THE ROLE OF β-CATENIN IN THE GONADOTROPE TRANSCRIPTIONAL NETWORK: INTERACTIONS WITH SF1 AND TCF

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation/thesis of APRIL KAY BINDER find it satisfactory and recommend that it be accepted.

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THE ROLE OF β-CATENIN IN THE GONADOTROPE

TRANSCRIPTIONAL NETWORK:

INTERACTIONS WITH

SF1 AND TCF

Abstract

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Gonadotropin-releasing hormone (GnRH) is necessary for the regulation of luteinizing hormone (LH) and follicle stimulating hormone (FSH) synthesized and secreted from gonadotrope cells of the anterior pituitary. GnRH signals to regulate genes through a transcriptional hierarchy, and herein we show that the co-factor β-catenin is a required component of this network. GnRH stimulates β-catenin accumulation and is required for expression of several gonadotrope specific genes through interactions with steroidogenic factor 1 (SF1) and T-cell factor (TCF). Regulation of the four gonadotrope specific genes, *Lhb*, *Fshb*, *Cga* and *Gnrhr* requires SF1 in addition to several immediate early genes that confer hormonal responsiveness including *Jun* and *Atf3*. The work presented here demonstrates that SF1 and β-catenin interactions are necessary for maximal regulation of *Lhb* subunit gene downstream of GnRH. This provides one input for β-catenin in the gonadotrope transcriptional network and suggests that the co-factor may be important in regulation of the other gonadotrope specific genes.

Beta-catenin was originally identified as a downstream mediator of WNT signaling through interactions with TCF proteins. A requirement for TCF in regulation of *Jun*/JUN downstream of GnRH provides a second input for β-catenin in the transcriptional network. TCF and β-catenin interactions are necessary for *Jun* mRNA accumulation, as well as transcriptional activity of a truncated *CGA* promoter reporter. These observations suggest that similar signaling pathways may mediate TCF-dependent transcription downstream of GnRH. We have found that similar signaling pathways regulate increased message of both *Jun* and *Atf3*. Furthermore, we found that the signaling pathway that mediates these events downstream of GnRH requires calcium mobilization and calcineurin activity. Increased cellular calcium and calcineurin activity is necessary for GnRH regulation of an NFAT responsive promoter, providing another possible site of action for β-catenin through direct or indirect interactions. These studies demonstrate that β-catenin is a key player in regulation of several gonadotrope genes, and interactions with SF1 and TCF are necessary for gonadotrope function.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iii

ABSTRACT v

LIST OF FIGURES x

ATTRIBUTIONS xii

CHAPTER

1. INTRODUCTION

Hypothalamic-Pituitary-Gonadal Axis 2

Function of β-catenin in gonadotropes 4

GnRH signaling in gonadotropes 6

NFAT gene regulation 7

Summary 8

References 10

2. WELCOMING β-CATENIN TO THE GONADOTROPIN-RELEASING HORMONE TRANSCRIPTIONAL NETWORK IN GONADOTROPES

Abstract 18

A short history of the GnRH transcriptional network in gonadotropes 19

GnRH regulation of the four signature genes requires steroidogenic factor 1 (SF1) 20

A surprising link between SF1, β-catenin, and GnRH regulated gene expression in gonadotropes 21

GnRH regulation of TCF/LEF dependent genes in gonadotropes: Another potential site of β-catenin action 23

Who carries the GnRH signal to β-catenin? 24

Putting the pieces back together 27

References 28

3. MAXIMAL ACTIVITY OF THE LHB PROMOTER REQUIRES β-CATENIN

Abstract 41

Introduction 42

Results 44

Discussion 52

Materials and Methods 56

Acknowledgements 60

References 61

4. GNRH REGULATED EXPRESSION OF JUN AND JUN TARGET GENES IN GONADOTROPES REQUIRES A FUNCTIONAL INTERACTION BETWEEN TCF/LEF FAMILY MEMBERS AND BETA-CATENIN

Abstract 72

Introduction 73

Results 75

Discussion 81

Materials and Methods 85

Acknowledgements 90

References 91

5. GNRH REGULATION OF TCF AND NFAT RESPONSIVE PROMOTERS REQUIRES MOBILIZATION OF INTRACELLULAR CALCIUM AND ACTIVATION OF CALCINEURIN

Abstract 103

Introduction 104

Results 107

Discussion 113

Materials and Methods 116

Acknowledgements 119

References 120

6. CONCLUSIONS/FUTURE DIRECTIONS

Conclusions 131

Future Directions 134

Regulation of β-catenin by GnRH 134

NFAT in the gonadotrope transcriptional network 135

In vivo role of β-catenin in mature gonadotropes 138

Summary 139

References 140

LIST OF FIGURES

1.1 Hypothalamic-Gonadal-Pituitary Axis 3

2.1 Selected overview of the GnRH transcriptional network in gonadotropes 36

2.2 Contributions from β-catenin enable ERG1 to act as a rheostat that regulates expression of LHB/Lhb 37

2.3 Potential signaling pathways for permitting crosstalk between WNT and GnRH 38

2.4 Moving β-catenin into the GnRH transcriptional network 39

3.1 Maximal transcriptional response of the LHB promoter to GnRH requires β-catenin 65

3.2 Beta-catenin enhances activity of EGR1 and SF1 66

3.3 siRNA against β-catenin reduces the activity of EGR1 and SF1 67

3.4 SF1 physically associates with β-catenin 68

3.5 The β-catenin binding site in SF1 is required for functional synergism between SF1 and EGR1 69

3.6 GnRH enhances the association of β-catenin with the proximal region of the endogenous *Lhb* promoter 70

4.1 GnRH regulates TCF/LEF-dependent transcription through β-catenin and TCF/LEF 97

4.2 GnRH stimulated increases in *Jun* expression are reduced in TCFDN expressing LβT2 cells 98

4.3 Endogenous β-catenin is required for maximal expression of *Jun* in GnRH treated cells 99

4.4 JUN protein levels are reduced in β-catenin deficient and TCFDN expressing cells 100

4.5 *CGA* transcription is compromised by TCFDN 101

5.1 JNK activity is required for GnRH regulation of TCF-dependent transcription and accumulation of *Jun* and *Atf3* transcripts 125

5.2 Ca^{2+} mobilization is necessary for GnRH activation of TCF-dependent transcription and *Jun* and *Atf3* accumulation 126

5.3 Calcineurin inhibition reduces GnRH mediated TOPflash activation and *Jun* and *Atf3* transcript accumulation 127

5.4 *Nfatc3* is expressed in LβT3 cells and GnRH regulates NFAT-dependent transcription 128

5.5 GnRH regulation of NFAT-dependent transcriptional activity requires Ca^{2+} and calcineurin activity 129

ATTRIBUTIONS

Chapter 2 is an invited mini-review that I co-authored with Travis Salisbury. It was published in 2008 by The Endocrine Society and is in the format of the journal *Molecular Endocrinology*. Travis and I wrote the paper together each focusing on a part of it, Travis wrote the SF1 section, and I wrote the TCF and signaling part of the paper. We worked together on the figures with our mentor, John Nilson, and to edit and review the manuscript for final publication.

Chapter 3 is work that I did during my laboratory rotation that I continued to pursue after I first joined the lab. I performed several preliminary experiments for the paper, the western blot presented in figure 1C and the co-immunoprecipitation shown in figure 4. I also helped with the statistics, assisted in writing, revising and preparing the final manuscript published in *Molecular Endocrinology*.

The paper presented in Chapter 4 is another co-authored paper published with Travis Salisbury. I performed the experiments and acquired the data presented in figures 1, 3, and 5 along with contributing toward writing sections of the paper where my experiments were described. The figures and figure legends were compiled and completed by me. I also assisted in writing and final preparation of the manuscript, which was published in the journal *Molecular Endocrinology* by The Endocrine Society.

Chapter 5 is written in the format for *Molecular Endocrinology* where the final manuscript will be submitted. Preparation of the manuscript and all experiments were presented were performed by me in consultation with my mentor John Nilson.

CHAPTER ONE

INTRODUCTION

Hypothalamic-Gonadal-Pituitary Axis

The pituitary gland is responsible for endocrine regulation of multiple processes including reproduction, lactation, growth, metabolism, aging and the stress response. The anterior pituitary consists of five distinct cell types that secrete six hormones, and each cell type is characterized by the hormone secreted. The five cell types are the corticotropes that secrete adrenocorticotropin hormone, somatotropes that secrete growth hormone, thyrotropes that secrete thyroid stimulating hormone, lactotropes that secrete prolactin and the gonadotropes that secrete the gonadotropins luteinizing hormone (LH) and follicle stimulating hormones (FSH) (1).

The various cell types of the anterior pituitary, and the hormones secreted, are under strict regulation by hypothalamic hormones that act specifically on each cell type. Gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus binds to its receptor on gonadotropes to regulate the synthesis and secretion of the gonadotropins (2;3). Normal reproductive function in mammals requires LH and FSH, and strict regulation is necessary to prevent undesirable consequences. Hypogonadotropic hypogonadism (2) and infertility (4;5) are common outcomes of reduced LH and FSH, while elevated LH has implications in breast cancer (6), and ovarian defects (7;8) including polycistic ovarian syndrome (PCOS) in humans (9;10). These effects demonstrate that precise regulation of LH and FSH is important for reproductive health and proper function of the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis is regulated through positive and negative feedback loops to maintain proper levels of these hormones as depicted in Figure 1.

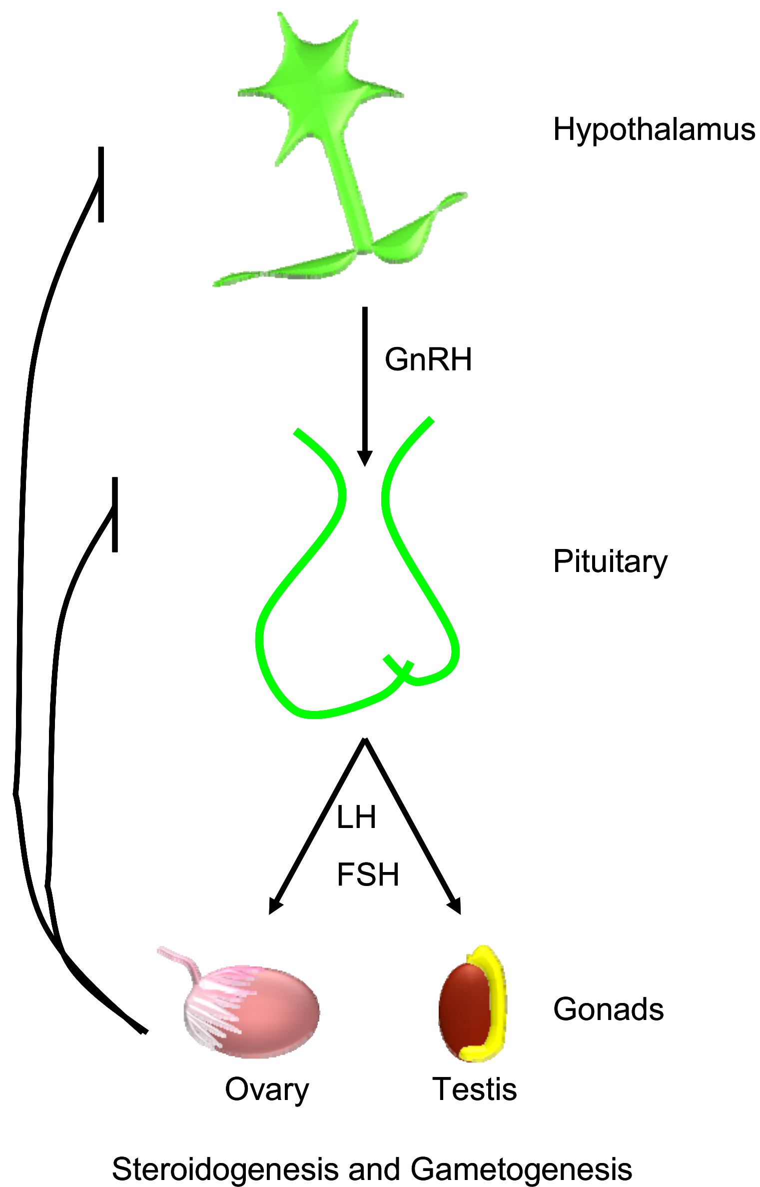

Figure 1: Hypothalamic-Gonadal-Pituitary Axis

GnRH secreted from the hypothalamus binds to its receptor on gonadotrope cells of the anterior pituitary to regulate the synthesis and secretion of LH and FSH. LH and FSH then bind to their respective receptors in the ovary/testis to regulate steroidogenesis and gametogenesis. Steroid hormones from the gonads can then act back at the hypothalamus and pituitary to inhibit GnRH, LH and/or FSH.

LH and FSH share a common alpha subunit (encoded by the gene *Cga*), while they each have specific beta subunits (encoded for by the genes *Lhb* and *Fshb*) that provide receptor specificity. These genes are regulated by GnRH, which also regulates its own receptor, encoded by the gene *Gnrhr*. Upon binding to its receptor, GnRH initiates a positive signaling cascade leading to increased transcription of the gonadotrope specific genes *Cga*, *Lhb*, *Fshb* and *Gnrhr* (reviewed in (11-13)). The α-glycoprotein subunit (encoded by *Cga*) then dimerizes with either LHβ or FSHβ to produce the glycoproteins LH and FSH. The gonadotropins then bind to their specific receptors in the ovary or the testis to regulate gametogenesis and steroidogenesis. The increased steroid production can then act to inhibit LH and FSH at both the hypothalamus and the pituitary.

LH and FSH stimulate several genes that are necessary for steroid biosynthesis in the Leydig cells of the testis, or the granulosa cells of the ovary leading to increased steroid production. Androgen can then be converted into testosterone or estrogen depending on the enzymes expressed. Increased levels of androgen (14;15) can then act to inhibit transcription of *Cga* independently of DNA binding through interactions with SF1. This provides multiple levels of control for gonadotrope specific gene expression and LH and FSH secretion (16). The regulation of this transcriptional network has been studied extensively, and is reviewed in Chapter 2.

Function of β-catenin in gonadotropes

Beta-catenin is a co-factor that has several roles in the cell, including at the cell membrane and in the nucleus (17;18). It functions at the cell membrane as a subunit of the cadherin complex (19), and was identified as a transcriptional co-activator downstream of WNT signaling (20-22). WNT signaling is important for embryogenesis and cellular differentiation of multiple tissues, including the pituitary. Development of the

pituitary is a complex process, and several members of the WNT signaling family have been implicated as important mediators of development (23-26). Beta-catenin within the WNT signaling pathway is important for pituitary growth and differentiation during development (23;27) and expansion of the gland (28). The signaling pathway has also been shown to be important for several cell types within the pituitary, including full proliferation and expansion of gonadotropes (23;26-30). While these studies have demonstrated that β-catenin is important during organogenesis, the role of the co-factor in mature pituitaries is unknown.

In the absence of WNT signals, cytoplasmic β-catenin is phosphorylated by Glycogen synthase kinase 3β (GSK3β), targeted for ubiquitination and proteasomal mediated degradation. This process is regulated by a complex of proteins including adenomatous polyposis coli (APC), Axin, and GSK3β that sequesters β-catenin and acts to facilitate Ser/Thr phosphorylation by GSK3β (31). In the absence of nuclear β-catenin, T-cell factor/Lymphoid enhancer factor (TCF/LEF) responsive genes are repressed through interactions of TCF/LEF with co-repressors such as groucho and histone deacetylases (17). WNT proteins are secreted glycoproteins that regulate cellular processes by binding to members of Frizzled (Fad) receptor family. Fzd receptors are seven transmembrane receptors that require the co-receptor Lipoprotein receptor-related proteins 5 and 6 (LRP5/6) (32). Secreted WNT binds to Fzd and activates Disheveled, a key intermediate, leading to inhibition of the degradation complex and accumulation of β-catenin in the cytoplasm (17;31).

Increased β-catenin is translocated into the nucleus where it displaces co-repressors and binds TCF/LEF increasing the transcriptional activity of target genes (20-22;31). In addition to WNT/Fzd activation, several G protein coupled receptors (GPCR) have been found to increase the nuclear localization of β-catenin leading to increased transcriptional activity, namely PKC (33), PKA (34;35) and PI3K (33). In addition to

TCF/LEF, β-catenin can bind and modulate the activity of nuclear receptors including the androgen receptor, and steroidogenic factor 1 (SF1) (20;36). This work investigates the role of β-catenin in regulation of gonadotrope specific genes, and finds that interactions with SF1 and TCF are essential for hormonal regulation by GnRH.

GnRH signaling in gonadotropes

GnRH is secreted in pulses from the hypothalamus and binds to its receptor, GnRHR on gonadotropes. GnRHR is a GPCR that signals through Gαq proteins activating phospholipase C leading to increased inosital-3-phosphate (IP3) and diacylglycerol (DAG) activity (reviewed in (12)). DAG activates PKC leading to activation of several members of the MAPK (37) family including ERK (38-40), JNK (38;40) and p38 MAPK (38;41). IP3 releases Ca^{2+} from intracellular stores leading to increased activity of calmodulin and calmodulin dependent proteins, including calmodulin kinase (CAMKI/CAMKII) and calcineurin (12). GnRH has also been linked to increased cAMP and PKA activation (42-44). As reviewed in Chapter 2, the four gonadotrope specific genes, *Lhb*, *Fshb*, *Cga* and *Gnrhr* require SF1 for basal expression. The presence of SF1 is required (45;46), but not sufficient (47;48), and each gene requires additional hormonal stimulus through increased transcription of the immediate early genes (IEGs), *Jun*, *Atf3* and *Egr1*. Increased transcription and translation of these IEGs carries the GnRH signal to the distal gonadotrope specific genes rendering them active and significantly increasing transcript levels (12;49).

Jun/JUN is an important component of the activating protein 1 (AP1) complex (50), and AP1 response elements have been identified in the proximal promoter regions of the genes encoding *Cga*, *Fshb* and *Gnrhr* (49;51;52). While JUN is necessary for regulation of these genes, additional factors are also required to facilitate differential expression downstream of GnRH. For example, JUN and ATF3 heterodimers are

necessary for regulation of the human *CGA* promoter (53), while JUN and FOS heterodimers are bound to the *Fshb* promoter (54). As such, multiple signaling pathways are activated downstream of GnRH, and converge on specific target genes. This is further demonstrated by concentration dependence of secondary messengers such as calcium.

GnRH stimulates Ca^{2+} mobilization from intracellular stores, as well as influx from voltage gated calcium channels found at the plasma membrane (55). In gonadotrope cells, the duration of Ca^{2+} pulses downstream of GnRH differentially regulates genes. *Cga* transcript is increased in response to fast pulses of Ca^{2+}, while *Fshb* responds to very slow pulses (56). The role of Ca^{2+} in *Lhb* transcription has been controversial depending on the cell type and species studied, as some groups report no change (38;40), while others suggest slow Ca^{2+} influx is sufficient (57;58). It has also been reported that Ca^{2+} is necessary for JNK and ERK activity, albeit through different channels (59). Furthermore, the IEGs JUN and ATF3 are Ca^{2+} targets, as are the calmodulin dependent kinases I and II (60-62), and the protein phosphatase calcineurin (62;63). Gonadotrope cells have multiple Ca^{2+} targets, and the regulation of Ca^{2+} is essential for GnRH signaling (as reviewed in (12)). JNK regulation by GnRH requires intracellular Ca^{2+} mobilization (59;64) suggesting that Ca^{2+} may be important for *Jun* mRNA accumulation and TCF-dependent transcription. The involvement of Ca^{2+} in TCF-dependent transcription is further examined in Chapter 5.

NFAT gene regulation

The calcium/calmodulin dependent phosphatase calcineurin plays a critical role in immune responses through activation of Nuclear Factor of Activated T cells (NFAT) proteins. In addition to expression in T cells and other immune cells, NFAT proteins have also been identified in various tissues including the brain, testis, ovary and heart

(reviewed in (65;66)). NFAT proteins are also important in regulation of *Nur77* (66), a GnRH target in αT3 cells (67) suggesting that GnRH may regulate the activity of NFAT-dependent transcription and *Nur77* is a possible NFAT target. NFATs are DNA binding transcription factors that show similarity in the DNA binding domain to the Rel-family proteins. NFATs are regulated post-transcriptionally through phosphorylation of N-terminal serine residues by GSK3β (65;66). Removal of these phosphate residues by calcineurin allows nuclear import of NFAT proteins and increased affinity for DNA binding, facilitating increased transcription of several cytokines, surface receptors and transcription factors including *NFκB1* (66), and *Nur77* (66;68).

NFAT proteins have been shown to cooperate with AP1 (JUN/FOS) to regulate transcription, in a promoter context dependent fashion (reviewed in (66;69;70). NFAT proteins have an AP1 interacting domain, and can interact with JUN when both are bound to DNA (69-71). Furthermore, NFAT has been shown to cooperate with JUN/ATF2 (72). AP1 sites are important for GnRH regulation of gonadotrope specific genes (12;49;51;52;54), suggesting that NFAT may cooperate with JUN downstream of GnRH and this possibility is further examined in Chapter 5.

Summary

The neurohormone GnRH activates multiple signaling pathways upon binding to its receptor on gonadotrope cells. These signaling pathways regulate the transcription and translation of several gonadotrope specific genes through a transcriptional hierarchy as described in Chapter 2. This dissertation aims to elucidate the role of the co-factor β-catenin in gonadotrope cells, and the signaling pathways that regulates differential gene transcription through interactions with SF1 and TCF. Specifically, in Chapter 3 we demonstrate the requirements for β-catenin and SF1 interactions in regulation of *Lhb*, and in Chapter 4 we show that TCF/β-catenin interactions are necessary for *Jun*

transcription. Finally, in chapter 5 we examine the signaling pathway that mediates TCF dependent transcription and begin to characterize the role of NFAT-dependent transcription downstream of GnRH in gonadotrope cells.

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CHAPTER 2

WELCOMING β-CATENIN TO THE GONADOTROPIN-RELEASING HORMONE TRANSCRIPTIONAL NETWORK IN GONADOTROPES

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Abstract

GnRH binds its G-coupled protein receptor, GnRHR, on pituitary gonadotropes and stimulates transcription of *Cga*, *Lhb*, and *Fshb*. These three genes encode two heterodimeric glycoprotein hormones, LH and FSH, that act as gonadotropins by regulating gametogenesis and steroidogenesis in both the testes and ovary. GnRH also regulates transcription of *Gnrhr*. Thus, regulated expression of *Cga*, *Lhb, Fshb,* and *Gnrhr* provides a genomic signature unique to functional gonadotropes. Steadily increasing evidence now indicates that GnRH regulates transcription of its four signature genes indirectly through a hierarchical transcriptional network that includes distinct subclasses of DNA-binding proteins that comprise the immediate early gene (IEG) family. These IEGs, in turn, confer hormonal responsiveness to the four signature genes. Although the IEGs confer responsiveness to GnRH, they cannot act alone. Instead, additional DNA-binding proteins, including the orphan nuclear receptor steroidogenic factor 1, act permissively to allow the four signature genes to respond to GnRH-induced changes in IEG levels. Emerging new findings now indicate that β-catenin, a transcriptional co-activator and member of the canonical WNT signaling pathway, also plays an essential role in transducing the GnRH signal by interacting with multiple DNA-binding proteins in gonadotropes. Herein, we propose that these interactions with β-catenin define a multicomponent transcriptional network required for regulated expression of the four signature genes of the gonadotrope, *Cga*, *Lhb, Fshb,* and *Gnrhr*. (*Molecular Endocrinology* 22(6): 1295–1303, 2008)

A short history of the GnRH transcriptional network in gonadotropes

GnRH signals through several mitogen-activated protein kinase (MAPK) cascades to regulate transcription of at least 75 genes (1-18). Genes regulated by GnRH organize into a tiered hierarchy (primary, secondary, and tertiary) based on the kinetics of their response to GnRH with selected examples depicted in **Figure 1**. Culmination of the GnRH transcriptional signal results in regulated expression of four tertiary gonadotrope signature genes: *Cga*, *Lhb*, *Fshb* and *Gnrhr*.

Egr1, *Jun,* and *Atf3* are members of the immediate early genes (IEG) family (19). EGR1 belongs to the zinc-finger C2H2 subfamily of IEGs whereas JUN, and ATF3 are members of the basic-leucine zipper (b-ZIP) subfamily. The IEGs typify primary response genes because detectable changes in transcription occur within one hour of GnRH stimulation (13;14). As DNA-binding proteins, their accumulation transfers GnRH-responsiveness to both secondary and tertiary genes that contain the appropriate DNA response elements. For example, as EGR1 accumulates it then regulates transcription of secondary response genes such as mitogen-activated protein kinase phosphatase 2 (*Mkp2),* also referred to as dual specificity phosphatase 4 *(Dusp4),* (20) and ultimately *Lhb* (21-27) (**Fig 1**). EGR1 also regulates primary response genes like *Atf3* (28;29) and displays a positive auto regulatory loop with its own gene (30;31). Thus, EGR1 confers GnRH responsiveness to a number of genes that comprise the hierarchical transcriptional network in gonadotropes including one of the signature genes, *Lhb*.

