Regulation of pituitary gonadotropin synthesis and fertility by gonadotropin-releasing hormone and activin signaling

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March 2014

Thesis submitted to McGill University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Abstract

Infertility is a major clinical problem, affecting up to 10% of couples of reproductive age worldwide. Infertility can be caused by dysfunctions in many organs that normally ensure homeostasis of the reproductive system. The pituitary gland, a small endocrine organ situated at the base of the brain, synthesizes and releases two dimeric glycoprotein hormones (so-called gonadotropins) that regulate reproductive function: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Abnormal production and secretion of LH and FSH by pituitary gonadotrope cells is commonly seen in infertile patients. Factors originating from the gonads (testicles and ovaries), the brain, and the pituitary regulate gonadotrope function. Among these, brain-derived gonadotropin-releasing hormone (GnRH) potently stimulates LH and FSH production and secretion. Activins, produced by the pituitary gland, selectively stimulate FSH synthesis. This effect is antagonized by gonad-derived hormones of the same family, inhibins. The mechanisms whereby gonadotrope cells transduce these various signals are poorly understood. In this thesis, I first used genetic tools in mice to probe the signaling mechanisms mediating activin regulation of FSH synthesis *in vivo*. Canonically, activins signal through the receptor-regulated SMAD proteins, SMAD2/3, which partner with SMAD4 to activate gene transcription in cooperation with interacting factors. Results from my gonadotrope-specific knockout studies indicate that FSH synthesis and fertility do not require SMAD2 or the DNA-binding activity of SMAD3, but depend on the individual and collective activities of SMAD4 and its cell-restricted interacting partner, forkhead box L2 (FOXL2). Then, using model cell lines, I explored the mechanism of GnRH-regulated human LH β subunit gene (*LHB*) transcription, and found that it is similar to other mammals. However, I observed that activin impairs GnRH-stimulated human *LHB* promoter activity, whereas the two hormones synergistically activate the murine *Lhb* promoter. The inter-species difference maps to divergent regulatory elements within the proximal *LHB/Lhb* promoters and involves physical and functional interactions between SMAD3 and a transcription factor crucial for LHβ synthesis, early growth response 1 (EGR1). Finally, by generating a knock-in mouse line, I found that a unique structural feature of the mammalian GnRH receptor is required for normal gonadotropin synthesis and female fertility. The novel insights obtained from this work contribute to our understanding of how gonadotropins are normally synthesized, and may guide the development of therapeutics to stimulate or inhibit their production.

Résumé

L'infertilité est un problème clinique majeur qui affecte jusqu'à 10% des couples qui tentent de concevoir un enfant. L'infertilité peut être causée par la dysfonction de plusieurs organes qui régissent le fonctionnement du système reproductif. L'hypophyse, un petit organe situé sous le cerveau, produit et sécrète les hormones «gonadotropines» qui régulent le système reproducteur: l'hormone lutéinisante (LH) et l'hormone folliculo-stimulante (FSH). Une production anormale de LH et FSH par les cellules gonadotropes est souvent observée chez les patients infertiles. Des hormones provenant des gonades (testicules et ovaires), du cerveau et de l'hypophyse agissent sur les gonadotropes. Parmi ceux-ci, l'hormone de libération des gonadotropines (GnRH), provenant du cerveau, stimule la production et la sécrétion de LH et FSH. Les activines, produites par l'hypophyse, stimulent sélectivement la production de FSH, un effet supprimé par les inhibines provenant des gonades. Les voies de signalisation engagées par ces hormones pour transmettre leurs effets demeurent obscures. Dans cette thèse, j'ai d'abord utilisé des outils génétiques chez la souris pour étudier comment les activines régulent la synthèse de FSH. Habituellement, les activines transmettent leur signaux intracellulaires par les protéines SMAD2 et SMAD3, qui se lient à SMAD4 et interagissent avec d'autres facteurs pour activer la transcription de gènes cibles. Les résultats d'inactivations génétiques restreintes aux cellules gonadotropes démontrent que la synthèse de FSH et la fertilité ne requièrent pas SMAD2 et la capacité de SMAD3 de se lier à l'ADN, mais dépendent des activités individuelles et concertées de SMAD4 et de son partenaire «forkhead box L2» (FOXL2). Par la suite, à l'aide de lignées cellulaires modèles, j'ai exploré les mécanismes par lesquels GnRH active l'expression du gène humain encodant la sous-unité β de LH, et observé que ceux-ci sont semblables aux autres mammifères. Par contre, l'activine inhibe l'effet stimulant de GnRH, au contraire de son effet chez la souris. Cela s'explique par des différences dans des éléments régulateurs du gène humain et de celui de la souris, impliquant une interaction physique et fonctionnelle entre SMAD3 et «early growth response 1» (EGR1), un facteur de transcription requis pour la synthèse de LHβ. Finalement, en générant un nouveau modèle de souris, j'ai observé qu'une particularité structurelle du récepteur de GnRH est requise pour la production normale des gonadotropines et la fertilité des femelles. Ensemble, ces travaux contribuent à notre compréhension de la fonction des gonadotropes et pourraient guider le développement de traitements visant à moduler la production des gonadotropines.

Publications

Fortin J, Boehm U, Deng CX, Treier M, Bernard DJ 2014. Follicle-stimulating hormone synthesis and fertility depend on SMAD4 and FOXL2. *Manuscript submitted*

Fortin J, Boehm U, Weinstein MB, Graff JM, Bernard DJ 2013. Follicle-stimulating hormone synthesis and fertility are intact in mice lacking SMAD3 DNA binding activity and SMAD2 in gonadotrope cells. *FASEB J. Epub Dec. 5th, 2013*

Fortin J, Kumar V, Zhou X, Wang Y, Auwerx J, Schoonjans K, Boehm U, Boerboom D, Bernard DJ 2013. NR5A2 regulates *Lhb* and *Fshb* transcription in gonadotrope-like cells *in vitro*, but is dispensable for gonadotropin synthesis and fertility *in vivo*. *PLoS ONE 2013;8(3):e59058*

Fortin J, Bernard DJ 2010. SMAD3 and EGR1 physically and functionally interact in promoter-specific fashion. *Cellular Signalling 22:936-943*

Fortin J, Lamba P, Wang Y, Bernard DJ 2009. Conservation of mechanisms mediating gonadotropin-releasing hormone 1 stimulation of human luteinizing hormone {beta} subunit transcription. *Molecular Human Reproduction 15(2):77-87*

Conference Abstracts

International conferences

Fortin J, Boehm U, Deng CX and Bernard DJ 2013. SMAD signaling in gonadotropes is critical for normal FSH synthesis and fertility in mice. *Oral presentation at the 95th Annual meeting of the Endocrine Society*, San Francisco, CA.

Fortin J, Bernard DJ 2012. SMAD2/3-Mediated Signaling in Gonadotropes Is Dispensable for Fertility and FSH Synthesis in Mice. *Oral presentation at the 94th Annual meeting of the Endocrine Society* Houston, TX.

Fortin J, Bernard DJ 2012. Female fertility and activin-stimulated *Fshb* expression do not require SMAD2/3 in murine gonadotropes. *Oral presentation at the 15th International Congress of Endocrinology and 14th European Congress of Endocrinology*, Florence, Italy.

Fortin J, Soto P, Boerboom D, Bernard DJ 2010. Regulation of follicle-stimulating hormone β subunit (*Fshb*) transcription by liver receptor homolog-1. *Poster presentation at the 92nd Annual meeting of the Endocrine Society*, San Diego, CA.

Fortin J, Wang Y, Lamba P, Bernard DJ 2009. Smad3 Partially Inhibits GNRH1 and EGR-1 Induction of the Human Luteinizing Hormone β Promoter. *Poster presentation at the 91st Annual meeting of the Endocrine Society*, Washington, DC.

Fortin J, Wang Y, Lamba P, Bernard DJ 2008. Transcriptional regulation of human luteinizing hormone β subunit transcription by GNRH1. *Oral presentation at the 90th Annual meeting of the Endocrine Society,* San Francisco, CA.

Local and regional meetings

Fortin J, Boehm U, Deng CX and Bernard DJ 2013. Gonadotrope expression of *Smad4* is required for normal FSH synthesis and fertility in mice. *Oral presentation at the 5th annual meeting of the Center for the Study of Reproduction*, Montreal, Quebec

Fortin J and Bernard DJ. Roles of SMAD2 and SMAD3 in murine gonadotrope function ex vivo and in vivo. *Oral presentation at the 4th Annual Symposium of the Réseau Québécois en Reproduction*, Montreal, Quebec

Fortin J, Wang Y, Lamba P and Bernard DJ 2008 Activin A antagonizes GNRH1-induced LHB transcription via SMAD3. *Poster presentation at the 9th Annual Joint Meeting of the Great Lakes G Protein-Coupled Receptor Retreat and le Club des Récepteurs a Sept Domaines Transmembranaires,* Bromont, Quebec

Fortin J, Wang Y, Lamba P, Bernard DJ 2008. Antagonism of GNRH1-stimulated Human Luteinizing β Promoter Activity by Activin A. *Oral presentation at the 1st Annual Symposium of the Réseau Québécois en Reproduction,* Montreal, Quebec

This thesis was assembled following the "Guidelines of Thesis Preparation" at McGill University. It is written in manuscript format, and comprises five manuscripts in chapter 2, chapter 3, chapter 4, chapter 5 and chapter 6. Three of these manuscripts have been published (chapters 2, 4 and 5). One manuscript is under revision (chapter 3), and one is in preparation (chapter 6).

Contribution of authors

Chapter 2

Fortin J, Boehm U, Weinstein MB, Graff JM, Bernard DJ 2013. Follicle-stimulating hormone synthesis and fertility are intact in mice lacking SMAD3 DNA binding activity and SMAD2 in gonadotrope cells. *FASEB J. Epub Dec. 5th, 2013*

I designed and performed all the experiments under DJB supervision, with the use of mouse lines provided by UB, MBW and JMG. I wrote the manuscript with DJB.

Chapter 3

Fortin J, Boehm U, Deng CX, Treier M, Bernard DJ. Follicle-stimulating hormone synthesis and fertility depend on SMAD4 and FOXL2. *Manuscript submitted*

I designed and performed all the experiments under DJB supervision, with the use of mouse lines provided by UB, CXD and MT. I wrote the manuscript with DJB.

Chapter 4

Fortin J, Lamba P, Wang Y, Bernard DJ 2009. Conservation of mechanisms mediating gonadotropin-releasing hormone 1 stimulation of human luteinizing hormone {beta} subunit transcription. *Molecular Human Reproduction 15(2):77-87*

I designed and performed all the experiments, with assistance and guidance from PL and YW, and under DJB supervision. I wrote the manuscript with DJB.

Chapter 5

Fortin J, Bernard DJ 2010. SMAD3 and EGR1 physically and functionally interact in promoter-specific fashion. *Cellular Signalling 22:936-943*

I designed and performed all the experiments under DJB supervision. I wrote the manuscript with DJB.

Chapter 6

Fortin J, Handley-Derry F, Zhou X, Lapointe E, Boerboom D and Bernard DJ 2013. Normal gonadotropin synthesis and female fertility depend on a unique structural property of the mammalian gonadotropin-releasing hormone receptor. *Manuscript in preparation*.

I designed and performed all the experiments, with contributions from FHD in Fig.6.1 and S6.1, assistance from XZ with ovariectomy and estrogen replacement experiments (Fig. 6.5), and assistance from EL with embryonic stem cell procedures. I generated the targeting vector and performed gene targeting in embryonic stem cells under DB guidance. The work was performed under the supervision of DJB. I wrote the manuscript with DJB.

Acknowledgements

I am deeply grateful to my mentor, Dr Daniel Bernard, who believed in my potential more than five years ago and allowed me to join his lab. Dan provided me with excellent training in all aspects of science, and I always felt that my progress and success was his top priority. More than anything else, it is Dan's superb guidance and support that made this work possible.

I would like to thank all the Bernard lab members, past and present, for sharing comments, expertise and help on a daily basis. Among them, I would like to especially thank: Dr Pankaj Lamba, for excellent training; Ying Wang, who is a figure of stability in the lab and contributed to chapter 4; Xiang Zhou, for being so nice and for help with the ovariectomy experiments described in chapter 2 and chapter 6. Finally, I particularly thank Dr Beata Bak, Vanessa Libasci, Tamara Ouspenskaia and Dr Stella Tran who, in addition to being remarkable colleagues, also became very good friends.

I thank Dr Derek Boerboom for guidance through the process of targeting vector construction and gene targeting in embryonic stem cells (chapter 6), and his students and postdocs for welcoming me in their lab. In particular, I thank Dr Evelyne Lapointe, who showed me all the practical aspects of embryonic stem cell manipulations and gene targeting. I also thank the McGill Transgenic Core for excellent work in generating chimeric mice, and for expert troubleshooting. I acknowledge the dedicated work of the McGill Comparative Medicine and Animal Resources Centre staff in maintaining the mouse colony.

I would also like to thank my Ph.D. committee members, for insightful comments, critique and encouragements: Dr Sarah Kimmins, Dr Stéphane Laporte and Dr Jacquetta Trasler.

I am thankful to a number of collaborators, for sharing genetically modified mouse lines. Without their contribution, most of this work would not have been possible: Dr Ulrich Boehm, Dr Chu-Xia Deng, Dr Jonathan Graff, Dr Mathias Treier and Dr Michael Weinstein.

Finally, I am deeply grateful to my family, for constant encouragement and for making me who I am. I also thank my "crew": Guillaume, Henri, Hugo, Jean-Gab, Pat and Pierre, for reminding me that there is a life outside of the lab. And, of course, I thank Frances for her unwavering love and support.

I would like to acknowledge the following sources of support:

The Canadian Institutes of Health Research, for a Banting and Best's Master Award (1 year; 17500$) and a Canada Graduate Scholarship (3 years; 90000$ + 15000$ research allowance)

The McGill Faculty of Medicine and the family of Dr Samuel Solomon for the Samuel Solomon Fellowship in Endocrinology (6 months; 10000$)

McGill University, for two Principal's Graduate Fellowship and a Provost's Graduate Fellowship (total 2 years; 7500$)

The Fonds de Recherche en Santé du Québec, for a Bourse de Maitrise (4 months; 5000$)

The Endocrine Society for a Summer Research Award (4500$)

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Abbreviations

ABP: androgen-binding protein

ACVR1B: activin receptor type 1B

ACVR1C: activin receptor type 1C

ACRVR2A: activin receptor type 2A

ACVR2B: activin receptor type 2B

Ad-Cre: adenovirus expressing Cre recombinase

Ad-GFP: adenovirus expressing green fluorescent protein

AF1: activation function 1

AF2: activation function 2

ANOVA: analysis of variance

AP-1: activator protein 1

AP-2: adaptor protein 2

AR: androgen receptor

ATF2: activating transcription factor 2

BGH: bovine growth hormone

BMP: bone morphogenetic protein

BMPR1A: BMP receptor type 1A

BMPR1B: BMP receptor type 1B

BMPR2: BMP receptor type 2

BMK(ERK5): big mitogen-activated protein kinase

CaM: calmodulin

CaMK: calmodulin kinase

cAMP: cyclic adenosine monophosphate

CBP: CREB binding protein

CGA: chorionic gonadotropin alpha

CGB: chorionic gonadotropin beta

CJUN: jun proto-oncogene

CFOS: FBJ osteosarcoma oncogene

ChIP: chromatin immunoprecipitation

COC: cumulus-oocyte complex

c-RAF(Raf1): v-raf-leukemia viral oncogene 1

CRE: cAMP response element

CREB: cAMP response element binding protein

CRTC1: CREB regulated transcription coactivator 1

CYP17A1: cytochrome P450, family 17, subfamily A, polypeptide 1

CYP19A1: cytochrome P450, family 19, subfamily A, polypeptide 1

CYP21A1: cytochrome P450, family 21, subfamily A, polypeptide 1

CYP26B1: cytochrome P450, family 26, subfamily B, polypeptide 1

CXCR4: C-X-C chemokine receptor type 4

DAG: diacylglycerol

DARE: downstream activin regulatory element

DBD: DNA-binding domain

DCA: downstream chromosomal arm

DMSO: dimethyl sulfoxide

DNAP: DNA precipitation

DTa: diphtheria toxin A chain

EC: extracellular loop

EDTA: Ethylenediaminetetraacetic acid

EGF: epidermal growth factor

EGR1: early growth response 1

ELISA: enzyme-linked immunosorbent assay

EMSA: electrophoretic mobility shift assay

ER: endoplasmic reticulum

ERα: estrogen receptor alpha

ERβ: estrogen receptor beta

ERK1/2: extracellular signal-regulated kinases type 1 and 2

*Esr*1: ERα gene

*Esr*2: ERβ gene

ETS: E26 transformation-specific

F2cKO: *Foxl2* conditional knockout

FACS: fluorescence-activated cell sorting

FBE: forkhead binding element

FGF: fibroblast growth factor

FGFR1: fibroblast growth factor receptor type 1

FOSB: FBJ osteosarcoma oncogene B

FOXH1: forkhead box H1

FOXL2: forkhead box L2

FSH: follicle-stimulating hormone

FSHβ: follicle-stimilating hormone β subunit

Fshb: FSHβ gene

FSHR: follicle-stimulating hormone receptor

FST: follistatin

GABA: gamma-aminobutyric acid

GATA2: GATA binding protein 2

$G\alpha_{12/13}$: G protein alpha subunit type 12/13

$G\alpha_{i}$: G protein alpha subunit type i

$G\alpha_{q/11}$: G protein alpha subunit type q/11

$G\alpha_{s}$: G protein alpha subunit type s

GDF: growth and differentiation factor

GDP: guanosine diphosphate

GnRH: gonadotropin-releasing hormone

GnRHR: gonadotropin-releasing hormone receptor

GPCR: G protein-coupled receptor

GPR54: G protein-coupled receptor 54

GRAS: GnRHR activation sequence

GRE: GnRH responsive element

GSE: gonadotrope-specific element

GTP: guanosine triphosphate

HA: Hemagglutinin

HESX1: homeobox expressed in embryonic stem cells 1

HPG: hypothalamic-pituitary-gonadal

Hpg: hypogonadal (mouse strain)

HS6ST1: heparan sulfate 6-O-sulfotransferase 1

IHH: idiopathic hypogonadotropic hypogonadism

IP: immunoprecipitation

IP_3: inositol 1,4,5-trisphosphate

IP_3R: IP_3 receptor

IVF: *in vitro* fertilization

JNK: jun-N-terminal kinase

JUNB: jun b proto-oncogene

LH: luteinizing hormone

LHβ: luteinizing hormone β subunit

Lhb: LHβ gene

LHR: luteinizing hormone receptor

LHX3: LIM homeobox protein 3

LHX3: LIM homeobox protein 2

LRH-1 (NR5A2): liver receptor homolog 1 (nuclear receptor, subfamily 5, group A, member 2)

Luc: luciferase

MAP3K: MAPK kinase kinase

MAP2K: MAPK kinase

MAPK: mitogen-activated protein kinase

MH2: Mad homology 2

MH3: Mad homology 3

MMLV: Moloney murine leukemia virus

Neo: neomycin resistance cassette

NFAT3: nuclear factor of activated T cells 3

NF-Y: nuclear factor Y

NUR77 (NR4A1): nuclear receptor 77 (nuclear receptor, subfamily 4, group A, member 1)

OTUD4: OTU domain containing 4

OTX2: orthodenticle homolog 2

PBE: PITX binding element

PBS: phosphate-buffered saline

PBX1A: pre B cell leukemia homeobox 1

PCOS: polycystic ovarian syndrome

PCR: polymerase chain reaction

PGR: progesterone receptor

PIP_2: phosphatidylinositol 4,5-bisphosphate

PITX: paired-like homeobox

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

PLB: passive lysis buffer

POU1F1 (PIT1): POU domain, class 1, transcription factor 1

PREP1: Pbx/knotted 1 homeobox

PROK2: prokineticin 2

PROKR2: prokineticin receptor type 2

PROP1: paired-like homeodomain factor 1

PSE: pituitary-specific element

qPCR: quantitative polymerase chain reaction

RIPA: radioimmunoprecipitation assay

RNF216: ring finger protein 216

RT-PCR: reverse transcription polymerase chain reaction

R-SMAD: receptor-regulated SMAD protein

S2/3cKO: *Smad2/3* conditional knockout

S4cKO: *Smad4* conditional knockout

S4F2cKO: *Smad4/Foxl2* conditional knockout

SBE: SMAD binding element

SDF-1: stromal cell-derived factor 1

SDS: sodium dodecyl phosphate

SF1 (NR5A1): steroidogenic factor 1 (nuclear receptor, subfamily 5, group A, member 1)

SHH: sonic hedgehog

SIX6: sine oculis-related homeobox 6

SMAD: Sma- and Mad-related protein

SOX2: SRY box 2

SOX3: SRY box 3

SOX9: SRY box 9

SP1: trans-acting transcription factor 1

SRF: serum response factor

SRY: sex-determining region Y

StAR: steroidogenic acute regulatory protein

Stra8: stimulated by retinoic acid 8

TAK1: TGFβ-activated kinase 1

TBS: tris-buffered saline

TBX19: T-box 19

TGFβ: transforming growth factor β

TGFBR1: TGFβ receptor type 1

TGFBR3: TGFβ receptor type 3

TM: transmembrane domain

TSS: translation start site

UCA: upstream chromosomal arm

VAX1: ventral anterior homeobox 1

Wnt: wingless/int

YFP: yellow fluorescent protein

General introduction

In vertebrates, the hypothalamic-pituitary-gonadal (HPG) (Fig. 1.1) axis controls reproductive function. This system relies on an intricate network of endocrine feed forward and feedback loops. At the "top" of the axis, gonadotropin-releasing hormone (GnRH), produced by neurons in the brain, acts on the gonadotrope cells of the pituitary gland to stimulate the production of the gonadotropin hormones: luteizining hormone (LH) and follicle-stimulating hormone (FSH). These factors enter the circulation and act on LH and FSH receptors (LHR and FSHR) in the gonads – the testes in males and the ovaries in females – to promote the production of fertilization-competent gametes (the spermatozoa in males and the oocytes in females). In addition, the gonads secrete hormones that feedback on the hypothalamus and pituitary to positively or negatively regulate their activity. The best characterized gonadal hormones are: estradiol (in females), progesterone (in females), testosterone (in males) and inhibins. Inhibins antagonize the action of activins or related ligands, which are produced within the pituitary and selectively stimulate FSH synthesis. Over the past century, an impressive amount of research has shed light on the function as well as dysregulation of the mammalian reproductive axis, from the systemic to the molecular level. The purpose of this introduction is to provide a comprehensive review of our current understanding of pituitary gonadotrope cell function and to emphasize the need for more research. First, I present an overview of the known causes of abnormal gonadotropin synthesis in humans as relates to infertility and their current treatment. Second, I outline the salient features of the main components of the HPG axis, starting with the hypothalamus, followed by the pituitary and the gonads. In the third part, I provide an in-depth description of mechanisms controlling gonadotropin hormone synthesis and release, with an emphasis on the signaling events directed by brain-derived GnRH and by the activin/inhibin hormonal system. I conclude by identifying specific gaps in our knowledge that are addressed by the experimental work presented in this thesis.

1. Abnormal gonadotropin production and its treatment

Proper function of many organs and regulatory systems is necessary for normal pituitary gonadotrope function. Because of this complexity, a large number of genetic or acquired anomalies result in abnormal gonadotropin production, leading to infertility or subfertility (1).

1.1. Hypogonadotropic hypogonadism

Mutations in several genes have been associated with central hypogonadotropic hypogonadism (HH), in which the primary defect is at the level of the brain or the pituitary gonadotropes (1, 2). HH is functionally divided in two main types: Kallmann syndrome, in which hypogonadism is associated with anosmia (absence of smell), and normosmic HH (2). Mutations found in Kallmann syndrome interfere with the maturation and migration of olfactory and GnRH neurons (described in Section 2.1.1.1.). By contrast, normosmic HH involves genetic anomalies that prevent normal function of gonadotropes, GnRH neurons, or upstream neural circuits (2). Among the genes mutated are components of the kisspeptin signaling system (see section 2.1.1.2.), which regulates the function of GnRH neurons (3-6). As well, rare mutations are found in the gene coding for GnRH (*GNRH1*) (7, 8) and for the GnRH receptor (*GNRHR*) (9). Some more recently identified genes, such as heparan sulfate 6-O-sulfotransferase 1 (*HS6ST1*), ring finger protein 216 (*RNF216*) and OTU domain containing 4 (*OTUD4*), are associated with complex and variable phenotypes, and no definitive functional link with the central control of reproduction in mammals has yet been made (10, 11). Less than 50% of the cases of congenital HH have a known genetic cause, suggesting that many more factors remain to be identified (2). Also, a proportion of HH cases involve compound mutations in two or more genes, adding further complexity to genetic studies of HH (12). In general, HH is associated with failure to activate the reproductive axis (absence of puberty), and deficiency of both LH and FSH (2). Rare inactivating mutations in the genes coding for the β subunits of LH and FSH (*FSHB* and *LHB* - see section 2.2.2.) cause distinct forms of hypogonadism due to selective loss of FSH or LH stimulation of the gonads (13-17). Overall, insights obtained from mutations causing HH have identified key regulators of GnRH neuron and pituitary gonadotrope function, thus guiding investigations of the mechanisms underlying gonadotropin production.

1.2. Syndromic infertility

In addition to the congenital anomalies outlined above, many well-characterized disturbances of the reproductive axis or reproductive tract occur in women of reproductive age (1). Among others, cases of anovulation (18) and endometriosis (19) are frequently seen in the fertility clinic. While some of these conditions clearly have a genetic component, their development and severity is also heavily influenced by acquired endocrine disorders (1, 20). One well-known example is polycystic ovarian syndrome (PCOS), the most common cause of anovulation, which affects up to 10% of women worldwide (20, 21). The etiology of PCOS is complex, but is most often accompanied by hyper-secretion of LH relative to FSH by the pituitary gland. (22). As proper function of the female reproductive axis depends on tightly regulated cyclic changes in LH and FSH levels (described in section 2.2.3.), gonadotropin imbalance leads to ovarian dysfunction (20, 21). Therefore, studying mechanisms underlying the regulation of LH and FSH production by the pituitary gland is important for understanding both inherited or acquired causes of infertility in humans.

1.3. Infertility treatments

One of the main goals of reproduction research is the development of treatments for infertility. Molecules that mimic or inhibit the activity of GnRH have been used clinically for several years. Native GnRH can be used to activate the reproductive axis in many cases of HH or delayed puberty (23-26). Synthetic analogues of GnRH are widely used to shut down GnRH signaling and downregulate the reproductive axis (27-31). These compounds are often used to induce “chemical castration” in cases of sex steroid-dependent cancer (e.g., prostate cancer) (32-34) and disorders such as endometriosis (35, 36), or precocious puberty (23, 37, 38). Gonadotropins and gonadotropin analogues are also extensively used in the fertility clinic (39). These are extracted from the urine of post-menopausal women (40-42), or produced by recombinant methods (43-46). Gonadotropins or analogues can be used in the treatment of pituitary insufficiency (e.g (47)). By far, the largest clinical application of GnRH and gonadotropin analogues is in the context of ovarian stimulation protocols, which aim at

retrieving oocytes for *in vitro* fertilization (IVF - including intracytoplasmic sperm injection (ICSI)). These treatments, which have been directly derived from our understanding of mammalian reproductive physiology, essentially recapitulate a controlled version of the normal cyclic hormonal profile in women (see section 2.2.3.) (48). Thus, understanding the normal function of the reproductive system is critical for the development of effective treatments for infertility. However, less than 25% of IVF cycles are successful (49), and undesirable or even life-threatening side effects arising from these protocols are common (48, 50). Furthermore, the procedure is cumbersome (requiring up to dozens of injections of GnRH and gonadotropin analogues) and expensive (48). Drugs like clomiphene, an estrogen receptor modulator which is used to increase endogenous FSH levels (see section 3.3.2. for more details) in anovulatory women - particularly those with PCOS – have been used for dozens of years (51-54). However, precise control of FSH production is hard to achieve with such treatment (55). Thus, there is a clear need for better drugs to modulate gonadotropin synthesis, release or activity. Overall, infertility is a major clinical problem, and a better understanding of the mechanisms controlling gonadotropin production is required to identify new therapeutic targets and strategies for effective treatment.

2. The mammalian reproductive axis from the top down

2.1 The hypothalamus

2.1.1. Gonadotropin-releasing hormone

The fact that a factor secreted by neurons in the brain controls the production of gonadotropins by the pituitary was first suggested by classical experiments showing that electrical stimulation of the hypothalamus, a region of the ventral brain situated above the pituitary gland, could stimulate ovulation in female rabbits - observations which were subsequently extended to other mammals (56, 57). Decades later, seminal physiological experiments established that direct exposure of the pituitary gland to hypothalamic extracts could trigger gonadotropin secretion (58, 59). This set off a race for the discovery of the gonadotropin-releasing factor, which was finally, after a herculean effort, purified in 1971 and identified as the 10 amino acid protein (decapeptide), GnRH (60, 61). The proof that GnRH not only stimulates,

but is in fact required for, gonadotropin production by the pituitary would come several years later following the discovery and analysis of a naturally occurring mutation in mice causing profound hypogonadism – the *hpg* mice (62, 63). *Hpg* mice carry a deletion on chromosome 14 encompassing part of the the *Gnrh1* gene (63). They completely fail to progress beyond infantile stages of reproductive maturation and produce extremely low levels of LH and FSH (62). These observations provided the first animal model of congenital hypogonadotropic hypogonadism and indicated that brain-derived GnRH is a master regulator of pituitary gonadotrope function.

2.1.1.1. Anatomy and developmental ontogeny of GnRH neurons

GnRH neurons are actually not born in the brain. Rather, they emerge from precursors in the nasal placode; the site of the future olfactory bulb (64-66). Indeed, as mentioned in section 1.1., many patients with HH also lack the sense of smell (Kallmann Syndrome) (67, 68). Because GnRH neurons fail to be born or migrate properly in these individuals, it is thought that olfactory and GnRH neurons rely on a shared set of cues for their specification, development and migration (68). The migratory journey of GnRH neurons from their birth place to their final position in the preoptic area and/or ventromedial hypothalamus is a complex process. Several factors required for their proper navigation have been identified from studies of naturally occurring mutations in humans and genetically engineered mouse models. Briefly, GnRH neurons use receptors for ligands or chemoattractant molecules secreted by surrounding neurons and supporting cells. These include: fibroblast growth factor 8 (FGF8) and its receptor FGFR1 (69, 70), prokineticin 2 (PROK2) and its receptor PROKR2 (71, 72), stromal cell-derived factor 1 (SDF-1) and its receptor C-X-C chemokine receptor type 4 (CXCR4) (73)). Cell adhesion/guidance molecules such as Eph/Ephrins and Semaphorins also play a role in GnRH neuron migration (74-76). The final GnRH-producing cell population has a size of approximately 800 neurons in mice and up to a few thousand in human (65, 77). Once in the hypothalamus, GnRH neurons extend long axons that reach the median eminence, adjoining the portal vasculature located at the interface between the ventral brain and the pituitary gland (78). There, GnRH neuron terminals release bursts of GnRH, which are transported over a short distance by the portal blood capillaries to their targets, the gonadotrope cells of the anterior pituitary gland.

2.1.1.2. GnRH synthesis and pulsatile release

GnRH is released in pulses into the pituitary vasculature, both in males and females (e.g., (79, 80)). The critical importance of this mode of GnRH secretion was demonstrated in monkeys with hypothalamic lesions that blocked GnRH release, causing hypogonadotropic hypogonadism. In these animals, delivery of exogenous GnRH in a pulsatile, but not continuous, manner rescued gonadotropin secretion (81). LH secretion from pituitary gonadotropes is highly coupled with pulsatile GnRH secretion (82). Whereas GnRH also stimulates FSH synthesis and release, FSH secretion is less obviously pulsatile than LH (82, 83). The system that produces episodic GnRH release (the so-called GnRH pulse generator) is located within the network that controls GnRH neuron activity. Indeed, bursting electrical activity within mediobasal hypothalamic neurons immediately precedes and is highly correlated with LH release from the pituitary gland (84, 85). Despite decades of studies, the molecular nature of this GnRH pulse generator remains somewhat elusive (86). Neurotransmitters, such as glutamate and gamma-aminobutyric acid (GABA) (87-89), as well as peptide hormones released from specialized neuronal populations, like kisspeptin (see below) (90, 91), and a variety of intracellular mechanisms (86) have been implicated in this process. Furthermore, GnRH neurons have intrinsic "pacemaker-like" activity, but how this rhythm is established and maintained remains unclear (86, 92, 93).

Arguably, the most important physiological regulator of GnRH synthesis and secretion in females is ovary-derived estradiol (94). Estradiol production by the ovary varies across the female reproductive cycle (detailed in section 2.2.3.). When estradiol is low, it suppresses the amplitude and frequency of GnRH release ("negative feedback") (94, 95). However, when estradiol is high, it enhances the amplitude and frequency of GnRH secretion ("positive feedback") (94, 95). In mammals, the positive feedback effect occurs once per reproductive cycle, immediately before ovulation. Outside of this period, the negative feedback effect prevails. It is important to note that the pituitary gonadotropes are also subjected to negative and positive regulation by estradiol (detailed in section 3.3.2.) (96-99). Ultimately, it is the combination of negative and positive feedback effects of estradiol on the brain and on the pituitary which determines estradiol's overall influence on gonadotropin production (95). The mechanisms underlying regulation of GnRH secretion by estradiol are not completely

understood. Canonically, estradiol influences target cell function through its cognate nuclear receptors, estrogen receptor (ER) α and β, which interact with the DNA and with co-factors to directly regulate gene transcription (100). Estradiol can also have rapid non-genomic (transcription-independent) action on GnRH neurons (101-103). In mice, both the positive and negative feedback effects of estradiol in the brain are mediated through ERα (104-106). However, GnRH neurons themselves do not express ERα (107, 108). Instead, the positive and negative feedback effects of estradiol appear to be mediated indirectly via kisspeptin neurons (109, 110). These cells release the peptide hormone, kisspeptin, and synapse onto GnRH neurons, which express the kisspeptin receptor (KISS1R or GPR54) (111). Mice or humans with inactivating mutations in the genes coding for kisspeptin or its receptor are profoundly hypogonadal (4, 5, 112). Overall, pulsatile GnRH secretion from hypothalamic neurons, which is dynamically regulated by estradiol, is a key stimulator of gonadotropin synthesis and release.

2.2. The pituitary gland

Gonadal homeostasis and the development of fertilization-competent germ cells is dependent on hormones secreted by the pituitary gland (Fig. 1.1), a fact established over 80 years ago by classical ablation/replacement experiments (113-115). Subsequently, it became evident that the pituitary gland produces two gonadotropin hormones, now known as LH and FSH (116). These hormones are exclusively produced by gonadotrope cells.

2.2.1. Pituitary cell lineage specification and gonadotrope development

Gonadotropes comprise a small proportion (around 5%) of the hormone-secreting cells of the anterior pituitary gland (Fig. 1.2A and ref. (117)). The other cell types (corticotropes, lactotropes, somatotropes, thyrotropes) secrete important regulators of growth, metabolism and lactation (114). The different pituitary cell types are specified from common precursors through a series of specification steps (Fig. 1.2B and ref.(118)).

2.2.1.1. Pituitary cell lineage specification

Development of the pituitary gland depends on reciprocal signaling between the oral ectoderm (roof of the mouth), from which the anterior pituitary tissue is derived, and the overlying ventral diencephalon – or infundibulum (the floor of the hypothalamus) (118). Several

transcription factors have been implicated in the specification and patterning of the infundibulum, including homeobox expressed in ES cells 1 (HESX1), orthodenticle homolog 2 (OTX2), sine oculis-related homeobox 6 (SIX6), ventral anterior homeobox 1 (VAX1) and Sex determining region Y-box 2 and 3 (SOX2 and SOX3) (119-124). Genes coding for at least three of these – *HESX1*, *SOX2* and *SOX3*, are mutated in human Septo-Optic Dysplasia, a heterogeneous disorder involving so-called combined pituitary hormone deficiency (120, 121, 124). Mechanistically, transcription factors regulating infundibulum development control the expression of secreted molecules of the fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) families, which act in concert with other ligands emanating from the adjacent oral ectoderm, such as BMP2 and Sonic Hedgehog (SHH), to induce anterior pituitary cell fate commitment (125-129). Though the precise signaling cascades activated by these ligands in developing pituitary cells are poorly characterized, they appear to establish a dorsoventral gradient of morphogens that lead to the selective expression of transcription factors controlling pituitary cell lineage specification (118, 127). Two of these, LIM homeobox protein 3 (LHX3) and *paired*-like homeodomain factor 1 (PROP1), are expressed sequentially at embryonic day (E) 9.5-10 and are required for the development of all pituitary cell types except corticotropes, which rely instead on T-box 19 (TBX19) (Fig. 1.2B) (130-132). Although still incomplete, a simple model for the subsequent differentiation of pituitary progenitors into specific lineages has been proposed. Briefly, PROP1 controls the expression of *POU domain, class 1, transcription factor 1* (*Pouf1*, also known as *Pit1*) (132), but a ventral-to-dorsal gradient of BMP2 induces GATA binding protein 2 (GATA2) expression, which then represses PIT1 activity when present in large amounts (133). GATA2 and PIT1 control the expression of cell-specific genes, including those coding for hormones or hormone subunits (133-137). Thus, GATA2+ cells become gonadotropes, GATA2+/ PIT1+ cells become thyrotropes, and PIT1+ cells become lactotropes and somatotropes (Fig. 1.2B) (118). Though this model provides an elegant mechanism for cell fate decision, it certainly does not account for all the events of pituitary lineage specification, which involves many other factors (138, 139).

2.2.1.2. Gonadotrope development

The first cell-specific event characterizing the gonadotrope fate is expression of steroidogenic factor 1 (SF1, also known as NR5A1), an orphan nuclear receptor, at E13.5 in

mice (Fig. 1.2B) (140). Global or pituitary-specific *Sf1* knockout mice have low or absent FSH and LH synthesis in the pituitary under basal condition (140, 141). Nevertheless, administration of exogenous GnRH to either mouse model increases gonadotropin synthesis, indicating that SF1 is, in fact, not absolutely required for gonadotrope lineage specification (141, 142). As described above, GATA2 has also been implicated in gonadotrope development, yet pituitary-specific *Gata2* knockout mice display only a mild hypogonadotropic phenotype (143). However, deletion of *Gata2* in this model may occur only at E12.5-E14.5, which is later than the onset of GATA2 expression (E10.5). (143). Other transcription factors are required for some aspects of gonadotrope maturation, such as selective synthesis of LH or FSH, but not for the full gonadotrope differentiation program ((144, 145) and see Section 3). To this day, no single factor has been identified that is absolutely and selectively required for commitment to the gonadotrope cell lineage (Fig. 1.2). It is quite possible that this process requires the cooperative activity of several factors, which may act redundantly. Therefore, although it is clear that the different pituitary lineages emerge from common precursors, the precise mechanisms governing terminal gonadotrope differentiation remain unknown.

The pituitary is generally considered to be a fairly quiescent tissue with low self-renewal and proliferative activity (146). Indeed, only about 1% of gonadotropes are in a proliferative state at any given moment under basal conditions in adult rodents (147). That said, it has been appreciated for quite some time that the adult gonadotrope population is not static *per se*. For example, there are substantial changes in the number of proliferating pituitary cells, including gonadotropes, across different stages of the estrous cycle in rodents (146-150). Furthermore, a recently described population of pituitary progenitors, expressing the pluripotency marker SOX2, has been shown to continuously supply the adult pituitary with new cells from all the pituitary lineages (151-156). Whereas most gonadotropes synthesize both LH and FSH, subsets express either LH or FSH during embryonic development and post-natally, suggesting heterogeneitity in gonadotrope function (157, 158). The life-span of gonadotropes, the mechanisms underlying their replacement throughout life, and how this process is regulated across physiological and pathological conditions, all remain unanswered questions. Intriguing recent evidence has shed light on hitherto unrecognized dynamic behavior of gonadotrope cells: they appear to maintain organized networks with selected pituitary lineages (159) and to move closer to or further from blood vessels depending on the estrous cycle stage and GnRH stimulation (160, 161).

Collectively, these observations suggest that the gonadotrope population remains highly dynamic in adult mammals.

2.2.2. Assembly, secretion, and bioactivity of the gonadotropins

The gonadotropins are peptide hormones. LH and FSH are dimeric glycosylated proteins comprised of a common α subunit, noncovalently linked to a unique β subunit, which confers biological specificity. The gonadotropin subunits are synthesized in gonadotrope cells from independent genes, denoted *Cga* (common α subunit), *Lhb* (LH β subunit), and *Fshb* (FSH β subunit) in rodents. In humans and primates, an additional gonadotropin, chorionic gonadotropin (CG), is synthesized by the placenta and comprised of CGA linked to a β subunit encoded by the *CGB* gene. CG has LH-like activity and is critical for corpus luteum maintenance and progesterone synthesis in pregnancy (162). Similar to other peptide hormones destined for secretion, the nascent gonadotropin subunit peptides have N-terminal signal sequences of 18-24 amino acids, which are cleaved during protein synthesis in the rough endoplasmic reticulum (ER) (163-165). In the ER, the gonadotropin subunits are folded by formation of disulfide bonds between cysteine residues, effectively creating loops that confer their characteristic "cysteine knot" structure (163, 166, 167). This arrangement is critical for assembly of the dimeric hormones: complementary cysteine loops of the folded α and β subunits slide into each other in a head-to-tail configuration like matching pieces of a puzzle; an organization beautifully revealed by the X-ray crystal structures of dimeric gonadotropins (168-172). This confers the dimeric hormones' remarkable structural strength, despite the non-covalent nature of the association between the subunits (173). Because α subunits are produced in excess, it appears that the amount of β subunit synthesized dictates the quantity of bioactive hormone generated (174, 175).

A hallmark of the gonadotropins is glycosylation of both the α and β subunits, which regulate their function. N-linked glycosylation of the gonadotropin subunits occurs in the ER, coincident with protein translation and folding (175-177). Two asparagine residues are N-glycosylated in the α subunit, one in the LH β subunit, and two in the FSH β subunit (178). Further processing of the carbohydrate chains occurs in the ER and the Golgi apparatus, yielding a large array of mature glycoforms (178). Importantly, LH and FSH differ in the predominant terminal groups on their attached carbohydrates: N-acetyl-galactosamine/sulfate for LH, and galactose/sialic acid in the case of FSH (179-182). The distinct mature carbohydrate chains

present on LH and FSH are important determinants of their half-lives in circulation, which is a few hours for FSH, compared with > 30 min. for LH (183-185). Specific recognition of the sulfated carbohydrates attached to LH by a receptor located on hepatic endothelial cells appears to underlie the rapid clearance of LH from the blood (186). Indeed, mice lacking the gonadotrope sulfotransferase responsible for this unique LH modification display increased circulating LH levels and hyperactivity of the reproductive axis (187). Because GnRH pulses stimulating LH secretion rarely occur more often than once every half hour (188) (see section 2.1.1.2.), rapid clearance of LH following its release likely explains the typical transient pulsatile pattern of circulating LH levels (186, 187). The glycosylation pattern of the gonadotropins varies with the reproductive cycles (see below) under normal conditions (189-191), and is disturbed in a number of pathological states (192-194). Thus, since the biological activity of the gonadotropins differs between glycoforms (195-198), abnormal glycosylation may contribute to the pathophysiology of infertility.

Subsequent to carbohydrate maturation in the Golgi apparatus, the gonadotropins are packaged for release. Dimeric LH is sorted to the regulated secretory pathway, whereas FSH is constitutively secreted (199). Indeed, within gonadotropes, LH is predominantly packed in small electron-dense compartments associated with the secretogranin II; hallmarks of vesicles relying on calcium signaling for exocytosis (200). By contrast, FSH is found mostly in larger vacuoles decorated with chromogranin A, features that may direct the vesicles towards immediate secretion following budding from the *trans*-Golgi network after cargo maturation (199-202). These observations have contributed to a central dogma of gonadotropin biology; namely, that circulating FSH levels mainly reflect how much FSH is synthesized at a given moment, whereas circulating LH levels instead reflect the strength and frequency of the stimulus regulating its release (199, 203). The exact nature of the signals regulating the differential sorting of LH and FSH is incompletely understood, but a seven amino acids stretch near the C-terminus of LHβ appears important for routing of LH to the regulated secretory pathway (204, 205). Thus, the β subunits of LH and FSH are differentially processed, modified and sorted during synthesis, impacting their secretion patterns and bioactivity.

2.2.3. Patterns of LH and FSH secretion in males and females

The dynamics of gonadotropin synthesis and release vary substantially depending on the species, sex, and age of the animals. In rodents, the ability of the pituitary to synthesize gonadotropins follows the maturation of GnRH neurons during late embryonic development (206-208). Thereafter, initiation of GnRH pulsatile secretion drives the onset of LH and FSH secretion (208-210). In the early postnatal period, a robust increase in circulating FSH, and to a lesser degree LH, takes place in females (211, 212). In males, a similar phenomenon may occur, but to lesser extent (211, 213, 214). This preferential increase in FSH secretion is thought to be due to a slow GnRH pulsatile stimulation – which favors FSH over LH synthesis (see section 3.2.1.) (215, 216) - coupled with a lack of negative feedback from ovary-derived estradiol (217, 218). After a few days, an increase in the GnRH pulse frequency and the establishment of steroid negative feedback leads to a decline in circulating FSH levels, whereas LH remains low until puberty (213). The role of this neonatal gonadotropin surge is unclear, as early ovarian follicle development progresses during the immediate postnatal period in the absence of FSH, LH, or their receptors in mice (219-223). In males, FSH secretion ramps up during the juvenile period, peaks before puberty and subsequently declines and stabilizes for the rest of adult life (211, 214, 224-226). Meanwhile, LH secretion remains highly pulsatile, with an increase in the frequency and amplitude of episodic LH release at puberty (211, 213, 224, 226-228).

In females, puberty onset is accompanied by a major shift in the pattern of gonadotropin release; highly dynamic, cyclical, and stereotyped changes in the levels of FSH and LH are established, and remain fixed for the remainder of reproductive life (Fig. 1.3). During the late juvenile period in rodents, an increase in LH and FSH release from the pituitary occurs (229-233). This "awakening" of the HPG axis is driven by increased hypothalamic GnRH secretion, though the exact trigger for this activation remains elusive (94, 234) (See section 2.1.1.2.). Increased FSH and LH levels induce the maturation of a cohort of "ovarian follicles", the functional units of the ovary (described in details below, in section 2.3.2.3.), which in turn produce estradiol (229, 231, 232, 235). Eventually, the positive feedback effect exerted by high estradiol leads to the first GnRH-driven LH surge (see section 3.1.1.) which triggers ovulation (236). Thereafter, robust cyclicity in the pattern of gonadotropin release is established (Fig. 1.3). The periodicity of the cyclical changes in gonadotropins varies between species, being about 4-6 days in rodents (the "estrous cycle") and 28-30 days in women of reproductive age (the "menstrual cycle") (237-239) (Fig. 1.3). LH is low during most of the cycle, except prior to

ovulation, when it dramatically peaks for a few hours in rodents (this day is called “proestrus”) and for longer in humans (240-245). At the same time, FSH also increases, driven by the large GnRH stimulus on pituitary gonadotropes (240-244). This episode of FSH release is widely considered to be an epiphenomenon, as blockade of LH alone at proestrus is sufficient to prevent ovulation in rats (246-248). On the morning of the following day (estrus), the so-called “secondary FSH surge” occurs, while LH returns to baseline (Fig. 1.3) (240, 242, 244). Increased FSH synthesis and secretion at that time drives the recruitment and maturation of a new cohort of ovarian follicles, for eventual ovulation during the next cycle. This was most elegantly demonstrated by classical experiments blocking the secondary FSH surge in rodents (249, 250). As discussed in more detail in section 3.2.1.4. , FSH production at this stage is driven by locally-produced activins or related TGFβ superfamily ligands. In humans, an analogous selective increase in FSH occurs during the first few days of the menstrual cycle, driving ovarian follicle maturation (“follicular phase”) (241, 243). After the secondary FSH surge occurs in rodents, a period of relative pituitary restraint ensues during metestrus and diestrus, lasting typically 2-3 days (Fig. 1.3) (240, 242, 244). During this time, ovarian follicles grow, secrete increasing amount of estradiol, which, on the day of proestrus, reach sufficiently high levels to trigger a new surge of LH and ovulation (Fig. 1.3). Overall, gonadotropin synthesis and release are highly regulated and dynamic processes which ensure proper function of the gonads.

2.3. The gonads

The main roles of the gonadotropins are to stimulate the production of gonadal hormones – especially sex steroids – and to promote the maturation of healthy fertilizable germ cells. The gonadotropins target somatic cells that surround the germ cells within the functional units of the gonads: the seminiferous tubules in the testes, and the ovarian follicles in the ovaries. The male and female gonads arise from common organ precursors which differentiate into either testes or ovaries during embryonic development (251).

2.3.1. The male gonad

Early development of the male gonad is associated with the specification of the Sertoli cells, a critical supporting cell lineage in the testis (252). Sertoli cells congregate around primordial (early) germ cells, creating tubular structures called "seminiferous tubules" (251). Later, an additional type of somatic cell envelops the seminiferous tubules: the Leydig cells, which are responsible for the production of the steroid hormone, testosterone (see section 2.3.1.2.). Thus, male germ cells are shielded in the specialized environment of the seminiferous tubules, where they will undergo replication and maturation.

2.3.1.1. Gametogenesis in males

Since the male and female gametes each contribute a complementary half of the genome to a developing embryo, mature spermatozoa and oocytes must carry a haploid genome (one copy of each chromosome) to yield diploid offspring after fertilization. Initially, though, early germ cells in the embryonic gonads are diploid, as they are derived from somatic cells. After their enclosure in tubular structures, male germ cells remain in a state of mitotic arrest as "spermatogonia" until postnatal day 10 (P10) in mice (253). Thereafter, germ cells undergo a series of maturation steps, which occur continuously throughout the life of the animal. This process, called "spermatogenesis", is divided into several stages based on cytological and functional criteria (254, 255). A large number of factors produced by the supporting cells of the testes control the process of spermatogenesis (256). Some of these factors, such as androgens (e.g., testosterone) produced by Leydig cells in the testicular interstitium, are synthesized in response to pituitary-derived gonadotropin hormones. In mice that do not produce gonadotropins or lack a pituitary gland, meiosis and germ cell development is blocked at the spermatocyte stage (257-259).

2.3.1.2. Somatic cell organization and function in the testis

In the testis, the Leydig and Sertoli cells provide the interface between circulating endocrine signals derived from the pituitary and developing germ cells in the seminiferous tubules. The main function of the Leydig cells is to produce testosterone (260). Testosterone is required not only for the maintenance of spermatogenesis, but also for the development of the male genital tract, maturation of secondary sexual characteristics, and the masculinization of behavior (260). Leydig cells increase production of testosterone late in embryonic development

in response to the onset of luteinizing hormone (LH) secretion by the pituitary gland (261). Testosterone is produced by the sequential enzymatic conversion of steroid precursors from cholesterol. Many of these steps occur in the mitochondria. LH induces the expression of Steroidogenic Acute Regulatory protein (StAR), which transports cholesterol from the cytoplasm to the mitochontria – the rate-limiting step in testosterone production (262). LH also stimulates the expression or activity of several steroidogenic enzymes (263-266). At puberty, the stronger LH stimulus (refer back to Section 2.2.3.) pushes testosterone production above the threshold required to drive full sexual maturation.

In addition to providing structural support to the germ cells in the seminiferous tubules, Sertoli cells provide a crucial link between the Leydig cells, the maturing gametes, and the pituitary gland. In particular, Sertoli cells are responsible for the delivery of testosterone produced by the Leydig cells to the germ cells *via* the androgen binding protein (ABP), which is expressed in response to follicle-stimulating hormone (FSH) (267-269). Another major product of Sertoli cells, in response to FSH stimulation, is the transforming growth factor β family member, inhibin B (270). In males, inhibin B may play some role in the negative endocrine feedback loop which controls FSH production by the pituitary, as detailed in section 3.2.1.3. In sum, Sertoli and Leydig cells respond to the gonadotropins to orchestrate the production of sex steroids and fertilization-competent male gametes.

2.3.2. The female gonad

The functional organization of the male and female gonads is similar: in both cases, somatic cells receive gonadotropin stimuli and direct germ cell maturation. In female embryos, the gonad differentiates towards an ovarian fate (251). Somewhat analogous the enclosing of male germ cells by Sertoli cells in the embryonic testis, early oocytes form small clusters comprising a few germ cells surrounded by somatic cells (271). These so-called “germ cell cysts” will remain undisturbed in the ovaries until the early postnatal period in mice. At that point, germ cell clusters are broken down, and individual oocytes are enclosed by a single layer of specialized somatic cells, the granulosa cell precursors (272). Each of these structures represents the earliest form of the functional units of the ovaries, the ovarian follicles. At this stage, they are called “primordial follicles” (Fig. 1.4). Thereafter, under the influence of a large number of intrinsic and extrinsic factors, granulosa cells grow and proliferate (272). Eventually,

the follicles are surrounded by an additional layer of supporting cells, the steroidogenic theca cells (Fig. 1.4). Ultimately, some follicles will mature to the point where they have the ability to release a fertilizable oocyte (ovulation), in a cyclical process termed "folliculogenesis" (Fig. 1.4) (272).

2.3.2.1. Gametogenesis in females

Unlike in males, where germ cells are mitotically arrested during embryonic development, oocytes enter meiosis immediately after ovarian fate commitment (273-276). However, they arrest at the prophase stage of meiosis I, and will not progress further until ovulation. Coincident with the release of the oocyte from its enclosing follicle at ovulation, meiosis resumes, but will not be fully completed until fertilization (277). Nevertheless, between its early maturation steps during embryonic development and ovulation in adulthood, the oocyte does not remain idle; it is transcriptionaly and translationaly active, accumulating in its large cytoplasm proteins and mRNAs which are absolutely crucial for the early development of the embryo after fertilization (278). Until ovulation, the oocyte remains enclosed in the protected milieu of the follicle; therefore, it is entirely dependent on surrounding theca and granulosa cells for reception of endocrine signals that direct its maturation (Fig. 1.4). Reciprocally, the oocyte has an instructive role for the development and homeostasis of follicular somatic cells (279).

2.3.2.2. Somatic cell organization and function in the ovary

Analogous to the role of Leydig cells in the testis, theca cells in the ovary have a prominent steroidogenic function. Theca cells are recruited to the ovarian follicle only when the latter reaches a certain developmental stage in the postnatal ovary (Fig. 1.4 and section 2.3.2.3.). Just as seen in Leydig cells, theca cells synthesize androgens in response to pituitary-derived LH, which induces the expression and activity of StAR and several enzymes required for steroidogenesis (280, 281). The main steroid product of the theca cells, androstenedione, diffuses to adjacent granulosa cells, where it is converted to estrone, and then to estradiol under the influence of FSH (282). The first important function of granulosa cells is to communicate with and "nurse" the oocyte, ensuring its maintenance and health. In particular, granulosa cells produce factors that ensure that the oocyte remains arrested in the prophase of meiosis I until ovulation (283). The second critical role of the granulosa cells is to respond to gonadotropin

stimulation to produce factors that regulate follicle maturation and/or that feedback on the hypothalamus and pituitary to regulate further gonadotropin production (272). One key product of the granulosa cells is estradiol, converted from estrone, itself produced from theca-derived androstenedione by the granulosa cell enzyme aromatase (encoded by the gene *Cyp19a1*). The expression of *Cyp19a1* is directly stimulated by FSH (284-286), which also plays a major role in stimulating granulosa cell proliferation through induction of *Cyclin D2* (287-289). The feedback effects of estradiol on hypothalamic GnRH neurons have been described in section 2.1.1.2., and those on pituitary gonadotropes are detailed in section 3.3.2. Other important endocrine products of the granulosa cells are inhibins, which feedback on the pituitary gland to negatively regulate the synthesis of FSH (290), as described in section 3.2.1.4.

2.3.2.3. Folliculogenesis

Shortly after ovarian follicle formation in the early postnatal period, a few primordial follicles undergo initial recruitment and grow to the primary and secondary stages (Fig. 1.4). During this transition, the granulosa cells, which are initially arranged in a single layer of flat cells surrounding the oocyte, grow and proliferate to form about four layers of cuboidal cells (Fig. 1.4) (272). By the secondary stage, theca cells surround the follicle, thus enabling subsequent supply of androgens to the granulosa cells (291). The initial recruitment of primordial follicle is continuous through life, until cessation of folliculogenesis at menopause. Despite decades of study, the factors responsible for the "decision" of primordial follicles to enter the growing pool of primary and secondary follicles remain unknown (272). What is clear is that the pituitary gonadotropins are not required for this to occur, as secondary follicles are observed in the ovaries of rodents lacking GnRH, FSH, LH, or after complete removal of the pituitary gland (220, 221, 292). Though the rate of initial follicle recruitment varies throughout life, there might be up to 50 secondary stage follicles at any given time in the ovary of young adult female mice (292). These follicles have the ability to respond to FSH and, if the stimulus is strong enough, will further develop into antral follicles (Fig. 1.4). This classification refers to the presence of an "antrum", a growing fluid-filled cavity within the follicle (293). From the antral stage onward, the follicles are absolutely dependent on FSH for their growth and maintenance (Fig. 1.4) (294). In response to FSH, the granulosa cells proliferate and secrete estrogens and other factors. If the follicles do not respond to FSH, or if the FSH stimulus is not sufficient, the follicles undergo

apoptosis and degenerate (a process called “atresia”) – and, indeed, this is the fate of most ovarian follicles (295, 296).

During the formation of the antral follicle, the granulosa cells that do not stay associated with the oocyte line the antrum underneath the theca layer, and become “mural” granulosa cells (Fig. 1.4). These cells differ from the cumulus granulosa cells in their function, in their ability to respond to endocrine stimuli, and in the types of factors they secrete (297). Late in the antral follicle stage, mural granulosa cells up-regulate expression of the LH receptor – until then restricted to the theca cells – under the influence of FSH (298-301). Coincidently, increases in the amount of estrogens released in the circulation by the preovulatory antral follicles (one in humans; up to 12-15 in mice) cause a surge of LH release by the pituitary gland (see section 3.1.1.). In response to LH, mural granulosa cells synthesize and secrete the epithelial growth factor (EGF)-like ligands, amphiregulin and epiregulin into the antrum (302-304). These factors act on the cumulus granulosa cells to promote the synthesis and secretion of an hyaluronic acid-rich extracellular matrix that intercalates between cumulus cells, thus causing “cumulus expansion” (272, 305, 306). At this stage, cumulus granulosa cells also produce a number of vasoactive factors and proteases required for ovarian follicle breakdown and the release of the oocyte (together with attached cumulus cells) from the ovary (Fig. 1.4) (272, 279, 283, 307-309). Following ovulation, and in response to LH, mural granulosa cells undergo dramatic morphological and functional changes, producing and releasing progesterone (310). Progesterone derived from these so-called “corpora lutea” (or the single “corpus luteum” in humans) (Fig. 1.4) is essential for embryo implantation and maintenance (311, 312). If the ovulated oocytes are not fertilized, the corpora lutea degenerate and cyclic ovarian follicle recruitment continues. Overall, theca and granulosa cells integrate LH and FSH stimuli to regulate ovarian follicle growth, oocytes maturation and ovulation.

3. Hormonal control of gonadotropin synthesis

Three major classes of hormones act directly on the gonadotropes to regulate gonadotropin synthesis and secretion: GnRH, the activin/inhibin system, and sex steroids. In this section, the physiological and molecular mechansisms underlying their effects on gonadotropes are described.

3.1. Gonadotropin-releasing hormone

3.1.1. GnRH and the LH surge

Arguably, the most remarkable event controlled by GnRH is the stimulation of the ovulation-inducing LH surge in females. Immediately prior to the LH surge, GnRH secretion dramatically increases (at least in rodents and sheep) and remains elevated for several hours (Fig. 1.5) (80, 188, 313). This drives a correspondingly massive production and release of LH by pituitary gonadotropes, which lasts at least several hours in rodents and sheep (e.g. (188, 313, 314)), and more than a day in humans (245). It is noteworthy that the LH surge is not exclusively driven by an increase in hypothalamic GnRH release. Indeed, there is substantial evidence showing that the sensitivity of the pituitary to GnRH dramatically increases at the time of the LH surge, an estrogen-dependent phenomenon probably attributable in large part to the up-regulation of GnRH receptors in pituitary gonadotropes (315-318). In fact, in humans with Kallmann's syndrome or in monkeys with hypothalamic lesions, an invariant regimen of pulsatile GnRH stimulation (i.e., same concentration and pulse frequency) can rescue menstrual cyclicity, including ovulation (319, 320). These observations call into question the requirement for an increase in endogenous GnRH release for LH surge generation in primates and humans. It is nevertheless clear that the full extent of the LH surge requires enhanced GnRH release, at least in sheep and rodents (321).

It is quite remarkable that the LH surge lasts for several hours, which has led many investigators to ask how much of the surge is actually required for ovulation. The LH surge regulates many events in the ovary required for ovulation of fertilization-competent eggs: resumption of meiosis in the oocytes, cumulus granulosa cell expansion, follicule rupture, and luteinization – processes that take several hours to be completed ((272, 307) and refer back to Section 2.3.2.3.). However, it is clear that the full extent of the LH surge (both in terms of amplitude and duration) is not required for ovulation and fertility. Indeed, mice with only 34% of

the normal complement of GnRH neurons exhibit normal fertility despite a blunted LH surge, whereas female mice with only 12% of the normal GnRH neuron number have poor fertility and essentially no LH surges (314). Furthermore, fertility can be rescued by transplanting *hpg* mice with only a few dozen GnRH neurons, despite the fact that their GnRH and LH surges are presumably much smaller than in normal animals (322, 323). In sheep, modest GnRH stimulation at the time of surge is sufficient for ovulation (324). The duration requirement of the surge across species remains unclear. It seems that rodents require between 1 and 2 h, whereas primates require more than 14 h of LH release to induce ovulation, which is shorter than their respective endogenous surges (325-327). Overall, these observations indicate that GnRH and LH are produced in excess at the time of the LH surge. As mentioned above in sections 2.1.1 and 2.2.3, GnRH is also a critical regulator of "baseline" LH and FSH synthesis and release during the rest of the estrous/menstrual cycle in females, and throughout life in males.

3.1.2. GnRH signaling at the cell surface

3.1.2.1. Structure and evolution of the GnRH receptor

The signaling events underlying GnRH regulation of LH and FSH synthesis and release is initated at the cell surface, by binding of GnRH to its receptor. The GnRH receptor (GnRHR) is a member of the G protein-coupled receptor (GPCR) superfamily, characterized by their seven transmembrane domains and their coupling to heterotrimeric guanine nucleotide-binding protein (G protein) complexes (328). In addition to mammals, the GnRHR is conserved in birds, amphibians and fish, and some species have more than one GnRHR-encoding gene (9, 329). Orthologs have also been identified in flies and worms, though it is not clear whether they are involved in reproductive function in those animals (9, 330). Elucidation of the tertiary structure of the GnRHR has, thus far, relied on homology modeling based on the projection and X-ray crystal structures of rhodopsin (9, 331-334). The positioning of the various transmembrane domains, intracellular, and extracellular loops was guided by the identification of two disulfide bonds – between transmembrane (TM) domain 3 and extracellular loop (ECL) 2, and between ECL2 and the N-terminal tail (335). Further, amino acids mediating physical interactions between various transmembrane domains (TM) are highly conserved between the GnRHR and other GPCRs of the same class (Class A), and are required for GnRHR stabilization (9, 334, 336). A ligand-binding pocket, involving direct contacts between the ligand and eight residues

within the extracellular loops or outer surface of TMs in GnRHR, has been defined by scanning mutagenesis studies (e.g., (9, 337-341)). Receptor activation likely involves changes in inter-TMs interactions and a rotation of TM3, similar to rhodopsin and other class A GPCRs (9, 342, 343). Finally, glycosylation of two extracellular residues in the murine receptor (and one in human) may affect receptor function and trafficking, though apparently not ligand binding affinity (344, 345). The recent description of the X-ray crystal structures of other class A GPCRs (346-349) will undoubtedly facilitate a refinement of GnRHR tertiary structure models, and perhaps pave the way for the experimental resolution of the GnRHR structure in the inactive and ligand-bound states. This could, in turn, facilitate the development of small molecule agonists or antagonists, which would be very useful clinically (refer back to section 1.3.).

3.1.2.2. G protein coupling of the GnRHR

GPCRs exist in equilibrium between ligand-bound/unbound and G protein-associated/dissociated states. The classical ternary model of GPCR function, to which the GnRHR appears to conform (9), proposes that agonist binding to the receptor promotes G protein association, and vice-versa (350, 351). Exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) bound to the G protein α subunit promotes G protein dissociation from the ligand-receptor complex (350, 351). Dissociated G protein subunits then activate downstream signaling mediators, and G_{α} hydrolyses GTP to return in the GDP-bound state (352). The nature of the signaling effectors activated varies between Gα subtypes, of which there are four main classes: $G_{\alpha s}$, $G_{\alpha q/11}$, $G_{\alpha i}$ and $G_{\alpha 12/13}$ (352). Even before the primary structure of the GnRHR was known, pioneering studies using nonhydrosylable GTP analogs established that the effects of GnRH in gonadotropes are mediated by G proteins (Fig. 1.6) (353-355). $G_{\alpha q/11}$ has consistently been identified as a crucial mediator of GnRHR signaling (356-360). $G_{\alpha s}$ and its canonical second messenger cAMP have also been implicated, but cAMP accumulation may occur downstream of $G_{\alpha q/11}$ (356, 357, 361-363). In heterologous cell lines, for example in prostate cancer cells, GnRH might signal through other G_{α} subunits – in particular $G_{\alpha i}$ – but it is not clear whether those pathways are also engaged in gonadotropes (364, 365). *In vivo* evidence supporting the involvement of any particular G_{α} subtype in GnRH signal transduction remains scarce. Mice lacking $G_{\alpha q}$ or $G_{\alpha 11}$ are fertile and have minimal defects in their response to GnRH agonists, strongly suggesting that they act redundantly, or perhaps that other G_{α} subtypes may

compensate in their absence (366). Thus, the G protein requirements for normal GnRH signaling in gonadotropes remain to be precisely defined.

3.1.2.3. GnRHR downregulation, desensitization and signal termination

After prolonged and/or intense stimulation, gonadotropes cease to respond to GnRH (188, 321). Loss of responsiveness to ligands or agonists that signal through GPCRs is commonly observed. In a prototypical GPCR, phosphorylation of the intracellular C-terminal tail (C-tail) or the third intracellular loop at serine or threonine residues by GPCR kinases (GRKs) occurs after ligand binding (367-370). This leads to the docking of β-arrestins, the recruitment of adaptor and coating proteins such as adaptor protein 2 (AP-2) and clathrins, the internalization of the receptors into vesicles, and finally their degradation or recycling (371-375). This process effectively desensitizes (and in the case of recycling, resensitizes) the cell to extracellular ligands acting via these receptors. However, the mammalian GnRHRs lack C-tails, which are present in all other GPCRs and non-mammalian GnRHRs described to date (376) (9). The functional implications of this unique property of the mammalian GnRHR have been extensively investigated. Though the C-tail of GPCRs might serve diverse functions (e.g., (377)), its best understood role is as a mediator of receptor downregulation following agonist stimulation (371, 378). Thus, the lack of a C-tail in GnRHRs was postulated to slow or impair receptor downregulation (379). This prediction was confirmed by a large number of studies in model cell lines. Mammalian GnRHRs internalize much more slowly and incompletely than their non-mammalian counterparts in response to agonist binding, whereas the addition of a C-tail (from another GPCR or a non-mammalian GnRHR) to a mammalian GnRHR accelerates its internalization (e.g (380-385)). The consequences of attenuated internalization in terms of signal transduction are unclear. Very few studies investigating this have employed homologous gonadotrope cell lines, and their conclusions depend on the signaling mediator measured (379, 380, 386, 387).

The functional role of slow and incomplete GnRHR downregulation in the mammalian reproductive system is highly intriguing. As described in Section 3.1.1., ovulation in mammals is driven by a protracted GnRH-driven LH surge. Generation of this surge requires that gonadotrope cells retain high sensitivity to intensive GnRH stimulation for several hours. Interestingly, species such as the domestic chicken, which have a GnRHR with a C-tail, generate

much shallower and shorter LH surges than mammals (388, 389). These observations have led to the proposal that the evolutionary loss of the C-tail in mammals may have enabled the production of protracted LH surges, upon which the ovulation process may have become reliant (390). However, this prediction remains to be formally tested *in vivo*. It is nevertheless clear, from the observation that the GnRH surge lasts longer than the LH surge (Fig. 1.5), that gonadotropes eventually desensitize to GnRH stimulation (80, 188, 313). The mechanisms underlying this desensitization are still largely unclear. This process does not necessarily involve cell surface downregulation of the receptors, and may instead be caused by the turnover of downstream signaling mediators (391-393). Overall, the gonadotrope response to GnRH desensitizes slowly, enabling potent and prolonged GnRH stimulation.

3.1.3. GnRH-regulated signaling patways in the cytoplasm

3.1.3.1. GnRH signaling pathways regulating gonadotropin release

In gonadotropes, GnRH activates two primary immediate biological responses: gonadotropin release and the activation of gonadotropin subunit gene expression (Fig. 1.6). Perhaps the most robust and reliably observed immediate response upon GnRH stimulation of gonadotropes is the production of inositol 1,4,5-trisphosphate (IP_3), which occurs within seconds after ligand binding to the GnRHR (394-399). IP_3 is produced by the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG) by phospholipase C (PLC) enzymes, themselves activated by GTP-bound $G_{\alpha q/11}$ proteins downstream of the GnRHR (Fig. 1.6) (353, 354, 400, 401). IP_3 then binds IP_3 receptors (IP_3R) on the membrane of the endoplasmic reticulum (ER), triggering the rapid efflux of calcium into the cytosol (Fig. 1.6) (376, 402-404). In gonadotrope cells, IP_3 production and intracellular calcium elevation is biphasic and oscillatory: a rapid phase of high-amplitude calcium release from the ER is followed by a prolonged and shallower phase, reflecting calcium influx from voltage-gated and voltage-independent calcium channels at the gonadotrope cell surface (405-408). As described in Section 2.2.2., LH is preferentially packaged in dense-core vesicles compared to FSH (200). Calcium binding to coat proteins induces vesicle priming and docking to the plasma membrane, followed by the extracellular release of luminal contents (Fig. 1.6) (409, 410). LH release and a large component of the FSH secretory reponse to GnRH are calcium-dependent (199). Remarkably, GnRH-induced LH secretion from gonadotropes is biphasic, precisely

following the pattern of IP_3 production and intracellular calcium elevation (411-413). Activation of protein kinase C (PKC) family members by DAG and calcium contribute to the second phase of intracellular calcium elevation and the associated sustained LH secretion, possibly through the modulation of calcium channel activity at the plasma membrane (414-417). Thus, calcium mobilization and influx in gonadotropes mediate rapid induction of gonadotropin secretion by GnRH.

3.1.3.2. Cytoplasmic GnRH signaling pathways regulating gene expression

In addition to stimulating gonadotropin secretion, the immediate signaling events induced by GnRH engage signaling cascades that activate gonadotropin subunit expression. PKCs comprise a family of at least eleven subtypes, categorized into three groups ("conventional", "novel" and "atypical") (418), and are canonical signaling mediators downstream of $G_{\alpha q/11}$. Their precise mechanism of activation and regulation varies between groups (418). In the case of conventional PKC isoforms, the enzymes are, basally, in an auto-inhibitory conformation, which is partially relieved by calcium binding (419, 420). This structural change facilitates cooperative binding of DAG (produced from PIP_2 hydrolysis by the action of PLC - see above) and calcium to the regulatory domain of the protein (419, 421, 422). DAG association with PKC creates a complex with high-affinity to phosphatidylserine, a lipid component of the plasma membrane that is required for full activation of PKC enzymatic activity (423, 424). Subsequently, PKC phosphorylates a wide variety of targets. As a result of its mechanism of activation, PKC accumulates near membranes after agonist-induced signaling (419). Several PKC subtypes are expressed in gonadotropes, with the exact complement varying between species and primary or immortalized cells (425-427). PKCs rapidly re-distribute to the plasma membrane in response to GnRH, consistent with the immediate production of IP_3 and the release of intracellular calcium stores after GnRHR activation (428-431). Several PKC subtypes are activated by GnRH, as defined by plasma membrane translocation and/or auto-phosphorylation (Fig. 6) (431-435). In addition, GnRH stimulates the expression of some PKC subtypes with varying kinetics (432). In gonadotrope-like cell lines and primary pituitary cell culture, PKC activation is required for GnRH-stimulated transcription of all three gonadotropin subunit genes (*Cga*, *Fshb* and *Lhb*) (436-439). Nevertheless, the identity of the PKC subtypes involved in regulating each of these responses remains unknown. Furthermore, there is as yet no *in vivo* evidence supporting a

necessary role for any PKC protein in gonadotrope function, though such studies would possibly be complicated by functional redundancy between subtypes [e.g. (440)].

Among the major signaling mediators activated downstream of PKC, mitogen-activated protein kinases (MAPKs) have been extensively implicated in GnRH-regulated gonadotropin subunit synthesis (Fig. 1.6) (441). There are four distinct MAPK pathways: extracellular signal-regulated kinases 1/2 (ERK1/2), cjun-N-terminal kinases (JNKs), p38, and big MAPK (BMK/ERK5) (442). All the MAPKs act downstream of linear phosphorylation cascades involving MAPK kinases (MAP2K) and MAP2K kinases (MAP3K) (Fig, 1.6) (442, 443). In the case of ERK1/2, well-characterized MAP2Ks and MAP3Ks are mitogen-activated protein kinase kinase 1/2 (MEK1/2) and v-raf-leukemia viral oncogene 1 (c-Raf or Raf1), respectively (443-445). Activated MAPKs can phosphorylate a wide variety of substrates, including transcription factors, thus providing a link between cytoplasmic signaling events and gene transcription (446). All four MAPKs are rapidly activated after GnRH stimulation in gonadotropes (447-453), in a PKC-dependent manner (438, 447, 452, 453). The link between PKC activation and MAPK pathway engagement in gonadotropes remains somewhat obscure. An obvious candidate is c-Raf (445, 454). Indeed, constitutively active c-Raf expression promotes ERK1/2 phosphorylation in gonadotropes (455, 456). However, pituitary-specific *c-Raf* knockout mice have no reproductive phenotype, and pharmacological inhibition of c-Raf does not prevent GnRH-induced ERK1/2 phosphorylation (455). Since MAPK pathways can be activated by a large number of effectors downstream of cell surface receptors (442), further studies are required to identify which ones couple GnRH receptor activation to MAPK phosphorylation. In primary pituitary cells and gonadotrope cell lines, GnRH-regulated *Lhb* expression depends on ERK1/2 and JNK, but not p38 (449, 457-459), whereas GnRH-induced *Fshb* expression appears to be dependent on ERK1/2, p38, and perhaps BMK, but not JNK (427, 448, 460-465) (Fig. 1.6). GnRH activates *Cga* expression through ERK1/2 (Fig. 1.6) (439, 466, 467). These cell-based findings were supported in part by the generation and analysis of pituitary-specific ERK1/2 knockout mice, which display low *Cga* and *Lhb* expression, impaired *Lhb* upregulation in response to gonadectomy or GnRH stimulation, and female infertility (468). These mice retain apparently normal basal *Fshb* expression and some *Fshb* upregulation in response to ovariectomy, but the interpretation of these results may be complicated by the crucial role played by activin signaling in the regulation of *Fshb* transcription (see Section 3.2.). Though these observations demonstrate

a necessary role for ERK1/2 signaling in mediating the effects of GnRH on gonadotropes *in vivo*, they do not rule out some contribution by other MAPKs, a possibility which has yet to be investigated.

In addition to MAPKs, GnRH activates other signaling pathways that may contribute to gonadotropin gene expression. For example, GnRH stimulation produces cAMP and activates PKA in gonadotropes (357, 362, 363, 469-474). This pathway has been implicated in the control of both *Lhb* and *Fshb* transcription (471, 473). Yet another pathway activated by GnRH signaling in gonadotropes is the calmodulin (CaM)/calmodulin-dependent kinase (CaMK) signaling cascade (475). When bound by calcium, CaM acts as a regulatory subunit for CaMKs and phosphatases such as calcineurin (476). By causing a large increase in intracellular calcium levels, GnRH stimulation activates CaM, and consequently CaMKs and calcineurin in gonadotropes (477-482). Both CaMKs and calcineurins regulate transcriptional responses to GnRH in gonadotrope-like cell lines (480-484). In the case of calcineurin, this may involve dephosphorylation (and, hence, activation) of the transcription factors nuclear factor of activated T cells 3 (NFAT3) and cyclic-AMP response element binding protein (CREB)-regulated transcription coactivator 1 (CRTC1) (483, 484). Overall, the evidence supporting roles for cAMP and CaM-dependent signals in mediating GnRH responses is preliminary, particularly in comparison to the well-established requirement for MAPKs. *In vivo* confirmation of their involvement, for example using appropriate knockout models, is required. Based on the most compelling evidence, GnRH regulation of gonadotropin subunit expression appears largely dependent on PKC and MAPK signaling in the cytoplasm.

3.1.4. GnRH regulation of gene expression

Integration of the signals controlling GnRH-stimulated gonadotropin subunit synthesis occurs mainly at the transcriptional level (485), though GnRH also regulates protein translation (486). Thanks to the extensive use of gonadotrope-like cell lines and to a limited number of *in vivo* genetic manipulations, major strides have now been made in our understanding of how *Cga*, *Lhb*, and *Fshb* are transcriptionaly activated downstream of GnRH-regulated cytoplasmic signaling cascades.

