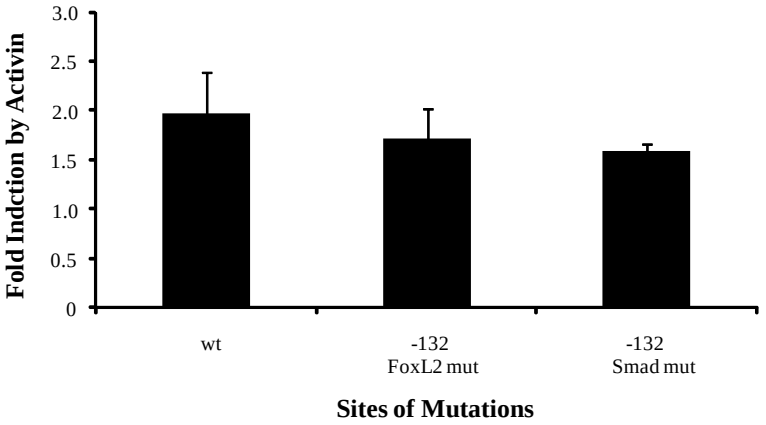
**A)**

```
Mouse     -350 ATATTTTGGTTT   -267 GTCTAGAC     -208 CAGATTCGGTTTG
Human     -360 ATTTTTTAATGC         --------     -223 CAAATTTAATTTG

Mouse     -153 GCATTTAG       -120 GTCTAAACAA
Human     -164 GCGTTTAG       -132 GTCTATCTAAACAA
```

```
human FSHβ wt -132    5′ GTCTATCTAAACAC 3′
   -Smad mutant:      5′ GTGAATCTAAACAC 3′
   -FoxL2 mutant:     5′ GTCTATAGAAACAC 3′
```

**B)**




**C)**

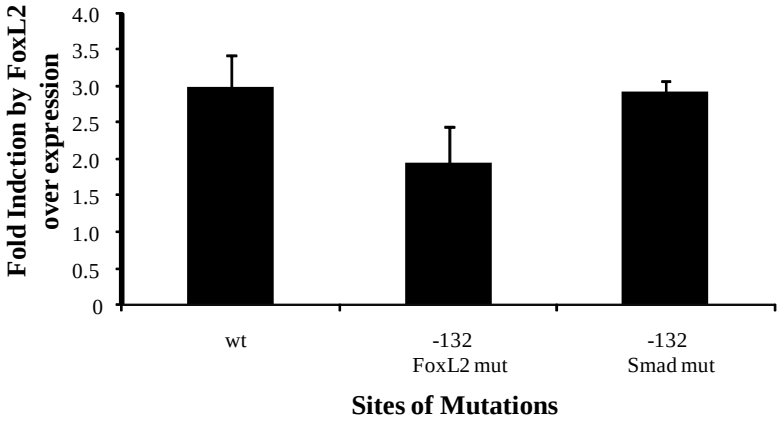



**Figure 9: Analysis of Smad half-site and FoxL2 site for FSHβ induction by activin**
A) An alignment of the mouse -120 site and the homologous human site at -132 and the mutations created in the human sequence to distinguish between Smad and FoxL2 sites. B) LβT2 cells were transfected with wildtype 1 kb human FSHβ promoter or the indicated mutations fused with a luciferase reporter gene and treated with 50 ng/ml activin for 24 hours. C) LβT2 cells were co-transfected with wildtype 1 kb human FSHβ promoter or the indicated mutations fused with a luciferase reporter gene, and overexpression vector for human FoxL2 cDNA.

# III

# Discussion

Normal reproductive function involves an intricate integration of hormonal signals from the hypothalamus, pituitary and gonads. Insight into the molecular mechanism of hormonal regulation of the pituitary glycoprotein follicle-stimulating hormone may provide further understanding of critical stages in the life cycle including fetal development, puberty, menstruation, pregnancy, and menopause. GnRH and activin are the most important regulators of FSH and play a critical role in its gene expression. Although FSH and LH are found in the same hormonal milieu, they experience differential regulation and expression by the hypothalamic hormones, as well as from gonadal feedback as observed in the menstrual/estrous cycle [3]. In humans, FSH bioactivity levels increase during the follicular phase and surge along with an even greater LH surge, which is required for ovulation. Then a secondary FSH rise is observed without a coinciding rise in LH, which is critical during folliculogenesis to recruit the following cohort of follicles [3].