GnRH also confers hormonal responsiveness to three (*Cga*, *Fshb*, and *Gnrhr*) of the four tertiary genes by acting through AP1 (3;4;32-36). AP1 heterodimers always contain a JUN subunit (37), and GnRH signals through JUN N-terminal kinase (JNK) to increase activity of AP1 (4;12;38). ATF3 also confers GnRH responsiveness to *Cga* by

forming a heterodimer with JUN and then binding to tandem cAMP response elements in the human promoter (4).

Expression of *Atf3* mRNA and activity of ATF3 protein is further enhanced by GnRH signaling through extracellular regulated kinase (ERK) and JNK (4). JUN, like EGR1, also establishes a positive auto regulatory loop with its own gene (39). In addition to these gene-gene relationships, the GnRH transcriptional signal also flows back to negatively regulate selected members of the MAPK cascades (20;40). For instance, GnRH stimulated increases in *Mkp2/Dusp4* culminates ultimately in inactivation of JNK and partial inactivation of ERK (40).

GnRH regulation of the four signature genes requires SF1

Response elements for the orphan nuclear receptor SF1 are found in the promoter regions of all four signature genes (32). SF1 plays a vital role in the GnRH transcriptional network (41-43) even though the orphan nuclear receptor appears refractory to regulation by the neurohormone (22;23;26;44;45). For instance, pulses of GnRH dynamically regulate transcription of mammalian *Lhb* genes (46-49). This dynamic regulation, however, represents a tertiary response to GnRH mediated by the more rapid induction of *Egr1* transcription and protein synthesis (13-16;22). Because levels of SF1 remain unresponsive to changes in GnRH, EGR1 is viewed as the primary determinant of hormone induced transcriptional fluxes of *Lhb* (22;23;26;44;45). Nevertheless, GnRH induction of EGR1 is not sufficient for ensuring that LH reaches the necessary levels required for physiological activity in transgenic mice with SF1-deficient gonadotropes (42;43). These mice are hypogonadal and fail to express detectable levels of *Cga, Lhb, Fshb,* and *Gnrhr* and are infertile (42;43). In contrast, conflicting phenotypes have been reported for EGR1 deficient males generated with different targeting constructs (50-52). LH levels are reduced whereas fertility is impaired in one

but not the other. While there are several possible explanations for this difference in fertility, lowered levels of LH and *Lhb* mRNA are consistent with the notion that SF1 acts permissively to render *Lhb* responsive to GnRH induced changes in EGR1. We predict that a permissive role of SF1 will extend to the three other signature genes that are regulated by GnRH induced changes in DNA binding proteins encoded by other IEGs.

A surprising link between SF1, β-catenin, and GnRH regulated gene expression in gonadotropes

Beta-catenin is a transcriptional co-activator typically associated with T cell factor (TCF)/lymphoid enhancer factor (LEF) responsive genes regulated by the WNT family of secreted glycoproteins (53;54). The binding of β-catenin to TCF/LEF causes release of histone deacetylase (HDAC) and co-repressors such as Groucho (officially GPRK2) and subsequent recruitment of additional co-activators and chromatin remodeling proteins like p300/CBP and BRG1, respectively (53-57). These collective interactions confer transcriptional competency to TCF/LEF (53-57).

Growing evidence indicates that β-catenin also co-activates a number of transcriptional proteins including IQGAP1 (58), PROP1 (55), PITX1 (59), AR (60;61), FOXO (62), members of the SOX family (63) and the bZIP proteins JUN and FOS (37). Additional reports indicate that β-catenin acts as a co-activator of SF1 when it transduces WNT signals to *Dax1* (officially *NR0B1*) and Inhibin α (officially *Inha*) (64;65). Co-activation of SF1 occurs through the binding of β-catenin to a cluster of amino acids (235–238) located in the first helix of the putative ligand-binding domain of the orphan nuclear receptor that also contains the activation function 1 domain (65;66). Beta–catenin, through an interaction with SF1, has also been shown to mediate FSH-stimulated increases in aromatase gene expression in granulosa cells (67). Together,

these reports suggest that β-catenin may serve as a required co-activator for many SF1-dependent genes.

Recently, we reported that GnRH regulation of *Lhb* gene expression in gonadotrope derived LβT2 cells requires a functional interaction between β-catenin and SF1 (44). Lines of evidence supporting this conclusion included the following: 1) reduction of β-catenin in LβT2 cells through overexpression of AXIN or through use of a pool of siRNA specific to β-catenin reduced GnRH-stimulated activity of an *LHB* promoter reporter construct; 2) overexpression of β-catenin increased the transactivation activity of SF1 and EGR1, as well as their functional interaction; 3) conversely, siRNA specific for β-catenin attenuated the activity of SF1 and EGR1 as well as their functional interaction; 4) GnRH increased accumulation of β-catenin and its physical association with SF1 when analyzed by co-immunoprecipitation; 5) an SF1 mutant lacking a β-catenin binding site acted in a dominant negative fashion and almost completely abolished the functional synergism normally exhibited between SF1 and EGR1 (22;26;27;45); and 6) GnRH enhanced the co-localization of β-catenin with the endogenous promoter region of the mouse *Lhb* gene that also binds SF1 and EGR1.

Together, the results enumerated above suggest that β-catenin serves as an essential co-activator of SF1 that renders the *Lhb* gene responsive to GnRH induced changes in EGR1 as modeled in **Figure 2**. In the absence of GnRH, we envision that the interaction between β-catenin and SF1, along with contributions from PITX1, another critical DNA-binding protein (22;68;69), maintains the *Lhb* gene in a poised state that has the potential to respond to GnRH. Levels of *Lhb* transcription in this poised state are low; analogous to the amount of light generated by a light dimmer when its controller is on but set to a low level. GnRH acts as a rheostat by increasing concentrations of EGR1 and thereby moving transcription from a poised to maximally active state of transcription. Since the GnRH stimulated increase in EGR1 is transient and dependent

on the concentration of the neurohormone that varies during pulsatile secretion (14;46;49;70), transcriptional activity of the *Lhb* gene must return to the poised state as the concentration of the zinc-finger IEG wanes.

As mentioned previously, expression of *Cga*, *Fshb* and *Gnrhr* also requires SF1 (42;43). Therefore, an important remaining question is whether these three gonadotrope genes also require a functional interaction between β-catenin and the orphan nuclear receptor. This question may be approachable through CRE-mediated recombination that targets deletion of β-catenin after embryonic specification of gonadotropes. If the transcriptional activity of SF1 requires its interaction with β-catenin *in vivo*; then mice harboring this deletion should lose expression of all four signature genes. As an alternative approach, mice that over express wild-type SF1 or a variant incapable of binding β-catenin should provide opposing phenotypes allowing further assessment of the physiological importance of the functional interaction between β-catenin and SF1.

GnRH regulation of TCF/LEF dependent genes in gonadotropes: Another potential site of β-catenin action

GnRH stimulates expression of several known TCF target genes including *Jun*, *Fra1*, and *Myc* (13;14;53;71;72). A recent report from Gardner and colleagues indicates that GnRH stimulated increases in the mRNAs encoded by these three genes in LβT2 cells is associated with increased nuclear accumulation of β-catenin and increased activity of TOPflash, an artificial TCF-dependent reporter construct (72). Together these data suggest that GnRH regulated expression of *Jun, Fra1, and Myc* requires a functional interaction between β-catenin and members of the TCF gene family. If so, then the JUN-responsive signature genes (*Cga*, *Fshb,* and *Gnrhr*) may be secondary targets of the GnRH-TCF pathway.

Clearly establishing the hierarchical placement of β-catenin and TCF will require additional experiments demonstrating that GnRH regulated expression of *Cga*, *Fshb,* and *Gnrhr* is secondary to β-catenin and TCF dependent regulation of *Jun* transcription. The likelihood of this possibility is reinforced by the observation that the promoter-regulatory region of *Jun* harbors response elements for TCF, JUN1 and JUN2 (71). *Jun* has also been identified as one of the most TCF/LEF responsive genes in hematopoietic and colon cancer cells (73;74). Within the context of the *Jun* promoter, β-catenin, JUN and TCF act cooperatively to stimulate transcription (71). These three proteins also bind to the *Jun* promoter when assayed using chromatin immunoprecipitation (71). Together these reports suggest that *Jun* may be a primary TCF/LEF gene target that is regulated by GnRH-stimulated changes in β-catenin. Pursuing this possibility is important as virtually all studies in gonadotropes reported to date have focused on signaling pathways that link GnRH to the terminal phosphorylation of JUN by JNK (4;8;18).

Who carries the GnRH signal to β-catenin?

Most GnRH signaling occurs through PKC which stimulates several MAPK cascades including JNK, ERK and p38 MAPK (1-12;17;18;49). Prior reports have linked SRC, CDC42 and MKK4/7 to neurohormonal regulation of JNK in gonadotrope-derived cell lines (6;8;18). GnRH induction of ERK is mediated by PKC and through MEK 1/2 because selective inhibition of this MAPK with either UO126 or PD98059 prevents ERK activation (3;17). Finally, GnRH activation of p38 MAPK proceeds through PKC and possibly MKK3/6 (2). A role for GnRH stimulated calcium influx through the plasma membrane for activation of ERK but not JNK has also been reported (18;75). These MAPK cascades activate many of the DNA-binding proteins that mediate the effect of GnRH on the primary, secondary, and tertiary response genes. As noted above, GnRH increases the nuclear accumulation of β-catenin (44;72) followed by augmented binding

of β-catenin and SF1 to the endogenous *Lhb* promoter-regulatory region in LβT2 cells (44). This begs the question of whether GnRH regulates accumulation of β-catenin through cross-talk that occurs between its G-protein coupled receptor (GPCR) pathway and the canonical WNT/β-catenin signaling pathway.

Transcriptional effects of β-catenin are usually linked to the canonical WNT/Frizzled/Dishevelled (Fz/Dvl) pathway that promotes inhibition of a multi-protein complex containing AXIN, adenomatous polyposis coli (APC), casein kinase I, and glycogen synthase kinase 3β (GSK3β) (53;54) (**shaded pathway**; **Fig. 3**). There is, however, emerging evidence indicating that multiple GPCR signaling pathways including those regulated by PKC (76), PKA (77;78) and PI3K (79) can "activate" β-catenin independent of WNT signaling (**Fig. 3**). Thus, PKC, PKA, and PI3K must be considered as candidates that mediate GnRH regulation of β-catenin in gonadotropes through cross-talk with the canonical WNT/β-catenin signaling pathway.

Recent evidence indicates that GnRH stimulation of the TOPflash reporter in LβT2 cells can be blocked by pretreatment with either antide, a competitive inhibitor of GnRH, or with a metabolic inhibitor of Gαq/11 (72). These results suggest that GnRH may regulate β-catenin via the PKC signaling pathway. This is consistent with another report indicating that PKC can enhance phosphorylation and thus inactivation of GSK3β (76). While, Gardner and colleagues were unable to observe GnRH-dependent changes in GSK3β phosphorylation in LβT2 cells (72), we have preliminary data indicating a strong correlation between GnRH induced increases in accumulation of β-catenin and S9 phosphorylation of GSK3β in LβT2 cells (unpublished data n=3), leaving open the possibility that GnRH may signal through the PKC pathway to inhibit activity of GSK3β. GnRH activation of calcium mediated single transduction through PKC is also a prominent signaling cascade activated by the neurohormone (18;48;75). Thus, a role for this signaling molecule for GnRH regulation of β-catenin may also exist in gonadotropes.

GSK3β can also be phosphorylated on S9 by AKT acting downstream of PI3K (76). As GnRH can activate both SRC and EGFR in αT3 cells (8;9;80;81), it is tempting to speculate these tyrosine kinases can lead to downstream activation of PI3K, phosphorylation and inactivation of GSK3β, and ultimately activation of β-catenin. However, there is little evidence indicating that GnRH activates PI3K in gonadotropes (82). In addition, metabolic inhibitors of PI3K fail to block GnRH-induced activity of a TOPflash reporter in LβT2 cells (72). Thus, if GnRH activation of SRC is involved in regulation of β-catenin, it may involve phosphorylation of GSK3β through CDC42 (**Fig. 3**). While a direct effect of GnRH on CDC42 activity has not been reported to date, the neurohormone does increase SRC activity which in turn has been shown to activate CDC42 in other cell systems (8;83). Moreover, overexpressed dominant negative CDC42 perturbs GnRH stimulated increases in JNK activity suggesting a functional role for this protein (8;18). This notion is further supported by the proposal that CDC42 regulation of GSK3β phosphorylation modifies cellular levels of β-catenin in astrocytes (84).

GnRH also signals through PKA in gonadotropes (85;86). In this regard, PKA-dependent phosphorylation of β-catenin on S675 and associated TOPflash activation has been reported in HEK293 and Cos-7 cells (77;78). PKA can also directly phosphorylate GSK3β on S9 leading to inactivation and subsequent stabilization of β-catenin (76). Thus, PKA provides another potential route that would allow GnRH to regulate β-catenin.

Finally, it is important to consider the possibility that GnRH may inhibit GSK3β through a mechanism independent of S9 phosphorylation (76). For instance, phenotypic analysis of homozygous knock-in mice suggests that WNT signaling is not compromised by mutation of S9 to A9 in GSK3β (87). One explanation is that GSK3β binding partners such as Frat can also regulate the activity of the kinase (76). For instance, Frat by

binding to GSK3β, perturbs the interaction between the kinase and AXIN which contributes to activation of β-catenin in WNT signaling (76). Alternatively, PGE2 stimulates activation of β-catenin in colon cancer cells through an interaction between Gαs and AXIN that occurs independently of cAMP and PKA (88). Thus, there are multiple signaling avenues and target sites whereby GnRH could regulate β-catenin, and expression of target genes such as *Jun*.

Putting the pieces back together

Beta-catenin has moved into the neighborhood of the GnRH transcriptional network. From all apparent appearances, β-catenin has established itself as a key player in enabling SF1 to act permissively in transducing the EGR1 signal from GnRH to *Lhb* in LβT2 cells (**Fig. 4**). Since *Cga*, *Fshb*, and *Gnrhr* also require permissive action of SF1 in responding to GnRH induced changes in AP1, we predict that the orphan nuclear receptor will require similar enabling from β-catenin. Like SF1, TCF also needs β-catenin to exert a positive transcriptional effect on its target genes. Given the known TCF responsiveness of *Jun* in colon cancer cells, this IEG is an odds on bet for serving as a natural GnRH-responsive target of TCF/β-catenin (**Fig. 4**). While it is tempting to speculate that *Atf3* may exhibit a similar dependency for TCF/β-catenin, it seems more likely that EGR1 will fill this need as *Atf3* expression is known to fall under the influence of this zinc-finger DNA-binding protein (**Fig. 4**). Clearly, there is a growing need for uncovering new members of the GnRH transcriptional network. Part will come from recognition of new genes that are responsive to SF1 or TCF and dependent on β-catenin. There is also a need to decipher just how GnRH signals to β-catenin and whether coupling pathways are few and restricted or broad and promiscuous. Whatever the outcome, the GnRH transcriptional network is an interesting neighborhood worthy of repeated visitation and exploration.

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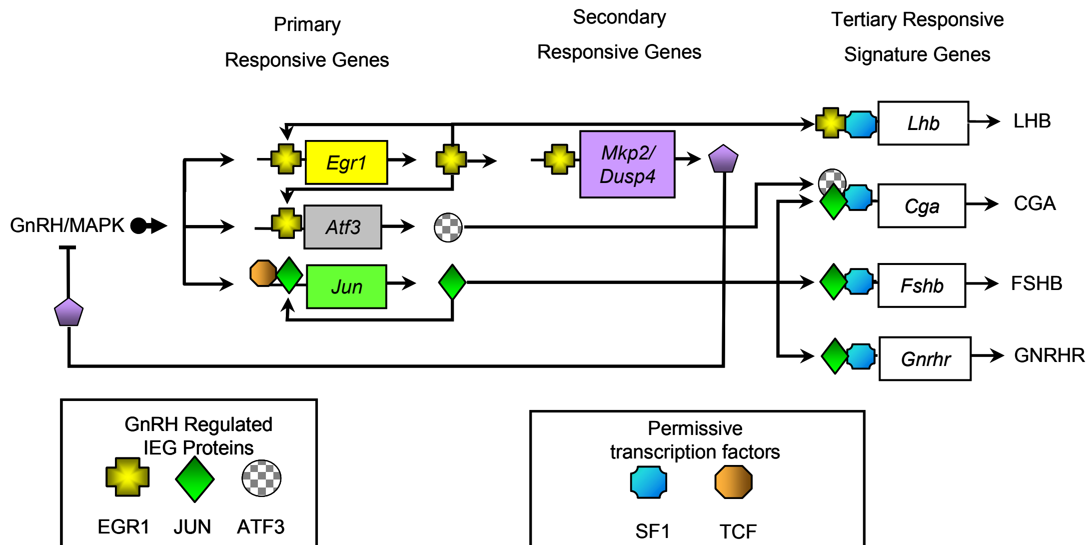

Figure 1: Selected overview of the GnRH transcriptional network in gonadotropes

GnRH activation of MAPK signaling cascades rapidly increases the transcription of several primary response genes including *Egr1*, *Atf3* and *Jun* (colored squares). These genes encode DNA-binding immediate early proteins (IEG; colored shapes) that confer GnRH responsiveness to secondary genes such as *Mkp2/Dusp4* and tertiary genes including *Lhb*, *Cga*, *Fshb*, and *Gnrhr*. The orphan nuclear receptor SF1 binds to the promoters of all four signature genes and acts permissively to render each gene responsive to GnRH. Recent evidence suggests that members of the TCF/LEF family of DNA binding proteins may also mediate the transcriptional effect of GnRH on select genes such as *Jun*.

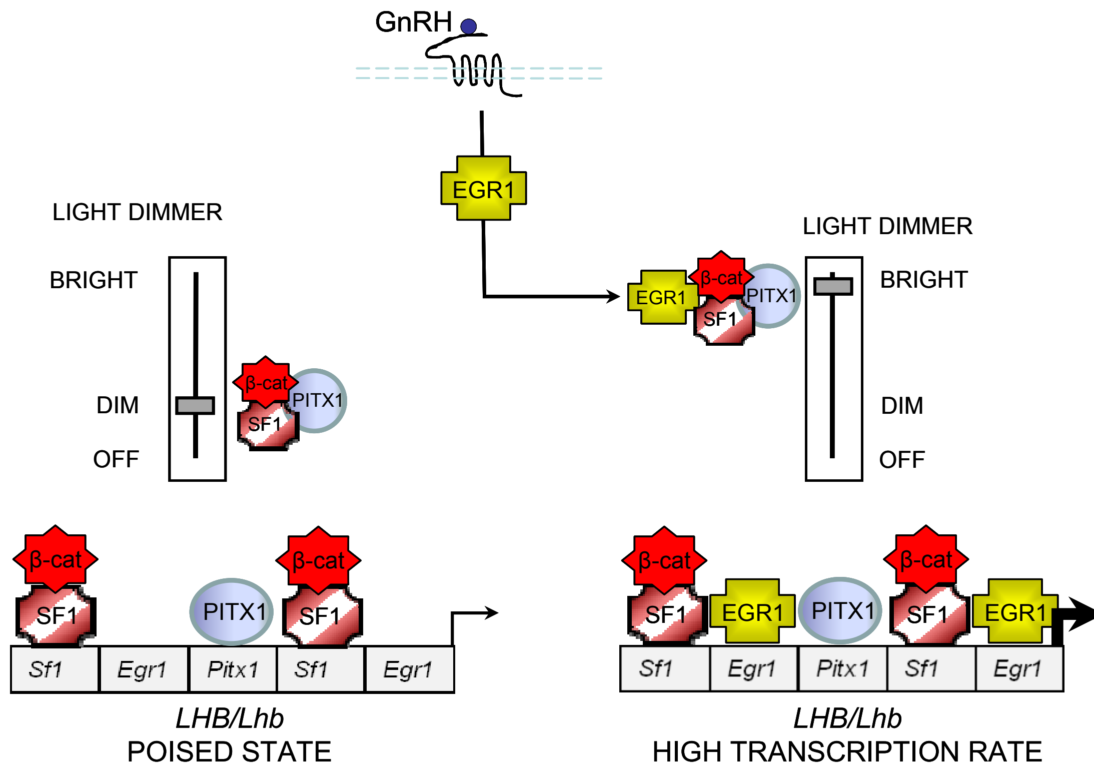

Figure 2: Contributions from β-catenin enable EGR1 to act as a rheostat that regulates expression of *LHB/Lhb*

Activity of SF1 requires binding of β-catenin. Contributions from this complex and other DNA-binding proteins such as PITX1 place the *LHB/Lhb* gene in a poised state with low transcriptional activity. As the concentration of EGR1 rises in response to the GnRH signal, transcriptional activity of the *LHB/Lhb* gene increases via synergistic interaction with β-catenin, SF1, and PITX1.

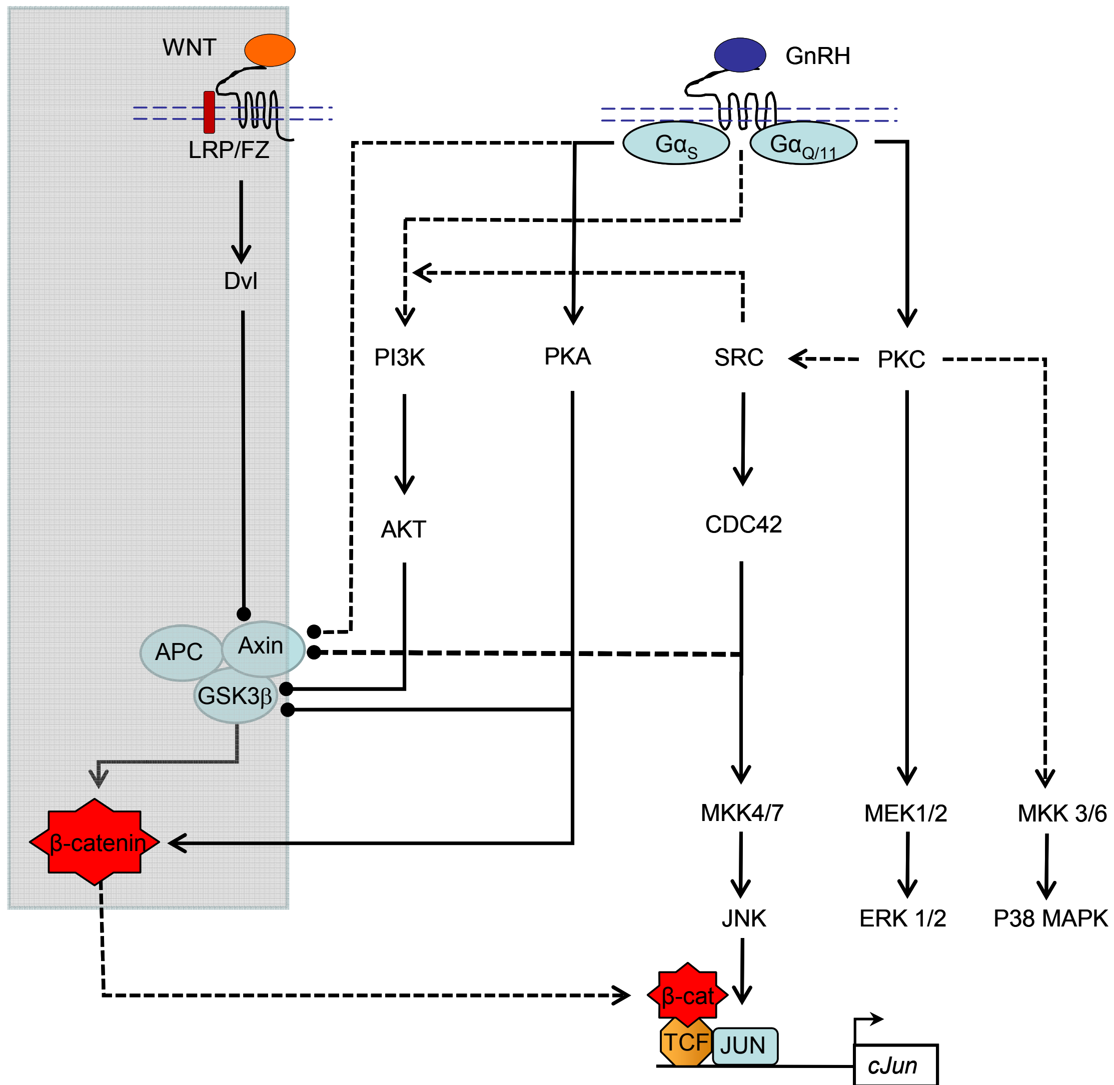

Figure 3: Potential signaling pathways for permitting crosstalk between WNT and GnRH

The shaded pathway represents the skeletal features of the canonical Wnt/β-catenin signal transduction pathway. Essential elements of known GnRH signaling cascades are depicted to the right of the shaded area. Solid arrows depict pathways where direct links have been established between known downstream components. Dotted arrows depict pathways where details that link upstream with downstream components remain incompletely characterized. We use the *Jun* promoter regulatory region to illustrate how GnRH signals through JNK and potentially through β-catenin to regulate activity of a TCF dependent promoter.