3.1.4.1. GnRH regulation of Cga transcription

The proximal *Cga* promoter contains several conserved *cis*-elements required for basal and GnRH-stimulated gene transcription (Fig. 1.7). A pituitary-specific element (PSE) (136), at -344/-300 directs basal *Cga* promoter activity to gonadotrope/thyrotope-like cell lines (here and after: "+1" is the transcription start site; unless otherwise noted, the base-pair positions correspond to the murine sequence) (487-489). LIM homeodomain transcription factors bind a consensus sequence within this element (490-492). Both LHX2 and LHX3 can bind and activate the PSE *in vitro*, and *Lhx3*-knockout mice have profoundly disrupted pituitary development, including near-complete absence of mature gonadotropes (131, 490-492). Another well-characterized and conserved basal gonadotrope-specific element (GSE), at -215/-207, is bound by SF1 (458, 488, 493, 494). Supporting the importance of this element, mice with global or pituitary-speficific deletion of *Sf1* have very low pituitary *Cga* expression (140, 141). In the human promoter, two cAMP-response elements (CREs) are very important for basal *CGA* transcription in cell lines (487, 495-498). This sequence, which is bound by c-Jun and ATF2 transcription factors (497, 499), is only partially conserved in other mammals (497, 500). A binding site for *paired*-like homeodomain transcription factors (PITX) at -398/-385 also contributes to basal *Cga* promoter activity (501).

GnRH responsiveness of the *Cga* promoter is conferred by the PSE described above (Fig. 1.7) (491, 502) and a GnRH response element (GRE) at position -406/-399, which contains a consensus binding site for E26 transformation-specific (ETS) transcription factors (Fig. 1.7) (439, 467, 502). Factors binding these two sites act cooperatively to mediate GnRH-stimulated promoter activity (502). One or more ETS family members act through the GRE downstream of GnRH and PKC/MAPK signaling (Fig. 1.7) (439, 467, 503, 504). However, the exact identity of the endogenous factor(s) involved remains unknown. One candidate is ELK1, an ETS transcription factor activated by ERK1/2 after GnRH stimulation (467, 505). Presumably, ETS factors bound to GRE physically (directly or indirectly) and functionally interact with LHX proteins bound to PSE in response to GnRH (485). The precise identify of the ETS and LHX transcription factors involved in this process remains unclear. A GnRH-responsive region has also been mapped in the human promoter (458, 487, 503). This *CGA* sequence comprises the conserved PSE, as well as an ETS transcription factor binding site (similar to the murine GRE)

(458, 485, 487, 503), suggesting that the general mechanism of GnRH-stimulated *Cga*/*CGA* promoter activation may be conserved. In sum, GnRH signaling appears to induce ETS proteins, which cooperate with LHXfactors to activate *Cga* transcription.

3.1.4.2. GnRH regulation of Lhb transcription

The rate limiting step for GnRH-stimulated *de novo* gonadotropin synthesis is the transcription of their β subunit genes. Basal and GnRH-regulated *Lhb* promoter activity depends on a segment, convering approximately 100-bp, that contains a well-defined set of five transcription factor binding sites conserved across mammals (Fig. 1.8) (485). Two "GSEs", located at -129/-122 and -57/-50, contain consensus binding sequences for SF1 (Fig. 1.8) (506-508). These elements contribute to both basal and GnRH-stimulated *Lhb* promoter activity in cell lines and transgenic mice (437, 506-513). As mentioned in section 2.2.1.2., SF1 is not absolutely required for GnRH-induced LHβ synthesis in mice (141, 142). The closely related transcription factor, liver receptor homolog-1 (LRH-1, or NR5A2) can also bind the *Lhb* promoter and stimulate its transcription, but is dispensable for normal *Lhb* expression and gonadotrope function *in vivo* (514, 515). A PITX binding site ("PBE") is located at -100/-95, and appears to be required for both basal and GnRH-stimulated *Lhb* promoter activation in immortalized gonadotropes and transgenic mice (Fig. 1.8) (510, 512, 513, 516-518). Two PITX proteins are expressed in gonadotropes (PITX1 and PITX2), and both can activate the *Lhb* promoter (516, 518, 519). *Pitx1* and *Pitx2* knockout mice display abnormal pituitary development, a lower number of gonadotropes and impaired *Lhb* expression (520, 521). However, gonadotrope-specific deletion of PITX2 in mice does not compromise LH synthesis or fertility, indicating either functional redundancy with PITX1, or a non-cell-autonomous role for the protein in gonadotrope function (522).

Two central elements, at -111/-103 and -48/-40, confer GnRH responsiveness to the *Lhb* promoter, and are located adjacent to the SF1 and PITX binding sites (145, 437, 509, 510, 513). These sites comprise a consensus binding sequence for the early-growth response 1 (EGR1) transcription factor (EBE) (Fig. 1.8). The unexpectedly critical role for EGR1 – a ubiquituously expressed transcription factor induced by a variety of stimuli (523) – in *Lhb* transcription was revealed by the analysis of *Egr1* knockout mice. These animals display female-specific infertility due to very low levels of LHβ synthesis and absence of ovulation (145, 524). *Egr1* mRNA and

protein levels are rapidly induced in response to GnRH in gonadotropes, a process which appears to rely on MAPK-dependent activation of ELK1, a direct activator of *Egr1* transcription (Fig. 1.8) (437, 505, 512, 513). Indeed, mice lacking ERK1/2 in gonadotropes are unable to upregulate *Egr1* expression in response to GnRH (468). EGR1 physically and functionally interacts with both SF1 and PITX1, which themselves also directly interact, to synergistically activate *Lhb* transcription (506, 509, 510, 512, 513). The proximity of the binding sites for all three factors within the *Lhb* promoter undoubtedlty facilitates their cooperative activity (485). Furthermore, the molecular link provided by EGR1 between SF1 and PITX1 likely explains the requirement of the later two proteins for GnRH-stimulated *Lhb* promoter activation, as they are not themselves induced by GnRH. EGR1 activity is potentiated by GnRH-induced CREB-binding protein (CBP) phosphorylation (p-CBP), and knock-in female mice lacking p-CBP are subfertile due to impaired GnRH-stimulated LH production and LH surge generation (525).

In addition to the core EGR1/SF1/PITX module regulating *Lhb* expression, other promoter elements and factors have been implicated in basal and GnRH-stimulated *Lhb* transcription. In the rat, two distally-located *trans*-acting transcription factor 1 (SP1) binding sites (also conserved in mouse) are required for the full basal and GnRH-stimulated induction of the *Lhb* promoter (Fig. 1.8) (439, 509, 526, 527). In cow, it is instead two NF-Y binding sites that are present at that location, one of which is required for basal promoter activity in transgenic mice (528). SP1 proteins might replace EGR1 binding to the more proximal EBE elements in some species (529). Similarly, the PITX element can be bound by orthodenticle homeobox (OTX) transcription factors (530). Nevertheless, roles for endogenous SP1 or OTX proteins in the regulation of *Lhb* transcription remain to be demonstrated. Finally, the co-activator β-catenin might be required for the full activity of the EGR1/SF1/PITX module, by physically associating with SF1 (531, 532). Overall, GnRH-stimulated *Lhb* expression is mainly mediated by ERK1/2-dependent induction of EGR1, which cooperates with SF1 and PITX1/2 to activate *Lhb* transcription.

3.1.4.3. GnRH regulation of Fshb transcription

Of the three gonadotropin subunit genes, *Fshb* is arguably the one whose regulation by GnRH is least understood, even though FSH synthesis is robustly GnRH-dependent *in vivo* (533, 534). Several promoter elements and transcription factors have been implicated in basal *Fshb*

transcription (203). Two putative "GSE" elements, at -341/-334 and -239/-231, bind SF1 and, when mutated in combination with and imperfect nuclear factor-y (NF-Y) site at -79/-69, decrease basal *Fshb* promoter activity (535). Consistent with a role for SF1 in *Fshb* expression, *Sf1* knockout mice have dramatically reduced pituitary *Fshb* and circulating FSH levels (140, 141). That said, SF1 overexpression does not directly activate the *Fshb* promoter (514). Since gonadotrope-specific *Sf1* knockout mice also have impared *Gnrhr* expression (141), the effect of loosing SF1 on FSH synthesis may be due to impaired GnRH signaling. A well-conserved PITX binding site, at -53/-49, contributes to both basal and activin-stimulated (see Section 3.2.4.1.) *Fshb* promoter activity in cell lines and in transgenic mice (501, 518, 536-540). Furthermore, knockdown of endogenous PITX1 or PITX2 proteins in immortalized gonadotrope-like cells impairs *Fshb* transcription (537). Whole-body *Pitx1* and *Pitx2* knockout mice have lower *Fshb* expression; but, as discussed above (see section 3.1.4.2.), this may reflect abnormal gonadotrope specification in those animals (520, 521). Gonadotrope-specific *Pitx2* knockout mice have normal *Fshb* expression (522), but definitive assessment of a role for PITX proteins would require removal of both *Pitx1* and *Pitx2*. LHX3 can activate *Fshb* transcription through up to three putative binding sites in the porcine promoter, and at least one in the human promoter (541, 542). Finally, pituitary-specific *Gata2* knockout mice have impaired FSH synthesis (143), but a direct role for GATA2 in regulating *Fshb* transcription remains to be demonstrated.

Unlike in the *Cga* and *Lhb* promoters, none of the "basal" *Fshb* elements outlined above have been clearly implicated in mediating the GnRH response. Many sequences required for GnRH activation are not fully conserved, which may reflect species-specific regulatory mechanisms (203). In the ovine promoter, two binding sites for activator protein-1 (AP-1) family members, at -120/-114 and -83/-77 (543), are required for GnRH induction in heterologous cells overexpressing GnRHR (544). Consistent with a role for AP-1 family proteins, GnRH stimulates the expression of the jun proto-oncogenes, CJUN and JUNB, as well as the FBJ osteosarcoma oncogenes, CFOS and FOSB, in immortalized gonadotropes (Fig. 1.9) (461, 462, 465). Heterodimers of FOS and JUN proteins form a functional transcription factor unit (545). GnRH appears to induce *c-jun* expression through activating transcription factor 2 (ATF2), and c-fos expression though serum response factor (SRF) and ELK1 (546, 547). Yet, mutation of the AP-1 sites minimally affects GnRH-stimulated ovine *Fshb* promoter activity in gonadotrope-like cells, and an ovine promoter lacking both sites shows normal regulation in transgenic mice – even

though it has lower GnRH responsiveness in primary pituitary cultures from the same animals (427, 460, 548). One of the AP-1 sites (-117/-111) is conserved in the human *FSHB* promoter, binds AP-1 proteins, and is required for GnRH induction (465). An additional AP-1 half site, present in both the murine (-71/-68) and human (-83/-80) promoters, also contributes to GnRH responsiveness (462, 465). Functional analyses using dominant-negative proteins supports a role for endogenous AP-1 proteins in immortalized gonadotropes (462, 465), but their requirement *in vivo* remains to be established. Mechanistically, the ERK1/2 and p38 pathways have been implicated in GnRH-induced AP-1 protein expression (Fig. 1.9) (453, 456, 461, 462, 465, 477, 547). However, mice lacking ERK1/2 in gonadotropes have normal basal, and midly impaired ovariectomy-induced *Fshb* expression, although the transcript or protein levels of AP-1 factors in the pituitary of these animals were not reported (468). NUR77 (also known as NR4A1), a transcription factor induced by GnRH (455, 549, 550) may also participate in GnRH-induced *Fshb* expression (482).

A GnRH regulatory mechanism involving AP-1 or NUR77 protein induction implies an indirect *Fshb* transcriptional response to GnRH stimulation. However, at least in rats, *Fshb* transcription occurs within a few minutes of GnRH stimulation (551), suggesting more immediate mechanisms. In the rat promoter, the AP-1 site instead acts as a cAMP-response element (CRE), bound by CREB, and is required to mediate approximately 50% of the GnRH response (Fig. 1.9) (552). Immediately adjacent to this site, upstream stimulatory factor (USF) proteins bind and activate *Fshb* transcription in cooperation with CREB (552), although this might be a rat-specific mechanism (Fig. 1.9) (203). Mechanistically, GnRH rapidly induces CREB phosphorylation in a cAMP/PKA-dependent manner (Fig. 1.9) (473), leading to the recruitment of transcription activating factors such as CBP (552). Whether cAMP production in response to GnRH reflects $G_{\alpha s}$ activation (357, 361, 362), or rather occurs downstream of $G_{\alpha q/11}$ and PKC (356, 363), remains to be established. Overall, each of the putative mechanisms of GnRH-stimulated *Fshb* transcription accounts only for a fraction of the GnRH response, and none have yet been validated *in vivo*.

3.2. Activins and Inhibins

3.2.1. The activin/inhibin system and the control of reproduction

As described in the previous sections, it is abundantly clear that GnRH potently stimulates the synthesis and release of both LH and FSH. However, LH and FSH are differentially regulated. For example, many mammals, including rodents and humans, display a period of selective FSH elevation during their estrous/menstrual cycles, which drives ovarian follicle maturation ((241, 243, 249, 250) ; see Section 2.2.3.). Furthermore, FSH increases faster than LH after gonadectomy (553), and is elevated prior to LH in pubertal girls (554) and peri-menopausal women (555, 556). Part of this differential regulation has been attributed to divergent sensitivity of LH and FSH synthesis to GnRH pulse frequency – with faster pulses favoring LH, and slower pulses favoring FSH (215, 216, 557-559). However, the importance of variations in GnRH pulse frequency for the regulation of FSH synthesis appears questionable: in *hpg* mice, continuous GnRH administration robustly rescues FSH synthesis (534). Also, in GnRH-deficient monkeys and humans, constant GnRH pulses allow the completion of folliculogenesis (319, 320). Furthermore, steady-state FSH synthesis does not depend as much on GnRH as LH synthesis does (560-563). These observations support the existence of a regulatory system, parallel to GnRH, controlling only FSH production. Such a system, involving inhibin and activin hormones, has now been well described (290, 564).

3.2.1.1. Discovery and purification of inhibins and activins

The existence of inhibins was first postulated 90 years ago, when hypertrophic pituitary cells (presumably gonadotropes) were observed in rats after testes irradiation – a procedure which damages seminiferous tubules but leaves androgen production by Leydig cells intact (565). This effect could be reversed by a water-soluble substance extracted from normal testes, suggesting the involvement of a non-steroidal, protein hormone ("inhibin") (566). The evidence, however, remained circumstantial and somewhat controversial until the mid-1970s (290). Crucial observations made at that time included the demonstration that Sertoli cell secretions can suppress FSH release by cultured pituitary cells (567), and that administration of testicular fluid extracts to castrated rats selectively suppressed FSH levels (568). Up to this point, the "inhibin effect" was considered male-specific. Subsequently, it was shown that the early post-castration

FSH increase in males and the secondary FSH surge (refer back to section 2.2.3.) in females could be suppressed by injection of steroid-depleted ovarian follicular fluid, strongly supporting the existence of "inhibin" in females as well (569, 570).

Follicular fluid can suppress FSH synthesis and secretion by gonadotropes in primary pituitary cultures (571). This provided a simple assay for the biochemical identification of inhibin. Its purification revealed that there is not one, but rather two forms of inhibin (A and B), and that both are dimeric proteins comprised of a common α (product of the *Inha* gene) and either of two similar β subunits (βA for inhibin A, and βB for inhibin B, encoded by the *inhba* and *inhbb* genes) (572, 573). Identification of the amino acid sequence of the subunits facilitated the subsequent cloning of their respective genes (574-577). During the process of inhibin biochemical characterization, it was surrendipitously observed that a follicular fluid fraction could stimulate the release of FSH by primary pituitary cells (578). Unexpectedly, further purification revealed that the responsible substance is composed of dimers of β subunits (579-581). The term "activins" was thus coined to describe these hormones (activin A, B or AB, depending on the identity of the two β subunits). The β subunits of inhibins and activins resemble those of transforming growth factor β (TGFβ), thus adding these proteins to the superfamily of TGFβ-related ligands (575). Overall, activins and inhibins are structurally related ligands which selectively and antagonistically regulate FSH synthesis and secretion.

3.2.1.2. Inhibins and activins: endocrine, paracrine, and autocrine actions

Inhibins act as classical endocrine hormones, participating in a long-range negative feedback loop with the pituitary gland. First, the inhibin subunits are abundantly expressed in testicular Sertoli cells and in granulosa cells of antral follicles (582-584). Second, gonadal expression of the α subunit and serum levels of inhibins are up-regulated in response to gonadotropin stimulation (577, 585-587), and inversely correlate with FSH secretion during the estrous cycle (see section 3.2.1.4.). Third, bioneutralization of circulating inhibins elevates FSH levels (588-591). Fourth, mice with a targeted deletion of the α subunit (*inha*) have elevated serum FSH levels (592). In fact, *inha* knockout animals develop aggressive testicular and ovarian tumors whose development is at least partially dependent on elevated FSH, as additional deletion of *Fshb* reduces tumor burden (593). That said, inhibin subunits are also synthesized by the pituitary gland (583) and may have additional autocine/paracrine effects on gonadotropes (594).

Activins, unlike inhibins, appear to primarily regulate the synthesis of FSH by autocrine/paracrine action on gonadotropes (595). Part of this view arose from the findings that follicular fluids contain another potent suppressor of FSH synthesis, follistatin (596, 597). Follistatin is not structurally related to inhibins or activins (596, 597) and binds almost irreversibly to activins, thus potently neutralizing their activity (598, 599).Thus, because they are bioneutralized by follistatin, gonad-derived activins are unlikely to provide an endocrine signal that regulates FSH levels under homeostatic conditions (600). Pituitary cells, including gonadotropes, express the activin βB subunit, in principle allowing autocrine/paracrine regulation of FSH synthesis (583, 601). Indeed, bioneutralization of activin B (dimers of βB subunits) released by primary pituitary cells inhibits the synthesis and secretion of FSH in culture (602). In addition, ovariectomy increases FSH synthesis by pituitary glands implanted under the kidney capsule of hypophysiectomized rats, and this effect is blocked by activin B antibodies, further supporting a role for autocrine/paracrine activin B in the regulation of FSH output *in vivo* (603). It is now well accepted that the potent FSH-suppressing effect of inhibins and follistatin observed in primary pituitary culture is not due to a direct signal induced by these factors in gonadotrope cells, but is rather explained by the blockade of locally-released activin B (595, 600). That said, *Inhbb* knockout mice, which do not produce activin B and activin AB, have normal-to-elevated circulating FSH levels (604). Complicating the interpretation of this phenotype, these mice also lack inhibin B, and activin A may compensate, though not from a gonadal source (604, 605). In fact, to this day, the identity of the activin or activin-related ligand(s) required for FSH synthesis *in vivo*, as well as their precise cellular origin, remains unknown. In sum, activins or related ligands, likely produced locally within the pituitary, stimulate FSH synthesis and release, and this effect is antagonized by gonad-derived inhibins.

3.2.1.3. Dynamics of the inhibin/activin system in males

Sertoli cells are the main source of inhibins in the circulation of male mammals (567, 606, 607). Unlike females, males usually produce only inhibin B (608-610). In rodents and humans, serum inhibin B levels peak during the postnatal period, as a consequence of increased pituitary FSH secretion and Sertoli cell proliferation (611-613). In rats, removal of the testes or bioneutralization of circulating inhibins at this age rapidly up-regulates FSH levels, supporting the existence of a functional FSH/inhibin B endocrine feedback loop (553, 612). Later in life,

however, serum inhibin B levels decline to less than a third of their postnatal levels (611-613), and blockade of inhibin activity at this stage no longer affects FSH levels in rats (612). Nevertheless, there is a robust inverse relationship between serum inhibin B and FSH in adult men, arguing that the inhibin feedback loop operates throughout life in humans (611).

3.2.1.4. Dynamics of the inhibin/activin system in females

In contrast to males, a critical role for the inhibin/activin system in the regulation of female reproductive function and fertility is well established (564). Inhibin production by the ovary and its feedback regulation of FSH synthesis begins at puberty (290, 553, 614). Thereafter, ovarian inhibin production is highly dynamic across reproductive cyles. In the rodent estrous cycle, inhibin A levels are low during metestrus and diestrus, peak at proestrus (coincident with the LH and primary FSH surge), and decline rapidly in the morning of estrus, at the time of the secondary FSH surge (Fig. 1.10) (610, 615). Inhibin B, on the other hand, is high at metestrus/diestrus, drops at proestrus, and remains low during early estrus (Fig. 1.10) (610, 615). Thus, there is a strong negative correlation between inhibin B and FSH levels across the estrous cycle (Fig. 1.10). A similar, though not identical, pattern is observed in the human menstrual cycle, where inhibin levels are lowest at the time of the selective FSH elevation at the beginning of the follicular phase (610, 615-617). At the molecular level, expression of the α and βA subunits is robustly detected only in the mural granulosa cells of antral follicles, but not at earlier stages or in corpora lutea (618). Accordingly, inhibin concentrations are highest in the fluid of large antral follicles (e.g (619-621)). These observations agree with circulating hormone measurements and correlate nicely with the maturation stages of ovarian follicles across the estrous cycle (refer back to Sections 2.2.3. and 2.3.2.3.). The presence of high inhibin A simultaneously with the primary FSH surge is consistent with a prominent role for GnRH in stimulating FSH (together with LH) synthesis and secretion at that time of the cycle (561, 622). On the other hand, the fact that both inhibins are depleted on the early morning of estrus, when FSH is selectively increased, suggests that minimal inhibin negative feedback enables the secondary FSH surge, which drives ovarian follicle maturation beyond the pre-antral stage (Fig. 1.10) (249, 250). Indeed, the estrus morning FSH surge can be blocked by inhibin-containing follicular fluid in rodents (570). Furthermore, the expression of inhibin/activin subunits in the pituitary shows little changes across the estrous cycle (623). Globally, these observations support

a model wherein low levels of inhibins provide a permissive environment for enhanced activin autocrine/paracrine action on pituitary gonadotropes in estrus (and early follicular phase in humans), thus leading to a surge in FSH synthesis that drives ovarian follicle maturation (203, 595, 600).

3.2.2. Activin signaling at the cell surface

The physiological observations outlined above have provided a clear incentive to elucidate the molecular mechanisms whereby activin signaling regulates FSH synthesis in gonadotropes. A model of activin signaling in gonadotropes, mostly based on *in vitro* observations, have now been described (203) (Fig. 1.11).

3.2.2.1. The activin receptors and their activation

Activins are members of the TGFβ superfamily, which comprises dozens of structurally related ligands including inhibins, bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs) (624). Most of these proteins share a common signaling mechanism at the level of their receptors and intracellular signaling effectors (625). TGFβ and activin ligands first bind simultaneously to two type II receptors at the cell surface (Fig. 1.11) (626). The type II receptors are constitutively active serine/threonine kinases, and their engagement by ligands facilitates the recruitment of two type I receptors into the complex (Fig. 1.11) (627, 628). The type II receptors phosphorylate a glycine/serine-rich region on the type I receptor, called the "GS box" (628-630). As a result, the serine/threonine kinase activity of the type I receptor is disinhibited, and the docking and phosphorylation of downstream effectors, the Sma and Mad-related (SMAD) proteins, is enabled (Fig. 1.11) (628, 631). BMP ligands serve as crucial platform for the interaction of type II – type I receptors, as revealed by structural analyses of BMP-bound receptor complexes (632). Activin A binding to type II receptors appears to fullfil a similar role (633, 634). Overall, the hexameric ligand-receptor complex is thought to form the functional signaling unit at the cell surface. Two type II receptors for activins have been identified: activin receptor type 2 A and B (ACVR2A and ACVR2B) (635, 636). As well, two *bona fide* activin type I receptors have been identified: the canonical receptor, activin receptor type 1B (ACVR1B, also known as ALK4), and activin receptor type 1C (ACVR1C, also known as ALK7), through which activin B can signal in gonadotropes (637-640). It is important to note

that these are not exclusive activin receptors, as BMPs and Nodal can also bind to and signal through some of these receptors (625). Furthermore, activins may signal through non-canonical type I receptors in some contexts (e.g. (641)) and through the BMP type II receptor BMPR2 in gonadotropes (642), providing additional complexity to the system.

3.2.2.2. Activin receptor function in gonadotropes

Acvr2a-null mice display female-specific infertility, low circulating FSH levels and low immunoreactivity for FSHβ in the pituitary (643). Thus, although both ACVR2A and ACVR2B are expressed in gonadotropes (644-646), ACVR2B is not able to fully compensate for the loss of ACVR2A. A potential contribution by ACVR2B *in vivo* has not be established so far, due to perinatal lethality resulting from global gene knockout (647). ACVR2A is also expressed in the ovary, where activins and related ligands have important functions, and the contribution of a gonad-autonomous defect in the sterility phenotype of *Acvr2a* mutant females cannot be excluded (648). Injection of the soluble ectodomain of ACVR2A, which bioneutralizes activins, robustly suppresses circulating FSH levels in post-menopausal women (649). Thus, activin signaling through ACVR2A appears to regulate FSH synthesis in humans as well. With respect to the type I receptor, activin induction of *Fshb* expression in immortalized and primary gonadotrope cells is mediated through one or a combination of ACVR1B, ACVR1C or transforming growth factor β receptor type I (TGFBR1) (644, 650-652). *Acvr1b* and *Tgfbr1* knockout mice die during embryonic development, thus precluding assessment of their pituitary function (653, 654). *Acvr1c*-null mice are viable and show mild reproductive dysfunction, including slightly lower circulating FSH in females (but normal pituitary *Fshb* expression), apparently due to ovarian and/or hypothalamic defects (655). Thus, gonadotrope-specific knockout of *Acvr1b* and *Tgfbr1* will be required to identify the relevant type I receptor(s) *in vivo*. Finally, multiple factors (e.g. Cripto, BMP and activin membrane-bound inhibitor (BAMBI) and SMAD7) act upstream, in parallel or downstream of the activin receptors to modulate ligand binding and/or receptor activity (reviewed in (625)), and their functional importance in gonadotrope cells remains ill-defined. In sum, activins regulate FSH synthesis via ACVR2A and one or more of ACVR1B, ACVR1C and TGFBR1.

3.2.3. Activin-regulated signaling pathways in the cytoplasm

3.2.3.1. SMAD proteins

Downstream of type I receptor activation, the canonical mediators of TGFβ superfamily ligand signaling are the SMAD proteins (Fig. 1.11) (625, 656-660). The receptor-activated SMAD proteins (R-SMADs) are divided in two classes: SMADs 2 and 3 are activated downstream of ACVR1B, ACVR1C and TGFBR1, whereas SMADs 1, 5, and 8 are activated downstream of ACVR1, ACVRL1, BMPR1A and BMPR1B (625, 656-660). The amino acid identity of a small stretch of residues on the R-SMADs (the L3 loop) and on the type I receptors (the L45 loop) mediate specific R-SMAD/receptor pairing (661-663). All R-SMADs are composed of two functional domains, Mad homology 1 (MH1) and 2 (MH2), separared by a "linker" region (664). Whereas the MH1 domain mediates DNA binding, the MH2 domains brokers interactions with other SMAD proteins and with co-factors (664). Upon their recruitment to ligand-bound receptor complexes, the kinase domain of the type I receptor phosphorylates two serine residues at the extreme C-terminus of R-SMADs (657, 658, 665). This event enables high-affinity interactions between R-SMADs, as well as their association with the "co-SMAD", SMAD4 (659, 664-669). The association of R-SMADs with SMAD4 facilitates SMAD complex accumulation in the nucleus, where they participate in transcriptional regulation of target genes (Fig. 1.11) (664, 665, 670, 671). Thus, SMAD4 is critically required for most of the biological responses downstream of TGFβ superfamily signaling (625). Interestingly, the full-length form of SMAD2 cannot bind DNA because of an extra amino acid stretch next to the β-hairpin structure that mediates MH1 binding to DNA (672, 673). However, a natural splice variant of SMAD2 (SMAD2Δexon3), which is expressed in gonadotropes (674), lacks these residues and can bind DNA similarly to SMAD3 (673).

In immortalized gonadotropes, SMAD2, SMAD3 and SMAD4 mediate activin-induced *Fshb* promoter activation (Fig. 1.11) (463, 539, 674-679). Whereas activin-stimulated rat *Fshb* promoter activity depends on SMAD3 (463, 539, 677), the mouse promoter appears to be regulated by both SMAD2 and SMAD3 (674, 676, 678). Global *Smad2* and *Smad4* knockout mice are embryonic lethal (680-682), requiring cell-specific inactivation to assess their function in gonadotropes *in vivo*. *Smad3* mutants, however, are viable (683-686). Several different *Smad3* knockout lines have been generated, with discordant phenotypes (683-686). Only one of these, in

which the 8th exon (of 9) was deleted (*Smad3*$^{\Delta ex8}$), shows clear reproductive anomalies (684, 687). Male mice homozygous for the *Smad3*$^{\Delta ex8}$ mutation have lower pituitary *Fshb* expression (688), yet the females have elevated circulating FSH levels (687). However, female *Smad3*$^{\Delta ex8}$ mice display intrinsic ovarian anomalies, consistent with a cell-autonomous requirement for SMAD3 in granulosa cell function (689), and complicating the interpretation of pituitary phenotypes (684, 687). Thus, the role of the canonical activin-regulated R-SMADs in gonadotropes, where they may very well act redundantly, remains unknown. In addition, SMAD1/5/8 proteins appear to mediate *Fshb* promoter activation downstream of BMP ligands in immortalized gonadotropes (690-692). Like SMAD2/3, their *in vivo* requirement remains to be established.

3.2.3.2. Non-canonical activin signaling

In addition to SMAD-mediated signaling, TGFβ family members activate biological responses through a variety of other pathways, especially involving MAPKs (reviewed in (693)). One of them involves the kinase TGFβ-activated kinase 1 (TAK1, also known as MAP3K7), which activates MAPK signaling (694, 695). One group has reported that activin-induced *Fshb* promoter activation in immortalized gonadotropes is TAK1 and p38-dependent (696). Yet, it was subsequently found that the TAK1 inhibitor used in that study has non-specific effects at the high concentrations used, that p38 antagonists inhibit ACVR1B kinase activity, and that activin stimulation does not trigger p38 phosphorylation in gonadotropes (697). Thus, at this point, there is no solid evidence implicating non-canonical activin signaling in mediating biological responses in gonadotropes. Overall, activins appear to engage a SMAD (probably SMAD2/3/4)-dependent pathway to regulate *Fshb* expression.

3.2.4. Activin regulation of gene expression

In the nucleus, SMAD2Δexon3, SMAD3, and SMAD4 can bind to a minimal 4 base-pair (bp) recognition sequence, GTCT or AGAC, to regulate gene expression (664, 698, 699). A palindromic arrangement of two such elements, which can accommodate in principle two SMAD proteins (699) forms an optimal binding site (700). As the minimal GTCT motif is encountered on average every 256 bp in the genome, additional elements are required to target SMAD proteins to specific loci and form functional transcriptional activation complexes (625, 664). This

was first revealed by the discovery that a physical interaction between SMAD2 and the forkead protein FOXH1 (also known as FAST1), enables transcriptional activation through a composite DNA element on the *Mix.2* promoter bound by SMAD4 and FOXH1 (670, 701, 702). Since then, a large number of additional SMAD-interacting transcription factor have been identified (703), and this general mode of regulation has been confirmed at the genome-wide level (704).

3.2.4.1 Activin regulation of Fshb transcription

In the mouse *Fshb* promoter, An 8-bp palindromic SMAD binding element (SBE, GTCTAGAC), at -266/-259 (Fig. 1.11), is required for transcriptional activation by activins (463, 539, 676, 677, 705). This site can be bound by a complex of SMAD2/3/4 proteins (676), and its mutation reduces – but does not abolish – activin induction of the *Fshb* promoter (463, 676, 705). Thus, the identification and analysis of the 8-bp SBE suggested that additional elements mediate activin responsiveness. An important breakthrough was achieved in recent years following comparative studies of the porcine and human *Fshb/FSHB* promoters. While both lack the 8-bp SBE observed in the rodent promoters, the porcine promoter is much more activin-responsive than its human counterpart despite ~90% sequence conservation (541, 706). The difference in activin responsiveness was mapped to a single bp, located in a putative forkhead transcription factor binding site (FBE) (706). Indeed, the porcine *Fshb* promoter is regulated by FOXL2, whose expression is restricted to gonadotropes and thyrotropes in the pituitary gland (706-708). Further investigation revealed the presence of a second FBE in the porcine promoter, and that both FBEs are adjacent to minimal (4-bp) SBEs, thus forming composite elements that are cooperatively activated by FOXL2 and SMAD proteins (706, 708). In addition, SMAD3 physically interacts with FOXL2 (708, 709), thus providing a molecular link between activin signaling and FOXL2. The more proximal of the two porcine FBE/SBE composite elements is conserved in the murine and human promoters (-115/-107 in mouse) and required for activin-induced murine promoter activity (Fig. 1.11) (678, 706). Accordingly, endogenous FOXL2 proteins are required in immortalized gonadotropes for murine *Fshb* transcriptional activation (706). Mechanistically, FOXL2 and either SMAD3 or SMAD4 – but not both – are required to bind to the DNA for functional complex formation, consistent with the sufficiency of a minimal 4-bp SBE (Fig. 1.11) (678). Two additional putative FBEs have been

identified in the murine promoter, but they are not adjacent to SBEs and appear less important than the composite FBE/SBE element for activin regulation (Fig. 1.11) (678, 710).

Humans with heterozygous germline mutations in the *FOXL2* gene show complex eyelid malformations, sometimes accompanied by premature ovarian failure (711, 712). Consistently, *Foxl2*-null mice have lethal craniofacial defects and profound ovarian dysfunction (713-715). Interestingly, these animals also have pronounced pituitary FSHβ deficiency around birth (144). However, due to perinatal lethality, definitive assessment of the role of FOXL2 in gonadotrope function required a conditional knockout approach, which was achieved recently (716). Mice with a gonadotrope-specific deletion of the *Foxl2* gene display a reduction in pituitary *Fshb* expression, low circulating FSH levels, and female subfertility (716). Collectively, these observations identified FOXL2 as a cell-restricted transcription factor critical for FSH synthesis. As mentioned above, an *in vivo* requirement for any of the SMAD proteins, alone or together with FOXL2, remains to be established.

In addition to the FBEs and SBEs, a binding site for PITX proteins, discussed in Section 3.1.4.3. (PBE), also appears to be important for activin-stimulated *Fshb* promoter activation (Fig. 1.11) (536, 537). Depletion of endogenous PITX proteins by RNA interference in immortalized gonadotropes impairs activin-stimulated *Fshb* transcriptional activation (537, 538). Because SMAD2/3/4 proteins can physically interact with PITX1 and PITX2, the PBE may serve as a platform for activation of transcription by an activin-induced SMAD/PITX complex (537, 538, 688). Another *Fshb* promoter element was previously suggested to bind the homeodomain transcription factors, pre B cell leukemia homeobox 1 (PBX1A) and Pbx/knotted 1 homeobox (PREP1), which can physically interact with SMAD2/3/4 (717). However, it was later shown that this element overlaps with the composite FBE/SBE site discussed above, thus casting doubt on the importance of PBX/PREP proteins in *Fshb* regulation (678). Based on the most compelling evidence, activin induction of *Fshb* transcription appears to be largely mediated by SMAD proteins their interacting cell-specific factor, FOXL2, acting through imperfectly conserved SMAD and SMAD/forkhead composite binding elements.

3.2.4.2. Activin regulation of Gnrhr, Lhb, and Fst transcription

Given that activins are primarily thought of as stimulators of FSH synthesis, mechanisms mediating activin-stimuated *Fshb* transcription have received the most attention. Nevertheless, *Fshb* is only one of many activin-responsive genes in gonadotropes, which include well-known regulators of reproductive function (718, 719). The gonadotropin-releasing hormone receptor gene (*Gnrhr*) is transcriptionaly stimulated by activins in primary pituitary cultures and immortalized gonadotrope cells (720-724). At least two regions of the *Gnrhr* promoter mediate activin responsiveness (721, 724-726). The first one, the "GnRHR activation sequence" (or "GRAS") is bound and activated by SMADs, AP-1 proteins, and FOXL2 (721, 724, 726). The second sequence, "downstream activin regulatory element" (or "DARE") is bound by the homeodomain transcription factor LHX2 and cooperates with GRAS to mediate activin responsiveness (725). However, mice with a whole-body deletion of *Avcr2a* or a gonadotrope-specific deletion of *Foxl2* have normal pituitary *Gnrhr* transcript levels, despite clearly impaired *Fshb* expression (716, 727). Thus, it is at present unclear whether activin regulation of *Gnrhr*, observed *in vitro*, is physiologically relevant. Whereas activins are usually considered "selective" inducers of FSH secretion, activin stimulates LH synthesis and secretion in primary pituitary cultures, immortalized gonadotropes, and *in vivo* (645, 688, 719, 728-731). Three minimal SBEs within the proximal rat *Lhb* promoter appear critical for activin induction, and male homozygous *Smad3*$^{\Delta ex8}$ knockout mice have lower pituitary *Lhb* expression (688).

Follistatin, a well-described activin antagonist, is expressed in the pituitary gland, including in gonadotropes (732, 733). Pituitary follistatin (*Fst*) expression varies with the estrous cycle, peaking prior to the primary FSH surge, and precipituously declining before the secondary FSH surge (623). Furthermore, follistatin expression and FSH synthesis negatively correlate in pituitary cell culture, and follistatin-overexpressing transgenic mice have low serum FSH levels (734, 735). Therefore, a decline in pituitary follistatin expression may facilitate activin-stimulated FSH production during the secondary FSH surge. Alternatively or in addition, since *Fst* is an activin-induced gene, its production may be part of a negative feedback loop that terminates the surge (acting along with increasing inhibin B – Fig. 1.10) (595, 736-739). The relevant intra-pituitary source of follistatin is unclear, as it is expressed in both gonadotropes and folliculostellate cells, which are abundant non-hormone producing pituitary cells (732, 740).

However, *Fst* expression might not be activin-regulated in folliculostellate cells (741), and further studies will be needed to address the cellular source and physiological importance of pituitary follistatin. Activin responsiveness of the *Fst* gene has been mapped to the first intron, which contains a minimal SBE (738). Interestingly, a nearby forkhead binding site is bound by FOXL2 (709). FOXL2 and SMAD3 act through the FBE and SBE to cooperatively activate *Fst* transcriptional activation (709). The relevance of this regulatory mechanism was validated, at least in part, by the analysis of global and gonadotrope-specific *Foxl2* knockout mice, which show impaired pituitary *Fst* expression (144, 716). While this should, in principle, enhance the autocrine/paracrine activin effect on gonadotropes, the low *Fshb* expression in these animals indicate a dominant role of FOXL2 in *Fshb* transcriptional regulation (144, 716). In sum, in addition to *Fshb*, activin signaling regulates the expression of many genes important for gonadotrope function, including *Lhb*, *Fst* and perhaps *Gnrhr*.

3.2.5. Mechanisms of activin antagonism by inhibins

As outlined in Section 3.2.1.4., cyclic fluctuations in inhibin production by the ovary provide a variably permissive or restrictive environment for activin autocrine/paracrine action on gonadotropes. Rather than inducing intracellular signaling (742), inhibins antagonize activin responses by blocking activin signaling at the receptor level (743).

3.2.5.1 Inhibin competitive binding to activin receptors

Because inhibins share β subunits with activins, it is not suprising that inhibins are also able to bind to the activin type II receptors (636, 744, 745). Yet, in contrast to activins, this interaction does not lead to activation of type I receptors (746-748). This suggests a competitive mode of activin antagonism by inhibins: an inhibin molecule can bind a type II receptor via its single β subunit, but fails to engage a second type II receptor (which would require a second β subunit). This precludes the recruitment of a pair of type I receptors and the formation of an active signaling complex ((742), and refer back to Section 3.2.2.). Nevertheless, inhibins bind to ACVR2A with much lower affinity (K_d~6 nM – (636)) than do activins (K_d ~0.2nM - (636)). Therefore, effective blockade of activin signaling by the sole engagement of the type II receptor would require a large excess of inhibin molecules. However, in rats, circulating inhibins are never higher than 10 pM for inhibin B, and 3 pM for inhibin A (610), which is much lower than

their K_d for ACVR2A. This has led to the proposal that high potency antagonism of activin signaling by inhibins may require the presence of a co-receptor, presumably engaged by the α subunit (742). So far, two candidate co-receptors have been identified (749, 750), but only one – TGFβ receptor type 3 (TGFBR3, also known as betaglycan) – has been validated as a *bona fide* inhibin co-receptor (750, 751).

3.2.5.2. Role of TGFBR3 in inhibin antagonism of activin signaling

TGFBR3 was initially identified as a heavily glycosylated co-receptor for TGFβ (752, 753). TGFβ binding to TGFBR3 enhances the affinity of ligand association for its type II receptor, and ectopic TGFBR3 expression potentiates TGFβ-induced biological responses (752, 753). Because TGFBR3's short intracellular tail lacks kinase activity – or any other recognizable signaling domain – present in other TGFβ superfamily receptors, it was concluded that TGFBR3 enhances TGFβ activity mainly by increasing ligand binding affinity to type II receptors (751-753). Several years after its cloning, TGFBR3 was identified as an inhibin co-receptor that enhances ligand binding to ACVR2A (Fig. 1.11) (750). Furthermore, TGFBR3 enables high-potency inhibin antagonism of activin signaling (IC_{50} ~9 pM, which is within the physiological inhibin circulatin concentration – see above) when ectopically expressed in cells that are normally insensitive to inhibins (750). Inhibins and TGFβ use different binding interfaces on TGFBR3 (754-757), and inhibin binding is mediated mainly through its α subunit (Fig. 1.11) (758). TGFBR3 is expressed in gonadotropes, and appears enriched at the plasma membrane at times of high inhibin tone during the estrous cycles (615, 750). Recent knockdown and bioneutralization experiments in immortalized gonadotropes and primary pituitary cells have confirmed that endogenous TGFBR3 is required for efficient inhibin A suppression of *Fshb* promoter activity, *Fshb* expression, and FSH secretion (759, 760). These observations suggest that TGFBR3 is required for inhibin regulation of FSH synthesis, but the reproductive consequences of its loss in gonadotropes *in vivo* remain to be established. Assessing this will require a cell-specific knockout approach, as *Tgfbr3*-null mice die during embryonic development with hematopoietic and cardiovascular defects (761, 762). Interestingly, inhibin B suppresses FSH synthesis and release more efficiently than inhibin A, despite the fact that it binds less efficiently to TGFBR3 (763). Added to the fact that crosslinked inhibin B binds a unique protein on the surface of immortalized gonadotropes (763), these observations suggest

that TGFBR3 may not be the exclusive inhibin co-receptor. In sum, inhibins antagonize activin signaling via competitive binding to ACVR2A, a process which apparently requires a co-receptor – most likely TGFBR3.

3.2.6. Bone morphogenetic proteins

BMPs, which are structurally similar to activins but signal through a distinct set of receptors and intracellular SMADs, are also candidate regulators of FSH synthesis, perhaps in synergy with activins or GnRH (644, 764-766). Several BMP ligands are expressed in the pituitary gland (644, 764), and they activate *Fshb* transcription to various extents, though less efficiently than activins in all cases (644). Furthermore, bioneutralization of BMPs in primary pituitary cell culture decreases FSH secretion (764). Inhibins can antagonize BMP signaling, supporting a possible dynamic regulation of BMP activity within the pituitary, akin to that of activins (767). BMP2 stimulation of *Fshb* promoter activity requires BMPR1A in immortalized cells (690), but a requirement for the ligand or its receptor in FSH synthesis has not yet been demonstrated *in vivo*.

3.3. Sex steroids

Steroid hormones produced by the gonads have long been recognized to exert context-dependent positive or negative feedback on the activity of the reproductive axis (94, 260, 768). Androgens, estrogens, and progestogens act on the brain to modulate GnRH release (see Section 2.1.1.2.), but also target gonadotrope cells to directly regulate gonadotropin subunit production.

3.3.1. Androgens

Direct negative regulation of LH production by testosterone at the level of the gonadotropes has been demonstrated in isolated pituitary cells and in castrated male rats treated with a GnRH antagonist (and thus devoid of hypothalamic input) (769, 770). In these experiments, testosterone administration suppressed the expression of both the α (*Cga*) and β (*Lhb*) subunits of LH (769, 770). Mechanistically, the *Cga*/*CGA* promoter (at least in humans) is negatively regulated by the ligand-bound androgen receptor (AR) through physical interaction between AR and the ATF2 and c-Jun transcription factors occupying the "CRE" elements (see Section 3.1.4.1.) (499, 771, 772). A similar mechanism appears to underlie regulation of *Lhb*

promoter by androgens, where the repressive activity of ligand-bound AR involves direct interactions between AR and the transcription factors SF1, SP1, and EGR1 (see Section 3.1.4.2.) (773-775). In contrast to *Cga* and *Lhb*, androgens directly stimulate pituitary *Fshb* expression in rats (776, 777). Functional AR binding sites are present within the *Fshb* promoter, but the identity of the site(s) mediating the androgen effect may be species-specific (778, 779). Interestingly, androgens and activins synergistically activate *Fshb* transcription, an interaction that requires a SMAD-binding element (adjacent to the proximal FOXL2 binding site –see Section 3.2.4.1.) and could be mediated by SMAD3-AR physical contact (652, 778, 780, 781). The consequences of the loss of gonadotrope AR signaling *in vivo* remain to be determined.

3.3.2. Estrogens

Extensive studies have sought to dissect the mechanisms underlying positive and negative feedback regulation of gonadotropin regulation by estrogens (782). Both the positive and negative feedback of estrogens are impaired in *Esr1*(encoding ERα)-null, but not (or minimally so) in *Esr2*(encoding ERβ)-null animals (104, 106, 783-786). Consequently, basal LH levels are dramatically elevated and LH surges are absent in *Esr1* knockout females (104, 785, 787). Mice lacking ERα in the brain (neuron-specific knockout) have impaired positive estrogen feedback (106). However, estrogen negative feedback appears largely intact in those animals. That is, they do not display the elevated basal LH levels and hypergonadotropic ovarian stimulation phenotypes seen in global *Esr1* knockout females (106, 782, 787). These observations suggest that negative feedback regulation of LH by estradiol is mediated at least in part at the level of the gonadotrope cells, which expresse both ERα and ERβ (99, 788). Indeed, pituitary-specific *Esr1* knockout mice show elevated basal LH levels and pituitary *Lhb* expression (99), and female subfertility (99) or infertility (97). That said, the increase in circulating LH in this model is not as high as in global *Esr1* null mice (787). Further, a GnRH antagonist can decrease LH levels in *Esr1* knockout animals (787) and the activity of an *Lhb* transgene in gonadectomized mice (775), pointing to a contribution of the brain to negative feedback as well. The molecular mechanisms underlying the negative regulation of LH subunit expression by estrogens in gonadotropes remain elusive (485). Unexpectedly, primary pituitary culture and promoter-reporter studies in immortalized gonadotropes have reported a stimulatory,

instead of the expected inhibitory, effect of estradiol on *Cga* and/or *Lhb* transcriptional activation (769, 789, 790).

Pituitary-specific *Esr1* knockout females are unable to mount LH surges (99), suggesting that positive feedback may also require estrogen action in the gonadotropes. This could be due to a requirement for estrogen-enhanced sensitivity of the pituitary to GnRH, involving for example up-regulation of *Gnrhr* expression at the time of the surge (315-318), although the precise mechanism remains to be elucidated. With respect to FSH, studies in primary pituitary cultures have reported species-specific negative regulation (e.g. (791)) or no effect (e.g. (548)) of estradiol on *Fshb* expression. Also, whereas global *Esr1* null mice may have elevated pituitary *Fshb* expression ((792), but see (104)), this is not seen in the pituitary-specific knockout models (97, 99). Thus, negative regulation of *Fshb* expression by estrogens may not occur in a cell-autonomous manner.

Despite our incomplete understanding of the mechanisms underlying estrogen regulation of gonadotropin synthesis, one of the most commonly used drugs to elevate FSH levels in PCOS women is an estrogen receptor modulator: clomiphene (refer back to Sections 1.2. and 1.3.). The effects of clomiphene have been attributed to moderate inhibition of estrogen receptor activity, which partially relieves the inhibitory effect of endogenous estradiol on LH and FSH synthesis and secretion at the hypothalamic and pituitary level (53, 793, 794). However, its precise mechanism of action remains somewhat elusive (48, 53, 795). A more recently developed class of drugs, the aromatase inhibitors, have a similar physiological effect, but instead act by blocking estradiol production by the ovarian granulosa cells (795, 796). To this day, clomiphene and aromatase inhibitors remain the only pro-fertility agents used clinically to specifically elevate endogenous FSH levels. In sum, both the positive and negative effects of estrogens on gonadotropin synthesis and secretion are mediated at least in part by direct actions on the gonadotropes, and this system can be targeted therapeutically to modulate gonadotropin levels.

3.3.3. Progesterone

Progesterone exerts both positive and negative feedback regulation of gonadotropin synthesis, and its receptor (PGR) is expressed in the hypothalamus and in pituitary gonadotropes (797-799). In ovariectomized animals, progesterone replacement suppresses elevated LH release

(800-802), and *Pgr*-null animals have increased basal serum LH levels (803). Limited experimental evidence suggests that this negative effect of progesterone is mediated at the level of the brain (804). By contrast, progesterone stimulates gonadotropin synthesis and secretion during at least two critical periods of the estrous/menstrual cycle, and both of these appear to involve a direct effect on gonadotrope cells (798). First, progesterone plays a role in LH surge generation, at last in rodents. Indeed, progesterone administration can trigger an LH surge in estrogen-primed animals (805). In addition, progesterone production by preovulatory follicles precedes ovulation in rats (245), and a PGR antagonist can block the LH surge (806, 807). The progesterone requirement for pre-ovulatory LH surge generation appears to involve a direct pituitary effect (798). Indeed, estrogens stimulate pituitary *Pgr* expression (808, 809), progesterone enhances pituitary sensitivity to GnRH at the time of the surge (810, 811), and this "priming" effect is absent in *Pgr*-null animals (812). The molecular events mediating this positive effect of progesterone remain to be clarified.

Second, progesterone appears to participate in the generation of the secondary FSH surge. Progesterone levels are high just prior to the secondary FSH surge (Fig. 1.3), and PGR inhibition prevents this incease in FSH even in a context of declining inhibin levels (813-816). Interestingly, a requirement for gonadotrope PGR activity during the secondary surge may be independent of progesterone itself (816), and instead be mediated by cooperativity between activin signaling and PGR (817). Indeed, SMAD3 can physically interact with PGR in a ligand-independent manner (652), providing a potential mechanism for activin signaling/progesterone receptor crosstalk. Up to five elements within the proximal *Fshb* promoter may be bound by PGR and required for transcriptional activation by progesterone, one of them appearing to be particularly critical in mouse (779, 818). FOXL2 binding sites and a direct physical interaction between FOXL2 and PGR contribute to cooperative activation of *Fshb* transcription by progesterone and activins (819). Ultimately, a more complete understanding of the role of progesterone in the positive and negative regulation of gonadotropin synthesis will require the analysis of brain- and gonadotrope-specific *Pgr* knockout mice. Overall, progesterone appears to have direct effects on the gonadotropes to regulate LH and FSH synthesis, and may play a critical role in driving the secondary FSH surge in rodents.

4. Rationale for the thesis

A better understanding of the molecular mechanisms controlling gonadotropin production and secretion is required for the identification of new therapeutic targets that can be exploited to effectively treat infertility. GnRH and the activin/inhibin system regulate LH and FSH synthesis and release. However, as outlined throughout the introduction, significant gaps remain in our knowledge of the underlying molecular mechanisms. The goal of this thesis is to address some of these outstanding issues. Specifically, this work focuses on activin regulation of FSH, and GnRH regulation of LH. Very little is known about the signaling pathways that mediate activin induction of FSH synthesis *in vivo*. In chapters 2 and 3, I examine the role of SMAD proteins and the cell-restricted SMAD co-factor FOXL2 in this process. While both GnRH and activin signaling regulate FSH, LH synthesis depends mostly on GnRH. GnRH regulation of LHβ subunit expression has been well-described, but it remains unclear if the prevailing model also applies to humans. In chapters 4 and 5, I assess the mechanisms underlying GnRH regulation of the human LHβ subunit gene transcription, and how it is modulated by activin signaling. GnRH-induced LH synthesis might be most important for the generation of the LH surge, which drives ovulation in mammals. In chapter 6, I examine the possibility that a unique structural feature of the GnRH receptor is required for this to happen.

Figure legends

Figure 1.1: Overview of the hypothalamic-pituitary gonadal axis. Gonadotropin-releasing hormone (GnRH), released from hypothalamic neurons, stimulates the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by gonadotrope cells of the anterior pituitary gland. LH and FSH target the gonads, leading the production of the sex steroids: testosterone in males; estrogens and progesterone in females. The sex steroids exert context-dependent positive or negative regulation of GnRH, LH and FSH synthesis and release. As well, inhibins produced by the gonads antagonize the positive effect of intra-pituitary activins on FSH synthesis.

Figure 1.2: Gonadotrope specification in the pituitary gland. **A**) Representative immunofluorescence staining for follicle-stimulating hormone β (FSHβ – top left) and luteinizing hormone β (LHβ – top right) subunits, as well as the nucleus-labeling DAPI stain (bottom left) and the merged image (bottom right) show the distribution of gonadotrope cells within the anterior pituitary gland in adult male C57Bl/6 mice. **B**) Schematic representation of transcription factor-driven fate-commitment and pituitary lineage specification. From common progenitors, ("pit stem cell"), T-box 19 (TBX19) induces the development of the corticotrope fate (expressing pro-opiomelanocortin; POMC), whereas LIM homeodomain transcription factor 3 (LHX3) and paired like homeodomain factor 1 (PROP1) sequentially direct the differentiation towards other lineages. Expression of GATA binding protein 2 (GATA2), steroidogenic factor 1 (SF1) and other factors (??) induce the development of gonadotropes (expressing luteinizing hormone; LH and follicle-stimulating hormone; FSH) from PROP1+ cells, whereas POU domain, class 1, transcription factor 1 (PIT1) directs commitment towards lactotropes (expressing prolactin; PRL), somatotropes (expressing growth hormone, GH) and, in conjunction with GATA2, thyrotropes (expressing thyroid-stimulating hormone; TSH).

Figure 1.3: Schematic representation of circulating hormone profile throughout the rodent estrous cycle. Top panel: follicle-stimulating hormone (FSH). Second panel from top: luteinizing hormone (LH). Third panel from top: estradiol. Bottom panel: progesterone. M/D: metestrus/diestrus; P: proestrus; E: estrus. 1°: primary FSH surge. 2°: secondary FSH surge.

Figure 1.4: Overview of ovarian folliculogenesis. Follicle growth up to the secondary stage occurs in the absence of gonadotropins. FSH stimulates the recruitment of follicles to the early antral stage and beyond. The granulosa cells are depicted as round-shaped cells; the steroidogenic theca cells are square-shaped. In pre-ovulatory follicles, a large fluid-filled antrum separates two functionally distinct populations of granulosa cells: "cumulus" cells, which immediately surround the oocytes, and "mural" cells, which line the antrum. Following ovulation, the ovarian follicle becomes the progesterone-producing corpus luteum.

Figure 1.5: The GnRH surge outlasts the LH surge. Schematic representation of GnRH (top panel) and LH (bottom panel) pulsatile release during the rodent estrous cycle. M/D: metestrus/diestrus; P: proestrus; E: estrus. Based on data from refs. (80, 313).

Figure 1.6: Integrated model of gonadotropin-releasing hormone (GnRH) signaling pathways activating LH release and gonadotropin subunits expression. GnRH receptor activation causes the dissociation of the α subunit from heteromeric G proteins. The α subunit activates membrane-bound phospholipase C (PLC), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG). IP_3 stimulates efflux of calcium (Ca^{2+}) from endoplasmic reticulum stores, leading to LH- and FSH-containing vesicle priming and exocytosis. DAG and Ca^{2+} activate protein kinase C (PKC), which in turn activates the mitogen-activated protein kinase (MAPK) cascade by the sequential activation of MAPK kinase kinases. MAPK kinases, and the MAPKs: extracellular signal-regulated kinase 1/2 (ERK1/2), jun-N-terminal kinase (JNK), p38 and big MAPK (ERK5). ERK1/2-mediated signaling activates the expression of the gonadotropin subunits, luteinizing hormone β (*Lhb*) and chorionic gonadotropin alpha (*Cga*) and, to a lesser extent, follicle-stimulating hormone β (*Fshb*). *Lhb* expression may also be partially JNK-dependent; whereas *Fshb* expression may also be partially p38-dependent. CGA subunits are produced in excess of LHβ and FSHβ subunits.

Figure 1.7: Model of gonadotropin-releasing hormone (GnRH)-induced chorionic gonadotropin α (*Cga*) expression. A gonadotrope-specific element (*GSE*), bound by steroidogenic factor 1 (SF1), and a pituitary-specific element (*PSE*), bound by LIM homeodomain transcription factor (LHX), contribute to basal *Cga* promoter activity. Extracellular-regulated kinase 1/2 (ERK1/2), downstream of a mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) cascade, phosphorylates an E26 transformation-specific (ETS) transcription factor which binds a GnRH-

response element (*GRE*). The subsequent *Cga* promoter activation may involve an interaction between ETS and LHX factors.

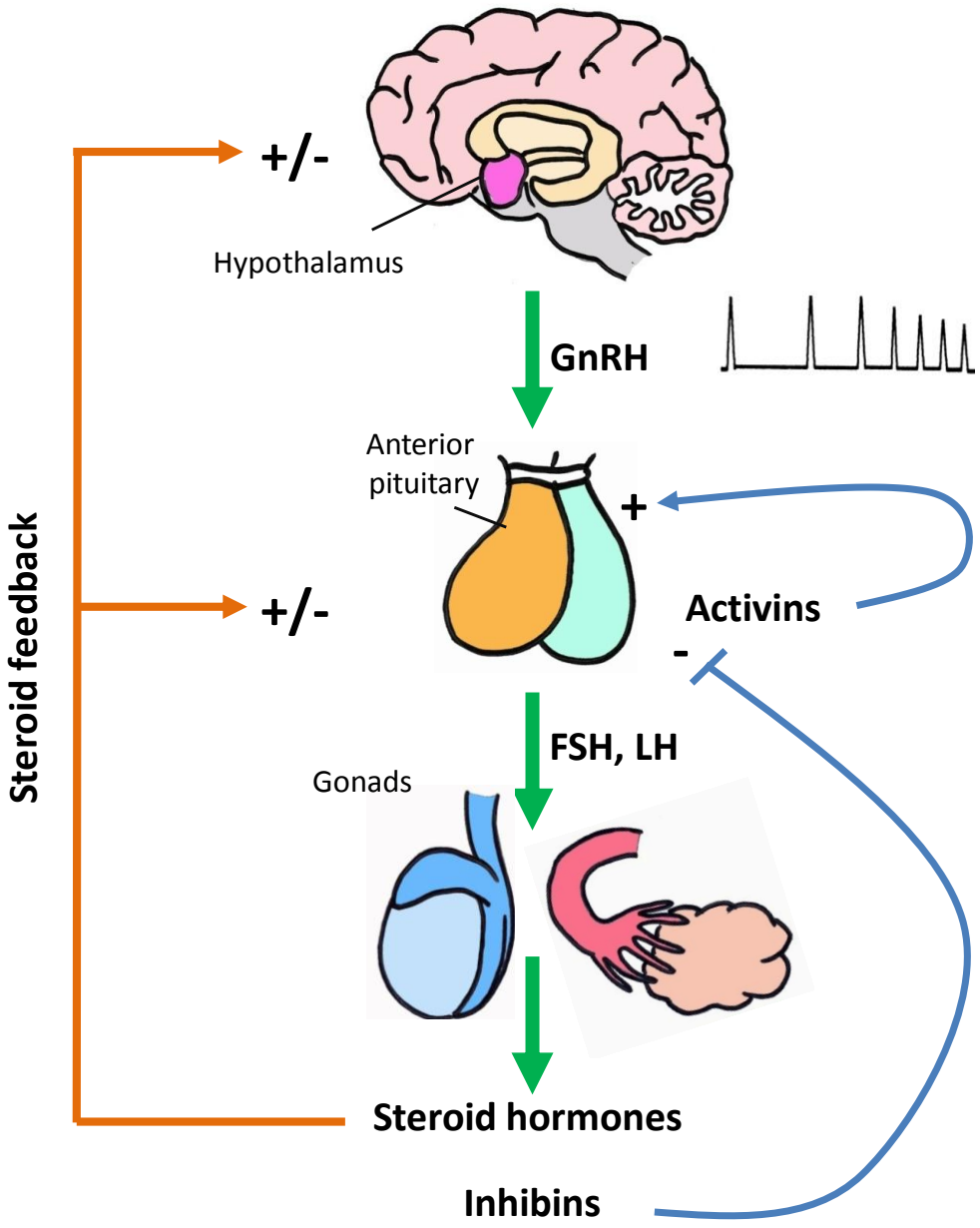
Figure 1.8: Model of gonadotropin-releasing hormone (GnRH)-induced luteinizing hormone β subunit (*Lhb*) expression. Two gonadotrope-specific elements (*GSE*), bound by steroidogenic factor 1 (SF1), and a paired-like homeodomain (PITX)-binding element (*PBE*), bound by PITX1 or PITX2, contribute to basal *Lhb* promoter activity. Extracellular-regulated kinase 1/2 (ERK1/2), downstream of a mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) cascade, activates the expression of early-growth response 1 (EGR1) *via* phosphorylation of the E26 transformation-specific (ETS) transcription factor ELK1 which activates *Egr1* transcription. EGR1 proteins bind to EGR1 binding elements (*EBE*) adjacent to the *GSE* and *PBE* site. Physical interactions between EGR1, SF1 and PITX proteins activate *Lhb* transcription. Distal elements bound by trans-acting transcription factor 1 (SP1) may also contribute to *Lhb* promoter activity.

Figure 1.9: Model of gonadotropin-releasing hormone (GnRH)-induced follicle-stimulating hormone β subunit (*Fshb*) expression. GnRH-stimulated *Fshb* promoter activation may be partially mediated by dimers of jun proto-oncogene (JUN) and FBJ osteosarcoma oncogene (FOS) proteins. *C-Jun* and *c-Fos* expression is activated by GnRH signaling cascades involving p38 and extracellular-regulated kinase 1/2 (ERK1/2), downstream of a mitogen-activated protein kinase kinases (MAPKK) and protein kinase C (PKC). p38 activates *c-jun* transcription via phosphorylation of activating transcription factor 2 (ATF2). ERK1/2 and calmodulin-dependent kinase II (CamKII) activate *c-fos* expression via phosphorylation of serum response factor (SRF) and E26 transformation-specific (ETS) transcription factor ELK1, respectively. Through a putative protein kinase A (PKA)-dependent signal, GnRH activates cyclic-AMP response element binding protein (CREB) phosphorylation. CREB cooperates with upstream stimulatory factor (USF) proteins to activate *Fshb* transcription, although this might be a rat-specific mechanism. The functional importance of either JUN/FOS or CREB-mediated *Fshb* expression remains unclear (indicated by "?").

Figure 1.10: The secondary FSH surge correlates with a decline in circulating inhibin A and inhibin B levels. Schematic representation of circulating FSH (top panel), inhibin A (middle panel) and inhibin B (bottom panel) levels across the rodent estrous cycle. M/D:

metestrus/diestrus; P: proestrus; E: estrus. 1°: primary FSH surge. 2°: secondary FSH surge. Based on data from refs. (610, 615).

Figure 1.11: Integrated model of activin signaling pathways regulating follicle-stimulating hormone β subunit (*Fshb*) expression in gonadotropes. At the cell surface, dimeric activin ligands engage two type II receptors (activin receptor type 2 - ACVR2A) and two type 1 receptors (either activin receptor type 1 B – ACVR1B or type 1 C – ACVR1C). This leads to type I receptor phosphorylation, which in turn phosphorylates the Sma- and Mad-related proteins, SMAD2 and SMAD3. SMAD2/3 partner with SMAD4 and accumulate in the nucleus. A SMAD complex binds to an 8-bp SMAD-binding element (*SBE*), which can accommodate two SMAD proteins. As well, a SMAD complex binds a composite *SBE*/forkhead binding element (*FBE*) along with forkhead box L2 (FOXL2). In this complex, SMAD3 and FOXL2 physically interact. Two additional *FBE* sites may contribute to promoter activation *via* FOXL2, with or without the involvement of SMAD proteins. Finally, paired-like homeobox (PITX) proteins bind a proximal site that contributes to *Fshb* promoter activity. PITX proteins may serve as a platform for SMAD proteins through physical interactions.



Original artwork by Beata Bak, used and modified with permission

Figure 1.1

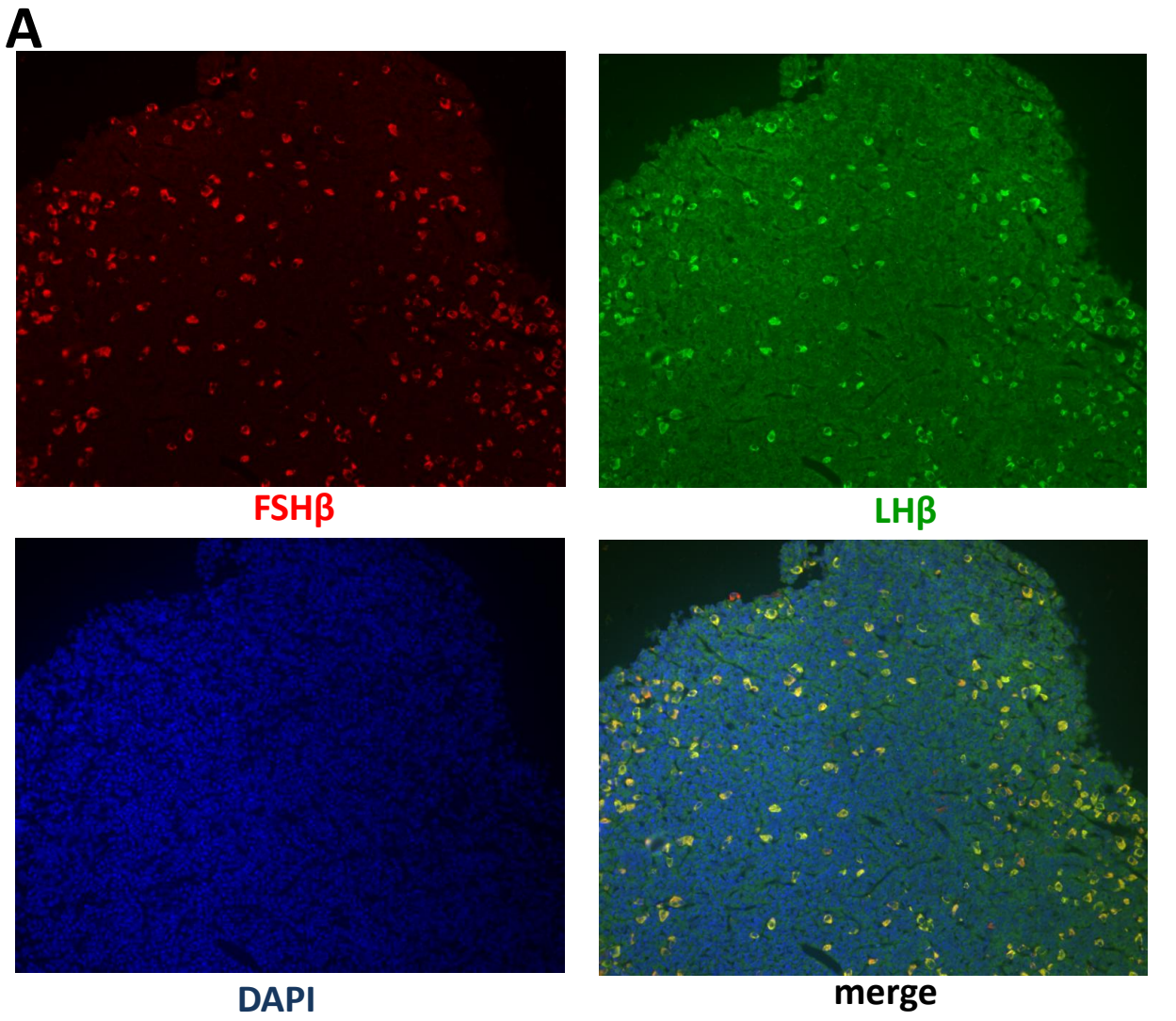

A
FSHβ
LHβ
DAPI
merge
B
Pit stem cell
LHX3
LHX3+
PROP1
PROP1+
PIT1
PIT1+
PRL
lactotrope
GH
somatotrope
TSH
thyrotrope
GATA2
SF1
??
LH
FSH
gonadotrope
TBX19
POMC
corticotrope

Figure 1.2

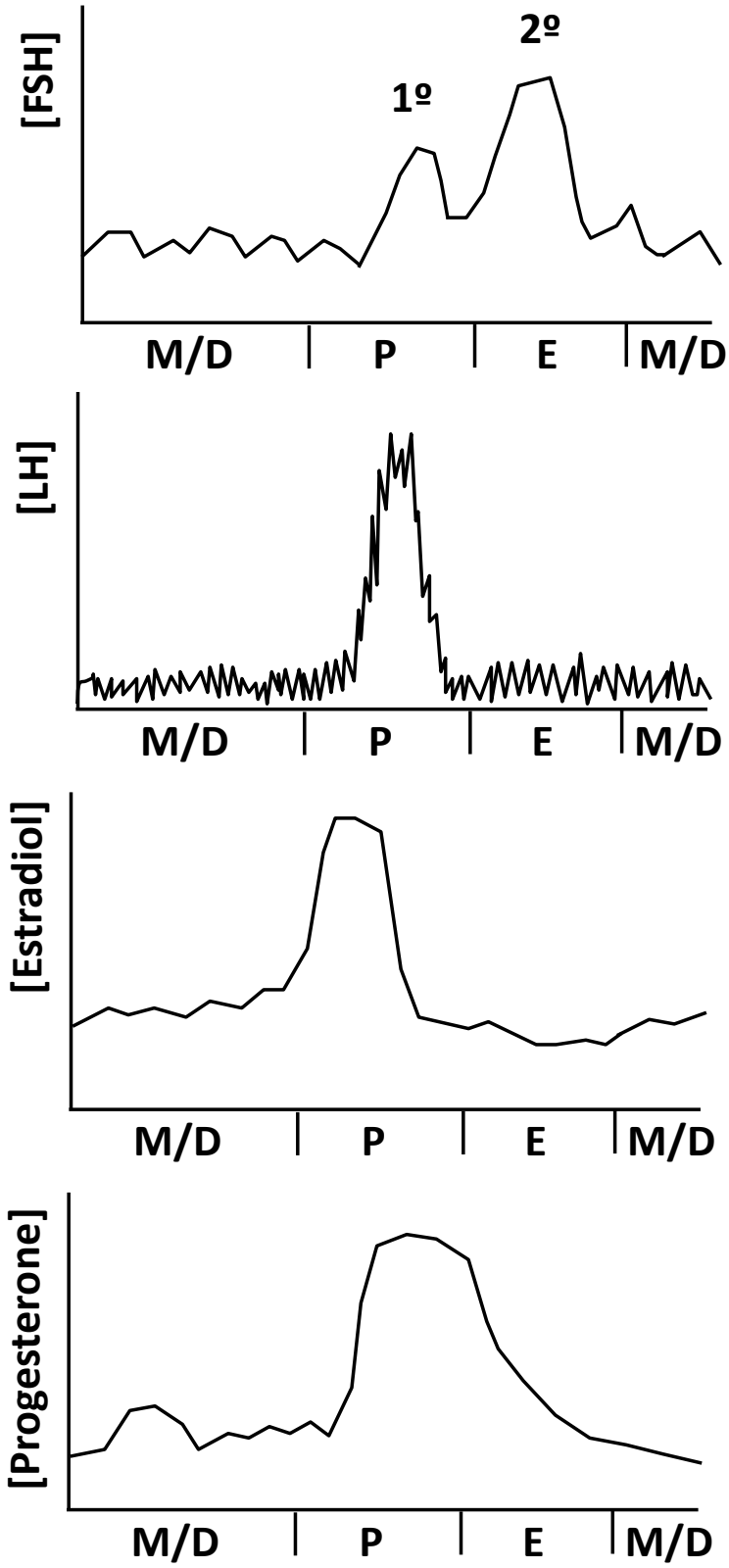

[FSH]
2º
1º
M/D
P
E
M/D
[LH]
M/D
P
E
M/D
[Estradiol]
M/D
P
E
M/D
[Progesterone]
M/D
P
E
M/D

Figure 1.3

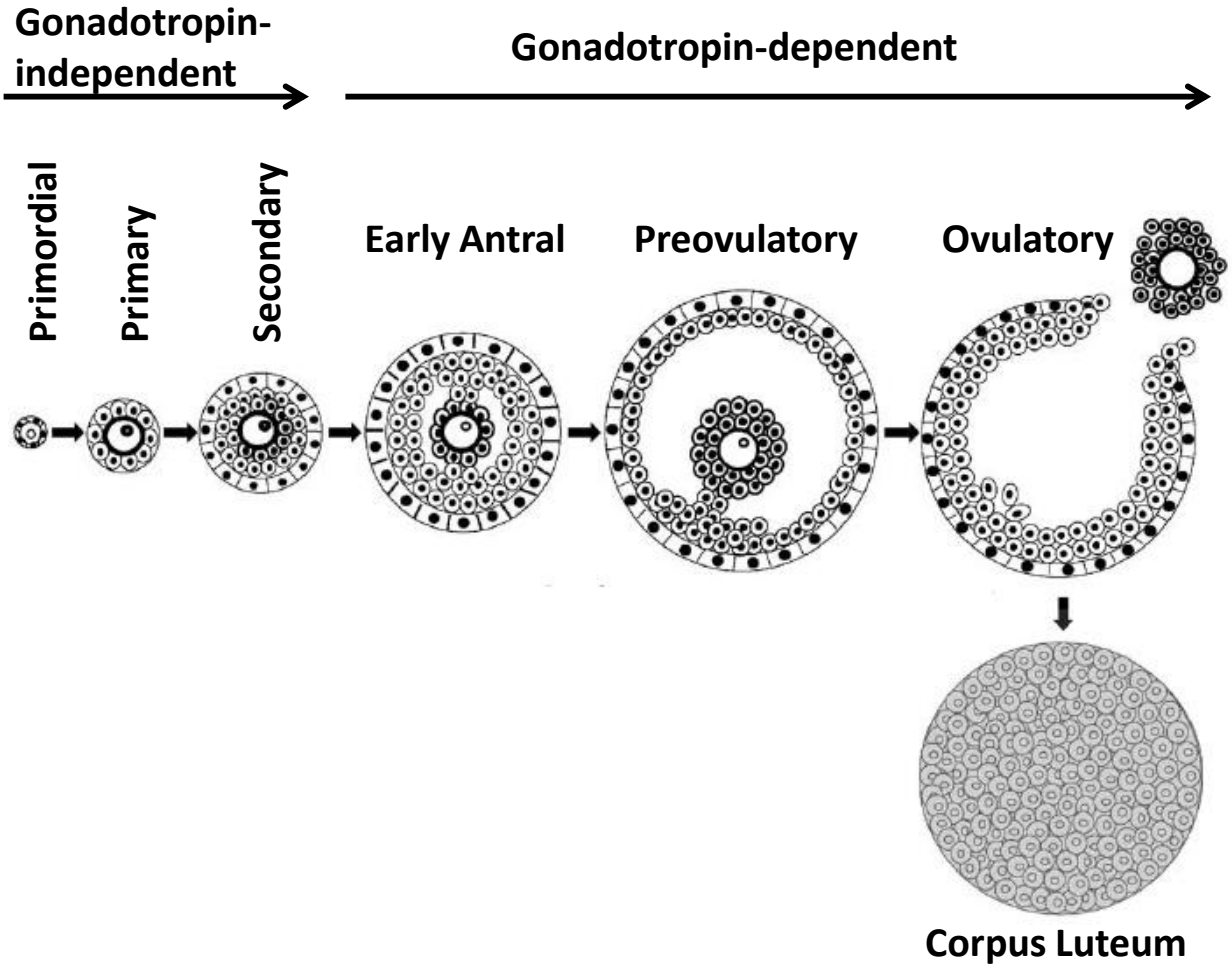
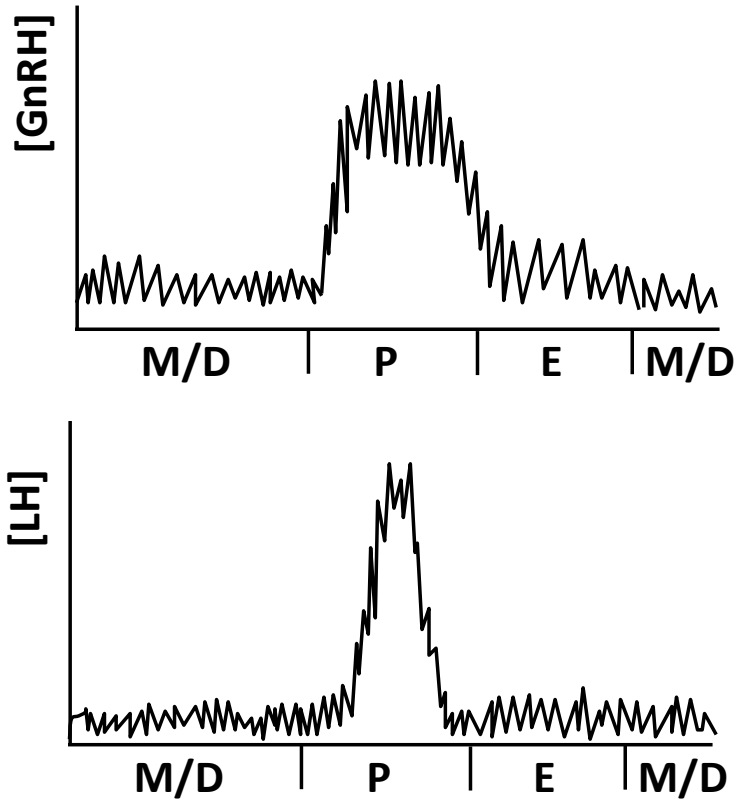


Figure reprinted and adapted from Molecular and Cellular Endocrinology, Vol. 225, Pangas SS and Matzuk MM "Genetic models for transforming growth factor beta superfamily signaling in ovarian follicle development", p.85. Copyright (2004), with permission from Elsevier.