In this study, we identify novel regulatory elements that participate in GnRH induction of the mouse FSHβ genes in pituitary cells, further elucidate the role of newly identified FoxL2 in activin induction of FSHβ genes, and provide insight into the synergistic induction of FSHβ genes by GnRH and activin. Previously, truncation

analysis of the mouse 1.5 kb FSHβ promoter mapped GnRH responsiveness downstream of -95 bp from the start site, and identified AP1 site at -76 as one of the critical GnRH-responsive elements in the promoter [29]. Although mutation of the AP1 site significantly decreases GnRH induction of FSHβ gene expression, it does not account for all the FSHβ gene response. Therefore, we sought to understand the mechanisms underlying the full response to GnRH regulation of FSHβ and its transcription.

Since the β-subunits of FSH and LH confer the biological specificity of these hormones, we analyzed whether the TATA-box is important for integration of hormonal response and basal expression of both the LHβ gene and FSHβ gene promoter. The TATA box, bound by TBP, is a predominant DNA element of core promoters that direct transcription initiation by RNA polymerase II. The TATA-box mutations reduced basal expression of the FSHβ gene, but not GnRH induction. Contrary to the FSHβ promoter, mutations within 5' TATA 3' TATA-box DNA sequence of LHβ significantly decreased GnRH responsiveness by 79% and basal expression by 56% (Fig. 3A, 3B). The differences observed regarding the involvement of TBP in gene regulation between FSH and LH β-subunits may be due to TBP interaction with early growth response factor-1 (Egr-1). Egr-1 is an inducible transcription factor of the immediate early response genes family that is known to be involved in cellular functions including proliferation, differentiation, apoptosis and gene regulation [66]. Egr-1 is further involved in reproduction since it upregulates LHβ and LH receptor expression [66]. GnRH induction of LHβ promoter results in Egr-1 interaction with tissue-specific and ubiquitous basal factors which then regulate LHβ expression [53]. There may also be an additional

interaction between TBP and Egr-1 which helps convey GnRH induction of LHβ promoter that is absent in the GnRH induction of FSHβ promoter. Whereas, GnRH induction of FSHβ promoter, which has been shown to exert its effects through AP1 and an unknown element at -23, may not utilize interaction with TBP. Thus, the presence of a functional TATA box seems to be important in GnRH responsiveness of the LHβ promoter, and this may be another component of the differential regulation of FSH and LH observed during the menstrual/estrous cycle.

Our experiments revealed that the 5' ATG 3' sequence is a novel element in the FSHβ promoter required for GnRH responsiveness. Analysis of the DNA sequence led us to postulate a potential consensus GATA motif. GATA factors are transcriptional regulators that play essential roles in cell differentiation, organ morphogenesis, and tissue-specific gene expression during development. They are expressed in the hematopoietic system, heart, gut, brain, placenta, pituitary, and gonads [67]. GATA1, 4, and 6 are expressed in the mammalian gonads [67], and the promoters of several steroidogenic genes, including SF-1, have been shown to contain consensus GATA regulatory motifs, suggesting that GATA factors likely play an essential role in the regulation of steroidogenesis [68]. In addition, GATA family members regulate reproductive gene expression in the hypothalamus and anterior pituitary gland. Studies have shown GATA4 localization to migrating GnRH neurons and that GATA2 and GATA3 are expressed in the developing pituitary gland [69]. Furthermore, there is evidence supporting gonadotropin regulation of gonadal GATA gene expression as observed in women who have mutations of the FSH receptor gene also have little GATA4 protein in their ovaries and their ovaries lack significant follicular development

[70]. In our efforts to identify transcription factors that may bind the 5' GATG 3' DNA sequence, we also confirmed that GATA proteins 2 and 4 are expressed in LβT2 cells and that our antibodies are specific (Fig. 5B). However, we did not observe any supershifts with our region of interest with the inclusion of GATA antibodies, suggesting that GATA proteins do not bind the 5' GATG 3' region of the FSHβ gene promoter (Fig. 5A). Thus, further investigation is required to determine the transcription factors involved with GnRH regulation of FSHβ gene promoter at this novel region.

Although we could not identify the factors that bind to this region, its internal deletion clearly demonstrates its importance in GnRH regulation. We sought to determine whether this newly identified region on the FSHβ promoter was also important for activin or synergistic regulation of FSHβ gene expression since activin is also a potent inducer of FSHβ gene expression. While both GnRH and activin individually stimulate expression of FSHβ gene, GnRH has been shown to stimulate activin-signaling pathways to result in greater FSHβ expression than the individual hormones combined [3, 57, 60]. Although the -30/-21 region of FSHβ promoter was not found to be critical for activin responsiveness, its deletion or specific mutation at the 5'ATG 3' site reduced synergistic induction of FSHβ gene by GnRH and activin (Fig. 6B). To better understand the synergistic regulation of GnRH and activin, we analyzed the novel activin responsive sites previously identified in our laboratory to determine their role in synergy.