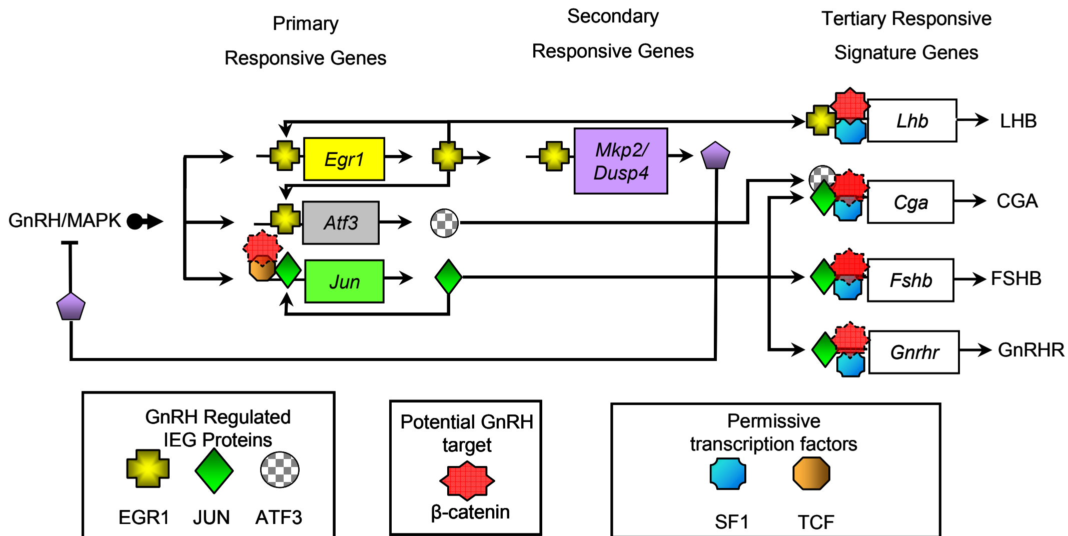

Figure 4: Moving β-catenin into the GnRH transcriptional network

Beta-catenin enables SF1 to act permissively in transducing an EGR1 signal from GnRH to *Lhb* in gonadotropes. Because *Cga*, *Fshb* and *Gnrhr* also require SF1 for GnRH responsiveness we predict that regulated expression of these tertiary response genes will also require β-catenin. Transcriptional activity of *Jun* is highly sensitive to TCF/β-catenin interactions in cancer cells. Thus, GnRH-responsiveness of *Jun* in gonadotropes will most likely depend on an interaction between TCF and β-catenin.

CHAPTER THREE

MAXIMAL ACTIVITY OF THE LHB PROMOTER REQUIRES β-CATENIN

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Abstract

GnRH regulates expression of *LHB* via transcriptional regulation of *EGR1*, an immediate early gene that encodes a zinc-finger DNA-binding protein. EGR1 interacts functionally with the orphan nuclear receptor SF1 and PITX1, a member of the paired-like homeodomain family. The functional synergism of this tripartite interaction defines the maximal level of *LHB* transcription that can occur in response to GnRH. Results presented herein provide new evidence that the interaction between SF1 and EGR1 also requires β-catenin, a transcriptional co-activator and member of the canonical WNT signaling pathway**.** For instance, targeted reduction of β-catenin attenuates activity of a GnRH-primed *LHB* promoter. Additional gene reporter assays indicate that overexpression of β-catenin, or its targeted reduction by siRNA, modulates activity of both SF1 and EGR1 as well as their functional synergism. Beta-catenin co-immunoprecipitates with SF1. Moreover, an SF1 mutant that lacks a β-catenin binding domain has compromised transcriptional activity and fails to interact synergistically with EGR1. Finally, GnRH promotes β-catenin co-localization with SF1 and EGR1 on the endogenous mouse *Lhb* promoter-regulatory region. Taken together, these data suggest that β-catenin binds to SF1 and that this interaction is required for subsequent functional interaction with EGR1. Thus, these data identify β-catenin as a new and required member of the basal transcriptional complex that allows the *LHB* promoter to achieve maximal activity in response to GnRH.

INTRODUCTION

Reproductive function and fertility require appropriate synthesis and release of luteinizing hormone (LH) (1-3). Appropriate level of LH is achieved through highly interactive feedback loops that regulate secretion of the hormone as well as transcription of the two genes that encode its subunits: common glycoprotein α (*CGA/Cga*) and luteinizing hormone β (*LHB*/*Lhb*). Secretion of LH occurs in pulses that are driven by hypothalamic secretion of gonadotropin releasing hormone (GnRH) and counterbalanced by gonadal steroids (4). GnRH and steroids also regulate transcription of both genes (5-8). Herein, we focus on a complex set of interactions that are required for maximal transcription of *LHB/Lhb* in the presence of GnRH.

Regulated *LHB/Lhb* gene expression requires combinations of regulatory elements that cluster in the proximal and distal regions of the promoter (8). The proximal domain is conserved across all mammals and contains a single central element that binds PITX1 (8-10). The single PITX1 element is flanked by pairs of elements that bind SF1 and EGR1 (11-16). Gonadotrope-specific expression of *LHB*/*Lhb* genes also occurs through contributions provided by a distal regulatory domain. This domain displays species-specific variation and includes arrays of elements ranging from tandem binding sites for NFY (17) to a tripartite composite element that contains two SP1 binding sites flanking a single CArG response element (18;19).

GnRH stimulates *LHB/Lhb* gene expression via transcriptional regulation of *EGR1* (11;20-23). The transcriptional contribution provided by GnRH induction of EGR1 is further amplified by subsequent functional interactions with SF1 and PITX1 (10-13;16;21;24). Indeed, physical interaction between these three transcription factors underlies their synergistic action (10;11).

In contrast to EGR1, levels of SF1 and PITX1 remain unaffected by GnRH (11;21). Consequently, contributions from SF1 and PITX1 set the basal transcriptional tone of the *LHB* promoter as well as serve as key amplifiers of GnRH signaling through their functional synergism with EGR1 (8;11;13;21;24).

While it is clear that EGR1 is a primary downstream target of GnRH (20), it alone is insufficient to allow for adequate transcription of the *LHB/Lhb* gene. This is best illustrated by recent studies with mice that harbor gonadotrope-specific, CRE-mediated deletion of *SF1* (25;26). Mice lacking SF1 in gonadotropes of the pituitary were hypogonadal with undetectable levels of *Lhb* and *Fshb* mRNA when assayed by PCR (25;26). Hence, SF1 plays an essential role in maximizing the transcriptional signal provided by GnRH induced EGR1.

In this study, we asked whether the synergistic interplay between SF1 and EGR1 requires a co-activator. The impetus for this line of investigation stems from two reports showing that β-catenin acts as a co-activator of SF1 when it transduces WNT signals to *Dax1* (officially *NR0B1*) and Inhibin α (officially *Inha*) promoters (27;28). Co-activation occurs through the binding of β-catenin to a cluster of amino acids (235-238) located in the first helix of the putative ligand binding domain of SF1 that also contains the activation function 1 (AF1) domain (28). Herein we report a new role for β-catenin in regulation of *LHB/Lhb* gene expression in gonadotropes.

RESULTS

Maximal transcriptional response of the *LHB* promoter to GnRH requires β-catenin

GnRH induction of EGR1 maximizes expression of the endogenous *Lhb* gene and *LHB*/*Lhb* reporter constructs in the LβT2 gonadotrope cell line (11;13;21). Accordingly, to assess the requirement for β-catenin, we targeted its reduction in LβT2 cells treated with either vehicle or GnRH via two different strategies and then assayed activity of a co-transfected *LHB* reporter construct.

The first strategy involved overexpression of AXIN. This protein promotes degradation of cellular β-catenin levels (29-31). Consequently, we co-transfected LβT2 cells with *LHB* (*LHB*-luciferase) and phRG-B-renilla reporter constructs along with a *CMV-Axin* expression vector. Controls included cells treated with vehicle instead of GnRH and cells co-transfected with an empty expression vector. Post transfection, LβT2 cells were treated for 24 hours with either vehicle or 10 nM GnRH.

As expected, activity of the *LHB* reporter is marginal in the absence of GnRH (Fig. 1A, lane 1) making detection of an AXIN effect on basal activity of the reporter problematic (Fig. 1A, lane 2). In contrast, AXIN strongly suppressed *LHB* promoter activity in LβT2 cells treated with GnRH ($p<0.01$, Fig. 1A, compare lanes 3 and 4). Although it is clear that overexpression of AXIN prevents the *LHB* promoter from achieving maximal activity after treatment with GnRH, we were unable to confirm reduction of β-catenin in these transient expression assays. Nevertheless, this outcome is consistent with the possibility that changes in β-catenin levels modulate activity of the *LHB* promoter.

To more directly examine a role for β-catenin in GnRH regulation of *LHB* promoter activity, we used a pool of four different siRNAs specific for β-catenin (Dharmacon SMARTpool) to reduce its levels in LβT2 cells. This experiment involved a

different transfection paradigm than that used in the AXIN overexpression studies. Pools of siRNA were introduced by reverse transfection (32) along with the *LHB*-luciferase construct over a 72 hour period. Cells were then treated with 10 nM GnRH for 24 hours and assayed for luciferase activity. To control for specificity, LβT2 cells were also reverse transfected with a pool of non-specific siRNA (Dharmacon).

In this reverse transfection paradigm, the siRNA specific for β-catenin (siRNAβ) reduced basal activity of the *LHB* reporter by approximately 50% when compared to cells treated with the control siRNA (Fig. 1B, compare lanes 1 and 2). This reduction in the absence of GnRH suggests that β-catenin contributes to basal promoter activity presumably through an interaction with one or several of the DNA-binding proteins that populate this region.

The pool of siRNA specific for β-catenin (βRNAi) also significantly attenuated the activity of *LHB* reporter in the presence of GnRH ($p<0.01$, Fig. 1B, compare lanes 3 and 4). The extent of the reduction in the presence of GnRH was 67%, slightly greater than that observed in the absence of GnRH. While it is tempting to suggest that β-catenin also alters GnRH responsiveness, it is difficult to make this conclusion based on a single time point. It is clear, however, that treatment with the β-catenin-specific siRNA prevents the *LHB* reporter from achieving maximal activity when exposed to GnRH.

Levels of total cellular β-catenin were also examined by immunoblot (Fig. 1C). Specific siRNA-mediated reduction of β-catenin was not detected in LβT2 cells treated with vehicle (Fig. 1C, lane 2 versus lane 1). In contrast, specific siRNA reduction of β-catenin was readily detected in LβT2 cells treated with GnRH. The reason for this apparent difference in siRNA efficiency remains unclear but could reflect specific effects of GnRH that facilitate one or several members of the siRNA pathway. Nevertheless, the siRNA-mediated reduction of β-catenin observed in the presence of GnRH, coupled

with the loss of *LHB*-reporter-activity, complements the results obtained with the AXIN paradigm. Together, these results suggest that β-catenin may be an essential determinant for establishing the basal transcriptional tone of the *LHB* promoter.

Beta-catenin enhances activity of SF1 and EGR1

Beta-catenin functions as a co-activator for DNA bound transcription factors in the canonical WNT signaling pathway (33). Therefore, it seemed likely that β-catenin could contribute to *LHB* promoter activity by interacting with any one of the primary transcriptional components of the proximal regulatory region (8). In LβT2 cells, overexpression of either EGR1 or SF1 increases activity of a *LHB* reporter (see below). In contrast, overexpression of PITX1 has no effect (data not shown). Consequently, we focused on examining whether β-catenin modulates activity of either SF1 or EGR1.

Transactivation properties of β-catenin, SF1, and EGR1 were assessed by transient co-transfection assays in LβT2 cells maintained in the absence of GnRH (Fig. 2). In this state, levels of EGR1 are extremely low and the endogenous *Lhb* gene is nearly silent (11). Since EGR1 is induced by GnRH, overexpression of EGR1 acts as surrogate of the neurohormone. In contrast to EGR1, levels and apparent activity of SF1 are unaffected by GnRH (11). Thus, effects of transfected SF1 depend on whether the levels of endogenous orphan receptor are limiting with respect to activity of the *LHB* promoter. As LβT2 cells contain ample β-catenin, detecting changes conferred by transfected β-catenin also depend on whether endogenous levels of the protein are limiting or in excess with respect to the co-transfected *LHB* promoter.

Overexpression of a constitutively active form of β-catenin (Δ90; (34)) only marginally increased activity of the *LHB* reporter (Fig. 2, lanes 1 and 2). This result would be expected if endogenous levels of β-catenin are close to that required for a

maximal transcriptional contribution. In contrast, overexpression of either SF1 or EGR1 increased activity of the *LHB* promoter ($p<0.01$, Fig. 2. lanes 3 and 5) indicating that their endogenous levels are limiting. Interestingly, ∆90 β-catenin significantly enhanced the transcriptional effects of both SF1 ($p<0.01$, Fig. 2, lanes 3 and 4) and EGR1 ($p<0.01$, Fig. 2, lanes 5 and 6). This result suggests that endogenous levels of β-catenin became limiting with the overexpression of either SF1 or EGR1. Together, these data underscore the notion that β-catenin acts as co-activator for either one or both of these DNA-binding proteins.

Functional synergism between EGR1 and SF1 also requires β-catenin

Our finding that β-catenin enhances the transcriptional effects of either EGR1 and SF1 suggests that the co-activator may also be required for the functional synergism that occurs when both factors are bound to the proximal *LHB* promoter. Thus, we explored the importance of β-catenin in modulating the synergistic interplay between SF1 and EGR1 by employing an siRNA strategy similar to that described earlier (Fig. 1).

LβT2 cells maintained in the absence of GnRH were co-transfected with either pooled control or β-catenin specific siRNA and with expression vectors encoding either SF1 or EGR1 or with both expression vectors. Transactivation of the *LHB* reporter by SF1 was significantly reduced when cells were transfected with siRNA specific for β-catenin ($p<0.01$, Fig. 3, lanes 3 and 4). EGR1 activity was also reduced in the presence of siRNA specific for β-catenin relative to cells co-transfected with EGR1 and control siRNA ($p<0.01$, Fig. 3, lanes 5 and 6). Most importantly, treatment with the β-catenin-specific siRNA significantly attenuated the functional synergism normally observed upon co-transfection of vectors encoding both SF1 and EGR1 ($p<0.01$, Fig. 3, lanes 7 and 8).

Together, these data suggest that the transcriptional synergism conferred by EGR1 and SF1 requires endogenous β-catenin.

β-catenin acts through SF1

While the transfection studies described in Figures 1-3 indicate that maximal activity of the *LHB* promoter requires β-catenin, they also suggest that both SF1 and EGR1 are potential functional targets of the co-activator. There are, however, steadily emerging reports indicating that SF1 and other steroid receptors bind β-catenin (28;35;36). In contrast, there are no reports to date indicating that β-catenin binds to EGR1. This led to the consideration that β-catenin contributions to EGR1 activity may be secondary to a primary interaction that occurs between β-catenin and SF1. We explored this possibility by determining whether β-catenin could physically interact with SF1 and whether removal of the known β-catenin binding site in SF1 would abrogate activity of the orphan receptor.

Physical interaction between SF1 and β-catenin was explored by co-immunoprecipitation assays. Nuclear extracts were prepared from LβT2 cells treated with either vehicle or GnRH for 60 minutes and then subject to immunoprecipitation with an antibody specific to β-catenin or control IgG. Immunoprecipitates were then subjected to immunoblot analysis employing antibodies specific for either β-catenin or SF1. In the absence of GnRH, only a marginal association between β-catenin and SF1 was observed (Fig. 4 lane 3). In contrast, GnRH treatment revealed a clear interaction between SF1 and β-catenin (Fig. 4, lane 6). Concomitant immunoblot analysis of whole cell lysates also indicated that GnRH treatment increased the accumulation of β-catenin (approximately two-fold, n=3, $P < 0.05$, data not shown).

Co-immunoprecipitation was also performed with an antibody specific for EGR1. While EGR1 was also found in a complex containing β-catenin when cells were treated with GnRH (data not shown), this would be expected given the known physical interaction between SF1 and EGR1 (11). Consequently, these co-immunprecipitation studies indicate the likelihood that SF1, EGR1, and β-catenin are found together in the same complex.

Nuclear receptors, including SF1, have been reported to interact with β-catenin (27;28;35;36). Indeed, Mizusaki and colleagues demonstrated that site-specific replacement with four alanines at amino acids 235-8 in SF1 (SF1 235-4A) prevents its physical interaction with β-catenin when tested by GST-pull down assays (28). This mutation also attenuates the transactivation function of the orphan receptor (28). As a consequence, we examined the impact of this mutant on activity of the *LHB* reporter in co-transfection assays that also employed vectors encoding SF1 and EGR1.

As observed earlier (Figs. 2 and 3), overexpression of SF1 stimulated *LHB* promoter activity relative to control transfected cells ($p<0.05$, Fig. 5, lanes 1 and 2). Transfected EGR1 also enhanced the activity of the *LHB* reporter vector ($p<0.01$, Fig. 5, lanes 1 and 4). Co-expression of EGR1 and SF1 resulted in maximal stimulation of *LHB* promoter activity ($p<0.01$, Fig. 5, lane 5). In contrast, SF1 235-4A virtually eliminated the functionally synergistic interaction between SF1 and EGR1 ($p<0.01$, Fig. 5, lanes 5 and 6). In essence, the SF1 mutant acts in a dominant negative fashion. This result is consistent with the known ability of SF1 235-4A to retain DNA-binding activity and limited functional activity when transfected at high concentrations (28). Thus, even if EGR1 interacts directly with β-catenin, this interaction cannot compensate for the absence of a β-catenin domain in SF1. Consequently, the dominant negative effect of SF1 235-4A

reinforces the notion that the synergistic interaction between SF1 and EGR1 requires a specific interaction between β-catenin and the orphan nuclear receptor.

GnRH enhances the association of β-catenin with the proximal region of the endogenous *Lhb* promoter

If β-catenin is required for maximal expression of the endogenous *Lhb* gene in LβT2 cells, then it should co-localize to the proximal promoter region. We examined this possibility by performing chromatin immunoprecipitation (ChIP) assays. Immunoprecipitated chromatin was subject to PCR using two primer sets. One primer set (Fig. 6) flanks a genomic fragment of the *Lhb* promoter containing response elements for EGR1, SF1 and PITX1. The other primer set flanks a 5' distal region of the mouse *Lhb* promoter and serves as a control.

Modest amounts β-catenin (Fig. 6, lane 4) and EGR1 (Fig. 6, lane 10) were associated with chromatin amplified by the primer set specific for the proximal promoter in the absence of a GnRH stimulus. By 60 minutes, GnRH increased the quantity of β-catenin ($p<0.05$, 2.74-fold ± 0.6 SEM, Fig. 6, lane 6) and SF1 ($p<0.05$, 2.8-fold ± 0.48 SEM, Fig. 6, lane 18) associated with the proximal region of the *Lhb* promoter. Consistent with prior reports, GnRH also increased the quantity of EGR1 ($p<0.05$, 3.86-fold ± 0.7 SEM, Fig. 6 lane 12) associated with the proximal region of the *Lhb* promoter (24). The binding of these three proteins to this region of the promoter was specific because they were not present on chromatin located ~3kb upstream (distal primers) from the transcriptional start site of the *Lhb* gene (Fig. 6, lanes 1-3, 7-9, and 13-15), In addition, normal rabbit IgG failed to precipitate chromatin (Fig. 6, lanes 4-6, 10-12, and lanes 16-18). Collectively, these data indicate that β-catenin co-localizes with EGR1

and SF1 on the endogenous *Lhb* promoter and that GnRH enhances their co-localization.

DISCUSSION

Pulses of GnRH dynamically regulate transcription of mammalian *LHB/Lhb* genes (37-39). This dynamic regulation, however, represents a secondary response to GnRH mediated by the more rapid induction of *EGR1* transcription and protein synthesis (11;20-23). Indeed, GnRH controls activity of the *EGR1* promoter-regulatory region through a protein kinase C/extracellular signal-regulated kinase pathway (11;20;22). Because levels of SF1 and PITX1 remain unresponsive to changes in GnRH (11;21), EGR1 is viewed as the primary determinant of hormone induced *LHB/Lhb* transcriptional fluxes. Nevertheless, as noted earlier, GnRH induction of EGR1 is not sufficient for ensuring that LH reaches the necessary levels required for physiological activity in transgenic mice with gonadotropes deficient in SF1 (25;26). Accordingly, SF1 has emerged as a pivotal synergistic partner of EGR1 that amplifies the response of *LHB/Lhb* genes to GnRH (13;16;21). We have now presented several lines of evidence indicating that activity of SF1 and its subsequent synergistic interaction with EGR1 requires β-catenin. This establishes a new and essential role for β-catenin; it is a critical co-activator of SF1 that maximizes transcription of *LHB/Lhb* genes in response to GnRH induced changes in EGR1.

We identified β-catenin as a co-activator of SF1 based on the following observations. Reduction of β-catenin in LβT2 cells through overexpression of AXIN or through use of a pool of siRNA specific to β-catenin reduced transcription of an *LHB* reporter construct to GnRH (Fig. 1). Although overexpression of β-catenin increased the transactivation activity of SF1 and EGR1, as well as their functional synergism (Fig. 2), we suspect that the response of EGR1 was secondary to the effect of β-catenin on endogenous SF1. In a reciprocal fashion, siRNA specific for β-catenin attenuated

activity of SF1 and EGR1 as well as their functional interaction (Fig. 3). GnRH increases accumulation of β-catenin (data not shown) and immunoprecipitation studies indicated that endogenous SF1, EGR1 and β-catenin physically associate in LβT2 cells (Fig. 4 and data not shown). Since the β-catenin binding pocket of SF1 is required for transactivation activity of the orphan receptor (Fig. 5), we suspect that the association of EGR1 is secondary to a primary interaction that occurs between SF1 and β-catenin. Although we cannot rule out the possibility that β-catenin also interacts with EGR1, this seems unlikely given the observation that the SF1 mutant lacking a β-catenin binding site acts in a dominant negative fashion and almost completely abolished the functional synergism normally exhibited between SF1 and EGR1 (Fig. 5). Finally, GnRH enhanced co-localization of β-catenin to the endogenous promoter region of the mouse *Lhb* gene that also binds SF1 and EGR1 (Fig.6). Together, these results support the notion that β-catenin is required for activity of SF1 and its subsequent functional synergism with EGR1.

The supposition that SF1 requires co-activation to contribute to GnRH regulation of the *Lhb* promoter was first proposed by Kaiser and colleagues to explain the concerted interaction between SP1, SF1, and EGR1 (13). Although unidentified, they proposed that a putative co-factor would interact with all three DNA-binding proteins and then bridge them to the core transcription complex. While such a co-factor may exist, our findings suggest a narrower and more specific role for β-catenin, namely in promoting a specific interaction with SF1 that unmasks its transactivation potential thereby permitting synergistic interaction with EGR1.

Although we emphasize the functional interaction between β-catenin and SF1 in transducing the GnRH transcriptional signal in gonadotropes, there are numerous other targets of the co-activator that play important roles in pituitary development and cell-type

specification. For example, β-catenin acts as co-factor for TCF/LEF transcription factors in fetal pituitaries that regulate expression of *Pitx2* (40). β-catenin has also recently been shown to act as a co-factor for Prop1 in regulating expression of *Pit1* and *Hesx1* that are required for specification of somatotropes, thyrotropes, and lactotropes in embryonic pituitaries (41). Additionally, transcripts for *Lhb* are substantially reduced in fetal mouse pituitaries that harbor targeted disruption of their β-catenin alleles (41). Together these data suggest that β-catenin can also act as an essential co-factor for multiple transcription factors that control pituitary cell-type specification.

While our study highlights the role for β-catenin as a required component of the GnRH transcriptional signal, it is likely that this co-activator facilitates the transcriptional effect of other signaling cascades initiated by peptide hormones binding to G protein coupled receptors (GPCRs). For instance, we have recently reported that follicle-stimulating hormone (FSH) regulation of aromatase (*Cyp19a1*) transcription in granulosa cells also requires a selective interaction between β-catenin and SF1 (42). GnRH is generally viewed to activate *Lhb* expression primarily via Gq/11 (43). In contrast, FSH signals via Gs (44). Consequently, the transcriptional effect of β-catenin may not be limited to specific classes of GPCRs. Moreover, the presence of GPCRs/β-catenin/SF1 transcriptional programs in pituitary and ovary suggests a broad and previously underappreciated role for this co-factor in the hypothalamic-pituitary-gonadal axis.