Figure 1.4



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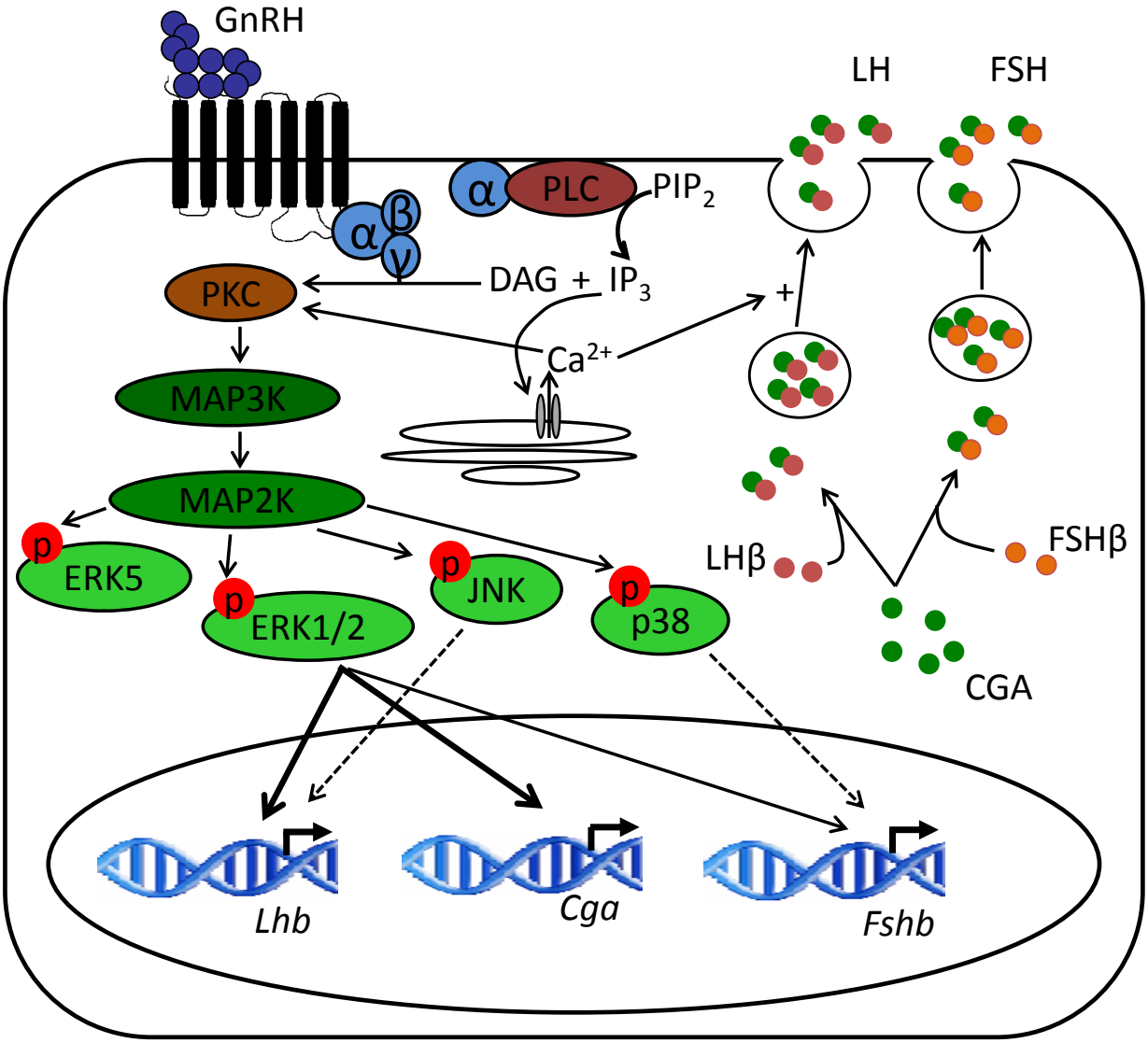

GnRH
LH
FSH
α
PLC
PIP2
α
β
γ
DAG + IP3
PKC
+
Ca2+
MAP3K
MAP2K
p
ERK5
p
ERK1/2
p
JNK
p
p38
LHβ
FSHβ
CGA
Lhb
Cga
Fshb

Figure 1.6

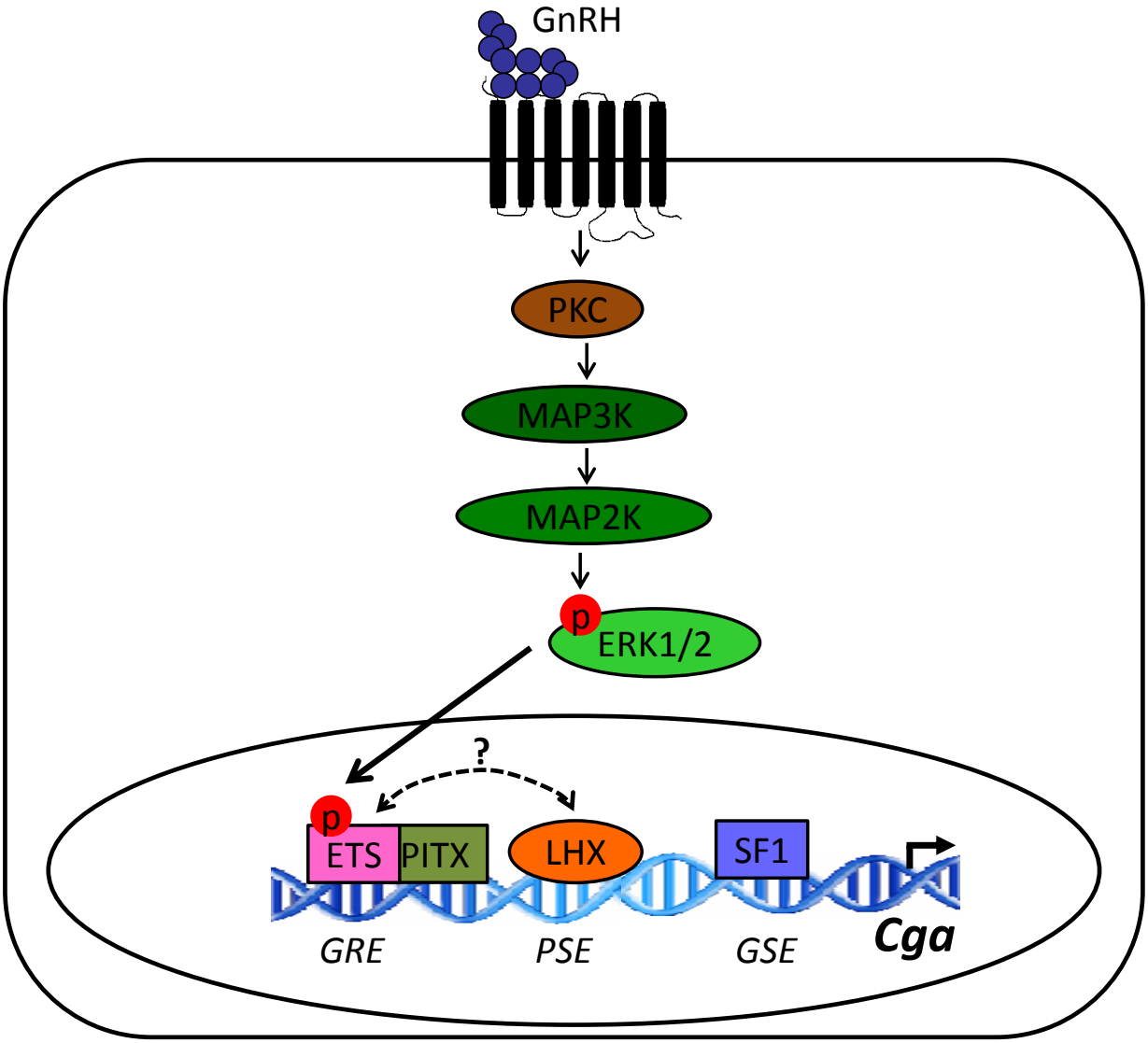


Figure 1.7

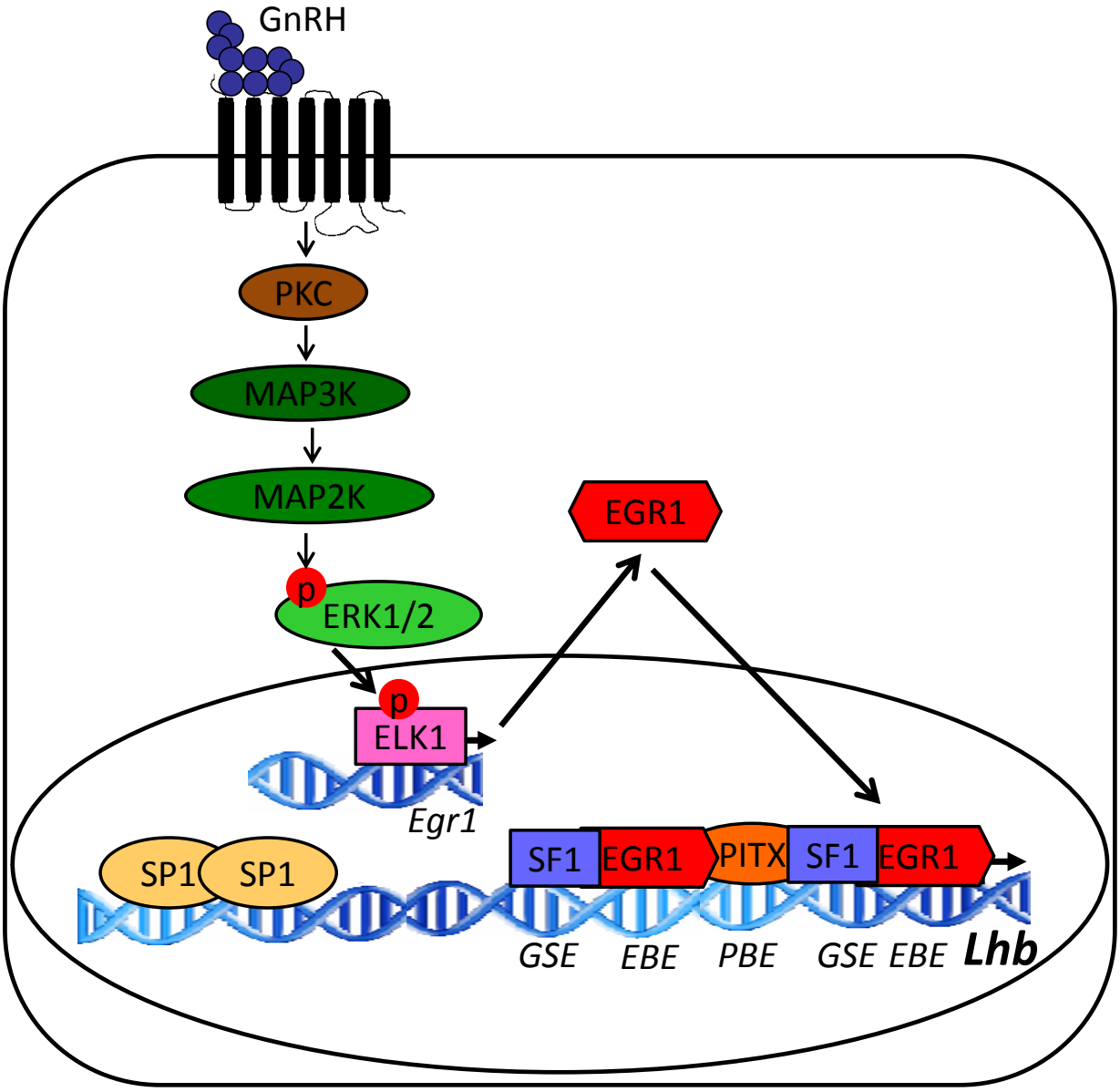
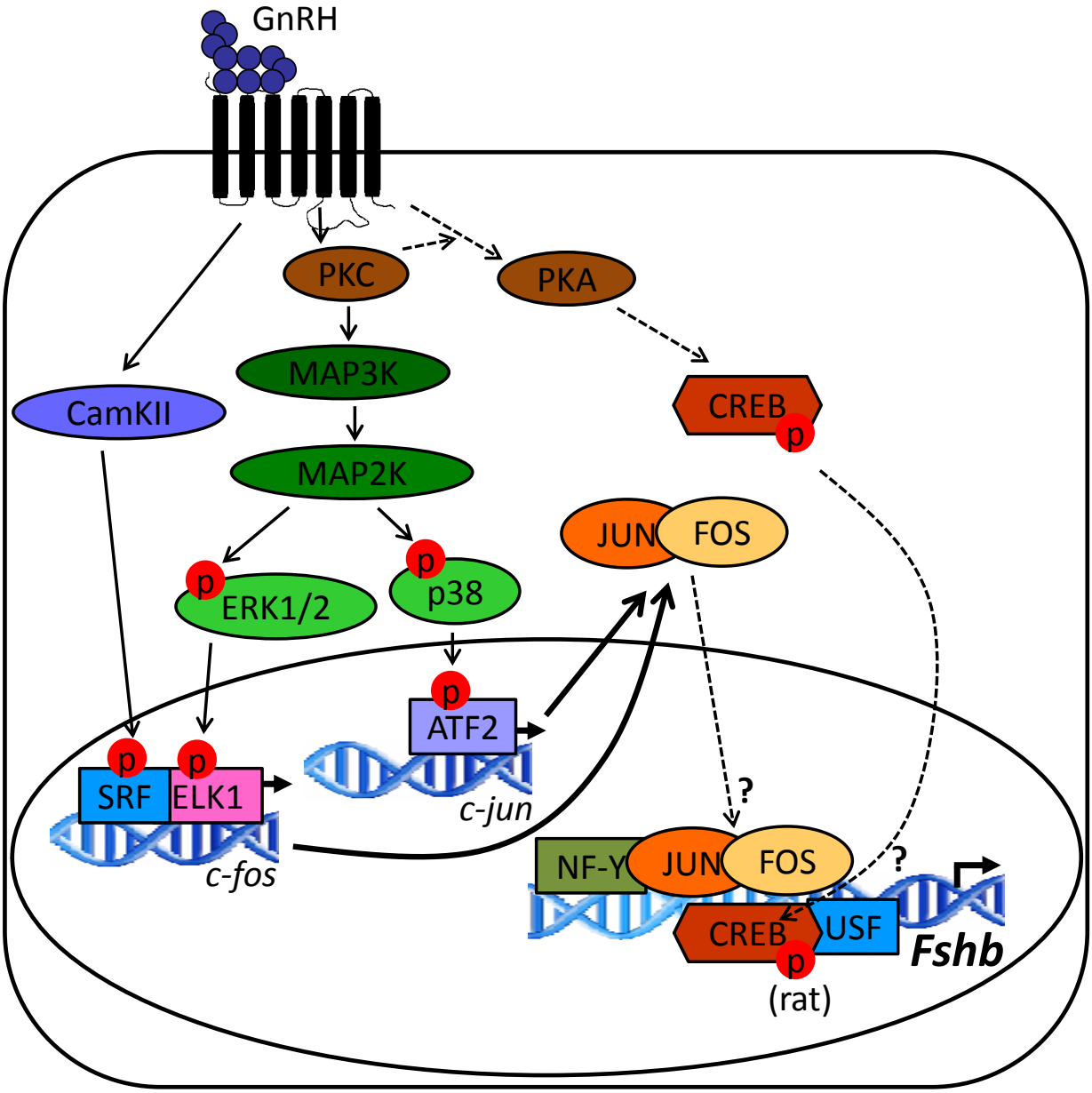

GnRH
PKC
MAP3K
MAP2K
p
ERK1/2
EGR1
p
ELK1
Egr1
SP1
SP1
SF1
EGR1
PITX
SF1
EGR1
GSE
EBE
PBE
GSE
EBE
Lhb

Figure 1.8


GnRH
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PKA
MAP3K
CamKII
CREB
p
MAP2K
JUN
FOS
p
ERK1/2
p
p38
p
ATF2
p
SRF
p
ELK1
c-jun
c-fos
?
NF-Y
JUN
FOS
?
CREB
USF
p
Fshb
(rat)

Figure 1.9

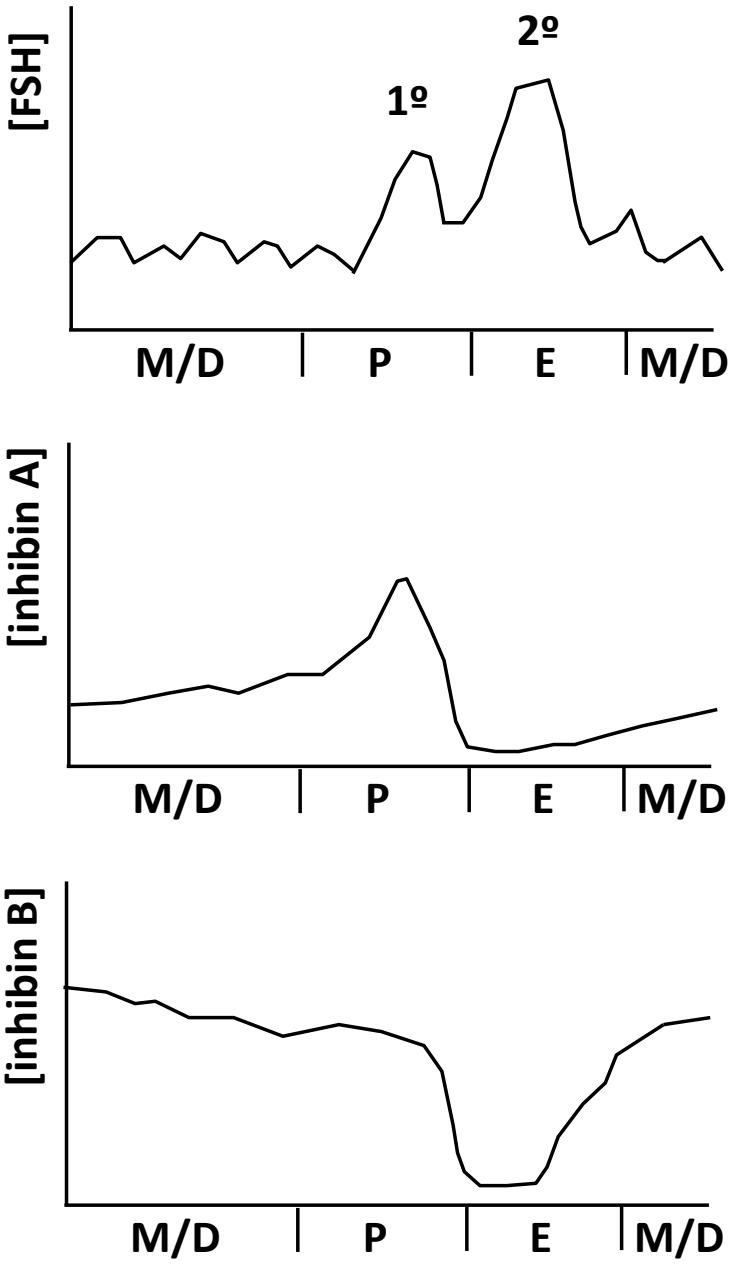

Figure 1.10

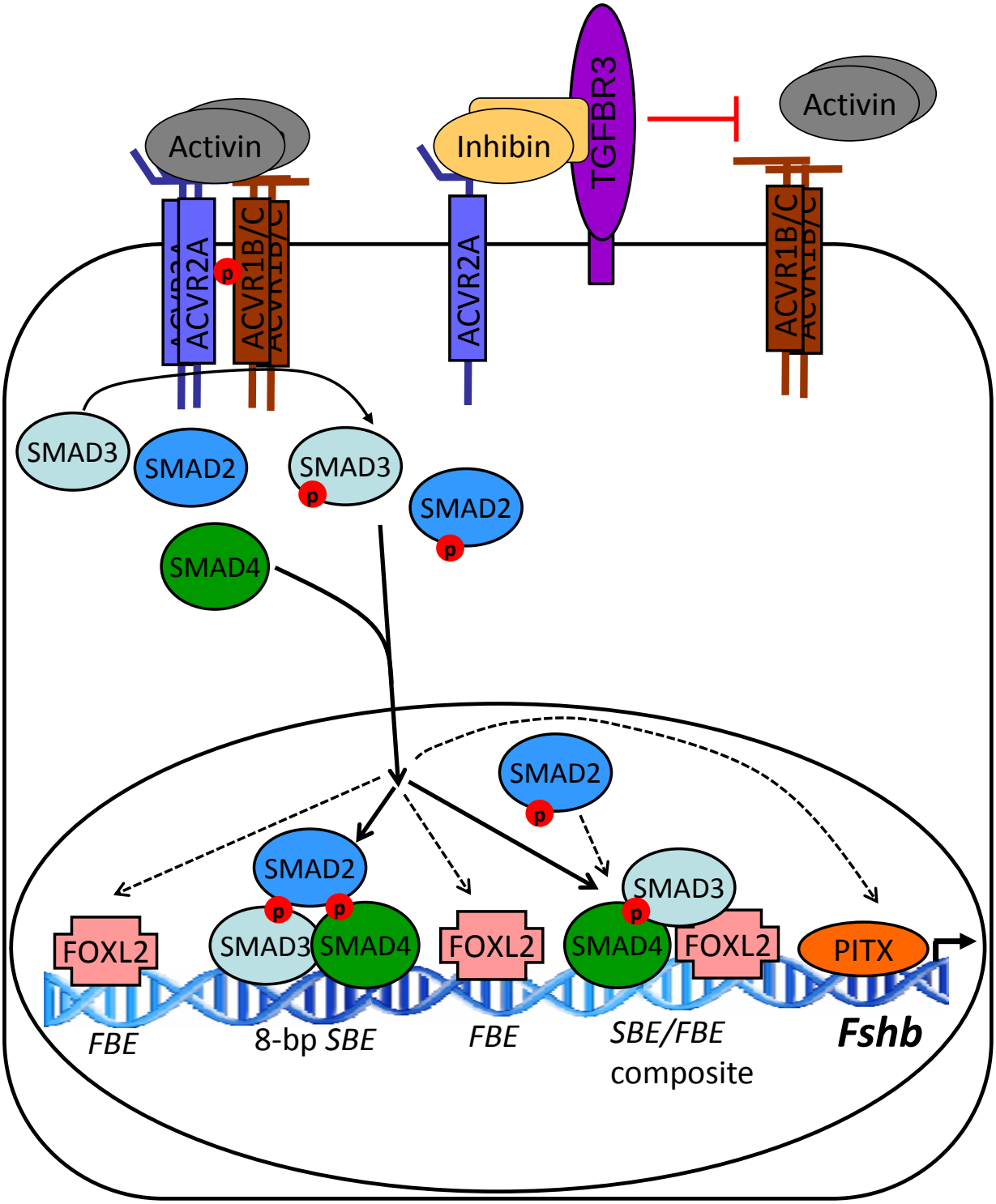
Activin
Inhibin
TGFBR3
Activin
ACVR2A
ACVR1B/C
ACVR2A
ACVR1B/C
p
SMAD3
SMAD2
SMAD3
p
SMAD2
p
SMAD4
SMAD2
p
SMAD2
p
p
SMAD3
p
FOXL2
SMAD3
SMAD4
FOXL2
SMAD4
FOXL2
PITX
FBE
8-bp SBE
FBE
SBE/FBE
composite
Fshb

Figure 1.11

Chapter 2

In recent years, the mechanisms controlling FSH production and release have been the focus of intensive investigation (203). We know that both GnRH and activins regulate *Fshb* transcription and FSH synthesis, but their relative contributions are still unclear. One way to examine this is to define the signaling pathways mediating the response to GnRH and activin, and assess the effect of ablating components of these pathways on FSH synthesis. Mice with a global deletion of the *Acvr2a* gene, which encodes a canonical activin type 2 receptor, exhibit FSH-deficiency and female sterility (643). These observations suggest that signaling mediators acting downstream of this receptor are required for FSH synthesis and fertility in mice. Thus far, the identification of candidate signaling mediators has relied principally on *in vitro* studies in model cell lines (immortalized gonadotrope-like cells and others). Several of these studies have supported a key role for the canonical effectors of activin signaling, SMAD2 and SMAD3 (463, 539, 674, 676-678, 708). Ultimately, a proof of their physiological importance must be obtained *in vivo.* In this chapter, I investigated the role of SMAD2/3-mediated signaling in FSH synthesis and fertility in a new mouse model. Because global *Smad2* deletion is embryonic lethal and the four extant *Smad3* knockout mouse lines have divergent phenotypes, including intrinsic ovarian defect in one of the lines (684, 687), I used a conditional knockout approach (Cre-lox system) to interrogate the function of SMAD2/3 specifically in gonadotropes. To do so, I employed a recently developed mouse line in which Cre expression is targeted specifically in gonadotropes (*Gnrhr*$^{IRES\text{-}Cre}$; or "GRIC" – (820)), as well as conditional ("floxed") alleles of *Smad2* (821) and *Smad3* (689). Here, I report the generation, validation, and phenotypic characterization of gonadotrope-specific *Smad2/3* knockout mice.

Title: Follicle-stimulating hormone synthesis and fertility are intact in mice lacking SMAD3 DNA binding activity and SMAD2 in gonadotrope cells

Short title: Regulation of gonadotrope function by SMAD2 and SMAD3

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Abstract

The activin/inhibin system regulates follicle-stimulating hormone (FSH) synthesis and release by pituitary gonadotrope cells in mammals. *In vitro* cell line data suggest that activins stimulate FSH β subunit (*Fshb*) transcription via complexes containing the receptor-regulated SMAD proteins, SMAD2 and SMAD3. Here, we used a Cre-*loxP* approach to determine the necessity for SMAD2 and/or SMAD3 in FSH synthesis *in vivo*. Surprisingly, mice with conditional mutations in both *Smad2* and *Smad3* specifically in gonadotrope cells are fertile and produce FSH at quantitatively normal levels. Importantly, however, we discovered that the recombined *Smad3* allele produces a transcript that encodes the entirety of the SMAD3 C-terminal Mad homology 2 (MH2) domain. This protein behaves similarly to full-length SMAD3 in *Fshb* transcriptional assays. As the truncated protein lacks the N-terminal MH1 domain, these results show that SMAD3 DNA-binding activity as well as SMAD2 are dispensable for normal FSH synthesis *in vivo*. Furthermore, the observation that deletion of proximal exons does not remove all SMAD3 function may facilitate interpretation of divergent phenotypes previously described in different *Smad3* knockout mouse lines.

Introduction

Follicle-stimulating hormone (FSH), a dimeric glycoprotein secreted by pituitary gonadotrope cells, is a critical regulator of gonadal function and is required for fertility in mammals (16, 220). Multiple factors derived from the brain, the gonads, and the pituitary regulate the expression of the FSH β subunit (*Fshb*), the rate-limiting step in FSH synthesis and release (203). Classical studies identified inhibins and activins as critical negative and positive regulators of FSH synthesis (572, 580, 581, 822, 823). Activins, derived from the pituitary gland, bind to their cognate receptors on the cell surface of gonadotrope cells, initiating a signaling cascade that culminates in the activation of *Fshb* transcription (203, 824). In contrast, inhibins, secreted from the gonads, suppress FSH synthesis by competitively binding to activin type II receptors (825).

Much effort has been directed toward the molecular dissection of the signaling pathways by which activins stimulate *Fshb* transcription. Canonically, activins signal through heteromeric assemblies of type I and type II receptors, which phosphorylate the effector proteins, SMAD2 and SMAD3. These proteins then partner with SMAD4 and accumulate in the nucleus, where they act as transcription factors (625, 826). Several lines of evidence implicate SMAD2/3/4 complexes as central components of activin-induced FSH synthesis. Studies in murine immortalized gonadotrope-like (LβT2) and heterologous cell lines indicate that SMAD2/3/4 complexes directly bind to the *Fshb* promoters of several mammalian species, including mouse, and activate their transcription (465, 539, 674-677, 679). Furthermore, a number of SMAD2/3-interacting transcription factors directly bind to the *Fshb* promoter and cooperatively activate *Fshb* transcription (537, 538, 678, 706). One such factor, forkhead box L2 (FOXL2), was recently confirmed to be a critical regulator of *Fshb* expression, FSH synthesis, and fertility *in vivo* in mice (144, 827).

Despite the large amount of data indicating that activins operate through a canonical SMAD-dependent signaling pathway to stimulate *Fshb* transcription *in vitro,* evidence demonstrating necessary roles for SMADs 2 and/or 3 in FSH synthesis *in vivo* is lacking. Mice harboring a global deletion of the 8th of the 9 exons in *Smad3* have modestly decreased pituitary *Fshb* transcript levels (688). However, *Smad3* is broadly expressed and these animals display a range of reproductive defects, including intrinsic gonadal dysfunction, making it difficult to

ascertain whether the *Fshb* deficiency is the result of cell-autonomous loss of SMAD3 function in gonadotropes (684, 687). To investigate the roles of SMADs 2 and 3 in FSH synthesis *in vivo*, we used a Cre-*loxP* approach to produce loss of function mutations in *Smad2* and/or *Smad3* selectively in gonadotrope cells of mice.

Materials and Methods

Mouse lines

The *Smad2*^{fl}, *Smad3*^{fl}, *Gnrhr*^{GRIC} and *ROSA26*^{eYFP} alleles and corresponding genotyping primers (Table S2.1) were described previously (689, 820, 821, 828). To generate S2/3cKO mice, *Smad2*$^{fl/+}$;*Smad3*$^{fl/+}$;*Gnrhr*$^{GRIC/+}$ females were mated with *Smad2*$^{fl/fl}$;*Smad3*$^{fl/fl}$ males, yielding S2/3cKO (*Smad2*$^{fl/fl}$;*Smad3*$^{fl/fl}$;*Gnrhr*$^{GRIC/+}$) and control (*Smad2*$^{fl/fl}$;*Smad3*$^{fl/fl}$;*Gnrhr*$^{+/+}$) mice at a frequency of 1/8 for each genotype. To generate mice with genetically labeled gonadotropes for subsequent FACS purification (see below), *Smad2*$^{fl/fl}$;*Smad3*$^{fl/fl}$;*ROSA26*$^{eYFP/eYFP}$ males were mated with *Smad2*$^{fl/fl}$;*Smad3*$^{fl/fl}$;*Gnrhr*$^{GRIC/+}$ females to yield *Smad2*$^{fl/fl}$;*Smad3*$^{fl/fl}$;*ROSA26*$^{eYFP/+}$;*Gnrhr*$^{GRIC/+}$ offspring at a frequency of 1/2. Control gonadotropes were obtained from *ROSA26*$^{eYFP/+}$;*Gnrhr*$^{GRIC/+}$ mice. All animal experiments were performed in accordance with federal guidelines and were approved by McGill University's Institutional Animal Care and Use Committee (Animal Use Protocol #5204).

Puberty and estrous cycle assessment

To determine the onset of puberty, female mice were examined daily for vaginal opening starting from the day of weaning (postnatal day 21). Estrous cyclicity was assessed for at least 21 consecutive days starting at 7 weeks of age. Vaginal cells, obtained every morning (9h00-10h00) using a cotton swab dampened with sterile saline, were smeared on glass slides, stained with 0.1% methyl blue and examined under a microscope. Stages were assigned following published guidelines (829). Because of high similarity in cell types, no distinction was made between metestrus and diestrus. A complete estrous cycle was defined as sequential mestrus/diestrus, proestrus, and estrus, regardless of the number of days spent in each stage.

Breeding trials

Mating trials were initiated one week after the completion of estrus cycle assessment. S2/3cKO or control females were paired with one 8 week-old C57BL/6J male for a period of 6 months. Starting from 20 days after pairing, cages were examined daily for the presence of newborn mice. As soon as a new litter was observed, pups were counted. Pups were left in the cage for two weeks before removal.

Organ analyses and sperm count

Reproductive organs were harvested from 10-week-old males (testes, seminal vesicles) or mestestrus/diestrus females (uterus, ovaries) and weighted on a precision balance. Homogenization-resistant epididymal sperm count was assessed as described (827). For ovarian histology, formalin-fixed tissues were paraffin-embedded and serial 5 µM sections collected after sectioning on a microtome. For corpora lutea (CL) counting, every 7^{th} section was Hematoxylin and Eosin (H&E)-stained and imaged by microscopy, which allowed tracking of individual CLs across several sections. One ovary was sectioned and analyzed per mouse. For testicular histology, testes were fixed in Bouin's overnight and washed in 95% and 70% ethanol prior to paraffin embedding. Seven µM transverse sections were obtained in the middle of the tissue, H&E-stained, and imaged by microscopy.

Pituitary and ovarian RNA extraction and quantitative PCR

Pituitaries and ovaries were collected from 10-week-old males and metestrus/diestrus females and immediately frozen on dry ice. Individual pituitaries and ovaries were homogenized in 500 µl TriZol and RNA extracted following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). One and a half (pituitaries) or two (ovaries) µg of RNA were reverse-transcribed using MMLV reverse transcriptase (Promega, Madison, WI, USA) as described previously (829) in a final volume of 40 µl. One µl of cDNA was assayed in triplicate qPCR reactions using Platinum qPCR Supermix-UDG (Invitrogen, Carlsbad, CA, USA) on a Corbett Rotorgene 6000 instrument. Gene expression was determined relative to that of the housekeeping gene *Rpl19* using the $2^{-\Delta\Delta Ct}$ method (830) and the primers described in Table S2.1.

Hormone assays

Blood was collected from 10-week-old males and metestrus/diestrus females by cardiac puncture, left to coagulate for 15 min at room temperature, and centrifuged at 3000xg for 10 min. Serum was collected and stored at -20^{o}C. LH and FSH levels were measured by multiplex ELISA at the Ligand Assay and Analysis Core of the Center for Research in Reproduction at the University of Virginia (Charlottesville, VA, USA).

Ovariectomy

Adult (≥ 8 week-old) S2/3cKO and control females in metestrus/diestrus morning were injected subcutaneously with 5 μg/g body weight of Carprofen. Mice were deeply anesthetized using isoflurane and placed on a heating pad. The back skin was shaved and cleaned, and a single midline incision was performed. Small incisions were made bilaterally through the muscle layer above the ovaries, through which the uterine horns were retrieved. The ovaries were removed by cauterization below the oviduct, and the incisions closed using Vicryl sutures (Ethicon, Blue Ash, OH, USA). Topical Carprofen was applied, and the skin incision was closed using wound clips (Reflex 7, CellPoint Scientific, Gaithersburg, MD, USA). The mice were left to recover on a heating pad. Sham-operated animals were processed in the same way, except that the ovaries were not removed. Mice were killed 7 hours post-surgery, and their pituitaries and serum collected for analyses.

Primary pituitary cell culture

Adult (≥ 8-week-old) mice were killed by CO_2 asphyxiation, and their pituitaries collected in M199 medium containing 10% fetal bovine serum (FBS). A single-cell suspension was prepared as previously described (514). Cells were seeded at a density of $4x10^5$/well in 48-well plates. All treatment conditions were performed in duplicate. For viral transduction, adenoviruses expressing eGFP or Cre-IRES-eGFP [Baylor College of Medicine Vector Development Laboratory (Houston, Texas)] were added 24 h after plating at a multiplicity of infection of 60. Twenty-four hours later, virus-containing media was removed and replaced with media containing 2% FBS with or without 1 nM activin A (R&D Systems). After 24 h incubation, cells from duplicate wells were harvested using 0.25% trypsin and pooled. RNA and DNA were extracted using the Qiagen Allprep DNA/RNA kit. RNA was eluted in 25 μl RNAse-free water and reverse-transcribed. The resulting cDNA was analyzed by qPCR.

Fluorescence-activated cell sorting (FACS)

For FACS, pituitaries were dissociated as above and the resulting cell suspensions passed through a 40 μm nylon mesh to eliminate cell clumps. YFP+ and YFP- cells were isolated on a FACSAria cell sorter at the flow cytomery core facility of the McGill University Life Sciences Complex. Approximately 5000-10,000 YFP+ cells (3-5% of all sorted cells) were routinely

obtained from each pituitary in these experiments. RNA and genomic DNA were isolated from sorted cells as described above for mixed pituitary cultures.

Cloning and expression vector construction

The primers used in the "primer walk" experiment are listed in Table 1. For expression vector construction, the full-length and truncated *Smad3* transcripts were amplified by PCR from S2/3cKO pituitary cDNA, using a sense primer 120 bp upstream of the canonical translation start site in exon 1 and an antisense primer immediately after the STOP codon in exon 9 (see Table S2.1). The resulting fragments were digested with *Hind*III and *BamH*I (engineered onto the 5' end of the primers) and ligated into the same sites in pcDNA3.0. To generate epitope-tagged constructs, the same strategy was used, except that the antisense primer replaced the stop codon with a *Cla*I restriction site. The resulting fragments were digested with *Hind*III and *Cla*I and ligated in-frame upstream of a 3X-HA tag in a previously modified pcDNA3.0 vector. Constructs were verified by sequencing (Genome Quebec, Montreal, Canada). The -846/+1 m*Fshb*-luc reporter, as well as the SMAD4 and FOXL2 expression vectors were described previously (676, 706).

Cell lines culture, transfections, reporter assays, and western blotting

LβT2 cells (a gift from Dr. Pamela Mellon, UCSD) were seeded at a density of 125,000 cells/well in 48-well plates one day prior to transfection. HeLa cells were cultured as previously described (514). Cells were transfected using Lipofectamine 2000 (Invitrogen) and reporter assays performed as described (465, 678, 706, 708). For protein analyses, confluent HeLa cells in 6-well plates were transfected with 2 μg expression vector using Lipofectamine/Plus reagent (Invitrogen), and harvested the next day in RIPA lysis buffer prior to western blot analysis (674). Antibodies used were mouse anti-HA (Sigma H9658), mouse anti-β actin (Sigma A2228) and goat anti-mouse IgG-HRP conjugate secondary antibody (Bio-Rad 170-6515).

Statistical analysis

Serum hormones, pituitary and ovarian transcripts, estrous cycle frequency, sperm counts, corpora lutea counts, and organ weights were compared using unpaired *t*-tests. Estrous cycle stages, gene expression in sorted cells and reporter experiments were analyzed using two-

way analysis of variance (ANOVA) followed by Tukey post-hoc tests. Ovariectomy experiments were analyzed using one-way ANOVA followed by Newman-Keuls multiple comparison test. Primary culture experiments were analyzed using one-way repeated measures ANOVA followed by Tukey post-hoc test. Data were log-transformed when variances were not equal between groups. Statistical analyses were performed using Systat 10.2 or GraphPad Prism 5. *P*-values < 0.05 were considered statistically significant.

Results

Generation of gonadotrope-specific Smad2/3 double knockout mice

To assess the role of SMAD2/3 signaling in FSH synthesis and fertility *in vivo*, we generated mice lacking the canonical forms of SMADs 2 and 3 in gonadotropes by crossing animals expressing Cre recombinase exclusively in gonadotropes [(*Gnrhr*-IRES-Cre, or $Gnrhr^{GRIC}$ (820)] with mice carrying conditional ("floxed") alleles of *Smad2* and *Smad3* (689, 821). The resulting double-knockout mice have the genotype $Smad2^{fl/fl};Smad3^{fl/fl};Gnrhr^{GRIC/+}$, and are hereafter referred to as *Smad2/3* conditional knockouts or S2/3cKO. Control mice were littermates carrying homozygous conditional alleles ($Smad2^{fl/fl};Smad3^{fl/fl}$). To assess the deletion efficiency of *Smad2/3* in the double-knockout mice, we crossed in the conditional $ROSA26^{eYFP}$ reporter allele (828) on the S2/3cKO background (hereafter S2/3cKO-YFP). In these animals, Cre recombinase expression in gonadotropes triggers both recombination of *Smad2/3* and expression of enhanced yellow fluorescent protein (eYFP), thus enabling the high efficiency purification of this cell population by fluorescence-activated cell sorting (FACS) (827, 831). PCR analysis of genomic DNA from sorted YFP+ and YFP- cells of S2/3cKO mice indicated essentially complete recombination of the *Smad2* and *Smad3* loci in gonadotropes (Fig. 2.1A-B). Consistent with this, quantitative PCR (qPCR) on cDNA prepared from the same cells, using primers directed at the deleted exons, showed a profound loss of *Smad2* and S*mad3* transcripts in S2/3cKO gonadotropes compared to those of $Gnrhr^{GRIC};ROSA26^{eYFP}$ (GRIC-YFP) controls (Fig. 2.1C-D). Interestingly, the same analysis indicated higher expression of both *Smad2* and *Smad3* in gonadotropes (YFP+) compared with other pituitary cell types (YFP-). Furthermore, downregulation of *Smad2* and *Smad3* transcripts (by about 30%) in whole pituitaries from S2/3cKO mice far exceeded the values one would expect if the genes were uniformly expressed across all pituitary cells types, as gonadotropes [and $Gnrhr^{GRIC}$-expressing cells (157, 820)] represent only 5-10% of the total cell population (Fig. S2.1A,B).

In addition to S2/3cKO mice, we also generated single gene knockout mice lacking either *Smad2* or *Smad3* in gonadotropes. As these mice showed no abnormalities in any of the experiments performed (data not shown), we focus here on the analysis of the double-knockout model.

Puberty and reproductive function in S2/3cKO females

To evaluate the effect of gonadotrope-specific loss of SMAD2/3 on hypothalamic-pituitary-gonadal (HPG) axis activity, we monitored reproductive maturation in S2/3cKO and control females. First, we assessed the day of vaginal opening (v.o.), an estrogen-dependent external marker of puberty onset in mice (829). S2/3cKO mice and control littermates showed a comparable onset of v.o. (Fig. 2.2A). Subsequently, we examined estrous cyclicity by daily vaginal smears in a cohort of seven S2/3cKO and seven control females over a period of three weeks. Mice of both genotypes exhibited cyclic variation in vaginal cytology, including several four- to five-days cycles, as is typical in mice (829). However, five out of seven S2/3cKO females showed one or more prolonged (four consecutive days or more) periods of estrus, whereas such events were never observed in control littermates (Fig. 2.2B). As a result, S2/3cKO females had a significant reduction in estrous cycle frequency and an increase in the proportion of time spent in estrus (Fig. 2.2C).

Next, we monitored fertility in the same cohort of animals by pairing them individually with wild-type C57BL/6J male mice for a period of six months. Unexpectedly, S2/3cKO females showed normal fertility. They produced litters of similar sizes and at a comparable frequency to control females, which was reflected in the cumulative number of pups delivered over the period of the breeding trial (Fig. 2.2D). To gain a more comprehensive view of HPG axis activity, we examined the reproductive organs of an additional cohort of 10 week-old females. The uteri and ovaries of S2/3cKO females were comparable in morphology and weight to those of control littermates (Fig. S2.1C,D and data not shown). Ovaries of S2/3cKO mice were histologically normal, with follicles at all stages of development. Moreover, quantitative analyses revealed similar numbers of corpora lutea in S2/3cKO and control mice (Fig. S2.1E). Ovarian expression of the FSH-responsive genes, *Ccnd2*, *Cyp19a1* and *Lhr* (832) did not differ between genotypes (Fig. S2.1F). Collectively, the data indicate that S2/3cKO females had increased variability in estrous cyclicity, but otherwise exhibited normal reproductive function.

Reduced testes weights and sperm production in S2/3cKO males

Although male mice do not require FSH for fertility, *Fshb* knockout males do exhibit gonadal dysfunction, most notably small testes and oligospermia (220). Furthermore, as the $Gnrhr^{GRIC}$ allele is active in male germ cells (820), deletion of the SMAD proteins therein might

affect sperm function or survival (833, 834). We therefore examined the reproductive organs of S2/3cKO males. Double knockouts had significantly decreased testes weights relative to controls (Fig. 2.3A). This was accompanied by a reduction in epididymal sperm counts (Fig. 2.3B). Testicular histology was largely unremarkable, although some seminiferous tubules appeared smaller in diameter in the testes of S2/3cKO males compared with controls (Fig. S2.1G). Seminal vesicle weight, a marker of circulating testosterone levels, was comparable between S2/3cKO male and control littermates (Fig. 2.3C). Consistent with their minor reproductive organ anomalies, S2/3cKO males exhibited normal fertility (data not shown).

Normal pituitary Fshb expression and FSH synthesis in S2/3cKO mice

In light of their unexpectedly mild reproductive abnormalities and normal fertility, we next asked whether FSH synthesis was impaired in S2/3cKO mice, as we predicted *a priori*. Surprisingly, serum FSH levels were equivalent in S2/3cKO mice and control littermates of both sexes (Fig. 2.4A). Indeed, in metestrus/diestrus female S2/3cKO mice, there was even a trend for increased circulating FSH (71% increase; p=0.058) (Fig. 2.4A). Consistent with the serum FSH values, pituitary *Fshb* transcript levels, analyzed in the same animals, were not significantly different between the genotypes; though, again, there was a non-significant increase in S2/3cKO females (64% increase; p=0.159) (Fig. 2.4B). Similarly, circulating LH levels were normal in these 10 week old S2/3cKO mice (Fig. S2.2A). However, we observed significantly higher serum LH, but not FSH, in older S2/3cKO than control females (metestrus/diestrus) retired from the breeding trials (Fig. S2.2B,C).

Next, we probed the ability of S2/3cKO females to up-regulate FSH synthesis following the removal of gonad-derived hormone negative feedback. In female rodents, an acute phase of increased FSH synthesis occurs within a few hours after ovariectomy. Because this increase is GnRH-independent, it is assumed to reflect increased activin-driven FSH production following the loss of ovarian inhibin feedback (835). Therefore, we ovariectomized (OVX) adult mestestrus/diestrus females and analyzed serum FSH and pituitary *Fshb* transcript levels after 7 h. In both control and S2/3cKO mice, there was a significant increase in circulating FSH levels and pituitary *Fshb* expression in OVX mice compared with sham-operated littermates (Fig. 2.4C). Collectively, these results suggest that activin-dependent *Fshb* expression and FSH synthesis are unimpaired in S2/3cKO mice.

Impaired Fshb transcription upon acute ablation of Smad2/3 in primary pituitary cells

Given the unexpectedly intact FSH secretion in S2/3cKO mice, we re-visited the roles of SMAD2 and SMAD3 in activin-stimulated *Fshb* transcription. Most of the evidence implicating these proteins in *Fshb* transcription regulation comes from overexpression and knockdown studies in immortalized gonadotrope-like and heterologous cell lines (13-18). To assess whether SMAD2/3 are similarly important in primary gonadotropes, we prepared pituitary cultures from mice homozygous for the *Smad2/3* conditional alleles (*Smad2*$^{fl/fl}$;*Smad3*$^{fl/fl}$) and infected these cells with Cre-expressing (Ad-Cre) or control (Ad-GFP) adenoviruses to induce recombination of *Smad2/3 ex vivo*. This procedure was highly efficient, as *Smad2* and *Smad3* mRNA levels were depleted by over 95% after transduction with Ad-Cre (Fig. 2.5A,B). Basal *Fshb* mRNA levels, which depend on autocrine/paracrine activin (or activin-like) signaling (602, 836), were reduced in Ad-Cre-infected male or female cultures (Fig. 2.5C). Furthermore, exogenous activin A stimulated *Fshb* expression in Ad-Cre-transduced cultures, but to a lesser extent than in Ad-GFP-infected cell (Fig. 2.5C). This difference was especially striking in cultures prepared from female mice. Deletion of *Smad2* or *Smad3* alone had similar, but generally milder, effects on basal and activin A-stimulated *Fshb* mRNA levels (Fig. S2.3A,B). Together, these data suggest that activin regulation of *Fshb* expression is at least partially SMAD2/3-dependent in cultured pituitary cells.

Retention of a transcript encoding a functional, truncated SMAD3 protein in S2/3cKO gonadotropes

The recombined *Smad2* allele used in our study produces a truncated protein (821). However, this protein cannot be C-terminally phosphorylated and does not have activity in functional assays (685). We therefore questioned whether, in contrast, functional *Smad3* transcripts might be retained in gonadotropes of S2/3cKO animals. Indeed, qPCR analysis of YFP+ and YFP- cells sorted from the pituitaries of control (GRIC-YFP) and S2/3cKO-YFP animals indicated a 2-fold upregulation of *Smad3* mRNA containing distal exons (exons 8 and 9) in S2/3cKO gonadotropes (Fig. 2.6A). This contrasted with a robust loss of *Smad3* transcripts containing the deleted exons (exons 2 and 3; see Fig. 2.1D). We further characterized retained *Smad3* transcripts in S2/3cKO gonadotropes using a "primer walk" PCR strategy on cDNA

obtained from FACS-sorted gonadotropes from control or S2/3cKO animals with an antisense primer in the terminal exon 9 and sense primers in exons 1 through 8. A single PCR product, of a smaller size than the full-length transcript (data not shown), was amplified from S2/3cKO (but not control) gonadotropes using the sense primer in exon 1. This transcript contained exon 1 spliced to exon 4, and then proceeded normally to include all the remaining exons of *Smad3* (Fig. 2.6B).

Translation of the SMAD3 protein normally initiates in exon 1 and the relevant translational start site (TSS) is retained in the truncated transcript. Removal of exons 2-3 introduces a frame-shift in exon 4, thus preventing the production of a functional protein from the canonical TSS. However, as the TSS in exon 1 does not conform to a consensus Kozak sequence (837), we considered the possibility that an alternative TSS, downstream of exon 3, might exist and be utilized in the novel *Smad3* transcript. We identified two potential AUG start codons in exon 4, one of which occurred in the context of a consensus Kozak sequence. This raised the possibility that the "knockout" transcript might encode a protein containing the entirety of the Mad homology 2 (MH2) domain in the event of leaky ribosomal scanning or translation reinitation (Fig. 2.6B).

To assess this possibility, we generated expression vectors for the full-length and truncated transcript (both starting upstream of the canonical TSS in exon 1) fused to a 3X-HA epitope tag at the C-terminus, and transfected them into HeLa and LβT2 (immortalized gonadotrope-like) cells. Western blot analysis of cells transfected with the wild-type SMAD3 expression vector revealed a protein product with a molecular mass of 55-60 kDa, consistent with the predicted size of full-length 3X-HA-tagged SMAD3 (Fig. 2.6C, lanes 2-6 and 9-13). In cells transfected with the truncated SMAD3 expression vector, the full-length protein was not observed; however, a novel, lower-abundance band, appeared at around 30 kDa (Fig. 2.6C, lanes 1 and 8). The size of this product was consistent with translation initiation in exon 4. Because this protein is predicted to retain the SMAD3 MH2 domain, but lack all of the MH1 domain and most of the "linker" region, it is hereafter referred to as "SMAD3(MH2)". Titration experiments indicated that a SMAD3:SMAD3(MH2) expression vector ratio of 1:5 in HeLa cells and 1:20 in LβT2 cells yielded comparable protein expression levels (Fig. 2.6C – compare lane 4 with lane 1, and lane 13 with lane 8, respectively). It should be noted that SMAD3(MH2) corresponds to a

naturally occurring variant previously described in murine pituitary and gonadotrope-like cells (838). This protein is phosphorylated and accumulates in the nucleus in response to activin A.

Using promoter-reporter assays, we assessed whether SMAD3(MH2) retains functional activity at the murine *Fshb* promoter. To directly compare the activity of SMAD3(MH2) and wild-type SMAD3, we transfected the amount of expression vector required to produce comparable levels of the two proteins, determined by our titration experiments described above (Fig. 2.6C). We previously reported that ectopic expression of FOXL2 and SMAD3 stimulates murine *Fshb* promoter activity in heterologous cells and that this effect is potentiated by SMAD4 (678). We reproduced these results here in HeLa cells using a -846/+1 murine *Fshb*-luciferase reporter (Fig. 2.6D). SMAD3(MH2) synergistically activated the *Fshb* promoter with FOXL2 and SMAD4 to the same extent as wild-type SMAD3 (Fig. 2.6D). To extend these analyses to a homologous system, we next employed LβT2 cells, which express endogenous FOXL2. Both wild-type SMAD3 and SMAD3(MH2) synergistically activated the -846/+1 murine *Fshb*-luc reporter with SMAD4 (Fig. 2.6E) and activin A (Fig. 2.6F). SMAD3(MH2) tended to show greater activity than wild-type in these assays, though this was not statistically significant. Collectively, these data indicate that the recombined *Smad3* allele encodes a truncated transcript and protein capable of activating *Fshb* transcription in cooperation with SMAD4 and FOXL2.

Discussion

SMAD2 and SMAD3 are the canonical activin-induced signaling molecules and both were previously implicated in activin-regulated *Fshb* transcription. We were therefore surprised to observe quantitatively normal FSH synthesis and fertility in gonadotrope-specific *Smad2/3* conditional knockout mice. Though the most parsimonious explanation for these results might be incomplete or insufficient recombination of the floxed alleles, at least three lines of evidence argue against this possibility. First, we observe robust (>90%) suppression of full-length *Smad2* and *Smad3* transcripts in genetically-labeled gonadotropes of S2/3cKO mice. Second, the *Gnrhr*^{GRIC} allele has demonstrated specificity and efficiency in other models (157, 820, 827). Third, we further decreased *Smad2/3* gene dosage by globally deleting one allele each of *Smad2* and *Smad3* such that only one floxed allele per gene required recombination in gonadotropes (*Smad2*$^{fl/-}$*;Smad3*$^{fl/-}$*; Gnrhr*$^{GRIC/+}$), but failed to observe additional phenotypes (data not shown). In light of these observations, we consider three alternative explanations for the absence of FSH deficiency in S2/3cKO mice: 1) compensation by a residual, but truncated form of SMAD3, SMAD3(MH2), 2) compensation by activin-dependent, but SMAD2/3-independent signaling, or 3) compensation by activin-independent signaling. We consider each of these possibilities in turn, with greatest emphasis on the first.

Our data clearly rule out necessary roles for SMAD3 DNA-binding activity and SMAD2 for quantitatively normal FSH synthesis *in vivo*. This result might have been anticipated for SMAD2 based on previous *in vitro* manipulations of SMAD2/3 levels (by knockdown or overexpression), which indicated a quantitatively more important role for SMAD3 than SMAD2 in *Fshb* transcriptional regulation (539, 674, 677, 678, 708). Furthermore, FOXL2, which is required for FSH synthesis and fertility *in vivo*, interacts more strongly with SMAD3 than SMAD2 (708, 709). In contrast with *in vivo* observations, but perhaps more in line with prior cell line data, we observed significant impairments of basal and activin A-stimulated *Fshb* expression following acute ablation of full-length *Smad2/3* in primary pituitary cells. Two, non-mutually exclusive, possibilities may explain these apparently discrepant results. First, the short time-frame of primary culture experiments may not have enabled the development of compensatory mechanisms similar to those established *in vivo*. Second, gonadotropes in dissociated cultures may rely more on ligand(s) signaling through SMAD2/3 to maintain *Fshb* expression than do

gonadotropes in the context of the intact gland and/or animal. Consistent with the first possibility, basal and activin A-stimulated *Fshb* mRNA expression were normal in pituitary cultures of female S2/2cKO mice (data not shown).

These *in vivo* and *in vitro* discrepancies aside, the most parsimonious explanation for the absence of FSH-deficiency in S2/3cKO mice was the failure of our mouse model to ablate all of SMAD3 function. That is, the recombined *Smad3* allele produces a transcript that encodes a truncated, but still functional SMAD3 protein: SMAD3(MH2). This protein is likely generated via translation re-initiation or leaky ribosomal scanning from the novel mRNA transcribed in these mice (837). The translation start site in exon 1, from which full-length SMAD3 is ordinarily derived, does not conform to a consensus Kozak sequence, with a thymine (uracil in the mRNA) rather than a guanine at the +4 position (with the adenine of the ATG/AUG denoted as +1). In the context of the mRNA lacking exons 2 and 3, translation appears to initiate at a consensus Kozak sequence in exon 4. Translation from this site is not unprecedented as an alternative *Smad3* transcript initiating in the third intron (generating a novel "exon 3a") was previously described in gonadotrope-like LβT2 cells (838). Though we were unable to confirm the presence of this particular transcript in control or S2/3cKO gonadotropes, the resulting protein would be indistinguishable from SMAD3(MH2) described here. As indicated above, the authors of the previous study showed that this truncated protein is phosphorylated and accumulates in the nucleus upon activin A stimulation. In contrast to what we report here, they suggested that the truncated protein acts as a dominant-negative when co-expressed with wild-type SMAD3. However, its independent actions were not assessed in their experiments and we propose that their results might be alternatively explained by hypomorphic (rather than dominant-negative) activity, as we observed in our reporter assays. That is, SMAD3(MH2) is expressed at lower levels than wild-type SMAD3 when equivalent amounts of expression vector are employed. We had to titrate the amount of wild-type vector to achieve equivalent expression of the two proteins in order to demonstrate their similar activities in transcriptional assays.

Based on previous observations, we predict that SMAD3(MH2) fulfills SMAD3 functions necessary for proper *Fshb* transcription. Indeed, we previously reported that either SMAD3 or SMAD4 (but not both) must bind DNA to stimulate murine *Fshb* transcription, suggesting that SMAD3 DNA-binding activity is dispensable for FSH synthesis (678). Furthermore, SMAD3 physically interacts with SMAD4 and FOXL2 via its MH2 domain, which is preserved in the

truncated protein described here. Consistent with these observations, SMAD3(MH2) activates the murine *Fshb* promoter in cooperation with SMAD4 and FOXL2 in *in vitro* assays. Supporting the important functionality of the SMAD3 MH2 domain, *Smad3*$^{\Delta exon8}$ knockout mice have reproductive anomalies and pituitary *Fshb* deficiency. It is important to note, however, that the reduction in *Fshb* expression in *Smad3*$^{\Delta exon8}$ KO mice is quantitatively modest (around 30%) (688) and females actually have elevated serum FSH levels (687). However, these observations may be confounded by ovarian abnormalities (684, 687) and/or partial compensation by SMAD2 in these mice.

In our *in vitro* experiments, transfection of the same amount of truncated and full-length *Smad3* expression vectors produced notably less SMAD3(MH2) than SMAD3 protein. This result is expected for proteins generated through leaky ribosomal scanning or translation reinitiation (837). Nevertheless, when expressed at the same protein level, SMAD3(MH2) was functionally equivalent to wild-type SMAD3. The implications of these observations for SMAD3(MH2)'s potential to compensate for the loss of full-length SMAD3 *in vivo* are hard to predict for at least two reasons. First, it is unclear what threshold level of SMAD3 activity (expression) is required to sustain quantitatively normal FSH synthesis *in vivo*. Second, we observed that transcripts containing distal exons were upregulated approximately 2-fold in S2/3cKO compared to control gonadotropes. Therefore, it is possible that the amount of SMAD3(MH2) protein in mutant gonadotropes is closer to wild-type levels than suggested by our *in vitro* over-expression studies. Unfortunately, due to the paucity of gonadotropes (we typically isolate 5,000-7,000 cells per pituitary), we are unable to obtain sufficient numbers of purified cells from GRIC-YFP and S2/3cKO-YFP mice for protein analysis. Therefore, establishing SMAD3's necessity in pituitary FSH synthesis will require cell-specific removal of all protein function, likely with a novel conditional *Smad3* allele.

Our observation that the recombined *Smad3* allele encodes a functional protein may also clarify discrepancies in the phenotypes of existing *Smad3* 'knockout' mouse lines. Indeed, global deletion of different *Smad3* exons has produced divergent phenotypes, with the only common observation being smaller body size (683-686). *Smad3*$^{\Delta exon2}$ knockout embryos produce an identical transcript (exon 1 splicing to exon 4) as the one reported here, presumably resulting in the production of the SMAD3(MH2) protein. Interestingly, this is apparently the only *Smad3*-null strain that develops fully penetrant colorectal cancer (686, 839). Unique phenotypes reported

in other *Smad3* knockout lines include mild forelimb malformation in *Smad3*$^{\Delta exon1}$ mice (683) and immune system dysfunction and aortic aneurysms in *Smad3*$^{\Delta exon8}$ mice (685, 840). The only allele that disrupts the coding sequence for the MH2 domain is *Smad3*$^{\Delta exon8}$. In light of our results, it is perhaps not surprising that this is the only *Smad3*-deficient strain in which reproductive defects and pituitary *Fshb* deficiency have been reported (684, 687). However, *Smad3*$^{\Delta exon8}$ mice also produce a truncated SMAD3 protein, comprising the MH1 domain, which can act as a dominant-negative in some contexts (685). Thus, as mentioned above, a novel conditional *Smad3* allele, which completely removes protein function and lacks dominant-negative activity, is needed to assess SMAD3's role not only in gonadotropes, but in all cell types. Indeed, such a mouse model will enable, if not necessitate, repetition of investigations using other modified *Smad3* alleles.

Although it seems likely that the retention of SMAD3(MH2) explains, at least in part, normal FSH levels in S2/3cKO mice, other mechanisms of compensation may exist. Other receptor-regulated SMADs (R-SMADs), in particular SMAD8, can activate *Fshb* transcription *in vitro* (644, 690, 691). Further, activins can signal through non-canonical R-SMADs in some contexts (641); though, the extent to which this also occurs in gonadotropes is unknown. In addition, it was proposed that activins may regulate *Fshb* transcription via a SMAD-independent mechanism involving the kinases TAK1 and p38 (696). However, a subsequent study showed that the small molecule TAK1 inhibitor 5Z-7-oxozeaenol non-specifically blocks activin type I receptor (ALK4) activity. This and other studies also failed to confirm a role for p38 in this system (675, 697, 765). Therefore, *in vitro* studies do not, at present, demonstrate SMAD-independent mechanism of FSH regulation by activins. This question could be definitively resolved, however, by disrupting all SMAD-dependent signaling by selectively ablating the common partner *Smad4* in gonadotropes. Indeed, preliminary data along these lines from our lab appear to confirm a necessary role for SMAD signaling in *Fshb* expression *in vivo* (841).

Signaling by hormones other than activins may regulate *Fshb* transcription and FSH synthesis *in vivo*, perhaps bypassing a requirement for SMAD2/3. For example, bone morphogenetic proteins (BMPs) regulate *Fshb* transcription and FSH synthesis in primary pituitary cells and gonadotrope-like cell lines (644, 690, 764-766). However, their contributions to FSH regulation *in vivo* remain to be determined. Another obvious candidate is gonadotropin-releasing hormone (GnRH), a well-established and potent stimulator FSH synthesis (203, 842).

Indeed, mice that lack GnRH or the GnRH receptor exhibit profound FSH deficiency (62, 171). However, because GnRH also regulates the expression of follistatin (736, 843), an activin antagonist, FSH phenotypes in GnRH-deficient mice may be attributable, at least in part, to dysregulation of activin or activin-like signaling. The relative roles of activins and GnRH in FSH regulation *in vivo* have not been clearly established; however, it is possible that GnRH might assume a more important and compensatory role in the absence of signaling via SMAD2/3. Finally, compensatory regulation by steroids may help sustain normal FSH production. In rodents, on the morning of estrus, a selective rise in FSH synthesis and secretion drives ovarian follicle recruitment and maturation (249). Although this FSH surge is thought to be driven by increased activin signaling in the face of lower inhibin levels (595, 610), it is also blocked by progesterone and glucocorticoid receptor antagonists (813-816). Thus, it is possible that the steroid milieu may enable appropriate FSH production in the absence of activin signaling.

In conclusion, our results demonstrate, for the first time, that *Fshb* transcription and FSH synthesis can occur independently of SMAD3 DNA-binding activity and SMAD2 *in vivo*. Further, they reveal that deletion of exons 2 and 3 of *Smad3* results in the production of a novel *Smad3* transcript, which encodes a functional protein. This latter observation has important implications for investigations (past, present, and future) of SMAD3 function using existing *Smad3* knockout mouse lines.

Acknowledgements

The authors acknowledge Xiang Zhou for help with the ovariectomy procedures and Ken McDonald for assistance with FACS. JF was supported by a Doctoral Research Award from the Canadian Institutes of Health Research (CIHR). This work was funded by CIHR operating grant MOP-89991 to DJB. DJB is a Senior Research Scholar of the Fonds de la recherche en Santé de Québec. UB is funded by DFG BO1743/2. The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core is supported by the National Institutes of Health NICHD (SCCPRR) Grant U54-HD28934.

Figure Legends

Figure 2.1: Generation and validation of gonadotrope-specific *Smad2/3* knockout mice. **A**-**B**) YFP+ and YFP- cells were isolated from GRIC-YFP and S2/3cKO-YFP mice by FACS. Genomic DNA was extracted and analyzed by genotyping PCR for the wild-type (WT), floxed (flox) and recombined (rec.) alleles of *Smad2* (**A**) and *Smad3* (**B**). Data are representative from one of three sorting experiments. **C-D**) cDNA was prepared from total RNA from sorted cells and analyzed by qPCR using primers overlapping the deleted exons in *Smad2* (**C** – forward primer in exon 10; reverse primers in exon 11) and *Smad3* (**D** - forward primer in exon 3; reverse primer in exon 4). *Smad2* and *Smad3* transcript levels were normalized to the levels of the housekeeping gene *Rpl19*. Data represent the mean +SEM from three independent sorting experiments assayed in triplicate. Bars with different symbols differ significantly.

Figure 2.2: Largely intact reproductive maturation and normal fertility in S2/3cKO females. **A**) Day of vaginal opening in S2/3cKO and control mice. Each dot represents an individual mouse. The horizontal line represents the group mean. **B**) Representative estrous cyclicity profile from two control (left) and two S2/3cKO (right) mice. M/D: metestrus/diestrus; P: proestrus; E: estrus. **C**) Proportion of time spent in each cycle stage (left) and estrus cycle frequency (right) for control and S2/3cKO mice (N=7 per genotype). **D**) Cumulative number of pups delivered per female over the course of a 6 month breeding trial, calculated at the end of each month. Data represent the means +/- SEM of 7 mice per genotype. *, significant at $p \leq 0.05$

Figure 2.3: Small reductions in testes weights and epididymal sperm counts in S2/3cKO males. **A**) Testes weight (sum of both testes), expressed as a percentage of body weight, in 10-week-old control and S2/3cKO male mice. Each point represents an individual mouse. The horizontal line represents the group mean. **B**) Caudal epididymal sperm count (per mL of homogenization buffer) in 10-week-old control and S2/3cKO male mice. **C**) Wet seminal vesicle (SV) weight, normalized to body weight, in the same animals as in **A**). n.s., not significant. *, significant at $p \leq 0.05$

Figure 2.4: Normal FSH synthesis and *Fshb* expression in S2/3cKO mice. **A**) Circulating FSH levels in 10-week-old control and S2/3cKO males (left) and metestrus/diestrus females (right). Data represent the mean +SEM of 12 mice per group. **B**) Relative pituitary *Fshb* mRNA levels,(with controls set to 1) assayed by qPCR in the same animals as in **A**). **C**) Adult metestrus/diestrus control and S2/3cKO females were ovariectomized (OVX) or sham-operated (sham). After 7 h, mice were killed, and their serum and pituitaries collected. Serum FSH (left) and pituitary *Fshb* mRNA levels (right) were analyzed in the same animals. Data represent the mean +SEM of 6-7 mice per group. Bars with different symbols differ significantly.

Figure 2.5: Partial dependence of basal and activin-stimulated *Fshb* expression on full-length SMAD2/3 in primary pituitary cultures. **A-B**) Primary pituitary cultures were prepared from *Smad2*$^{fl/fl}$*;Smad3*$^{fl/fl}$ male (left) and female mice (right), and infected for 24 h with adenoviruses expressing Cre recombinase (Ad-Cre) or green fluorescent protein (Ad-GFP). Cells were stimulated for 24 h with 1 nM activin A, or left untreated (no ligand) prior to RNA extraction. *Smad2* (**A**) and *Smad3* (**B**) mRNA levels were assessed by qPCR. **C**) Expression of *Fshb* in the same samples as in **A-B**). In all panels, data represent the mean +SEM of five independent experiments measured in triplicate (n=5). Bars with different symbols differ significantly.

Figure 2.6: Retention of a truncated *Smad3* transcript coding for a functional protein in S2/3cKO mice. **A**) Distal *Smad3* mRNA levels (forward primer in exon 8; reverse primer in exon 9), analyzed by qPCR in YFP+ and YFP- cells from the same experiment as in Figure 1. Data represent the mean +SEM of three independent sorting experiments measured in triplicate (n=3). **B**) Top: Schematic representation of the wild-type SMAD3 protein. Amino acids are numbered. MH1: Mad homology 1 domain; MH2: Mad homology 2 domain. The "SSVS" sequence at the C-terminus contains the serine residues phosphorylated by the type I receptor. Middle: *Smad3* gene structure with numbered exons. Splicing of the wild-type and recombined *Smad3* alleles is depicted with dashed lines. Arrowheads indicate the start of translation in exon 1, and the putative alternative translation initiation site in exon 4. The nucleotide sequence surrounding the AUG sequence (underlined) of both sites is indicated. Bases which conform to the consensus Kozak sequence, -3RCCAUGG+4, at positions -3 and +4 are indicated in black italics. Bottom: Representation of the truncated protein, SMAD3(MH2), arising from the recombined *Smad3*

allele. **C**) Western blot analysis of whole cell protein lysates from HeLa (lanes 1-6) or LβT2 (lanes 8-13) cells transfected with 2 μg expression vector encoding C-terminally tagged 3X-HA tagged truncated SMAD3 (SMAD3-MH2; lane 1 and 8) or differing amounts of wild-type SMAD3-HA expression vector (lanes 2-6 and 9-13: 1:1 – 2 μg ; 1:2 – 1 μg ; 1:5 – 0.4 μg ; 1:10 – 0.2 μg; 1:20 – 0.1 μg). The blot was probed with anti-HA (IB: HA - top) and anti-β actin (IB: actin – bottom) antibodies. Arrows indicate the relative migration of full-length SMAD3 and SMAD3-MH2 products. **D**) HeLa cells were transfected with 225 ng/well of the -846/+1 murine *Fshb*-luc reporter, with 25 ng of FOXL2 and/or SMAD4 expression vectors, together with 25 ng of SMAD3 or 5 times more (125 ng) SMAD3(MH2) expression vectors. Whole cell lysates were subjected to luciferase assays. The amount of transfected vectors was balanced across all conditions with pcDNA3.0. **E**) LβT2 cells were transfected with 225 ng/well of the -846/+1 murine *Fshb*-luc reporter, with 25 ng of SMAD4 expression vector, together with 25 ng of SMAD3 or 20 times more (500 ng) SMAD3(MH2) expression vector as indicated. The amount of transfected vectors was balanced across all conditions with pcDNA3.0. Whole cell lysates were subjected to luciferase assays. **F**) LβT2 cells were transfected with 225 ng/well of the -846/+1 murine *Fshb*-luc reporter, along with 25 ng SMAD3 or 20 times more (500 ng) SMAD3(MH2) expression vector. The amount of transfected vectors was balanced across all conditions with pcDNA3.0. Cells were stimulated with 1 nM activin A for 24h, or left untreated, prior to assaying luciferase activity. Data represent the mean +SEM of seven (**D)** or three **(E, F**) independent experiments performed in triplicate. Bars with different symbols differ significantly.

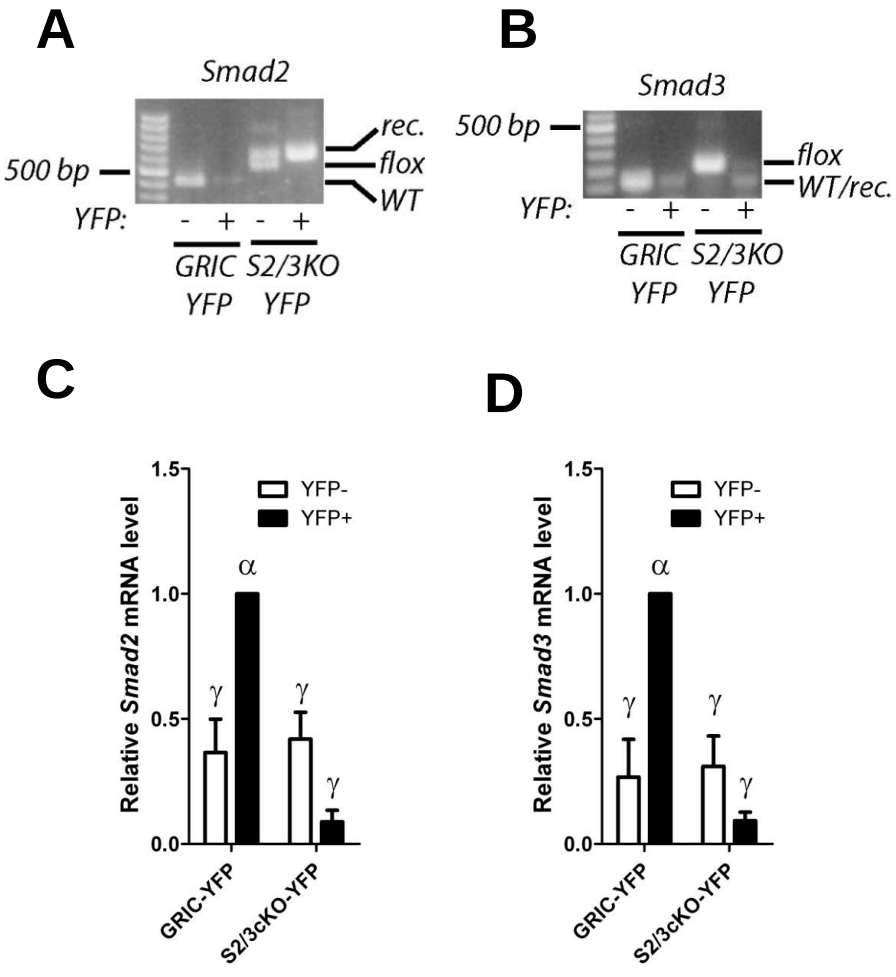
Supplementary Figure Legends

Figure S2.1: Decreased pituitary *Smad2* and *Smad3* transcript levels and normal reproductive organs in S2/3cKO mice. cDNA was prepared from total pituitary RNA and analyzed by qPCR using primers overlapping the deleted exons in *Smad2* (**A** – forward primer in exon 10; reverse primer in exon 11) and *Smad3* (**B** - forward primer in exon 3; reverse primer in exon 4). *Smad2* and *Smad3* transcript levels are shown relative to controls set to 1. Data represent the mean +SEM from 12 mice per group assayed in triplicate. *, significant at $p \leq 0.05$. Mean ovarian (**C**) and uterine (**D**) weights of 10-week-old metestrus/diestrus females, expressed as a percentage of body weight. Each point represents an individual mouse. Means are shown with the horizontal

lines. The data are from the same animals used in Fig. 4. n.s., not significantly different. **E**) Ovarian sections (left) and corpora lutea counts (right) in ovaries from control and S2/3cKO females (n=6 per genotype; 1 ovary/mouse). Scale bars: 0.5 mm. n.s., not significantly different. **F**) Expression of *Cyp19a1*, *Ccnd,2* and *Lhr* in the ovaries of control and S2/3cKO females, assessed by qPCR. Shown are means +SEM (n=6 per genotype). **G**) Testicular histology of control and S2/3cKO males. Shown are representative sections from n=3 males analyzed per genotype. Scale bars: 0.5 mm.

Figure S2.2: Serum LH levels are normal in young adult S2/3cKO males and females, but elevated in older S2/3cKO females. **A**) Serum LH levels from 10-week-old male (left) and metestrus/diestrus female (right) control and S2/3cKO mice. The data are from the same animals used in Fig. 4 (N=12 mice per group). Serum LH (**B**) and FSH (**C**) from 10-month-old metestrus/diestrus females retired from the breeding trial (see Figure 2). Each dot represents an individual animal. Means are shown with the horizontal lines.