The newly identified activin responsive sites include -350, -208, -153, and -120 of the mouse FSHβ promoter. All of these sites significantly reduce fold induction due to synergy when mutated (Fig. 7C) and all of the sites have been shown to bind the

transcription factor, FoxL2 [38]. Forkhead-domain transcription factor L2 (FoxL2) is expressed mainly in somatic cells of female gonads, pituitary cells and developing eyelids [71, 72]. It is a new member of the forkhead family and is considered essential for ovarian folliculogenesis and granulose cell development. Loss of function mutations of FoxL2 cause the dominant autosomal disorder, BPES, which results in eyelid dysplasia and depending on the type, may also present with premature ovarian failure. Furthermore, it has been suggested to play a role in early pituitary development, as it is one of the earliest markers expressed, and also in gonadotrope cell differentiation and regulation of glycoprotein hormone production [53, 72]. In the mouse and porcine pituitary cells, FoxL2 has been suggested to play a role in activin regulated FSHβ transcription [52, 72]. Furthermore, human FoxL2 is highly homologous to mouse with 90% sequence identity [71, 72]. It has been demonstrated that FoxL2 and Smads interact and synergistically upregulate follistatin expression in pituitary cells [73]. In addition, our lab recently demonstrated that FoxL2 is also required for activin induction of both mouse and human FSHβ [38]. FoxL2 is constitutively bound to the promoter and its binding does not change following activin treatment. Thus, the question regarding how activin through activin receptors activates FoxL2 is yet unclear. It has been suggested that activated Smads are recruited to the promoter by interaction with FoxL2 and that is why FoxL2 sites are critical. Indeed FoxL2 sites contain Smad half sites or Smad half sites are juxtaposed to Forkhead elements in FSHβ promoter. However, the molecular mechanism of FoxL2 induction of FSHβ promoter has yet to be determined.

Although there is sequence homology between mouse, human and porcine FSHβ promoters, there are species-specific differences in the affinity of FoxL2 binding to each

of these regions. For instance, while the mouse -350 site binds FoxL2 with high affinity, FoxL2 does not bind the homologous human -360 element and mutation of this -360 region of the promoter has no effect on activin response. However, the human FSHβ promoter binds FoxL2 with high affinity at the -223 (homologous to mouse -208) and -164 regions (homologous to mouse -153) [38]. Furthermore, mouse -120, which is homologous to porcine -134 and human -132, is highly conserved and it was determined that Ptx1/Prep1 and Smad4 protein complexes bind the site and are critical for activin response of FSHβ gene promoter [41]. In the mouse FSHβ promoter, -350 and -120 were determined to be most critical for FoxL2 role in regulation of FSHβ gene expression by activin (Fig. 8). The -120 DNA sequence, 5' GTCTAAACAA 3', in the mouse promoter, contains both a Smad half-site (5' GTCT 3') and a FoxL2 site (5' CTAAACAA 3'), which are overlapping. Therefore, it has not been determined whether the FoxL2 or Smad site is more critical for activin induction of FSHβ gene in this more proximal region. We focused on the human FSHβ promoter at the -132 region (homologous to mouse -120) since the Smad site and FoxL2 are not overlapping. Since human FSHβ promoter is still responsive to activin although to a lesser degree, and yet lacks the classical Smad site at -267 and is Smad-independent [38], we believed that FoxL2 site may be critical for activin induction of human FSHβ gene expression. Our preliminary results (Fig. 9) indicate that although FoxL2 site at -132 is important for FoxL2 induction of the FSHβ promoter, mutations of FoxL2 do not completely eliminate activin response. Interestingly, mutations of Smad site also do not completely eliminate human FSHβ response to

activin, suggesting that Smad and FoxL2 may interact to convey activin responsiveness in some instances but require other unknown elements in other instances.

The data reported here can contribute to understanding the mechanism of differential regulation of the gonadotropin during the follicular and luteal phase of the menstrual cycle. At the end of the luteal phase, the hormones estrogen and progesterone, which provide the negative feedback signals, drastically decrease. With the loss of the negative feedback, the hypothalamus secretes more GnRH. The secondary rise of FSH that does not coincide with an increase in LH, is a hallmark of the luteal to follicular transition in the menstrual cycle and is essential for the recruitment of follicles for the next cycle. In rodents, activin regulation of FSHβ occurs independently of GnRH during the secondary rise of FSH. However, in humans, activin may require GnRH signaling to exert its effects. Furthermore, in rodents, while activin regulation may require a Smad half-site to be adjacent to the newly identified FoxL2 sites, in humans, there may be an interaction between Smads and FoxL2 to exert activin effects, or there may be a more complex mechanism involved with the synergistic regulation of human FSHβ by GnRH and activin.

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