In summary, normal gonadal function requires continuous pulsatile secretion of both GnRH and LH (2;4;6). Sustained pulsatile secretion of LH exerts a transcriptional demand on the genes that encodes its subunits (37-39). Our results have exposed a new and critical role for β-catenin where it functions as a co-activator of SF1 rendering the orphan receptor capable of functionally synergizing with GnRH-induced EGR1. The synergistic interdependency of β-catenin, SF1 and EGR1 provides a means of

amplifying the transcriptional response of *LHB/Lhb* genes to GnRH to ensure that sufficient amounts of LH are available for sustained pulsatile secretion, a requirement for fertility in both males and females.

MATERIALS AND METHODS

Chemicals

GnRH, PMSF, Triton X-100, Igepal CA-300, salmon sperm, sodium deoxycholate, and Nonidet P-40 were purchased from Sigma Chemical Co.; SDS was from BioRad Laboratories; formaldehyde was from Fisher Scientific; glycine was from MP Biomedics.

DNA constructs and siRNA

The -779 bp bovine *LHB* reporter construct has been described (9;17;45). The *Egr1* expression vector (CMVNGF1A) was kindly provided by Dr. Jeffery Milbrandt (Washington University Medical School, St. Louis, MO) (46); the murine *Axin1* in a pcS2+MT expression vector was generously provided by Dr. Frank Costantini (Columbia University, New York, NY, (30)); and the murine ∆ 90 β-catenin in the pUHD10-3 vector was provided by Dr. James Nelson (Stanford University of Medicine, Palo Alto, CA) (34). The pCMX-*SF-1* and *SF-1* 235-4A expression vectors were kindly provided by Dr. Ken-Ichirou Moroshashi (University of Tsukuba, Japan) (28). Plasmids encoding N terminally myc tagged *Sf1* or *Sf1* 235-4A were prepared by digesting pCMX-*Sf1* or *Sf1* 235-4A with EcoRI (Invitrogen) and ligated into the EcoRI site of pCMVTag3B (Stratagene). Smart pools of interfering RNA for *Catnb* and non-targeting interfering RNA were obtained from Dharmacon (Lafayette, CO).

Cell Culture and Transient Transfections

LβT2 cells were maintained at 37 C with 5% CO_2 in high-glucose DMEM supplemented with 10 % fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen Life Technologies). Prior to transfection, LβT2 cells were plated (24 well

plates, 250,000/well) and maintained in DMEM supplemented with 10% FBS. After 24 hours, cells were washed with phosphate buffered saline (PBS) (Invitrogen Life Technologies) and a transfection cocktail containing DNA, lipofectamine (2µl/well; Invitrogen Life Technologies) and DMEM was added. Twelve-16 hours later an equal volume of DMEM supplemented with 20% FBS was added. Twenty-four hours later cells were assayed for reporter gene activity (Dual Luciferase Reporter assay kit; Promega Corporation). The amount of DNA was maintained constant by adding an empty vector containing the cytomegalovirus (CMV) promoter.

For siRNA experiments with GnRH, a suspension of 250,000 LβT2 cells/per well was transfected with lipofectamine (2µl/well), siRNA, and DNA in DMEM, and cells were allowed to attach to a 24-well plate. Six hours post transfection, DMEM containing 20% FBS was added to each transfection well. Seventy-two hours post transfection cells were treated with either vehicle or 10 nM GnRH for 24 hours and reporter activity was measured.

To examine the activity of EGR1 and SF1 in the presence of siRNA, pre-plated LβT2 cells (250,000 cells per well in a 24 well plate) were transfected (lipofectamine 2µl/well) with DNA and siRNA and reporter activity was assayed 72 hours post transfection.

Immunoprecipitation and immunoblot analysis

For immunoprecipitation experiments, LβT2 cells were plated into 150 mm plates in complete media. Once cells were 70% confluent they were dosed with either GnRH (10 nM) or vehicle (PBS) in complete media. Sixty minutes post GnRH or vehicle, cells were washed with cold PBS and crude nuclear lysate was isolated as described by Gummow et al. 2003 (27). Nuclear extracts (1 mg) were then incubated with 15 µg of agarose-conjugated IgG (sc-2346) or β-catenin beads (sc-1496 AC) (Santa Cruz

Biotechnology) overnight at 4°C while rotating. Protein complexes where collected following a brief centrifugation and washed 3 times with PBS. Complexes were boiled in sample buffer and assayed by immunoblot.

For immunoblot analysis of siRNA mediated knockdown of β-catenin 4 μg of transfection lysate was subjected to SDS-PAGE and transferred to PVDF membrane (BioRad Laboratories). Membranes were then blocked for 1 hour at room temperature in Tris- buffered saline solution containing 0.05% Tween 20 (TBST) and 5 % nonfat-dry milk. Membranes were then probed with either anti-β-catenin (BD Transduction laboratories, cat # 610153) antibody diluted 1:5,000 or anti-AKT (Cell Signaling Technology) antibodies diluted 1:2,000 in TBST containing 5% nonfat-dry milk for either 1 hour at room temperature or overnight at 4° C. Membranes were then rinsed twice for 5 minutes each in TBST and then incubated for 1 hour at room temperature with either an anti-mouse or an anti-rabbit IgG secondary antibody conjugated to a horseradish peroxidase enzyme (Amersham Pharmacia Biotech) diluted 1:40,000. The membrane was then rinsed 3 times in TBST and subjected to ECL (Amersham Pharmacia Biotech).

Immunoblot analysis of IP reactions were performed in a similar fashion, except that membranes were probed with either anti-SF1 (Upstate Biotechnology/Millipore) diluted 1:5,000 or an anti β-catenin (H-102; Santa Cruz Biotechnology) diluted 1:5,000 in TBST containing 5% non-fat dry milk overnight at 4° C.

Chromatin immunoprecipitation assays

Ten million LβT2 cells were plated into 100 mm plates for 24 hours, treated with vehicle or 10 nM GnRH as indicated above, and then cross-linked with formaldehyde at a final concentration of 1% for 10 minutes at room temperature. Glycine was then added to a final concentration of 125 mM. After 5 minutes cells were scraped and centrifuged at 400Xg for 5 minutes at 4 C. Cell pellets were lysed with 1 ml of SDS lysis buffer (1%

SDS, 10mM EDTA, 50 mM pH 8 Tris-HCL) plus protease inhibitors (Complete mini Tabs; Roche Diagnostics) on ice for 10 minutes and sonicated (DNA length of approximately 500 kb). One hundred-fifty µl of chromatin per immunoprecipitation was diluted 1:10 in dilution buffer (16.7 mM Tris-HCL, pH 8, 167 mM NaCL, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100,) and incubated with 5 µg of anti-Egr-1 (C-19/C-588; Santa Cruz Biotechnology), anti-β-catenin (H102; Santa Cruz Biotechnology), anti-SF1 (Upstate Biotechnology/Millipore), or control rabbit IgG (Santa Cruz Biotechnology) by rotation at 4 C overnight with 60 µl of Protein G Agarose (Upstate Biotechnology/Millipore). Protein G complexes were collected by centrifugation and beads were rinsed 1-3 times with buffer A (20 mM Tris-HCl, pH 8, 150 mM NaCl, 2.0 mM EDTA, 0.1% SDS, 1.0% Triton X-100) and once with buffer B (same as buffer A except without 500 mM NaCl). Complexes were eluted with 450 µl of elution buffer (0.1 M $NaHCO_3$, 1% SDS) for 30 minutes. Resulting supernatants were collected and NaCl (final concentration of 0.3M) and proteinase K (20 µg/ml) were added to eluates and incubated at 65 C for 5 hours. DNA was isolated using phenol chloroform extraction and resuspended in 30 µL of H_2O. One µL of DNA was subjected to 30 cycles of standard PCR using primers designed to amplify a distal fragment 5’ cctccttggtgttggagaaa 3’ and 5’ gagagtgggaggtggctaga 3’ and a proximal fragment 5’ tcaccttctccttgggtgtc 3’ and 5’ gtcctcccctgctgtgttta 3’ of the *Lhb* gene.

Statistics

Reporter activity was analyzed by one way ANOVA and differences among treatments were determined with the Newmans-Keuls multiple comparison test (Figs. 2, 4 and 5). Reporter activity was analyzed by two-tailed Student *t* tests in Figs. 1 and 3. Chromatin immunoprecipitation assays in Fig. 6 were analyzed by a one-paired Students *t* test.

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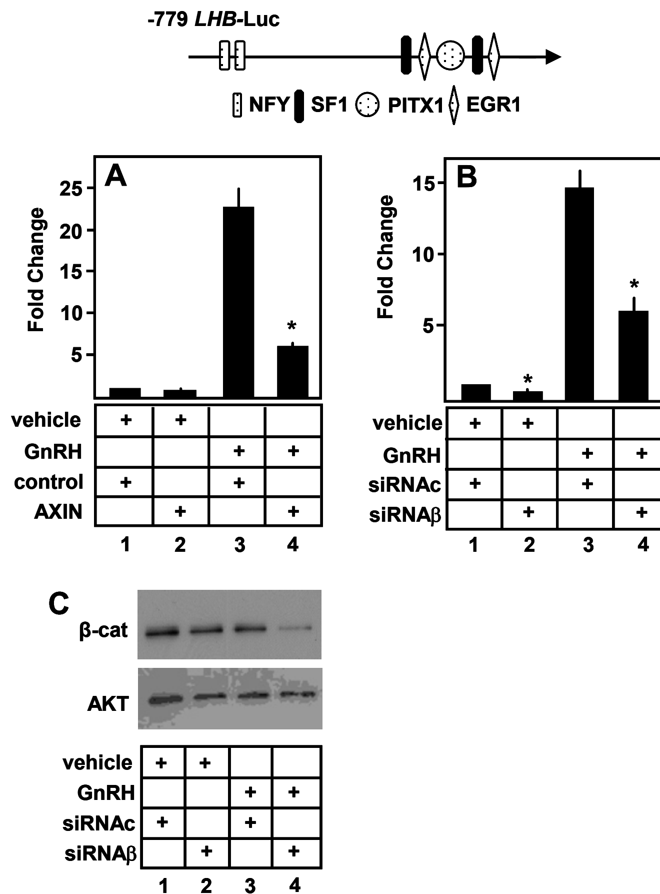

Figure 1: Maximal transcriptional response of the *LHB* promoter to GnRH requires β-catenin

(A) LβT2 cells were transiently co-transfected with -779/+10 bovine *LHB* promoter-luciferase (200 ng) and phRG-B-renilla reporter constructs (10 ng) and an expression vector encoding AXIN (100 ng) and then treated with either GnRH (10 nM) or vehicle. Luciferase activities for each data point were normalized against renilla activities. To calculate fold change within an experiment, *LHB* promoter activities were expressed relative to the maximal amount of *LHB* promoter activity, assigned a value of 1.0, in cells transfected with an empty CMV expression vector and treated with GnRH. Values were then averaged from three experiments performed in triplicate. Data shown are the means ± SEM of these three experiments. *, $P<0.01$ *(B)* LβT2 cells were transiently transfected using a reverse transfection protocol (32) with -779/+10 bovine *LHB* promoter-luciferase vector and 100 nM of non-targeting control interfering RNA (siRNAc) or β-catenin interfering RNA (siRNAβ) and subsequently treated with either vehicle or GnRH (10 nM). Luciferase activities for each data point were normalized to total amount of protein. Fold change represents *LHB* promoter activities expressed relative to the maximal amount of *LHB* promoter activity, assigned a value of 1.0, in cells transfected with cRNAi and treated with GnRH. Values were averaged from three experiments performed at least in duplicate. Data shown are the means ± SEM of three experiments. *, $P<0.01$ *(C)* Transfection lysates from experiments in Fig. 2, panel B were probed by immunoblot analysis of β-catenin and AKT, the latter as a loading control. Results shown are representative of two experiments.

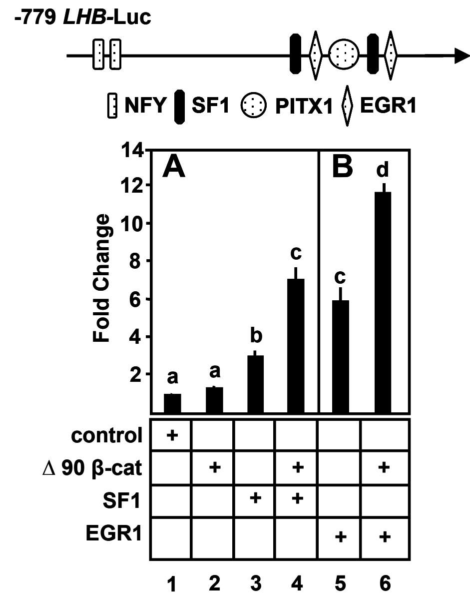

Figure 2: β-catenin enhances activity of EGR1 and SF1

Transient co-transfection assays were performed in LβT2 cells with -779/+10 bovine *LHB* promoter-luciferase and phRG-B-renilla reporter constructs. The activity of these reporter constructs was assayed upon their co-transfection with expression vectors encoding either Δ 90 β-catenin (100 ng), SF1 (20 ng), or EGR1 (10 ng). Luciferase activities for each data point were normalized against renilla activities. To calculate fold change, *LHB* promoter activities were expressed relative to the amount of *LHB* promoter activity in the control group (cells transfected with empty CMV expression vectors and reporter vectors). Data shown are the means ± SEM from three experiments performed in triplicate. Significant differences ($P<0.01$) are indicated between groups that do not share any letters in the superscript.

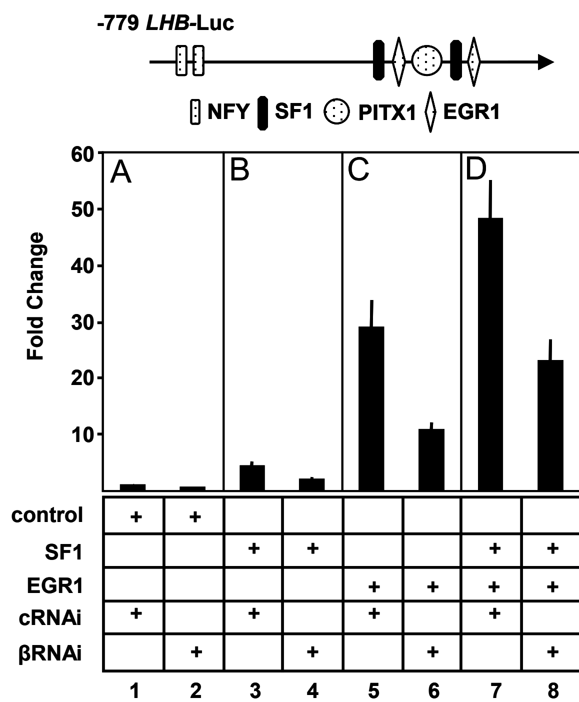

Figure 3: siRNA against β-catenin reduces the activity of EGR1 and SF1

LβT2 cells were transiently co-transfected with -779/+10 bovine *LHB* promoter-luciferase and either 20 nM of siRNAc or 20 nM of siRNAβ with expression vectors encoding either SF1 or EGR1. To calculate fold change, *LHB* promoter activities were expressed relative to the maximal amount of *LHB* promoter activity, assigned a value of 1.0, in cells co-transfected with siRNAc and expression vectors encoding SF1 and EGR1. Calculated values were then averaged from four experiments performed in triplicate. Data shown are the means ± SEM of four experiments. *, $P<0.01$

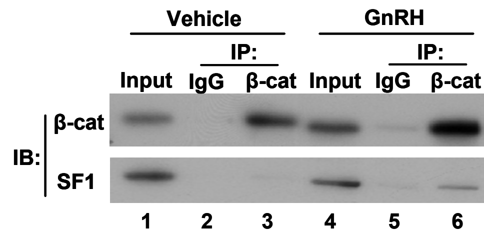

Figure 4: SF1 physically associates with β-catenin

Nuclear extracts isolated from LβT2 cells treated with either vehicle (lanes 1-3) or 10 nM GnRH (lanes 4-6) for 60 minutes were immunoprecipitated with either anti-β-catenin antibodies or control rabbit serum. Precipitates were then subjected to immunoblot blot (IB) analysis with SF1 and β-catenin antibodies. Results are representative of three separate experiments.

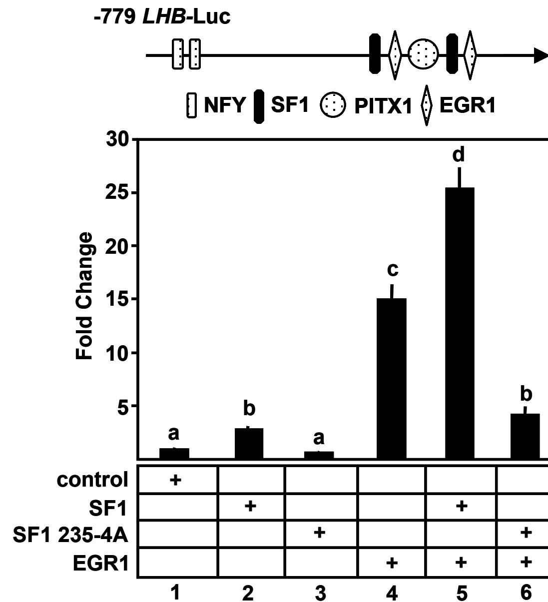

Figure 5: The β-catenin binding site in SF1 is required for functional synergism between SF1 and EGR1

Transient co-transfection assays were performed in LβT2 cells with -779/+10 bovine *LHB* promoter-luciferase and phRG-B-renilla reporter constructs. The activity of these reporter constructs was assayed upon their co-transfection with expression vectors encoding either SF1 (20 ng), SF1 235-4A (50 ng), or EGR1 (10 ng). Luciferase activities for each data point were normalized against renilla activities. To calculate fold change, *LHB* promoter activity for each experimental group is expressed relative to that of "Control" cells that is arbitrarily set at 1. Data shown are the means ± SEM of three experiments. Significant differences ($P<0.05$) are indicated between groups that do not share any letters in the superscript.

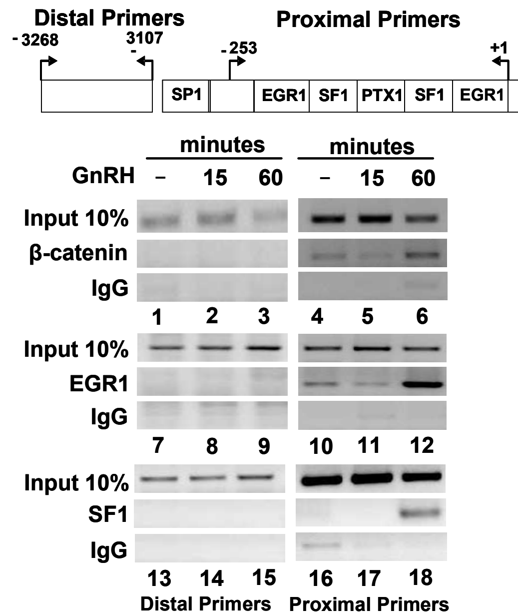

Figure 6: GnRH enhances the association of β-catenin with the proximal region of the endogenous *Lhb* promoter

Chromatin immunoprecipitation assays were performed with chromatin prepared from LβT2 cells prior to and post a GnRH stimulus (15 and 60 minutes) and immunoprecipitated with anti-β-catenin antibodies (lanes 1-6), anti-EGR1 antibodies (lanes 7-12), anti-SF1 antibodies (lanes 13-18), or control rabbit serum (lanes 1-18). Precipitated chromatin was amplified with primers that span response elements for EGR1, SF1 and PITX1 (proximal primer set) and with primers that span a distal fragment of *Lhb* promoters that lack sites for these transcription factors (distal primer set). To calculate fold change in signals from cells treated with vehicle versus GnRH for 60 minutes, arbitrary densitometric units for cells treated with GnRH were expressed relative to values in vehicle treated cells, arbitrarily assigned a value of 1. Statistical analyses were performed on three distinct experiments performed on different days and results are presented in text as means $\pm$ SEM.

CHAPTER FOUR

GNRH REGULATED EXPRESSION OF *JUN* AND JUN TARGET GENES IN GONADOTROPES REQUIRES A FUNCTIONAL INTERACTION BETWEEN TCF/LEF FAMILY MEMBERS AND BETA-CATENIN

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ABSTRACT

GnRH regulates gonadotrope function through a complex transcriptional network that includes three members of the immediate early gene family: *Egr1*, *Jun* and *Atf3*. These DNA binding proteins act alone or in pairs to confer hormonal responsiveness to *Cga*, *Lhb*, *Fshb*, and *Gnrhr*. Herein we suggest that the transcriptional response of *Jun* requires a functional interaction between the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family of DNA-binding proteins and β-catenin (officially CTNNB1), a co-activator of TCF/LEF. Supporting data include demonstration that GnRH increases activity of TOPflash, a TCF/LEF-dependent luciferase reporter, in LβT2 cells, a gonadotrope derived cell line. Additional co-transfection experiments indicate that a dominant negative form of TCF7L2 (TCFDN) that binds DNA, but not β-catenin, blocks GnRH induction of TOPflash. Overexpression of AXIN, an inhibitor of β-catenin, also reduces GnRH stimulation of TOPflash. Transduction of LβT2 cells with TCFDN adenoviruses diminishes GnRH stimulation of *Jun* mRNA without altering expression of *Egr1* and *Atf3,* two other immediate early genes that confer GnRH responsiveness. Reduction of β-catenin in LβT2 cells, through stable expression of short hairpin RNA, also selectively compromises GnRH regulation of *Jun* expression and levels of JUN protein. Finally, overexpression of TCFDN attenuates GnRH regulation of *Cga* promoter activity, a known downstream target of JUN. Together, these results indicate that GnRH regulation of *Jun* transcription requires a functional interaction between TCF/LEF and β-catenin and that alteration of either impacts expression of JUN downstream targets such as *Cga*.

INTRODUCTION

The hypothalamic neurohormone, gonadotropin-releasing hormone (GnRH), acts through a specific G-protein coupled receptor (GnRHR) to regulate synthesis and secretion of LH and FSH from gonadotropes in the anterior pituitary (1-6). LH and FSH are heterodimers; their synthesis requires expression of three genes, *Cga*, *Lhb* and *Fshb* (1;5). *Cga* encodes an α subunit common to both hormones (1;5). *Lhb* and *Fshb* encode β-subunits unique to each hormone (1;5). Once secreted, LH and FSH bind to distinct G-protein coupled receptors in male and female gonads to regulate gametogenesis and steroidogenesis (7-9). Together, these organs and hormones comprise a hypothalamic-pituitary-gonadal axis characterized by positive and negative feedback loops essential for maintaining proper reproductive function (1-9).

GnRH regulates synthesis of LH and FSH primarily at the level of transcription (1;5). Upon binding of GnRH to its receptor, transduction of the transcriptional signal flows through parallel cascades of multiple kinase families that phosphorylate a number of downstream targets including several DNA-binding proteins (10-15). Activation of these signaling cascades culminates in the regulated transcription of at least 75 other genes including *Cga*, *Lhb*, *Fshb*, and *Gnrhr*, four signature genes that endow gonadotropes with full functionality (16-19).

The accumulation of mRNAs regulated by GnRH, along with their encoded proteins, occurs in three distinct waves designated as primary, secondary, or tertiary (16-20). *Cga*, *Lhb*, *Fshb*, and *Gnrhr* reside within the tertiary network of GnRH responsive genes. They respond more slowly to GnRH because changes in their transcription depend on the proteins encoded by the primary and secondary response genes (20). For example, three members of the primary GnRH response network are the immediate early genes (IEGs) *Egr1*, *Atf3* and *Jun*; each encodes a unique DNA-binding protein that ultimately confers GnRH responsiveness to secondary and tertiary genes. Thus, EGR1 confers GnRH responsiveness to *Lhb* and to *Atf3* (21;22). In addition, EGR1 autoregulates transcription of its own gene (23;24). In contrast, JUN, as a component of AP1, confers GnRH responsiveness to *Cga*, *Fshb* and *Gnrhr* (25-30).

Moreover, JUN also positively autoregulates it own expression (31). Finally, ATF3 contributes to the GnRH responsiveness of *Cga* by forming a heterodimer with JUN (15).

GnRH has recently been reported to stimulate the nuclear localization of β-catenin (officially CTNNB1) and modulate the activity of TOPflash, an artificial TCF/LEF-dependent luciferase reporter in LβT2 cells (32). These changes were associated with parallel increases in GnRH-dependent mRNAs specific for *Jun*, *Fra1* and *Myc* (32). These three genes are known to be regulated in cancer and hematopoietic cells by members of the TCF/LEF family and β-catenin (33;34). In addition, recent work has also revealed instances in which the full transcriptional activity of JUN on the *Jun* promoter requires TCF7L2 and β-catenin (33). Thus, these collective findings suggest that GnRH regulation of *Jun* expression requires a functional interaction between TCF/LEF family members and β-catenin and that alteration of either would have a secondary impact on the ability of JUN to confer GnRH-responsiveness to downstream tertiary gene targets. The experiments reported herein address this possibility.