Figure S2.3: Mildly impaired basal and activin A-stimulated *Fshb* transcription in primary pituitary cultures lacking *Smad2* or *Smad3*. Primary pituitary cultures were prepared from *Smad2*$^{fl/fl}$ (**A**) or *Smad3*$^{fl/fl}$ (**B**) males (left) and females (right), and infected for 24 h with adenoviruses expressing Cre recombinase (Ad-Cre) or green fluorescent protein (Ad-GFP). Cells were stimulated for 24 h with 1 nM activin A, or left untreated (no ligand) prior to RNA extraction. *Fshb* mRNA levels were assessed by qPCR. Data represent the mean +SEM of five (male and female *Smad3*), four (male *Smad2*) and three (female *Smad2*) independent experiments assayed in triplicate. Bars with different symbols differ significantly.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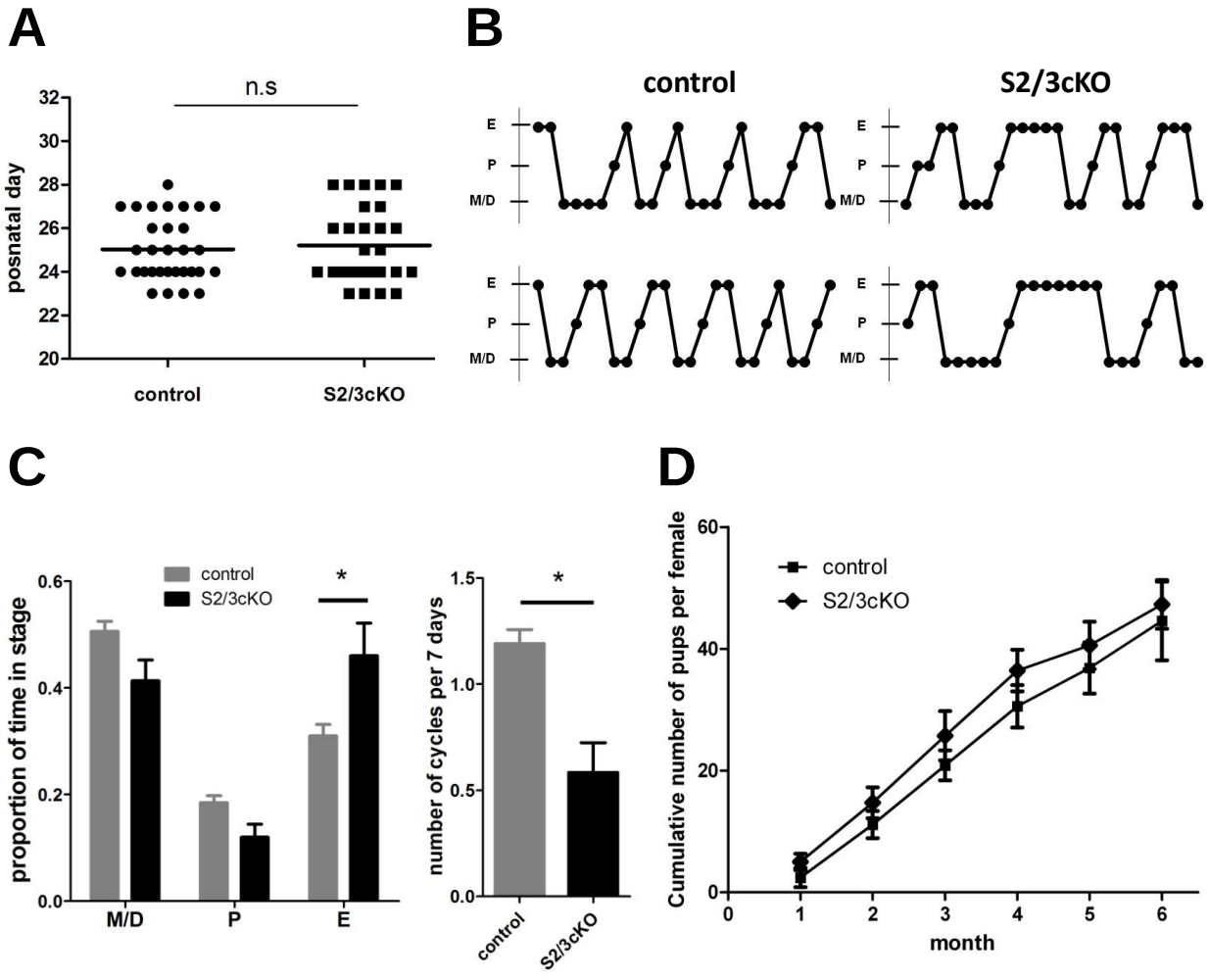
Smad2
500 bp
rec.
flox
WT
YFP: - + - +
GRIC YFP
S2/3KO YFP
B
Smad3
500 bp
flox
WT/rec.
YFP: - + - +
GRIC YFP
S2/3KO YFP
C
Relative Smad2 mRNA level
YFP-
YFP+
1.5
1.0
0.5
0.0
α
γ
γ
γ
GRIC-YFP
S2/3cKO-YFP
D
Relative Smad3 mRNA level
YFP-
YFP+
1.5
1.0
0.5
0.0
α
γ
γ
γ
GRIC-YFP
S2/3cKO-YFP

Figure 2.1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n.s
posnatal day
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30
28
26
24
22
20
control
S2/3cKO
B
control
S2/3cKO
E
P
M/D
C
control
S2/3cKO
*
proportion of time in stage
0.6
0.4
0.2
0.0
M/D
P
E
number of cycles per 7 days
1.5
1.0
0.5
0.0
control
S2/3cKO
D
Cumulative number of pups per female
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40
20
0
control
S2/3cKO
0
1
2
3
4
5
6
month

Figure 2.2

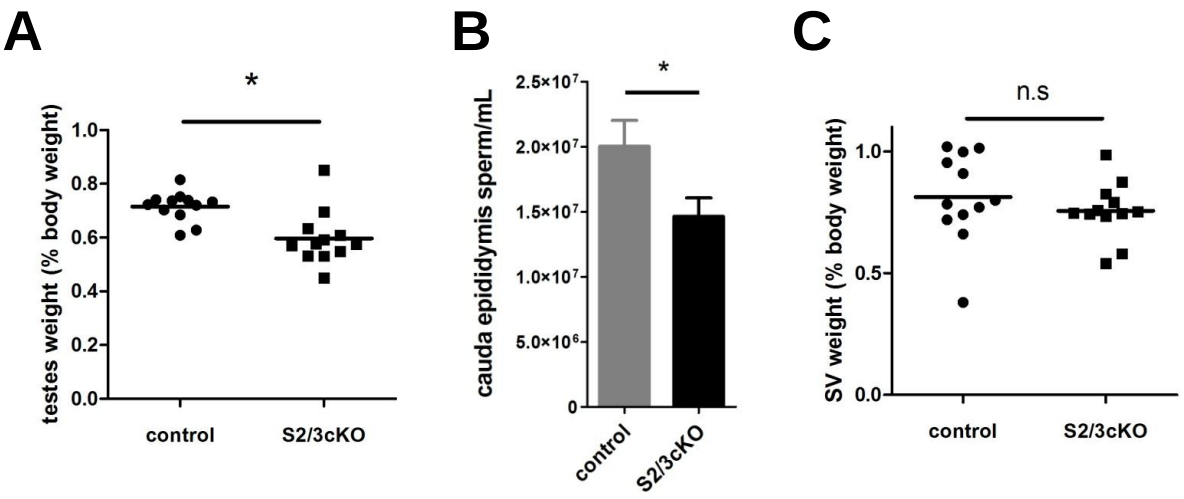
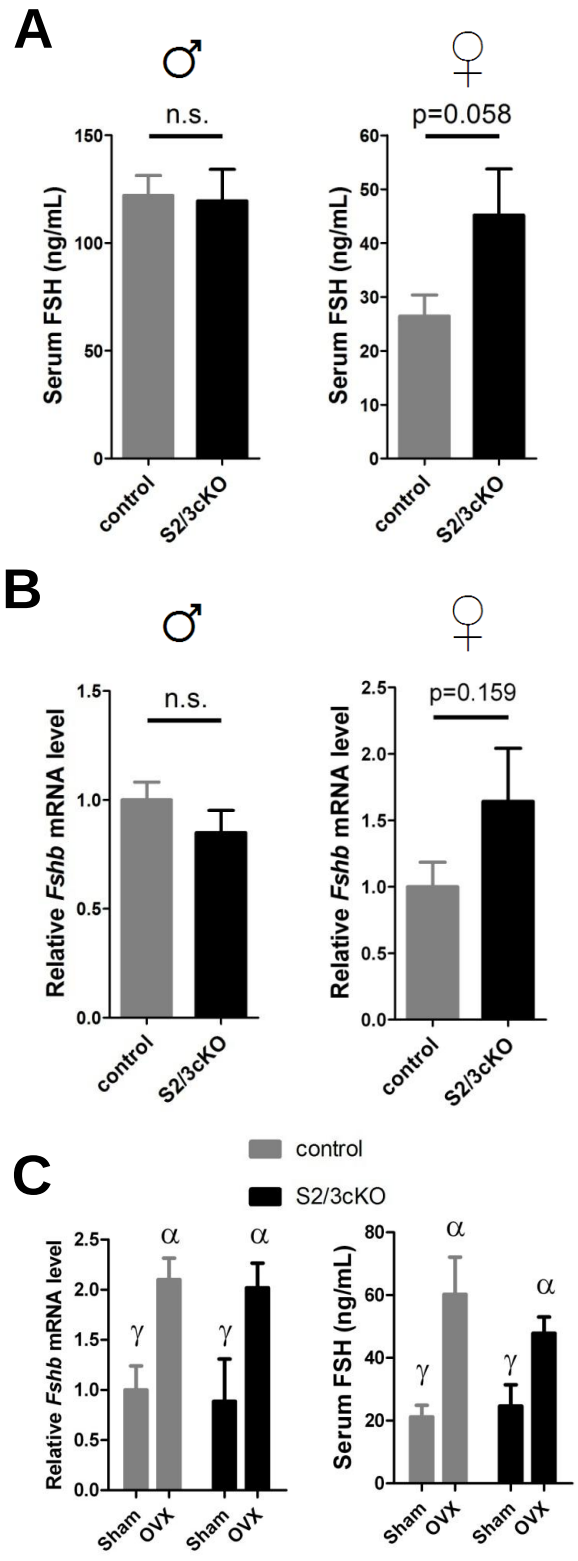

A
*
testes weight (% body weight)
1.0
0.8
0.6
0.4
0.2
0.0
control
S2/3cKO
B
*
cauda epididymis sperm/mL
2.5×10^7
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
0
control
S2/3cKO
C
n.s
SV weight (% body weight)
1.0
0.5
0.0
control
S2/3cKO

Figure 2.3


A
♂
n.s.
Serum FSH (ng/mL)
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100
50
0
control
S2/3cKO
♀
p=0.058
60
50
40
30
20
10
0
control
S2/3cKO
B
♂
n.s.
Relative Fshb mRNA level
1.5
1.0
0.5
0.0
control
S2/3cKO
♀
p=0.159
2.5
2.0
1.5
1.0
0.5
0.0
control
S2/3cKO
C
control
S2/3cKO
Relative Fshb mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
α
α
γ
γ
Sham
OVX
Sham
OVX
Serum FSH (ng/mL)
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60
40
20
0
α
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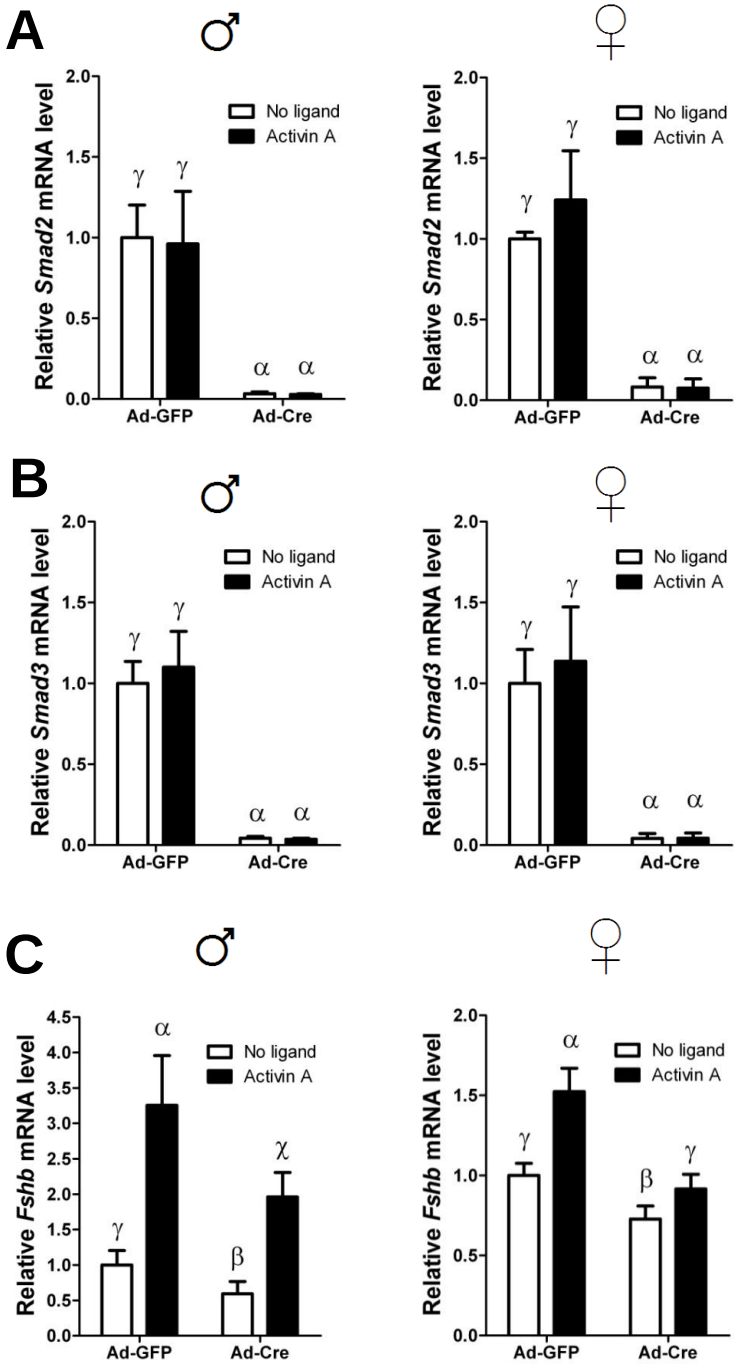
γ
γ
Sham
OVX
Sham
OVX

Figure 2.4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♂
♀
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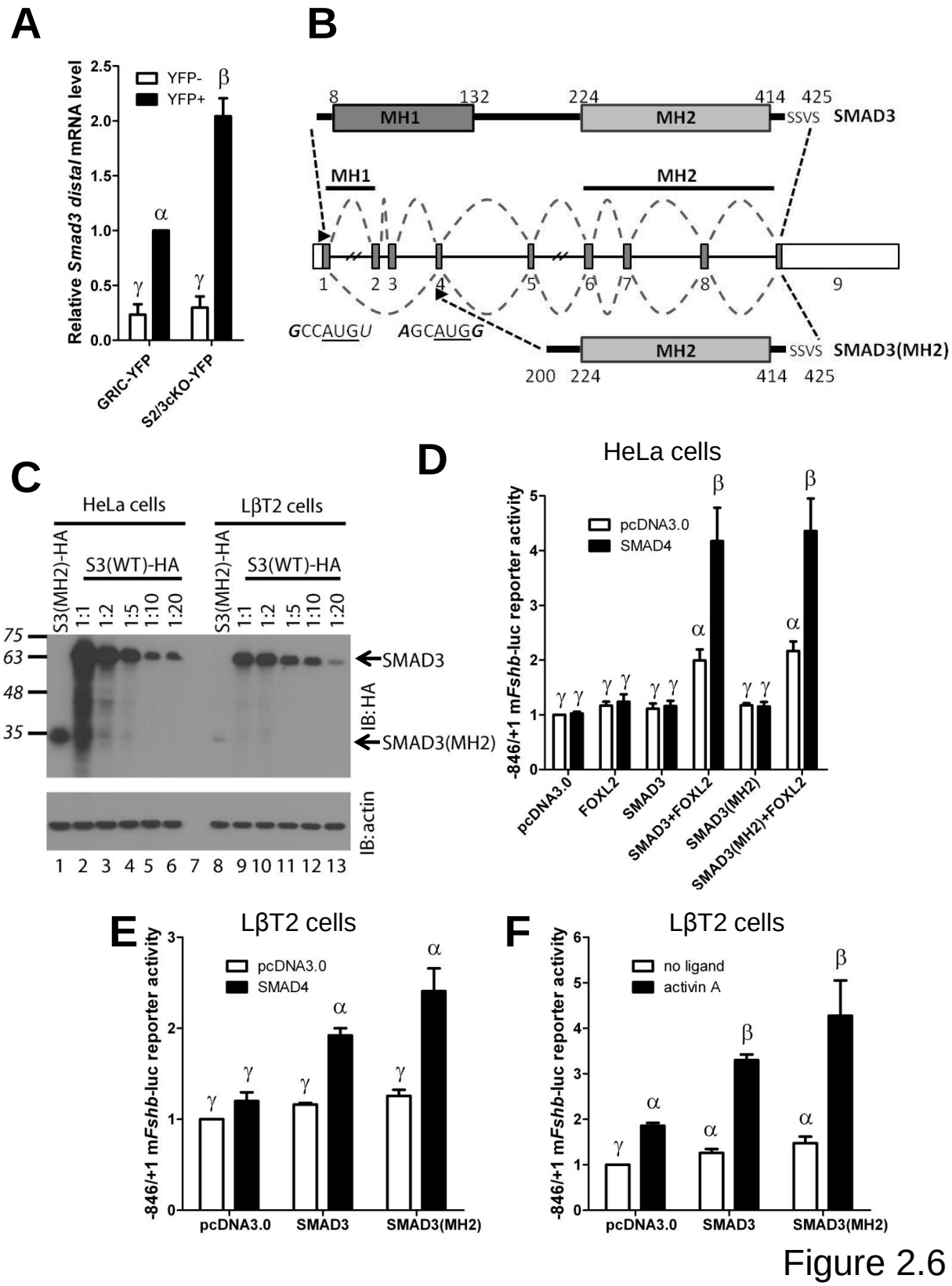
Ad-Cre
B
Relative Smad3 mRNA level
C
Relative Fshb mRNA level

Figure 2.5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YFP+
GRIC-YFP
S2/3cKO-YFP
B
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MH2
SSVS
SMAD3
SMAD3(MH2)
GCCAUGU
AGCAUGG
C
HeLa cells
LβT2 cells
S3(MH2)-HA
S3(WT)-HA
SMAD3
SMAD3(MH2)
IB:HA
IB:actin
D
HeLa cells
pcDNA3.0
SMAD4
-846/+1 mFshb-luc reporter activity
pcDNA3.0
FOXL2
SMAD3
SMAD3+FOXL2
SMAD3(MH2)
SMAD3(MH2)+FOXL2
E
LβT2 cells
F
LβT2 cells
no ligand
activin A

Figure 2.6

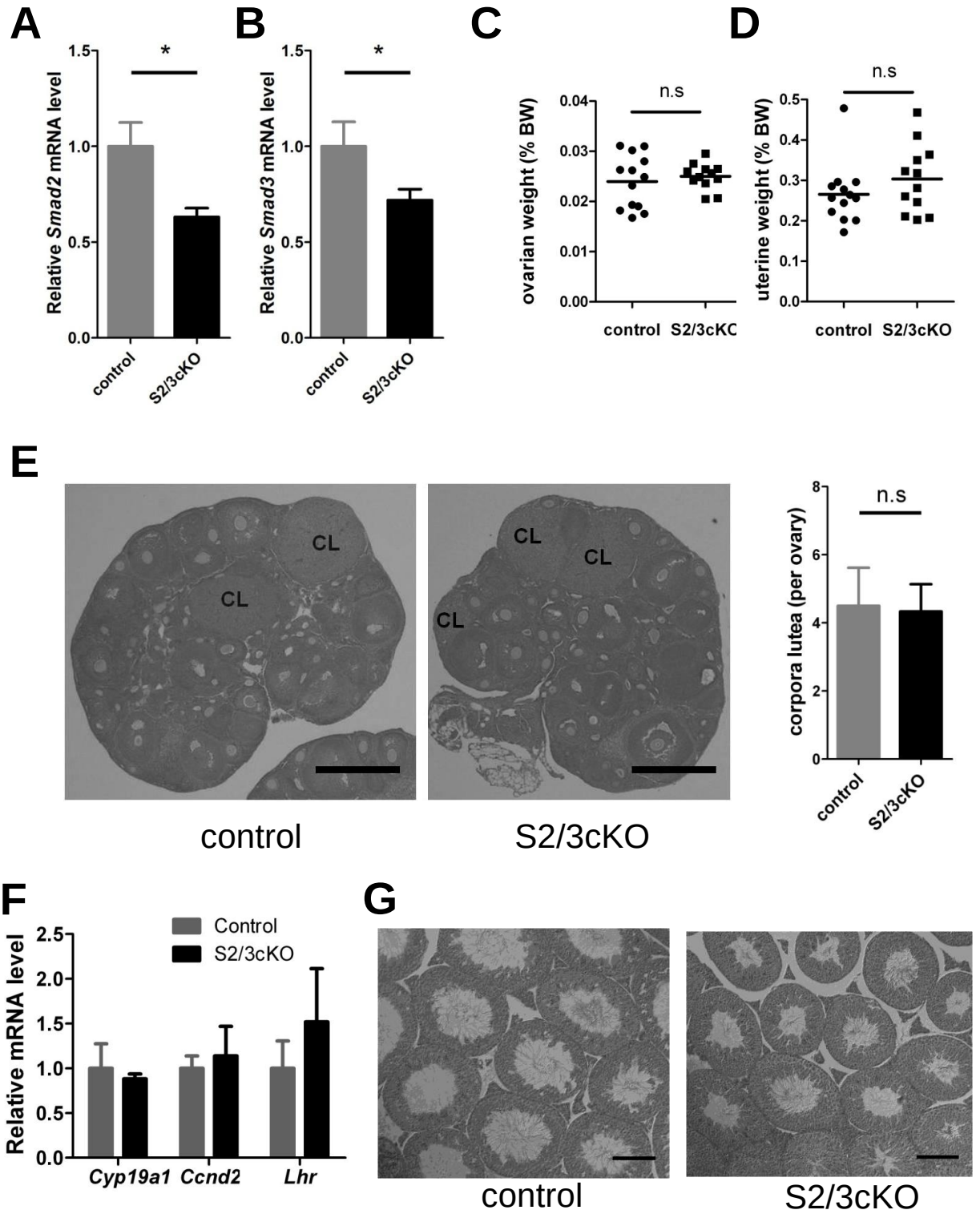
A
B
C
D
E
F
G
Relative Smad2 mRNA level
Relative Smad3 mRNA level
ovarian weight (% BW)
uterine weight (% BW)
corpora lutea (per ovary)
control
S2/3cKO
n.s
*
CL
Control
Relative mRNA level
Cyp19a1
Ccnd2
Lhr

Figure S2.1

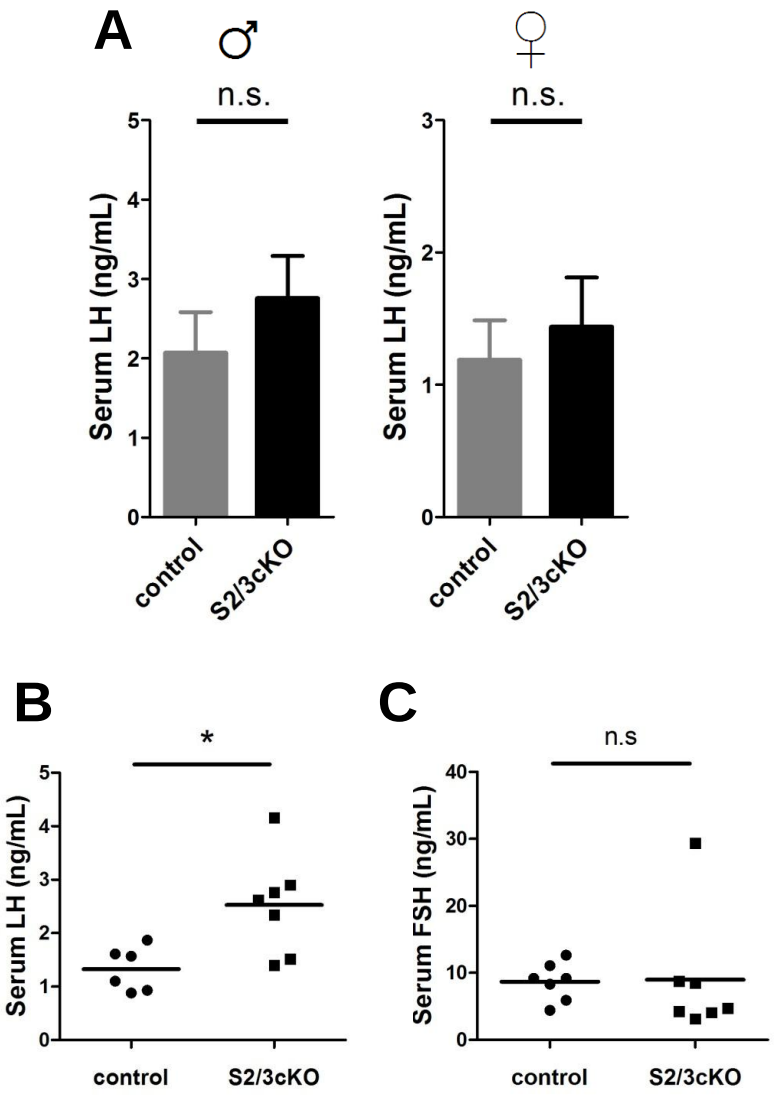
A
♂
n.s.
Serum LH (ng/mL)
control
S2/3cKO
♀
n.s.
Serum LH (ng/mL)
control
S2/3cKO
B
*
Serum LH (ng/mL)
control
S2/3cKO
C
n.s
Serum FSH (ng/mL)
control
S2/3cKO

Figure S2.2

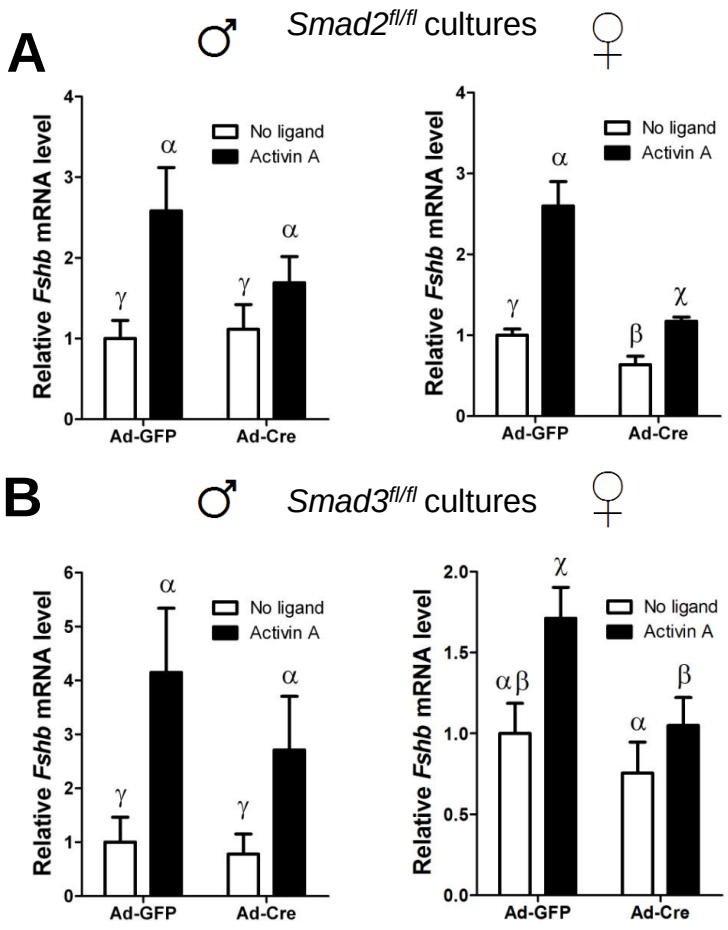
A
♂
Smad2fl/fl cultures
♀
Relative Fshb mRNA level
No ligand
Activin A
Ad-GFP
Ad-Cre
B
♂
Smad3fl/fl cultures
♀
Relative Fshb mRNA level
No ligand
Activin A
Ad-GFP
Ad-Cre

Figure S2.3

Primer and purpose	Sequence
Smad2flox genotyping, forward	5’-TACTTGGGGCAATCTTTTCG-3’
Smad2flox genotyping, reverse	5’-GTCACTCCCTGAACCTGAAG-3’
Smad2recombined genotyping, forward	5’-GAGCTGCGCAGACCTTGTTAC-3’
Smad3flox genotyping, forward	5’-CTCCAGATCGTGGGCATACAGC-3’
Smad3flox genotyping, reverse	5’-GGTCACAGGGTCCTCTGTGCC-3’
Smad3recombined genotyping, forward	5’-TCGTCGATCGACCTCGAATAAC-3’
Gnrhr WT genotyping, forward	5-GAACTACAGCTGAATCAGTC-3’
Gnrhr WT genotyping, reverse	5’-CTCTAACAAACTCTGTACA-3’
Gnrhr GRIC genotyping, forward	5’-GGACATGTTCAGGGATCGCCAGCC-3’
Gnrhr GRIC genotyping, reverse	5’-GCATAACCAGTGAAACAGCATTGCTG-3’
ROSA26 WT/eYFP genotyping, forward	5’-AAAGTCGCTCTGAGTTGTTAT-3’
ROSA26 WT genotyping, reverse	5’-GCGAAGAGTTTGTCCTCAACC-3’
ROSA26 eYFP genotyping, reverse	5’-GGAGCGGGAGAAATGGATATG-3’
Smad2 qPCR, exon 10, forward	5’-ATCAGCTAACCCGAATGTGC-3’
Smad2 qPCR, exon 11, reverse	5’-AAGGGGATCCCATCTGAGTT-3’
Smad3 qPCR, exon 3, forward	5’-CATTCCATTCCCGAGAACAC-3’
Smad3 qPCR, exon 4, reverse	5’-ATGCTGTGGTTCATCTGGTG-3’
Smad3 qPCR, exon 8, forward	5’-CATCCGTATGAGCTTCGTCA-3’
Smad3 qPCR, exon 9, reverse	5’-CATCTGGGTGAGGACCTTGT-3’
Fshb qpCR, forward	5’-GTGCGGGCTACTGCTACACT-3’
Fshb qpCR, reverse	5’-CAGGCAATCTTACGGTCTCG-3’
Rpl19 qpCR, forward	5’-CGGGAATCCAAGAAGATTGA-3’
Rpl19 qpCR, reverse	5’-TTCAGCTTGTGGATGTGCTC-3’
Cyp19a1 qPCR, forward	5’-GACAGGCACCTTGTGGAAAT-3’
Cyp19a1 qPCR, reverse	5’-GAGGTTCACGCCACCTACTC-3’
Ccnd2 qPCR, forward	5’-AGCTGTCCCTGATCCGCAAG-3’
Ccnd2 qPCR, reverse	5’-GTTCACTTCATCATCCTGCTG-3’
Lhr qPCR, forward	5’-CGTCCCATTGAATGCATGG-3’
Lhr qPCR, reverse	5’-TGTAACACAGGCATCCGGA-3’
Smad3 primer walk, exon 1, forward	5’-AGTTGGACGAGCTGGAGAAG-3’
Smad3 primer walk, exon 2, forward	5’-CACAGCCACCATGAATTACG-3’
Smad3 primer walk, exon 3, forward	5’-CATTCCATTCCCGAGAACAC-3’
Smad3 primer walk, exon 3a, forward	5’-GTCAACGCGTTAGGATCCAG-3’
Smad3 primer walk, exon 4, forward	5’-CCTCCTGGCTACCTGAGTGA-3’
Smad3 primer walk, exon 5, forward	5’-TGTCCCCAGCACACAATAAC-3’
Smad3 primer walk, exon 6, forward	5’-CTGGGCCTACTGTCCAATGT-3’
Smad3 primer walk, exon 7, forward	5’-TGTGCGGCTCTACTAGACCG-3’
Smad3 primer walk, exon 8, forward	5’-CATCCGTATGAGCTTCGTCA-3’
Smad3 primer walk, exon 9, reverse	5’-CATCTGGGTGAGGACCTTGT-3’
Smad3 transcript cloning, forward	5’-GCAAGCTTTTCTCCAGAGTTAAAAGCGAAG-3’
Smad3 transcript cloning, reverse	5’-GCGGATCCCTAAGACACACTGGAACAGC-3’
Smad3 transcript cloning, HA tag, reverse	5’-GCATCGATAGACACACTGGAACAGCGGA-3’

Table S2.1

Chapter 3

In chapter 2, I showed that mice lacking SMAD2 and the DNA-binding domain of SMAD3 have preserved FSH synthesis and fertility. Thus, these results demonstrate that SMAD2 is dispensable for normal FSH synthesis and that SMAD3, if it is required, does not need to bind DNA to produce its actions. In the latter case, it seems that the residual truncated SMAD3 protein (SMAD3-MH2) cooperatively activates *Fshb* transcription with SMAD4 and FOXL2. However, as detailed in the discussion of chapter 2, there are at least three other possibilities: 1) activin-stimulated *Fshb* expression is mediated by other receptor-regulated SMAD proteins (SMAD1/5/8); 2) activin-stimulated *Fshb* expression depends on a non-canonical, SMAD-independent signaling pathway; or 3) activin signaling is altogether dispensable for FSH synthesis and fertility *in vivo*. All of these alternatives would challenge dogma and force a re-consideration of the prevailing models of activin-regulated *Fshb* expression. The third possibility is particularly disconcerting: could we have been misled for more than 30 years in thinking that activin signaling is required for FSH synthesis *in vivo*? Thus, it is fundamentally important to verify whether SMAD-dependent signaling, of any kind, is necessary for FSH synthesis and fertility. All the receptor-activated SMADs function in association with the common SMAD, SMAD4, to regulate gene transcription (625). Therefore, deleting the *Smad4* gene should block all SMAD-dependent signaling. In this chapter, I generated mice with a gonadotrope-restricted deletion of *Smad4* and evaluated their FSH synthesis and fertility. The prevailing model of *Fshb* transcriptional regulation by activins predicts cooperative induction of *Fshb* expression by a combination of independent and interdependent activities of SMADs and FOXL2 (844). Indeed, mice with a gonadotrope-specific deletion of *Foxl2* are FSH-deficient and subfertile, but still synthesize some FSH, presumably accounting for their partially preserved fertility (716). However, this may also reflect the maximal defect that can result from ablation of activin signaling components in gonadotropes, with the residual FSH synthesis (and fertility) being controlled by GnRH signaling. To discriminate between these possibilities, I generated mice simultaneously deficient in SMAD signaling and FOXL2 in gonadotropes (gonadotrope-specific *Smad4/Foxl2* double knockout mice).

Title: Follicle-stimulating hormone synthesis and fertility depend on SMAD4 and FOXL2

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Short title: Regulation of FSH synthesis by SMAD4 and FOXL2

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Abstract

Follicle-stimulating hormone (FSH) is an essential regulator of gonadal function and fertility. Loss-of-function mutations in the *FSHB/Fshb* gene cause hypogonadotropic hypogonadism in humans and mice. Both gonadotropin-releasing hormone (GnRH) and activins, members of the transforming growth factor β (TGFβ) superfamily, stimulate FSH synthesis; yet, their relative roles and mechanisms of action *in vivo* are unknown. Here, using conditional gene-targeting, we show that the canonical mediator of TGFβ superfamily signaling, SMAD4, is absolutely required for normal FSH synthesis in both male and female mice. Moreover, when the *Smad4* gene is ablated in combination with its DNA binding co-factor *Foxl2* in gonadotrope cells, mice make essentially no FSH and females are sterile. Indeed, the phenotype of these animals is remarkably similar to that of *Fshb* knockout mice. Not only do these results establish SMAD4 and FOXL2 as essential master regulators of *Fshb* transcription *in vivo*, they also suggest that activins may play more important roles in FSH synthesis than GnRH.

Introduction

Approximately 10% of couples are infertile. Though both male and female factors contribute to the problem, the underlying causes are frequently unknown, stemming from our incomplete understanding of the physiological processes controlling reproduction. Gonadal (testicular and ovarian) function is regulated by hormonal signals from the brain and pituitary gland. Impairments in the synthesis, secretion, or action of these hormones can cause hypogonadotropic hypogonadism (HH), which usually manifests as delayed or absent puberty (7, 8, 14, 16, 17, 845-848). Though there are many causes of HH, impaired gonadal function ultimately results from insufficient stimulation by the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Circulating LH and FSH levels are also perturbed in other forms of infertility, including polycystic ovarian syndrome and premature ovarian failure (20, 849).

LH and FSH are dimeric glycoproteins produced by gonadotrope cells of the anterior pituitary. They share a common α subunit (now called chorionic gonadotropin alpha or CGA) noncovalently linked to hormone-specific β subunits (LHβ and FSHβ). Both the hypothalamic decapeptide GnRH (7, 8, 62, 441) and intrapituitary activins (203) have been implicated as the primary stimulators of FSHβ synthesis.

Activins are members of the TGFβ superfamily and were discovered (and named) for their abilities to stimulate FSH secretion (203, 290, 580). Analyses in model cell lines have uncovered candidate mechanisms of activin action *in vivo* (203, 850). In brief, activins bind to receptor serine/threonine kinases on the plasma membrane of gonadotropes, leading to phosphorylation of the intracellular signaling proteins SMAD2 and SMAD3. Phosphorylated SMADs 2 and 3 dissociate from the receptors and associate with the obligate co-factor, SMAD4 (625). SMAD complexes then accumulate in the nucleus and activate transcription of the FSHβ subunit gene (*Fshb*), the rate-limiting step in FSH synthesis (203, 674, 679). *In vitro* data indicate that SMADs partner with the cell-restricted forkhead transcription factor, FOXL2 (678, 706, 708, 710), and perhaps other molecules (537, 538, 540), to regulate transcription via both conserved and species-specific *cis*-regulatory elements in the proximal *Fshb* promoter.

Consistent with this model, gonadotrope-specific ablation of *Foxl2* causes selective FSH-deficiency and subfertility in mice (716). Conversely, however, mice lacking SMAD2 and the DNA-binding domain of SMAD3 have normal FSH levels and fertility (851). Thus, it is

presently unclear whether activins – or other TGFβ superfamily ligands – signal through SMADs or rather a non-canonical signaling pathway to regulate *Fshb* expression and FSH synthesis *in vivo*. To discriminate between these possibilities, we selectively ablated *Smad4* in gonadotrope cells *in vivo* using conditional gene-targeting in mice. As SMAD4 mediates the actions of all TGFβ superfamily ligands (625), this approach enabled us to assess whether or not SMAD-dependent signaling is required for FSH synthesis in the intact murine pituitary gland. Furthermore, we ablated *Smad4* and *Foxl2* in combination to determine whether the two proteins function cooperatively and/or independently to regulate FSH *in vivo*.

Materials and Methods

Mice

The *Smad4*^{fl}, *Foxl2*^{fl}, *Gnrhr*$^{IRES\text{-}Cre(GRIC)}$, and *ROSA26*^{eYFP} alleles, as well as corresponding genotyping protocols, were described previously (715, 820, 828, 852). To generate mice with the experimental genotypes, the *Gnrhr*^{GRIC} allele was always introduced from the females, due to germline Cre activity in males (157). To obtain S4cKO (*Smad4*$^{fl/fl}$;*Gnrhr*$^{GRIC/+}$) mice, *Smad4*$^{fl/fl}$ males were crossed with *Smad4*$^{fl/+}$;*Gnrhr*$^{GRIC/+}$females. *Smad4*$^{fl/fl}$ littermates were used as controls. To obtain S4F2cKO mice (*Smad4*$^{fl/fl}$;*Foxl2*$^{fl/fl}$;*Gnrhr*$^{GRIC/+}$), *Smad4*$^{fl/fl}$;*Foxl2*$^{fl/fl}$ males were crossed with *Smad4*$^{fl/+}$;*Foxl2*$^{fl/+}$;*Gnrhr*$^{GRIC/+}$ females. *Smad4*$^{fl/fl}$;*Foxl2*$^{fl/fl}$ littermates were used as controls. To obtain S4cKO-YFP mice (*Smad4*$^{fl/fl}$;*ROSA26*$^{eYFP/+}$;*Gnrhr*$^{GRIC/+}$), *Smad4*$^{fl/fl}$; *ROSA26*$^{eYFP/eYFP}$ males were crossed with *Smad4*$^{fl/+}$;*Gnrhr*$^{GRIC/+}$females. To obtain GRIC-YFP mice (*Gnrhr*$^{GRIC/+}$;*ROSA26*$^{eYFP/+}$), *ROSA26*$^{eYFP/eYFP}$ males were crossed with *Gnrhr*$^{GRIC/GRIC}$ females. All animal experiments were performed in accordance with institutional and federal guidelines and approved by the McGill University Institutional Animal Care and Use Committee.

Fluorescence-activated cell sorting (FACS)

Dissociated pituitary cell suspensions were prepared from adult (> 8-week-old) S4cKO-YFP and GRIC-YFP mice as previously described (514, 716). Sorting was performed on a FACSAria cell sorter at the flow cytomery core facility of the McGill University Life Sciences Complex. RNA was obtained from the YFP+ and YFP- cell populations using the Total RNA Mini Kit (Geneaid) following the manufacturer's instructions.

Reproductive organ analysis, testicular/ovarian histology, and sperm and follicle counting

Reproductive organs were collected from adult mice at the indicated ages and weighed on a precision balance. Formalin-fixed ovaries were paraffin-embedded and cut in 5 μm sections. Every 5th section was H&E stained and analyzed by transmitted light microscopy. This allowed tracking of corpora lutea and antral follicles across several sections. Follicle staging and counting was performed following published guidelines (853, 854). The number of pre-antral follicles in each section was estimated by counting the number of oocytes. For testicular histology, testes

were fixed in Bouin's with gentle motion overnight. The testes were then washed with 95% and 70% ethanol and paraffin-embedded. Seven μm transverse sections were obtained in the middle of the tissue, H&E-stained, and examined by light microscopy. For sperm counting, cauda epididymides were dissected and immediately frozen on dry ice. Homogenization-resistant sperm count was performed as described (716).

Fertility assessment

To assess fertility, 10-week-old male or female experimental and control mice were paired with C57BL/6J mice (Charles River) of the opposite sex. Starting from 20 days after pairing, the cages were inspected daily for the presence of newborn mice. As soon as a litter was present, pups (living or dead) were carefully counted and put back into the cage. Pups were separated from the mother at postnatal day 15 to avoid interfering with the following pregnancy. The mating trial was performed for 6 months in the case of S4cKO and control animals, and 4 months in the case of S4F2cKO and control females. S4F2cKO females, who never delivered a litter, were carefully inspected at several time points for signs of pregnancy, which were never observed.

Puberty and estrous cyclicity assessment

Starting from the day of weaning (postnatal day 21), females were inspected daily for vaginal opening following published guidelines (829). At 7 weeks of age, estrous cyclicity was assessed daily in the morning (~10 a.m.). A cotton swab wet with sterile saline was introduced approx. 5 mm into the vagina, and collected cells were smeared on a glass slide. The smears were stained with 0.1% methyl blue, and examined by light microscopy. Staging was assessed according to published guidelines (829). One cycle was defined as the sequential appearance of all estrous cycle stages, regardless of the number of days spent in each stage. For estrus morning experiments, cyclicity was first assessed for at least two complete cycles to facilitate stage assignment. Thereafter, blood and pituitaries were collected at 7 a.m. the day following a clear proestrous smear.

Superovulation

Juvenile (postnatal day 23-25) females were injected intraperitoneally (IP) with 5 IU pregnant mare serum gonadotropin (PMSG; Sigma) at 11 a.m. on day 1. On day 3, mice were injected IP with 5 IU of human chorionic gonadotropin (hCG; Sigma) at 7 a.m. Fourteen hours later, mice were killed, and cumulus-oocyte complexes (COCs) retrieved by puncturing the oviduct under a dissecting microscope. The COCs were transferred to phosphate-buffered saline (PBS). Oocytes were dissociated from COCs by a 10 min treatment with 2 mg/mL hyaluronidase in PBS at 37°C and counted under a light microscope.

Hormone analyses

Blood was collected by cardiac puncture and left to clot at room temperature for 20 min. Serum was obtained following centrifugation at 3000 x g and stored at -20°C until analysis. All hormone assays were performed at the Ligand Assay and Analysis Core of the Center for Research in Reproduction at the University of Virginia (Charlottesville, Virginia). LH and FSH were measured by multiplex ELISA. Testosterone was measured by radioimmunoassay.

Quantitative PCR

Pituitaries were frozen on dry ice immediately upon dissection and stored at -80°C. Single pituitaries were homogenized in 500 µL TriZol reagent (Invitrogen, Carlsbad, CA, USA) and RNA isolated following the manufacturer's protocol. One and a half µg of RNA were reverse-transcribed using MMLV reverse transcriptase (Promega, Madison, WI, USA) as described previously (674) in a final volume of 40 µL. For qPCR analysis, 1 µL of cDNA was used in triplicate reactions and assayed on a Corbett Rotorgene 6000 instrument using Platinum qPCR Supermix-UDG (Invitrogen, Carlsbad, CA, USA). Gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (830) relative to the expression of the housekeeping gene *Rpl19*, using the primers described previously (514, 716, 851)

Primary pituitary culture

Primary pituitary cultures were prepared as previously described by our lab (651, 716). Cells were seeded at a density of 400,000 cells/well in 48-well plates. For "*in vivo* recombination" experiments, cells from S4cKO or control littermates were cultured in 10%

serum-containing media for 24 h and then in 2% serum-containing media supplemented with 1 nM activin A, where indicated, for an additional 24 h. For "*ex vivo* recombination" experiments, pituitary cells from *Smad4*$^{fl/fl}$ or *Smad4*$^{fl/fl}$;*Foxl2*$^{fl/fl}$ mice were cultured for 24 h after plating and then infected with adenoviruses expressing GFP (Ad-GFP) or Cre-IRES-GFP (Ad-Cre) [Baylor College of Medicine Vector Development Laboratory (Houston, Texas)] at a multiplicity of infection (MOI) of 60 in 10% serum-containing media for 24 h. Cells were further cultured for 24 h in 2% serum-containing media in the presence or absence of 1 nM activin A. Cells were harvested with 0.25% Trypsin and RNA was extracted using the Total RNA Mini Kit (Geneaid) following the manufacturer's instructions.

Immunofluorescence

Deeply anesthetized adult mice were perfused transcardially with PBS, followed by 4% paraformaldehyde (PFA) buffered in PBS. The pituitaries were post-fixed in 4% PFA overnight at 4°C, transferred to PBS, and embedded in paraffin. Five μm pituitary sections were progressively re-hydrated using a graded series of ethanol solutions and subjected to antigen retrieval in boiling 10 mM sodium citrate (pH 6.0) for 35 minutes. Sections were blocked in 5% BSA-containing PBS supplemented with 1% Tween-20 (PBST) for 1 h at room temperature and incubated with primary antibodies overnight at 4°C in 5% BSA-PBST. After a series of washes in PBST, fluorophore-conjugated secondary antibodies were added in 5% BSA-PBST at a concentration of 1:500 and incubated at room temperature for 1 h. For the Cre antibody, sections were incubated with 1:150 biotinylated anti-rabbit IgG, washed in PBST, and further incubated in fluorophore-conjugated streptavidin at a 1:400 dilution. After another series of PBST washes, sections were mounted with DAPI-containing Prolong Gold reagent (Invitrogen) and imaged by epifluorescence on a Zeiss Axioplan 2 microscope. Primary antibodies used were: anti-FSHβ (NIDDK AFP7798-1289, 1:500, raised in rabbit), anti-LHβ (Santa Cruz sc-7824, 1:500, raised in goat) and anti-Cre (a kind gift from Dr. Jacques Drouin - Novagen 69050, 1:500, raised in rabbit). Secondary antibodies used were: Alexa fluor 594-conjugated anti-rabbit (Invitrogen, A21-207, raised in donkey), Alexa fluor 488-conjugated anti-goat (Invitrogen, A-11055, raised in donkey), Alexa Fluor 594-conjugated anti-goat (Invitrogen, A-11058, raised in donkey), biotinylated anti-rabbit (Vector, BA-1100, raised in horse), and Alexa fluor 488-conjugated Streptavidin (Invitrogen, S-11223).

Statistical analyses

Reproductive organ weights, sperm counts, ovarian follicle counts, ovulated oocyte counts, onset of puberty, estrous cyclicity, pituitary gene expression, litter size, and serum hormones were analyzed using unpaired *t*-tests. In the case of pituitary *Fshb* transcripts and serum FSH levels in S4F2cKO females, Mann-Whitney U tests were used due to high variability in the control group. Primary culture experiments were analyzed using two-way ANOVA, followed by Tukey post-hoc tests. Data were log-transformed when variances were unequal between groups. Statistical analyses were performed using Systat 10.2 or GraphPad Prism 5. *P*-values < 0.05 were considered statistically significant.

Results

Generation of gonadotrope-specific Smad4 knockout mice

To generate mice lacking *Smad4* specifically in gonadotropes (hereafter "S4cKO"), we crossed mice carrying conditional alleles of *Smad4* (*Smad4*$^{fl/fl}$) (852) with GnRHR-IRES-Cre (GRIC) mice, which express Cre recombinase in gonadotropes (820). To quantitatively assess the extent of *Smad4* deletion in the gonadotropes of S4cKO animals, we crossed in the Cre-dependent *ROSA26*^{eYFP} reporter allele (828) on the S4cKO background, generating *Smad4*$^{fl/fl}$*;Gnrhr*$^{GRIC/+}$*;ROSA26*$^{eYFP/+}$ mice (S4cKO-YFP), thereby enabling purification of gonadotropes by FACS (514, 716, 851). Similar to what we reported in other models using the GRIC allele, there was a robust (~90%) loss of *Smad4* mRNA in S4cKO-YFP gonadotropes (green bars in Fig. 3.1A) compared with gonadotropes from control mice (*Smad4*$^{+/+}$*;Gnrhr*$^{GRIC/+}$*;ROSA26*$^{eYFP/+}$; white bars).

Conditional Smad4 knockout mice are hypogonadal and subfertile

S4cKO mice developed normally and appeared healthy. However, examination of the reproductive organs in adult (10-week-old) animals revealed hypogonadism. Male S4cKO mice had markedly decreased testicular weights (~40% less than controls), which was accompanied by a 50% reduction in epididymal sperm counts (Fig. 3.1B-D). Histology revealed grossly normal testicular morphology, though the seminiferous tubules were generally smaller, and many had narrow lumina (supplementary material Fig. S3.1A). In contrast, seminal vesicle weights and circulating testosterone levels did not differ between S4cKO and control mice (Fig. 3.1E and supplementary material Fig. S3.1B). S4cKO males also exhibited normal fertility (supplementary material Fig. S3.1C).

In contrast, S4cKO females were subfertile (Fig. 3.2A), producing significantly fewer pups compared with control mice over the course of a 6 month breeding trial (supplementary material Fig. S3.2A). This was due to a smaller number of pups produced per litter (Fig. 3.2A); litter frequency was comparable between genotypes (supplementary material Fig. S3.2B). Comprehensive assessment of reproductive maturation in S4cKO females revealed normal puberty onset and robust estrous cyclicity (supplementary material Fig. S3.2C-E), indicating that the subfertility was unlikely caused by abnormal activation of the reproductive axis.

To identify the basis of the subfertility in S4cKO females, we first examined their reproductive organs. Adult S4cKO females had smaller ovaries, but normal uterine weights on diestrus compared to controls (Fig. 3.2B-C and supplementary material Fig. S3.2F). Histological analysis indicated that, while follicles at all stages of development were present, there was a significant decrease in the number of corpora lutea in the ovaries of S4cKO animals (Fig. 3.2Di). To gain further insight into this phenotype, we counted follicles at different stages of development in S4cKO and control ovaries. We noted a progressive decline in the number of follicles beyond the pre-antral stage in S4cKO mice, whereas there were no measurable differences in follicle numbers between genotypes at earlier stages (Fig. 3.2Dii-iv). To rule out intrinsic ovarian defects in S4cKO mice, juvenile females (post-natal days 23-25) were stimulated with PMSG to induce follicle growth and, 48 h later, with hCG to trigger ovulation. S4cKO and control females ovulated a comparable number of oocytes, demonstrating normal ovarian responsiveness to exogenous gonadotropins (Fig. 3.2E).

Conditional Smad4 knockout mice are FSH deficient

Next, we asked whether gonadotropin synthesis was impaired in S4cKO mice. Indeed, there was a profound (~90%) reduction in circulating FSH levels in S4cKO males compared with controls, along with a 50% reduction in LH (Fig. 3.3Ai, iii). We also observed FSH-deficiency in diestrous females, though the decrease was more variable and of a lower magnitude (~50%) than in males; LH was unaffected (supplementary material Fig. S3.3A). We therefore examined serum gonadotropins on the early morning of estrus, at the time of the activin-dependent ("secondary") FSH surge (249). Mean serum FSH levels were reduced ~4-fold in S4cKO mice and robust secondary surges were absent (Fig. 3.3Aii). LH levels did not differ between genotypes (Fig. 3.3Aiv). Next, we examined whether the reduction in circulating gonadotropins was due to impaired gonadotropin subunit expression in gonadotropes. First, we analyzed pituitary sections doubly stained with antibodies directed against the LHβ and FSHβ subunits. There was a notable decrease in the intensity of FSHβ staining in both male and female S4cKO pituitaries compared with control littermates (Fig. 3.3B). LHβ staining appeared normal, suggesting that LH deficiency in S4cKO males does not result from impaired LHβ synthesis (see below) or gonadotrope specification (Fig. 3.3B). Next, we measured gonadotropin subunit mRNA levels by qPCR. *Fshb* expression was significantly lower in S4cKO males and females

(morning of estrus) compared with control littermates (Fig. 3.4A). In contrast, LHβ subunit (*Lhb*) expression was normal in S4cKO males and estrus morning females, but modestly increased in diestrous females (Fig. 3.4A and supplementary material Fig. S3.3B). Expression of *Cga*, which encodes the common gonadotropin α subunit, was unchanged in females but reduced in S4cKO males, perhaps contributing to the lower LH levels in the latter (Fig. 3.4A and supplementary material Fig. S3.3B). We also analyzed the pituitary expression of additional genes encoding important regulators of gonadotrope function. Interestingly, gonadotropin-releasing hormone receptor (*Gnrhr*) transcripts were up-regulated in both male and female S4cKO mice regardless of estrous cycle stage (Fig. 3.4A and supplementary material S3.3B). The activin antagonist follistatin (*Fst*) was modestly down-regulated, but only in estrous morning S4cKO females (Fig. 3.4A and supplementary material S3.3B). Expression of the canonical activin type I and type II receptors (*Acvr1b*, *Acvr2a*) or the SMAD co-factor *Foxl2* did not differ between genotypes (Fig. 3.4A and supplementary material Fig. S3.3B). Collectively, these results strongly suggest that FSH-deficiency in S4cKO mice derives primarily from impaired *Fshb* mRNA expression.

Activin regulation of Fshb expression is impaired in Smad4 knockout pituitaries

The marked reduction of circulating FSH and pituitary *Fshb* mRNA levels in S4cKO females on estrous morning suggested that gonadotropes lacking SMAD4 may be impaired in their ability to up-regulate *Fshb* expression in response to activins. To directly address this possibility, we measured activin-stimulated *Fshb* expression in primary pituitary cultures from control and S4cKO mice. Basal *Fshb* mRNA levels were dramatically depleted (> 98%) in cultures from either male or female S4cKO mice (Fig. 3.4B). Basal *Fshb* expression in such cultures is heavily dependent on autocrine/paracrine activin signaling (602, 651). Note that the more profound reduction in *Fshb* transcripts observed in S4cKO pituitary cultures compared with S4cKO pituitaries *in vivo* (Fig. 3.4A) may reflect the absence of GnRH (or additional endocrine signaling) signaling in the former. Interestingly, exogenous activin A was able to stimulate *Fshb* expression in both control and S4cKO cells, but the absolute magnitude of *Fshb* induction was much lower in the latter (Fig. 3.4B). Therefore, the *Fshb* gene in S4cKO gonadotropes retains some ability to respond to activins, but this is insufficient to produce wild-type levels of *Fshb* mRNA.

Conditional Smad4/Foxl2 knockout females are hypogonadal and sterile

The reproductive phenotypes of S4cKO mice are remarkably similar, though not identical, to those of gonadotrope-specific *Foxl2* knockout animals (F2cKO) (716). In both models, females are FSH-deficient and subfertile. This contrasts with the phenotype of female *Fshb* knockout mice, which completely lack the dimeric hormone and are sterile (220). In both the S4cKO and F2cKO models, incomplete recombination of the floxed alleles could explain their residual FSH production. That said, both models show >90% reductions in the targeted mRNAs. It therefore seems likely that another mechanism accounts for the ability of these mice to produce sufficient FSH to stimulate modest follicle growth. We turned our attention back to the current model of activin-regulated *Fshb* expression (844), where SMAD4 and FOXL2 form part of the same transcriptional complex binding to a composite SMAD/forkhead *cis*-element at -115/-107 of the murine *Fshb* promoter (678). In addition, SMAD4 binds an 8-bp SMAD binding element at -266/-259 independently of FOXL2 (676), whereas FOXL2 binds independently of SMAD4 at a non-canonical forkhead binding element at -350 (710). Therefore, residual *Fshb* production in the individual *Smad4* and *Foxl2* knockout models might reflect the fact that the two proteins have both interdependent and independent functions.

To test this hypothesis, we generated mice lacking both *Smad4* and *Foxl2* selectively in gonadotropes (*Smad4*$^{fl/fl}$*;Foxl2*$^{fl/fl}$*;Gnrhr*$^{GRIC/+}$; hereafter S4F2cKO). S4F2cKO females exhibited pale and barely patent vaginas and smears were usually devoid of cells. Therefore, we could not reliably assess the onset of puberty or estrous cyclicity in these animals. In a four month breeding trial, S4F2cKO females did not produce any pups and none showed evidence of pregnancy. In contrast, control (*Smad4*$^{fl/fl}$*;Foxl2*$^{fl/fl}$) females showed normal fertility (Fig. 3.5A). Consistent with their sterility, S4F2cKO females had dramatically reduced ovarian weights and hypoplastic (thread-like) uteri (Figs. 3.5B-D). Ovarian histology revealed the complete absence of corpora lutea and pre-ovulatory follicles, and the presence of only a few early antral follicles in young adult S4F2cKO females (10-week-old) (Fig. 3.5E). In 6-month-old females, which we analyzed after the conclusion of the breeding trial, ovarian tissue was abnormal in S4F2cKO females, with few immature follicles and evidence of tubulostromal hyperplasia (supplementary material Figs. S3.4A-B).

Male S4F2cKO mice were similarly hypogonadal, with a 50% reduction in testicular mass relative to controls (Figs. 3.5F-G). This was a greater decrease than observed in S4cKO

(Figs. 3.1B-C) or F2cKO mice (716). Testes of S4F2cKO mice showed normal histology, though their seminiferous tubules were smaller in diameter compared with controls (supplementary material Fig. S3.4C). In contrast, their seminal vesicle weights were normal (Figs. 3.5F and H).

Conditional Smad4/Foxl2 knockout mice are FSH deficient

Consistent with their reproductive phenotypes, S4F2cKO mice had dramatically impaired serum FSH levels, even more pronounced than observed in S4cKO or F2cKO mice. Values in males and in most females were near the detection limit of the assay (Fig. 3.6Ai/ii). Pituitary FSHβ immunoreactivity and *Fshb* mRNA were barely detectable in S4F2cKO mice (Figs. 3.6B and 3.7A). In control males and females, LHβ+ cells were all co-labeled with FSHβ (FSHβ+), whereas some cells were uniquely FSHβ+ (Figs. 3.6B). Strikingly, in both male and female S4F2cKO mice, LHβ+ cells were devoid of FSHβ staining (Figs. 3.6B). Therefore, gonadotropes lacking *Smad4* and *Foxl2* are specified normally, but synthesize very low, if any, FSHβ. That said, some FSHβ-only cells remained scattered throughout S4F2cKO pituitaries, and presumably account for the residual *Fshb* mRNA and serum FSH in these animals (Figs. 3.6Ai/ii and B). We explored the possibility that these cells continue to synthesize FSHβ because they do not express the Cre recombinase enzyme. However, the FSHβ + cells were also Cre+ (supplementary material Fig. S3.5). Moreover, some Cre+ cells were LHβ-, indicating that there is a subpopulation of FSHβ+/LHβ- gonadotropes that expressed *Gnrhr* (and hence Cre) at some point during their lifetime (supplementary material Fig. S3.5). Therefore, *Fshb* expression in these cells appears to be SMAD4/FOXL2-independent, but insufficient to maintain reproductive axis activity.

Conditional Smad4/Foxl2 knockout mice show gender-specific alterations in LH secretion

In contrast to FSH, serum LH levels were increased more than 5-fold in female S4F2cKO mice compared to controls (Fig. 3.6Aiv). However, LH was decreased by about 50% in S4F2cKO males (Fig. 3.6Aiii). In both sexes, pituitary *Lhb* mRNA levels were increased by 3-5 fold (Fig. 3.7A). LHβ immunoreactivity appeared normal, if not elevated, in knockout pituitaries (Figs. 3.6B). Pituitary *Cga* mRNA levels were significantly depleted in S4F2cKO males, but not females, perhaps contributing to the sex difference in serum LH levels (Fig. 3.7A). Interestingly, *Gnrhr* expression, which was elevated in S4cKO mice, was normal in S4F2cKO animals (both

genders). *Fst*, *Acvr1b*, and *Acvr2a* mRNA levels did not differ between genotypes (Fig. 3.7A). *Foxl2*, as expected, was significantly reduced as in (716).

Basal and activin regulated Fshb expression are abolished in Smad4/Foxl2-depleted pituitaries

Above, we showed that *Smad4* deletion impaired basal and activin-stimulated *Fshb* expression in primary pituitary cultures (Fig. 3.4B). We also observed a similar, albeit milder, effect upon acute deletion of *Smad4* (supplementary material Fig. S3.6A-B) or *Foxl2* (716) in primary pituitary cultures from *Smad4*$^{fl/f}$ or *Foxl2*$^{fl/fl}$ mice infected with a Cre-expressing adenovirus (Ad-Cre). Therefore, we tested the hypothesis that the residual activin response was FOXL2- or SMAD4-dependent, respectively, by examining the effects of acute recombination of both *Smad4* and *Foxl2* conditional alleles in pituitary cultures from *Smad4*$^{fl/fl}$*;Foxl2*$^{fl/fl}$ mice. In Ad-Cre infected cultures, we observed a dramatic decrease in basal *Fshb* transcription and the complete loss of activin responsiveness (Fig. 3.7B and supplementary material Fig. S3.7A-B). These results indicate that SMAD4 and FOXL2 are required for basal and activin-stimulated *Fshb* expression in adult gonadotropes. In Ad-Cre infected cultures, *Lhb* expression was normal, whereas *Cga* was mildly downregulated in males (supplementary material Fig. S3.7Aiii/iv and S3.7Biii/iv). This suggests that changes in *Cga* but not *Lhb* expression in S4F2cKO pituitaries (Fig. 3.7A) may be partially explained by cell-autonomous mechanisms. The increases in *Lhb* in contrast may represent endocrine (indirect) effects.

Discussion

Loss-of-function mutations in the *FSHB/Fshb* gene cause hypogonadotropic hypogonadism in humans and mice, demonstrating an essential role for FSH in reproductive development and fertility (14, 16, 220). GnRH and activins are regarded as the primary stimulators of FSH synthesis in mammals; yet, their relative roles and mechanisms of action are poorly understood, particularly *in vivo*. Here, we show that the canonical mediator of TGFβ superfamily signaling, SMAD4, is absolutely required for normal FSH synthesis in both male and female mice. Moreover, when the *Smad4* gene is ablated in combination with the gene encoding its most thoroughly characterized DNA binding co-factor in gonadotrope cells, *Foxl2*, mice make essentially no FSH and females are sterile. The phenotype of these animals is remarkably similar to that of *Fshb* knockout mice. Not only do these results establish SMAD4 and FOXL2 as essential regulators of *Fshb* transcription, they suggest that activins (or related TGFβ ligands) may play more important roles in FSH synthesis than GnRH, at least in mice. Indeed, the normal or elevated *Lhb* and *Gnrhr* expression in these animals indicates that GnRH receptor signaling is intact in these animals. At present, there is no evidence that GnRH regulates *Fshb* transcription via SMAD4 or FOXL2 (679, 706). However, if GnRH requires underlying activin signaling to stimulate FSH, then the loss of SMAD4 and FOXL2 could impair both activin (directly) and GnRH (indirectly) action.

Though both SMAD4 and FOXL2 can bind the *Fshb* promoter directly (463, 539, 676, 678, 706, 708), neither is a direct target of activin or other TGFβ receptors. In addition, the two proteins do not directly interact (708, 709). Therefore, another protein (or proteins) must provide the link between receptor activation and SMAD4/FOXL2 at the level of the *Fshb* promoter. Although SMAD3, and to a lesser extent SMAD2, are the obvious candidates, we recently showed that SMAD2 and the DNA-binding activity of SMAD3 are dispensable for FSH synthesis and fertility in mice (851). We postulated that the C-terminal MH2 domain of SMAD3, which is retained in these mice, can provide the molecular link between activin receptor activation and *Fshb* expression in the nucleus (Fig. 8) because it is both phosphorylated by the activin type I receptor and interacts with SMAD4 and FOXL2 (678, 706, 708, 709). Indeed, *in vitro* data show that SMAD4's DNA binding activity is sufficient to confer synergistic activation of *Fshb* transcription by DNA-binding-deficient SMAD3 and FOXL2 (678). Nonetheless, this

and other possibilities, such as non-canonical signaling through SMADs 1, 5, or 8 (690), require investigation using appropriate knockout models.

SMADs require co-factors to bind target genes with high specificity and affinity (625). The first identified SMAD-interacting partner fulfilling this role was the forkhead transcription factor FOXH1 (701, 702). The data presented here demonstrate the functional synergism between SMAD4 and one of its forkhead protein partners *in vivo*. At the same time, because mice lacking both *Smad4* and *Foxl2* exhibit a more robust phenotype than mice lacking either alone [this study and (716)], the data suggest that the two proteins regulate *Fshb* expression both cooperatively and independently. Indeed, based on *in vitro* studies, our current model (Fig. 3.8) predicts that, in the absence of either SMAD4 or FOXL2, some activin signaling to the *Fshb* promoter can be maintained. That is, without SMAD4, activin-regulated SMAD3 can still activate *Fshb* transcription in synergy with FOXL2 through at least two *cis*-elements in the proximal murine *Fshb* promoter (678, 706, 708, 710) (Fig. 3.8ii). In the absence of FOXL2, in contrast, SMAD complexes can still stimulate *Fshb* via an 8-bp SMAD binding element (Fig. 3.8iii) (676). In both scenarios, the residual signaling can maintain FSH levels above a threshold required to drive maturation of some ovarian follicle to the pre-ovulatory stage (Fig. 3.8ii-iii). In the absence of both SMAD4 and FOXL2, however, all activin responsiveness is lost, rendering animals incapable of generating threshold levels of FSH (Fig. 3.8iv). Consistent with this idea, the double knockout mice do not cycle and exhibit an arrest in follicle development at the preantral stage.

Though our results clearly demonstrate necessary roles for SMAD4 and FOXL2 in FSH synthesis in mice, it is presently unclear whether the human *FSHB* gene is similarly regulated. Nonetheless, activins are indirectly implicated in human FSH secretion, as circulating inhibins negatively correlate with FSH levels (600) and a soluble ectodomain of ACVR2A suppresses FSH in women (649). Importantly, FOXL2 is expressed in human gonadotropes (855) and the composite *cis*-element through which SMAD4 and FOXL2 regulate murine *Fshb* is present in the human *FSHB* promoter (706). Therefore, it seems likely that we have identified a fundamental and conserved mechanism underlying FSH synthesis in all mammals.

Aknowledgements

We thank Ken McDonald for assistance with FACS, as well as the Goodman Cancer Research Center Histology Facility and the Center for Bone and Periodontal Research Histology Platform for the preparation of histological sections. We also thank Dr. Alfredo Ribeiro-da-Silva for generously providing access to the Zeiss Axioplan 2 microscope, and Dr. Derek Boerboom for insightful comments on ovarian histology. JF was supported by a Doctoral Research Award from the Canadian Institutes of Health Research (CIHR) and the Samuel Solomon Fellowship in Endocrinology from the Endocrine Division and McGill Faculty of Medicine. This work was funded by CIHR operating grants MOP-89991 and 123447 to DJB. DJB was a Senior Research Scholar of the Fonds de la Recherche en Santé de Québec. UB is funded by DFG BO1743/2. The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core is supported by the National Institutes of Health NICHD (SCCPRR) Grant U54-HD28934.

Figure Legends

Figure 3.1: Hypogonadism male S4cKO mice. **A**) YFP- and YFP+ cells were sorted from *ROSA26*$^{eYFP/+}$*;Gnrhr*$^{GRIC/+}$ (GRIC-YFP; controls) or *Smad4*$^{fl/fl}$*;ROSA26*$^{eYFP/+}$*;Gnrhr*$^{GRIC/+}$ (S4cKO-YFP) male and female mice by fluorescence-activated cell sorting (FACS). *Smad4* expression was assessed by qPCR and measured relative to *Rpl19* expression. N=3 independent sorting experiments. Bars represent means +s.e.m. Bars with different symbols differ significantly. **B**) Representative testes from 10-week-old control and S4cKO mice. **C**) Testicular weights (expressed as % body weight) in 10-week-old control and S4cKO mice. Body weights did not differ. Here and below, individual data points are plotted as circles or squares and means are shown by horizontal lines. *: $p < 0.05$. **D**) Cauda epididymal sperm counts in adult control and S4cKO males (one epididymis analyzed per mouse). Bars represent means +s.e.m. *: $p < 0.05$. **E**) Seminal vesicle weights, expressed as % body weight (which did not differ), in 10-week-old control and S4cKO males. n.s.: non significant.

Figure 3.2: Subfertility and abnormal ovarian follicular maturation in S4cKO female mice. **A**) Average litter size delivered by control and S4cKO females during a 6 month breeding trial. Bars represent means +s.e.m. *: $p < 0.05$. **B**) Representative H&E-stained histological sections from 10-week-old control and S4cKO females ovaries. Scale bars = 0.5 mm. **C**) Diestrous ovarian weights, expressed as % of body weight (which did not differ), in 10-week-old control and S4cKO female mice. Each dot represents an individual animal; horizontal bars show means. *: $p < 0.05$ **D**) Corpora lutea (i), late antral/preovulatory (ii), early antral (iii) and pre-antral (iv) follicle counts in ovaries from 10-week-old control and S4cKO females (one ovary examined per mouse). Bars represent means +s.e.m. *: $p < 0.05$. n.s.: non significant. **E**) Number of oocytes recovered from both oviducts in response to PMSG/hCG stimulation in juvenile (P23-25) control and S4cKO females.

Figure 3.3: Impaired FSH synthesis in S4cKO mice. **A**) Serum FSH (i, ii) and LH (iii, iv) in 10-week-old male (i, iii) and > 10-week-old female (ii, iv; assessed on estrous morning) control and S4cKO mice. *: $p < 0.05$; n.s: non-significant. **B**) Immunofluorescence for LHβ (green) and

FSHβ (red) in the pituitaries of 10-week old-control and S4cKO male (left) and female (right) mice. Scale bars = 25 μm.

Figure 3.4: *Fshb* deficiency and impaired activin-stimulated *Fshb* expression in S4cKO pituitaries. **A**) Expression of selected genes in the pituitaries of 10-week-old male (top) and > 10-week-old estrous morning female (bottom) control and S4cKO mice, assessed relative to the expression of the housekeeping gene *Rpl19*. The animals are the same as in figure **3A**). *Lhb*: luteinizing hormone β subunit. *Cga*: gonadotropin α subunit. *Gnrhr*: gonadotropin-releasing hormone receptor. *Fst*: follistatin. *Acvr1b*: activin receptor type 1B. *Acvr2a*: activin receptor type 2A. *Foxl2*: forkhead box L2. n=14 per group in males; n=9 per group in females. Bars represent means +s.e.m. *: $p < 0.05$; n.s: non-significant. **B**) Primary pituitary cells prepared from control or S4cKO male (left) or female (right) mice and treated for 24 h with 1 nM activin A or left untreated. *Fshb* transcripts were assessed by qPCR, relative to the expression of *Rpl19*. Males: N=4; females: N=3 independent experiments. Bars represent means +s.e.m. Bars with different symbols differ significantly.

Figure 3.5: Profound hypogonadism and female sterility in S4F2cKO mice. **A**) Average litter size delivered by control and S4F2cKO females during a 4 month breeding trial. S4F2cKO females delivered no litters. Bars represent means +s.e.m. **B**) Representative reproductive tracts from 10-week-old control and S4F2cKO mice. **C-D**) Ovarian (**C**) and uterine (**D**) weights, expressed as % body weight (which did not differ), in > 10-week old female control and S4F2cKO mice. *: $p < 0.05$. **E**) Representative H&E-stained histological sections from 10-week-old control and S4F2cKO female ovaries. Scale bars = 0.5 mm. **F**) Representative testes and seminal vesicles from 10-week-old control and S4F2cKO males. **G-H**) testicular (G) and seminal vesicle (H) weights, expressed as % body weight (which did not differ) in 10-week-old male control and S4F2cKO mice. Each dot represents an individual animal; horizontal bars show means. *: $p < 0.05$. n.s.: non significant.

Figure 3.6: Profoundly impaired FSH synthesis in S4F2cKO mice. **A**) Serum FSH (i, ii) and LH (iii, iv) in 10-week-old male (i, iii) and adult > 10-week-old female (ii, iv) control and S4F2cKO

mice. Each dot represents an individual animal; horizontal bars show means. *: $p < 0.05$; n.s: non-significant. **B**) Immunofluorescence for LHβ (green) and FSHβ (red) in pituitaries of 10-week-old control and S4F2cKO male (left) and female (right) mice. Scale bars = 25 μm.

Figure 3.7: *Fshb* deficiency in S4F2cKO pituitaries and loss of activin-stimulated *Fshb* expression upon acute deletion of *Smad4* and *Foxl2*. **A**) Expression of selected genes in the pituitaries of 10-week-old male (top) and > 10-week-old female (bottom) control and S4F2cKO mice assessed relative to *Rpl19*. n=6 per group in males; n=9 and 7 for control and S4F2cKO female mice, respectively. Bars represent means +s.e.m. *: $p < 0.05$; n.s: non-significant. **B**) Primary pituitary cells prepared from $Smad4^{fl/fl};Foxl2^{fl/fl}$ male (left) or female (right) mice, infected with Cre-expressing (Ad-Cre) or control (Ad-GFP) adenoviruses, and treated for 24 h with 1 nM activin A or left untreated. *Fshb* transcripts were assessed by qPCR, relative to the expression of the housekeeping gene *Rpl19*. N=3 independent experiments. Bars represent means +s.e.m. Bars with different symbols differ significantly.

Figure 3.8: Model of hormonal regulation of *Fshb* expression in gonadotropes *in vivo*. Left column: Proposed mechanisms of *Fshb* transcriptional regulation in (i) wild-type mice or mice lacking (ii) *Smad4* (S4cKO), (iii) *Foxl2* (F2cKO), or (iv) both *Smad4* and *Foxl2* (S4F2cKO) in gonadotropes. Middle column: Resulting circulating FSH levels across the estrous cycle (1°: GnRH-induced primary FSH surge; 2° activin-induced secondary FSH surge). Right column: Observed fertility outcomes in females of the indicated knockout strains. **i)** In wild-type mice, activin signaling through SMAD3 (S3) and its obligatory partner SMAD4 (S4) activate *Fshb* transcription in cooperation with FOXL2 (F2) *via* a composite SMAD/FOXL2 proximal binding site, an 8-bp SMAD-responsive element, and a distal FOXL2 binding site. Mechanisms of GnRH signaling to the murine Fshb promoter are poorly described and denoted by "?". **ii)** In S4cKO animals, both FOXL2 binding sites can still confer some activin responsiveness, presumably *via* activin-regulated SMAD3. **iii)** In F2cKO mice, the activity of the 8-bp SMAD binding element should be preserved. In both cases, this results in a decrease in activin responsiveness of the *Fshb* promoter, a shallower secondary FSH surge, and reduced fertility. **iv)** In S4F2cKO mice, activin responsiveness is completely lost. As a result, these mice cannot generate a secondary FSH surge and are sterile. A primary FSH surge should also be absent due

to a block of antral follicle growth, resulting in the absence of an estrogen-stimulated GnRH surge.

Supplementary Figure Legends

Figure S3.1: Reduced testicular size, but normal fertility in S4cKO male mice. **A**) Representative H&E-stained histological sections from 10-week-old control (left) and S4cKO (right) male testes. Scale bar = 0.1 mm. **B**) Serum testosterone levels in 10-week-old S4cKO and control males. Each dot represents and individual animals. Horizontal bars show means. n.s.: non significant. **C**) Average litter size delivered by wild-type females paired with control and S4cKO males during a 6 month breeding trial. Bars represent means +s.e.m. n.s.: non significant.

Figure S3.2: Subfertility, but normal reproductive maturation in S4cKO females. **A**) Average cumulative number of pups delivered by control and S4cKO females over a 6 month breeding trial. Bars represent means +s.e.m. *: $p < 0.05$. **B**) Average (+s.e.m.) frequency of litters (number per month) delivered by control and S4cKO females over a 6 month breeding trial. **C**) Age at vaginal opening ("0" being the day of birth), used as a marker of puberty onset, in control and S4cKO females. N=20 and 25 for control and S4cKO animals, respectively. **D**) Representative estrous cyclicity profiles from two control (left) and two S4cKO (right) females. Individual points represent consecutive days. **E**) Quantification of the proportion of time spent in each estrous cycle stage in control and S4cKO females. Control: n=7. S4cKO: n=10. E: estrus. P: proestrus. M/D: mestestrus/diestrus. Bars represent means +SEM. n.s.: non significant **F**) Uterine weights, expressed as a % body weight (which did not differ), in 10-week-old control and S4cKO female mice. N=11 and 17 for control and S4cKO females, respectively. Each dot represents an individual animal; horizontal bars show means. n.s.: non significant.

Figure S3.3: Reduced serum FSH levels and increased pituitary *Lhb* and *Gnrhr* expression in S4cKO diestrous females. **A**) Serum FSH (i) and LH (ii) in 10-week-old female control and S4cKO mice, assessed in diestrus. N= 13 and 17 for control and S4cKO animals, respectively. Each dot represents an individual animal; horizontal bars show means. *: $p<0.05$. n.s.: non significant. **B**) Expression of the indicated genes in the pituitaries of 10-week-old diestrous

control and S4cKO females, assessed relative to the expression of the housekeeping gene *Rpl19*. The animals are the same as used for hormone analyses in panel **A**. *Lhb*: luteinizing hormone β subunit. *Cga*: gonadotropin α subunit. *Gnrhr*: gonadotropin-releasing hormone receptor. *Fst*: follistatin. *Acvr1b*: activin receptor type 1B. *Acvr2a*: activin receptor type 2A. *Foxl2*: forkhead box L2. Bars represent means +s.e.m. *: $p < 0.05$. n.s.: non significant.