RESULTS

β-catenin mediates GnRH stimulation of a synthetic TCF-dependent promoter

To begin to examine whether GnRH signals through β-catenin to modulate the activity of TCF/LEF proteins, we conducted transient expression experiments in LβT2 cells with TOPflash, a luciferase containing construct that harbors a synthetic TCF/LEF dependent promoter that contains several adjacent TCF regulatory elements (35;36). TOPflash is also widely used as a read out of β-catenin activity (35;36). To attribute possible increases in TOPflash activity to a functional interaction between TCF proteins and β-catenin, a subset of cells were transfected with expression vectors that encode a dominant negative form of TCF7L2 (TCFDN) that contains a DNA binding domain but lacks a β-catenin binding domain (37;38). Controls included cells transfected with a CMV expression vector that does not encode a protein. LβT2 cells were transiently transfected and treated with either vehicle or 100 nM GnRH for 24 hours.

GnRH stimulated a large increase in TOPflash activity when compared to vehicle treated controls (Fig. 1A). Overexpression of TCFDN in the absence of GnRH only marginally impacts the activity of TOPflash when compared to vehicle treated cells. In contrast, TCFDN almost completely blocked GnRH stimulation. Gardner et al have also reported that TCFDN blocks GnRH stimulated increases in TOPflash activity (32). Together, these results suggest that GnRH stimulates activity of TCF/LEF dependent promoters and that TCF7L2 requires a β-catenin binding domain for maximal activity.

Gardner and colleagues also reported that GnRH stimulates nuclear accumulation of β-catenin in LβT2 cells (32). This is consistent with the notion that GnRH stimulated increases in the amount and possibly activity of β-catenin relieve TCF/LEF-dependent promoters from repression (39). To provide additional evidence that GnRH stimulated TOPflash through β-catenin, we co-transfected LβT2 cells with TOPflash and an expression vector encoding AXIN. Overexpression of AXIN reduces the transcriptional effects of β-catenin by promoting

degradation of the cofactor (40;41).

AXIN significantly reduced GnRH stimulated increases in TOPflash activity ($P<0.05$; Fig. 1B) but only marginally affected basal TOPflash activity when compared to control cells. While it is clear that AXIN reduces GnRH regulated increases in TOPflash activity, we are unable to verify reduced levels of β-catenin in these transient transfection experiments. This result is not surprising because LβT2 cells exhibit low transient transfection efficiency. Nevertheless, the AXIN-mediated reduction in GnRH regulation of TOPflash is consistent with the TCFDN result (Fig. 1A) and suggests GnRH regulation of a synthetic TCF-dependent promoter requires β-catenin. Immunoblot analysis of nuclear extracts from LβT2 cells indicated that levels of TCF7L2 are unchanged after treatment with GnRH (Fig. 1C). This result further supports the notion changes in β-catenin underlie changes in TCF7L2 activity.

Overexpression of TCFDN selectively attenuates GnRH stimulation of *Jun* expression without affecting expression of *Atf3* or *Egr1*

GnRH induction of TOPflash suggests that the neurohormone signals through β-catenin to modulate the activity of a synthetic TCF/LEF-dependent promoter. The question remains, however, whether GnRH also regulates expression of endogenous genes in gonadotropes that are dependent on TCF/LEF and β-catenin. To address this possibility, we focused on *Jun* based on recent reports indicating that its expression in cancer and hematopoietic cell lines is TCF/LEF and β-catenin dependent (33;34).

To examine whether GnRH signals through TCF/LEF and β-catenin to increase expression of *Jun*, we transduced LβT2 cells with TCFDN expressing adenovirus. LβT2 cells were then treated with 100 nM GnRH for 1 hour and resulting changes in gene expression were examined by real time PCR after reverse transcription of gene products. Control cells were transduced with a GFP expressing adenovirus.

Transduced TCFDN mRNA was easily detected when compared to control cells transduced with the GFP adenovirus when examined by reverse transcription endpoint PCR (Fig. 2A). Note that the TCFDN adenovirus encodes human TCF7L2 that lacks the binding domain for β-catenin. Thus, there is no PCR signal in the GFP control since the primers used for real time PCR are specific to human but not mouse *Tcf7l2* mRNA. TCFDN transduced LβT2 cells also exhibited high levels of TCFDN protein when examined by western blot (Fig. 2B).

As expected, GnRH dramatically stimulated expression of *Jun* mRNA when compared to the GFP control group (Fig. 2C). In contrast, transduced TCFDN significantly dampened the stimulatory effect of GnRH on *Jun* expression ($P<0.05$; Fig. 2C). Moreover, transduced TCFDN had no significant effect in the absence of GnRH suggesting that TCF7L2 contributes to the hormonal responsiveness of *Jun* transcription.

As noted earlier, GnRH also stimulates a rapid increase in the accumulation of *Atf3* and *Egr1* mRNA (16-19). In contrast to the results reported for *Jun*, transduced TCFDN had no impact on GnRH stimulation of *Atf3* or *Egr1* mRNA accumulation (Fig. 2D & E). Collectively, these results suggest that GnRH regulation of *Jun* expression is unique in its requirement for a functional form of TCF7L2 when compared to the other two IEGs.

Targeted reduction of endogenous β-catenin compromises GnRH regulated expression of *Jun*

The finding that TCFDN disrupts GnRH stimulated increases in *Jun* expression implies that GnRH signals through β-catenin to increase *Jun* expression. To examine this possibility, we stably transfected LβT2 cells with recombinant lentivirus that expresses short hairpin (sh) RNA specific for β-catenin. LβT2 cells that stably express non-silencing shRNA were also generated and served as controls. These new lines of LβT2 cells were treated with either vehicle or 100 nM GnRH for one hour and gene expression was assayed using real time PCR after reverse transcription of gene products.

Stable expression of shRNA specific for β-catenin significantly reduced levels of its mRNA independently of GnRH when compared to the non-silencing shRNA groups (Fig. 3A). As expected, in the absence of GnRH, levels of *Jun* mRNA were low and not significantly impacted by shRNA specific for β-catenin (Fig. 3B). In contrast, reduced levels of β-catenin mRNA correlated with an attenuated response of *Jun* mRNA to GnRH ($P<0.05$; Fig. 3B). A concurrent reduction in *Jun* and *β-catenin* expression is consistent with observed reduction in TOPflash activity in the presence of AXIN (Fig. 1B). Together, both experimental paradigms suggest that GnRH regulation of TCF dependent promoters, both natural and synthetic, requires β-catenin. Similar to the experiments with TCFDN, GnRH stimulated increases in *Egr1* and *Atf3* were unaffected by the reduced levels of β-catenin mRNA (Fig. 3C & D). Together, these results suggest that β-catenin mediates GnRH transcriptional responsiveness of *Jun* but not that of *Egr1* or *Atf3*.

JUN protein levels are reduced in TCFDN expressing and β-catenin deficient cells

To ensure that changes in the mRNAs for *Tcf7l2*, *β-catenin*, and *Jun* correlated with changes in their cognate proteins, we performed immunoblot analysis of whole cell extracts from LβT2 cells transduced with TCFDN adenovirus or from stably transfected cell lines that express either the non-silencing or β-catenin shRNAs. Levels of JUN protein were significantly lower in cells that were transduced with TCFDN and then exposed to GnRH (100 nM, 60 min) when compared to their control counterparts ($P<0.05$; Fig. 4A & B), consistent with the notion that maximal stimulation of JUN requires TCF7L2 with an intact β-catenin binding domain.

With respect to β-catenin protein, GnRH (100 nM, 60 min) stimulated accumulation of the co-activator in the stable cell line that expresses the non-silencing shRNA (Figure 4C). While levels of β-catenin protein were lower and refractory to stimulation by GnRH in the stable cell lines that express shRNA specific for the co-activator (Fig. 4C), the silencing of β-catenin protein was not as robust relative to its cognate mRNA (Fig. 3A). This may be attributed to a

requirement of β-catenin for growth (37), which would favor the selection and propagation of clones with moderate reductions in β-catenin protein versus clones with dramatic reductions in the co-activator. Additionally, β-catenin is also a required component of the cadherin cell adhesion complex (42;43), and alterations in cell morphology have been demonstrated downstream of GnRH (43;44) providing another role for the co-activator in gonadotropes. Nevertheless, the concurrent reduction of β-catenin and JUN protein ($P<0.05$; Fig. 4C & D) is consistent with the possibility that GnRH signals through TCF7L2 and β-catenin to regulate transcription of *Jun*, an event that leads to subsequent accumulation of JUN protein.

Transduced TCFDN comprises activity of a JUN responsive promoter in LβT2 cells

Prior reports indicate that full activity of the human *CGA* requires binding of JUN to tandem cAMP response elements (15;27). Since GnRH stimulated JUN confers hormonal responsiveness to *Cga* in LβT2 cells, then TCFDN dependent reductions in JUN should correlate with attenuated response of a *CGA* luciferase reporter after a GnRH challenge. To examine this possibility, LβT2 cells were transiently co-transfected with a *CGA* luciferase reporter and an expression vector encoding TCFDN. After transient transfection, LβT2 cells were treated with GnRH (100 nM) for 24 hours and then assayed for luciferase activity.

TCFDN had a marginal effect (~2-fold reduction) on *CGA* promoter activity in vehicle treated LβT2 cells (Fig. 5A). In contrast, TCFDN significantly reduced GnRH stimulated increases in *CGA* promoter activity by over 4-fold ($P<0.01$; Fig. 5A). Since GnRH stimulation of *Egr1* occurs independently of TCF/LEF (Fig. 2), we also examined whether a murine *Egr1* luciferase reporter (45) is refractory to TCFDN. As expected, GnRH increased the activity of the *Egr1* promoter reporter (Fig. 5B). While TCFDN appeared to reduce GnRH stimulated increases in *Egr1* promoter activity, the effect was not statistically significant (Fig. 5B). Together, these data indicate a strong correlation between TCFDN induced reductions in *Jun* expression (Fig. 2) and JUN protein (Fig. 4) and reduced activity of a JUN regulated promoter

reporter in GnRH treated LβT2 cells. These data also suggest that the requirement for TCF/LEF is specific for *Jun* but not for the other two IEGs that confer GnRH responsiveness to downstream gene targets.

DISCUSSION

Prior reports have revealed that GnRH regulates transcription of a large network of genes (16-19). Genes within this regulated network have been categorized as primary, secondary or tertiary genes based on how rapidly levels of their mRNAs change in response to a GnRH stimulus (16-19). Primary genes that encode DNA binding proteins play a vital role in the GnRH network because, as DNA binding proteins, they confer hormonal responsiveness to secondary and tertiary genes that contain the appropriate regulatory elements. The findings presented herein have provided greater insight into how GnRH coordinates activation across this transcriptional network. Specifically, our data indicate maximal levels of *Jun* expression in response to GnRH requires a functional interaction between TCF7L2 and β-catenin. Moreover, alterations in the activity of TCF7L2 or levels of β-catenin have a secondary impact of the GnRH-responsiveness of JUN-dependent downstream promoters such as *Cga.* Finally, the requirement for TCF7L2 and β-catenin is selective for *Jun* because neither the transcription factor or co-activator are required for GnRH regulated expression of *Egr1* or *Atf3*, two other members of the primary network of neurohormone responsive genes .

Historically, β-catenin has been viewed as a member of the WNT canonical signaling cascade that plays important roles during development (46-50). For instance, *Wnt4* is important for the growth of the embryonic pituitary (49;50), where as *Wnt5* regulates the shape of the endocrine gland during development (47). TCF7L2 seems to restrict the growth of the pituitary, because embryonic pituitary glands of *Tcf7l2* null mice are enlarged and exhibit excessive proliferation (48). Beta-catenin, by binding to the DNA binding protein PROP1, induces the expression of *Pit1*, resulting in the differentiation of pituitary precursor cells into somatotropes, lactotropes and thyrotropes (51). Levels of *Lhb* gene expression were also reduced in embryonic pituitary glands devoid of β-catenin (51). In addition to roles in WNT signaling and development, β-catenin is also an important cofactor in the cadherin cell adhesion complex (42). Recently it has been shown that GnRH induces changes in gonadotrope cell morphology by

altering the actin cytoskeleton (44) suggesting concurrent changes in the cadherin complex. While it is clear that WNT and β-catenin are important for development and cell adhesions, recent data (32), including our own, indicates that GnRH has hijacked components of the WNT signal transduction cascade to regulate transcription in differentiated gonadotropes.

While we have focused on GnRH, FSH and prostaglandin E2 (PGE2) have also been shown to signal through β-catenin to regulate transcription (52-54). For instance, FSH stimulated increases in aromatase expression in ovarian granulosa cells are mediated by β-catenin (52). PGE2, by binding to its heterotrimeric G protein coupled receptor (GPCR) on colon cancer cells, stimulates the accumulation of β-catenin and increases TCF/LEF transcriptional activity (53;54). GnRH is thought to primarily signal through activation of Gq/11 (55). In contrast, FSH and PGE2 both signal through activation of Gs (7;53), suggesting that the role of β-catenin in mediating responsiveness to hormones that bind to GPCRs is not restricted to one type of G protein .

Glycogen synthase kinase β (GSK3β) may serve as an important signaling intermediate that links GnRH to increases in β-catenin levels or activity (39;56). WNT inhibition of GSK3β activity prevents the phosphorylation of residues in the N-terminus of β-catenin, resulting in the accumulation of the co-activator (39;56). Recently, Gardner and colleagues reported that GnRH stimulates the rapid phosphorylation of GSK3β on serine 9, leading to inhibition of the kinase (32). We have also observed a strong correlation between GnRH-stimulated increases in GSK3β phosphorylation and increased activity of β-catenin in LβT2 cells (unpublished data, n=3). In colon cancer cells, PGE2 inhibits GSK3β activity via phosphorylation of serine 9, an event that contributes to increases in TOPflash activity (53;54). In addition to serine 9, recent work has revealed that p38 MAPK can phosphorylate and inactivate GSK3β through serine 389 (57). This inactivation stimulated the accumulation of β-catenin in mouse embryonic fibroblasts and ES cells (57). This result may be relevant to GnRH regulation of β-catenin because the neurohormone signals through p38 MAPK (13). Together these reports suggest that inhibition

of GSK3β activity may serve as a broad mechanism for hormones to modulate β-catenin downstream of a GPCR. Further studies will be required to reveal the signaling cascade that links GnRH to induced phosphorylation of GSK3β and concomitant changes in the levels and activity of β-catenin.

Thus far, we have emphasized the selective requirement for TCF7L2 and β-catenin in GnRH regulated expression of *Jun* along with their indirect impact on JUN downstream targets such as *Cga.* It is important to note, however, that β-catenin plays another distinct role in the network of GnRH-responsive genes. Recently, we reported that β-catenin can also act independently of GnRH to support the permissive role of the nuclear receptor SF1 (officially NR5A1) in allowing *Lhb* to respond to GnRH stimulated rises in EGR1 (58). Moreover, SF1 is required for expression of *Cga*, *Fshb* and *Gnrhr*. For example, mice that lack SF1 in gonadotropes are hypogonadal and infertile due to insufficient synthesis and secretion of LH and FSH that in turn are a consequence of attenuated expression of *Gnrhr*, *Fshb* and *Lhb* (59;60). This suggests that β-catenin may act independently of GnRH to support the permissive role of SF1 in allowing all four gonadotrope signature genes to respond to GnRH. This enabling role of β-catenin may also explain why FSH stimulated increases in aromatase gene expression in granulosa cells requires a functional interaction between SF1 and β-catenin (52). Interestingly, FSH also stimulates increases in *Jun* expression, suggesting that β-catenin may play both permissive and hormone responsive roles in granulosa cells as it does in gonadotropes (61). Finally, there is a growing number of reports indicating that transcriptional activity of a number of nuclear receptors, including SF1, require a functional interaction with β-catenin (62). Thus, the permissive requirement for β-catenin may be extended generally to members of the nuclear receptor family.

A prior report has revealed that a splice variant of *Tcf7l2* originally identified in embryonic pituitary may influence whether β-catenin interacts with TCF/LEF family members or SF1 (38;63). This *Tcf7l2* isoform (also known as TCF-4N) lacks a DNA binding domain, but

retains an N terminal β-catenin interaction domain (38;63). TCF-4N was found to inhibit β-catenin regulated increases in TOPflash activity (38). Conversely, TCF-4N increased synergistic interactions between β-catenin and SF1 on the Inhibin α promoter (38). While it is tempting to suggest that TCF-4N would favor *Lhb* gene expression and inhibit *Jun* expression in gonadotropes, our immunoblot experiments suggest that LβT2 cells only express full length TCF7L2 (data not shown).

In summary, this report indicates that GnRH signals through β-catenin and TCF7L2 to increase expression of *Jun*. Although the requirement for β-catenin and TCF7L2 appears to be limited to one of the three IEGs that confer hormonal responsiveness to secondary and tertiary genes of the GnRH network, the potential impact is significant as three of the four signature genes (*Gnrhr*, *Fshb* and *CGA*) respond to GnRH mediated changes in JUN (25-30). Consequently, blockade of any point along the signaling route that links GnRH to β-catenin is likely to wreak havoc with gonadotrope function.

MATERIALS AND METHODS

Chemicals and GnRH

Luteinizing hormone releasing hormone (GnRH), Triton X-100, Igepal CA-300, sodium deoxycholate, and Nonidet P-40 were purchased from Sigma Chemical Co. (St. Louis, MO); sodium dodecyl sulfate (SDS) was from Bio-Rad Laboratories (Hercules, CA); glycine was from J. T. Baker (Phillipsburg, NJ). TRIzol and Lipofectamine were purchased from Invitrogen (Carlsbad, CA).

DNA constructs

The –1500bp/+45 human *CGA* promoter-Luciferase promoter has been described previously. The -1381/+79 mouse *Egr1*-Luciferase promoter was a kindly provided by Dr. Larry Jameson (Northwestern University, Chicago IL). The TOPflash luciferase reporter vector was purchased from Upstate Biotechnology, Inc (Lake Placid, NY). TCFDN was cloned into pcDNA3 and was kindly provided by Dr. Frank McCormick (University of California School of Medicine, San Francisco, California). The murine *Axin1* in a pcS2_MT expression vector was generously provided by Dr. Frank Costantini (Columbia University, New York, NY) .

Transient Transfections

LβT2 cells maintained at 37°C with 5% CO_2 in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) (complete media) (Invitrogen) were plated into a 24 well plate (250,000 cells/well). Twenty-four hours later LβT2 cells were washed with Phosphate Buffered Saline (PBS), (Sigma Chemical Co.) and a transfection cocktail containing DNA constructs, DMEM, and Lipofectamine (1.6μl/well) was added in accordance with the manufacturer’s protocol. Transfection media was removed following an overnight incubation and cells were treated with vehicle or 100 nM GnRH diluted in DMEM supplemented with 1% P/S for 24 hours. Luciferase activity was examined using the

Dual Luciferase Reporter assay kit [Promega Corporation, (Madison, WI)]. The amounts of DNA constructs used are in the figure legends.

Short hairpin expressing LβT2 cells and GnRH treatment

Expression Arrest™ GIPZ lentiviral shRNAmir vectors (non-silencing and β-catenin) were purchased from Open Biosystems (Huntsville, AL). Lentiviral packaging was done in TLA-HEK293T cells in accordance with the Trans-Lentiviral GIPZ packaging system. Five hours following transfection, the packaging cocktail was aspirated and replaced with complete media. Seventy-two hours following transfection, HEK293T cells were lysed and lentiviral containing supernatants were isolated following centrifugation (3000 rpm for 20 min).

LβT2 cells in 6 well plates (2.5 x 10^6 per well) were transduced with lentivirus diluted in 2 mL of DMEM containing 8 µg/mL Sequabrene. Six hours later an equal amount of DMEM supplemented with 20% FBS was added to each well. The following day the transfection cocktail was removed and replaced with complete media. Puromycin (350 ng/mL) diluted in complete media was used to select for shRNA expressing cells. Cells were then plated and maintained in complete media in 96 well plates to cultivate individual clones. Levels of β-catenin expression in shRNA expressing cells were examined by Quantitative Real-time (QRT) PCR (Fig. 3A) and by western blot (Fig. 4A).

For GnRH treatment, serum starved (overnight) shRNA expressing cells in 6 well plates (80% confluent) were treated with either vehicle (PBS) or GnRH for 60 min. Treatments were removed and 1 mL of TRIzol was added.

Adenoviral transduction of LβT2 cells and GnRH treatment

TCFDN was excised from pcDNA3 with EcoR1 and BamH1, and subcloned into EcoR1 and BrgI II sites of the pDC316(io) shuttle vector and TCFDN adenoviruses were constructed using the AdMax system (Microbix Biosystems, Tronto, ON, Canada). LβT2 cells [1x10^6 per

well, (80% confluent)] in 6 well plates were transduced with adenoviruses diluted in 1 mL of DMEM supplemented with 2% FBS and 1% P/S. Twelve to fourteen hours post transduction, adenoviral containing media was removed and new media (DMEM containing 2% FBS and 1% P/S) was added. At 48 hrs post transduction, an overnight serum starve was initiated, and approximately 16 hrs later transduced cells were treated with vehicle or GnRH (100 nM) for 60 min. Treatments were then removed and 1 mL of TRIzol was added to each well. Viral particles per milliliter (VPM) was determined by measuring sample optical density (OD) at 260 and the relationship of 10^{12} VPM per OD. The amount of adenovirus used (denoted in the figure legends) was based on levels of TCFDN expression and functional outcome.

RNA isolation and cDNA synthesis

Total RNA was isolated from TRIzol in accordance with the manufacturer's protocol. All RNA work was done with DEPC treated water. Prior to cDNA synthesis, total RNA (0.5 µg) was incubated with: 1 µL of DNAse [DNAse I, RNAase-free, (Roche Diagnostics, Indianapolis, IN)], 2 µL of 5X First Strand Buffer (Invitrogen, Carlsbad) in a total volume of 10 µL for 15 min at room temperature. One µL of EDTA (25mM) was then added to each reaction and incubated at 70º C for 15 min. The reaction was then placed on ice for 1 min and 4 µL of qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg,MD) was added per sample. Twenty µL cDNA reactions were then carried out following the qScript cDNA SuperMix protocol. Samples were then diluted 1:10 in DEPC treated water and stored at –70º C.

Quantitative Real-Time PCR

Relative mRNA levels were determined using Quantitative Real-Time (QRT) PCR and the 7000 ABI prism sequence detection software system (Applied Biosytems, FosterCity, CA). Primer Express 2.0 software (Applied Biosystems, Foster City, CA) was used to design intron

spanning primers (except *Jun* which lacks apparent introns). Primer sets were optimized using a range of primer concentrations and 300 nM was optimal for *Atf3*, *Jun* and *cyclophilin B* (officially *Ppib*), while 150 nM was optimal for *β-catenin* and *Egr1* primer sets. Primer efficiency was then determined over a range of cDNA dilutions (1:1, 1:10, 1:100, 1:1000). The amplification efficiency of the QRT reaction for each gene was between 93% and 105% and therefore relative levels of expression were calculated using the comparative method ($\Delta\Delta C_T$). *Cyclophilin B* expression was used to normalize samples. Samples were assayed in triplicate.

For each QRT-PCR reaction, a master mix containing: 12.5 μL of Platinum® SYBR® Green qPCR SuperMix-UDG, 0.5 μL of ROX reference Dye [Invitrogen, (Carlsbad, CA)], and primers were added to 5 μL of cDNA. The final volume of a QRT-PCR reaction was 25 μL. QRT-PCR reaction conditions were: 50^0 C for 2 min, 95^0C for 10 min; then 40 cycles of 95^0C for 15 sec, and 60^0 for 1 min. Primer sequences for real time PCR include: *cJun* [(F) 5'-AGTTCTTGTGCCCCAAGAACG-3', (R) 5'-AAGCGTGTTCTGGCTATGCAG-3'], *Atf3* [(F) 5'-TTACCGTCAACAACAGACCCCT-3', (R) 5'-CGCCTCCTTTTCCTCTCATCTT-3'], *Egr1* [(F) 5'-GAACCCCTTTTCAGCCTAGTTCA-3', (R) 5'-AGGATGAAGAGGTCGGAGGATT–3'], *cyclophilin B* [(F)5'-CAAAGACACCAATGGCTCACAG-3', (R) 5'-CCACATCCATGCCCTCTAGAAC-3'], *β-catenin* [(F) 5'-CGCAAGAGCAAGTAGCTGATATTG, (R) 5'-CGGACCCTCTGAGCCCTAGT-3'].

Western blot

Protein extracts were prepared one of three ways. Nuclear extracts were prepared as previously described (58;64) or whole cell lysates were collected using the following two methods. Cells were rinsed twice in cold PBS and lysed in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.5% deoxycholate, 1% Triton X-100. Lysis buffer was supplemented with the Mini Protease Inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Cell homogenates were then cleared by centrifugation at 16,000 xg for 10 min

and supernatants analyzed. For samples that were lysed in TRIzol, following RNA isolation, the protein was extracted by the modified protocol provided for Tri Reagent (Molecular Research Center, Inc, Cincinnati, OH). Extracted proteins were solubilized in 5 M urea, 1% SDS, 25 mM DTT and heating at 50 °C. Protein concentrations were determined by Coomassie Plus Protein Assay (Pierce, Rockford IL) using BSA as a standard. Protein samples were separated on 12% polyacrylamide SDS gels. Proteins were then transferred to a PVDF membrane (BioRad) in Towbin buffer. Membranes were blocked in 5% non-fat milk in Tris buffered (pH 8.) saline containing 0.05% Tween 20 (TBST). Membranes were incubated for either 1 hour at room temperature or overnight at 4°C using primary antibodies for β-catenin (BD Transduction), JUN, AKT (Cell Signaling), TCF7L2, total CREB (Upstate), tubulin (Abcam), or actin (Sigma) diluted in TBST containing 5% non-fat milk. Membranes were rinsed 3 times with TBST and then incubated in HRP conjugated secondary antibody (Pierce) for 1 hour at room temperature. The membrane was rinsed 3 times with TBST and antigen-antibody complexes were detected by chemiluminescence (Immobilon HRP, Millipore, Billerica, MA).

Statistics

Levels of luciferase activity, gene expression, and arbitrary densitometric units were analyzed by one-way ANOVA, and differences between groups were determined with the Tukey post hoc test. Levels of arbitrary densitometric units in figure 4B were analyzed by a two-tailed Student’s t test.

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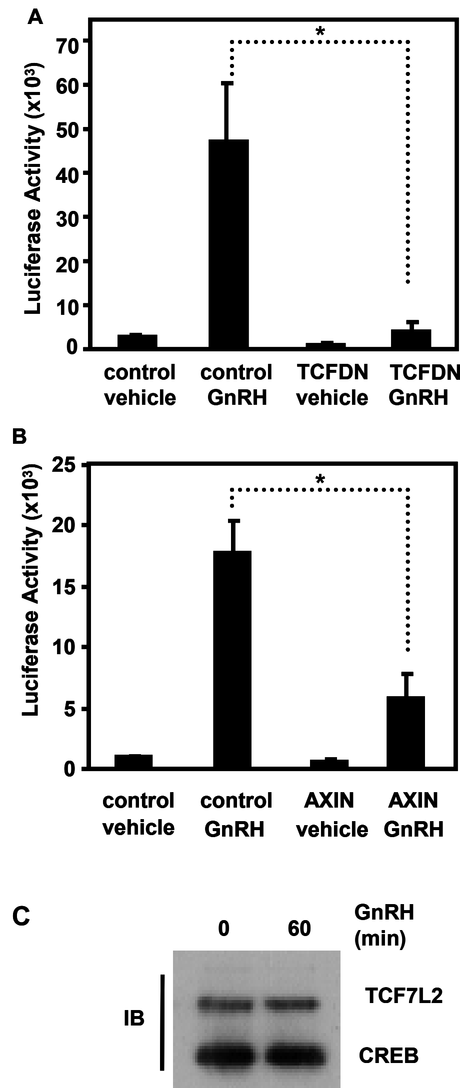

Figure 1: GnRH regulates TCF/LEF dependent transcription through β-catenin and TCF/LEF

A, LβT2 cells were co-transfected with TOPflash (50 ng) and either an empty CMV expression vector (control) (100 ng) or a TCFDN encoding expression vector (100 ng), treated with vehicle or GnRH (100 nM) for 24 hours and resulting luciferase activity was measured. B, LβT2 cells were co-transfected with TOPflash (50 ng) and either an empty CMV expression vector (control) (100 ng) or an expression vector encoding AXIN (100 ng), treated with vehicle or 100 nM GnRH for 24 hours and resulting luciferase activity was measured. C, Nuclear extracts were isolated from LβT2 cells treated with vehicle or 100 nM GnRH for 1 hour followed by immunoblot (IB) using antibodies specific for TCF7L2 and CREB (loading control). Data shown are the means +/- SEM from three independent experiments performed in triplicate. (*, $P<0.05$)

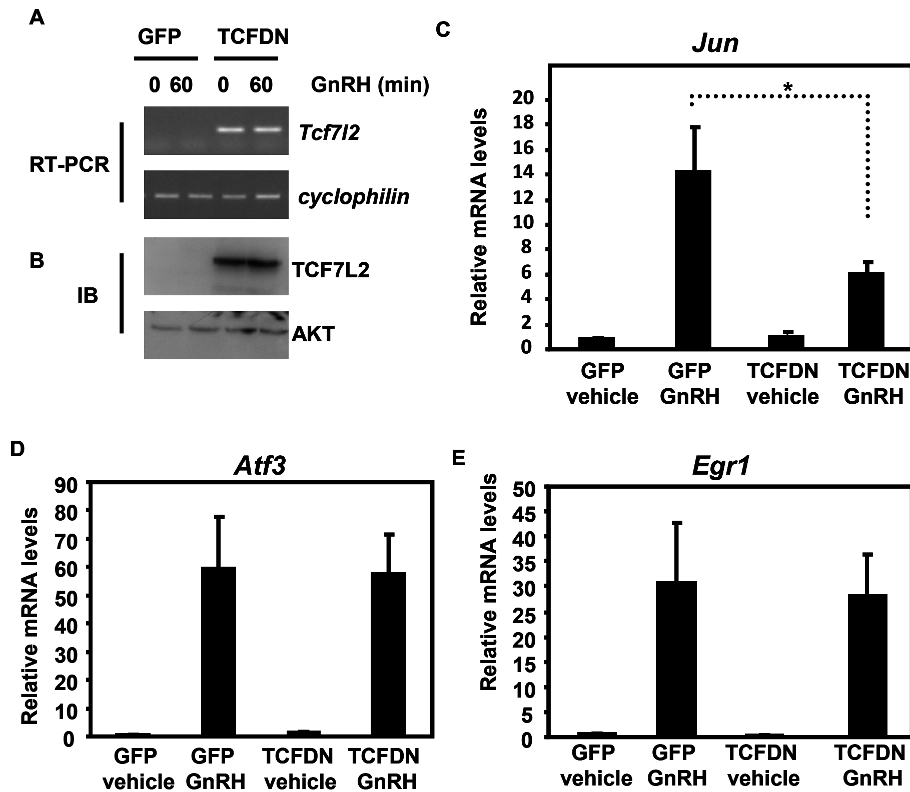

Figure 2: GnRH stimulated increases in *Jun* expression are reduced in TCFDN expressing LβT2 cells

LβT2 cells were transduced with either GFP or TCFDN expressing adenoviruses [1×10^{11} virus particles milliliter (vpm)] and subsequently treated with either vehicle or 100 nM GnRH for 1 hour. A, Is a representative endpoint reverse transcription-PCR experiment using primers that span either the DNA binding domain of *TCF7L2* or *cyclophilin B*. B, Whole cell extracts were isolated from GFP and TCFDN transduced LβT2 cells and subject to western blot analysis using antibodies specific for TCF7L2. Quantitative real-time PCR was used to determine relative levels of *Jun* (C), *Atf3* (D) or *Egr1* (E) expression. Levels of *cyclophilin* expression were used to normalize samples. The expression levels of each gene are expressed relative to the amount of expression in vehicle treated GFP expressing LβT2 cells. Data shown are the means +/- SEM from distinct three experiments in which each sample was assayed in triplicate (*, P<0.05).

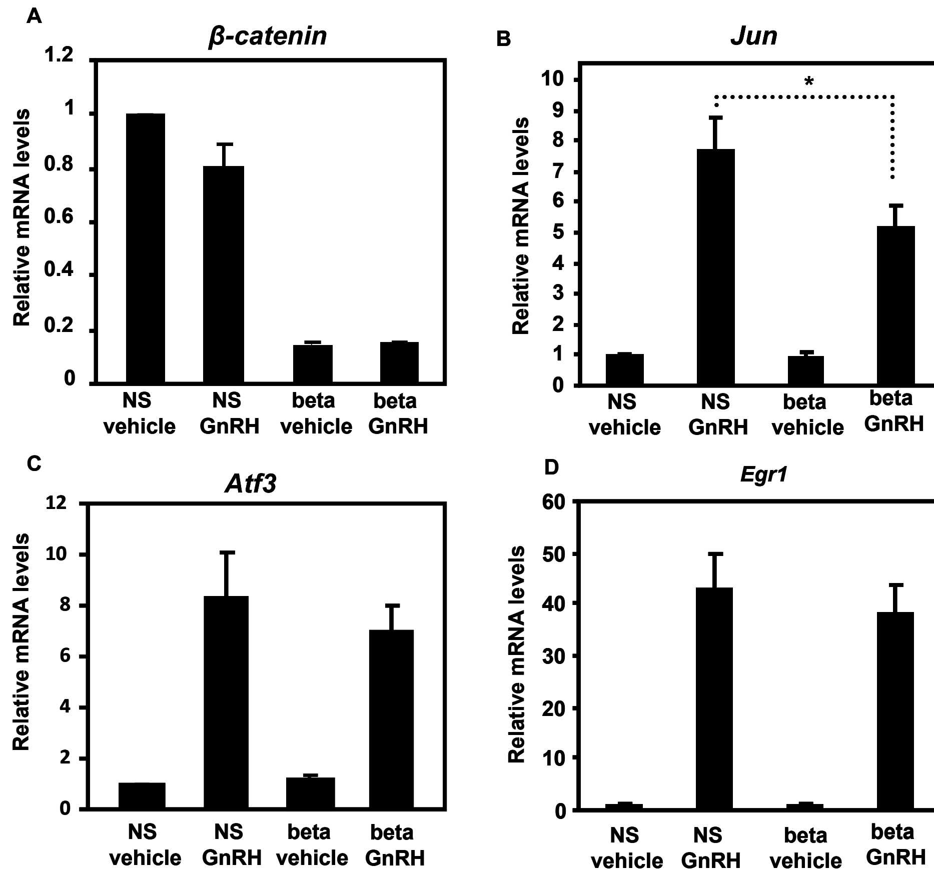

Figure 3: Endogenous β-catenin is required for maximal expression of *Jun* in GnRH treated cells

LβT2 cells that stably express either non-silencing short hairpin (sh) RNA (NS) or shRNA to β-catenin (beta) were treated with either vehicle or 100 nM GnRH for 1 hour. Quantitative real-time PCR was used to determine relative levels of *β-catenin* (A), *Jun* (B), *Atf3* (C) or *Egr1* (D) expression. Levels of *cyclophilin* expression were used to normalize samples. The expression levels of each gene are expressed relative to the amount of expression in vehicle treated cells that express non-silencing shRNA. For (A) data shown is the mean +/- SEM; n=3. For B-D, data shown are the means +/- SEM; n=6 (*, $P<0.05$).

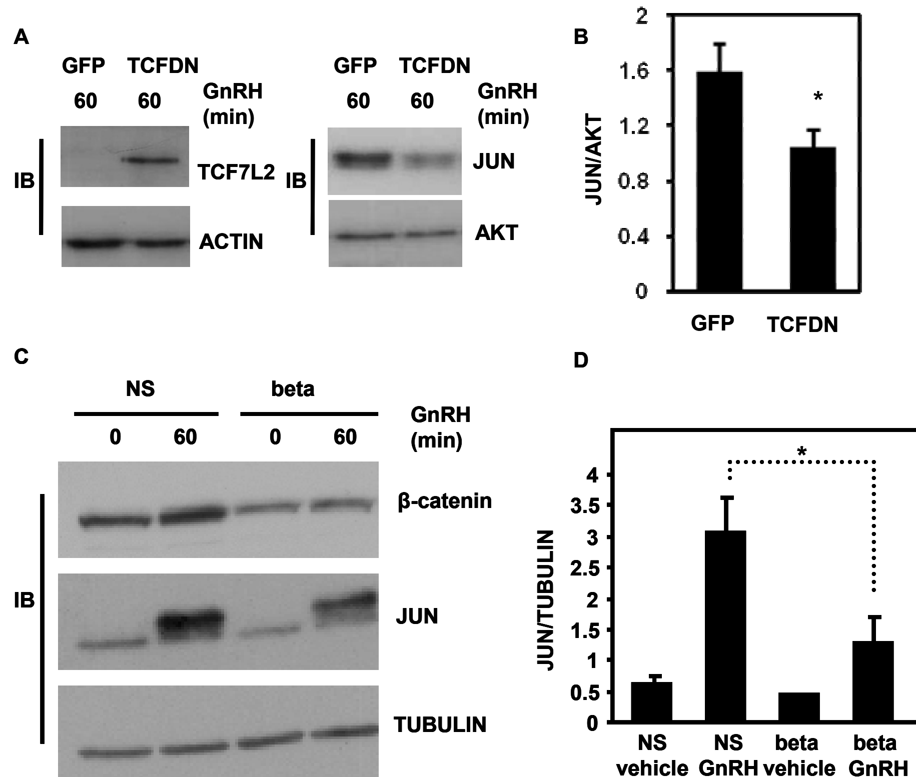

Figure 4: JUN protein levels are reduced in β-catenin deficient and TCFDN expressing cells

A, Whole cell extracts isolated from LβT2 cells transduced with either GFP or TCFDN expressing adenoviruses [$5{\times}10^{10}$ (vpm)] and treated with 100 nM GnRH for 1 hour, were subject to immunoblot analysis using antibodies specific for TCF7L2, JUN, ACTIN (loading control) and AKT (loading control). B, Arbitrary densitometric units of JUN divided by arbitrary densitometric units of AKT. Data shown in panel B are the means +/- SEM from three distinct experiments (*, $P<0.05$). C, Whole cell extracts were isolated from LβT2 cells stably expressing non-silencing short hairpin (sh) RNA (NS) or shRNA to β-catenin (beta) and subjected to immunoblot analysis using antibodies specific for β-catenin, JUN and Tubulin (loading control). D, Arbitrary densitometric units of JUN divided by arbitrary densitometric units of Tubulin. Data shown in panel D are the means +/- SEM from three distinct experiments (*, $P<0.05$).

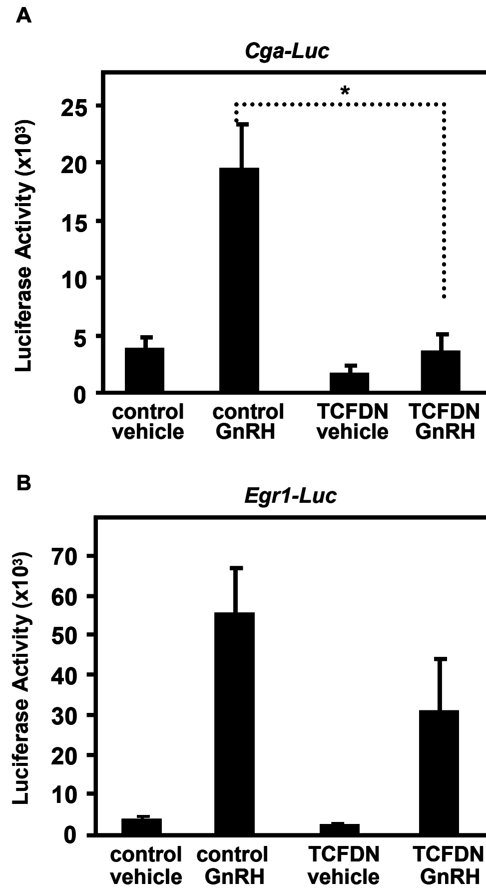

Figure 5: *CGA* transcription is compromised by TCFDN

A, LβT2 cells were co-transfected with a human *CGA*-Luc promoter reporter (50 ng) and either an empty CMV expression vector (control) (100 ng) or a TCFDN expression vector (100 ng) and then treated with vehicle or 100 nM GnRH for 24 hours. B, LβT2 cells were co-transfected with an *Egr1*-Luc promoter reporter (50 ng) and either an empty CMV expression vector (control) (100 ng) or a TCFDN expression vector (100 ng) and then treated with vehicle or GnRH for 24 hours. Data shown are the means +/- SEM from three independent experiments performed in triplicate. (*, $P<0.05$)

CHAPTER FIVE

GNRH REGULATION OF TCF AND NFAT RESPONSIVE PROMOTERS REQUIRES MOBILIZATION OF INTRACELLULAR CALCIUM AND ACTIVATION OF CALCINEURIN

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ABSTRACT

Gonadotropes of the anterior pituitary synthesize and secrete the gonadotropins LH and FSH that act on the gonads to regulate gametogenesis and steroidogenesis. The genes that encode these two hormones are regulated by GnRH that is secreted from the hypothalamus and binds to its receptor on gonadotropes. GnRH activates JUN N-terminal kinase (JNK) and intracellular Ca^{2+} mobilization. Recent work from our laboratory has shown that β-catenin is a downstream target of GnRH and required for hormonal regulation of several genes including *Jun*. GnRH regulation of *Jun* also requires T-cell factor (TCF)/β-catenin interactions. In this study we test the hypothesis that GnRH regulates β-catenin dependent genes through activation of JNK and an increase in intracellular Ca^{2+}. Pretreatment of LβT2 cells with SP600125, a JNK specific inhibitor, reduces GnRH mediated activation of TOPflash, an artificial β-catenin dependent reporter, and expression of *Jun* and *Atf3*. Pretreatment of LβT2 cells with BAPTA-AM, an intracellular Ca^{2+} chelator, or cyclosporin A, an inhibitor of the Ca^{2+} dependent protein phosphatase calcineurin, reduces GnRH mediated activation of TOPflash and expression of *Jun* and *Atf3*. Nuclear factor of T cells (NFAT) proteins are regulated by calcineurin, and *Nfatc3* was found expressed in LβT2 cells. Furthermore, GnRH stimulated an NFAT-dependent promoter reporter and this activation required intracellular Ca^{2+} and calcineurin activation. In summary, Ca^{2+} and calcineurin are important for regulation of TCF- and NFAT-dependent transcription and *Jun*/*Atf3* accumulation. NFAT and JUN cooperate to regulate several cytokines suggesting they may also be necessary for GnRH regulation of gonadotrope specific genes.

INTRODUCTION

Normal reproductive function requires the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) that are synthesized and secreted from gonadotrope cells of the anterior pituitary. LH and FSH are heterodimeric glycoproteins composed of a common alpha subunit (encoded by *Cga*) and a beta subunit (encoded by *Lhb* and *Fshb* respectively), that confers receptor specificity. Gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus binds to its receptor (encoded by *Gnrhr*) and activates multiple signaling pathways ultimately leading to increased expression of the gonadotrope specific genes *Cga*, *Lhb*, *Fshb* and *Gnrhr* (1;2). Hormonal responsiveness of the gonadotrope specific genes also requires several immediate early genes (IEGs): *Jun*, *Atf3* and *Egr1* which act to communicate the GnRH signal by binding to their response elements on *Fshb* and *Gnrhr* (JUN), *Cga* (JUN/ATF3) and *Lhb* (EGR1) (reviewed in (1;3-5)).

GnRH signals through Gαq proteins ultimately leading to increased activity of several members of the mitogen activated protein kinase (MAPK) family (6). These include the extracellular regulated kinase (ERK) (7-9), c-JUN N-terminal kinase (JNK) (7;9) and p38 MAPK (7;10-12). GnRH also increases Ca^{2+} mobilization and influx through voltage gated calcium channels (VGCC) (12-16) suggesting there may be cross-talk between multiple signaling pathways. Recent work has demonstrated that expression of *Jun*/JUN requires an interaction between T-cell factor (TCF) 4 and the co-factor β-catenin for full transcriptional activity downstream of GnRH (17). Additionally, it has been demonstrated that GnRH regulates an artificial TCF-dependent promoter reporter TOPflash (17;18); however the signaling pathway that regulates TCF has yet to be elucidated.

Calcium is an important second messenger that has a plethora of intracellular targets. GnRH signaling leads to increased Ca^{2+}, which in turn interacts with calmodulin

leading to activation of calmodulin-dependent protein kinase type I and type II (CaMKI/CaMKII) (19-21). CaMKI has been shown to repress transcription of *Fshb* via phosphorylation of HDACs within gonadotrope cells (21). In LβT2 cells GnRH regulates CaMKII phosphorylation leading to increases in *Cga* (19;20) and rat *Lhb* promoter reporter activity (15;16). A second Ca^{2+}/calmodulin target in gonadotrope cells is the calcium dependent protein phosphatase calcineurin (21-23). Calcineurin is regulated in αT3 cells (21), and its activity is required for ATF3 protein accumulation in conjunction with both JNK and ERK (22). The role of Ca^{2+} in regulation of gonadotrope specific genes such as *Lhb* is controversial, and investigation into the relationship between gonadotrope gene expression and Ca^{2+} has provided inconsistent results depending on the species studied or cell line utilized in the studies (13-16;24;25).

There is known cross-talk between the MAPK family members and Ca^{2+} signaling targets (4;13;26;27). It has been demonstrated that Ca^{2+} influx through VGCC affects ERK activation, while intracellular Ca^{2+} mobilization affects JNK in gonadotrope derived cell lines (13;24). Since *Jun/*JUN is a target of TCF/β-catenin and JNK, and GnRH has been shown to regulate TCF-dependent transcription (17), we hypothesized that similar signaling pathways acting downstream of the GnRH receptor may regulate transcription of TCF-dependent genes such as *Jun*. If so, then JNK and Ca^{2+} may be a point of convergence upstream of TCF. To test this hypothesis, we investigated the role of JNK and Ca^{2+} in regulating TOPflash and the IEGs. This approach provided two mechanisms for looking at TCF-dependent transcription; the first being an artificial TCF-dependent transcriptional reporter and the second being examination of expression of the IEGs including *Jun*, a TCF target, and *Atf3* and *Egr1.* It also provided a negative control; as *Egr1* transcript requires ERK phosphorylation that is not affected by Ca^{2+} mobilization (24;28). During the course of our studies we found that calcineurin is required for TOPflash activation, and *Jun* and *Atf3* transcription downstream of GnRH. The

requirement of calcineurin suggested that NFAT proteins may be activated downstream of GnRH (29;30). Herein, we demonstrate that LβT2 cells express *Nfat4* (officially *Nfatc3*), and that its transcriptional activity is regulated by intracellular Ca^{2+} and calcineurin activation downstream of GnRH. These findings demonstrate a previously unknown role of NFAT in GnRH signaling, and suggest additional downstream Ca^{2+}/NFAT targets within gonadotrope cells such as *Jun* or other AP1 dependent genes.

RESULTS

JNK activation is required for TCF-dependent regulation of *Jun* and *Atf3* by GnRH in LβT2 cells

GnRH has been shown to regulate TOPflash, an artificial TCF-dependent promoter (17;18), and this regulation requires Gαq proteins (18). To further examine the signaling pathways that regulate TCF-dependent transcription in gonadotropes, we utilized this promoter reporter. Members of the MAPK signaling family are key components of GnRH signaling and regulation of gonadotrope specific genes (31), therefore we first investigated the role of several MAPK family members. Gonadotrope derived LβT2 cells were transfected with TOPflash and then pretreated with the JNK inhibitor SP600125 (25 uM) for one hour. Cells were then treated with vehicle or 100 nM GnRH followed by cell lysis and luciferase assay. GnRH increased TOPflash activity as previously reported, and pretreatment with SP600125 significantly reduced GnRH mediated activation (Fig 1A) of the reporter. Inhibitors specific for ERK (PD98059) and p38 MAPK (SB203580) did not reduce the activation of TOPflash by GnRH (data not shown) suggesting that JNK activation is specific for TCF-dependent transcription in LβT2 cells.

JNK phosphorylates JUN leading to increased transcriptional activity of the oncogene and is also necessary for ATF3 accumulation (32). Most work in LβT2 cells examines the post-transcriptional accumulation of ATF3 and phosphorylated JUN (10;22;32), while the transcriptional regulation of the genes is rarely studied. To investigate the role of JNK in *Jun* and *Atf3* transcription, LβT2 cells were serum starved, and then pretreated with 25 uM SP600125 or DMSO control for an hour prior to GnRH treatment. Gene products were reverse transcribed and real-time PCR was performed using primers specific for the IEGs *Jun*, *Atf3* and *Egr1*. Inhibition of JNK significantly reduced *Jun* transcription (70% reduction) downstream of GnRH (Fig. 1B). *Atf3* was

also reduced (Fig. 1C), although to a lesser extent (24% reduction). The increase of *Egr1* mRNA following GnRH treatment remained unaffected (Fig. 1D). These results demonstrate that JNK activation contributes to TCF-dependent activation of TOPflash and maximum accumulation of *Jun*, both of which also require TCF (17;18) and *Atf3* mRNA accumulation. This suggests involvement of another signaling pathway due as the JNK inhibitor did not completely inhibit mRNA accumulation.