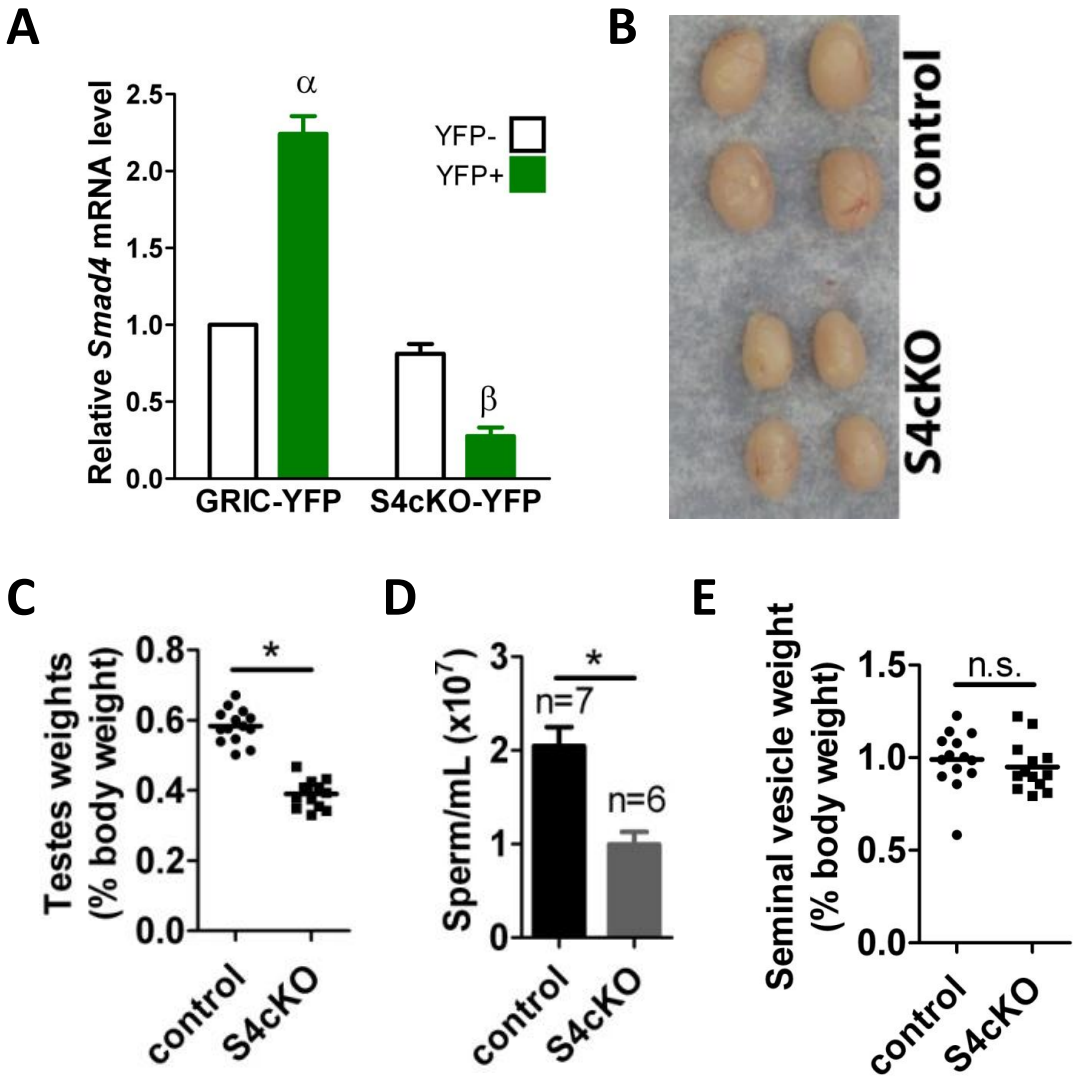
Figure S3.4: Disrupted ovarian architecture and tubulostromal hyperplasia in 6-month-old S4F2cKO females. **A**-**B**) Representative low (2.5X; left panels) and high magnification (20X; right panels) H&E-stained histological sections from two 6-month-old retired S4F2cKO breeder ovaries (note that the animals were in breeding trials, but were sterile). The area shown in higher magnification is delineated by a box. Scale bars: 2.5X = 0.5 mm. 20X = 20 μm. **C**) Representative H&E-stained histological sections from 10-week-old control (left) and S4F2cKO (right) males. Scale bars = 0.1 mm.

Figure S3.5: Cre expression in FSHβ-only gonadotropes in S4F2cKO mice. **A**-**B**) Representative immunofluorescence images of Cre (green; top), FSHβ (**A** - red; middle) or LHβ (**B**- red; middle) and merged Cre/FSHβ (**A**) and Cre/LHβ (**B**) staining (bottom) from 10-week-old S4F2cKO mouse pituitaries. Arrows in **A**) indicate Cre-positive, FSHβ-positive cells. Arrows in **B**) indicate Cre-positive, LHβ-negative cells. Scale bars = 0.1 mm.

Figure S3.6: Lower basal and activin A-induced *Fshb* expression upon acute deletion of *Smad4* in primary pituitary cultures. Primary pituitary cells were prepared from adult $Smad4^{fl/fl}$ male (**A**) or female (**B**) mice, infected with Cre-expressing (Ad-Cre) or control (Ad-GFP) adenoviruses, and treated for 24 h with 1 nM activin A or left untreated. *Fshb* (**Ai, Bii**) and *Smad4* (**Aii, Bii**) transcripts were assessed by qPCR, relative to the expression of *Rpl19*. N=10 (**A**) or 5 (**B**) independent experiments. Bars represent means +s.e.m. Bars with different symbols differ significantly.

Figure S3.7: Effect of acute *Smad4* and *Foxl2* deletion on *Smad4*, *Foxl2*, *Lhb*, and *Cga* expression in primary pituitary cultures. Primary pituitary cultures from male (**A**) and female (**B**) $Smad4^{fl/fl}$;$Foxl2^{fl/fl}$ mice were infected with Cre-expressing (Ad-Cre) or control (Ad-GFP)

adenoviruses, and treated for 24 h with 1 nM activin A or left untreated. *Smad4* (**Ai, Bi**), *Foxl2* (**Aii, Bii**), *Lhb* (**Aiii, Biii**) and *Cga* (**Aiv, Biv**) mRNAs levels measured by qPCR. Data are from the same experiments presented in Fig. **7B**. Bars represent means +s.e.m. Bars with different symbols differ significantly.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S4cKO-YFP
B
control
S4cKO
C
Testes weights (% body weight)
0.8
0.6
0.4
0.2
0.0
*
control
S4cKO
D
Sperm/mL (x10^7)
3
2
1
0
*
n=7
n=6
control
S4cKO
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1.0
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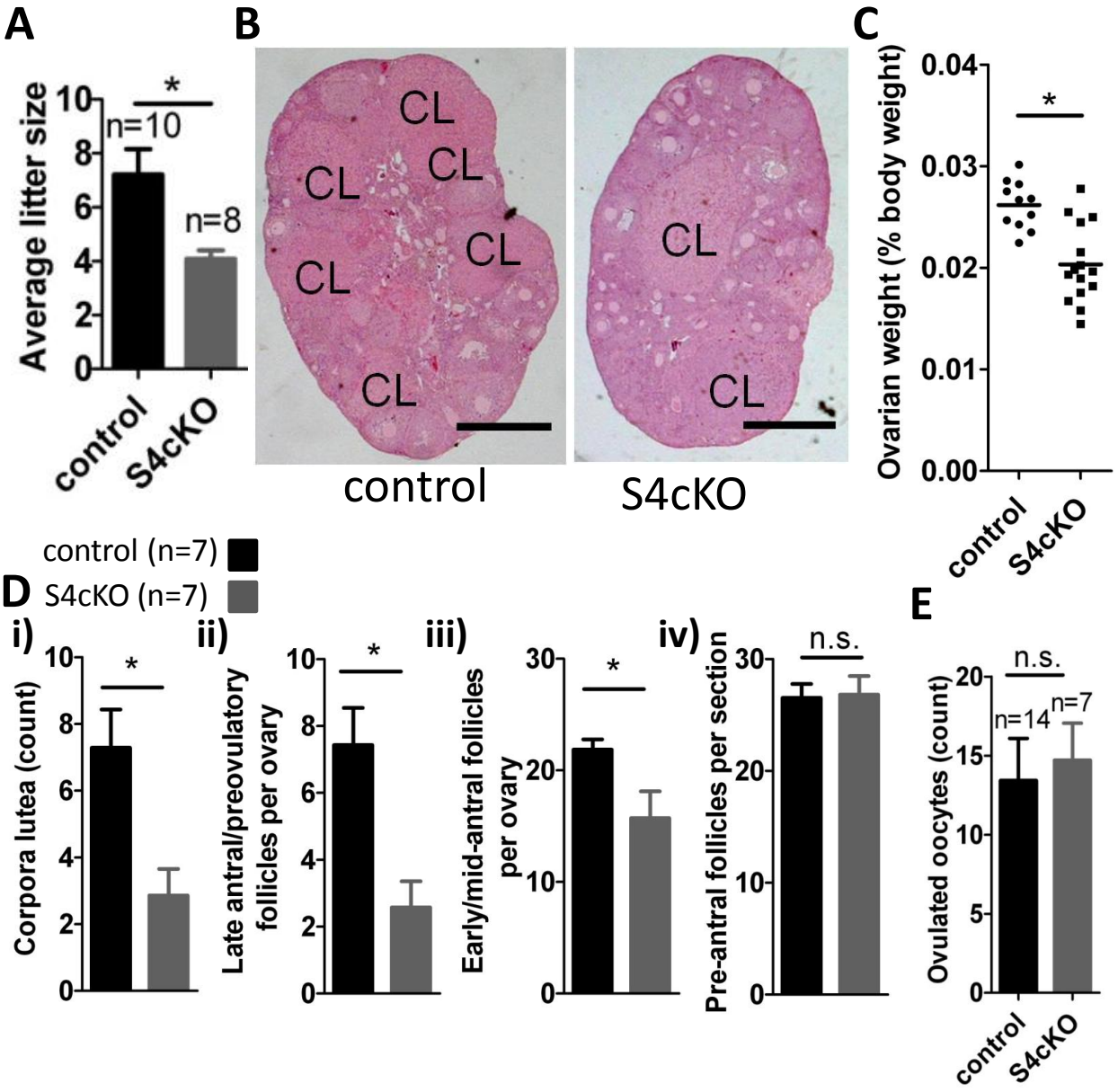
control
S4cKO

Figure 3.1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*
control
S4cKO
B
CL
control
S4cKO
C
Ovarian weight (% body weight)
*
control
S4cKO
D
control (n=7)
S4cKO (n=7)
i)
Corpora lutea (count)
ii)
Late antral/preovulatory follicles per ovary
iii)
Early/mid-antral follicles per ovary
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n.s.

Figure 3.2

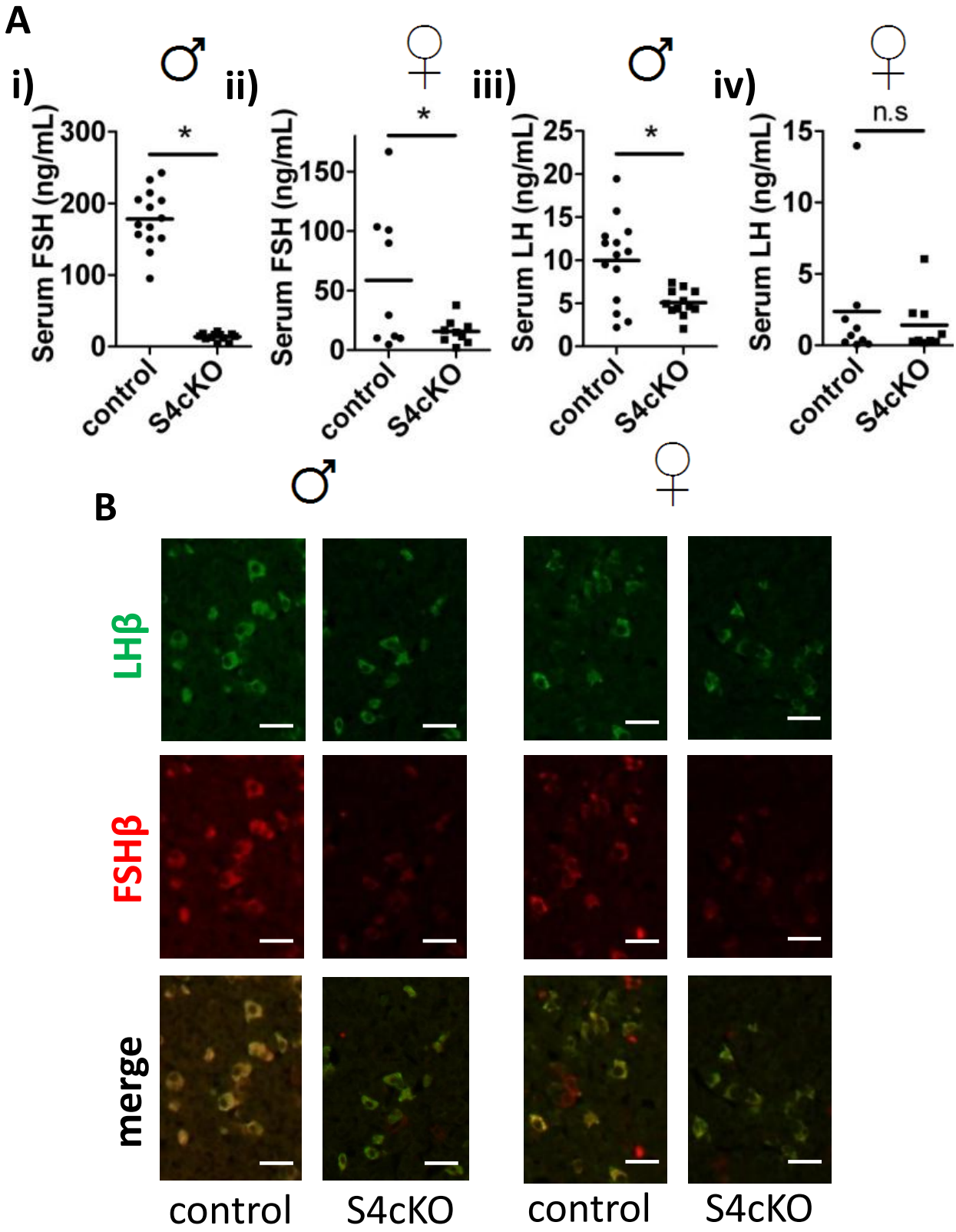
A
i)
ii)
iii)
iv)
Serum FSH (ng/mL)
Serum LH (ng/mL)
control
S4cKO
n.s
B
LHβ
FSHβ
merge
control
S4cKO
control
S4cKO

Figure 3.3

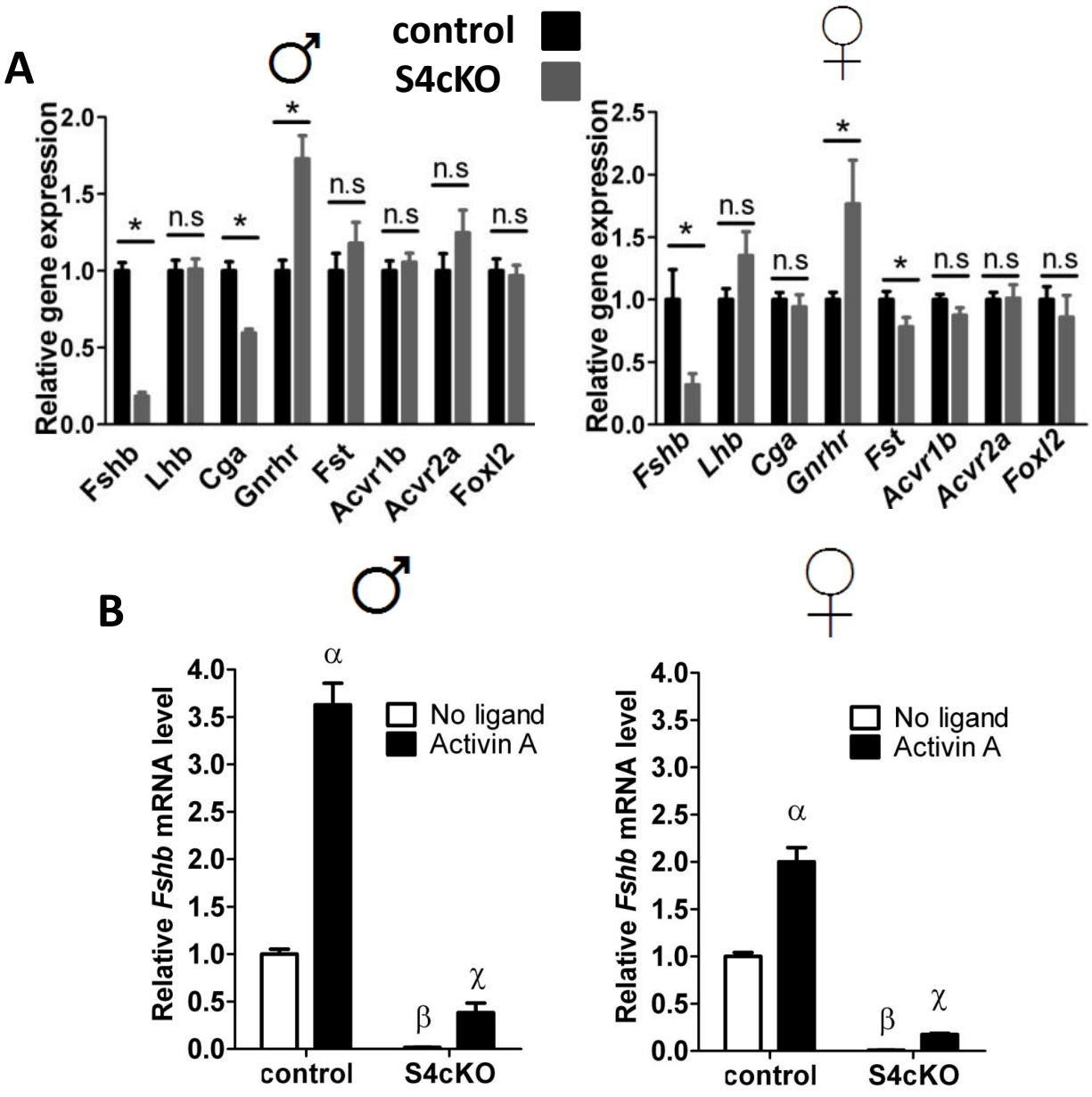
A
control
S4cKO
Relative gene expression
Fshb
Lhb
Cga
Gnrhr
Fst
Acvr1b
Acvr2a
Foxl2
n.s
B
Relative Fshb mRNA level
No ligand
Activin A
control
S4cKO

Figure 3.4

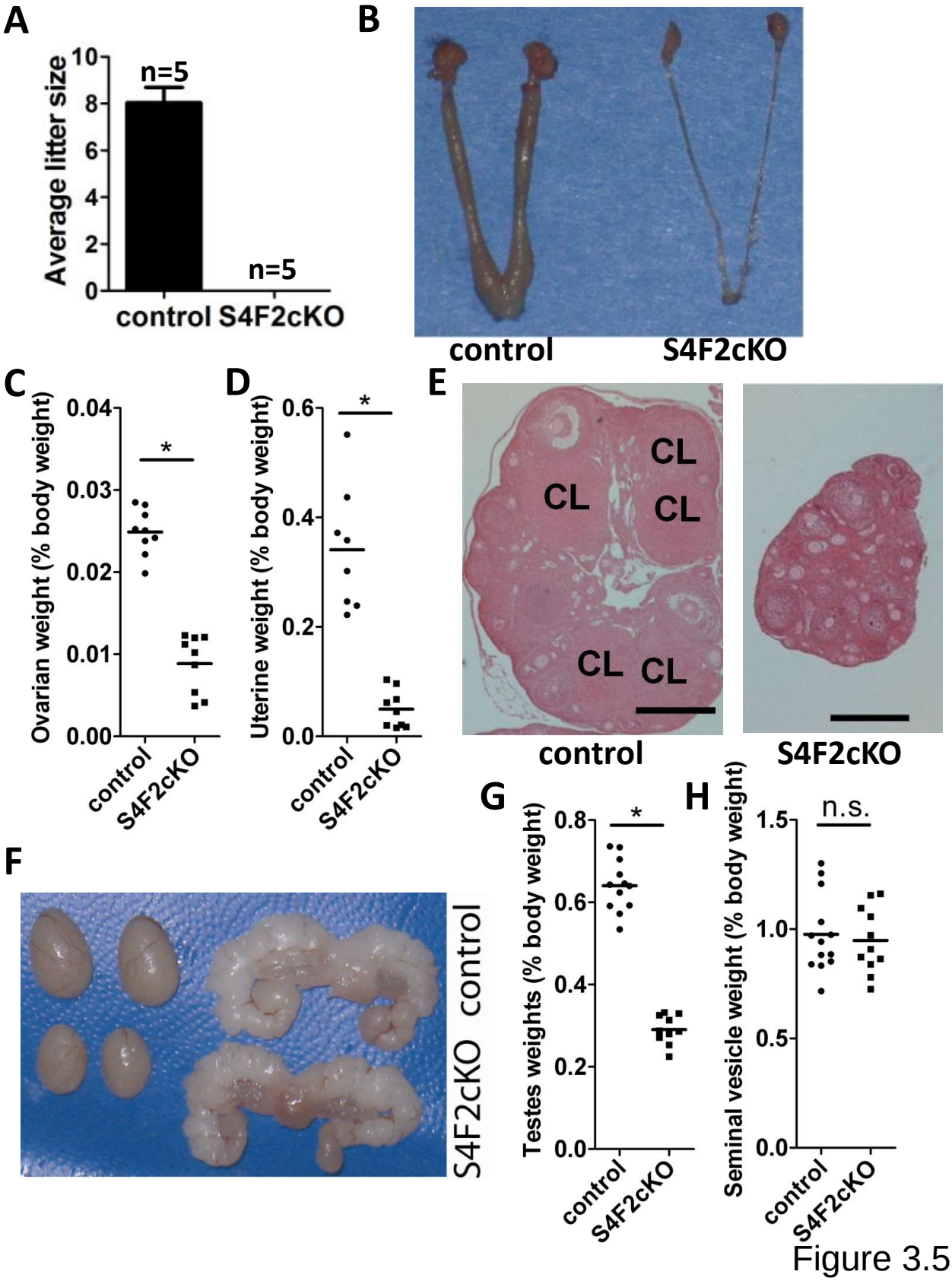
A
Average litter size
10
8
6
4
2
0
n=5
n=5
control
S4F2cKO
B
control
S4F2cKO
C
Ovarian weight (% body weight)
0.04
0.03
0.02
0.01
0.00
*
control
S4F2cKO
D
Uterine weight (% body weight)
0.6
0.4
0.2
0.0
*
control
S4F2cKO
E
CL
CL
CL
CL
CL
control
S4F2cKO
F
S4F2cKO
control
G
Testes weights (% body weight)
0.8
0.6
0.4
0.2
0.0
*
control
S4F2cKO
H
Seminal vesicle weight (% body weight)
1.5
1.0
0.5
0.0
n.s.
control
S4F2cKO

Figure 3.5

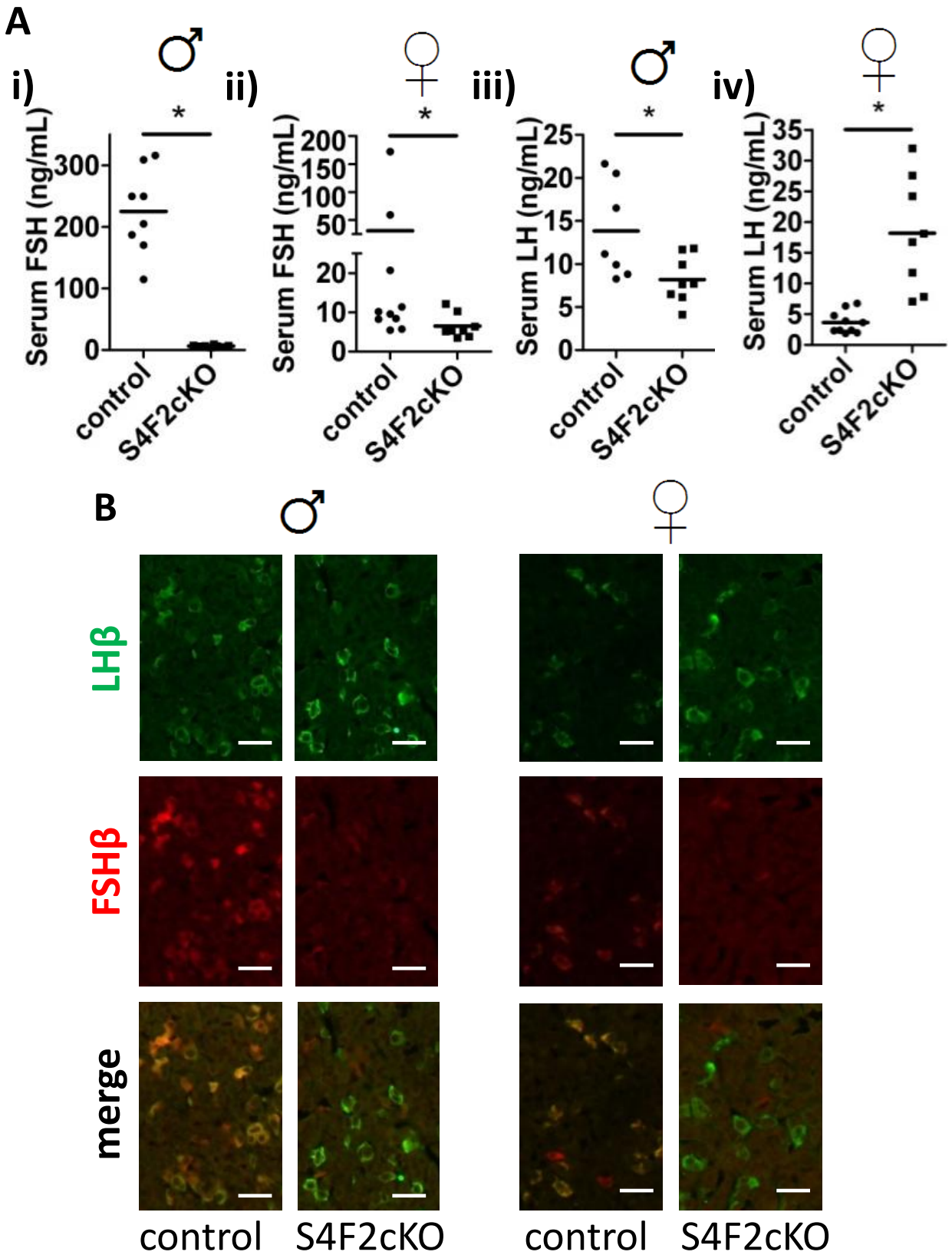
A
i)
ii)
iii)
iv)
♂
♀
♂
♀
Serum FSH (ng/mL)
Serum FSH (ng/mL)
Serum LH (ng/mL)
Serum LH (ng/mL)
300
200
100
0
200
150
100
50
20
10
0
25
20
15
10
5
0
35
30
25
20
15
10
5
0
*
*
*
*
control
S4F2cKO
control
S4F2cKO
control
S4F2cKO
control
S4F2cKO
B
♂
♀
LHβ
FSHβ
merge
control
S4F2cKO
control
S4F2cKO

Figure 3.6

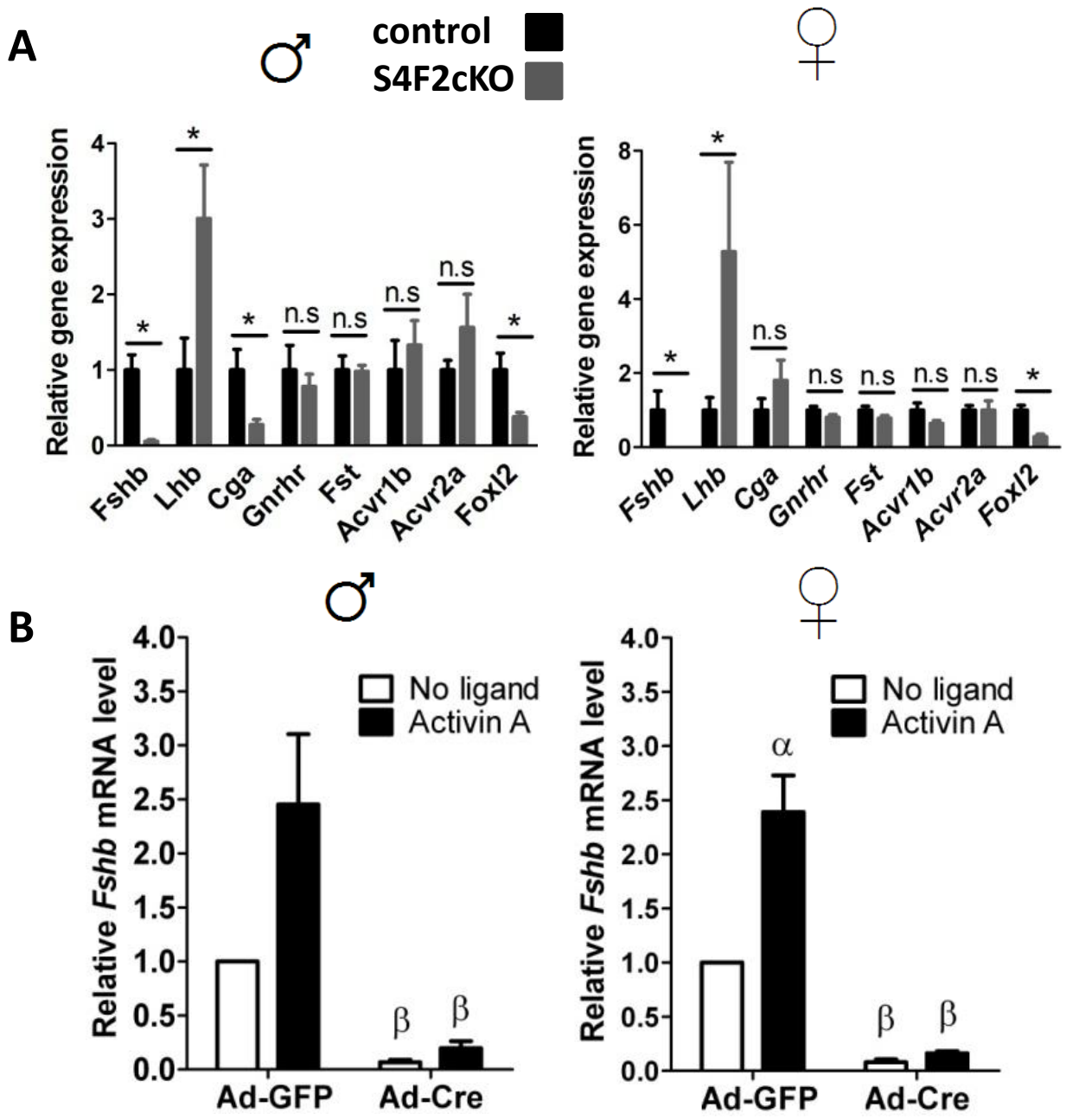
A
♂
control
S4F2cKO
♀
Relative gene expression
Fshb
Lhb
Cga
Gnrhr
Fst
Acvr1b
Acvr2a
Foxl2
*
n.s
B
Relative Fshb mRNA level
No ligand
Activin A
Ad-GFP
Ad-Cre
β
α

Figure 3.7

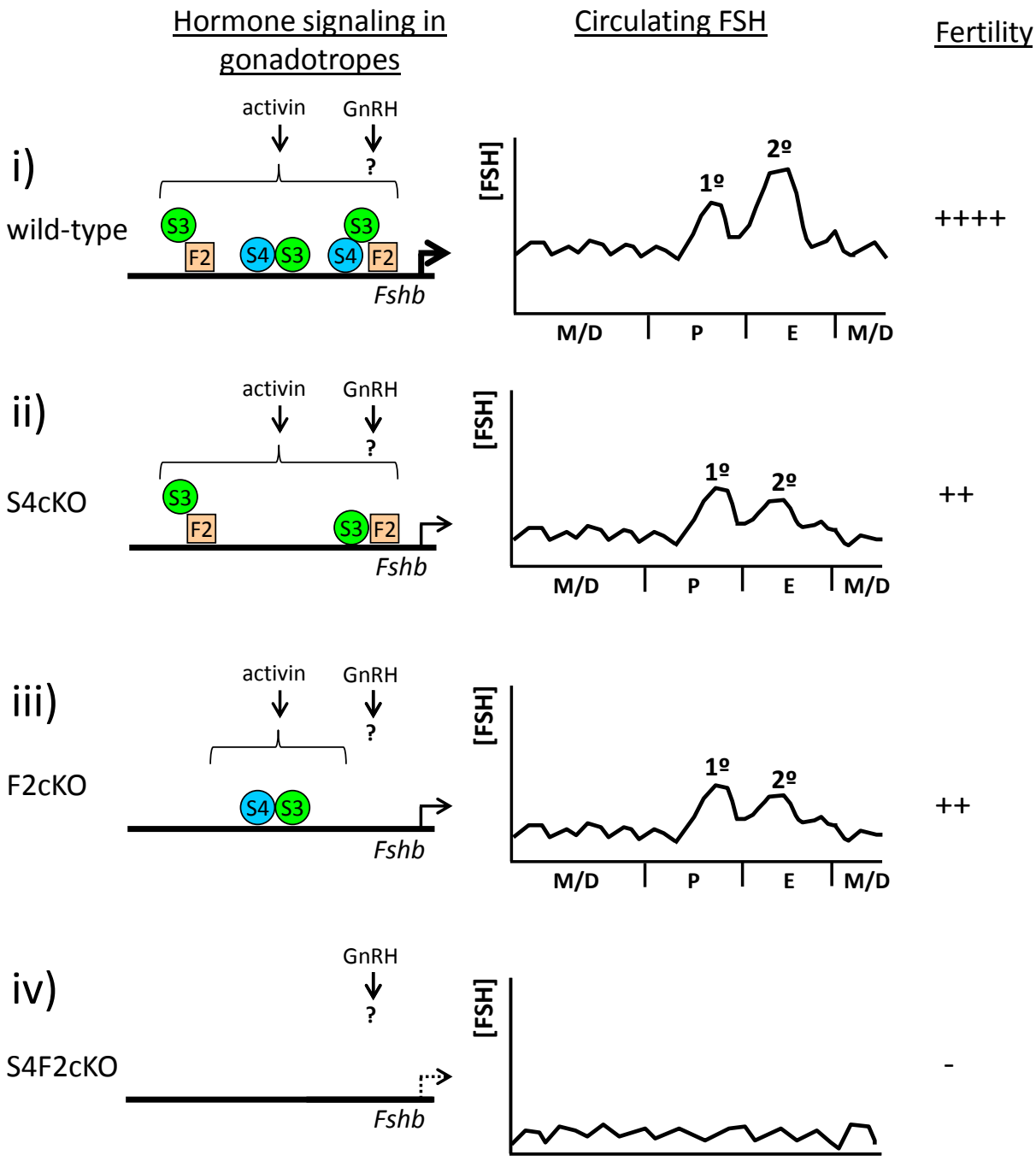
Hormone signaling in gonadotropes
Circulating FSH
Fertility
i)
wild-type
activin
GnRH
?
S3
F2
S4
S3
S3
S4
F2
Fshb
[FSH]
1º
2º
M/D
P
E
M/D
++++
ii)
S4cKO
activin
GnRH
?
S3
F2
S3
F2
Fshb
[FSH]
1º
2º
M/D
P
E
M/D
++
iii)
F2cKO
activin
GnRH
?
S4
S3
Fshb
[FSH]
1º
2º
M/D
P
E
M/D
++
iv)
S4F2cKO
GnRH
?
Fshb
[FSH]
-

Figure 3.8

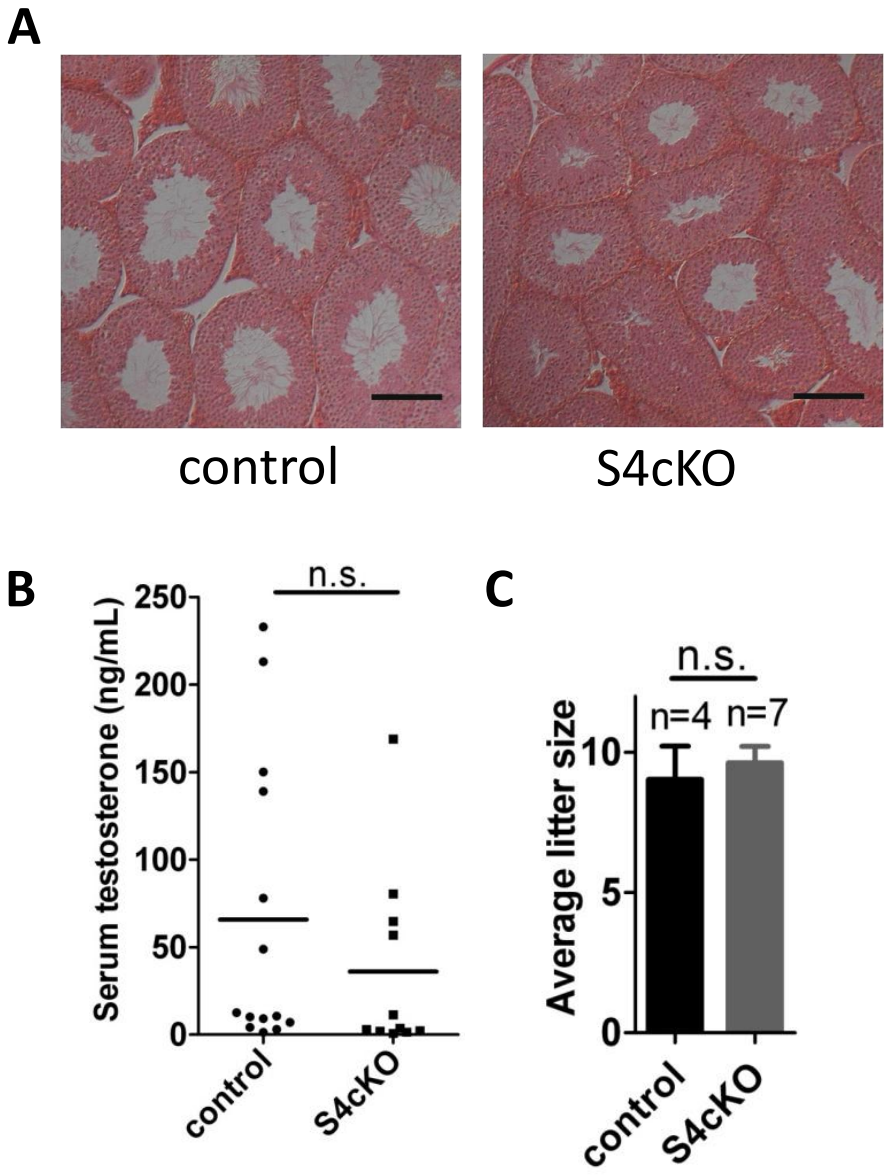
A
control
S4cKO
B
n.s.
Serum testosterone (ng/mL)
250
200
150
100
50
0
control
S4cKO
C
n.s.
n=4
n=7
Average litter size
10
5
0
control
S4cKO

Figure S3.1

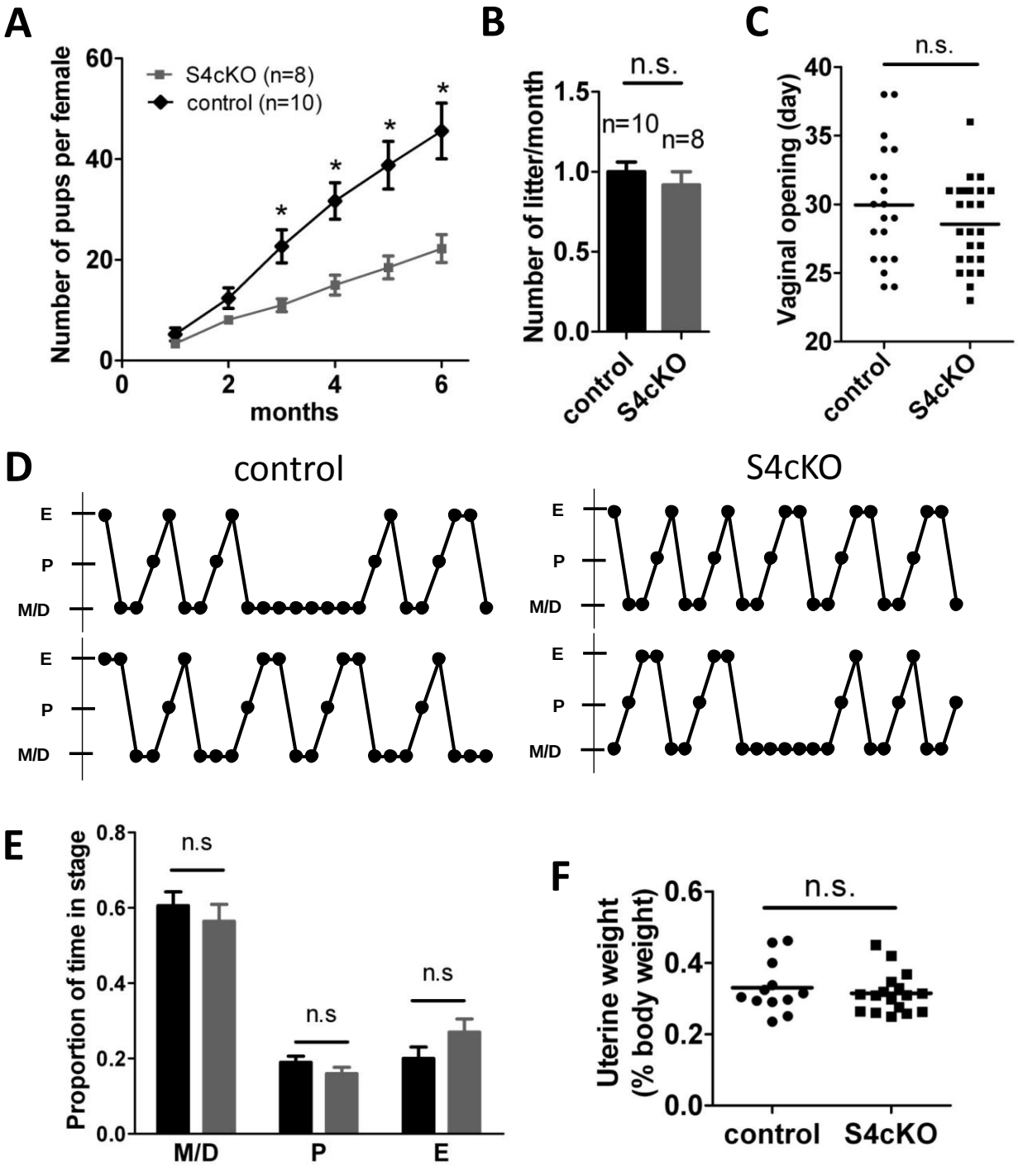
A
Number of pups per female
S4cKO (n=8)
control (n=10)
months
B
Number of litter/month
n.s.
n=10
n=8
control
S4cKO
C
Vaginal opening (day)
n.s.
control
S4cKO
D
control
S4cKO
E
P
M/D
E
Proportion of time in stage
n.s
M/D
P
E
F
Uterine weight (% body weight)
n.s.
control
S4cKO

Figure S3.2

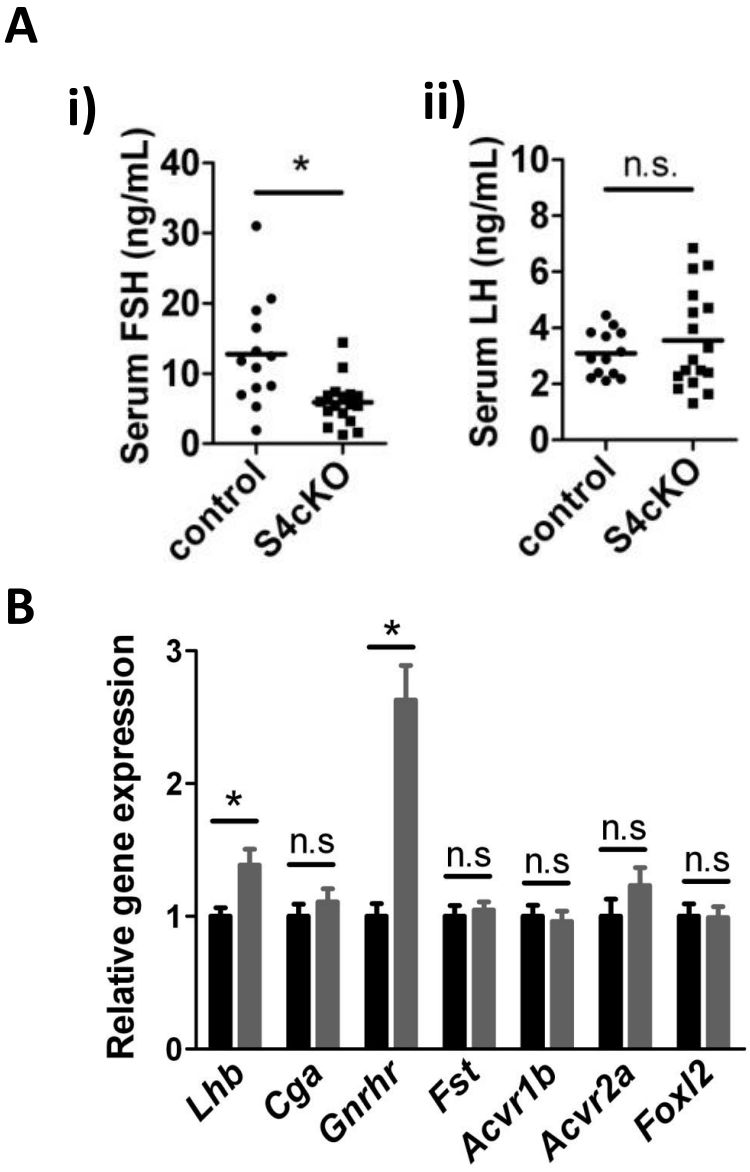
A
i)
Serum FSH (ng/mL)
40
30
20
10
0
*
control
S4cKO
ii)
Serum LH (ng/mL)
10
8
6
4
2
0
n.s.
control
S4cKO
B
Relative gene expression
3
2
1
0
*
n.s
*
n.s
n.s
n.s
n.s
Lhb
Cga
Gnrhr
Fst
Acvr1b
Acvr2a
Foxl2

Figure S3.3

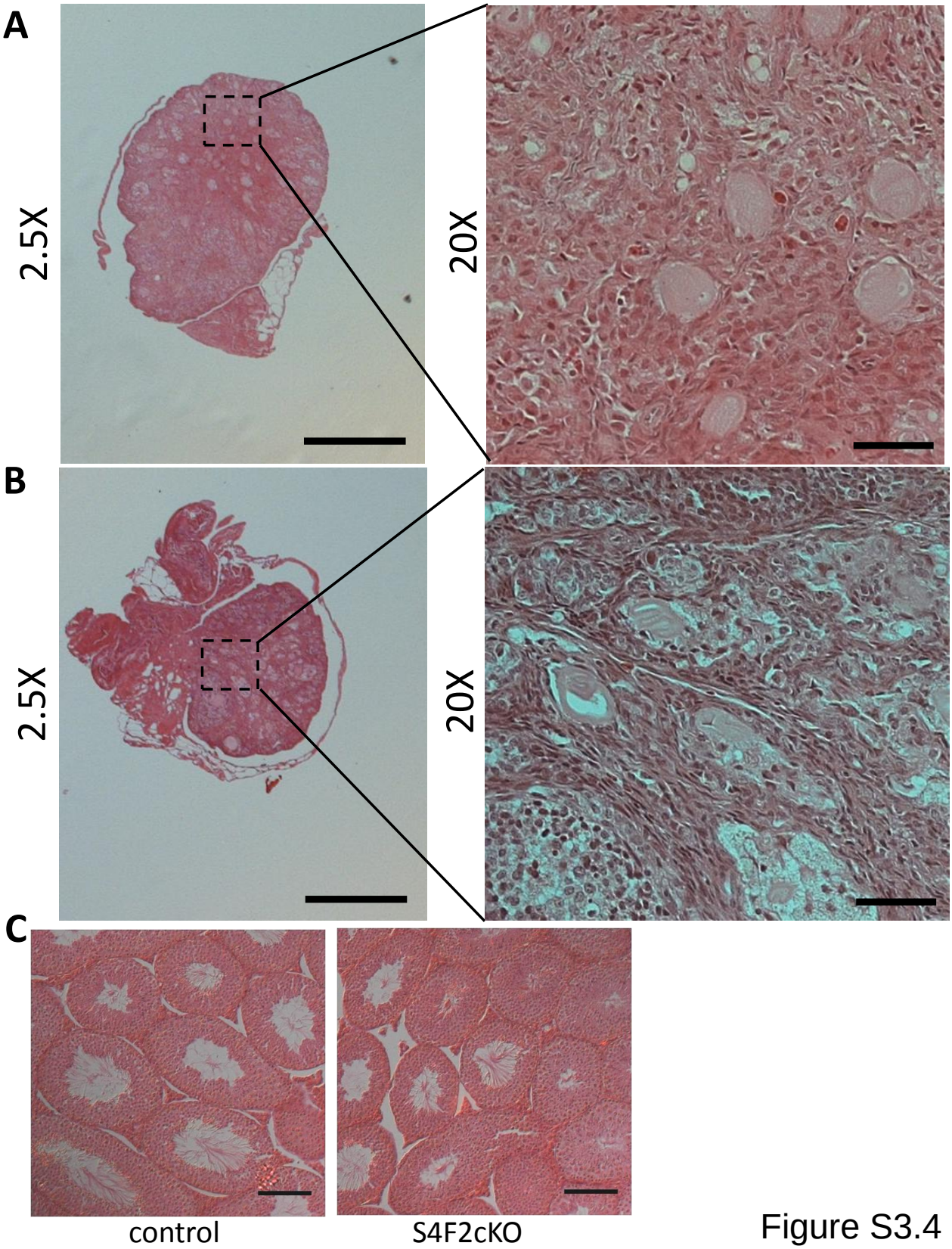

Figure S3.4

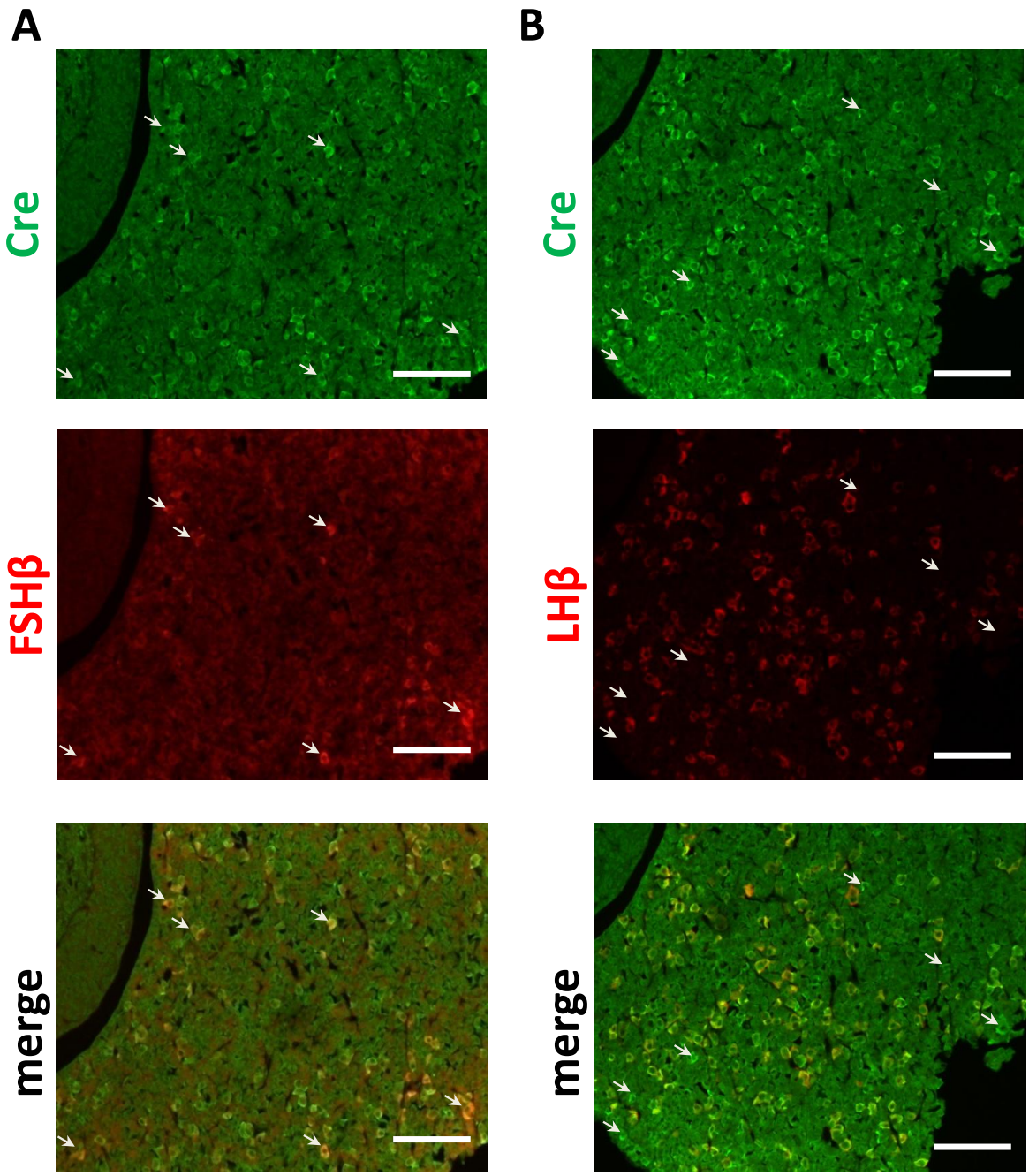

Figure S3.5

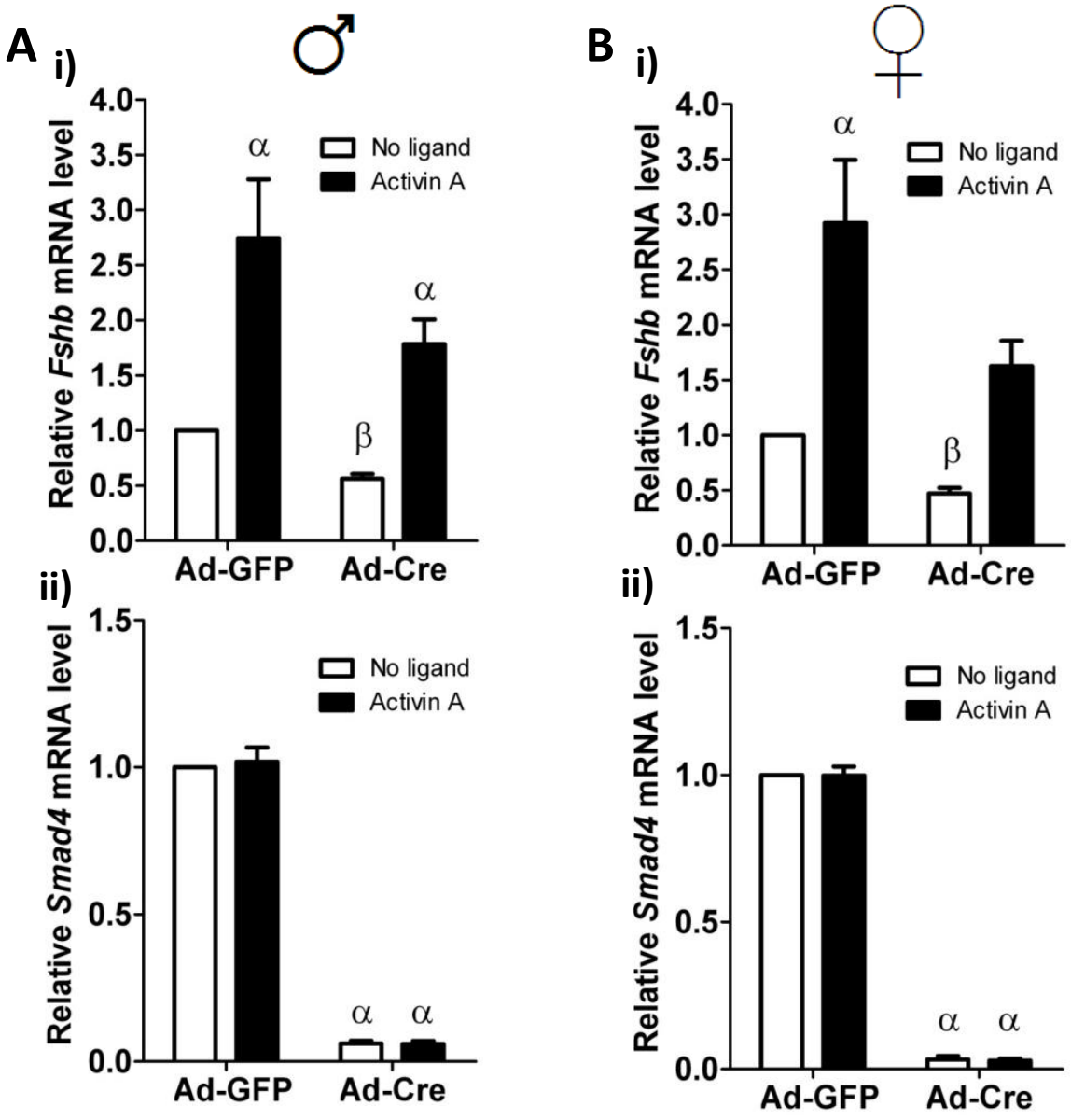
A
i)
♂
Relative Fshb mRNA level
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
α
β
α
No ligand
Activin A
Ad-GFP
Ad-Cre
ii)
Relative Smad4 mRNA level
1.5
1.0
0.5
0.0
α
α
No ligand
Activin A
Ad-GFP
Ad-Cre
B
i)
♀
Relative Fshb mRNA level
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
α
β
No ligand
Activin A
Ad-GFP
Ad-Cre
ii)
Relative Smad4 mRNA level
1.5
1.0
0.5
0.0
α
α
No ligand
Activin A
Ad-GFP
Ad-Cre

Figure S3.6

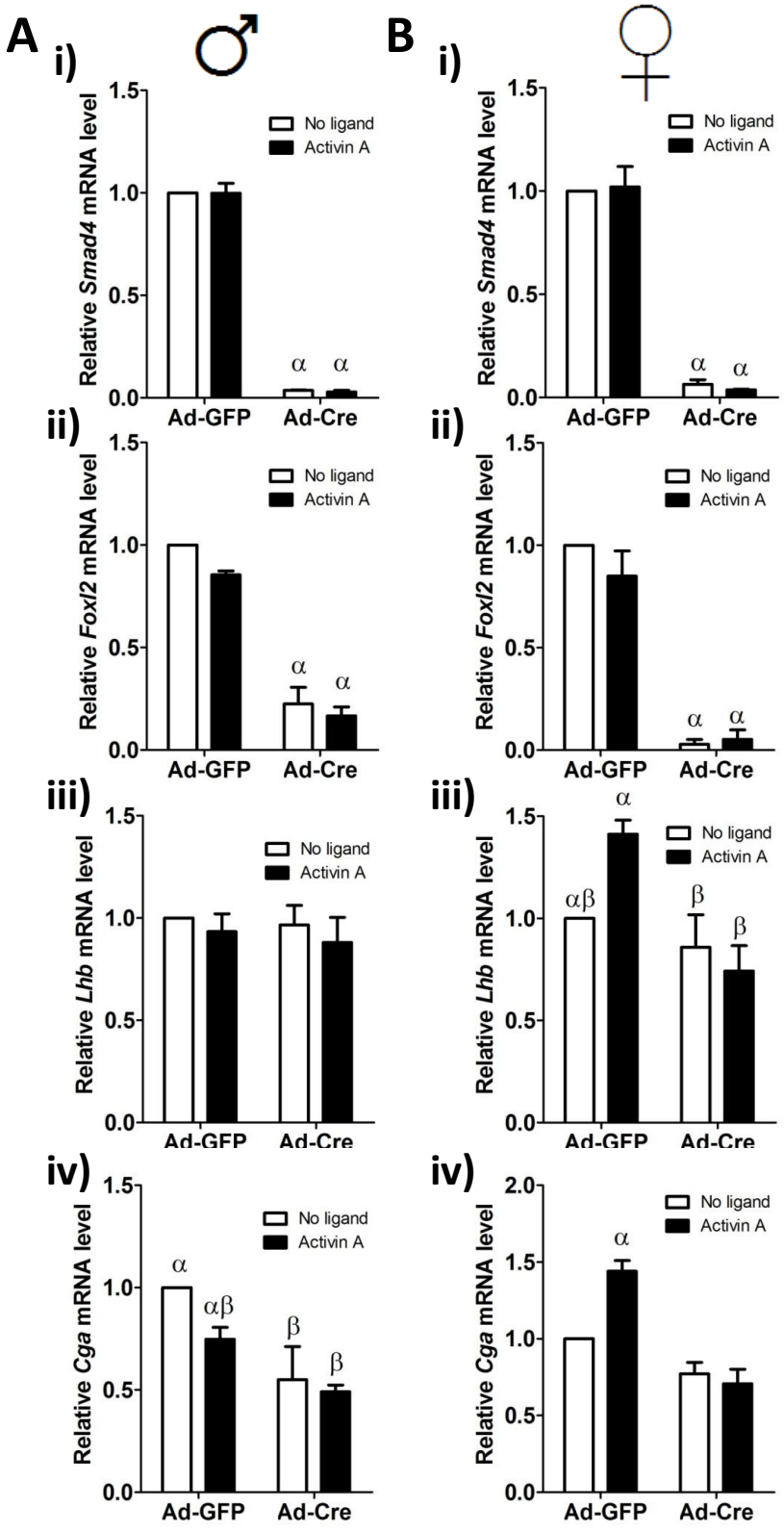
A
B
i)
ii)
iii)
iv)
No ligand
Activin A
Relative Smad4 mRNA level
Relative Foxl2 mRNA level
Relative Lhb mRNA level
Relative Cga mRNA level
Ad-GFP
Ad-Cre
α
αβ
β

Figure S3.7

Chapter 4

In chapters 2 and 3, using conditional knockout mouse models, I identified a requirement for SMAD signaling and FOXL2 for activin-regulated *Fshb* transcription, FSH synthesis, and fertility. Female mice with a gonadotrope-specific deletion of *Smad4* and *Foxl2* are sterile and exhibit ovarian follicle growth arrest at the pre-antral stage, strongly suggesting that they are incapable of mounting a secondary FSH surge. These observations are consistent with an absolute requirement for FSH for female fertility, most clearly demonstrated in *Fshb* knockout mice (220). Equally important for mammalian fertility is the other pituitary-derived gonadotropin, LH. LH stimulates gonadal steroidogenesis, and a mid-cycle LH surge provides the critical trigger for ovulation in females. While FSH synthesis is both GnRH and activin-dependent (the data presented in chapter 3 would argue that activin signaling might be more important), there is general agreement that GnRH is the primary regulator of LH synthesis and secretion in mammals (485). Mechanisms of GnRH-stimulated LH synthesis have been thoroughly investigated. Of particular importance, GnRH stimulates expression of the transcription factor EGR1, which then physically interacts with its cell-restricted partners SF1 (NR5A1) and *paired*-like homeobox (PITX) proteins to stimulate *Lhb* transcription (485). Indeed, global *Egr1* or gonadotrope-specific *Nr5a1* knockout mice are profoundly LH-deficient and sterile (141, 145). Is this model useful at all for understanding normal and abnormal LH synthesis in humans? Answering this question has important therapeutic and clinical implications, but the mechanisms underlying GnRH-stimulated human *LHB* promoter activation have never been studied in details. In this chapter, using a combination of overexpression, knock-down, and promoter mutation analyses, I assessed to what extent the mechanisms of GnRH-stimulated *Lhb* transcription are conserved in the human *LHB* promoter.

Title: Conservation of mechanisms mediating gonadotropin-releasing hormone 1 stimulation of human luteinizing hormone β subunit transcription

Short title: GNRH1 regulation of human *LHB* transcription

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Abstract

BACKGROUND: Gonadotropin-releasing hormone (GNRH1) regulates pituitary luteinizing hormone (LH). Previous studies delineated a mechanism for GNRH1-induced LHβ subunit gene (*Lhb*) transcription, the rate-limiting step in LH production. GNRH1 induces expression of early growth response 1 (EGR1), which interacts with steroidogenic factor 1 (SF1) and *paired*-like homeodomain transcription factor 1 (PITX1) to regulate *Lhb* promoter activity. Though the *cis*-elements for these factors are conserved across species, regulation of human *LHB* transcription has not been thoroughly investigated. METHODS and RESULTS: We characterized *LHB* transcriptional regulation by GNRH1 using promoter-reporter analyses in LβT2 cells. GNRH1 stimulated *LHB* transcription via an ERK1/2 pathway. EGR1 bound to two binding sites and this binding was increased by GNRH1. Mutation of either site or knockdown of endogenous EGR1 decreased basal and/or GNRH1-regulated promoter activity. The human *LHB* promoter contains low and high affinity *SF1* binding sites. Mutation of these elements or depletion of endogenous SF1 impaired basal and ligand-induced transcription. Knockdown of PITX1 or PITX2 isoforms impaired GNRH1 induction, and endogenous PITX1 bound to the candidate *PITX* binding site. CONCLUSIONS: The mechanism described for GNRH1 regulation of *Lhb* in other species is conserved for human *LHB*. We also uncover a previously unappreciated role for PITX2 isoforms in this process.

Introduction

Luteinizing hormone (LH) is a dimeric pituitary glycoprotein comprised of the unique LHβ (*LHB*) subunit and a common α subunit (CGA), which it shares with follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH) and, in humans, chorionic gonadotropin (hCG). LH and FSH are produced and secreted by the same cells in the pituitary gland, gonadotropes, and expression of their β subunits is the rate-limiting step in their synthesis. The primary stimulus for both LH release and *LHB* transcription is pulsatile gonadotropin-releasing hormone (GNRH1) secretion from the hypothalamus. *Lhb* mRNA levels are increased within minutes after GNRH1 stimulation in immortalized gonadotropes (437, 464, 510, 513).

Results from several groups working on the *Lhb* promoters in rat, cow, and horse, as well as data from knockout mouse models, have converged to suggest a general model of *Lhb* transcriptional regulation by GNRH1 [Reviewed in (485)]. GNRH1 rapidly stimulates early growth response 1 (*Egr1*) expression within 30 minutes (510). Upon translation, the EGR1 protein then binds the proximal *Lhb* promoter via two conserved *cis*-elements (506, 529, 856), both of which are critical for induction of the *Lhb* gene in various species (437, 506, 509, 510, 513, 856, 857). The importance of EGR1 *in vivo* was demonstrated in female *Egr1* null mice, which are infertile due to the loss of *Lhb* expression (145, 524).

EGR1 acts in concert with the nuclear receptor steroidogenic factor 1 (SF1, NR5A1), which binds to conserved elements occurring in tandem with the two *EGR* sites in the *Lhb* promoter from various species. Both *SF1* sites are required for maximal induction of *Lhb* by GNRH1 (437, 506-510, 513). Targeted deletion of *Sf1* in gonadotropes results in significant reduction of LH production in mice (141, 858), confirming the important role for SF1 in *Lhb* expression *in vivo*. Over-expression analyses in heterologous cells show that EGR1 and SF1 act together through their tandem response elements to stimulate *Lhb* transcription (506, 509, 510, 513).

Several observations suggest that a binding site for *Bicoid*-related homeodomain transcription factors (hereafter '*PITX*' element), which occurs between the tandem *EGR/SF1* sites, is also important for maximal induction of the *Lhb* promoter by GNRH1 (510, 516, 517). The exact identity of the protein(s) binding this element has not be unequivocally determined (530), though evidence from several groups implicates *paired*-like homeodomain transcription

factor 1 (PITX1) or the related PITX2 (510, 512, 516-518). Mice with homozygous deletion of *Pitx1* die after birth, precluding an assessment of PITX1 in LH synthesis in adult animals (521, 859). Mice with gonadotrope-specific deletion of *Pitx2* are fertile (860), though it is possible that PITX1 can compensate for loss of PITX2 in these animals. Nonetheless, several studies show that PITX1 and PITX2 isoforms can independently and synergistically regulate *Lhb* transcription with SF1 and EGR1 (437, 506, 508-510, 513, 517). Thus, the current model holds that GNRH1 stimulates *EGR1* expression, which then acts in concert with SF1 and PITX1 to regulate *Lhb* transcription through the proximal promoter, which contains a *Pitx* binding site flanked by tandem *EGR/Sf1* elements (485)

Most investigations on the transcriptional regulation of the *Lhb* gene have used the bovine or rodent promoters. In contrast, transcriptional regulation of the human *LHB* promoter has received considerably less attention. One report indicated that both *EGR* sites and the proximal *SF1* site in the human promoter have higher affinity for their respective transcription factors than do the comparable sites in the rat or bovine promoters (529). In addition, the distal *SF1* element in the human promoter was reported to be of much lower affinity than in other species (529). However, the functional relevance of these sites in the context of basal or GNRH1-regulated transcription were not reported. Further, the role of the putative *PITX* site in the *LHB* promoter and the identity of the protein(s) binding there are unknown. Sequence alignment of the *LHB/Lhb* promoters from several species reveals single base-pair differences in the *EGR*, *SF1*, and *PITX* elements (Fig. 4.1), which may be functionally significant. Therefore, we characterized transcriptional regulation of the human *LHB* promoter by GNRH1. Collectively, the data suggest that the primary mechanisms by which GNRH1 regulates the *Lhb/LHB* promoter are conserved between humans and other species.

Materials and Methods

Reagents

Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate was from Wisent (St. Bruno, QC). DMEM/F-12 Ham's media (1:1) with 2.5 mM L-Glutamine, 15 mM HEPES was from HyClone (South Logan, UT). Fetal bovine serum (FBS), Lipofectamine, Lipofectamine 2000 and gentamycin were purchased from Invitrogen (Burlington, ON, Canada). Polyclonal anti-Flag (F7425) and anti-c-myc (M5546) antibodies, aprotinin, leupeptin, pepstatin, PMSF, GNRH1 (LHRH) and SP600125 were from Sigma (St. Louis, MO). SB202190 was from Calbiochem (San Diego, CA). Deoxynucleotide triphosphates (dNTPs), T4 DNA ligase, T4 polynucleotide kinase, restriction endonucleases, 5X Passive Lysis Buffer (PLB) and U0126 were from Promega (Madison, WI). DNA polymerases (*Pfu* Ultra and Turbo) were purchased from Stratagene (La Jolla, CA). [γ-^{32}P] ATP was from PerkinElmer (Boston, MA). *Egr1* (D-040286-01, *Sf1* (D-051262-01, *Pitx1* (D-043250-03), *Pitx2* (D-058287-01) and control (D-001210-05) siRNAs were purchased from Dharmacon (Lafayette, CO). The SF1 rabbit polyclonal antibody (PA1-800) was from Affinity Bioreagents (Golden, CO). PITX1 N-15 (sc-18922X) and EGR1 C-19 (sc-189X) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Normal rabbit IgG (12-370) was from Upstate (Lake Placid, NY). Protease inhibitor tablets (Complete Mini) were purchased from Roche (Indianapolis, IN). Oligonucleotides were synthesized by IDT (Coralville, IA). ECL-plus reagent were purchased from Amersham Biosciences (GE Healthcare, Piscataway, NJ)

Constructs

The *LHB* luciferase reporters were produced by PCR amplification from genomic DNA (for primers see Table S4.1) as described earlier for the 0.2 kb construct and ligated into pA3-luc (861). Mouse EGR1 (NGFIA) in pJDM464 and NR5A1 (SF1) in pCMV5 were generous gifts from Drs. Jeffrey Milbrandt (Washington University School of Medicine, St Louis, MO) and Keith Parker (UT Southwestern Medical Center, Dallas, TX), respectively. Murine PITX1, Flag-PITX1, myc-PITX1 and PITX2 expression vector were described earlier (519, 537). To make Flag tagged EGR1 and SF1, the parental constructs were sub-cloned using strategies described

earlier for Flag-PITX1 (537). Constitutively active MKK6 was a gift from Dr. David Engelberg (Hebrew University, Jerusalem, Israel), and Raf-CAAX was from Dr. Linda Van Aelst (Cold Spring Harbor Lab). The mutant promoter-reporter and siRNA resistant expression vectors were constructed using the QuikChange protocol (Stratagene) using the primers described in Table S4.1. All constructs were verified by sequencing (Genewiz, South Plainfield, NJ).

Cell culture, transfections, and reporter assays

LβT2 cells were gift from Dr. Pamela Mellon (University of California, San Diego, CA). CHO and CV1 cells were provided by Dr. Patricia Morris (Population Council, New York, NY). All cells were cultured and transfected as described previously (537, 861). Briefly, LβT2 cells were transfected over-night with 450 ng reporter/well and the indicated amounts of plasmid DNA or siRNA. Total DNA transfected was balanced across all conditions. Control siRNAs used in our experiments consistently had non-specific effects on reporter activity, and therefore could not be used as a valid negative control. Indeed, the manufacturer (Dharmacon) cautioned that several of their control siRNAs may have unwanted effects in some contexts (http://www.dharmacon.com/catalog/Item.aspx?Product=31197). The following day, transfection medium was replaced with serum-free DMEM, and cells starved overnight. Next, cells were treated with GNRH1 and harvested in PLB. Luciferase assays were performed as described previously (861). For experiments using pharmacological inhibitors, compounds were applied 30 min before GNRH1 treatment. CV1 cells were transfected with the indicated plasmids using the calcium-phosphate method and harvested the following day for luciferase assays. All reporter experiments were performed a minimum of three times with duplicates or triplicates of all treatments.

Electrophoretic mobility shift, DNA affinity pull-down, and immunoblot assays

For EMSA and DNA affinity pull-down (DNAP) experiments, LβT2 cells were grown until confluent in 10-cm plates. Cells were stimulated or not with 10^{-7} M GNRH1 for 1 h prior to collection of nuclear or whole cell lysates. CHO cells in 10-cm plates were transfected with the indicated plasmid DNA using Lipofectamine, and cells harvested the following day. Nuclear extracts were prepared and gel shift assays were performed as described previously (676). Briefly, the binding reactions were composed of 10 mM KCl, 25 mM HEPES (pH 7.2), 5 mM

dithiothreitol (DTT), 20% glycerol, 500 ng of salmon sperm DNA and equivalent amounts of protein. Where appropriate, cold competitor probe or antibodies were added and reactions incubated 10 min at room temperature. Following the addition of 0.05 pmol of ^{32}P-labeled double-stranded probe and incubation for 15 min at room temperature, protein:DNA complexes were resolved on 5% native polyacrylamide gels at 4˚C. DNA affinity pull-down assays using streptavidin-coupled Dynabeads® M-280 (Dynal, Invitrogen) were performed as previously described (537, 676) using the biotinylated probes. Following elution from the beads, proteins were resolved on 10% SDS-PAGE gels as described previously (674). Sequences of the probes used for gel shift and DNAP assays are described in Table S4.1.

Statistical analysis

The data presented are the mean (± SEM) of representative experiments. Differences between means were compared using one-, two-, or three-way analyses of variance (ANOVA), followed by pair-wise comparisons using the Tukey post-hoc test where appropriate (Systat 10.2, Richmond, CA). In some experiments, data were log transformed when the variances were unequal between groups. Significance was assessed relative to $p < 0.05$

Results

The proximal LHB promoter is time- and dose-dependently stimulated by GNRH1 in LβT2 cells

LβT2 cells express both the α and β subunits of LH as well as the GNRH1 receptor, and produce LH in response to GNRH1 stimulation (862). Because no human gonadotrope cell lines are currently available, we used LβT2 cells as a model system to study the regulation of the human *LHB* promoter. LβT2 cells were transfected with human *LHB* promoter-reporter constructs containing approximately 0.2, 0.5 and 1 kb of 5' flanking region cloned upstream of the luciferase reporter gene. GNRH1 (6 h at 10^{-7} M) stimulated reporter activity 15-21 fold with all three reporters (Supplementary Fig. S4.1A). The promoterless vector was not regulated by GNRH1 [data not shown and (861)]. Thus, the major elements mediating GNRH1 responsiveness were contained within the proximal 200 base-pairs. GNRH1 regulation of the 0.2 kb construct was time and dose-dependent (Supplementary Fig. S4.1B). At doses of 10^{-7} and 10^{-6} M, the maximal GNRH1 response was observed at 8 h and declined thereafter. This suggested that sustained stimulation desensitized the response to GNRH1 in these cells. For further experiments, we used the 10^{-7} M GNRH1 concentration and 6 h treatment, which is on the rising phase of the promoter activity.

GNRH1 stimulates transcriptional activity at the LHB promoter through an ERK, but not JNK or P38-mediated pathway

GNRH1 activates the extracellular signal-regulated kinase 1/2 (ERK1/2), mitogen-activated protein kinase 14 (p38) and c-jun N-terminal kinase (JNK) MAPK pathways in LβT2 cells (449, 458, 863) (data not shown). The ERK1/2 and JNK branches of the MAPK cascades have been implicated in the regulation of the rat *Lhb* promoter by GNRH1 (458, 459). To assess the requirements for GNRH1-mediated *LHB* promoter activation, we antagonized all three pathways using previously validated inhibitors at validated concentrations (861). The p38 (SB202190) and JNK (SP600125) inhibitors did not affect the fold GNRH1 response (Supplementary Fig. S4.2A). In contrast, pre-treatment with the MEK1 inhibitor, U0126, markedly suppressed GNRH1-stimulated transcriptional activity by almost 70%. None of the inhibitors had a significant effect on basal transcriptional activity of the promoter.