BAPTA blocks GnRH mediated activation of TCF-dependent transcription as well as *Jun* mRNA accumulation

JNK activation downstream of GnRH has been shown to require Ca^{2+} mobilization, while ERK activation requires Ca^{2+} influx from VGCC (13). To investigate the role of Ca^{2+} mobilization in GnRH regulation of TCF-dependent genes, we utilized the intracellular Ca^{2+} chelator BAPTA-AM. GnRH stimulation increases TOPflash activity while pretreatment with BAPTA blocks this activation (Fig. 2A) demonstrating Ca^{2+} mobilization is necessary for TCF-dependent transcription in LβT2 cells.

Next, we wanted to examine the role of intracellular Ca^{2+} on GnRH regulation of the IEGs. LβT2 cells were pretreated with Ca^{2+} chelator BAPTA-AM or DMSO control followed by treatment with vehicle or 10 nM GnRH for an hour. Cells were then lysed and real-time PCR performed with primers specific for the IEGs *Jun*, *Atf3* and *Egr1*. It has previously been reported that BAPTA-AM blocks activity of a *Jun*-Luc reporter construct in αT3 cells (13), yet endogenous gene expression in LβT2 cells has not been examined. Calcium mobilization was also shown to be necessary for ATF3 protein accumulation (21), however the mechanism of regulation whether it be protein stabilization or gene transcription was not explored. *Jun* (Fig. 2B) and *Atf3* (Fig. 2C) transcription downstream of GnRH was partially inhibited by BAPTA (74% and 59% reduction respectively), while *Egr1* transcription was reduced (40% reduction), although

the difference was not statistically significant (Fig 2D). This confirms previous work (13;22) and demonstrates that GnRH regulation of TCF-dependent transcription, as well as *Jun* and *Atf3* mRNA accumulation requires Ca^{2+} while *Egr1* does not demonstrating that not all IEGs require intracellular Ca^{2+}. Moreover, this suggests downstream Ca^{2+} targets, including calcineurin, may also be necessary for IEG transcription.

Calcineurin activity is required for *Jun* and *Atf3* transcription downstream of GnRH

Increased calcium can activate several downstream effectors including the Ca^{2+}-dependent protein phosphatase calcineurin (21;22;33). To further characterize the effect of Ca^{2+} on TCF transcriptional targets, LβT2 cells were transiently transfected with TOPflash, and then pretreated with the calcineurin inhibitor cyclosporin A (cycloA) or EtOH control followed by treatment with vehicle or 100 nM GnRH. Calcineurin inhibition reduced TOPflash activity downstream of GnRH (Fig. 3A), demonstrating that the phosphatase is necessary for TCF-dependent transcription of an artificial promoter.

To examine endogenous gene transcription, LβT2 cells were serum starved, pretreated with cycloA followed by treatment with vehicle or 10 nM GnRH for an hour. Real-time PCR was performed using primers specific for the IEGs as described above. CycloA reduced the amount of *Jun* transcript (51% reduction) downstream of GnRH (Fig. 3B), suggesting a role for the phosphatase in the regulation of *Jun* message. It has previously been reported that inhibition of calcineurin reduces ATF3 protein levels (22), and herein we demonstrate that *Atf3* transcript is also reduced (59%) (Fig. 3C), while *Egr1* message remains unaffected (Fig. 3D). These findings demonstrate that GnRH activates calcineurin; presumably downstream of Ca^{2+} and that this signaling cascade is necessary for *Jun* and *Atf3* transcriptional regulation but not all the IEGs. Increased *Egr1* mRNA is necessary for *Lhb* mRNA accumulation, while *Jun* and *Atf3* confer

hormonal responsiveness to the other gonadotrope specific genes, *Cga*, *Gnrhr* and *Fshb* suggesting that the Ca^{2+} response is specific for *Jun* and *Atf3* targets.

Nfat4 is expressed in LβT2 cells and GnRH increases the transcriptional activity of an NFAT responsive promoter

Calcineurin is a calcium dependent protein phosphatase that regulates the transcriptional activity of NFAT proteins (29;30;34-36). Microarray analysis indicated that LβT2 cells express one member of the NFAT family, *Nfat4* (official name *Nfatc3*) (unpublished data). To verify the microarray data, PCR was performed using primers specific for each of the 4 classical members of the NFAT family (37). LβT2 cells were serum starved overnight and then treated with vehicle or 10 nM GnRH for one hour followed by reverse transcription of the gene products. As shown in Figure 4A, LβT2 cells express *Nfat4*, while the other family members do not appear to be expressed. Furthermore, the transcript levels do not appear to be increased by GnRH stimulation (Fig. 4A), however quantitative PCR needs to be done to confirm this.

The transcriptional activity of NFAT proteins is regulated by post-translational phosphorylation. In the absence of signal, NFAT proteins are phosphorylated leading to accumulation in the cytoplasm and exclusion from the nucleus. These phosphate groups are removed by the Ca^{2+} dependent protein phosphatase calcineurin, allowing nuclear translocation of the NFAT proteins and increased transcriptional activity (29;29;30;34;36). To examine whether GnRH alters the transcriptional activity of NFAT, we utilized an NFAT responsive promoter reporter (3 NFAT response elements from the promoter of the IL-2 gene fused to luciferase) (kindly provided by Neil Clipstone (38)). LβT2 cells were transfected with the NFAT-Luc reporter and then treated with vehicle or 100 nM GnRH. In the absence of hormone the activity of the NFAT-Luc was low, while GnRH significantly increased the amount of luciferase activity (Fig 4B). These results

demonstrate that GnRH regulates activity of an NFAT responsive promoter in a gonadotrope cell line.

GnRH regulation of an NFAT responsive promoter requires Ca^{2+} mobilization and calcineurin activity

To confirm that GnRH regulation of an NFAT responsive promoter requires intracellular Ca^{2+} release, LβT2 cells were transfected with an NFAT-Luc reporter and then pretreated with Ca^{2+} chelator BAPTA-AM. The cells were then treated with vehicle or 100 nM GnRH followed by a luciferase assay. GnRH induced transcriptional activation of NFAT-Luc promoter reporter, and pretreatment with BAPTA-AM abolished luciferase activity (Fig. 5A). This demonstrates that GnRH regulation of the NFAT responsive promtoer requires intracellular Ca^{2+} and suggests downstream targets such as calcineurin may also be necessary.

To examine if the activation of the NFAT-Luc reporter is dependent on calcineurin, cells were transfected and the pretreated with cycloA for an hour followed by treatment with vehicle or 100 nM GnRH. After treatment cells were lysed and a luciferase assay was performed. GnRH mediated activation of an NFAT responsive promoter requires calcineurin activation (Fig. 5B, while inhibitors specific for other Ca^{2+} targets such as CaMKK (Fig. 5C) or CaMKII (data not shown) do not affect luciferase activity.

We also examined the role of JNK in NFAT-dependent transcriptional activation by GnRH. Cells were transfected with NFAT-Luc and then pretreated with SP600125 (25 uM) for an hour followed by vehicle or GnRH and luciferase assay. Inhibition of JNK did not reduce luciferase activity (Fig. 5D) suggesting that JNK is not required for NFAT-dependent transcription downstream of GnRH. These findings demonstrate that GnRH activates NFAT-dependent transcription downstream of Ca^{2+} and calcineurin, which is

the first report of NFAT transcriptional regulation downstream of GnRH. It also provides evidence that activity of an NFAT promoter occurs independently of JNK, while a TCF promoter requires Ca^{2+}, calcineurin and JNK activity.

DISCUSSION

We have demonstrated that JNK activation is required for TCF-dependent transcription, as well as *Jun* and *Atf3* transcription downstream of GnRH. GnRH also increases intracellular Ca^{2+} concentrations, and increased Ca^{2+} can crosstalk with members of the MAPK signaling family (4;13;26;27). It has been shown that Ca^{2+} mobilization from intracellular stores affects JNK activity, while Ca^{2+} influx affects ERK phosphorylation and activity necessary for *Egr1* (14;24). TOPflash activation by GnRH requires JNK activation (Fig.1), while inhibition of ERK had no effect on the promoter reporter (data not shown). As such we investigated the role of intracellular Ca^{2+} mobilization on TCF-dependent transcription and IEG regulation downstream of GnRH. Removal of intracellular Ca^{2+} fully blocked GnRH regulation of TOPflash, as well as significantly reduced *Jun* and *Atf3* transcript levels while *Egr1* was not significantly affected. Furthermore, we have shown that GnRH regulates an NFAT responsive promoter in LβT2 cells, and hypothesize that NFAT is required for full GnRH activation of *Jun* mRNA accumulation.

GnRH activation of JNK takes approximately 30 minutes to reach its peak activity level (8;12). Interestingly, JNK has also been shown to export NFAT4 from the nucleus in BHK cells (39), suggesting that JNK may antagonize NFAT4 gene products. The data presented here suggest that calcineurin activation downstream of GnRH may regulate NFAT-dependent transcription that in turn facilitates NFAT4 nuclear import and increased transcriptional activity. Therefore, it is possible that GnRH stimulates nuclear import that is followed soon thereafter by export. This is intriguing as *Jun*/JUN responds to GnRH pulses, such that it needs to be rapidly upregulated and then downregulated in order to be primed to respond to the next GnRH pulse. Specifically, calcineurin may stimulate nuclear localization of NFAT, while JNK is important for export providing both positive and negative controls for *Jun* transcription. Further studies using

immunocytochemistry to examine cellular localization will be necessary to determine import/export patterns and could provide more insight into the regulation of *Jun* transcription.

NFAT proteins have been shown to cooperate with JUN bound to AP1 sites on the promoter of several genes important for the immune response, including *IL-2*, *IL-3*, *IL-5* and *FasL* (reviewed in (30;40)). The ability of JUN and NFAT proteins to cooperate suggests NFAT may also contribute to the transcriptional regulation of JUN dependent genes in the gonadotrope including *Jun*, *Cga*, *Gnrhr* and *Fshb*. Interestingly, maximal transcription of *FasL* involves calcineurin and activation of NFAT and AP1 (41;42). Furthermore, cooperation has also been demonstrated between PKCθ and calcineurin to induce JNK and activate *IL-2* (43). These findings suggest that calcineurin and JNK may regulate multiple genes through JUN and NFAT. We have demonstrated that both are important for maximal *Jun* and *Atf3* transcript levels, suggesting that both genes may be targets of NFAT. Future studies are needed to elucidate downstream NFAT targets in gonadotrope cells, including *Jun* and the gonadotrope specific genes *Cga*, *Gnrhr* and *Fshb*.

GnRH has been shown to transcriptionally regulate calcineurin in αT3 cells (21), however an endogenous target gene was not identified. Herein, we demonstrate the necessity of calcineurin for *Jun*, *Atf3* and NFAT-dependent gene transcription. Calcineurin has been demonstrated to be necessary for *Fshb* regulation through an association with HDAC although direct targets remain unknown. *Fshb* was shown to be repressed by HDAC, leading to differences in *Lhb* and *Fshb* transcriptional regulation (21), which is important particularly during the LH surge. Most work is done in αT3 cells to investigate GnRH signaling; however there are some differences between the cell lines and signaling pathways activated. It is also possible that GnRH regulates expression of *Fshb* through multiple mechanisms, providing more control for secretion of

the hormone. We utilized the LβT2 cell line to look at calcium dependent signaling and have shown that GnRH may use calcineurin as a regulator of NFAT target genes. It is unknown if calcineurin is regulated by GnRH in LβT2 cells, however the ability of cyclosporin A to inhibit *Jun*, *Atf3* and NFAT-dependent transcription provides evidence that the activity of calcineurin is modulated by the neurohormone and that NFAT targets genes are also regulated.

There are multiple signaling pathways activated once GnRH binds to its receptor on gonadotrope cells (reviewed in (4)). The mobilization of Ca^{2+} regulates TCF-dependent transcription, both of an artificial reporter promoter and the endogenous TCF-dependent gene *Jun*. Furthermore, *Jun* and *Atf3* are regulated differently than *Egr1*, as Ca^{2+} and calcineurin activation are only required for *Jun* and *Atf3,* not *Egr1*. Calcineurin activation is also necessary for TCF-dependent gene transcription, as well as regulation of an NFAT responsive promoter downstream of GnRH. Both TCF and NFAT are important transcription factors for T-cells in the immune system (34;44), and herein we report that reporter constructs dependent on the two are regulated through similar pathways. Additionally, the cooperation of NFAT: AP1 proteins in immune cells (45), and the requirement of AP1 sites on several gonadotrope specific genes (32;46-49) suggests that they may be targets of NFAT4. Work is currently in progress to examine the role of NFAT4 in transcription of *Jun*, and possibly JUN targets *Cga*, *Gnrhr* and *Fshb*. We hypothesize that *Jun* transcription will require a functional interaction between Ca^{2+}/calcineurin and NFAT in addition to TCF/β-catenin and JNK downstream of GnRH in gonadotrope cells.

MATERIALS AND METHODS

Chemicals

GnRH, DMSO, BAPTA-AM and PBS were purchased from Sigma Chemical Company (St. Louis, MO); TRIzol and Lipfectamine were purchased from Invitrogen (Carlsbad, CA); the p38 MAPK inhibitor SB203580, CamKII inhibitors KN62 and KN93/92, CAMKK inhibitor STO609, and cyclosporin A were purchased from Calbiochem (EMD Biosciences Inc, La Jolla, CA); ERK inhibitor PD98059 was purchased from Alexis Biochemicals (San Diego, CA) and the JNK inhibitor SP600125 was purchased from Biosource International Inc. (Camarillo, CA).

DNA Constructs

The TOPflash luciferase reporter vector was purchased from Upstate Biotechnology Inc. (Lake Placid, NY) and previously described (17). The NFATΔNEO-Luc construct was kindly provided by the laboratory of Dr. Neil A. Clipstone (Loyola University Medical School, Chicago, IL). The construct was modified by removal of the SV40 ori from NFAT-Luc as described previously (38).

Cell Culture and Transient Transfections

LβT2 cells (provided by P. Mellon, UCSD, San Diego CA) were maintained at 37 °C with 5% CO_2 in high-glucose DMEM supplemented with 10% fetal-bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen Life Technologies) for the duration of experiments. For transient transfection LβT2 cells were plated (250,000 cells/well in a 24-well plate) in complete media for 24 hours. Cells were then washed with PBS and a transfection mixture containing DNA, lipfectamine (1.6 uL/well; Invitrogen Life Technologies) and DMEM was allowed to form liposomes for 30 minutes and then

added to the cells. Twelve to sixteen hours later, vehicle or inhibitor was added to the cells (in DMEM/1%penicillin-streptomycin) for 60 minutes followed by treatment with PBS or 100 nM GnRH for eight hours. Cells were maintained in the presence of the inhibitor listed for the duration of the experiment. After 8 hours, cells were washed with PBS, lysed and reporter gene activity was assayed (Single Luciferase Reporter Assay Kit, Promega Corp., Madison WI).

For examination of RNA, LβT2 cells were plated (500,000 cells/well, 35 mm plates) and incubated in complete media overnight. Twenty-four hours after plating, cells were serum starved overnight (12-16 hours), followed by pretreatment with the inhibitor for 60 minutes. Cells were then treated with 10 nM GnRH for one hour in the presence of inhibitor before being washed with PBS and 1 mL TRIzol® was added.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from the cells following the manufacturers' protocol using diethylpyrocarbonate (DEPC) treated water. One half ug total RNA was used to synthesize cDNA using 4 uL Quanta cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) and 20 uL reactions (total volume) were performed following the manufacturers protocol. cDNA samples were diluted 1:10 in DEPC treated water at stored at -20 C.

End point PCR

To investigate the expression of the *Nfat* family members, standard PCR was performed using 5 uL of diluted cDNA using primers specific for *Nfat1*, *Nfat2*, *Nfat3* and *Nfat4* as described by (50). Standard PCR methods were followed and 27 cycles were run. Samples were then run on a 1.5% agarose gel and subjected to UV light.

Quantitative Real-Time PCR

Relative mRNA levels were determined using Quantitative Real-Time (QRT) PCR and the 7000 ABI prism sequence detection software system (Applied Biosytems, Foster City, CA). Primer Express 2.0 software (Applied Biosystems, Foster City, CA) was used to design intron spanning primers (except *Jun* which lacks apparent introns). Primer sets were optimized using a range of primer concentrations and 300 nM was optimal for *Atf3*, *Jun* and *cyclophilin B* (officially *Ppib*), while 150 nM was optimal for *β-catenin* and *Egr1* primer sets. Primer efficiency was then determined over a range of cDNA dilutions (1:1, 1:10, 1:100, 1:1000). The amplification efficiency of the QRT reaction for each gene was between 93% and 105% and therefore relative levels of expression were calculated using the comparative method ($\Delta\Delta C_T$). *Cyclophilin B* expression was used to normalize samples. Samples were assayed in triplicate.

For each QRT-PCR reaction, a master mix containing: 10 μL of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and primers were added to 5 μL of cDNA. The final volume of a QRT-PCR reaction was 20 μL. QRT-PCR reaction conditions were: 50^0 C for 2 min, 95^0C for 10 min; then 40 cycles of 95^0C for 3 sec, and 60^0 for 30 sec. Primers specific for *Jun*, *Atf3*, *Egr1* and cyclophilin B (to normalize cDNA samples) were used as previously described (17).

Statistics

Reporter activity and differences in gene expression during the various treatment protocols were analyzed using one-way ANOVA and differences were determined using the Tukey post hoc test. P values for each experiment are listed in the figure legends.

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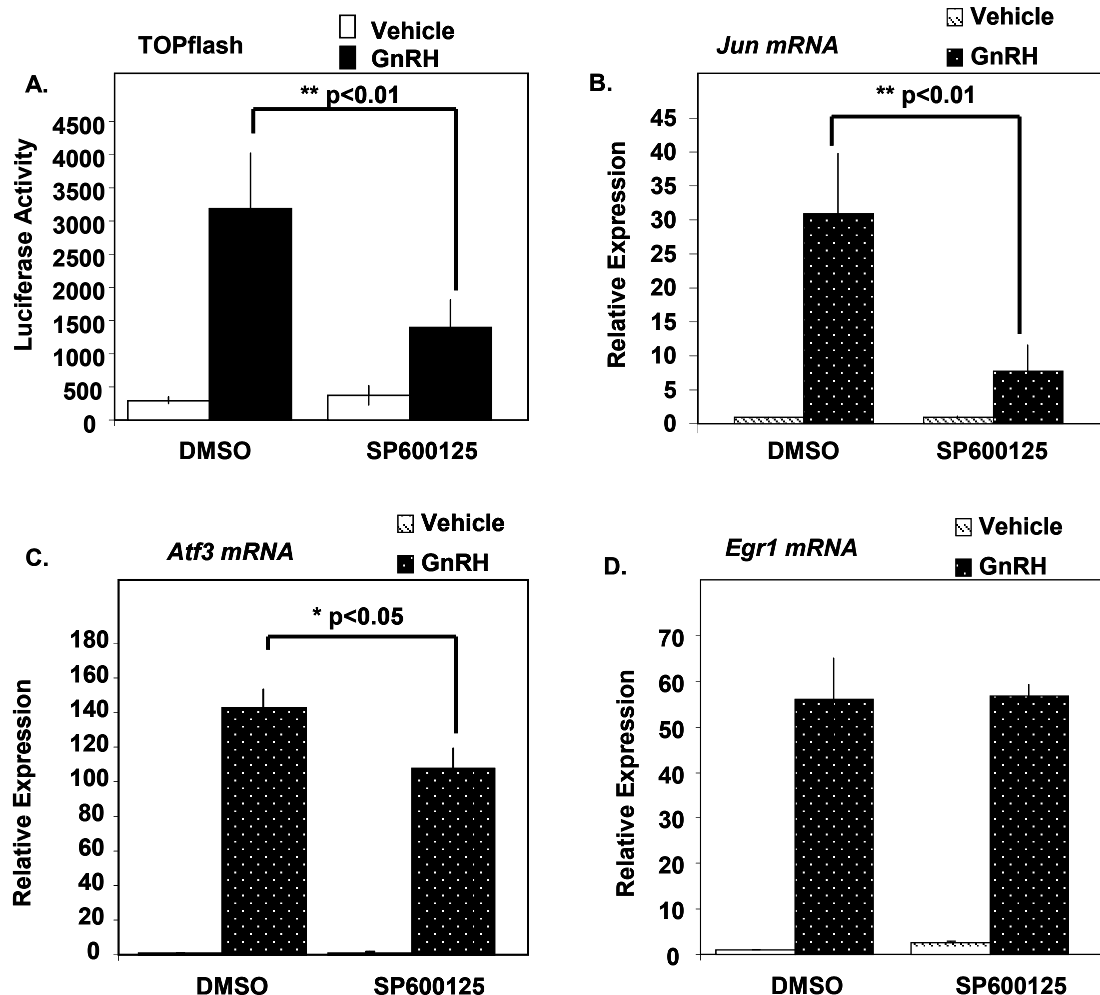

Figure 1: JNK activity is required for GnRH regulation of TCF-dependent transcription and accumulation of *Jun* and *Atf3* transcripts

A.) LβT2 cells were transiently transfected with TOPflash (50 ng) and then pretreated with vehicle (DMSO) or the JNK inhibitor SP600125 (25 uM) for one hour. The cells were then treated with vehicle (PBS) or 100 nM GnRH for 8 hours, and luciferase activity was measured. The data shown are the means +/- SEM from three independent experiments performed in triplicate. B-D.) LβT2 cells were serum starved overnight and then pretreated with vehicle (DMSO) or the JNK inhibitor SP600125 (25 uM). Cells were then treated with vehicle (PBS) or 10 nM GnRH for an hour and then RNA was isolated. After reverse transcription of cDNA, real-time PCR was performed using primers specific for *Jun* (B), *Atf3* (C) or *Egr1* (D). Data shown averages +/- SD from two independent experiments. *, $p<0.05$, **, $p<0.01$

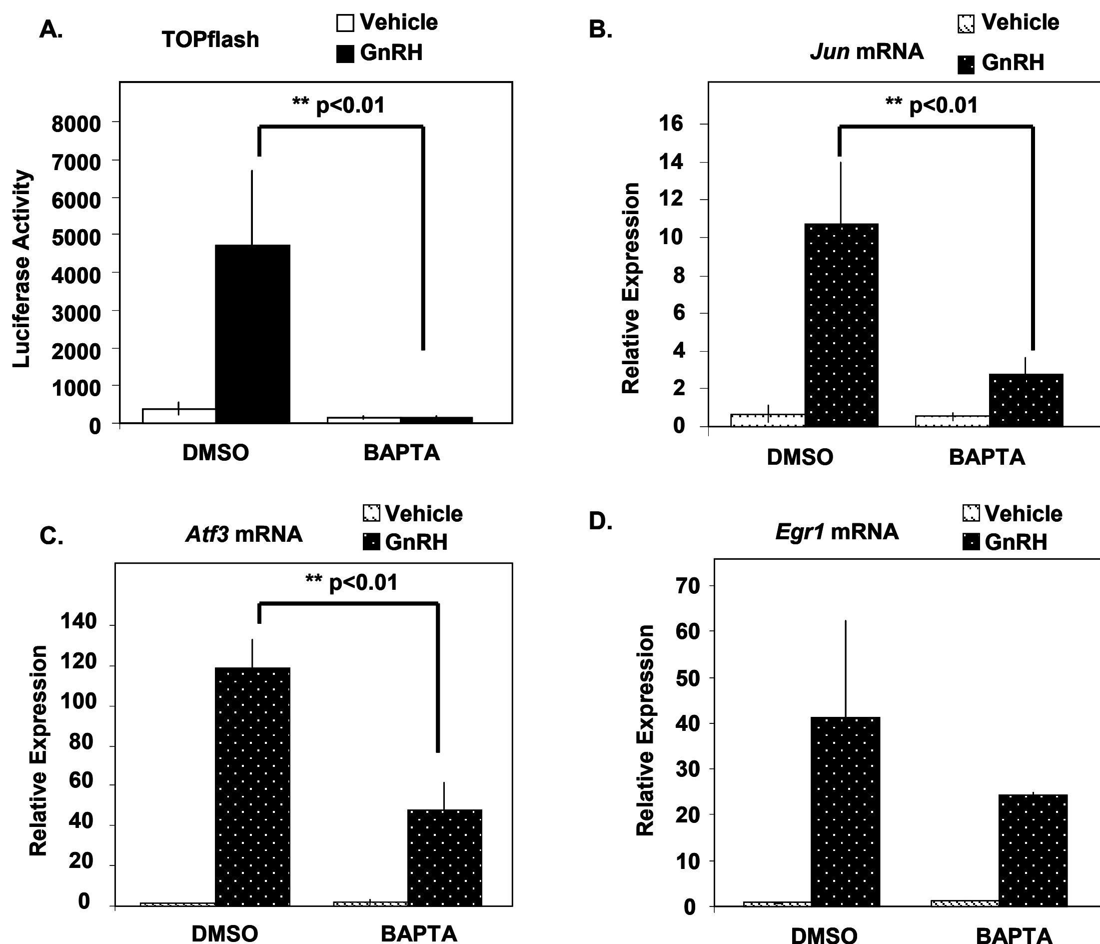

Figure 2: Ca^{2+} mobilization is necessary for GnRH activation of TCF-dependent transcription and *Jun* and *Atf3* accumulation

A.) LβT2 cells were transfected with TOPflash (50 ng) and then pretreated with vehicle (DMSO) or Ca^{2+} chelator BAPTA-AM (50 nM) for an hour. After treatment the cells were then stimulated with vehicle (PBS) or 100 nM GnRH for 8 hours followed by a luciferase assay. B-D.) Serum starved LβT2 cells were pretreated with vehicle (DMSO) or BAPTA-AM (50 uM) for an hour. The cells were treated with vehicle (PBS) or 10 nM GnRH for an hour before RT-PCR was performed using primers specific for *Jun* (B), *Atf3* (C) or *Egr1* (D). Data shown are the means +/- SEM from three independent experiments (A,B) or average +/- SD from two independent experiments (C,D). **, $p<0.01$.