To confirm a role for ERK (MEK1) signaling, we co-transfected the minimal *LHB*-luc construct with expression vectors for constitutively active (ca-) forms of MKK6 and Raf1 (Raf-CAAX), upstream kinases of p38 and MEK1, respectively. Whereas Raf-CAAX potently stimulated reporter activity, ca-MKK6 had no effect when expressed alone and did not alter the Raf-CAAX effect (Supplementary Fig. S4.2B). Together, these data indicate that GNRH1 stimulates expression of human *LHB* through an MEK1 (ERK1/2), but not p38 or JNK-dependent pathway in LβT2 cells.

Two Egr1 binding sites are critical to confer GNRH1 responsiveness to the LHB promoter

Having mapped GNRH1 responsiveness of the *LHB* promoter to within the proximal 0.2 kb, we next sought to identify critical *cis*-elements. Two conserved *EGR* response elements, at -111/-103 ("distal", d) and -49/-41 ("proximal", p), are present in the human promoter (Fig. 4.1). These two elements, which mediate the GNRH1-induced *trans*-activation of the *Lhb* promoter by EGR1 in other species (510, 513, 856), are perfectly conserved with those in the bovine promoter, but differ from the rat's proximal and distal sites at one and two base-pairs, respectively (Fig. 4.1). To assess the role of these sites in the human promoter, we mutated each, either alone or in combination. Corresponding mutations have been shown to functionally inactivate the conserved elements in the rat and bovine promoter (510, 513). Mutation of either the distal or proximal site decreased basal reporter activity (Fig. 4.2A). The proximal, but not distal site mutation also decreased the fold GNRH1 response. The mutations together further decreased the fold GNRH1 induction. These data indicated that the two conserved *EGR1* sites are critical for basal and GNRH1-regulated human *LHB* promoter activity.

Two SF1 binding sites and a PITX binding site are required for maximal induction of the LHB promoter by GNRH1

In the rodent and bovine *Lhb* promoters, two binding sites for SF1 are located 5' to the two *EGR* elements and are important for *trans*-activation (437, 506-510, 513). These sites are also present in the human promoter (at -130/-123 and -58/-51), although the distal element differs from those in the bovine or rodent promoters and diverges from the consensus binding sequence for SF1 relative to the other species (Fig. 4.1). Mutation of the distal *SF1* site alone had no effect on either the basal or GNRH1-stimulated *LHB* promoter activity (Fig. 4.2B). In

contrast, inactivation of the proximal site decreased the basal reporter activity, without altering the fold induction by GNRH1. The two mutations in combination further decreased basal activity and significantly impaired the fold GNRH1 response.

Between the two tandem *SF1/EGR* elements, at -100/-95, is a binding site for *paired*-like homeodomain transcription factors, which has been implicated in transcription of the *Lhb* promoter of various species (510, 516, 517, 530). This site is also present in the human promoter; but, unlike in the other species, perfectly matches the consensus site for Pitx proteins (GGATTA (864)) (Fig. 4.1). Introducing a mutation in the element dramatically decreased the basal, but not GNRH1-induced transcriptional activity (Fig. 4.2C). These results indicate that none of the *SF1* or *PITX* elements alone are required for GNRH1 responsiveness, but all contribute to basal activity and therefore maximal induction of transcription by GNRH1. At the same time, GNRH1 induction of the promoter requires at least one intact *SF1* site.

EGR1 and SF1 interact with the LHB promoter via two tandem elements

We examined the proteins binding to the putative *EGR*, *SF1*, and *PITX* sites. First, we performed gel-shift assays using two probes containing the distal or proximal tandem *SF1/EGR* elements and nuclear extracts from LβT2 cells treated or not with GNRH1 for 1 h. We detected four specific complexes (Fig. 4.3A, lane 1, labelled ‘a’ through ‘d’) binding the proximal *SF1/EGR* tandem element, which were competed by 100-fold excess cold homologous probe (lane 3). GNRH1 stimulation markedly increased the intensity of complex a (lane 2), which was competed by 100-fold excess wild-type probe (lane 3), but not by a probe containing the inactivating mutation in the presumptive *EGR* site (lane 5). This complex was super-shifted by an EGR1 antibody (lanes 8 and 9) but not by control IgG (lane 6) or an SF1 antibody (lane 7). A strong complex (complex ‘d’) present under both basal and GNRH1-stimulated conditions (lanes 1 and 2) was competed by 100-fold excess of homologous cold probe (lane 3), but not by a probe containing the inactivating mutation in the putative *SF1* element (lane 4). This complex was super-shifted by an SF1 antibody (lane 7), but not by control IgG (lane 6) or the EGR1 antibody (lanes 8 and 9). There was a slight increase in intensity of the SF1-containing complex with GNRH1 treatment (compare lanes 1 and 2). Binding by complexes ‘b’ and ‘c’ was competed by 100-fold excess of probe with an mutant *EGR* (lane 5) but not *SF1* site (Lane 4). The intensity of

both complexes was mildly decreased by an SF1 antibody (lane 7), but their identities remain to be determined.

We next performed a similar analysis with the distal *SF1/EGR* tandem element. Using nuclear extracts from LβT2 cells stimulated or not with GNRH1, we could not clearly detect any complexes containing SF1 or EGR1 (not shown). To determine whether these observations related to differences in affinities of the proteins for the distal versus proximal sites, we performed competition assays with the radio-labeled proximal probe and varying amounts of cold homologous and distal probes. As little as 10-fold excess cold homologous probe markedly inhibited binding of both SF1 and EGR1 to the proximal *SF1* and *EGR* elements (Fig. 4.3B, lane 3), and complex formation was completely abolished by 50-fold excess cold probe (lane 4). In contrast, although increasing amounts of cold distal probe were able to compete for binding of both SF1 and EGR1 to the proximal elements, complex formation was incompletely abolished even in the presence of 500-fold excess cold probe (lanes 7 through 10). Nonetheless, introduction of the inactivating mutations in the distal elements blocked their abilities to compete for binding to SF1 (compare lanes 10 and 13) and EGR1 (compare lanes 10 and 14). Together, these data indicate that the proximal *SF1* and *EGR* elements are higher affinity binding sites for their respective transcription factors in the *LHB* promoter than are the more distal sites.

Endogenous PITX1 binds to the LHB promoter

The putative *PITX* element in the *Lhb*/*LHB* promoter could potentially bind several homeodomain transcription factors, and studies in others species have yielded conflicting results regarding the identity of the endogenous proteins occupying this site (530). In EMSAs, we detected the formation of two specific complexes (Fig. 4.4A, lanes 1 and 2, labelled ‘a’ and ‘b’) under both basal and GNRH1-stimulated conditions with a probe containing the *PITX* element. Complex binding was competed by 100-fold cold homologous probe (lanes 3 and 4), but not by a probe containing the inactivating mutation in the *PITX* site (lanes 5 and 6). Further, complex formation was disrupted by a PITX1 antibody (lanes 9 and 10), but not by control IgG (lanes 7 and 8). To confirm that these two complexes contained PITX1 proteins, we incubated the probe with nuclear extracts from CHO cells transfected with a myc-tagged PITX1 construct (lanes 12-14). We observed the formation of two complexes co-migrating with the two complexes obtained with the LβT2 nuclear extracts, and both were super-shifted by an anti-myc antibody (lane 14).

To confirm these results, we performed DNA affinity pull-down experiments using a biotinylated probes (Fig. 4.4B). We pulled down endogenous PITX1 from lysates of control and GNRH1-treated LβT2 cells with a wild-type probe more readily than with a probe containing the inactivating mutation in the *PITX* site.Together, these results indicate that endogenous PITX1 can bind the *LHB* promoter.

Because PITX2 proteins bind the same consensus sequence as PITX1 and all known PITX2 isoforms are expressed in LβT2 cells (519), we next evaluated the possibility that these proteins might be recruited to this element. Using nuclear extracts from transfected CHO cells, we detected binding of all five PITX2 variants to the *LHB* promoter (not shown). However, none of the complexes clearly co-migrated with the endogenous complexes observed using LβT2 nuclear extracts in gel shifts. Also, we no longer have PITX2 antibodies of sufficient quality to use in the DNAP analyses.

EGR1, SF1, PITX1, and PITX2 mediate trans-activation of the LHB promoter

To confirm the roles for EGR1, SF1, PITX1, and PITX2 (isoforms) in the basal and GNRH1-induced *LHB* transcriptional activity, we first knocked down expression of the proteins in LβT2 cells using short interfering RNAs (siRNA). siRNAs targeting *Egr1* or *Sf1* mRNAs markedly decreased both basal reporter activity and fold stimulation by GNRH1 (Fig. 4.5A). Depletion of PITX1 markedly decreased GNRH1-induced activity and also appeared to inhibit basal reporter activity, but the latter effect was not statistically significant (Fig. 4.5B).

Notably, knockdown of PITX1 had less of an effect on reporter activity than did the mutation of the *PITX* response element (Fig. 4.2C). This could be attributable to incomplete knockdown and/or to functional compensation by PITX2 proteins. We therefore knocked down PITX2 expression using two siRNAs, one (#1) targeting the first coding exon (exon 2) [expected to affect the PITX2A, B1 and B2 isoforms] , and the other (#2) targeting the 3' end of the coding region (exon 6) [common to all five PITX2 isoforms] (519). *Pitx2* siRNA #2 had a more dramatic effect on *LHB* promoter activity than *Pitx2* siRNA #1 (Fig. 4.5C). Whereas *Pitx2* siRNA #2 consistently decreased basal transcriptional activity, this did not reach statistical significance. The GNRH1-stimulated activity, in contrast, was significantly reduced. Together, these results suggested that endogenous PITX1 and PITX2 proteins in LβT2 cells participate in

the *trans*-activation of the *LHB* promoter. Control experiments confirmed the efficacy and sequence specificity of the siRNAs (Fig. S4.3).

Finally, we used over-expression in heterologous CV-1 cells to examine functional cooperation between EGR1, SF1 and PITX1 at the *LHB* promoter. Expression of EGR1 or PITX1, but not SF1, by themselves stimulated transcriptional activity of the 0.2 kb promoter-reporter (Figs. 4.6A and B). Further, PITX1 synergistically induced reporter activity with either EGR1 or SF1. SF1 did not further potentiate the combined effects of PITX1 and EGR1, but instead partially inhibited their actions (data not shown). These results indicate that the transcription factors binding the proximal *LHB* promoter can cooperate to enhance transcriptional activity.

Discussion

Previous studies delineated a mechanism by which GNRH1 signaling induces *Lhb* transcription. Here, we show that this mechanism is largely conserved in the human *LHB* promoter. GNRH1 signals through the ERK1/2 MAPK signaling cascade to regulate *LHB* transcription and does so primarily through the proximal 200 base-pairs. As in rodents, cow, and horse, basal and/or GNRH1-regulated human *LHB* transcription is dependent upon the coordinated activities of EGR1, SF1 and PITX1 acting through conserved *cis*-elements within this proximal promoter region. RNA interference experiments confirmed roles for the endogenous proteins in basal and/or GNRH1 regulated promoter activity and further suggest a potential role for PITX2 isoforms.

The data show that GNRH1 induces transcriptional activity of the *LHB* promoter primarily through an ERK1/2-mediated pathway. Although both the ERK1/2 and JNK MAPK cascades have been implicated in GNRH1 regulation of the *Lhb* promoter in other species (458, 459), a more critical role has been attributed to ERK1/2 (449, 464). GNRH1 stimulates *Egr1* expression through the ERK1/2 pathway (505, 865), and EGR1 appears to be the primary transducer of the GNRH1 signal to the *Lhb* promoter (510, 513). Indeed, our data confirm a critical role for EGR1 in regulation of the human *LHB* promoter through two conserved *cis*-elements.

In the rat *Lhb* promoter, a distal region containing at least two Sp1 sites (-450/-441 and -410/-402) contributes significantly to GNRH1 induction (509, 526, 527, 857). Only one of the putative Sp1 sites is partially conserved in the human promoter. Though we noted differences in basal activity between the 1 kb, 0.5 kb and 0.2 kb *LHB* promoter-reporters, the fold-induction by GNRH1 was similar among the three. This suggests that distal elements do not significantly contribute to GNRH1 induction of the human *LHB* gene, at least under the experimental conditions used here.

As in other species, the *EGR*, *SF1* and *PITX1* sites are required for maximal induction of the *LHB* promoter by GNRH1. Mutation of the proximal *EGR* element or both *SF1* sites strongly attenuated the GNRH1 response. We confirmed binding of EGR1 and SF1 binding to their respective sites. Binding to the proximal elements was potentiated following GNRH1 treatment, particularly for EGR1. These results are consistent with the fact that EGR1 levels are markedly

increased in gonadotropes upon GNRH1 stimulation (510, 513) (data not shown). Although it has been reported that SF1 levels are unaffected by GNRH1 stimulation in gonadotropes, (510, 513) we observed a slight increase in intensity of SF1 binding to the proximal promoter element upon GNRH1 treatment. Therefore, this change in binding might reflect post-translational modifications in SF1 induced by GNRH1 signaling, such as phosphorylation (866, 867), and/or potentiation of binding through cooperation with induced EGR1. The data show that the proximal *SF1*/*EGR* elements have higher affinity for their respective transcription factors and contribute more than the distal *SF1/EGR* sites to overall *cis*-activation of the *LHB* promoter. Fold GNRH1 induction was decreased when the proximal *EGR* site was ablated, but was maintained in the presence of a mutated distal *EGR* element. Inactivation of the distal *SF1* site did not affect transcriptional activity either basally on in response to GNRH1. Therefore, this site is likely dispensable for *LHB* promoter activation. In contrast, inactivation of this element alone prevents normal GNRH1 induction of the bovine *Lhb* promoter in transgenic mice (508), though this was not the case in LβT2 cells (510). In the rat *Lhb* promoter, this site contributes significantly to basal activity and shows similar affinity for *SF1* compared with the proximal element (506, 508, 513, 529). In cow and rat, the distal *SF1* element is a perfect match to the consensus site, whereas the human element differs at positions 3 and 6 (Fig. 4.1). Nevertheless, our data suggest that the distal *EGR* and *SF1* elements can partially compensate for the loss of the proximal sites. Indeed, mutation of the two *EGR1* or *SF1* elements impairs transcriptional activity to a greater extent than inactivation of the proximal sites alone.

In transgenic mice, there is a clear requirement for the *Pitx* binding site for activation of the bovine *Lhb* promoter by GNRH1 (517). Results from mutation analyses reported here similarly indicate a critical role for this site in maximal induction of the human *LHB* promoter. We also showed binding of endogenous PITX1 to the *LHB* promoter by gel shift and DNA affinity pull-down assays, which has not been unequivocally demonstrated in other species (516, 517, 530). This may be explained by the fact that the human *PITX* binding site conforms perfectly to the consensus site [5'-GGATTA-3", (864)], whereas the corresponding sites in the rodent or bovine promoters do not (5'-AGATTA-3'). Structural analyses indicate that the GG nucleotides are critical for PITX2 binding to the *PITX* response element (868). Because the homeodomains of PITX2 and PITX1 are 97% identical (869), this requirement most likely also applies to PITX1.

Though several studies have implicated PITX1 in the regulation of the *Lhb* promoter (510, 512, 516, 517), possible roles for PITX2 isoforms have been largely overlooked despite the observations that they can *trans*-activate the bovine *Lhb* promoter in heterologous cells (518). Results from RNA interference experiments shown here suggest roles for both PITX1 and PITX2 proteins in basal and GNRH1-regulated *LHB* promoter activity. However, it was recently reported that targeted deletion of *Pitx2* in terminally differentiated gonadotropes had no effect on *Lhb* expression and fertility in mice (860), suggesting either that PITX2 proteins play no role in *Lhb* regulation in mice or that PITX1 can compensate for their loss. Additional experiments in which *Pitx1* is ablated alone or together with *Pitx2* in differentiated gonadotropes will be needed to address these ideas. At the same time, the difference in the *PITX* binding site between mice and humans leaves open the possibility that different proteins may bind these elements in the two species or that the same proteins may bind with different affinities. As such, results in mice may not be entirely predictive of what occurs in humans. Though the siRNA experiments here suggest a role for PITX2 proteins in regulation of the human *LHB* gene, we were unable to confirm binding of any endogenous PITX2 protein isoforms in our analyses. Unfortunately, we exhausted the PITX2 antibody we used previously (519), which precluded super-shift and DNA affinity pull-down analyses of the kind we employed with PITX1.

In summary, our results indicate that the primary mechanisms of GNRH1-induced *LHB* transcription are conserved between humans and other species. This contrasts with what we have reported for regulation of the *FSHB/Fshb* in humans and other species (676, 861). In the latter case, we argued that inter-species differences in transcriptional regulation may relate to observed differences in FSH dynamics in different organisms. When viewed in this light, one might predict conservation of *LHB/Lhb* transcriptional regulatory mechanisms. That is, in all mammalian species studied to date, GNRH1 pulses are followed faithfully and rapidly by LH pulses. Given the slower kinetics of increases in *LHB* transcription, one might view this response as a compensatory mechanism to replenish intracellular LH stores in advance of subsequent GNRH1 pulses. This may be particularly important in the context of the LH surge, where GNRH1 pulse frequency and amplitude are elevated, increasing the demand for releasable LH. Given that the dynamics of LH surge generation are common among mammalian species, it is perhaps not surprising that the mechanisms for *LHB/Lhb trans*-activation would be similarly conserved.

Acknowledgements

The authors thank Drs. Pamela Mellon (UCSD) and P. Morris (Population Council) for the LβT2 and CHO cells, respectively. The murine EGR1 (NGFIA) expression vector was a generous gift from Dr. Jeffrey Milbrandt (Washington University School of Medicine, St Louis, MO). Dr. Keith Parker (UT Southwestern Medical Center, Dallas, TX) provided the SF1 (NR5A1) expression vector. Constitutively active MKK6 and Raf-CAAX were gifts from Drs. David Engelberg (Hebrew University, Jerusalem, Israel) and Linda Van Aelst (Cold Spring Harbor Lab), respectively. This research was supported by grants from the NIH (HD047794) and CIHR (89991) to DJB

Figure Legends

Figure 4.1: Aligment of proximal *Lhb/LHB* promoters from human, rat, and cow. In all cases, +1 refers to the transcription start site. Nucleotides that differ from the consensus are shaded. The conserved *SF1*, *EGR* and *PITX* elements are boxed. 'd': distal, 'p': proximal.

Figure 4.2: Schematic representations of the proximal *LHB* promoter are shown at the left of each graph. The *SF1*, *EGR*, and *PITX* elements are represented by squares, triangles and a circle, respectively. Black symbols indicate mutated sites. A) LβT2 cells were transfected with 450 ng/well of the indicated *LHB*-luc reporters. (WT, wild-type; xd*EGR*, mutated distal *EGR* site; xp*EGR*, mutated proximal *EGR* site; 2x*EGR*, both *EGR* elements mutated). Cells were treated or not with 10^{-7} M GNRH1 for 6 h. B) LβT2 cells were transfected as above with either WT 0.2 kb *LHB*-luc reporter or mutant constructs with the inactivated distal (xd*SF1*), proximal (xp*SF1*) or both (2x*SF1*) SF1 sites. Where indicated, GNRH1 treatment was given for 6 h. C) LβT2 cells were transfected as above with either WT *LHB*-luc reporter or a construct with a mutated *PITX* element (x*PITX*). Differences in reporter activity were measured after 6 h GNRH1 treatment. The fold induction by GNRH1is indicated at the bottom of the graph. Bars with different symbols differ significantly. N=3 for all treatments.

Figure 4.3: A) Nuclear extracts from LβT2 cells treated (+) or not (-) with 10^{-7} M GNRH1 for 1 h were incubated with a radio-labeled probe corresponding to -66/-33 of the *LHB* promoter. Where indicated, the binding reactions contained 100-fold excess of cold homologous wild-type probe (WT; lane 3) or probes with mutated proximal *SF1* (xp*SF1*; lane 4) or *EGR* (xp*EGR*; lane 5) elements. Control IgG (lane 6), or SF1 (lane 7) or EGR1 (lanes 8-9) antibodies were added as indicated. Asterisks denote supershifted complexes. B) Nuclear extracts from LβT2 cells treated (+) or not (-) with 10^{-7} M GNRH1 for 1 h were incubated with a radio-labeled probe corresponding to the -66/-33 region of the *LHB* promoter. Ten, 50, 100 or 500-fold excess homologous cold probe (-66/-33; lanes 3-6), cold probe containing the putative distal *SF1* and *EGR* elements (-134/-103; lanes 7-10), or cold probe with mutated proximal or distal *SF1* or *EGR* elements (500x only; lanes 11-14) were added where indicated.

Figure 4.4: A) Nuclear extracts from LβT2 cells treated (+) or not (-) with 10^{-7} M GNRH1 for 1 h (lanes 1-10), or nuclear extracts from CHO cells transfected with empty vector (pcDNA3; lane 11) or Myc-PITX1 (lanes 12-14) were incubated with a radio-labeled probe corresponding to the -104/-79 region of the *LHB* promoter. Where indicated, the binding reactions contained 100-fold excess of cold homologous WT probe (lanes 3-4) or probe with a mutated *PITX* site (PITX mut; lanes 5-6), control IgG (lanes 7-8, 13), PITX1 antibody (lanes 9-10) or Myc antibody (lane 14). B) DNAP was perfomed using the probes described above. Whole cell lysates (Total) or proteins interacting with the probes were subjected to immunoblot (IB). Cells were treated (+) or not (-) with 10^{-7} M GNRH1 for 1 hour.

Figure 4.5: A) LβT2 cells were co-transfected with 450 ng/well of WT 0.2 kb *LHB*-luc reporter and 10^{-8} M of A) *Egr1* or *Sf1*, B) *Pitx1*, or C) *Pitx2* siRNAs. In all cases, 1X siRNA buffer was used as control. Cells were treated or not with 10^{-7} M GNRH1 for 6 h prior to collection of lysates for luciferase assays. Fold induction by GNRH1 is indicated at the bottom of the graphs. Bars with different symbols differ significantly. N=3 per treatment.

Figure 4.6: A) CV-1 cells were transfected with 900 ng/well of the 0.2 kb *LHB*-luc reporter along with 30 ng/well of EGR1 and/or PITX1 expression vectors or empty vector (pcDNA3). After overnight recovery, reporter activity was measured. The average fold stimulation, indicated at the bottom of the graph, was normalized to the reporter activity measured in presence of only the empty vector. B) CV-1 cells were transfected as in panel A with 30 ng/well of SF1 and/or PITX1 expression vectors or empty vector (pcDNA3). Reporter activity was measured and normalized as above. Bars with different symbols differ significantly. N=3 per treatment.

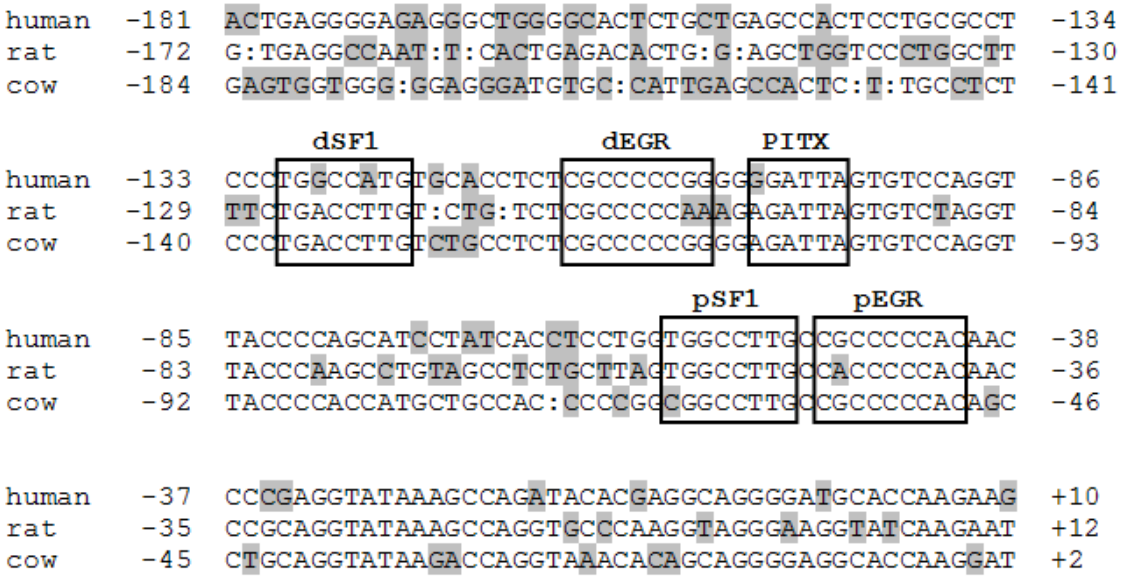
Supplementary Figure Legends

Figure S4.1: A) LβT2 cells were transfected with 450 ng/well of the indicated *LHB*-luc reporters and treated for 6 h with 10^{-7} M GNRH1. Fold induction by GNRH1 for each reporter is indicated at the left of the graph. Bars with different symbols differ significantly. N=3 for all treatments. B) The 0.2 kb *LHB*-luc construct was transfected in LβT2 cells as above. Cells were treated with 10^{-8} (closed circles), 10^{-7} (open circles) or 10^{-6} M (closed triangles) GNRH1 for 2, 4, 8 or 24

hours. N=2 for all treatments. Experiments were repeated three or more times with similar results each time.

Figure S4.2: A) LβT2 cells were transfected as in Fig. 2B. After overnight starvation in serum-free DMEM, cells were pre-treated with 5 x 10^{-6} M of the MEK inhibitor U0126, 10^{-5} M of the p38 inhibitor SB202190 or 2.5 x 10^{-5} M of the JNK inhibitor SP600125 for 30 min followed by treatment with 10^{-7} M GNRH1 for 6 h. The fold induction by GNRH1 is indicated at the bottom of the graph. B) LβT2 cells were transfected with 450 ng/well 0.2 kb *LHB*-luc reporter and 200 ng/well of constitutively active Raf1 (Raf-CAAX) and/or ca-MKK6 vectors. Bars with different symbols differ significantly. N=3 for all treatments.

Figure S4.3: CHO cells were transfected with WT or siRNA-resistant (Res.) forms of Flag-tagged EGR1 (A), SF1 (B) or Pitx1 (C), with 10^{-8} M control, *Egr1*, *Sf1* or *Pitx1* siRNAs or 1X siRNA buffer. Whole-cell lysates were collected and subjected to anti-Flag western blot analyses. Arrowhead and asterisk in panel A indicate specific and non-specific bands, respectively.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dEGR
PITX
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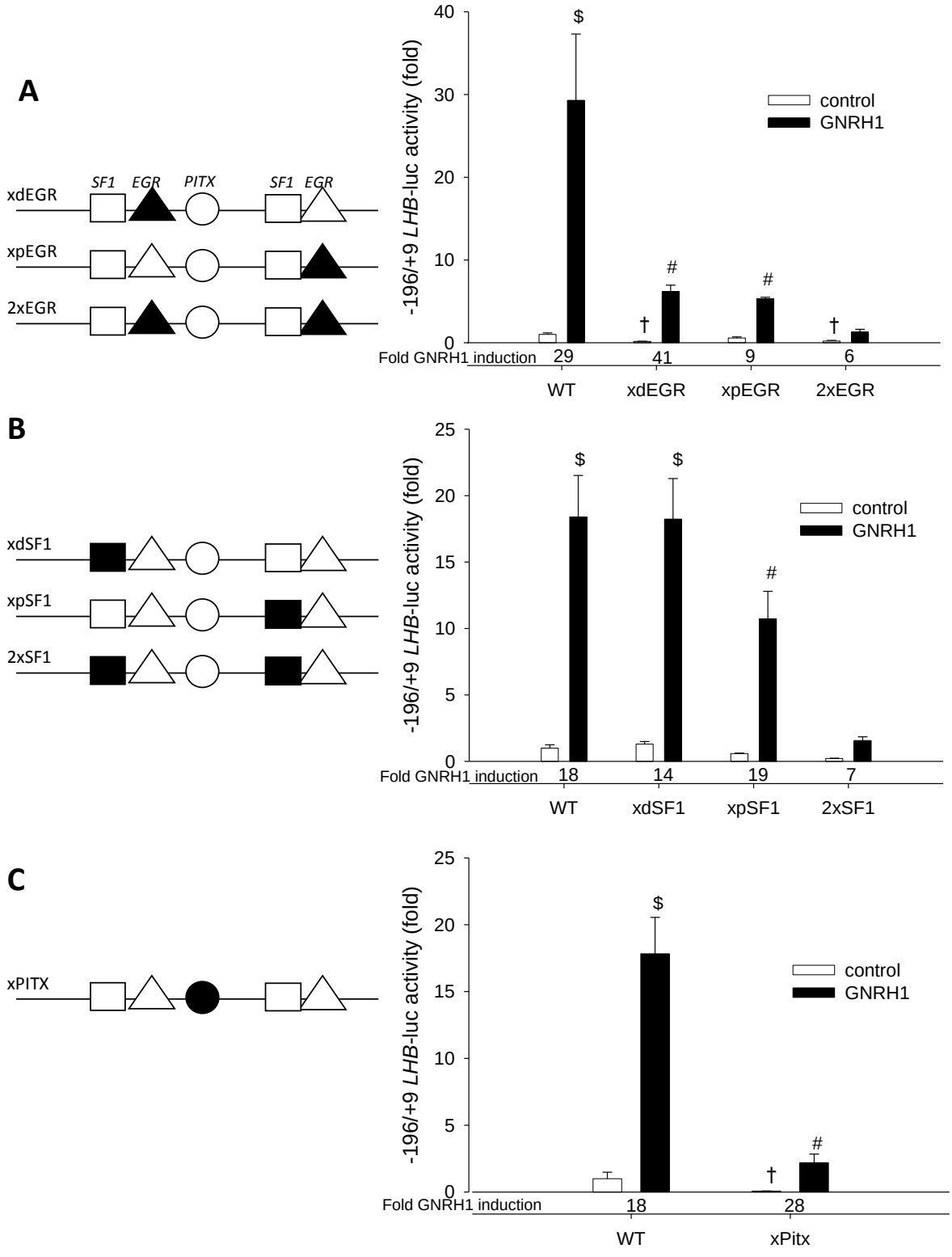
Figure 4.1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SF1
EGR
PITX
SF1
EGR
xdEGR
xpEGR
2xEGR
-196/+9 LHB-luc activity (fold)
control
GNRH1
Fold GNRH1 induction
29
41
9
6
WT
xdEGR
xpEGR
2xEGR
B
xdSF1
xpSF1
2xSF1
-196/+9 LHB-luc activity (fold)
control
GNRH1
Fold GNRH1 induction
18
14
19
7
WT
xdSF1
xpSF1
2xSF1
C
xPITX
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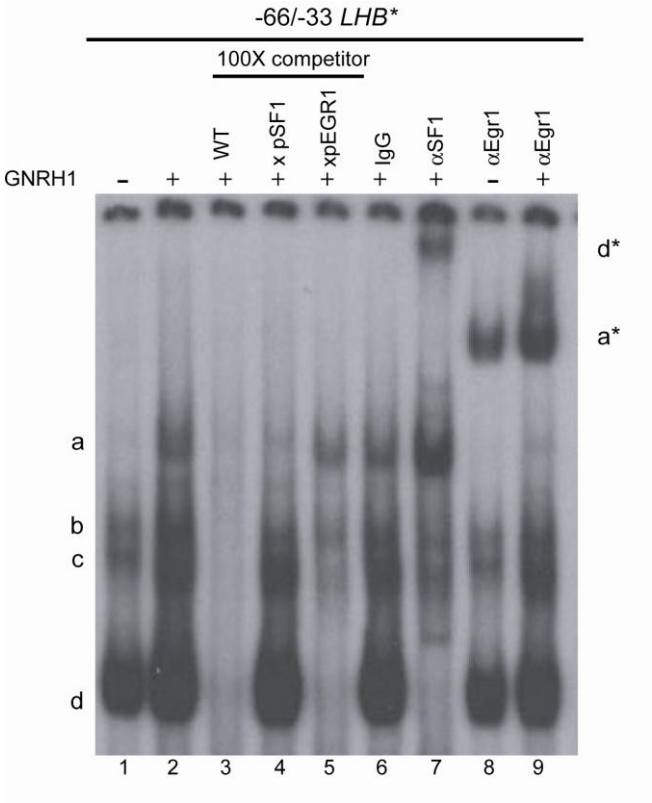
Fold GNRH1 induction
18
28
WT
xPitx

Figure 4.2

A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100X competitor
GNRH1
- + WT + x pSF1 + xpEGR1 + IgG + αSF1 - αEgr1 + αEgr1
d*
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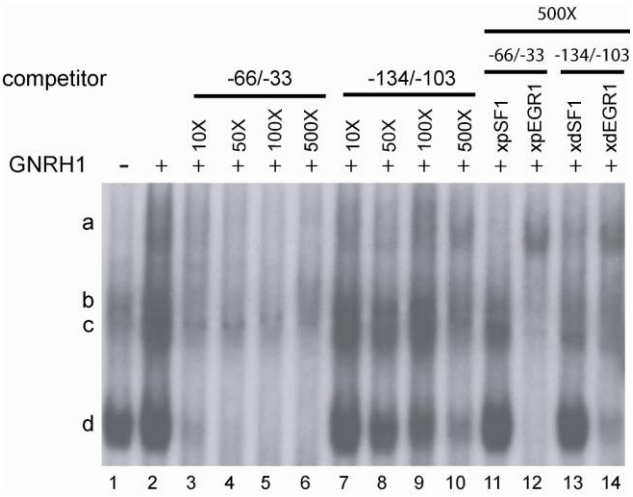
b
c
d
1 2 3 4 5 6 7 8 9

B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-66/-33
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GNRH1
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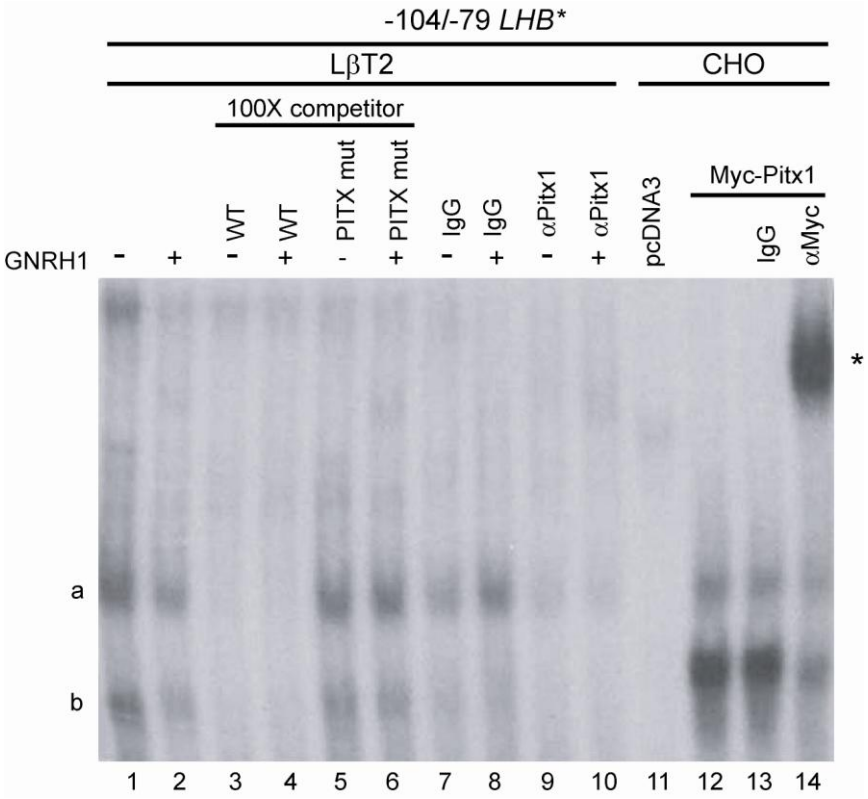
a
b
c
d
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 4.3

A


-104/-79 LHB*
LβT2
CHO
100X competitor
GNRH1
- + - WT + WT - PITX mut + PITX mut - IgG + IgG - αPitx1 + αPitx1 pcDNA3 Myc-Pitx1 IgG αMyc
*
a
b
1 2 3 4 5 6 7 8 9 10 11 12 13 14

B

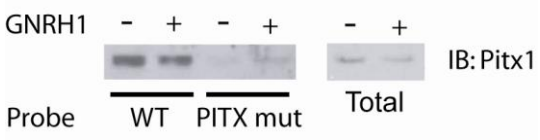

GNRH1 - + - + - +
IB: Pitx1
Probe WT PITX mut Total

Figure 4.4

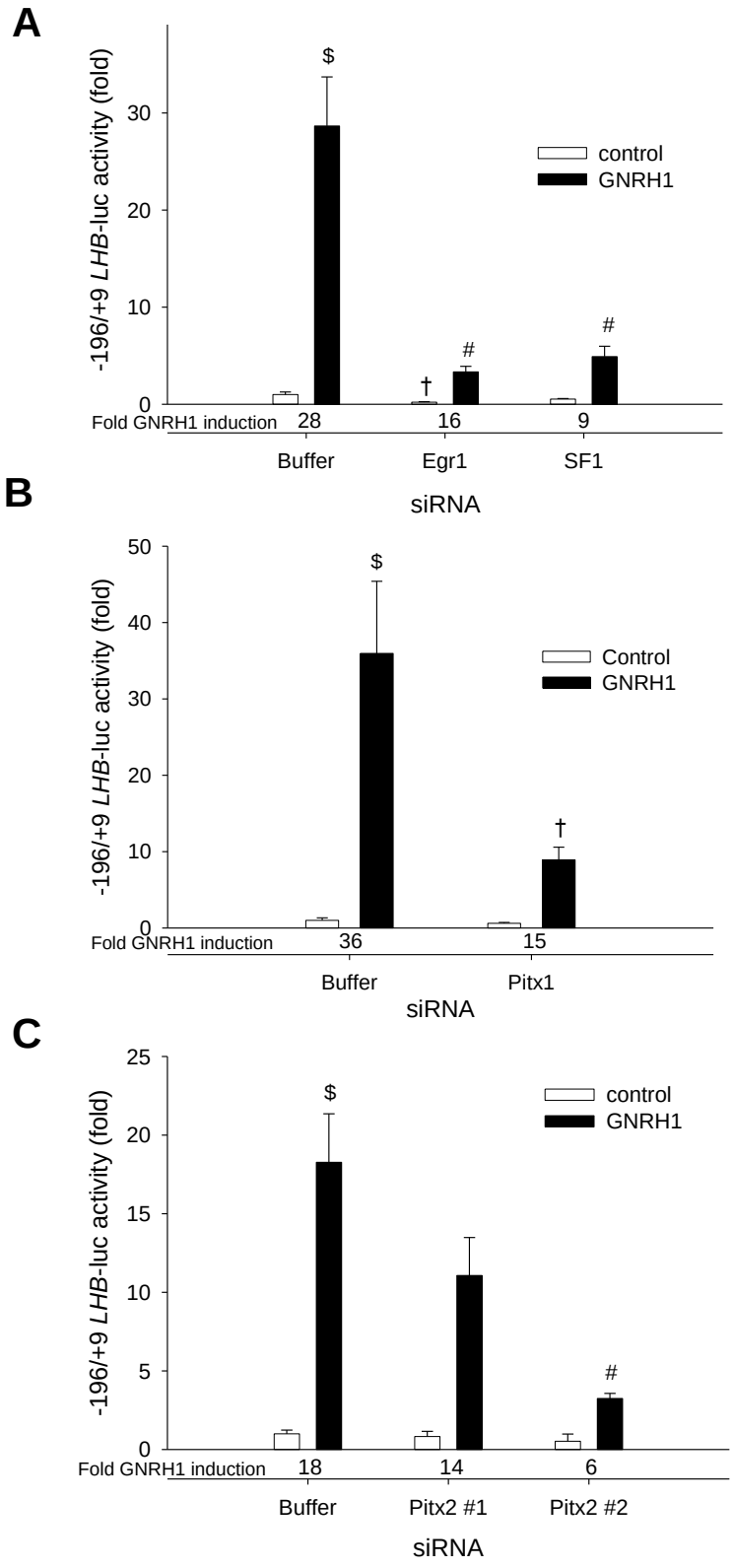

A
-196/+9 LHB-luc activity (fold)
0
10
20
30
$
#
†
#
control
GNRH1
Fold GNRH1 induction
28
16
9
Buffer
Egr1
SF1
siRNA
B
-196/+9 LHB-luc activity (fold)
0
10
20
30
40
50
$
†
Control
GNRH1
Fold GNRH1 induction
36
15
Buffer
Pitx1
siRNA
C
-196/+9 LHB-luc activity (fold)
0
5
10
15
20
25
$
#
control
GNRH1
Fold GNRH1 induction
18
14
6
Buffer
Pitx2 #1
Pitx2 #2
siRNA

Figure 4.5

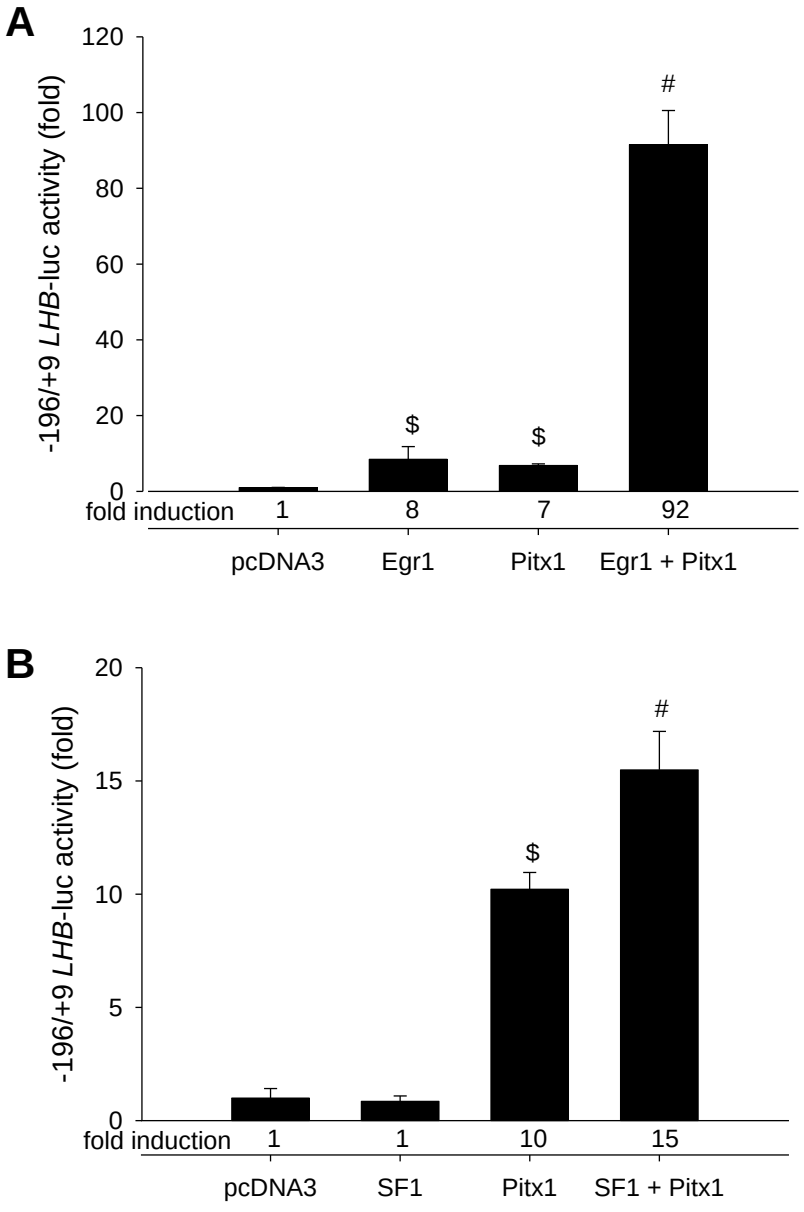

A
-196/+9 LHB-luc activity (fold)
120
100
80
60
40
20
0
#
$
$
fold induction
1
8
7
92
pcDNA3
Egr1
Pitx1
Egr1 + Pitx1
B
-196/+9 LHB-luc activity (fold)
20
15
10
5
0
#
$
fold induction
1
1
10
15
pcDNA3
SF1
Pitx1
SF1 + Pitx1

Figure 4.6

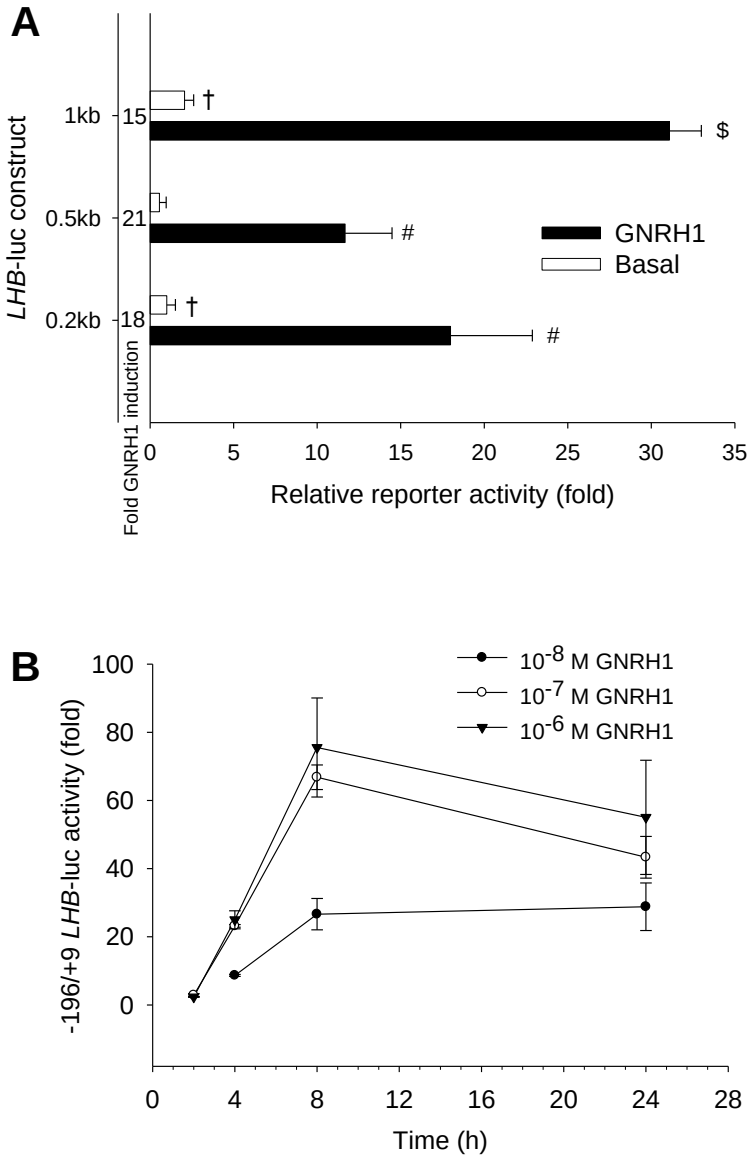

A
LHB-luc construct
1kb
0.5kb
0.2kb
15
21
18
†
†
$
#
#
GNRH1
Basal
Fold GNRH1 induction
0
5
10
15
20
25
30
35
Relative reporter activity (fold)
B
10-8 M GNRH1
10-7 M GNRH1
10-6 M GNRH1
-196/+9 LHB-luc activity (fold)
100
80
60
40
20
0
0
4
8
12
16
20
24
28
Time (h)

Figure S4.1

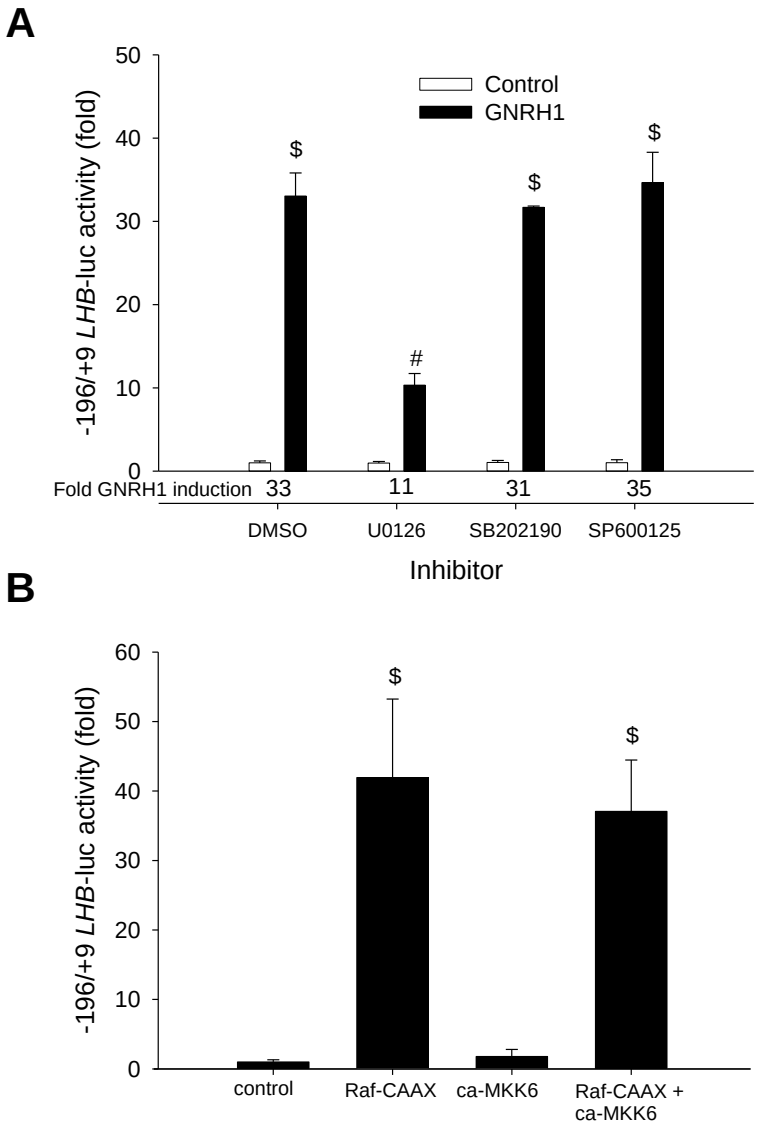
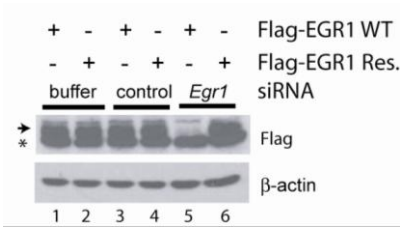
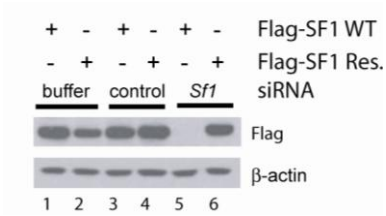

A
-196/+9 LHB-luc activity (fold)
50
40
30
20
10
0
Control
GNRH1
$
$
$
#
Fold GNRH1 induction
33
11
31
35
DMSO
U0126
SB202190
SP600125
Inhibitor
B
-196/+9 LHB-luc activity (fold)
60
50
40
30
20
10
0
$
$
control
Raf-CAAX
ca-MKK6
Raf-CAAX +
ca-MKK6

Figure S4.2

A



B



C

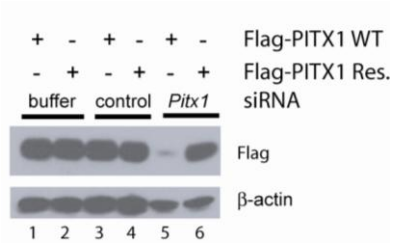


Figure S4.3

Promoter-reporter cloning#	
-197/-178.hLHB.F	GCGGGTACCCTCACCTCTGGCGCTAGACC
+8/-12.hLHB.R	CGGAAGCTTCTTGGTGCATCCCCTGCCTC
-500/-481.hLHB.F	CGGGGTACCCATCTGGGTCAAGTGGCTTC
-1068/-1049.hLHB.F	CGGGGTACCGCCCTGTCTCTGGCTCAGGA
Reporter mutagenesis†	
hLHB.xdSF1.F	CCTGCGCCTCCCTGGaatTGTGCACCTCTCGCC
hLHB.xdEGR.F	CTGCGCCTCCCTGGCCATGTGCACCTCTtagtaCtcGGGGGATTAGTGTCCA
hLHB.xPITX.F	CTCTCGCCCCCGGGGGttgTAGTGTCCAGGTTACC
hLHB.xpSF1.F	TCACCTCCTGGTGGaaTTcCCGCCCCCACAACC
hLHB.xpEGR.F	CTATCACCTCCTGGTGGCCTTGCgGttCttAtAACCCCGAGGTATAAAGCCAGAT
gel shift†	
-134/-103 LHB	TCCCTGGCCATGTGCACCTCTCGCCCCCGGGG
-134/-103 xdSF1 LHB	TCCCTGGaatTGTGCACCTCTCGCCCCCGGGG
-134/-103 xdEGR LHB	TCCCTGGCCATGTGCACCTCTtagtaCtcGGG
-104/-79 LHB	GGGGATTAGTGTCCAGGTTACCCCAG
-104/-79 PITX mut LHB	GGGttgTAGTGTCCAGGTTACCCCAG
-66/-33 LHB	CTCCTGGTGGCCTTGCCGCCCCCACAACCCCG
-66/-33 xpSF1 LHB	CTCCTGGTGGaaTTcCCGCCCCCACAACCCCG
-66/-33 xpEGR LHB	CTCCTGGTGGCCTTGCgGttCttAtAACCCCG

Restriction sites are underlined.
† Only sense strand is shown. Mutations are in lowercase.

Table S4.1

Chapter 5

In chapter 4, I demonstrated that the mechanisms of GnRH-stimulated *Lhb/LHB* transcription are largely conserved between humans and other mammals, at least *in vitro*. This is great news, because it suggests that model species like rodents can offer insights into aberrant LH synthesis in humans and can provide a valuable pre-clinical platform for the development of therapeutics that modulate LH production. While it is clear that GnRH is the primary stimulator of LH synthesis and secretion, other hormonal inputs may regulate *Lhb/LHB* transcription, either alone or through modulation of GnRH signaling. Such roles have been described for sex steroids and activins (824). Indeed, studies in immortalized gonadotrope-like cell lines and primary pituitary cells indicate that murine *Lhb* is an activin-responsive gene (645, 688, 728, 731). In apparent contrast, activin treatment has no effect of the human *LHB* promoter by itself, and antagonizes GnRH-stimulated *LHB* promoter induction (465). Resolving this discrepancy is crucial to understand the extent to which studying mice can tell us about the mechanisms regulating LH synthesis in humans. In this chapter, building on the analysis of GnRH regulation of the human promoter performed in chapter 4, I use a comparative approach to elucidate the mechanistic basis for the interspecies difference in activin modulation of GnRH signaling.

Title: SMAD3 and EGR1 physically and functionally interact in promoter-specific fashion

Short title: SMAD3-EGR1 interaction

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Abstract

Gonadotropin-releasing hormone (GNRH1) stimulates luteinizing hormone β subunit (*LHB*/*Lhb*) transcription. The transforming growth factor β superfamily ligand activin A partially inhibits this effect on the human *LHB* promoter while potentiating GNRH1-induction of the murine *Lhb* gene. Here, we investigated the mechanisms underlying the species-specific modulation of the GNRH1 response by actvin signalling. GNRH1 stimulates *LHB/Lhb* transcription via induction of early growth response 1 (EGR1), which binds to the proximal promoter of both species. Activin A decreased GNRH1-induced recruitment of EGR1 to the human, but not murine, promoter. We hypothesized that the activin A signaling protein, SMAD3, might play a role in this system. Indeed, we observed both physical and functional interactions between SMAD3 and EGR1. The two proteins interacted via the SMAD3 MH2 domain and the EGR1 DNA binding domain. Analogous to the species-specific activin A effect on the GNRH1 response, SMAD3 over-expression partially inhibited EGR1-induction of the human promoter, while potentiating EGR1-induced murine *Lhb* promoter activity. The proximal murine *Lhb* promoter contains three minimal SMAD binding elements (SBEs) that are absent from human *LHB*. Introduction of the SBEs into the human promoter converted SMAD3 from an inhibitor to a stimulator of EGR1-induced transcription. The converse was observed when the SBEs in the murine promoter were replaced by the corresponding human sequences. Together, our results suggest a model in which activin A inhibits GNRH1-induction of human *LHB* transcription via an interaction between SMAD3 and EGR1 that inhibits the latter's recruitment to the proximal promoter. In contrast, in mouse, the presence of SBEs in the promoter allows SMAD3 and EGR1 to function synergistically to regulate *Lhb* transcription. The basis for their functional cooperativity is not completely clear, but may involve enhancement of EGR1's physical interaction with other important co-factors, including *paired*-like homeodomain transcription factor 1 (PITX1).

Introduction

Activins play essential roles in the control of reproductive and other biological processes (604, 870, 871). Like other transforming growth factor β (TGFβ) superfamily ligands, activins signal through hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors (625). In the canonical activin signalling cascade, type II receptors phosphorylate and activate type I receptors, which then phosphorylate the receptor-regulated SMADs (R-SMADs), SMAD2 and SMAD3. The R-SMADs then homo- and hetero-oligomerize with the co-SMAD, SMAD4, and accumulate in the nucleus where they regulate gene transcription through DNA binding and protein-protein interactions (703, 872-874). In many instances, SMAD proteins and interacting factors bind adjacent promoter elements to activate gene transcription (e.g. (702, 875)). In some contexts, this type of interaction leads to the recruitment of co-repressor complexes and, hence, transcriptional repression (e.g., (876)). SMAD proteins can also repress transcription by interfering with the recruitment of transcription factors to their target promoters (e.g., (877)).

One important site of activin action is the pituitary gonadotrope cell (564, 600, 878, 879). In these cells, activins regulate the transcription of several cell-specific genes, including the follicle-stimulating hormone (FSH) and to a lesser extent luteinizing hormone (LH) β subunits (539, 645, 674, 688, 728, 731). Expression of the *FSHB*/*Fshb* and *LHB*/*Lhb* genes is rate-limiting in the synthesis of the mature dimeric glycoprotein hormones. The primary stimulus for *LHB*/*Lhb* expression is the hypothalamic decapeptide gonadotropin-releasing hormone (GNRH1). GNRH1 induces *LHB*/*Lhb* transcription via the immediate-early gene, early-growth response 1 (EGR1) (145, 510, 524, 880). *In vivo* studies clearly demonstrate the necessity for EGR1 in LH synthesis and fertility (145, 524). EGR1 binds to and activates the proximal *LHB*/*Lhb* promoter in cooperation with other transcription factors, such as *paired*-like homeodomain transcription factor 1 (PITX1) (reviewed in (485)).

Previously, it was demonstrated that GNRH1 and activin A synergistically activate the rat *Lhb* promoter (485). In contrast, we recently reported that activin A partially inhibits GNRH1-stimulated activation of the human *LHB* promoter (465). Here, we examined the mechanism underlying species-specific modulation of GNRH1-stimulated *LHB*/*Lhb* promoter activity by

activins. The data suggest that functional and physical interactions between SMAD3 and EGR1 may underlie activin A modulation of GNRH1 signalling to the *LHB/Lhb* promoter.

Materials and Methods

Reagents

DMEM with 4.5g/liter glucose and L-glutamine, with or without sodium pyruvate, was from Wisent (St-Bruno, Quebec, Canada). Lipofectamine, Plus reagent, Lipofectamine 2000, gentamycin, SYBR green quantitative PCR master mix, and fetal bovine serum were obtained from Invitrogen (Burlington, Ontario, Canada). Anti-FLAG antibody (F7425), EZview Red M2 FLAG affinity beads, FLAG peptide and chemicals were from Sigma (St. Louis, MO). Taq polymerase, T4 DNA ligase, restriction endonucleases, deoxynucleotide triphosphates and 5X Passive Lysis Buffer (PLB) were purchased from Promega (Madison, WI). ECL-plus reagent and protein markers were from GE Healthcare (Piscataway, NJ). Protease inhibitor tablets (Complete-Mini) were from Roche (Indianapolis, IN). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Anti-SMAD2/3 antibody (07-408) and normal rabbit IgG (12-370) were from Millipore (Billerica, MA), anti-SMAD3 (51-1500) was from Invitrogen (Burlington, Ontario, Canada), anti-phospho-SMAD2 (3101) was from Cell Signaling (Danvers, MA), anti-nucleoporin p62 (610498) was from BD Biosciences (San Jose, CA) and anti-calnexin (SPA-860) was from StressGen (Assay Designs, Ann Arbor, MI). Anti-EGR1 C-19 antibody (sc-189) and Protein A/G PLUS-Agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Constructs

The murine -232/+5 *Lhb* luciferase reporter was produced by PCR amplification of murine genomic DNA and ligated in pA3-luc, as described earlier for the human -196/+9 *LHB*-luc reporter (465). Mutant promoter-reporter constructs were generated using the QuikChange system (Stratagene). Murine EGR1 (NGFIA) in pJDM464 was a generous gift from Dr. Jeffrey Milbrandt (Washington University School of Medicine, St Louis, MO). Full-length FLAG-tagged EGR1 in pcDNA3 was described earlier (880). To generate FLAG-tagged truncated EGR1 constructs, the regions corresponding to previously-delineated functional domains (881)

were amplified by PCR, using the full-length murine EGR1 as template. The amplicons were cloned in-frame downstream of a FLAG tag coding sequence in pcDNA3. Full-length murine SMAD2 and SMAD3 expression vectors were described previously (674), and full-length human FLAG-SMAD3 and FLAG-SMAD2 in pCAGGS were gifts from Dr. Elizabeth Roberston (Oxford University, UK). FLAG-tagged SMAD3 sub-domains were obtained from Addgene (Cambridge, MA), and are described in detail in (671). Primer sequences are available upon request. All the constructs were verified by sequencing (Genewiz, South Plainfield, NJ, or GenomeQuebec, Montreal, Canada).

Cell culture, transfections and reporter assay

LβT2 cells, a gift from Dr. Pamela Mellon (University of California, San Diego, CA), were cultured as previously described (674). Briefly, for reporter experiments, cells were seeded in 48-well plates, and transfected with 225 ng reporter per well, along with the indicated amount of expression plasmid DNA using Lipofectamine 2000. The next day, transfection media was replaced with serum-free media, and cells cultured overnight before ligand treatment as indicated. HEK293 cells (a gift from Dr. Terry Hébert, McGill University, Montréal, Canada) were cultured in DMEM without sodium pyruvate, supplemented with 10% FBS. Cells were seeded in 48-wells plate at a density of 30,000 cells/well, and transfected the next day as described for the LβT2 cells. Twenty-four h later, transfection media was replaced with serum-free media, and cell lysates were harvested the next day. Total DNA transfected was balanced across each condition using the appropriate empty vector. Whole cell lysates were assayed for luciferase activity as previously described (465). CHO cells (gift from Patricia Morris, Population Council, New York, NY) were cultured in DMEM/F12 supplemented with 10% FBS.

Co-immunoprecipitation analyses and western blotting

Lysates from CHO or LβT2 cells were cultured, transfected, and processed for co-IP using anti-FLAG M2 agarose affinity beads as previously described (537). Nuclear and cytoplasmic extracts were prepared following published methods (882). Western blotting was performed as described in (674).

Chromatin immunoprecipitation

LβT2 cells in 10-cm dishes were transfected with the human -196/+9 *LHB*-luc construct, and treated for 2 h with 10^{-7} M GNRH1 and/or 25 ng/mL activin A. After treatment, formaldehyde was added to a final concentration of 1%, and crosslinking performed for 10 min at room temperature. The crosslinking reaction was quenched with 125 mM glycine for 5 min. Cells were then lysed in 1 mL lysis buffer [1% SDS, 1 mM EDTA, 50 nM Tris-HCl (pH 8), protease inhibitors]. Half of the lysate was sonicated with six 5-sec pulses at power 0.5 using a Misonix Sonicator 3000 (Misonix, Farmingdale, NY). The sonicated chromatin was spun for 10 min at 13,000 rpm to pellet cellular debris. Two hundred µL of sonicated chromatin was added to 1800 µL dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8, 16.7 mM NaCl, protease inhibitors) and pre-cleared for 30 min at 4°C with 75 µL protein A/G-agarose bead slurry (1:3 volume of protein A/G-agarose beads in a solution containing 10 mM Tris, 1 mM EDTA, 0.1% BSA, 0.27 µg/µL salmon sperm DNA). Beads were pelleted at 3,000 x g for 5 min at 4°C. One-twentieth of the volume of pre-cleared chromatin was removed and kept as "input" chromatin. The remaining chromatin was divided in two, and each half incubated overnight with 60 µL of the protein A/G-agarose beads slurry (see above) and 5 µg anti-EGR1 antibody or normal rabbit IgG. The next day, beads were sequentially washed with low salt buffer (0.1%SDS, 1% Triton X-100, 2 mM EDTA, 20 nM Tris pH 8, 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 nM Tris pH 8, 500 mM NaCl), LiCl buffer (250 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris pH 8), and twice in TE buffer (10 mM Tris pH 8, 1 mM EDTA). Each wash was performed for 5 min at 4°C, followed by a 30-sec spin at 3,000 x g. DNA was eluted for 15 min at room temperature in 480 µl elution buffer (1M $NaHCO_3$, 1%SDS). NaCl was added to a final concentration of 0.3 M and protein:DNA complexes were reverse-cross-linked overnight at 65°C. The next day, samples were incubated for 30 min at 37°C with 20 ng/µl RNase A. Tris-HCl (pH 6.8) and EDTA were added to final concentrations of 400 mM and 100 mM, respectively, and samples incubated at 45°C for 2 h with 20 ng/µl proteinase K. DNA was extracted with phenol:chloroform and precipitated with ice-cold ethanol for 30 min in presence of 30 pg/µl tRNA at -80°C. DNA was pelleted at 13,000 rpm at 4°C for 15 min, washed with 75% ethanol, dried, and dissolved in clean water. One-thirtieth of each immunoprecipitation product and 1/15,000 of each input sample was analyzed in triplicate by real-time quantitative

PCR with SYBR Green qPCR Master Mix using a Corbett Rotor-Gene 6000 instrument. Primer sequences are available upon request. For quantification, the calculated chromatin concentration (determined with the relative standard curve method) obtained with IgG was subtracted from that obtained with anti-EGR1, and divided by the input chromatin specific for each condition.

Statistical analysis

The data presented are the mean (+/- SEM) of at least three independent experiments. Differences between means were compared using one-, two- or three-way analyses of variance (ANOVA), followed by pair-wise comparisons using the Tukey post-hoc test where appropriate (Systat 10.2, Richmond, CA). Data were log-transformed when variances were unequal between groups. Significance was assessed relative to $p<0.05$.

Results

Species-specific modulation of GNRH1-stimulated LHB/Lhb promoter activity by activin A

Similar to previous reports for the rat *Lhb* promoter (688, 731), we observed that activin A stimulated a small, but significant 1.5- to 2-fold increase in murine -232/+5 *Lhb* promoter activity in immortalized LβT2 murine gonadotropes (Fig. 5.1A). Further, activin A and GNRH1 synergistically stimulated the murine promoter (Fig. 5.1A). In contrast, but consistent with our previous findings (465), activin A treatment had no effect by itself on the activity of the human -196/+9 *LHB* reporter, but inhibited GNRH1-stimulated promoter activity by about 25% (Fig. 5.1B).

Based on the observation that activin responsiveness of the rat *Lhb* promoter is mediated by three minimal SMAD binding elements (SBEs) (688), we hypothesized that the synergistic action of activin A and GNRH1 on the murine promoter may require the same elements, which are conserved in mouse, but not human (double underlined sequences in Fig. 5.1C). To test this idea, we mutated the three SBEs to the corresponding sequence in the human promoter (Fig. 5.1C). In contrast with the wild-type (WT) murine reporter, activin A neither independently nor synergistically (with GNRH1) stimulated the mutant promoter (3SBEmut) (Fig. 5.1A). Next, we tested whether the absence of the three SBEs in the human promoter explained the antagonism of the GNRH1 response by activin A. To this end, we introduced the three rodent SBEs into the human promoter (3SBE+). Though this manipulation did not confer activin A responsiveness to the human promoter, it abolished the partial inhibition of GNRH1-stimulated reporter activity by activin A seen with the WT construct (Fig. 5.1B).

SMAD3 and EGR1 functionally interact at the LHB/Lhb promoter

To explain the observed modulation of the GNRH1response by activin A, we hypothesized that one or both of the canonical transducers of activin signalling, SMAD2 and SMAD3, may modulate GNRH1 signalling. Indeed, over-expression of SMAD3, and to a lesser extent SMAD2, partially inhibited GNRH1-stimulated human *LHB* promoter activity in LβT2 cells (Fig. S5.1B). There was a trend for SMAD3 to potentiate GNRH1 induction of the murine *Lhb* promoter, but this was not statistically significant (p=0.09) (Fig. S5.1A).

Because induction of the *LHB*/*Lhb* promoter by GNRH1 is largely EGR1-dependent (145, 510, 524, 880)., we hypothesized that SMAD2 and/or SMAD3 might functionally interact with EGR1. To address this possibility, we examined the effects of over-expressed SMAD2 or SMAD3 on EGR1-stimulated murine or human reporters in LβT2 cells. Transfection of EGR1 stimulated both promoters (Figs. 5.2A and 5.2B). Neither of the SMAD proteins alone significantly modulated the activity of either promoter. However, SMAD3 co-transfection potentiated or partially inhibited the effect of EGR1 on the murine (Fig. 5.2A) or human (Fig. 5.2B) promoter-reporters, respectively. Similarly, SMAD2 enhanced activation of the murine promoter and inhibited activation of the human promoter by EGR1, albeit to a lesser extent than SMAD3 (Figs. 5.2A and B). To determine whether the observed effect of SMAD2/3 on EGR1-mediated *LHB*/*Lhb* promoter activation is dependent on the presence or absence of the SBEs, we employed the mutant human (3SBE+) and murine (3SBEmut) promoters described above. Strikingly, in both species, the effect of SMAD3 on EGR1-stimulated promoter activation was completely reversed in the mutant promoters. That is, SMAD3 markedly enhanced EGR1 induction of the 3SBE+ human promoter (Fig. 5.2D), whereas SMAD3 attenuated EGR1-stimulated 3SBEmut murine promoter activity (Fig. 5.2C). SMAD2 had no significant effect on EGR1-stimulated activation of either the mutant murine or human promoter (Fig. 5.2C and D),

To further explore functional interactions between SMAD proteins and EGR1, we employed HEK293 cells. This system avoided possible confounding effects of endogenous EGR1- and SMAD-interacting factors regulating the proximal *LHB*/*Lhb* promoter in homologous cells, such as paired-like homeodomain (PITX) transcription factors (510, 537, 539), Expression of EGR1 was sufficient to activate both the murine and human *Lhb/LHB* promoters in HEK293 cells (Figs. 5.3A and B), though to a lesser extent than in homologous cells. Co-expression of SMAD3, but not SMAD2, enhanced EGR1-mediated activation of the murine promoter (Fig. 5.3A). In contrast, SMAD3 partially inhibited EGR1-mediated activation of the human *LHB* promoter (Fig. 5.3B). SMAD2 failed to modulate EGR1-mediated activation of the human reporter.

SMAD3 physically interacts with EGR1

To gain greater insights into the mechanism(s) underlying the functional interaction between EGR1 and SMAD3, we asked whether the two proteins can physically interact. We first

performed co-immunoprecipitation (co-IP) experiments in CHO cells. Cells were transfected with FLAG-tagged SMAD2 or SMAD3 along with untagged EGR1 or empty vector (pcDNA3). SMAD proteins and EGR1 were expressed at similar levels across conditions (Fig. 5.4A, 'Total' panels), and both SMAD proteins were immunoprecipitated with similar efficiency (Fig. 4A, second panel, lanes 1-2). Importantly, EGR1 was co-precipitated with FLAG-SMAD3, but not FLAG-SMAD2 (Fig. 5.4A, top panel, compare lanes 1 and 2). EGR1 was not detected in the immunoprecipitates from cells co-transfected with empty vector (Fig. 5.4A, top panel, lane 3). Similar results were obtained when we performed the reverse co-IP experiment. That is, FLAG-EGR1 was able to co-IP untagged SMAD3, but not SMAD2 in transfected CHO cells (Fig. 5.4B, first panel, compare lanes 1 and 2). To assess whether the interaction between EGR1 and SMAD3 was preserved in the context of the homologous gonadotrope cell line, LβT2 cells were transfected with FLAG-SMAD3 or empty vector and stimulated with GNRH1 to induce expression of endogenous EGR1 (510). GNRH1 treatment for 1 h led to robust induction of EGR1 protein expression (Fig. 5.4C, third panel, compare lanes 1 and 3 with lane 2). Endogenous EGR1 was co-precipitated with FLAG-SMAD3, confirming that the two proteins can interact in LβT2 cells (Fig. 5.4C, top panel, lane 3).

Next, we mapped the sub-domains of each protein mediating their interaction. Co-IP was performed with lysates of CHO cells transfected with FLAG-tagged SMAD3 sub-domains along with full-length untagged EGR1. EGR1 was detected in the immunoprecipitates from cells co-transfected with each of the SMAD3 sub-domains (Fig. 5.5A, top panel, lanes 2-6), but not from cells co-transfected with empty vector (Fig. 5.5A, top panel, compare lane 1 to 2-6). However, the interaction was strongest with fragments containing the SMAD3 C-terminus (i.e., the MH2 domain; Fig. 5A, top panel, lanes 5 and 6). For some of the SMAD3 sub-domains (L-MH2 and MH2), western blot analysis of total cell lysates and of the immunoprecipitated fraction revealed multiple, rather than single bands (Fig. 5.5A, second and fourth panels, lanes 5-6). Though the exact nature of these products is currently unknown, the pattern we observed is consistent with previous results with the same constructs (671).

We performed a similar analysis to determine which domain(s) of EGR1 mediates its physical interaction with SMAD3 (Fig. 5.5B). To this end, we generated constructs coding for FLAG-tagged fragments of EGR1 corresponding to its main functional sub-domains (881). SMAD3 interacted most robustly with the isolated DNA-binding domain (DBD) of EGR1 (Fig.

5.5B, top panel, lane 4), and to a lesser extent with a fragment comprising the DBD and the AF2 domain (Fig. 5.5B, top panel, lane 5). A very faint interaction between SMAD3 and the N-terminal portion of EGR1 encompassing the AF1 domain and the DBD was detected (Fig. 5.5B, top panel, lane 3). No SMAD3 was detected in the immunoprecipitates from cells co-transfected with the isolated FLAG-tagged AF1 domain or empty vector (Fig. 5.5B, top panel, lanes 1-2). In this experiment, interaction between SMAD3 and full-length EGR1 or the isolated C-terminal AF2 domain could not be compared to other EGR1 trunctations because these constructs were expressed at much lower levels in CHO cells, even when an excess of expression vector was transfected (data not shown). Taken together, these co-IP analyses indicated that SMAD3 and EGR1 interact predominantly through the SMAD3 MH2 domain and the EGR1 DBD.

Effect of activin A on GNRH1-stimulated EGR1 recruitment at the LHB/Lhb promoter

Because we observed that SMAD3 and EGR1 physically and functionally interact, we hypothesized that activin signalling may modulate the function of endogenous EGR1 in LβT2 cells, resulting in the observed changes in GNRH1-stimulated *LHB/Lhb* transcription. First, we verified whether activin A co-administration modulates the expression, stability, or sub-cellular localization of EGR1 produced following GNRH1 stimulation in LβT2 cells. However, up to 6 h after GNRH1 application, there was no effect of activin A on the magnitude or kinetics of EGR1 protein induction or on its nuclear accumulation (Fig. S5.2). GNRH1 induction of *LHB*/*Lhb* transcription is dependent on EGR1 protein expression and binding to the proximal promoter (510, 880, 883). Therefore, we asked whether activin A signalling modulates the recruitment of EGR1 to the murine and human promoters. To assess this possibility, we performed chromatin immunoprecipitation (ChIP) assays in LβT2 cells transfected with the human *LHB* promoter and treated with GNRH1, activin A, or the two in combination. GNRH1 substantially enhanced EGR1 binding to both the transfected human *LHB* promoter and the endogenous murine *Lhb* promoter, whereas activin A had no effect by itself on EGR1 occupancy of either promoter (Figs. 5.6A and 5.6B). Interestingly, in all four replicates of the experiment, EGR1 binding to the human promoter was lower when the cells we co-stimulated with GNRH1 and activin A compared with GNRH1 alone (Fig. 5.6A). In contrast, there was no difference in the occupancy of the murine *Lhb* promoter by EGR1 in cells stimulated with GNRH1 alone compared with cells stimulated with GNRH1 plus activin A (Fig. 5.6B). Thus, activin A attenuated EGR1 recruitment

to the human but not murine promoter, perhaps explaining its partial antagonism of GNRH1-stimulated human *LHB* promoter activity.

SMAD3 enhances the association between EGR1 and PITX1

As activin A did not appear to enhance GNRH1-stimulated EGR1 recruitment to the murine *Lhb* promoter, we examined alternative mechanisms for activin A potentiation of GNRH1-induced murine *Lhb* promoter activity. EGR1 acts in concert with *paired*-like homeodomain transcription factor 1 (PITX1) to regulate *LHB/Lhb* transcription in a variety of species (510, 880). Interestingly, PITX1 can physically interact with both EGR1 and SMAD3 (510, 537, 688). We therefore hypothesized that SMAD3 might enhance the association between EGR1 and PITX1. CHO cells were transfected with FLAG-tagged PITX1 along with untagged EGR1 and untagged SMAD2, SMAD3, or pcDNA3 (empty vector). Consistent with previous observations (510, 537, 688), both EGR1 and SMAD3 were co-precipitated with FLAG-PITX1 (Fig. 5.7, top panel, lane 2 and second panel, lane 3). Importantly, in presence of SMAD3, but not SMAD2, the interaction between FLAG-PITX1 and EGR1 was enhanced (Fig. 5.7, top panel, compare lanes 3 and 4 with lane 2).

Discussion

Using murine and human *LHB*/*Lhb* promoters as a model, we report species-specific modulation of GNRH1-stimulated promoter activation by activin A. In particular, we describe novel functional and physical interactions between SMAD3, a canonical transducer of activin signalling, and EGR1, a mediator of GNRH1-stimulated *LHB*/*Lhb* promoter activation. Globally, our data indicate that: 1) species-specific modulation of the GNRH1/EGR1 response by activin A/SMAD3 is dependent upon the presence or absence of three non-conserved SMAD binding elements (SBEs) in the proximal promoter; 2) addition or removal of the SBEs reverses the effect of SMAD3 on EGR1-induction of the *LHB*/*Lhb* promoters; 3) SMAD3 physically interacts with EGR1 and has a more pronounced effect on EGR1 function than SMAD2; and 4) activin A signalling modulates GNRH1-stimulated EGR1 binding to the human *LHB*, but not to the murine *Lhb* promoter.

Whereas activin potentiates GNRH1-stimulated murine *Lhb* promoter activation, it inhibits induction of the human promoter by GNRH1. Our data suggest that this difference stems, at least in part, from the presence of three SBEs within the proximal murine promoter, which are not conserved in human. Indeed, mutation of the murine SBEs to the corresponding human nucleotides abolishes the synergy between activin A and GNRH1. Conversely, introduction of SBEs into the human promoter prevents inhibition of the GNRH1 response by activin A. Interestingly, although these mutations block the activin A effect on both promoters, they do not confer one species-typical response to the other. Therefore, the SBEs are necessary but not sufficient for activin A potentiation of the GNRH1 response. At the same time, the SBEs are sufficient to prevent antagonism of the GNRH1 stimulation by activin A signalling when introduced in the human promoter. These results suggest that additional elements within the murine promoter, apparently absent (or different) in the human promoter, are required for synergism between activin A and GNRH1 signalling. Identifying these elements will further our understanding of the mechanistic basis for species-specific responsiveness to the combined action of GNRH1 and activin A.

Our data suggest that the effect of activin A on the GNRH1 response is mediated, at least in part, by an interaction between SMAD3 (an activin A effector) and EGR1 (a GNRH1 effector). That is, SMAD3 (and SMAD2 to a lesser extent) modulation of EGR1-stimulated

LHB/*Lhb* promoter activity recapitulates the effect of activin A on the GNRH1 response. However, unlike the case with ligand-stimulated promoter activity, the SBE manipulations completely reverse the effect of the SMAD3 on EGR1 function. Indeed, SMAD3 inhibits EGR1-stimulated induction of the murine promoter lacking SBEs whereas it potentiates EGR1-induction of the human promoter possessing SBEs. These results indicate that the SBEs are both necessary and sufficient for SMAD3 potentiation of the EGR1 response. Importantly, the responses of the wild-type and mutant constructs were similar, though not identical for SMAD3/EGR1 versus activin A/GNRH1. Further investigation is required to explain the different transcriptional responses. At a minimum, it seems likely that the modulation of GNRH1 signalling by activin A includes factors in addition to SMAD3 and EGR1, which directly or indirectly regulate the *LHB*/*Lhb* promoters, possibly in species-specific fashion.

At a mechanistic level, one way whereby activin A signalling could modulate GNRH1-stimulated *LHB*/*Lhb* promoter activation is by regulating EGR1 function. In the case of the human promoter, our previous data (880) predicts that reduced EGR1 activity or binding would attenuate the GNRH1 response. We observe that activin A fails to modulate GNRH1-stimulated EGR1 expression or nuclear translocation. Rather, the chromatin immunoprecipitation (ChIP) analyses indicate that activin A partially inhibits recruitment of EGR1 to the proximal human promoter. Perhaps, in the absence of SBEs, a physical interaction between SMAD3 and EGR1 may interfere with the binding of EGR1 to the human promoter. That the interaction occurs through the EGR1 DNA binding domain makes this a distinct possibility. In the murine promoter, the presence of SBEs may enable collaborative binding of EGR1 and SMAD3 to adjacent promoter elements and/or a means for SMAD3 to tether EGR1 to the promoter in the event that direct EGR1 binding is impaired. Though the ChIP data indicate that GNRH1-stimulated EGR1 association with the promoter is the same in the presence or absence of activin A, the analysis does not permit an assessment of the nature of the binding (i.e., direct vs. tethering). Nonetheless, these data do suggest that the synergism is not explained simply by enhanced EGR1 binding to the murine promoter.

How, then, might activin A potentiate GNRH1-induced promoter activity? One possibility is activin A enhancement of EGR1 cooperation with other transcription factors that regulate the *Lhb* promoter (reviewed in (485)). Consistent with this possibility, we observe that SMAD3 potentiates the physical interaction between EGR1 and PITX1. Because all three factors can bind

adjacent elements in the murine promoter (Fig. 5.1C), its activation may be amplified as a result. This could involve enhanced recruitment of co-factors, such as p300 (884).

Although both SMAD2 and SMAD3 modulate EGR1-mediated *trans*-activation of the *LHB*/*Lhb* promoter, the effect of SMAD2 over-expression is weaker than that of SMAD3, and SMAD2 has no effect on EGR1 induction of the SBE mutant promoters. Further, in heterologous HEK293 cells, SMAD3, but not SMAD2, was able to functionally interact with EGR1. The absence of functional interaction between SMAD2 and EGR1 in these cells was not due to the lack of DNA-binding activity of SMAD2, because a DNA-binding splice variant, SMAD2$^{\Delta ex3}$ (673), was similarly ineffective (data not shown). Instead our novel observation that SMAD3, but not SMAD2, can physically associate with EGR1 may explain their different activities. The structural basis for this selectivity is not yet clear as SMAD3 interacts with EGR1 principally via its MH2 domain, which is highly conserved in SMAD2 (97% identity in mouse). Nonetheless, our observations indicated that SMAD2 and SMAD3 are distinct in their abilities to modulate EGR1 function. This is consistent with a large body of evidence showing that SMAD2 and SMAD3 can each serve distinct roles in transcriptional regulation by interacting with a unique complement of transcription factors (703, 885).

The results of the experiments described here may have significance beyond regulation of the *LHB*/*Lhb* gene. Indeed, crosstalk between SMAD-mediated signalling and EGR1-regulated gene transcription has been reported in other contexts. For example, TGFβ signalling up-regulates EGR1 expression via SMAD3 in primary human fibroblasts (however, we did not see a similar effect by activin A in LβT2 cells), enhancing EGR1-mediated type I protocollagen gene transcription (886). Recently, it was reported that EGR1 can repress TGFβ-stimulated smooth muscle α-actin transcription by competing for SMAD2 and SMAD3 binding to the gene promoter (887). Our results demonstrate that SMAD3 and EGR1 can physically associate in cells and this interaction leads to either potentiation or attenuation of target gene transcription depending on the nature of the promoter. Because SMAD3 and EGR1 are activated by a range of stimuli and regulate transcription of a large number of genes, we speculate that uncovering their physical and functional interaction will be of broad interest.

Acknowledgements

The authors thank Ying Wang for important observations during the early part of this work. The authors also thank several investigators for their generous gifts of reagents, as detailed in the *Materials and Methods*. Dr. Terry Hébert provided valuable comments on an earlier draft of this manuscript. This work was supported by the Canadian Institutes of Health Research (CIHR) (grant no. MOP-89991). DJB is a Chercheur-boursier of the Fonds de recherche en santé du Québec (FRSQ). JF was supported by studentships from the FRSQ and CIHR, as well as a Summer Research Fellowship from the Endocrine Society.

Figure Legends

Figure 5.1: Modulation of GNRH1-stimulated LHB/Lhb promoter activation by activin A. A) LβT2 cells were transfected with 225 ng/well of the wild-type murine -232/+5 *Lhb*-luc reporter (WT) or the same reporter with the three SMAD-binding elements mutated to the corresponding bases found in the human promoter (3SBEmut). Cells were treated for 6h with 10^{-8} M GNRH1 and/or 25 ng/mL activin A. Ligand-stimulated reporter activity was normalized to basal activity ('control') specific for each construct, and data from the WT and 3SBEmut reporters were analyzed separately. Bars with different symbols differ significantly. Data represent the mean +/- SEM of six independent experiments conducted in triplicate. B) LβT2 cells were transfected with 225 ng/well of the wild-type human -196/+9 *Lhb*-luc reporter (WT) or the same reporter mutated to introduce the three SMAD-binding elements of the murine promoter (3SBE+). Cells were treated for 6h with 10^{-8} M GNRH1 and/or 25 ng/mL activin A. Ligand-stimulated reporter activity was normalized to basal activity ('control') specific for each construct, and data from the WT and 3SBE+ reporters were analyzed separately. Bars with different symbols differ significantly. Data represent the mean +/- SEM of seven independent experiments conducted in triplicate. C) Alignment of proximal *LHB/Lhb* promoters from human and mouse. Bases are numbered relative to the transcriptional start site (+1; not shown). Nucleotides that differ between the species are shaded. The conserved EGR1, SF1 and PITX binding sites are boxed. SMAD-binding elements (SBEs) are numbered according to (688) and double-underlined. 'p', proximal; 'd', distal.

Figure 5.2: Functional interaction between SMAD3 and EGR1 at the LHB/Lhb promoters in LβT2 cells. LβT2 cells were transfected with 225 ng/well of the murine -232/+5 *LHB*-luc (A) or human -196/+9 *Lhb*-luc (B) reporters along with 50 ng/well EGR1 expression vector and/or 50 ng/well FLAG-SMAD3, FLAG-SMAD2, or empty vector (pcDNA3). Bars with different symbols differ significantly. Data represent the mean +/- SEM of four (A) or six (B) independent experiments conducted in triplicate. LβT2 cells were transfected with 225 ng/well of the murine -232/+5 *LHB*-luc (C) or human -196/+9 *Lhb*-luc (D) reporters along with 50 ng/well EGR1 expression vector and/or 50 ng/well FLAG-SMAD3, FLAG-SMAD2, or empty vector

(pcDNA3). Bars with different symbols differ significantly. Data represent the mean +/- SEM of four independent experiments conducted in triplicate.

Figure 5.3: Functional interaction between SMAD3 and EGR1 at the LHB/Lhb promoters in HEK293 cells. HEK293 cells were transfected with 225 ng/well of the murine -232/+5 *Lhb*-luc (A) or human -196/+9 *LHB*-luc (B) reporters along with 50 ng/well EGR1 expression vector and/or 0.5 µg/well FLAG-SMAD3, FLAG-SMAD2 or empty vector (pcDNA3). Bars with different symbols differ significantly. Data represent the mean +/- SEM of three independent experiments conducted in triplicate.

Figure 5.4: Physical interaction between SMAD3 and EGR1. A) CHO cells were co-transfected with 4 µg EGR1 along with 4 µg FLAG-SMAD2 or FLAG-SMAD3 expression vectors or empty vector (pcDNA3). Whole-cell lysates were collected and subjected to FLAG immunoprecipitation (IP) using anti-FLAG M2 affinity beads. Eluted proteins or whole cell lysates (Total) were analyzed by immunoblot (IB) using the indicated antibodies. B) CHO cells were co-transfected with 4 µg FLAG-tagged EGR1 or empty vector (pcDNA3) along with 4 µg SMAD2 or SMAD3 expression vectors. Whole cell lysates were processed and analyzed as in (A). C) LβT2 cells were transfected with FLAG-SMAD3 or empty vector (pcDNA3) and treated where indicated with 10^{-7} M GNRH1 for 1 h. Whole cell lysates were processed and analyzed as in (A).

Figure 5.5: Sub-domains of SMAD3 and EGR1 mediating their physical interaction. A) CHO cells were co-transfected with EGR1 expression vector along with empty vector (pcDNA3), full-length FLAG-tagged SMAD3 (full) or the indicated truncated FLAG-SMAD3 expression vectors (MH1, N-terminal MH1 domain; L, linker region; MH2, C-terminal MH2 domain). Whole-cell lysates were subjected to FLAG IP and immunoblotting as in Fig. 3. B) CHO cells were co-transfected with SMAD3 expression vector along with empty vector (pcDNA3) or the indicated truncated FLAG-tagged EGR1 expression vectors (AF1, N-terminal AF1 domain, amino acids 1-281; DBD, DNA-binding domain, amino acids 275-425; AF2, C-terminal AF2 domain, amino acids 420-533). The amount of vector transfected was titrated to achieve similar expression

levels. Total DNA transfected was balanced with pcDNA3 across all conditions. Whole cell lysates were processed and analyzed as in Fig. 3.

Figure 5.6: Effect of activin signalling on GNRH1-stimulated EGR1 recruitment to the LHB/Lhb promoters. A and B) LβT2 cells were transfected with the human -196/+9 *LHB*-luc reporter, and stimulated where indicated with 10^{-7} M GNRH1 and/or 25 ng/mL activin A for 2 h. After ligand treatment, cells were cross-linked and harvested. Sonicated chromatin was immunoprecipitated with IgG or EGR1 antibody. Input and precipitated DNA were analyzed by quantitative PCR using primers amplifying the transfected proximal human *LHB* promoter (A) or the endogenous murine *Lhb* promoter (B). The qPCR signal was normalized within each condition as described in the *Materials and Methods*. Data represent the mean +/- SEM of the binding observed for each condition relative to the GNRH1-stimulated condition in four independent experiments.

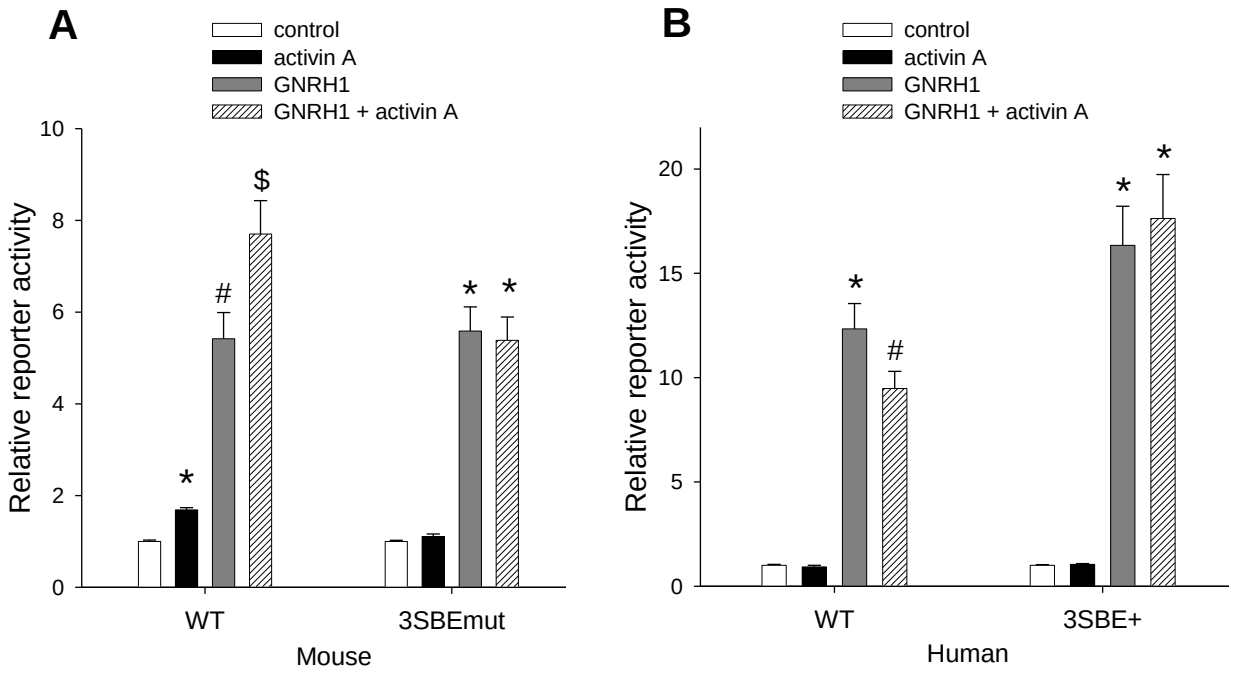
Figure 5.7: SMAD3 enhances the association of EGR1 and PITX1. CHO cells were co-transfected with 4 μg EGR1 along with 4 μg FLAG-PITX1 and 4μg SMAD2 or SMAD3 expression vectors or empty vector (pcDNA3). Whole-cell lysates were collected and subjected to FLAG immunoprecipitation (IP) using anti-FLAG M2 affinity beads. Eluted proteins or whole cell lysates (Total) were analyzed by immunoblot (IB) using the indicated antibodies.

Supplemental Figure Legends

Figure S5.1: Effect of SMAD2/3 overexpression on GNRH1-stimulated LHB/Lhb promoter activation. LβT2 cells were transfected with 225 ng/well of the murine -232/+5 *Lhb*-luc (A) or human -196/+9 *LHB*-luc (B) reporters, along with 50 ng/well SMAD2 or SMAD3 expression vectors, or empty vector (pcDNA3). Cells were treated for 6 h with 10^{-8} M GNRH1. Bars with different symbols differ significantly. Data represent the mean +/- SEM of five independent experiments conducted in triplicate.

Figure S5.2: Effect of activin signalling on GNRH1-stimulated EGR1 protein expression, stability, and sub-cellular localization. LβT2 cells were stimulated with 10^{-7} M GNRH1 and/or 25 ng/mL activin A for the indicated times, or left untreated. Cells were collected, and the

cytoplasmic and nuclear fractions separated. The fractions were analyzed by immunoblot using the indicated antibodies.



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Human  -133 CCCTGGCCATGTGCACCTCTCGCCCCCGGGGGGATTAGTGTCCAGGT  -86

                                              pSF1     pEGR1
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Figure 5.1

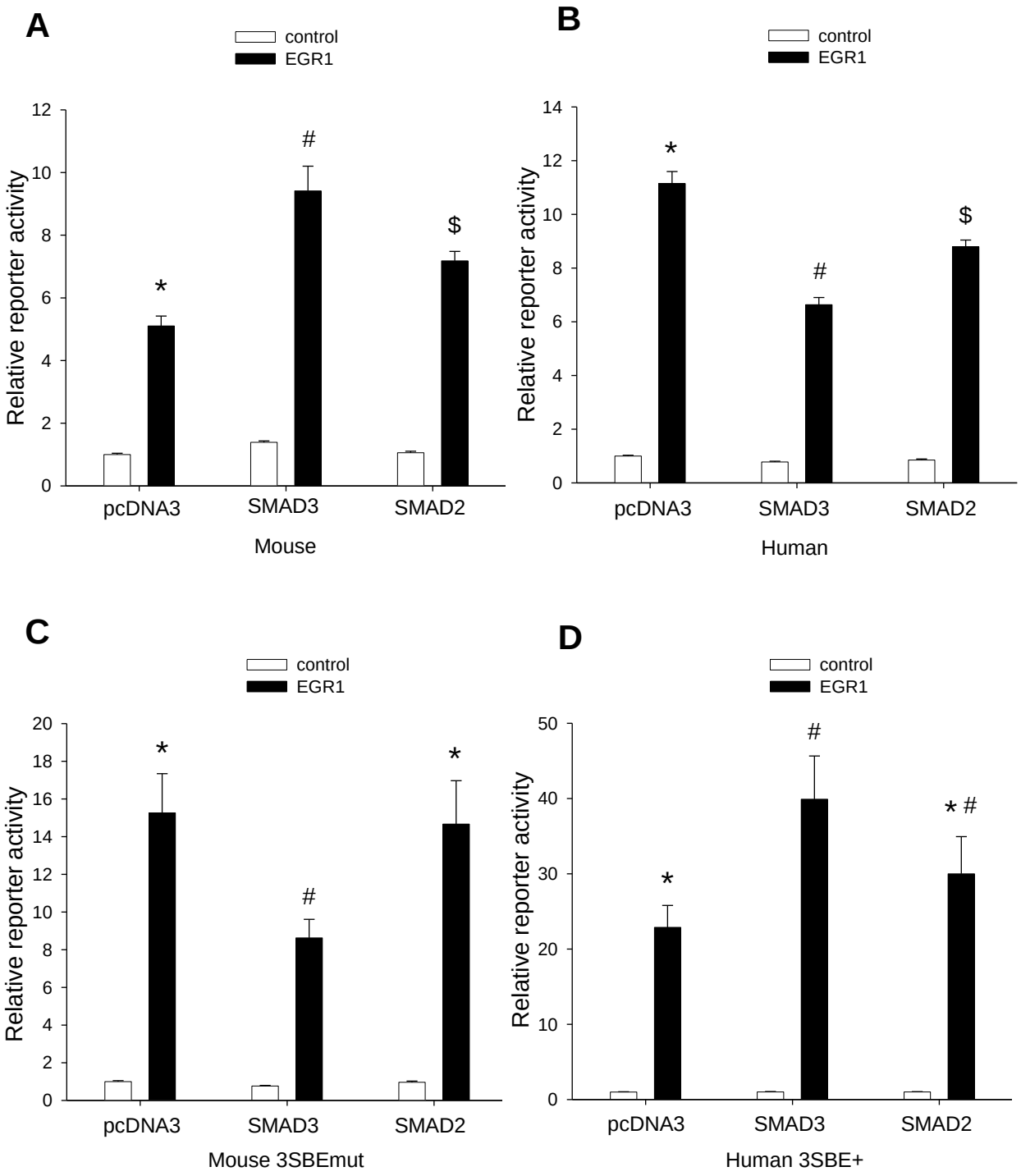
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Figure 5.2

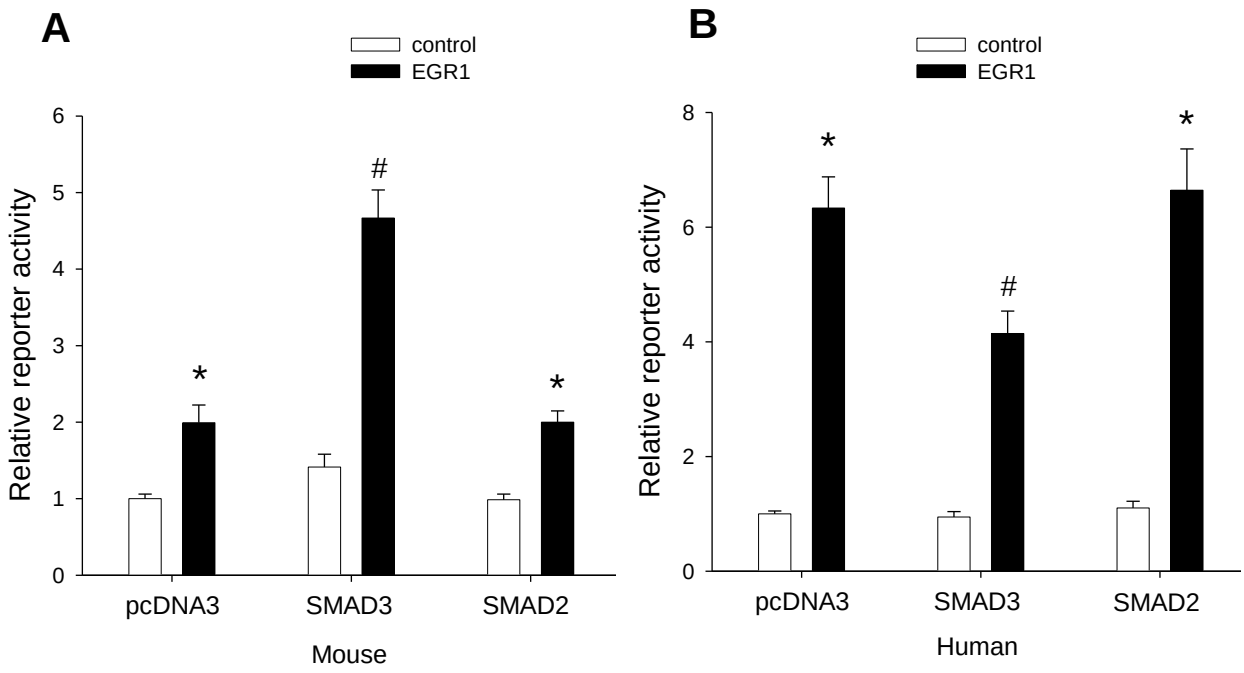
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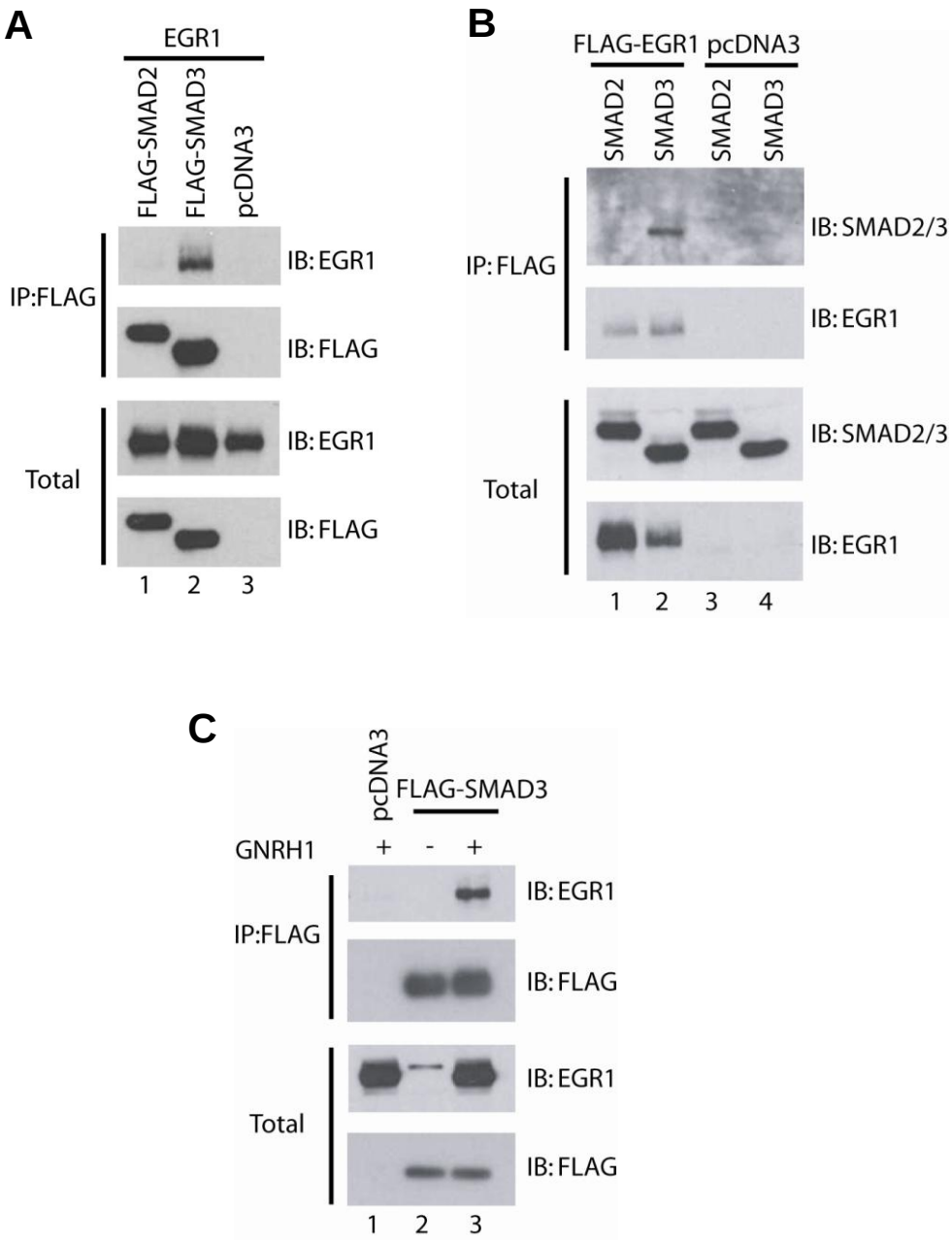
Figure 5.3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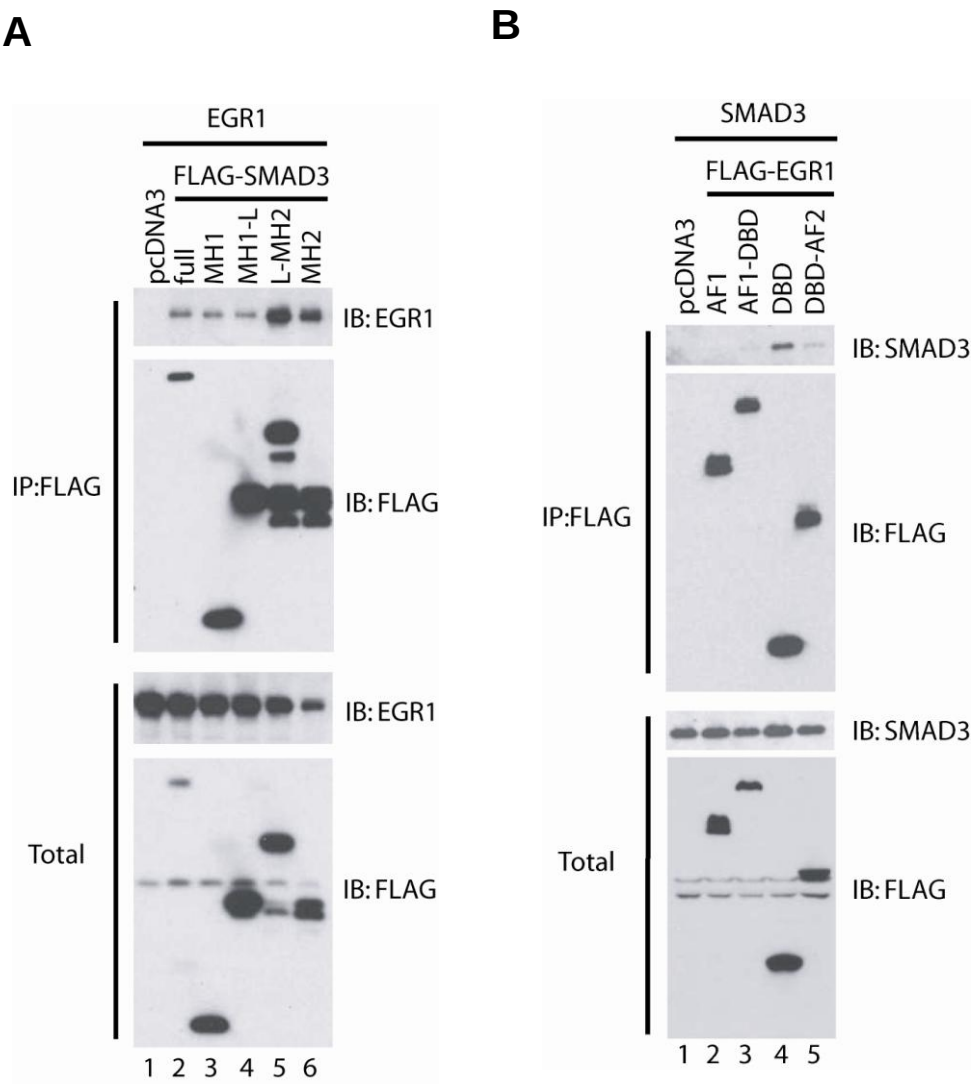
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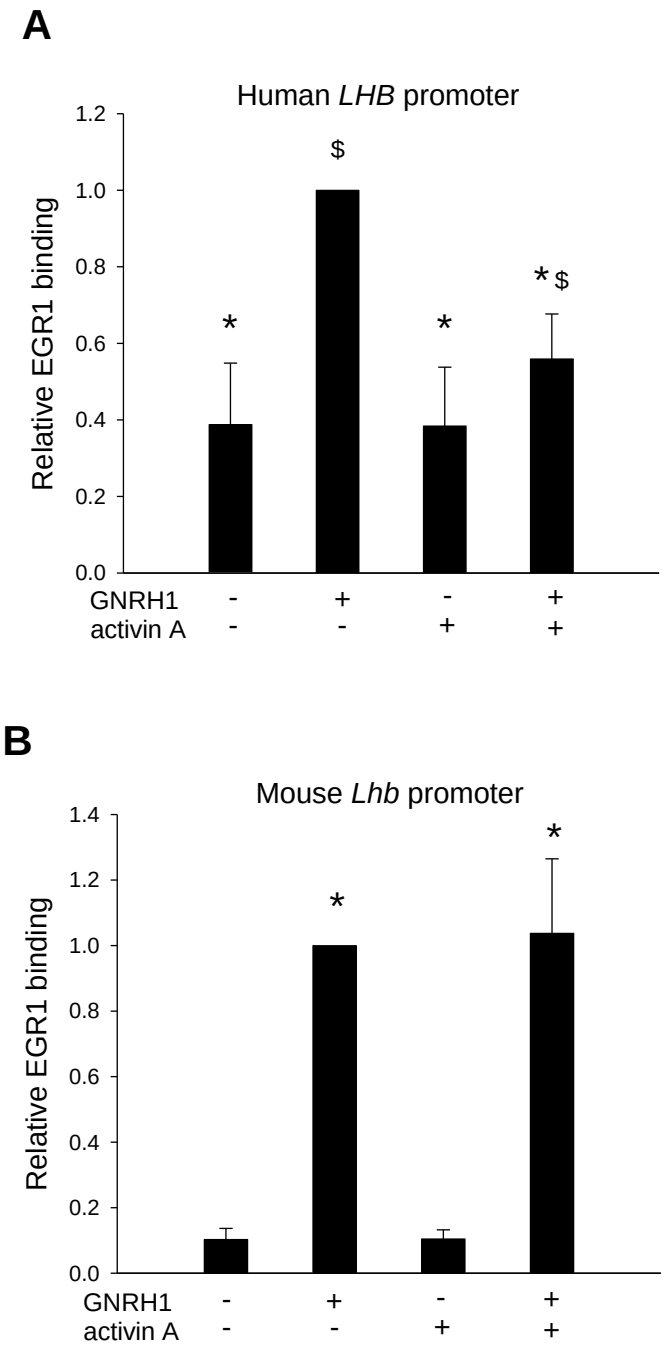
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GNRH1 - + - +
activin A - - + +
B
Mouse *Lhb* promoter
Relative EGR1 binding
1.4
1.2
1.0
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0.4
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GNRH1 - + - +
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Figure 5.6

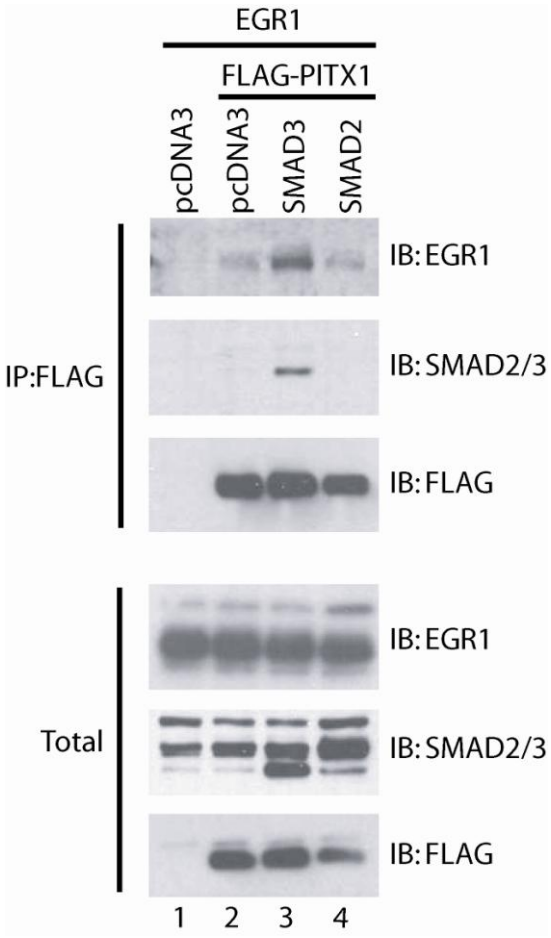
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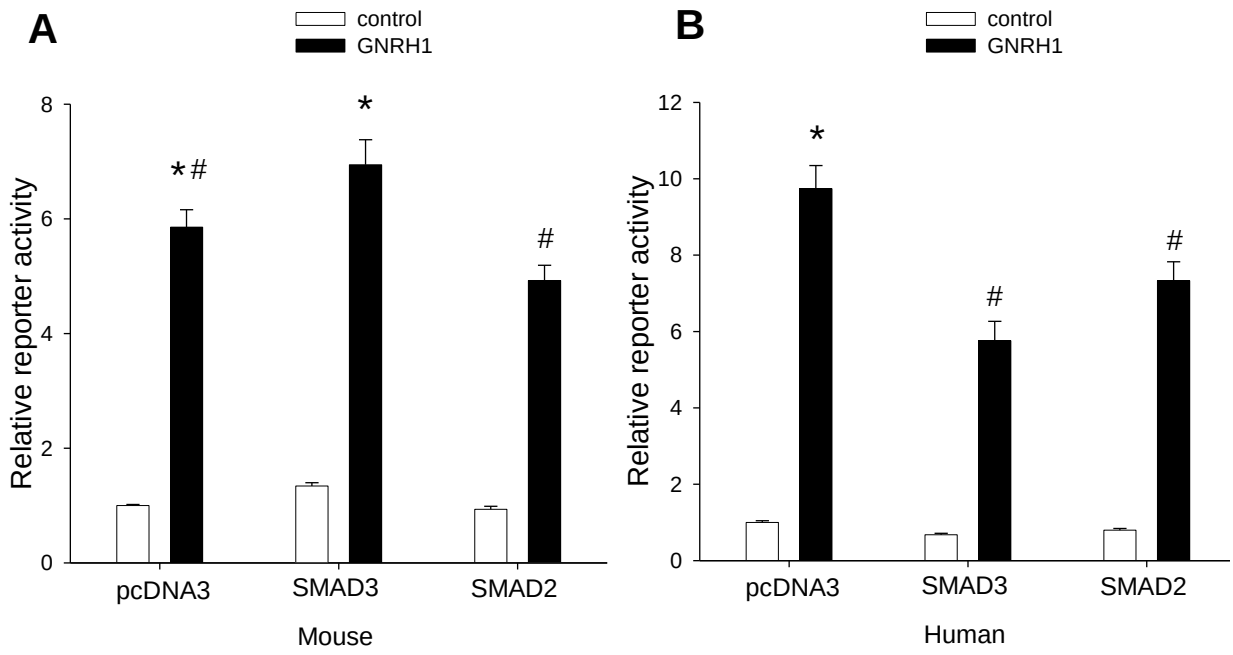
Figure 5.7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Relative reporter activity
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*
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pcDNA3
SMAD3
SMAD2
Human

Figure S5.1

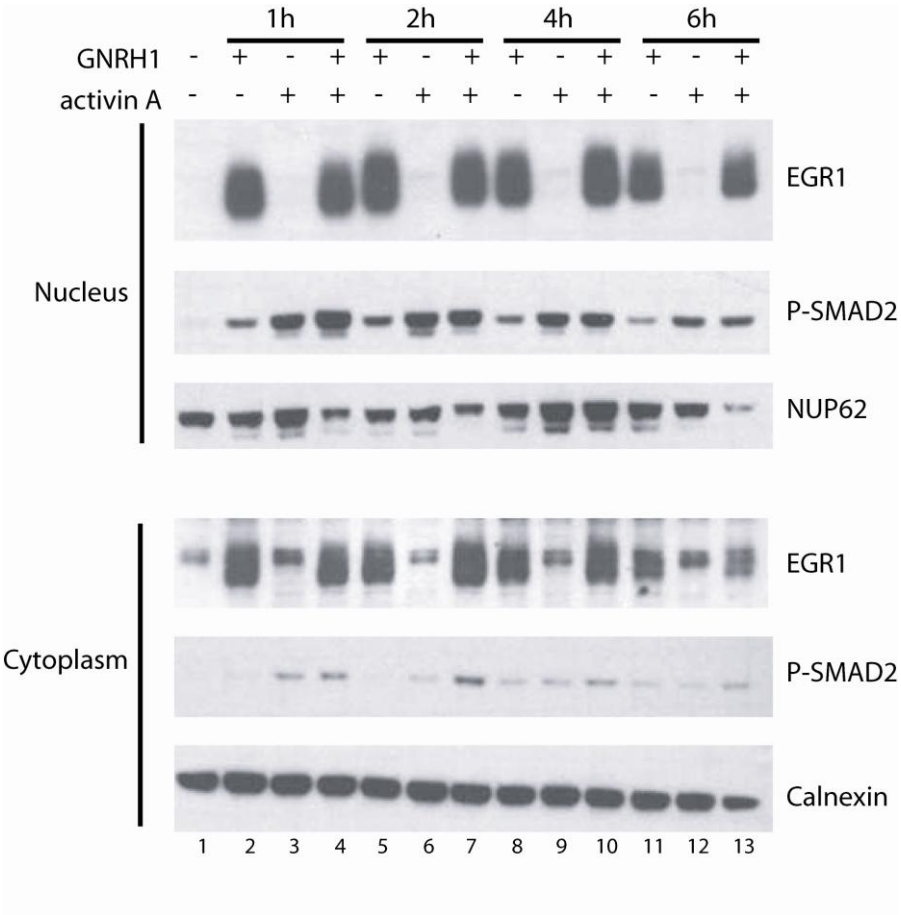
1h
2h
4h
6h
GNRH1
activin A
Nucleus
EGR1
P-SMAD2
NUP62
Cytoplasm
EGR1
P-SMAD2
Calnexin
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure S5.2

Chapter 6

In chapters 2 and 3, I showed that SMAD4/FOXL2-dependent FSH synthesis is required for ovarian follicle maturation in females. Once mature (pre-ovulatory) follicles develop, the high levels of estradiol they secrete positively feedback on the hypothalamus and pituitary to stimulate GnRH release and/or action. This results in a surge of LH synthesis and release, triggering ovulation. These protracted LH surges occur in all mammals, including humans. In chapter 4, I showed that the mechanisms underlying GnRH-stimulated *Lhb/LHB* transcription are conserved between humans and other species. Such conservation suggests that GnRH-stimulated LH synthesis is similarly important in all mammals. Yet, it is well known that a given GnRH pulse releases only a small fraction of the pool of available LH packaged in dense-core secretory granules (888-890). Therefore, gonadotrope cells can probably respond to many GnRH pulses before becoming depleted of LH stores. Why, then, is GnRH-stimulated *Lhb/LHB* transcription and LH synthesis so important? At the time of the LH surge, robust GnRH induction of *Lhb/LHB* transcription may be required for rapid replenishment of intracellular LH stores and to increase the amount of releasable LH (891). Thus, during the LH surge, it may be necessary to keep GnRH signaling capability intact for a long period of time. The GnRH receptor (GnRHR) is a member of the G protein-coupled receptor (GPCR) superfamily, which typically internalize rapidly after ligand binding (371). However, the mammalian GnRHR lacks an intracellular C-terminal "tail", a critical requirement for receptor internalization. Interestingly, non-mammalian species, in which no – or attenuated – LH surges occur, have C-tails on their GnRHRs. Was the loss of the GnRHR C-tail an evolutionary adaptation enabling prolonged gonadotrope responsiveness to GnRH at the time of the LH surge? In this chapter, I explored this intriguing question by generating and analyzing a new conditional knock-in mouse line expressing a chimeric GnRHR fused to the C-tail of the chicken GnRHR.

Title: Normal gonadotropin synthesis and female fertility depend on a unique structural property of the mammalian gonadotropin-releasing hormone receptor.

Short title: Role of a unique structural property of the mammalian GnRHR.

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Abstract

Gonadotropin-releasing hormone (GnRH) is a critical regulator of reproductive function and fertility in vertebrates. The hormone is released in pulses by hypothalamic neurons and acts on pituitary gonadotrope cells to stimulate the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone. In females, once per reproductive cycle, GnRH triggers a protracted LH surge, which in turn acts on the ovaries to induce ovulation. The LH surge depends on enhanced GnRH release, augmented pituitary sensitivity to the hormone, or some combination of the two. Regardless, it is clear that the GnRH receptor (GnRHR) can continue to signal in response to sustained agonist stimulation. Indeed, clinically, long-term agonist treatment is required to downregulate GnRHR signaling. Mammalian GnRHRs, unlike their non-mammalian counterparts, lack intracellular carboxyl tails (C-tails). This domain mediates homologous desensitization of other G protein-coupled receptors. We therefore hypothesized that the absence of a C-tail on their GnRHRs may enable mammals to generate large LH surges. To test this idea, we generated a knock-in mouse line, in which the endogenous *Gnrhr* gene encodes a chimeric GnRHR fused to the chicken GnRHR C-tail. *In vitro*, this receptor was impaired in its ability to mediate agonist-induced extracellular regulated kinase 1 and 2 signaling, which underlies GnRH-induced LH synthesis. Knock-in females displayed abnormal estrous cyclicity and subfertility. Importantly, they appeared impaired in their ability to generate estrogen-induced LH surges. Collectively, our results suggest that the loss of the GnRHR C-tail may represent an evolutionary adaptation enabling robust LH surge generation and maximizing reproductive success in mammals.

Introduction

The hypothalamic decapeptide, gonadotropin-releasing hormone (GnRH), is a critical regulator of vertebrate reproduction (441). Mice and humans with inactivating mutations in the genes encoding GnRH or its receptor (GnRHR) are hypogonadal and sterile (7, 8, 62, 63, 846, 848, 892). GnRH is released in pulses from nerve terminals in the median eminence and travels via the portal vessels to the anterior pituitary gland (893, 894). There, the hormone binds the GnRHR on pituitary gonadotrope cells, initiating signaling events that culminate in the synthesis and secretion of the heterodimer glycoproteins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (9, 441). LH and FSH (collectively known as the gonadotropins) travel via the systemic circulation to regulate gonadal function, including gametogenesis and steroid hormone production.

GnRH stimulates LH release from secretory granules via Ca^{2+}-dependent exocytosis (412). Though a typical GnRH pulse triggers the secretion of only a small proportion of the available hormone, (888, 889, 891) it simultaneously promotes *de novo* LH synthesis (441), presumably to replace what was secreted. GnRH induces the production of the transcription factor, early growth response 1 (EGR1), via an extracellular regulated kinase 1/2 (ERK1/2)-dependent pathway (145, 449, 457, 468, 505, 510, 524). EGR1 then forms complexes with steroidogenic factor 1 (SF1 or NR5A1) and *paired*-like homeodomain transcription factors (PITX1/2) to stimulate transcription of the LHβ subunit gene (*Lhb*) via conserved proximal promoter *cis*-elements (437, 506-510, 513, 516, 517, 880).

GnRH-regulated LH synthesis may be most critical at the time of the preovulatory LH surge. That is, in females, GnRH stimulates a massive release of LH, which in turn drives several periovular events, including resumption of meiosis, expansion of cumulus granulosa cells, luteinization of mural granulosa cells, and ultimately follicle rupture (307). In rats, pituitary LH content remains stable throughout the LH surge, despite the marked increase in hormone secretion (895). This suggests that the pituitary upregulates LH synthesis to compensate for the increased secretion. Indeed, *Lhb* mRNA levels increase in association with (if not before) the LH surge (895). Surge generation depends upon increased GnRH secretion (at least in some species) as well as enhanced sensitivity of pituitary gonadotropes to the hormone. The latter is due, at least in part, to up-regulation of the GnRHR (80, 188, 313, 315-317). Regardless, given the

length of the LH surge, lasting several hours in sheep and rodents and more than a day in humans (188, 245, 313), it is surprising that gonadotropes remain sensitive to protracted GnRH signaling. Though the underlying mechanisms are poorly understood, a unique characteristic of the GnRHR may provide some insight.

The mammalian GnRHR is unusual among G protein-coupled receptors (GPCRs) in that it lacks an intracellular carboxyl-terminal tail (C-tail) (9). In other GPCRs, ligand-dependent phosphorylation of the C-tail by GPCR kinases promotes the recruitment of adaptor proteins that direct receptor internalization, diminishing cellular sensitivity to further agonist exposure (so-called homologous desensitization) (368, 371-373, 896). By virtue of lacking C-tails, mammalian GnRHRs internalize slowly in response to agonist (379, 390). In contrast, non-mammalian GnRHRs (e.g., in birds, fish, and amphibians) have C-tails and show rapid agonist-dependent desensitization (384, 897-900). Fusion of a C-tail, either from a non-mammalian GnRHR or from unrelated GPCRs, to mammalian GnRHRs accelerates their ligand-induced internalization (380, 381, 383-385, 393). These observations suggest that the absence of a C-tail on GnRHRs may endow mammalian gonadotrope cells with the capacity to respond to sustained GnRH stimulation, as it occurs at the time of the LH surge.

As the GnRHRs of non-mammalian vertebrates possess C-tails (as do all other GPCRs), it is likely that the mammalian GnRHR lost its tail (e.g., due to the acquisition of a nonsense or frame-shift mutation) during evolution. Since all mammals have a tailless receptor, this change may have conferred some advantage over the ancestral form of the receptor. It seems possible that slow GnRHR desensitization kinetics enable the generation of high amplitude, long duration LH surges. These characteristics of the surges may be required for ovulation. Here, using a novel conditional knock-in mouse line, we tested the hypothesis that the addition of a C-tail to a mammalian GnRHR will disrupt LH surge generation and thereby impair fertility.

Materials and Methods

Plasmid construction, cell culture, and transfection

To construct the flag-tagged murine GnRHR and GnRHR-Ctail expression vectors, the coding sequence of the murine *Gnrhr* gene was amplified by PCR from immortalized gonadotrope (LβT2 cells a generous gift from Dr Pamela Mellon, UCSD) cDNA, using a forward primer introducing an *EcoRI* restriction site and omitting the translation initiation codon and reverse primer introducing an *XbaI* restriction site (Table S6.1). The resulting fragment was ligated in-frame downstream of a flag tag coding sequence preceded by a translation initiation codon in pcDNA3.0, yielding Flag-GnRHR. To generate Flag-GnRHR-Ctail vector, the STOP codon in Flag-GnRHR was replaced with a *ClaI* restriction site by site-directed mutagenesis. The C-tail coding sequence from the chicken *Gnrhr* gene was amplified by PCR from chicken embryonic genomic DNA (extracted from a chicken embryo provided by Dr Aimee Ryan, McGill University) using primers incorporating *ClaI* sites at both ends. This fragment was inserted into the *ClaI* site created at the end of the *Gnrhr* coding sequence. Correct orientation determined by sequencing. Chinese hamster ovary (CHO) cells (a gift from Dr. Patricia Morris, The Population Council, New York, NY) were cultured in DMEM-F12 media supplemented with 10% fetal bovine serum (both from Gibco, Life Technologies, Carlsbad, CA, USA). Cells were seeded at a density of 1.5 X 10^5 cells/well in 6-well plates two days prior to transfection. One µg of Flag-GnRHR or the Flag-GnRHR-Ctail expression vector was transfected in each well using Lipofectamine (Invitrogen, Life technologies, Carlsbad, CA, USA). The next day, the growth medium was replaced with 1 mL serum-free medium per well, and GnRH (human GNRH1, Sigma, St Louis, MO, USA) was added to each well for the specified amount of time at a final concentration of 60 nM.

Western blotting

Following GnRH treatment, whole cell lysates were prepared on ice using radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors (Complete

Mini, Roche Applied Science, Indianapolis, IN, USA). Protein concentrations were assayed using the BCA protein assay kit (Pierce, Thermo Scientific, Waltham, MA, USA). Equal amounts of protein per sample were denatured in 50 mM Tris (pH 6.8), 2% SDS, 1% glycerol, 0.04% bromophenol blue, and 2% β-mercaptoethanol for 5 min at 99°C and loaded on 10% 37:5:1 polyacrylamide gels overlayed with 5% polyacrylamide stacking gels. Gels were subjected to electrophoresis at 90 volts for 30 min followed by 150 volts for 90 minutes in 25nM Tris, 250mM glycine and 0.1% SDS. Proteins were transferred to nitrocellulose membranes (Optitran BA-S 85, Whatman) at 25 volts for 90 minutes in 25 mM Tris, 192 mM glycine and 20% methanol. The membranes were then blocked for 1 h at room temperature with gentle agitation in Tris-buffered saline + 1% Tween-20 (TBST) containing 5% non-fat powdered milk. Membranes were then incubated overnight in primary antibodies (see below) in TBST-5% milk. Following three 15-min washes in TBST, membranes were incubated in 1:3000 secondary antibody diluted in TBST-5% milk at room temperature for 1 h. Following three more washes in TBST, protein-antibody complexes were revealed by adding ECL-Plus reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and exposing to film (Hyblot CL, Denville Scientific, Metuchen, NJ). Quantification was performed using Image J (National Institutes of Health, Bethesda, Maryland). Primary antibodies used were: Anti-Flag (1:2000, antibody #F3165, Sigma), anti-phospho ERK1/2 (1:1000, antibody #9102, Cell Signaling, Danvers, MA, USA), and anti-ERK1/2 (1:1000, antibody #05-1152, Millipore, Billerica, MA, USA). The secondary antibody was goat anti-rabbit IgG-HRP conjugate (antibody #170-6515, Bio-Rad, Hercules, CA, USA).

Targeting vector construction

To generate the downstream chromosomal (DCA) arm, a 6.7 kb DNA fragment starting 1 kb upstream of exon 3 was amplified by PCR using the Expand Long Template PCR System (Roche) from 129SvEv genomic DNA using primers incorporating 5' *XmaI* and 3' *NotI* restriction enzyme sites (all primers listed in Table S6.1). The fragment was cloned in pGEM-T easy. The STOP codon in exon 3 was replaced with a *ClaI* restriction enzyme site by site-directed mutagenesis. The *ClaI*-flanked C-tail coding sequence from the chicken *Gnrhr* (also used for the Flag-GnRHR-Ctail construct described above) was inserted, and correct orientation

was verified by sequencing. The whole DCA containing the chimeric exon 3 was ligated between the *XmaI* and *NotI* sites, 3' of the *Frt* flanked neomycin (neo) selection cassette, in pKOII (901). We used a two-step process to generate the upstream chromosomal arm (UCA) and the "floxed" exon 3 regions. First, a genomic DNA fragment starting 1 kb upstream of exon 3 and terminating immediately after the STOP codon in exon 3 was amplified by PCR using a 5' primer introducing a *XmaI* restriction site and a *loxP* site, and a 3' primer introducing a *PmeI* restriction site. This amplicon, along with a *PmeI-XhoI* fragment comprising the bovine growth hormone (BGH) polyA sequence (obtained by PCR from the pcDNA3.0 cloning vector) were ligated in a 3-part ligation between the *XmaI* and *XhoI* restriction sites of pBluescript II KS. To complete the UCA, a 3.6 kb fragment spanning exon 2 and terminating 1 kb upstream of exon 3 (the position of the upstream *loxP* site) was amplified by PCR using primers incorporating 5' *KpnI* and 3' *XmaI* sites, and joined to the *XmaI-XhoI* construct (in pBluescript II KS) described above. The whole UCA was then ligated into the *KpnI* and *XhoI* restriction sites in the pKOII vector containing the DCA, 3' of the negative selection cassette diphtheria toxin A (DTa). Sequencing was performed to ensure the integrity of the targeting vector and the absence of mutations in and around exons and splice-junctions (Genome Québec, Montreal, Canada). The targeting vector was linearized with *KpnI*, phenol-chloroform extracted, and resuspended at a final concentration of 1 μg/μl in Tris-EDTA.

Targeting in ES cells and knock-in mouse generation

Twenty-five μg of linearized targeting vector were electroporated into 10 million R1 ES cells (129/SvEv-derived) in triplicate, and each electroporated sample plated on primary mouse embryonic fibroblasts in two 10-cm dishes. The following day, culture media was supplemented with 200 μg/mL G418 for positive selection of clones incorporating the targeting vector. After 8 days of selection, 420 clones were picked manually under a dissecting microscope, dissociated in trypsin, and transferred to individual wells of 96-well plates. Cells were cultured for 5 days and then split into three separate plates. Two of plates were frozen at -80°C after the addition of 10% DMSO. Cells in the remaining plate were grown to confluence. Genomic DNA was extracted, cleaned with a series of 75% ethanol washes and digested overnight with *Xma*I. Homologous recombination events were screened by Southern blot using sequential hybridization with 5' and

3’ probes external to the homology arms (see Table S6.1 for the primers used to generate the probes). C57BL/6J blastocysts were microinjected with cells from two correctly targeted clones and transferred into pseudopregnant mothers at the Transgenic Core Facility of the Life Science Complex at McGill University. Resulting chimeric males were bred to C57BL/6J females and germline transmission of ES cell-derived DNA monitored by coat color. Brown pups were genotyped by PCR for the presence of the modified allele (denoted *Gnrhr*^{Ctailfloxneo}) and later confirmed by Southern blot. The *neo* cassette was removed *in vivo* by breeding *Gnrhr*$^{Ctailfloxneo/+}$ mice to “flp deleter” mice (*B6.129S4-Gt(ROSA)26Sor*$^{tm1(FLP1)Dym}$*/RainJ*, obtained from The Jackson Laboratory (902)). The resulting *Gnrhr*$^{Ctailflox/+}$ mice were bred to *EIIa::Cre* transgenic mice (*B6.FVB-Tg(EIIa-cre)C5379Lmgd/J*, obtained from the Jackson Laboratory (903)) to yield *Gnrhr*$^{Ctail/+}$ mice. Experimental (*Gnrhr*$^{Ctail/Ctail}$) and control littermates (*Gnrhr*$^{+/+}$) mice were then obtained by crossing heterozygotes. Mice were maintained on a 12 hours light/dark cycle (lights on: 7 a.m.; lights off: 7 p.m.). All animal experiments were performed in accordance with institutional and federal guidelines, and approved by the McGill University Institutional Animal Care and Use Committee (protocol #5204).

RT-PCR

Pituitaries from adult (> 8-week-old) male mice were quickly dissected and immediately frozen on dry ice. Total RNA was isolated using TriZol (Invitrogen) and quantified using a nanodrop spectrophotometer. One µg of RNA was treated with 1 U of RQ1 DNase I (Promega) for 30 min at 37°C. The RNA was incubated 100 µg of random primers for 10 min at 70°C. First-strand cDNA synthesis was then performed at 37°C for 60 min and 70°C for 5 minutes in a 40 µl reaction containing 100 U of MMLV-RT, 20 U of RNAsin, 400 µM dNTPs. and 1X MMLV-RT buffer. One µL of cDNA was then used for PCR using the primers listed in Table S6.1. Each 50 µl PCR reaction contained 1X Green GoTaq Flexi Buffer (Promega), 1.5mM $MgCl_2$, 200 µM dNTPs, 5% DMSO, 400mM of each primer and 2.5U of GoTaq Flexi polymerase (Promega). The PCR cycle was: 5 min at 95°C, 35 cycles of (30 sec at 95°C, 30 sec at 60°C and 1 min at 72°C), and 10 min at 72°C.

Puberty, estrous cyclicity, and fertility assessment

Puberty onset was determined by daily examination of vaginal opening starting from weaning (postnatal day 21). For estrous cyclicity assessment, vaginal smears were collected daily (~ 10 a.m.) for at least three consecutive weeks starting at postnatal day 50, using a cotton swab wetted with sterile saline. Cells were smeared on glass slides, stained with 0.1% methyl blue in saline, and examined by light microscopy. Stages were assigned following published guidelines (829). Fertility was monitored by pairing individual 10 week old females with age-matched control C57BL/6J male mice for a period of 6 months. Starting from day 20 after pairing, cages were inspected daily for the presence of newborn pups. As soon as a litter was observed, pups were carefully counted and placed back in the cage. Pups were separated from the mother 15 days after birth to avoid interference with the subsequent litter.

Reproductive organ dissection and blood collection

Mice were euthanized with CO_2 and blood collected by cardiac puncture. Blood was left to clot at room temperature for 20 min and spun at 3000 x g for 10 min. Serum supernatant was recovered and frozen at -20°C until analysis. Seminal vesicles, testes, uteri and ovaries were dissected and weighted on a precision balance.

Ovariectomy and experimental LH surge induction

Ovariectomy and estrogen replacement were performed with modifications of published protocols (904, 905). For ovariectomy ("day 0"), mice were injected subcutaneously with 5 μg/g body weight with carprofen and deeply anesthetized using isoflurane (Abbott Laboratories, Abbott Park, IL, USA). The back skin was shaved and cleaned, and a single midline incision was performed. Small bilateral incisions were made in the muscle layer, through which the ovaries and tip of the uterine horns were exposed. The ovaries were removed by cauterization at the level of the oviduct, and the incisions were closed using Vicryl sutures (Ethicon, Blue Ash, OH, USA). A subcutaenous pocket was created rostral to the mindline skin incision by separating tissues using a haemostat. Alzet osmotic minipumps (model 1007D, Durect, Cupertino, CA,

USA) filled with a 7.5 ng/μl 17β-estradiol (Sigma) in 1X phosphate-buffered saline (prepared from a solution of 750 ng/μl 17β-estradiol dissolved in ethanol) were implanted in the pocket. Topical carprofen was applied and the skin incision closed with surgical wound clips (Reflex 7, CellPoint Scientific, Gaithersburg, MD, USA). The animals were left to recover on a heating pad. Mice received carprofen (5 μg/g body weight) on the morning of day 1 and day 2 after ovariectomy. On day 2, 50 μl of blood was collected by submandibular venipuncture at 11 a.m., 4 p.m., and 6 p.m. Terminal blood collection (by cardiac puncture) was performed at 8 p.m (1 hour after lights off). Serum (approximately 20-25 μl) was isolated and stored as above. LH and FSH multiplex ELISA assays were performed at the Ligand Assay and Analysis Core of the Center for Research in Reproduction at the University of Virginia (Charlottesville, Virginia).

Statistical analysis

Estrous cycle and fertility parameters, reproductive organ weights and serum hormones were analyzed using unpaired *t*-tests. LH levels at 11 a.m. and 8 p.m. in individual animals were compared with paired *t*-tests. Western blot quantification data were analyzed by separate one-way ANOVAs on the "Flag-GnRHR" and "Flag-GnRHR-Ctail" conditions, followed by Dunnett's post-hoc test to compare all groups with the "no ligand" condition. Statistical analysis was performed using GraphPad Prism 5 or Systat 10.2. $p < 0.05$ was considered statistically significant.

Results

Addition of a C-terminal tail to the murine GnRHR decreases GnRH-induced ERK1/2 phosphorylation

To probe the functional significance of the absence of a C-tail on mammalian GnRHRs, we first set out to identify a species whose GnRHR C-tail could modify the signaling properties of the murine GnRHR in response to agonist. We selected the domestic chicken (*Gallus gallus*) for several reasons. First, birds are the vertebrates that are evolutionarily most closely related to mammals (906). Second, the genome of the chicken has been fully sequenced, and the *Gnrhr* gene characterized (907, 908). Third, chickens generate shorter and shallower GnRH surges than mammals (388, 389). Finally, addition of the chicken GnRHR C-tail to the human GnRHR dramatically accelerates the receptor's internalization kinetics upon agonist stimulation in heterologous cells (384). However, the effects of fusing the chicken C-tail to the closely related murine GnRHR had not been investigated.

ERK1 and 2 are rapidly phosphorylated after GnRH treatment and are absolutely required for GnRH-stimulated LH synthesis in mice (468). We assessed the amplitude and kinetics of ERK1/2 activation after GnRH stimulation of heterologous CHO cells ectopically expressing Flag-tagged wild-type murine GnRHR or a Flag-tagged chimera of the murine GnRHR fused to the chicken GnRHR C-tail. The chimeric GnRHR was expressed to a similar level as the wild-type receptor (Fig. 6.1A and S6.1A-B). In several independent experiments (Fig. 6.1A and S6.1A-C), GnRH rapidly (within 5 min.) stimulated ERK1/2 phosphorylation in cells transfected with either the wild-type or the chimeric GnRHR (lanes 2 and 9 in Fig. 6.1A and S6.1A-C; quantification in Fig. 6.1B). In the absence of transfected receptors, GnRH did not induce ERK1/2 phosphorylation (data not shown). In cells transfected with the wild-type GnRHR, phospho-ERK1/2 remained elevated for at least 4 h after ligand treatment (lanes 3-6 in Fig. 6.1A and S6.1A-C; quantification in Fig. 6.1B). In contrast, in cells expressing the chimeric GnRHR, phospho-ERK1/2 returned to baseline levels 1-2 hours after GnRH treatment (lanes 10-13 in Fig. 6.1A and S6.1A-C; quantification in Fig. 6.1B). Thus, addition of the chicken GnRHR C-tail to the murine GnRHR accelerates ERK1/2 signal termination following receptor activation.

Generation and validation of a conditional knock-in mouse model expressing murine/chicken chimeric GnRHR

The results above are consistent with earlier *in vitro* observations showing that the addition of various C-tails modifies mammalian GnRHR signaling properties (380, 381, 383-385, 393). Neither those nor our analyses, however, provide insight into the functional consequences of C-tail attachment *in vivo*. To address this shortcoming, we generated a novel conditional knock-in mouse model. Specifically, we modified the endogenous murine *Gnrhr* gene on Chr. 5 in embryonic stem (ES) cells. We flanked the terminal exon (exon 3) of *Gnrhr* with *loxP* sites and introduced an additional, but modified form of exon 3 downstream (3') of the second *loxP* site (Fig. 6.2A). The modified exon contained the murine exon 3 coding sequence with the transcription termination codon removed and the coding sequence for the chicken GnRHR C-tail fused in-fame. In principle, this allele allows conditional removal of the endogenous exon 3 by the action of *Cre* recombinase, and its replacement with the modified exon 3. To prevent transcriptional read-through after the endogenous exon 3, we also introduced a strong transcriptional STOP cassette (from the bovine growth hormone gene) immediately after the STOP codon in the endogenous exon 3 (Fig. 6.2A). Two of 420 ES clones surviving positive/negative selection showed correct targeting, as revealed by Southern blotting with both 5' and 3' probes. Both clones were injected into blastocysts and generated high-grade chimeras; one chimera transmitted the modified allele ($Gnrhr^{CtailfloxNeo}$) via the germline when crossed to C57BL/6 mice (Fig. 6.2B). The neomycin resistance cassette (Neo+), which was flanked by Frt sites, was excised *in vivo* by breeding $Gnrhr^{CtailfloxNeo/+}$ mice to "deleter" mice expressing the flippase recombinase (902), yielding $Gnrhr^{Ctailflox/+}$ mice.

To determine whether these mice could produce the chimeric GnRHR following Cre-mediated recombination, we crossed $Gnrhr^{Ctailflox}$ animals with the *EIIa::Cre* deleter strain, which expresses Cre early in embryonic development (903) (Fig. 6.2A). The resulting $Gnrhr^{Ctail/+}$ mice were then interbred to produce $Gnrhr^{+/+}$ (control), $Gnrhr^{Ctail/+}$, and $Gnrhr^{Ctail/Ctail}$ (experimental) mice. Genotyping (Fig. S6.2A) indicated that $Gnrhr^{Ctail/Ctail}$ animals were obtained at a normal mendelian ratio (Fig. S6.2B). In RT-PCR analyses, we observed wild-type *Gnrhr* transcript in $Gnrhr^{+/+}$ and $Gnrhr^{Ctail/+}$ pituitaries (Fig. 6.2C, top panel, lanes 2-3). As expected, the chimeric transcript was observed in $Gnrhr^{Ctail/+}$, but not in $Gnrhr^{+/+}$ pituitaries

(Fig. 6.2C, bottom panel, lanes 2-3). No PCR product was observed when mock reverse-transcribed ("RT-") samples were used as templates (Fig. 6.2C, top and bottom panels, lanes 4-5). Thus, the *Gnrhr*^{Ctail} allele encodes the chimeric transcript.

Female Gnrhr$^{Ctail/Ctail}$ *mice display normal puberty onset, but disrupted estrous cyclicity*

To probe the potential effects of the *Gnrhr*^{Ctail} modification on reproductive axis function, we first examined vaginal opening, a marker of puberty onset (829, 909), in a cohort of control and experimental females. The mean day of vaginal opening did not differ significantly between genotypes, suggesting that puberty onset is unaffected by the *Gnrhr*^{Ctail} allele (Fig. 6.3A). Next, we assessed estrous cyclicity by examining vaginal cytology in *Gnrhr*$^{Ctail/Ctail}$ and *Gnrhr*$^{+/+}$ control littermates over a period of at least three weeks, starting at seven weeks of age. Mice of both genotypes exhibited cytology characteristic of all estrous cycle stages (Fig. 6.3B). However, most *Gnrhr*$^{Ctail/Ctail}$ females exhibited prolonged periods of vaginal cornification (indicative of estrus), ranging from three to 15 consecutive days (Fig. 6.3B). A total of 26 such events were observed in 12 of 15 *Gnrhr*$^{Ctail/Ctail}$ mice, whereas only three cases (once in each of three mice) were seen in 10 control mice. As a result, *Gnrhr*$^{Ctail/Ctail}$ mice had reduced estrous cycle frequency (Fig. 6.3C) and spent significantly more time in estrus (and less in the other stages) than control mice (Fig. 6.3D). This phenotype, though even more pronounced, was also observed in older females retired from breeding studies (see below). Indeed, four of seven females assessed at that age (9-month-old) exhibited persistent vaginal cornification, lasting from one to three months uninterrupted (as long as we assessed them). This was not observed in any of the controls. Together, these data suggest that estrous cyclicity is perturbed in *Gnrhr*$^{Ctail/Ctail}$ mice.

Gnrhr$^{Ctail/Ctail}$ *females are subfertile and have reduced ovarian weight*

To assess the impact of the *Gnrhr*^{Ctail} modification on fertility, we entered *Gnrhr*$^{Ctail/Ctail}$ and control females into 6 month long breeding trial with wild-type C56BL/6J male. Whereas mice from both genotypes were fertile, the *Gnrhr*$^{Ctail/Ctail}$ females produced significantly smaller litters than control littermates (Fig. 6.3E). This phenotype varied in severity, but all *Gnrhr*$^{Ctail/Ctail}$ females delivered litters of a smaller average size than all but one of the control females (Fig. S6.3A). Furthermore, the significant difference between genotypes was preserved even if one

infertile *Gnrhr*$^{Ctail/Ctail}$ mouse and another that delivered only a single pup were removed from the analysis (Fig. S6.3B). The inter-litter interval was not significantly different between genotypes (Fig. S6.3C). Analysis of the reproductive organs of retired breeders revealed significantly smaller ovaries in *Gnrhr*$^{Ctail/Ctail}$ females compared with controls (Fig. 6.4A). Uterine weights did not differ significantly between the two groups, although the four *Gnrhr*$^{Ctail/Ctail}$ females that were in persistent estrus had relatively bigger uteri than other mice (Fig. 6.4B). Ovaries from 3-6 month old females, removed at ovariectomy (see below), were also smaller in *Gnrhr*$^{Ctail/Ctail}$ compared with control mice (Fig. S6.3D).

Gnrhr$^{Ctail/Ctail}$ *males have reduced testes weights*

Although the *Gnrhr*^{Ctail} modification was predicted to disrupt GnRHR function only in the context of persistent GnRH stimulation (i.e., at the time of the GnRH/LH surge), we also examined reproductive axis activity markers in *Gnrhr*$^{Ctail/Ctail}$ males. The external genitalia of *Gnrhr*$^{Ctail/Ctail}$ males appeared normally masculinised at weaning and in adulthood, indicative of normal reproductive maturation (data not shown). Next, we examined the reproductive organs of adult (10-week-old) *Gnrhr*$^{Ctail/Ctail}$ males. Compared with control or heterozygous littermates, *Gnrhr*$^{Ctail/Ctail}$ mice showed a significant reduction in testicular weight, though the decrease was quantitatively small (~ 13%) and not fully penetrant (Fig. 6.4C). However, seminal vesicle weights were normal, suggesting a normal testosterone tone (Fig. 6.4D). Given that mice with null mutations in *Gnrh* or *Gnrhr* display profound hypogonadism (62, 892), these observations suggest the presence of a functional GnRH signaling system in *Gnrhr*$^{Ctail/Ctail}$ males.

Impaired LH surge generation Gnrhr$^{Ctail/Ctail}$ *females*

To evaluate whether gonadotrope cells expressing the chimeric GnRHR are impaired in their capacity to respond to persistent GnRH stimulation, we assessed the ability of *Gnrhr*$^{Ctail/Ctail}$ and control females to generate LH surges. To do so, we employed a well-established paradigm of experimentally-controlled LH surge generation (904, 905). In ovariectomized mice receiving estrogen replacement, robust LH surges are observed around the time of lights off for several consecutive days (904). Therefore, we ovariectomized *Gnrhr*$^{Ctail/Ctail}$ and control females, and implanted them subcutaneously with osmotic minipumps delivering a high dose (90 ng/day) of estrogen (Fig. 6.5A). On the second day after ovariectomy, we collected small volumes of blood

for serum LH analysis from the animals at 11 a.m. (baseline) and at the presumed time of the surge in late afternoon/evening (Fig. 6.5A). Surprisingly, *Gnrhr*$^{Ctail/Ctail}$ mice had significantly higher baseline LH levels compared with controls (Fig. 6.5Bi). Closer inspection of the data indicated that this was attributable to 5 *Gnrhr*$^{Ctail/Ctail}$ animals that displayed much higher LH levels than the 5 other *Gnrhr*$^{Ctail/Ctail}$ and all the control mice (Fig. 6.5Bii). Previous reports using a similar paradigm have reported a measurable LH surge before lights off (314, 904). In our experiments, most animals in both groups failed to display a clear LH surge 3 h and 1 h before lights off (data not shown). However, LH levels measured 1 h later (8 p.m.) were significantly higher than at 11 a.m in both *Gnrhr*$^{Ctail/Ctail}$ and control mice (Fig. 6.5Bii/iii). The absolute LH levels did not differ between genotypes at 8 p.m. (Fig. 6.5Biv). However, when normalized to their individual baseline (11 a.m.) circulating LH levels, the mean magnitude of LH "surges" generated by *Gnrhr*$^{Ctail/Ctail}$ mice was considerably smaller than controls (about 4-fold - Fig. 6.5Bv). This may have been explained in large part by the 5 *Gnrhr*$^{Ctail/Ctail}$ mice that had high LH levels at 11 a.m. (Fig. 6.5Biii). Unexpectedly, in the same animals, serum FSH levels were markedly lower in *Gnrhr*$^{Ctail/Ctail}$ animals compared with controls at all time points (Fig. 6.5Ci/ii). Together, these results suggest that *Gnrhr*$^{Ctail/Ctail}$ mice are impaired in their ability to generate LH surges and have defective FSH synthesis.

Discussion

GnRH signaling is required for gonadotropin synthesis and release, and fertility in mammals, including humans (7, 8, 62, 63, 846, 848, 892). In females, GnRH stimulates the LH surge that drives ovulation at mid-cycle. The functional effects of the lack of a C-tail on the mammalian GnRHR have been extensively documented *in vitro* (380, 381, 383-385, 393). However, the physiological role of this feature, unique among all known GPCRs, remains unknown. It has been proposed that slow desensitization of the GnRHR due to the absence of the C-tail enables the generation of an LH surge (390). Before the present study, this prediction remained untested. Here, by generating a new knock-in mouse line, we show that addition of a C-tail to the endogenous GnRHR may impair, but does not preclude, LH surge generation and fertility in female mice.

Both male and female *Gnrhr*$^{Ctail/Ctail}$ mice show apparently normal reproductive axis maturation and puberty. Because inactivating mutations in *Gnrh* or *Gnrhr* cause complete failure of reproductive maturation in mice (62, 892), addition of a C-tail to the murine GnRHR does not prevent GnRH signaling. That said, GnRH action is altered in these mice. *Gnrhr*$^{Ctail/Ctail}$ females display abnormal estrous cyclicity, characterized by prolonged periods of estrus. The physiological basis for this phenotype remains unclear. Intriguingly, half of the *Gnrhr*$^{Ctail/Ctail}$ females subjected to E_2-induced LH surge experiments had higher baseline LH levels than controls. In principle, this could be due to ineffective E_2 replacement by the osmotic minipumps in this subset of mice, a possibility that can be addressed my measuring their serum E_2 levels. Alternatively, this could reflect abnormal estrogen feedback on the hypothalamus or pituitary in *Gnrhr*$^{Ctail/Ctail}$ mice. It is also possible that these animals just happened to be sampled at the time of an LH pulse. It will be important to verify whether gonadotropin levels are also dysregulated in intact *Gnrhr*$^{Ctail/Ctail}$ animals. In E_2-induced LH surge experiments, most *Gnrhr*$^{Ctail/Ctail}$ mice appeared able to generate an LH surge. Consistent with these observations, *Gnrhr*$^{Ctail/Ctail}$ females are fertile, indicating that they secrete sufficient LH to stimulate ovulation. However, the amplitude of the LH surges generated by *Gnrhr*$^{Ctail/Ctail}$ mice appeared diminished compared with controls. Because the LH levels measured at one time point (8 p.m.) may not represent the peak of the surge, the extent by which the LH surge is disrupted in *Gnrhr*$^{Ctail/Ctail}$ females is unclear. Could impaired LH surges account for the subfertility of *Gnrhr*$^{Ctail/Ctail}$ females? A shallow LH

surge is sufficient for normal fertility in mice (314). However, how long the surge must last is somewhat unclear. It seems that rodents require a surge lasting at least 1 h, but less than 2 h, which is shorter than the duration of the endogenous surge (325, 326). That said, the surge may need to last longer if its amplitude is decreased. Unfortunately, the results of our E_2-induced LH surge experiments do not reveal whether the duration of the LH surge is altered in *Gnrhr*$^{Ctail/Ctail}$ females. Our *in vitro* experiments suggest that addition of the C-tail to the GnRHR affects both the amplitude and the duration of GnRH-induced ERK1/2 signaling. It is conceivable that *Gnrhr*$^{Ctail/Ctail}$ mice may exhibit both shallower and shorter LH surges than control. This, in turn, may drive the ovulation of fewer oocytes.

Intriguingly, in E_2-induced LH surge experiments, *Gnrhr*$^{Ctail/Ctail}$ females had markedly lower FSH levels compared with controls. In rats, the immediate post-OVX rise in serum FSH is GnRH-independent, but is attenuated by a GnRH antagonist 2 days later (835). Therefore, lower FSH levels in ovariectomized *Gnrhr*$^{Ctail/Ctail}$ females may reflect impaired GnRH signaling. It will be important to measure FSH levels in intact *Gnrhr*$^{Ctail/Ctail}$ females, as lower FSH could contribute to the subfertility phenotype. However, the secondary FSH surge, which determines the number of ovarian follicles growing to the pre-ovulatory stage, depends on activins (or related ligands) rather than GnRH (203, 570). Decreased circulating FSH levels could also contribute to the reduction in testicular weights in *Gnrhr*$^{Ctail/Ctail}$ males.