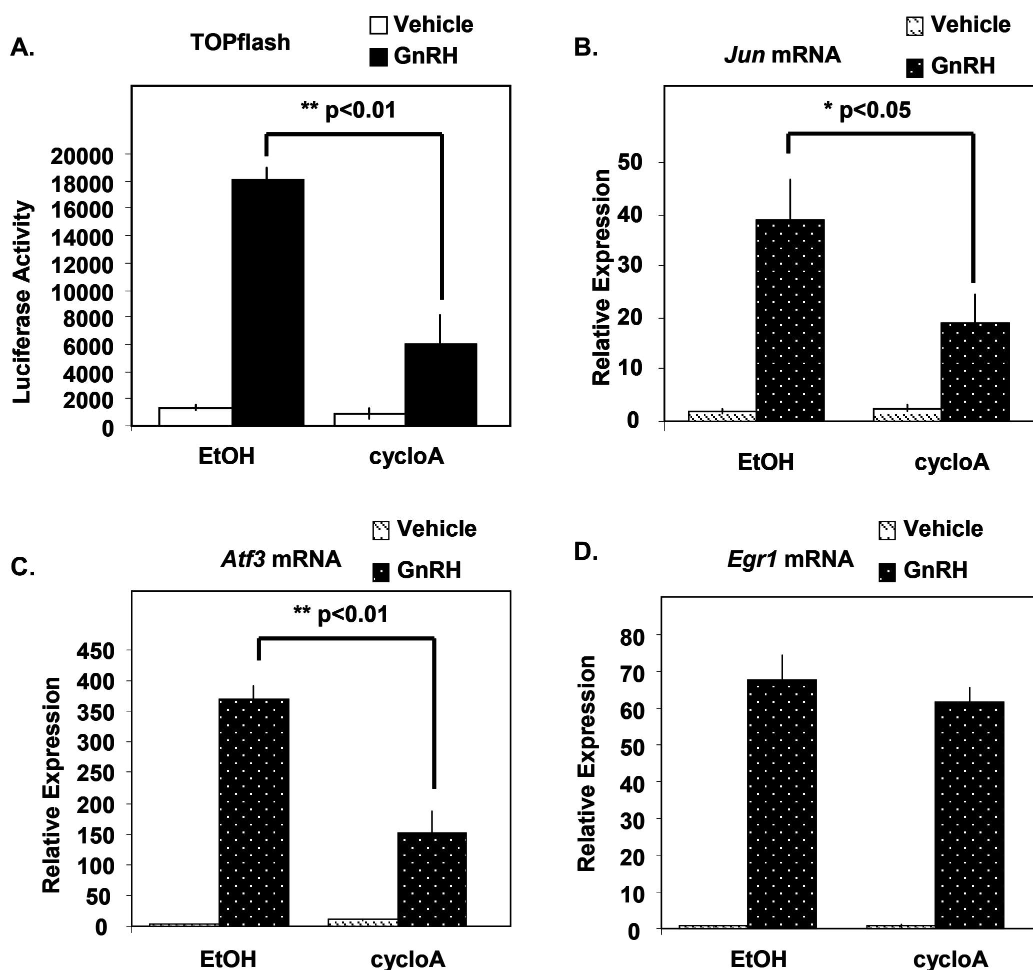

Figure 3: Calcineurin inhibition reduces GnRH mediated TOPflash activation and *Jun* and *Atf3* transcript accumulation

A.) TOPflash was transected into LβT2 cells and then treated with vehicle (EtOH) or calcineurin inhibitor cyclosporin A (200 nM) for an hour followed by vehicle or 100 nM GnRH treatment for 8 hours. After cell lysis, luciferase activity was measured. Data shown is average +/- SD of two independent experiments each performed in triplicate. B-D). LβT2 cells were serum starved and then pretreated with vehicle (EtOH) or cycloA (200 nM) for an hour. Cells were then stimulated with vehicle (PBS) or 10 nM GnRH for an hour and RT-PCR was done using primers specific for *Jun* (B), *Atf3* (C) and *Egr1* (D). Data shown are the means +/- SEM from three independent experiments. *, p<0.05, **, p<0.01.

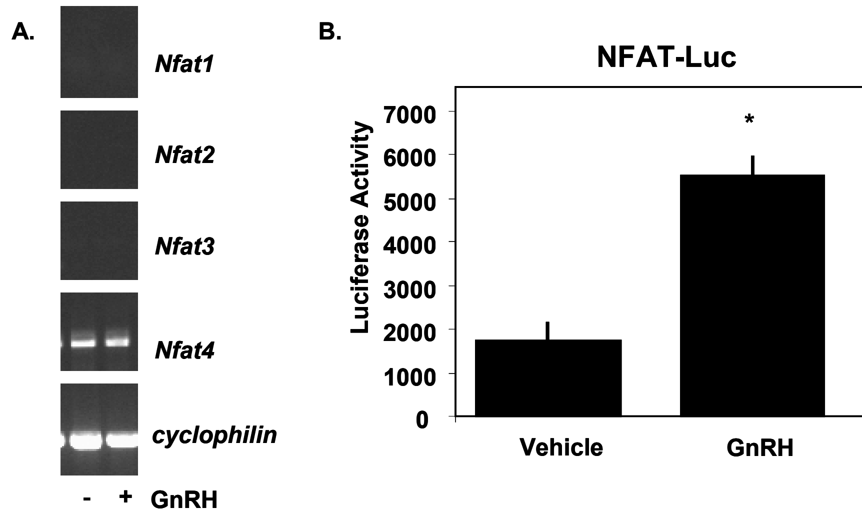

Figure 4: *Nfat4* is expressed in LβT2 cells and GnRH regulates NFAT-dependent transcription

A.) LβT2 cells were serum starved and then treated with vehicle (PBS) or 10 nM GnRH for one hour. PCR was performed on reverse transcribed RNA using primers specific for *Nfat1*, *Nfat2*, *Nfat3* and *Nfat4* and cyclophilin. Data shown is from two independent experiments. B.) LβT2 cells were transfected with NFAT-Luc, treated with vehicle (PBS) or 100 nM for 8 hours before luciferase activity was measured. Data shown is the mean +/- SEM from three independent experiments each performed in triplicate. *, $p<0.01$.

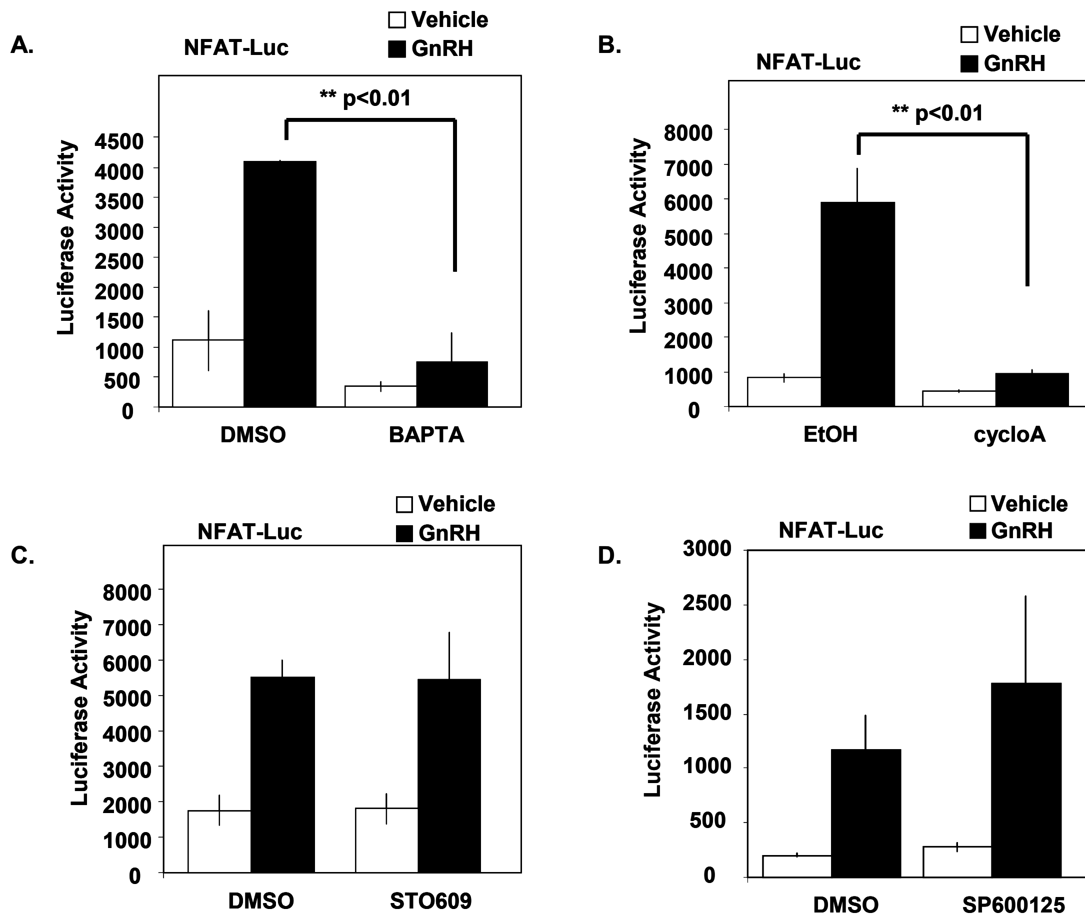

Figure 5: GnRH regulation of NFAT-dependent transcriptional activity requires Ca^{2+} mobilization and calcineurin activity

A-D.) LβT2 cells were transfected with NFAT-Luc (200 ng) and then pretreated with the inhibitor listed for an hour. After pretreatment, cells were stimulated with vehicle (PBS) or 100 nM GnRH for 8 hours and luciferase activity was measured. A.) Cells were treated with vehicle (DMSO) or BAPTA-AM (50 uM). Data shown is the average +/- SD from two independent experiments each performed in triplicate. B.) LβT2 cells were pretreated with vehicle (EtOH) or cyclosporin A (200 nM). C.) Cells were pretreated with the Calmodulin Kinase Kinase inhibitor STO609 (10 uM). D.) Cells were pretreated with vehicle (DMSO) or JNK inhibitor SP600125 (25 uM). Data shown are the means +/- SEM from three independent experiments performed in triplicate. *, p<0.05, **, p<0.01.

CHAPTER SIX

CONCLUSIONS/FUTURE DIRECTIONS

CONCLUSIONS

GnRH regulation of the four gonadotrope signature genes, *Lhb*, *Fshb*, *Cga* and *Gnrhr* requires activation of multiple signaling pathways and transcription factors (1;2). Steroidogenic factor 1 (SF1) is necessary for transcription of all four genes (3;4) however, it is not sufficient to induce expression (5;6). In addition to SF1, increased transcription and translation of several immediate early genes (IEGs) are required to convert the GnRH signal to the distal responsive genes (reviewed in (7)). A good example of this is the regulation of *Lhb/LHB* downstream of GnRH. While SF1 alone was able to increase transcriptional activity of the bovine *LHB*-Luc construct, maximal activity was not observed until both SF1 and EGR1 were expressed. Furthermore, the synergistic properties of SF1 and EGR1 required SF1 and β-catenin interactions (Chapter 3, (8)).

Beta-catenin is a co-factor important for cellular proliferation, differentiation and hormone response (9;10). Work presented in Chapter 3 demonstrates the necessity of β-catenin and SF1 interactions for GnRH regulation of *Lhb/LHB* gene expression. We demonstrated that removal of β-catenin from LβT2 cells reduces the synergy normally observed between SF1 and EGR1 in regulating the bovine *LHB*-Luc promoter reporter. GnRH also regulates the mass of β-catenin (8;11) and the localization of the co-factor on the *Lhb* murine promoter (8). Beta-catenin is an essential transcriptional co-factor for WNT signaling through interactions with T-cell factor/lymphoid enhancer factor (TCF/LEF) target genes (11-13). The regulation of β-catenin by GnRH suggests that TCF- dependent genes may also be important for GnRH signaling.

To investigate the role of TCF in GnRH signaling TOPflash, an artificial TCF-dependent promoter reporter, was transfected into LβT2 cells. GnRH treatment increased the transcriptional activity of TOPflash (Chapter 4, 5 (12;14)) and this

activation was shown to require Gαq (14) and β -catenin (12;14). The ability of GnRH to regulate TOPflash in a manner dependent on G proteins suggests TCF/β-catenin interactions may also be necessary for downstream targets of the neurohormone. In this regard, TCF and β-catenin interactions were found to be necessary for *Jun*/JUN accumulation downstream of GnRH. Furthermore, reduction of β-catenin protein levels within LβT2 cells blocked maximal accumulation of Jun/JUN. Finally, the loss of TCF and β-catenin interactions reduced the transcriptional activity of *CGA*-Luc, a JUN target gene (Chapter 4 (12)).

The signaling pathway that regulates TOPflash and *Jun*, an endogenous TCF/β-catenin target, was investigated in Chapter 5. Activation of JUN N-terminal kinase (JNK) is necessary for TOPflash and increased *Jun/Atf3* transcript. JNK activation has been reported to require Ca^{2+} (15;16), so we examined the role of the second messenger in LβT2 cells. Intracellular calcium mobilization and calcineurin activation were found to be necessary for TOPflash activation by GnRH as well as *Jun* and *Atf3* mRNA accumulation but not *Egr1*. Nuclear factor of T-cells (NFAT) proteins are common targets of Ca^{2+} and calcineurin, and *Nfat4* (officially *Nfatc3*) was found to be expressed in LβT2 cells. Furthermore, GnRH was able to activate a NFAT responsive promoter reporter suggesting that NFAT targets may be regulated through Ca^{2+} mobilization and calcineurin activation in LβT2 cells (Chapter 5).

The work presented here provides new insights into the GnRH transcriptional network; however the complete story is still unknown. For example, GnRH regulates TCF-dependent transcription downstream of both JNK and calcineurin, yet it is still unknown if this same pathway increases the abundance of β-catenin or if another player is involved. We have also demonstrated that *Nfat4* is expressed and the transcriptional activity of NFAT is regulated by GnRH, yet endogenous targets are still unknown.

Future work to determine how β-catenin is regulated by GnRH, identify NFAT target genes and calcineurin targets, and examine the role of the proteins *in vivo* gonadotrope function with an intact HPG axis would provide a more complete story of β-catenin and NFAT in the anterior pituitary.

FUTURE DIRECTIONS

Regulation of β-catenin by GnRH

Interactions of β-catenin with both SF1 and TCF suggest the co-factor is important for GnRH signaling. Beta-catenin is also regulated by GnRH and the results presented by Gardner and colleagues (14) and in this dissertation suggest that JNK and Ca^{2+}/calcineurin are important downstream effectors (Chapter 5). While we know some of the target genes for regulation of TCF-dependent transcription, the signaling pathway that increases the mass of β-catenin has yet to be fully elucidated. Beta-catenin is important for both SF1- and TCF-dependent transcription, however the roles that these two proteins play is different. SF1 is important for basal transcription of the gonadotrope specific genes (3;4) and is not regulated by GnRH (5;6;8), while TCF is necessary for GnRH mediated increases in *Jun*/JUN (12). Regulation of the GnRH transcriptional network is complex, and requires input from multiple signaling pathways (17;18).

As an original member of the WNT signaling pathway, β-catenin has also been shown to be regulated downstream of PKC (19), PKA (20;21), and SRC (22). GnRH activation of JNK is also reported to involve activation of SRC (23-26). This suggests that SRC may be a potential point of convergence for the regulation of β-catenin and TCF-dependent transcription. In this respect, we have unpublished data suggesting that SRC inhibition reduces GnRH mediated activation of TOPflash. SRC inhibitors are very unspecific (27;28), therefore further investigation into the role of SRC in GnRH regulation of *Jun* and TOPflash would best be examined using dominant negative adenoviral constructs. Additionally, the role of Ca^{2+} and calcineurin in regulation of β-catenin abundance and cellular localization could be further examined using cellular fractionation and western blotting. Immunocytochemistry including live cell microscopy could also be utilized to explore this possibility in LβT2 cells.

In the absence of signal, β-catenin is phosphorylated by glycogen synthase kinase 3β (GSK3β) and targeted for degradation (19;29). GnRH regulates the phosphorylation of GSK3β in HEK cells expressing the *Gnrhr* (14) and in LβT2 cells (our unpublished data). Interestingly, NFAT is also a target of GSK3β (30;31), suggesting multiple roles for the kinase in gonadotrope cells. Phosphorylation of NFAT maintains cytoplasmic stores of the protein, removal of these phosphate groups by calcineurin leads to nuclear import (30;31). GnRH may control NFAT dependent transcription at two levels, both through inhibition of a kinase and activation of a phosphatase. Dual regulation such as this has been demonstrated in granulosa cells downstream of LH (32). This may allow two unique branches of the GnRH signaling pathway to converge and allow both β-catenin and NFAT to localize in the nucleus and increase transcriptional activity. Examining the nuclear localization of both β-catenin and NFAT using immunocytochemistry under varying cell conditions could provide insight into how GnRH regulates the two pathways. Additionally, the role of GSK3β can be examined using siRNA or constitutively active constructs to establish the role of the kinase in regulation of both β-catenin and NFAT.

NFAT in the Gonadotrope Transcriptional Network

GnRH regulation of NFAT-dependent artificial promoter suggests that NFAT target genes may be important in the gonadotrope transcriptional network (Chapter 5). We have demonstrated that this regulation requires both intracellular Ca^{2+} mobilization and calcineurin activation, while other Ca^{2+} targets downstream of GnRH do not affect NFAT transcriptional activity. Calcium and calcineurin activity were also found to be necessary for *Jun* and *Atf3* mRNA accumulation downstream of GnRH (Chapter 5). This suggests that *Jun* and *Atf3* may be endogenous NFAT targets. Furthermore, TCF-

dependent transcription was also shown to require Ca^{2+} and calcineurin suggesting that TCF- and NFAT- dependent transcription may be regulated through similar mechanisms. This suggests that GnRH regulation of *Jun* occurs through at least three distinct pathways – TCF/β-catenin, Ca^{2+}/calcineurin/NFAT, and JNK. Full activation of JUN may be regulated directly by JNK and require a functional interaction with NFAT. While functional interactions of TCF/β-catenin and JNK/JUN have been shown to regulate the expression of *Jun*, we have demonstrated that Ca^{2+}/calcineurin are necessary and suggest that NFAT is also required.

We have shown that TCF-dependent transcription requires calcineurin and JNK, while NFAT only requires Ca^{2+} and calcineurin activity. The promoter regulatory region of *Jun* contains an AP1 response element (7;33;34) and JUN autoregulates its own transcription through phosphorylation and activation by JNK (35). NFAT proteins cooperate with JUN to regulate target genes (31;36-38); suggesting that GnRH regulation of *Jun* may require a functional interaction between β-catenin, TCF and NFAT. To further examine this, reduction of *Nfat4* should reduce *Jun* mRNA expression. This would suggest that NFAT is necessary for GnRH regulation of the oncogene. Co-immunoprecipitation studies could provide evidence for cooperation and interaction between the proteins. For example, an immunoprecipitation with an NFAT4 specific antibody should pull down JUN protein as examined by western blot, demonstrating that these two proteins interact downstream of GnRH. Furthermore, Chromatin-immunoprecipitation (ChIP) to look at the recruitment of NFAT to the promoter region of the *Jun* gene would indicate a novel NFAT target in gonadotrope cells, and provide multiple inputs for gene regulation.

AP1 response elements are also necessary for regulation of *Cga*, *Fshb* and *Gnrhr* (7;33;34) suggesting cooperation between NFAT and JUN may also be important

for distal responsive genes. It is also possible that genes not dependent on JUN are NFAT dependent, and *Nur77* falls into this category. *Nur77* is an NFAT target gene in T-cells and thymocytes (39), and has been found to be regulated by calcineurin (40) and PKA downstream of GnRH (41). The dependence of NFAT downstream of GnRH could be studied using a dominant negative NFAT adenovirus or siRNA specific for *Nfatc3* as described above. It is also possible that this interaction is cell specific and NFAT works through activation of other responsive genes to confer hormonal responsiveness.

Calcineurin is an important downstream target of Ca^{2+} downstream of GnRH signaling (40;42) and we have demonstrated that it is an important mediator of *Jun* and *Atf3* mRNA accumulation and regulation of TCF and NFAT responsive promoters. Calcineurin is a protein phosphatase with many targets suggesting that GnRH may regulate other proteins through calcineurin. Several approaches could be taken to examine other calcineurin targets, including overexpression of a constitutively active calcineurin (42), expression of a dominant negative form of the protein, or reduction using siRNA. Microarray under these various cellular conditions could provide novel calcineurin dependent genes that are important for gonadotrope function and regulation of LH and FSH.

The work presented here demonstrated that *Jun* and *Atf3* are calcineurin targets in LβT2 cells. Since both JUN and ATF3 confer hormonal responsiveness to gonadotrope specific genes, we expect *Cga*, *Fshb* and *Gnrhr* to require calcineurin activation. It is unknown if this is a direct affect on NFAT proteins, or if other calcineurin targets are activated. The specificity of calcineurin on NFAT proteins could be examined following pretreatment with cyclosporin A or a broader phosphatase inhibitor and then examine the phosphorylation state of NFAT following co-immunoprecipitation, or through immunocytochemistry and the localization of the transcription factor.

***In Vivo* Role of β-catenin in Mature Gonadotropes**

The work presented in this dissertation demonstrates the importance of β-catenin in *in vitro* cell cultures systems, and suggests that it may also be necessary for *in vivo* regulation of gonadotropins. This could be verified using a gonadotrope cell specific *Cre recombinase* mouse model crossed with a floxed β-catenin animal as was used to reduce granulosa cell specific expression of the co-factor in the ovary. Beta-catenin is essential for pituitary development (43;44); it will be necessary to reduce expression after development of the gland. This could be accomplished using a *Cre*-driven promoter that turns on after development of the gland to study the *in vivo* effects of reduction. A gonadotrope specific *LHB*-cre driven promoter was recently developed in the laboratory of Sally Camper (45) that expresses *Cre*-recombinase after e16.5. We have obtained these animals and set up crosses between male *LHB-cre* positive/floxed β-catenin animals with homozygous floxed β-catenin females expecting 25% of the offspring to be homozygous floxed/*LHB-cre* positive knock-out animals. In examining over 180 animals, we have identified only 2 knockout animals (unpublished data). The *Lhb-cre* animals were also shown to have testis specific activity of CRE recombinase (45) suggesting that reduction of β-catenin in germ cells may be lethal to the spermatids and reduce the population of sperm. The role of β-catenin in testis biology and spermatogenesis is currently being further examined in the laboratory. Another possible approach would be to use a hemizygous floxed β-catenin animal as done in granulosa cells (46) to reduce the number of recombination events necessary to produce knock-out animals. Without completing these studies, it is difficult to place β-catenin as an essential piece of the HPG axis *in vivo*, however the *in vitro* studies presented here (8;12) and demonstrated by Gardner and colleagues (14) suggest it is.

Summary

Beta-catenin has become an important co-factor necessary for gonadotrope gene regulation through interactions with two different transcription factors. Additionally, we have welcomed NFAT into the neighborhood. Much work has been done to look at the regulation of the IEGs and further downstream gonadotrope specific genes by GnRH, but we believe the story is not complete. Identification of NFAT targets and calcineurin targets within the gonadotrope transcriptional network can provide more insight into the differential regulation of gonadotrope gene regulation by GnRH.

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