Based on the available data, it is difficult to identify the precise molecular defect(s) causing the observed phenotypes. The C-tail functions first and foremost as a mediator of homologous desensitization, but has other roles as well. For example, deletion of the CXCR4 C-tail affects both ligand-induced internalization and G protein coupling in mice (377). In other GPCRs, residues in the C-tail are required for proper maturation and trafficking after receptor synthesis in the endoplasmic reticulum (910). At present, it is unknown whether the addition of the chicken GnRHR C-tail alters the signaling properties of the murine GnRHR independently of its effect on ligand-induced internalization. There might be reduced receptor expression at the cell surface of gonadotropes; for example, because of enhanced constitutive internalization, or defects in recycling to the membrane or in anterograde trafficking after *de novo* synthesis. Also, we cannot rule out effects of the targeted modification on expression of the *Gnrhr* gene. To

clarify these issues, it will be important to assess the pituitary mRNA and protein expression of the chimeric GnRHR, as well as GnRH binding to pituitary membranes from *Gnrhr*$^{Ctail/Ctail}$ mice.

The absence of a C-tail in all mammalian GnRHRs, coupled with its presence in non-mammalian species suggests that it underlies divergent reproductive processes between these animal classes. The loss of the C-tail may have been caused by a spontaneous nonsense mutation at the end of the coding sequence for the last transmembrane domain in the *Gnrhr* gene. Such a mutation could have been positively selected because it increased responsiveness of the pituitary gonadotropes to GnRH, and hence conferred robustness to the reproductive system. Over time, mammals may have become more reliant on this evolutionary adaptation. For example, a larger and/or longer LH surge may have become required for successful ovulation. That said, it is clear that the simple addition of a C-tail to the mammalian GnRHR is insufficient to completely block reproduction – at least in mice. In the evolutionary time since mammals lost their GnRHR C-tail, the *Gnrhr* genes in other species have undergone changes as well. Thus, it is likely that the chicken *Gnrhr* has evolved in parallel, while maintaining the reproductive requirements of this species. Because chickens do generate LH surges, it would be interesting to study the functional consequences of modifying the murine GnRHR with the C-tail from other species that may have even lower LH requirements for ovulation, such as amphibians or fish.

In conclusion, we show that the addition of a C-tail to the mouse GnRHR alters its signalling properties *in vitro* and reproductive physiology *in vivo*. Notably, females expressing the chimeric receptors may be impaired in their ability to mount normal LH surges, have defective FSH synthesis, display abnormal estrous cycles, and are subfertile. Thus, the loss of a C-tail on the GnRHR, a unique property among GPCRs, appears to have been co-opted during evolution to confer robustness to the mammalian reproductive system.

Figure legends

Figure 6.1: Addition of a C-tail to the murine GnRHR impairs ERK1/2 activation. **A**) Representative western blot analysis of whole cell lysates from CHO cells transfected with the wild-type Flag-tagged murine GnRHR (lanes 1-6) or the chimeric murine/chicken GnRHR (lanes 8-13). Cells were treated for 5 min (lanes 2 and 9), 1 h (lanes 3 and 10), 2 h (lanes 4 and 11), 4 h (lanes 5 and 12) or 6 h (lanes 6 and 13) with 60 nM GnRH, or left untreated (lanes 1 and 8). The blots were probed with anti-phospho-ERK1/2 (top) or anti-Flag (bottom) antibodies. The smeary pattern detected with the Flag antibody is expected because of receptor glycosylation. **B**) Quantification of the intensity of the phospho-ERK1/2 signal, relative to the Flag signal (indicative of the level of receptor expression) in the blot shown in A) and in supplementary Figs. S1A-B. Bars represent the means (+SEM). N=3 independent experiments for the "no ligand", "5 min", "1h" and "2h" time points, and N=2 for the "4h" and "6h" time points (which were absent in the experiment shown in Fig. S1B) *: significantly different ($p<0.05$) from the "no ligand" condition.

Figure 6.2: Generation and validation of *Gnrhr*$^{Ctail/Ctail}$ conditional knock-in mice. **A**) Targeting strategy. The wild-type locus (**i**), the targeting construct (**ii**), the targeted locus (**iii**), and the outcomes of Flp-mediated (**iv**) and Cre-mediated recombination (**v**) are shown. Exons are shown as white boxes, with their corresponding numbers above. The asterisks (*) indicate STOP codons. "*X*" refer to *XmaI* restriction sites. The positions of the 5' and 3' Southern blot probes are shown below the wild-type locus (**i**) and targeted allele (**iii**). The sizes of the *XmaI* restriction fragments detected by the 5' and 3' Southern blot probes are indicated above the wild-type locus (**i**) and below the targeted allele (**iii**). The *loxP* sites are indicated with open triangles, and the *Frt* sites with black (leftward-facing) triangles. "3+C-tail": chimeric murine exon 3 fused to the coding sequence of the chicken GnRHR C-tail. "pA": bovine growth hormone polyA signal sequence. "neo": neomycin resistance cassette. "DTa": diphtheria toxin A chain negative selection marker. **B**) Southern blot analysis with the 5' and 3' probes depicted in **A**) on genomic DNA obtained from a wild-type mouse (*Gnrhr*$^{+/+}$) and a mouse carrying the targeted allele (*Gnrhr*$^{CtailfloxNeo/+}$). **C**) PCR analysis on reverse-transcribed (RT+; lanes 2-3) or mock reverse-transcribed (RT-; lanes 4-5) RNA from *Gnrhr*$^{+/+}$ (lanes 2 and 4) and *Gnrhr*$^{Ctail/+}$ (lanes 3 and 5)

adult male mouse pituitaries. PCR was performed with primers amplifying the *Gnrhr*$^{+}$ transcript (top panel) or the *Gnrhr*^{Ctail} transcript (bottom panel). Shown on top are schematics of the relevant portions of the *Gnrhr* alleles, using the same symbols as in **A**).

Figure 6.3: Abnormal estrous cyclicity and subfertility in female *Gnrhr*$^{Ctail/Ctail}$ mice. **A**) Day of vaginal opening in *Gnrhr*$^{+/+}$ *and Gnrhr*$^{Ctail/Ctail}$ females (postnatal day 21 is the day of weaning). Each dot represents an individual animal; means are shown with horizontal bars. n.s.: non significant. **B**) Representative estrous cyclicity profiles from three *Gnrhr*$^{+/+}$ (left) and three *Gnrhr*$^{Ctail/Ctail}$ females. Each dot represents one day. **C**) Quantification of the estrous cycle frequency in *Gnrhr*$^{+/+}$ *and Gnrhr*$^{Ctail/Ctail}$ females. *: $p<0.05$. **D**) Quantification of the proportion of time spent in each estrous cycle stage in *Gnrhr*$^{+/+}$ *and Gnrhr*$^{Ctail/Ctail}$ females. In panels B and D, M/D: metestrus/diestrus. P: proestrus. E: estrus. *: $p<0.05$. **E**) Average litter size delivered by *Gnrhr*$^{+/+}$ *and Gnrhr*$^{Ctail/Ctail}$ females over a 6 month breeding trial. *: $p<0.05$.

Figure 6.4: Reduced ovarian and testicular weights in *Gnrhr*$^{Ctail/Ctail}$ mice. **A-B**) Ovarian (**A**) and uterine (**B**) weights in *Gnrhr*$^{+/+}$ and *Gnrhr*$^{Ctail/Ctail}$ retired breeders (10-month-old), normalized to body weights (which did not differ). Each dot represents an individual animal. Horizontal bars show means. The four *Gnrhr*$^{Ctail/Ctail}$ mice in persistent estrus are indicated. *: $p<0.05$. n.s.: non significant. **C-D**) Testicular (**C**) and seminal vesicle (SV) (**D**) weights in 10-week-old *Gnrhr*$^{+/+}$, *Gnrhr*$^{Ctail/+}$ and *Gnrhr*$^{Ctail/Ctail}$ males, normalized to body weights (which did not differ). Each dot represents an individual animal. Horizontal bars show means. *: $p<0.05$. n.s.: non significant.

Figure 6.5: Impaired LH surge generation in *Gnrhr*$^{Ctail/Ctail}$ females. **A**) Schematic representation of the ovariectomy (OVX) and estrogen (E_2) replacement procedure (left) and experimental protocol (right) for LH surge induction and assessment. **B**) **i**) Mean (+SEM) serum LH levels at 11 a.m. in *Gnrhr*$^{+/+}$ (grey bars) and 10 *Gnrhr*$^{Ctail/Ctail}$ mice (black bars) subjected to the OVX + E_2 replacement paradigm described in **A**. **ii-iii**) Serum LH levels at 11 a.m. and 8 p.m. in individual in *Gnrhr*$^{+/+}$ (**ii**) and *Gnrhr*$^{Ctail/Ctail}$ (**iii**) mice. Connecting lines link the data points from each animal. **iv**) Mean (+SEM) serum LH levels measured at 8 p.m. in the same animals at in **i-iii**). **v**) LH surge magnitude, computed as the ratio of serum LH levels at 8 p.m. to the levels at 11 a.m. in each animal. *: $p<0.05$. n.s.: non significant. N=10 mice per group. **C**) Serum LH

levels were measured at 11 a.m. (**i**) and 8 p.m. (**ii**) the same animals as in **B**). *: $p<0.05$. n.s.: non significant.

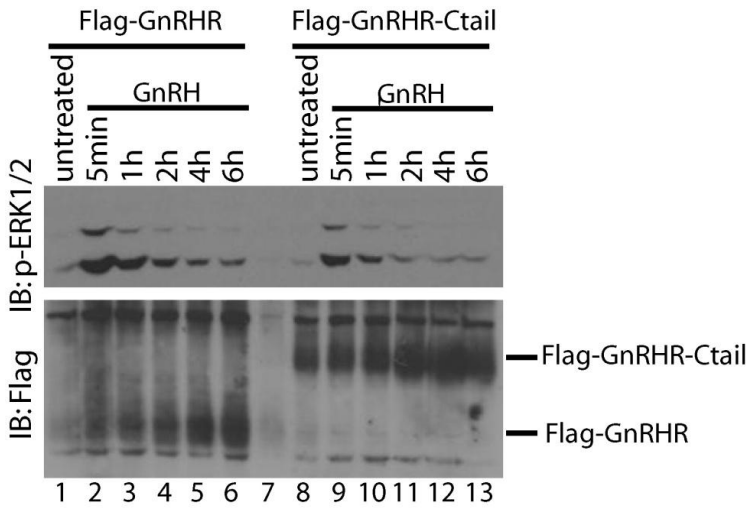
Figure S6.1: Addition of a C-tail to the murine GnRHR impairs ERK1/2 activation. **A**) Western blot analysis of whole cell lysates from CHO cells transfected and treated as described in Fig. **1A**. **B**) Western blot analysis of whole cell lysates from CHO cells transfected as described in Fig. **1A** and **S1A**, but treated for different periods of time: 5 min (lanes 2 and 9), 10 min (lanes 3 and 10), 30 min (lanes 4 and 11), 1 h (lanes 5 and 12), 2 h (lanes 6 and 13) or left untreated (lanes 2 and 8). **C**) Western blot analysis of whole cell lysates from CHO cells transfected and treated as described in Fig. **1A** and **S1A**. The blots were probed with anti-phospho-ERK1/2 (top) or anti-ERK1/2 (bottom) antibodies. The blots in **A**) and **B**) and the blot shown in Fig. **1A** were used for the quantitative analysis shown in Fig. **1B**.

Figure S6.2: *Gnrhr*$^{Ctail/Ctail}$ mice are obtained at the expected Mendelian ratio. **A**) Genotyping strategy to identify the *Gnrhr*$^{+}$ and *Gnrhr*^{Ctail} alleles. Top: schematic of the wild-type (*Gnrhr*$^{+}$) and targeted (*Gnrhr*^{Ctail}) alleles, along with the position of the genotyping primers (arrows) and the expected size of the amplicons. *XmaI* restriction sites ("X"), a *loxP* site (open triangle) and an *Frt* site (leftward-facing black triangle), remnant of the targeting and Flp/Cre-mediated recombination events, are shown in the *Gnrhr*^{Ctail} allele (see also Fig. **2A**). Bottom: representative genotyping results from a litter of 10 pups obtained from a *Gnrhr*$^{Ctail/+}$ X *Gnrhr*$^{Ctail/+}$ cross (lanes 2-11). The genotypes are indicated above the gel image. Lane 1: ladder. **B**) Number, observed proportion (% observed) and expected proportion (% expected) of *Gnrhr*$^{+/+}$, *Gnrhr*$^{Ctail/+}$ and *Gnrhr*$^{Ctail/Ctail}$ mice, from crosses of *Gnrhr*$^{Ctail/+}$ X *Gnrhr*$^{Ctail/+}$ parents, genotyped at weaning (postnatal day 21).

Figure S6.3: Subfertility and reduced ovarian weights in *Gnrhr*$^{Ctail/Ctail}$ females. **A**) Average litter size delivered by *Gnrhr*$^{+/+}$ and *Gnrhr*$^{Ctail/Ctail}$ females over a 6 month breeding trial. Each dot represents an individual female. These data are presented in bar graph format in Fig. **3E**. *: $p<0.05$. **B**) Same data as in **A**), but with one infertile *Gnrhr*$^{Ctail/Ctail}$ female and one that delivered a single pup removed from the analysis. *: $p<0.05$. **C**) Inter-litter interval in *Gnrhr*$^{+/+}$ and *Gnrhr*$^{Ctail/Ctail}$ females during the 6 month breeding trial. n.s.: non significant. **D**) Ovarian weights

in *Gnrhr*$^{+/+}$ and *Gnrhr*$^{Ctail/Ctail}$ adult females (> 10-week-old) at ovariectomy. These ovaries were retrieved from females subjected to the LH surge induction protocol detailed in Fig. **5**. Each dot represents an individual animal. Horizontal bars show means. *: $p<0.05$.

A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1h
2h
4h
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Flag-GnRHR
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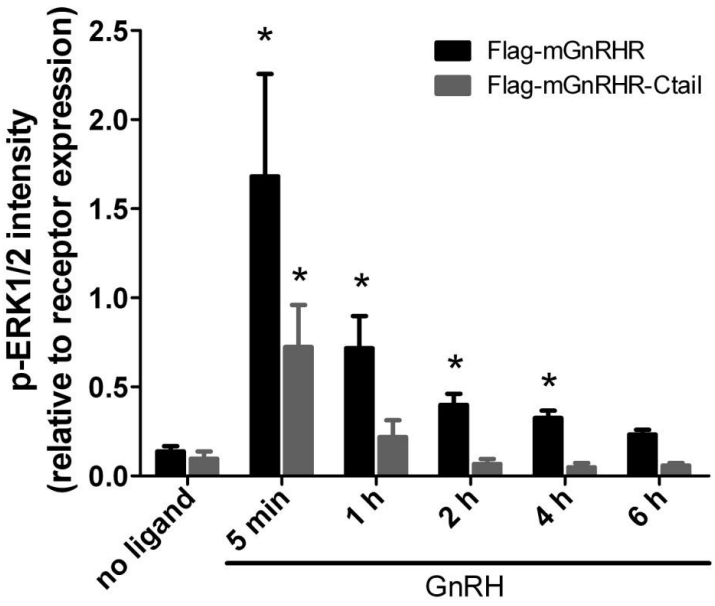
B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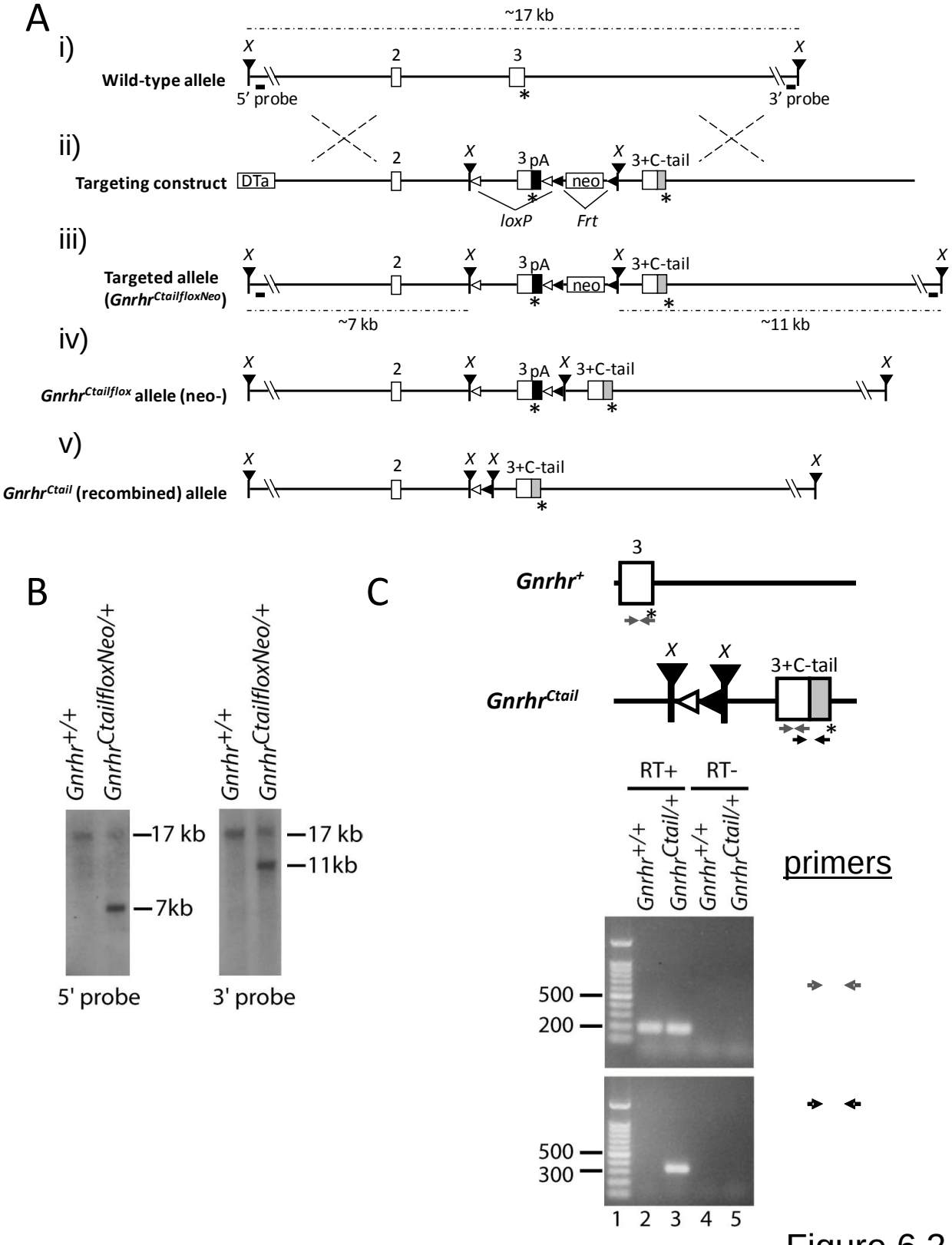
1 h
2 h
4 h
6 h
GnRH

Figure 6.1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3' probe
ii)
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neo
loxP
Frt
3+C-tail
iii)
Targeted allele
(Gnrhr Ctailfloxneo)
~7 kb
~11 kb
iv)
Gnrhr Ctailflox allele (neo-)
v)
Gnrhr Ctail (recombined) allele
B
Gnrhr+/+
GnrhrCtailfloxNeo/+
17 kb
11kb
7kb
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RT-
primers
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200
300
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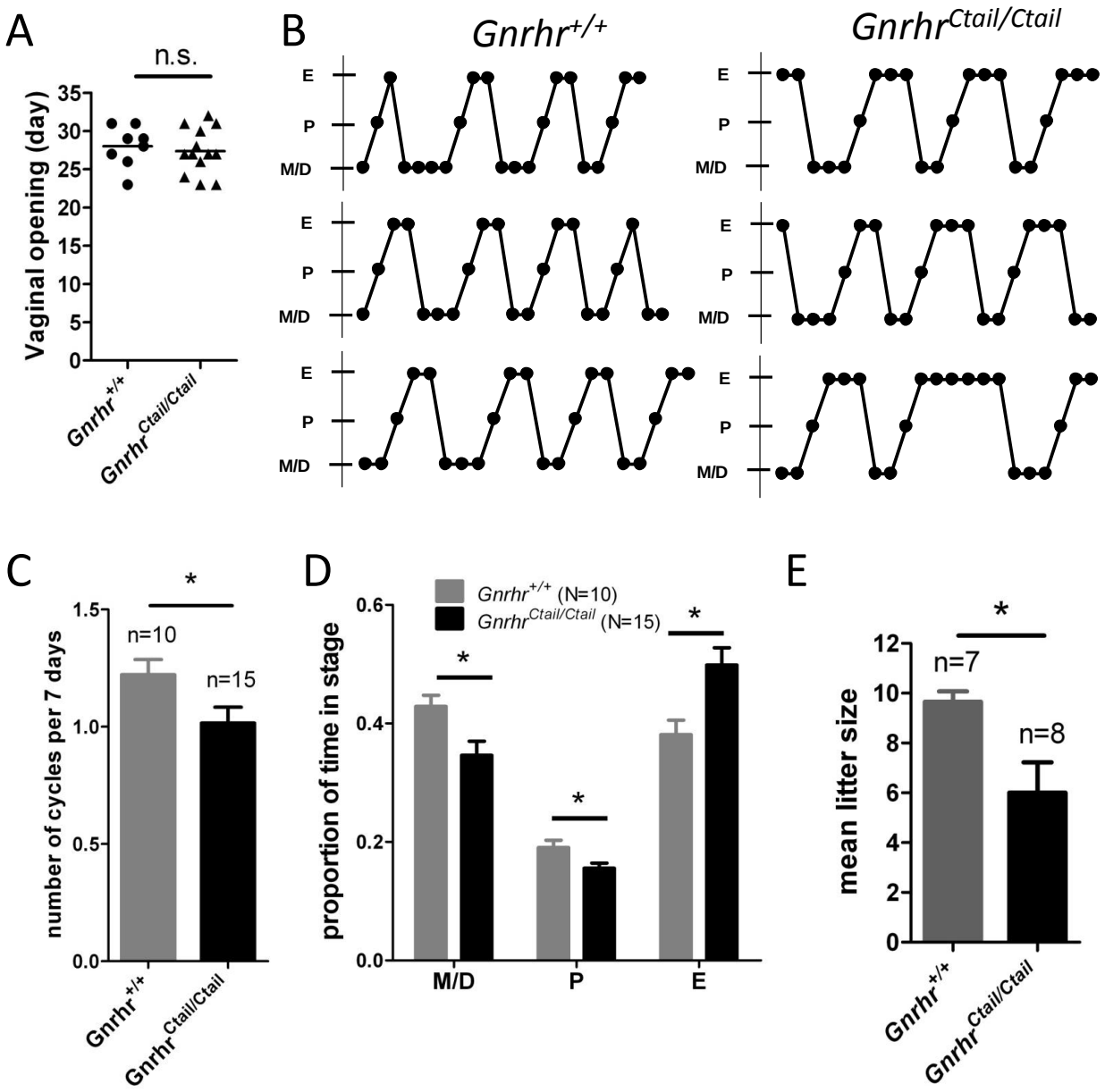
Figure 6.2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30
25
20
15
10
5
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GnrhrCtail/Ctail
B
Gnrhr+/+
GnrhrCtail/Ctail
E
P
M/D
C
*
number of cycles per 7 days
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1.0
0.5
0.0
n=10
n=15
Gnrhr+/+
GnrhrCtail/Ctail
D
Gnrhr+/+ (N=10)
GnrhrCtail/Ctail (N=15)
proportion of time in stage
0.6
0.4
0.2
0.0
*
M/D
P
E
E
*
mean litter size
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6
4
2
0
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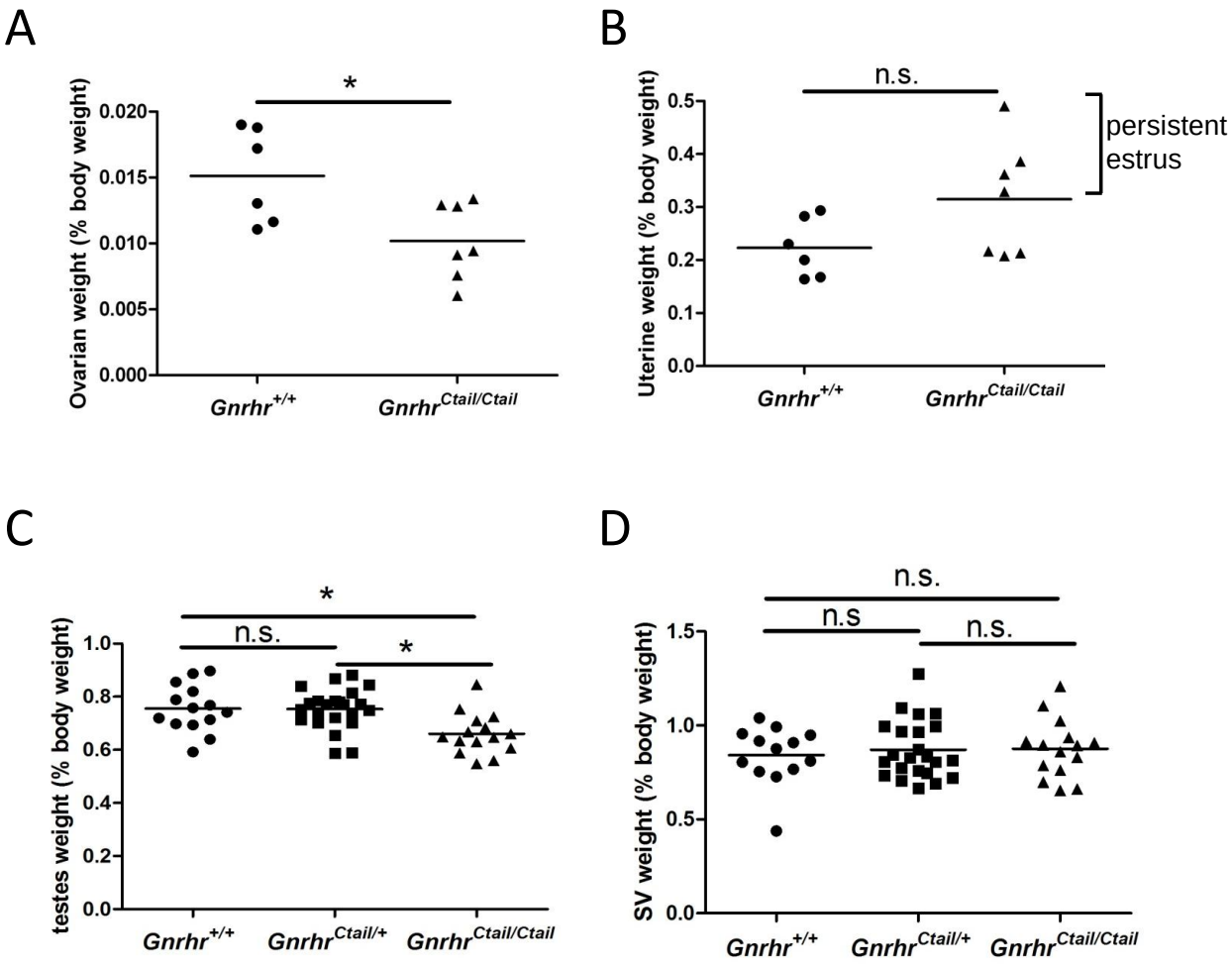
Gnrhr+/+
GnrhrCtail/Ctail

Figure 6.3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Uterine weight (% body weight)
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0.4
0.3
0.2
0.1
0.0
n.s.
persistent estrus
Gnrhr+/+
GnrhrCtail/Ctail
C
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0.6
0.4
0.2
0.0
*
n.s.
*
Gnrhr+/+
GnrhrCtail/+
GnrhrCtail/Ctail
D
SV weight (% body weight)
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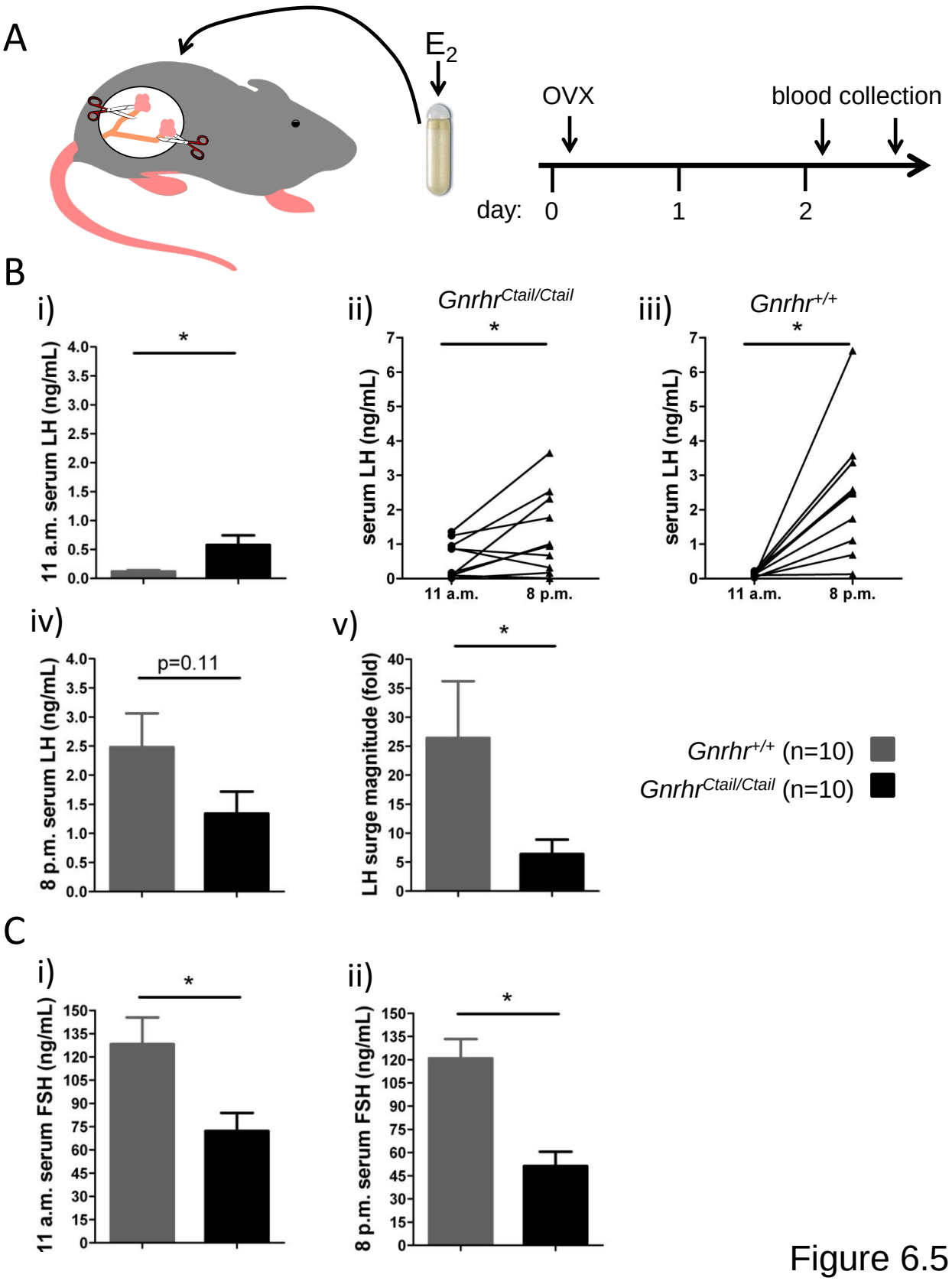
Figure 6.4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ii)
Gnrhr Ctail/Ctail
iii)
Gnrhr +/+
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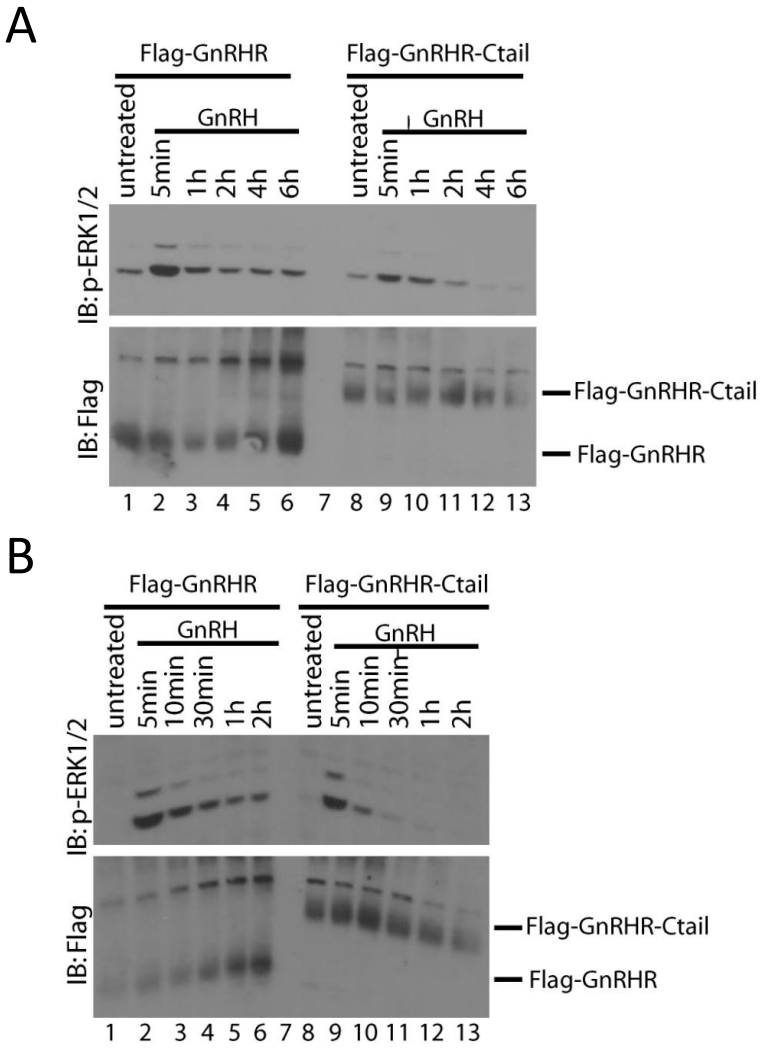
serum LH (ng/mL)
11 a.m.
8 p.m.
iv)
p=0.11
8 p.m. serum LH (ng/mL)
v)
LH surge magnitude (fold)
Gnrhr +/+ (n=10)
Gnrhr Ctail/Ctail (n=10)
C
i)
ii)
11 a.m. serum FSH (ng/mL)
8 p.m. serum FSH (ng/mL)

Figure 6.5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5min
1h
2h
4h
6h
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1h
2h
4h
6h
IB: p-ERK1/2
IB: Flag
Flag-GnRHR-Ctail
Flag-GnRHR
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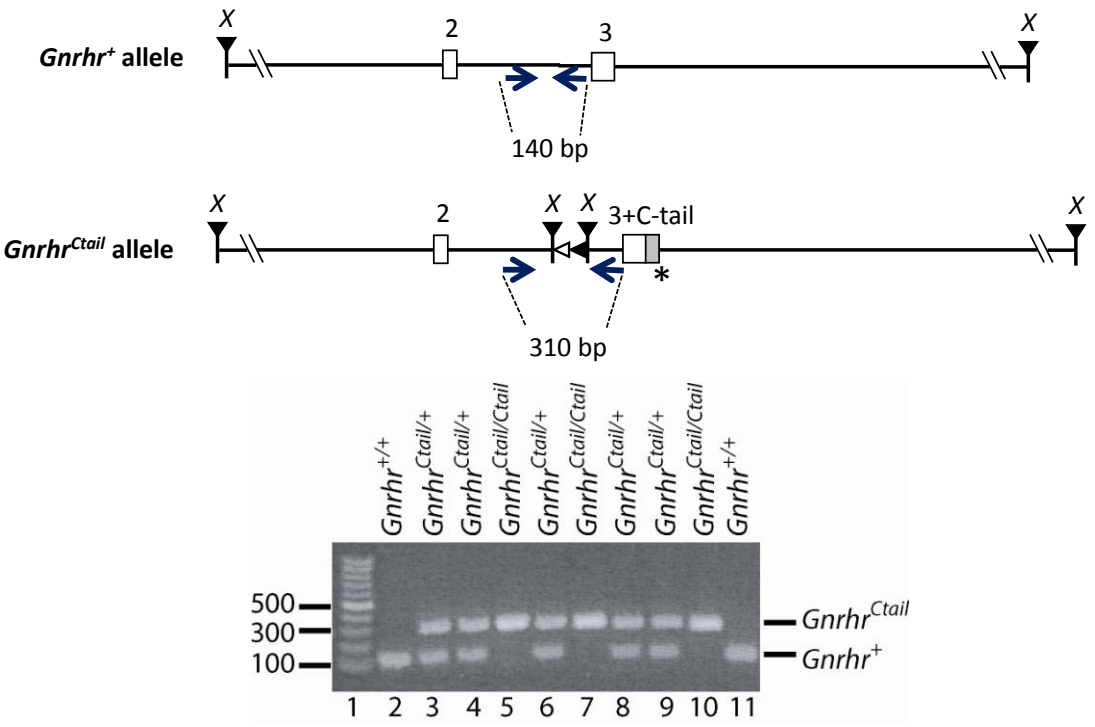
B
Flag-GnRHR
Flag-GnRHR-Ctail
GnRH
GnRH
untreated
5min
10min
30min
1h
2h
untreated
5min
10min
30min
1h
2h
IB: p-ERK1/2
IB: Flag
Flag-GnRHR-Ctail
Flag-GnRHR
1 2 3 4 5 6 7 8 9 10 11 12 13
C
Flag-GnRHR
Flag-GnRHR-Ctail
GnRH
GnRH
untreated
5min
10min
30min
1h
2h
untreated
5min
10min
30min
1h
2h
IB: p-ERK1/2
IB: ERK1/2
1 2 3 4 5 6 7 8 9 10 11 12 13

Figure S6.1

A



B

genotype	*Gnrhr*$^{+/+}$	*Gnrhr*$^{Ctail/+}$	*Gnrhr*$^{Ctail/Ctail}$
number	37	75	36
% observed	25%	50.7%	24.3%
% expected	25%	50%	25%

Figure S6.2

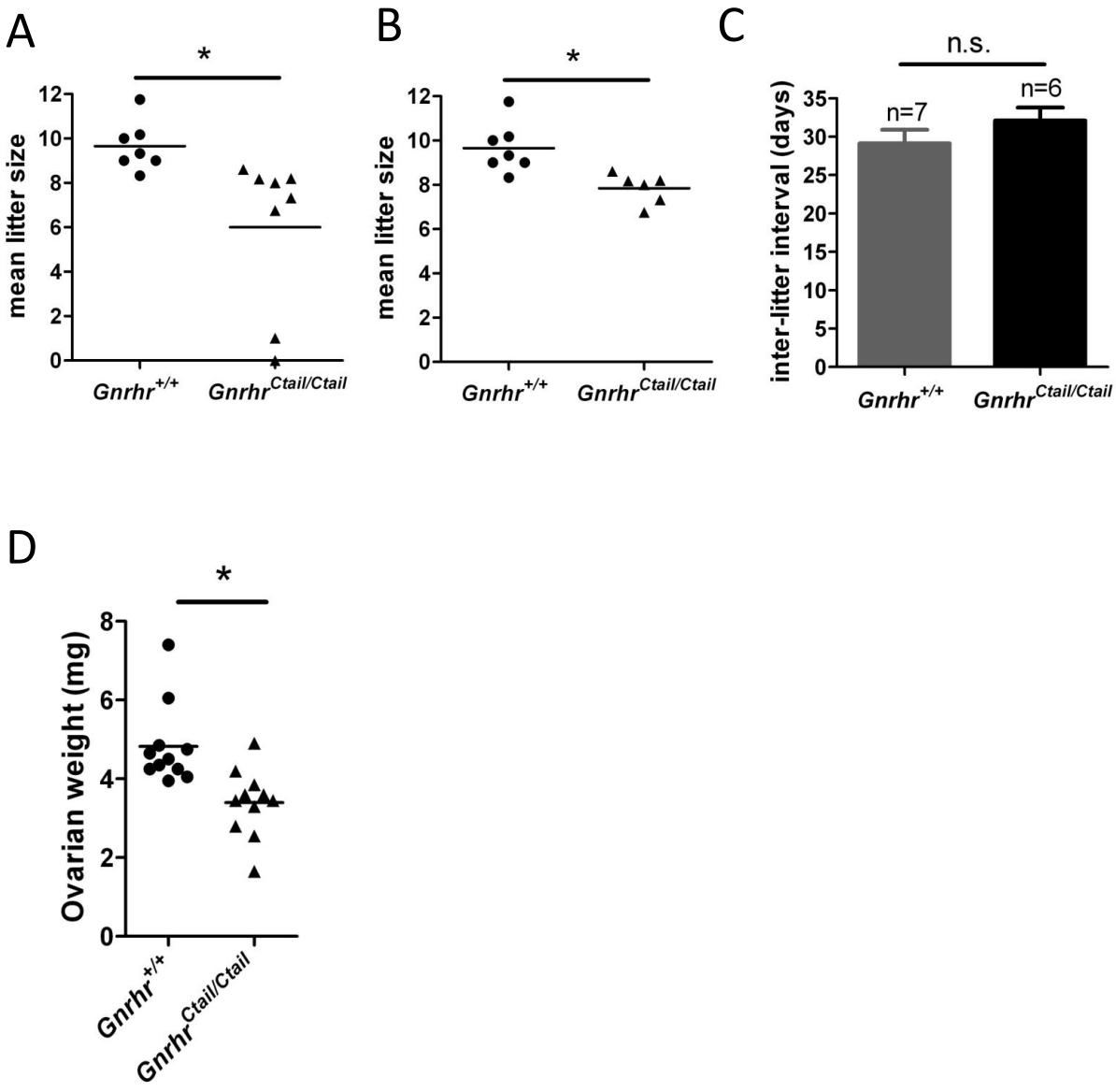

A
*
mean litter size
12
10
8
6
4
2
0
Gnrhr+/+
GnrhrCtail/Ctail
B
*
mean litter size
12
10
8
6
4
2
0
Gnrhr+/+
GnrhrCtail/Ctail
C
n.s.
n=7
n=6
inter-litter interval (days)
35
30
25
20
15
10
5
0
Gnrhr+/+
GnrhrCtail/Ctail
D
*
Ovarian weight (mg)
8
6
4
2
0
Gnrhr+/+
GnrhrCtail/Ctail

Figure S6.3

Primer	Purpose	Sequence
GnrhrCtail_geno_F	genotyping	5’-CATGGAGATCCTTGCTGACA-3’
GnrhrCtail_geno_R	genotyping	5’-CACCTGGGGGCTAGTCTGT-3’
Gnrhr_WTtranscript_ex3_F	RT-PCR	5’-CTCGGCTGAGAACGCTAAAG-3’
Gnrhr_WTtranscript_ex3_R	RT-PCR	5’-CCCATATATGAGTGGGTCGAA-3’
Gnrhr_Ctailtranscript_ex3_F	RT-PCR	5’-TTCGCTACCTCCTTTGTCGT-3’
Gnrhr_Ctailtranscript_Ctail_R	RT-PCR	5’-TGTTAACGGTTGTCCCATTG-3’
Gnrhr__UCA_F	cloning	5’-CGGGGTACCTATAACTCATTAGCTGATTCAAACTT-3’
Gnrhr_UCA_R	cloning	5’-CGGCCCGGGCAGTTCTGACAGACTAGCCCCC-3’
Gnrhr_floxedregion_F	cloning	5’-CGGCCCGGGCGATAACTTCGTATAATGTATGCTATACGAAGTTATCAGGATTCACCTCACCATGG-3’
Gnrhr_floxedregion_R	cloning	5’-CGGGTTTAAACCTACAAAGAGAAATACCCAT-3’
Gnrhr_DCA_F	cloning	5’-CGGCCCGGGCAGGATTCACCTCACCATGG-3’
Gnrhr_DCA_R	cloning	5’-CGGGCGGCCGCAATTGAAGATCACAGTGTTTGGA-3’
ChickenCtail_F	cloning	5’-CGGATCGATCGTTTCGGGAGGACGTGCAA-3’
ChickenCtail_R	cloning	5’-CGGATCGATTCAGCACACCGTGTTAACGG-3’
BGHpolyAtail_F	cloning	5’-TAAGTTTAAACCGCTGATCAGC-3’
BGHpolyAtail_R	cloning	5’-CGGCTCGAGCCATAGAGCCCACCGCATC-3’
Gnrhr_STOP-to-ClaImut_sense	mutagenesis	5’-TGCACCCACTCATATATGGGTATTTCTCTTTGATCGATGGAGACTACACAAGAACTCAGATAGAAATAAG-3’
Gnrhr_STOP-to-ClaImut_antisense	mutagenesis	5’-CTTATTTCTATCTGAGTTCTTGTGTAGTCTCCATCGATCAAAGAGAAATCACCATATATGAGTGGGTCGA-3’
Gnrhr expression vector_F	cloning	5’-CGGAATTCGCTCACAATGCATCTCTTGAG-3’
Gnrhr expression vector_R	cloning	5’-ACTCTAGATCTCCAAAGAGAAATACCCATATA-3’
Gnrhr_expression vector_STOP-to-ClaImut_sense	mutagenesis	5’-GACCCACTCATATATGGGTATTTCTCTTTGATCGATTAGAGGGCCCTATTCTATAGTGTCACCTA-3’
Gnrhr_expression vector_STOP-to-ClaImut_antisense	mutagenesis	5’-TAGGTGACACTATAGAATAGGGCCCTCTAATCGATCAAAGAGAAATACCCATATATGAGTGGGTC-3’
Gnrhr 5’ Southern probe_F	Southern blot	5’-CTTCAACCCGCCCTCTAGT-3’
Gnrhr 5’ Southern probe_R	Southern blot	5’-AGCCGGTCTAAGAATCCTCTC-3’
Gnrhr 3’ Southern probe_F	Southern blot	5’-CAAAGTGCCCACAGATTTTG-3’
Gnrhr 3’ Southern probe_R	Southern blot	5’-GCCTGGTGTTCTGAGAGACTG-3’

Table S6.1

Chapter 7: general discussion

The experimental work presented in this thesis provides new insights into the molecular mechanisms underlying GnRH and activin-regulated gonadotropin synthesis. Below, the implications of these results and some outstanding questions are discussed. In the first part of the thesis, I assessed the importance of some intracellular activin signaling components in regulating gonadotrope function, using conditional gene knockout in mice. The results show that *Fshb* expression, FSH synthesis and fertility do not require SMAD2 and the DNA-binding activity of SMAD3 (chapter 2), but are absolutely dependent on the individual and concerted actions of SMAD4 and FOXL2 (chapter 3). These observations extend previous *in vitro* data and establish SMAD4 and FOXL2 as critical regulators of *Fshb* transcription *in vivo* (see section 7.1.1.), a role which may be conserved in humans (see section 7.1.2.). The FSH-deficiency and hypogonadism phenotypes observed in mice lacking *Smad4* and/or *Foxl2* in gonadotropes are presumably caused by impaired signaling by activins or related ligands, which are yet to be precisely defined (see section 7.1.3.). Importantly, the results suggest that activin signaling is a least as important – if not more so – than GnRH in regulating FSH *in vivo* (see section 7.1.4.). Loss of *Smad4* and *Foxl2* may affect other aspects of gonadotrope function, but FSH-deficiency is clearly the main contributor to the hypogonadal phenotype (see section 7.1.5.). The second experimental part focused on elucidating the mechanisms underlying GnRH-regulated human *LHB* promoter activity, which appear to be conserved with other species (chapters 4). These results suggest a similar role for GnRH-induced LH synthesis in all mammals, which might be particularly important at the time of the pre-ovulatory LH surge in females (see section 7.2). By contrast, activin differentially modulate GnRH-induced mouse and human *Lhb/LHB* promoter activity (chapter 5), hinting at species-specific fine-tuning regulation of LH synthesis (see section 7.2). Finally, I studied the functional significance of a unique characteristic of the mammalian GnRH receptor – the lack of an intracellular C-terminal tail – in a new knock-in mouse model (chapter 6). Mice expressing a chimeric receptor harboring the chicken GnRHR C-tail show female subfertility, and possible defects LH surge generation. These results suggest that the loss of a C-tail might have conferred an evolutionary advantage to the mammalian reproductive system (see section 7.3).

7.1 Activin regulation of FSH synthesis

7.1.1. SMADs and FOXL2 as master regulators of Fshb transcription

Gonadotropes lacking SMAD4 and FOXL2 show very little – if any – pituitary *Fshb* expression and FSH synthesis. Because the *Gnrhr*^{GRIC} allele is transcriptionally activated as early as E12.75 in mice (157), the gonadotropes of S4F2cKO animals lost SMAD4 and FOXL2 prior to their terminal differentiation. Thus, it is possible that SMAD4/FOXL2-deficient gonadotropes develop abnormally, precluding *Fshb* expression. If this is the case, the effect is very specific because robust LHβ expression remains. Nevertheless, it will be important to investigate whether SMAD4 and FOXL2 are required for *Fshb* expression during a defined developmental time window, or whether they are necessary for ongoing FSH synthesis throughout life. The fact that acute deletion of both factors blocks activin induction of *Fshb* transcription in primary pituitary cultures strongly supports the latter possibility. This prediction could be formally tested *in vivo* by selectively deleting the *Smad4* and *Foxl2* genes in adult animals. However, a mouse line enabling temporal control over Cre-mediated recombination in gonadotropes has yet to be described.

While FSHβ is markedly diminished or absent in the LHβ-positive gonadotropes of S4F2cKO mice, it is retained in some LHβ-negative cells. In principle, this could be due to a lack of Cre activity in some FSHβ-positive cells. However, in juvenile *Gnrhr*^{GRIC} mice, essentially all the FSHβ-positive cells show Cre activity (157). Accordingly, we found that *Cre* is expressed in all the remaining FSHβ-positive cells in adult S4F2cKO mice. Thus, rather than incomplete Cre-mediated recombination, it seems more likely that there is a minor population of "FSHβ-only gonadotropes" in which SMAD4 and FOXL2 are not required for FSH synthesis. The existence of molecularly distinct populations of gonadotropes has been suggested by several observations (144, 157, 158). In particular, a small subset of gonadotropes retains intense FSHβ expression in juvenile *Foxl2* knockout mice (though these cells are also LHβ-positive) (144). Also, pituitary cell ablation directed by an *Lhx3* enhancer eliminates LHβ/FSHβ-double positive cells, while sparing the FSHβ-only cells (158). Thus, it is possible that FSHβ-only gonadotropes have distinct transcriptional requirements for *Fshb* expression, or do not respond to activins. The molecular basis for this heterogeneity would be an interesting focus for further investigation.

Nevertheless, it is clear that the vast majority of *Fshb* expression depend on SMAD4 and FOXL2 in mice.

7.1.2.Relevance of SMAD4/FOXL2 in the regulation of FSHB transcription in humans

Can the observations made in S2/3cKO, S4cKO and S4F2cKO mice be extended to humans? Several physiological and clinical observations support a role for the activin/inhibin system in FSH regulation in humans (e.g., (616, 617, 649, 911, 912)). However, activin regulation of the *Fshb/FSHB* promoter appears to differ significantly between species (203). Notably, the porcine and murine promoters are much more activin responsive than the human promoter in immortalized gonadotropes (676, 706). Indeed, the 8-bp SMAD binding element (SBE) in the proximal murine *Fshb* promoter is absent in humans, and its ectopic introduction in the human promoter increases activin responsiveness (676). The porcine promoter also lacks the 8-bp SBE, yet is very activin responsive. This was the basis for the identification of the composite forkhead/SMAD binding sites that mediate activin induction of the porcine and murine *Fshb* promoters (678, 706, 708). Limited promoter-reporter studies suggest that FOXL2 also regulates the human *FSHB* promoter (710). Indeed, mutation of two FOXL2 binding sites in the *FSHB* promoter (different than the composite elements found in the murine or porcine promoters) impairs basal and (weak) activin-stimulated transcriptional activation (710). Coupled with the fact that FOXL2 is expressed in human gonadotropes (855, 913), these observations suggest a possible role for FOXL2 (and perhaps SMADs) in human *FSHB* expression and FSH synthesis.

FOXL2 mutations are found in humans with blepharophimosis-ptosis-epicanthus inversus syndrome (BPES), which is characterized by complex eyelid malformations, sometimes accompanied by premature ovarian failure (711). FSH levels appear normal – or even elevated – in BPES patients; however, those individuals almost invariably carry heterozygous mutations, and the few reported homozygous mutations produce proteins that retain some transcriptional activity (914-916). Furthermore, heterozygous loss of *Foxl2* in the gonadotropes does not impair FSH synthesis in mice (716). Heterozygous germline mutations in *SMAD4* cause juvenile polyposis syndrome (JPS), associated with pre-malignant growths (polyps) in the intestinal tract (917). No homozygous mutations have been reported, consistent with a requirement for SMAD4 in early embryonic development (682). Thus, we cannot reach any definitive conclusions

regarding the role of SMAD4 or FOXL2 in human FSH synthesis from the described BPES or JPS patients.

Might the inter-species differences in activin regulation of *Fshb/FSHB* transcription explain evolutionary divergence in reproductive physiology? The selective FSH increase that drives ovarian follicle maturation - at estrus in rodents, following estrus in pigs, and at the beginning of the follicular phase in humans - is arguably of greater magnitude in mice and pigs compared to humans (243, 244, 918). Experimental (in mice and pigs) or correlative (in humans) evidence indicates that the selective FSH increase is activin-driven in all three species (570, 616, 617, 919). FSH levels during the secondary surge correlate with the number of ovulated oocytes or pups delivered in mice and pigs (chapter 3 and (249, 918)). Similarly, there appears to be a dose-dependent effect of circulating FSH on the number of ovulated oocytes in humans: First, multiple pregnancies are observed in ~10% of women taking clomiphene, a drug designed to increase FSH levels in the follicular phase (55, 796). Second, administration of supra-physiological doses of FSH in IVF or ovulation induction protocols drives the maturation of several (up to dozens) of preovulatory follicles (48). Thus, the interspecies differences in the magnitude of activin-dependent FSH surge may explain, at least in part, why mice and pigs deliver much larger litters (10 pups and more) than humans (typically a single baby). Taking all this evidence together, it is tempting to equate greater activin responsiveness of the *Fshb* promoter with greater FSH levels and higher number of follicles recruited per cycle. If this is true, the human *FSHB* gene should not be able to sustain normal FSH synthesis and fertility in mice. However, a human FSHβ transgene can fully rescue fertility in *Fshb* knockout females (920). This transgene comprises the region upstream of the transcription start site, that is regulated by SMADs and FOXL2 in other species, as well as the entire gene and some 3' flanking sequence (921). Does this mean that human *FSHB* can be induced by activin signaling just as well as the mouse gene? Not necessarily. These animals carry multiple copies of the transgene (921), so it is possible that the fertility rescue occurs in the context of lower activin induction of a larger number of *FSHB* "genes". The consequences of the lower activin responsiveness of the human *FSHB* gene, observed *in vitro*, on FSH synthesis *in vivo* might be best studied by generating knock-in mice carrying a "humanized" *FSHB* promoter. When coupled with cell-specific inactivation of SMAD proteins and/or FOXL2, such a model (or the transgenic rescue model mentioned above) could also be used to verify the requirement for

SMADs and FOXL2 in *FSHB* transcriptional regulation. In addition, the development of a human gonadotrope cell line, from a gonadotrope adenoma or immortalized primary gonadotropes, would be very valuable for these studies.

7.1.3.Signaling pathways upstream of SMAD4 and FOXL2

A critical role for SMAD4 and FOXL2 in the regulation of FSH synthesis *in vivo* implies a requirement for activin/activin-related ligands, their cell surface receptors, and intracellular signaling mediators acting upstream of SMAD4 and FOXL2. However, their identities remain ill-defined. Perplexingly, while activins have been purified almost 30 years ago, it is still unclear whether they are actually required for normal FSH synthesis *in vivo*. Cultured pituitary cells produce one or many ligand(s) that stimulate FSH synthesis and secretion, and that are antagonized by inhibin, follistatin and a pharmacological inhibitor of ACVR1B/ACVR1C/ TGFBR1 (SB-431542) (572, 573, 596, 597, 651, 652). The source of the ligand(s) may be the gonadotropes themselves, because SB-431542 suppresses *Fshb* expression in isolated primary gonadotropes (X Zhou *et al*, unpublished observations). If so, the relevant ligand may be activin B, but not activin A or activin AB, as gonadotropes express the βB, but not the βA activin subunit (601, 644). In support of this possibility, an activin B bioneutralizing antibody suppresses FSH secretion by cultured pituitary cells (602) and circulating FSH levels in rats (603). However, the relevant ligand(s) regulating FSH, at least *in vivo*, are not necessarily activins. Indeed, mice with a targeted disruption of the gene encoding the activin βB subunit (*inhbb*) have normal FSH levels (604). There are at least three possible explanations for this observation: First, the ligand(s) regulating FSH may come from other pituitary sources than the gonadotropes cells. Activin A and various members of the TGFβ superfamily (including several BMPs) are expressed in the pituitary (644). They may exert paracrine effects on gonadotropes and compensate for the absence of activin B. Second, the relevant ligand(s) could be derived from extra-pituitary sources. If the ligands are activins, they do not come from the gonads in females because simultaneous ablation of *inhbb* in the whole body and of the gene encoding the βA subunit (*inhba*) in the ovary causes <u>increased</u> FSH levels (605). Third, FSH synthesis may not require an extracellular activin/activin-related ligand at all. Activin signaling is thought to be important for the generation of the secondary FSH surge in rodents. However, in rats, the secondary FSH surge can be suppressed by a progesterone receptor antagonist (813-816).

Because SMADs and FOXL2 physically and functionally interact with the progesterone receptor (PGR) to activate *Fshb* transcription, their involvement in regulating FSH synthesis could be activin-independent (652, 819). Alternatively, the secondary FSH surge may be induced by the synergistic actions of activin and progesterone signaling (652, 817).

Although it is formally possible that activin/activin-related ligands are not required for normal FSH synthesis, at least one key observation suggests an important role for ligand-regulated signaling: mice lacking the activin type II receptor, ACVR2A, have clear FSH-deficiency (643). That said, the reduction in circulating FSH levels in *Acvr2a*-null mice is apparently milder (50-60% in males and females (643, 727)) than in S4cKO (90% in males; 75% in estrus morning females) and S4F2cKO (97% in males; 80% in females) mice (chapter 3)). The milder phenotype observed in *Acvr2a*-null mice could be due to partial compensation by ACVR2B. Alternatively, the differences between the models may indicate that SMAD4 and FOXL2 have activin-independent roles in regulating FSH synthesis, as mentioned above. In any case, a role for ACVR2A in the regulation of FSH strongly suggests that a ligand is required. The question is: which one? No less than 19 TGFβ superfamily ligands can signal through ACVR2A (922). A ligand-independent function for ACVR2A in gonadotropes cannot be excluded, but such a mechanism of action has not yet been described.

Because of the large number of ligands and the broad range of possible cellular source, it might be simpler to start by identifying the type I receptor(s) requirement in gonadotropes. As mentioned above, signaling through ACVR1B, ACVR1C and/or TGFBR1 is necessary for normal *Fshb* expression, at least in primary pituitary culture (651). Targeted inactivation of *Acvr1b* and *Tgfbr1* result in embryonic lethality (653, 654), and their roles in gonadotropes have yet to be assessed. Global *Acvr1c*-null mice have normal pituitary *Fshb* expression and slightly reduced circulating FSH levels, but also show hypothalamic and ovarian defects (655). Therefore, gonadotrope ACVR1C plays only a minor role, at best, in the regulation of FSH synthesis. It is possible that the type I receptors may act redundantly, in which case genetic inactivation of either type I receptor alone may not have a major impact on FSH levels and fertility. The same could be true for the ligands. Ultimately, the complexity of ligand-receptor and receptor-receptor pairing in the TGFβ superfamily (625, 922) is likely to make their functional dissection in gonadotropes challenging. For example, if ACVR1B is required for FSH

synthesis, its ligand could be any of activin A, activin B, GDF1, GDF3, GDF9, GDF11, myostatin, BMP3B or nodal (922). If TGFBR1 is required, its ligand could be any of TGFβ1, TGFβ2, TGFβ3, GDF11 or myostatin (922). Redundancy between the receptors and between the ligands would further increase the number of possibilities. Nevertheless, as demonstrated in this thesis, double or even triple conditional genetic knockout, which may be necessary to assess type I receptor requirements, is feasible with the *Gnrhr*^{GRIC} allele. Then, assessing the expression pattern of the TGFβ superfamily members that can signal through this/these receptor(s) (for example, determining which ones are expressed in gonadotropes and in the pituitary) may help narrow down the list of possible ligand(s).

In addition to the ligands and receptors, the identity of the intracellular signaling mediators acting upstream of SMAD4 and FOXL2 remains to be established. Based on several *in vitro* overexpression and knockdown studies, SMAD2 and SMAD3 are the most likely candidates (463, 539, 674-678). Of these two proteins, SMAD3 may play a more important role, at least in rodents (674, 677). Indeed, compared with SMAD2, depletion of endogenous SMAD3 has a stronger effect on activin-induced murine *Fshb* promoter activation in immortalized gonadotropes (674). Furthermore, SMAD3, but not SMAD2, physically interacts with FOXL2 (708, 709). Accordingly, SMAD3, but not SMAD2, synergistically activate the murine *Fshb* promoter with SMAD4 and FOXL2 (678). The experiments performed in chapter 2 could not confirm the predicted requirement for SMAD3 due to incomplete inactivation of its function, so the development of a true conditional null allele of *Smad3* will be required. Although SMAD2 appears dispensable by itself, it will be interesting to see if it can compensate (partially or completely) for the loss of SMAD3. SMAD1/5/8, which regulate *Fshb* transcription *in vitro* (690-692), may compensate for the loss of SMAD2/3. However, since they do not interact physically or functionally with FOXL2 ((708) and N Zhu *et al*, unpublished), and do not usually signal downstream of ACVR1B, ACVR1C or TGFBR1, this seems unlikely. Overall, the identification of the ligand(s), type I receptor(s) and receptor-regulated SMAD protein(s) acting upstream of SMAD4 and FOXL2 should be considered high priority experiments that will provide key insights in the mechanisms underlying FSH synthesis.

7.1.4. GnRH versus activin/activin-like regulation of FSH synthesis

In addition to activin/activin-like signaling, there is little doubt that GnRH is important for normal *Fshb* transcription and FSH synthesis. This is perhaps best demonstrated by GnRH-deficient *hpg* mice, which have low circulating FSH levels (62). In chapter 6, I observed that addition of a C-tail to the GnRHR blunts the ovariectomy-induced FSH increase in mice, most likely as a result of altered GnRH signaling. In fact, GnRH is widely considered to be the primary physiological regulator of FSH synthesis and release (842). In principle, the differential sensitivity of LH and FSH to GnRH pulse frequency could account for most of the selective FSH or LH synthesis: faster pulses favor LH at the time of the pre-ovulatory surge, and slower pulses favor FSH in estrus (or early follicular phase in humans) (842, 923). However, as outlined in the Introduction (section 3.2.1.), the GnRH pulse frequency may not be critical for FSH regulation. The data presented in this thesis support the view that activin/activin-like signaling (regardless of the ligand requirement) is at least as much – if not more – important than GnRH in the regulation of FSH synthesis. While the mechanisms underlying GnRH regulation of *Fshb* transcription remain obscure (203), there is no evidence that SMAD4 or FOXL2 play a role in this process (679, 706). Thus, their deletion likely affects only activin/activin-like signaling. GnRH stimulation appears insufficient to overcome the effect of loss of SMAD4 and FOXL2. What then is the role of GnRH in FSH synthesis? There are at least 3 possibilities: 1) GnRH is required for developmental activation of FSH synthesis; 2) a GnRH stimulus provides a baseline level of *Fshb* transcription, which is potentiated by activins; 3) GnRH pulses modulate the local synthesis of follistatin and/or activin subunits, permitting activin-driven selective FSH surges (924). Further investigations are required to discriminate between these possibilities, and to identify the intracellular signaling pathways engaged by GnRH to regulate *Fshb* transcription and FSH synthesis.

7.1.5. SMADs and FOXL2 regulation of other gonadotrope genes

Other than *Fshb*, S4cKO (chapter 3), F2cKO (716), and S4F2cKO (chapter 3) mice have altered expression of additional genes important for gonadotrope function. *Lhb* expression was dramatically increased in S4F2cKO mice, and moderately elevated in diestrus S4cKO females. Though *Lhb* is positively regulated by activins *in vitro* (645, 688, 719, 728, 731), a likely explanation for increased *Lhb* expression is a lower negative feedback by gonad-derived sex steroids on the pituitary and/or on GnRH release by the hypothalamus (99, 770, 787). That said,

estradiol and testosterone levels appeared unchanged in both S4cKO and S4F2cKO animals (chapter 3 and data not shown). However, measurement of sex steroids at a single time point may not be very useful to determine whether the animals have impaired steroid production. That is, the highly pulsatile patterns of sex steroid production, which explains the very variables measurements between animals, may have obscured chronic changes in their circulating levels (925). Altered sex steroid levels and/or enhanced GnRH stimulation might also explain elevated *Gnrhr* mRNA in S4cKO mice, even though activins stimulate its expression (at least, *in vitro*) (720-724). Interestingly, *Gnrhr* expression was normal in S4F2cKO mice. This could reflect the opposing effects of enhanced GnRH signaling and loss of FOXL2, a positive regulator of *Gnrhr* promoter activity (726), though the F2cKO mice show normal *Gnrhr* expression (716). The increased circulating LH levels in S4F2cKO females probably contribute to certain aspects of the phenotype. For example, older S4F2cKO females show evidence of ovarian tubulostromal hyperplasia, with hypertrophic luteal cells, a sign of LH hyperstimulation (926). Nevertheless, FSH deficiency appears to be the primary cause of ovarian failure, leading to increased LH and further ovarian anomalies.

In contrast with females, S4cKO and S4F2cKO males have decreased serum LH levels. This likely reflects lower *Cga* expression, which, in the case of S4F2cKO animals, offsets the *Lhb* increase. The striking sex-specific change in *Cga* expression suggests the involvement of sex steroids. Indeed, the *Cga* promoter is bound and regulated by the androgen receptor, but not by the estrogen receptor (771). While there have been conflicting observations regarding how activins might regulate *Cga* expression, FOXL2 stimulates *Cga* transcription *in vitro* (645, 696, 707, 722, 728). It is possible that, since the *Cga* promoter is regulated by androgens but not estrogens, loss of SMAD/FOXL2 signaling affects *Cga* expression only in males - whether or not testosterone levels are altered.

Follistatin (*Fst*) expression is decreased in S4cKO (estrus morning) and F2cKO females. This is consistent with activin stimulation of *Fst* expression being SMAD and FOXL2-dependent (709, 738). *Fst* downregulation in S4cKO females in estrus morning, but not in diestrus, may reflect activin sensitivity of *Fst* expression (595, 737). Indeed, no change in *Fst* expression was detected in S4F2cKO females when compared with diestrous controls. Because *Fst* is an activin antagonist, its downregulation would be expected to facilitate activin signaling. Therefore, it is

unlikely that the observed changes in *Fst* expression contribute to the FSH-deficiency phenotype. Are there other genes whose altered expression in the absence of SMAD4 and/or FOXL2 might affect gonadotrope function? Although no obvious candidate comes to mind that would selectively affect FSH synthesis, an unbiased screen for gene expression changes in the pituitaries or purified gonadotropes from S4cKO, F2cKO, or S4F2cKO mice would reveal the complete transcriptional program regulated by SMAD4 and FOXL2.

7.2. Regulation of the human LHB promoter by GnRH and activin signaling

In contrast to FSH, the mechanisms underlying GnRH regulation of LH synthesis and release have been well described (441). The data presented in chapter 4 suggest that GnRH activates the human *LHB* promoter in a similar manner as in other species: GnRH induces EGR1 expression, which then synergizes with SF1 and PITX proteins to directly activate the *Lhb/LHB* promoter. This suggests that GnRH-stimulated LH synthesis (*Lhb/LHB* expression being the rate-limiting step) may play similar roles in all mammals. How important is LH synthesis in the regulation of circulating LH levels? Since a given GnRH pulse appears to release a fraction – as opposed to a fixed amount - of the LH pool available for secretion (96, 888-891), smaller LH stores might result in a lower amount of LH released by GnRH. If this is true, defects in *Lhb/LHB* expression (and, hence, LH synthesis) should result in lower circulating LH levels. Unfortunately, serum LH measurements have not been systematically reported in mice with defective GnRH-regulated *Lhb* expression. Pituitary-specific male *Sf1* knockout mice have low serum LH (measurements in females were not reported) (141), whereas LH levels are low in many, but not all, *Egr1* null males (again, no female data) (145, 524, 927). In pituitary-specific *Erk1/2* knockout mice, serum LH appears normal under basal conditions, but the gonadectomy-induced upregulation is impaired (468). Globally, these observations suggest that impaired GnRH-dependent regulation of *Lhb* expression results in lower LH in the circulation. In females, GnRH-regulated LH synthesis may be particularly important at the time of the LH surge. At that time, the amount of LH that can be released by a GnRH pulse increases substantially (889). In this context, *de novo* LH synthesis may be important to replenish LH stores – which, in fact, remain constant across the surge (895) – and meet the demand for increased secretion. Thus, it is possible that EGR1/SF1/PITX-driven *Lhb/LHB* transcription ensures rapid and efficient LH production, which might be critical for ramping up circulating LH levels and generate a surge.

The fact that all mammals generate an LH surge may explain why the mechanism underlying GnRH-regulated *Lhb/LHB* transcription has been well conserved.

In contrast with the largely conserved mechanism of GnRH stimulation, activin modulation of GnRH-induced *Lhb/LHB* promoter activity is divergent between mouse and human (chapter 5). The presence of three minimal SMAD-binding elements within the proximal murine promoter, and their absence in the human promoter, contribute to the difference. Because SMAD3 and EGR1 physically interact, it seems that the two factors can cooperate to activate transcription when binding adjacent DNA elements in the mouse *Lhb* promoter. However, in the human *LHB* promoter, SMAD3 cannot bind the DNA and may instead interfere with EGR1 recruitment. What might be the physiological relevance of crosstalk between the GnRH and activin pathways? In rodents, based on the circulating inhibins pattern (610, 615), substantial activin/activin-like signaling probably occurs only in the morning of estrus. At that time, pituitary LH contents are lower than the previous day, during the LH surge (928). Thus, synergistic induction of *Lhb* transcription by activin and GnRH may help replenish LH stores after the surge. Whether there might be a need for increased LH synthesis in estrus is unclear because LH secretion (which, as explained above, likely depends on the size of the LH stores) remains relatively low until the next LH surge, 2-3 days later (240, 244). S4cKO and S4F2cKO mice have normal and elevated *Lhb* expression, respectively (chapter 3), although a positive role for SMAD proteins in *Lhb* regulation may have been masked by possible changes in the steroid milieu and/or in the intensity of GnRH stimulation. Unfortunately, the conditional *Smad3* knockout model presented in chapter 2 cannot be used to probe the functional significance of the EGR1/SMAD3 interaction, because these mice retain SMAD3's MH2 domain, which meditates the physical interaction with EGR1. A new conditional null allele of *Smad3* is required for these investigations. In humans, it is conceivable that antagonism of GnRH signaling by activins in the early follicular phase might keep LH levels "in check" while enabling a selective increase in FSH, but this remains speculative. Pharmacological manipulation of the inhibin/activin system in humans – which has not yet been reported – may help to probe this further.

7.3. Functional significance of the lack of a C-tail in the mammalian GnRHR

While GnRH regulation of LH synthesis and release is well described, the mechanisms surrounding GnRH-induced LH surge generation and termination remain somewhat obscure. The

work presented in chapter 6 provides a platform to gain new insights into the signaling requirements for the production of the surge. That is, the unique absence of the C-tail on the mammalian GnRHRs, which slows down receptor downregulation after agonist stimulation (380, 381, 383-385, 393) has been predicted to enable the generation of a protracted LH surge (390). This hypothesis can now be tested using the new *Gnrhr*$^{Ctail/Ctail}$ mouse line. Based on the data collected so far, it seems that addition of a C-tail on the mouse GnRHR may impair LH surge generation. However, the extent to which the surge might be disrupted is unclear. While the ovariectomy and estradiol replacement experiment was designed to obtain measures of the amplitude and duration of the surge, the results provided neither. Evidence for an LH surge was seen only 1 hour after lights off (8 p.m.), which is later than what others have reported using similar protocols (314, 904, 905, 929). Notably, however, the three groups that described complete LH surge profiles in mice used silastic implants to deliver estradiol (314, 904, 929), whereas another group that used osmotic minipumps (like us) reported data for a single time point (905). It is possible that the mode of estradiol delivery influences LH surge generation (930). The light/dark cycle (14 h light/10 h dark in (904, 905, 929); 12 h light/12 h dark in (314) and in our experiments) may also have contributed to the timing difference. Because the surge was observed only at 8 p.m., it is unclear whether LH levels measured at that time represent the peak of the surge. Therefore, the available data may under-represent the real difference between the *Gnrhr*$^{Ctail/Ctail}$ and control mice. Notably, the magnitude of the difference between LH levels at 8 p.m. and those at 11 a.m. were much lower in the *Gnrhr*$^{Ctail/Ctail}$ mice than controls, but this appeared to be explained by a subgroup of *Gnrhr*$^{Ctail/Ctail}$ mice that had high LH at 11 a.m. This may represent a real effect of the GnRHR modification, or a technical limitation of the experiment (LH pulsatility, or inefficient estradiol replacement). Therefore, it will be important to measure baseline LH levels in intact males and females, and to verify whether all animals subjected to the LH surge induction experiment achieved similar levels of circulating estradiol. As a complementary experiment, natural LH surges could be measured in intact proestrus mice. Reliably assessing estrous cyclicity in mice based on vaginal cytology can be challenging, but post-hoc examination for the presence of oocytes in the oviducts and corpora lutea in the ovaries would confirm that the mice were indeed in proestrus. As well, the ability of the *Gnrhr*$^{Ctail/Ctail}$ mice to respond to GnRH stimulation could be assessed by measuring the amount of LH released in response to a GnRH/GnRH agonist challenge.

Interestingly, after ovariectomy, *Gnrhr*$^{Ctail/Ctail}$ mice have lower FSH levels compared with controls. In rats, the increase in circulating FSH levels observed two days after ovariectomy is clearly GnRH-dependent (835). Therefore, *Gnrhr*$^{Ctail/Ctail}$ mice may have defective GnRH-induced FSH synthesis. How might the addition of a C-tail to the GnRHR affect GnRH regulation of FSH? One possibility is that the *Gnrhr*$^{Ctail/Ctail}$ mice simply have fewer GnRH receptors. In fact, this might also affect GnRH-regulated LH synthesis and release, and explain potential defects in LH surge generation. At this point, whether the targeted mutation affected *Gnrhr* expression in *Gnrhr*$^{Ctail/Ctail}$ mice is unknown. Regardless of the *Gnrhr* mRNA levels, it will be important to compare the number of GnRH receptors at the cell surface of gonadotropes. This can be achieved by performing binding experiments with a radio-labeled GnRH analogue on pituitary membrane preparations (931). The extent to which decreased *Gnrhr* expression or receptor number may affect LH or FSH synthesis is unclear. Heterozygous *Gnrhr*-deficient mice are fertile and overtly normal (932), but comparisons of serum gonadotropins with wild-type animals in that model, or in other point-mutants (892, 933) have not been reported. Another possibility is that the GnRH-dependent FSH up-regulation after ovariectomy requires sustained signaling through GnRHR, akin to the generation of the LH surge at the time of ovulation. Addition of a C-tail could cause higher receptor down-regulation in that context, thus impairing FSH synthesis and release. Finally, the C-tail might affect the signaling properties of the GnRHR – for example, G protein coupling – which, in turn, could impair GnRH-dependent FSH regulation.

Despite possible defects in gonadotropin synthesis and LH surge generation, female *Gnrhr*$^{Ctail/Ctail}$ females display only mild sub-fertility. What might be the evolutionary advantage of having a "tailless" receptor? It is possible that the production of protracted LH surges confers a high degree of robustness to the reproductive system. The activity of the reproductive axis depends on a number of environmental factors, for example stress or nutritional status (e.g. (934, 935)). All the experiments described in chapter 6 were conducted in the well-controlled environment of an animal care facility, where mice have access to food and water *ad libitum*, and do not have to actively defend a territory or seek a mate. In less optimal conditions like those found in the wild, a higher gonadotrope response to GnRH might be particularly beneficial. This idea could be tested by challenging *Gnrhr*$^{Ctail/Ctail}$ mice, for example with different dietary

conditions. These experiments could provide clues about why the lack of a C-tail, a unique feature among all known GPCRs, might have been positively selected during evolution.

Conclusion

These are exciting days for students of gonadotrope biology. While much has been learned about the role of gonadotropins in reproduction through physiological studies, many questions remain regarding the molecular mechanisms that regulate their synthesis and release. Answering these questions is critically important to understand both normal function and dysfunction of the reproductive axis. In turn, this will enable the development of more efficient treatments for infertility – a major unmet clinical need. In particular, the advent of sophisticated mouse genetic tools now affords unprecedented opportunities to probe the inner workings of gonadotrope cells *in vivo*. New insights obtained from these models may reveal new therapeutic targets that can be exploited to design effective pro- or anti-fertility drugs that selectively target FSH or LH synthesis.

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