THE ROLES OF PIN1 IN THE PITUITARY GONADOTROPES

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TABLE OF CONTENTS

SUMMARY

Pin1 is a peptidyl-prolyl *cis/trans* isomerase which catalyzes the isomerization of phosphorylated Ser/Thr-Pro peptide bonds. Pin1 knockout mice have marked abnormalities in their reproductive development and function. However, the molecular mechanisms underlying their reproductive defects are poorly understood. In this study, it has been demonstrated that Pin1 is required for both basal and GnRH-induced gonadotropin β subunit gene transcription through physical and functional interactions with the transcription factors SF-1, Pitx1, and Egr-1. Pin1 activates transcription of the gonadotropin β subunit genes synergistically with these transcription factors, either by modulating their stability or by increasing their protein–protein interactions. Notably, it has been shown that Pin1 is required for the Ser 203 phosphorylation-dependent ubiquitination of SF-1, which facilitates SF-1–Pitx1 interactions and therefore results in an enhancement of SF-1 transcriptional activity. It has also been demonstrated that in gonadotrope cells sufficient levels of activated Pin1 are maintained through transcriptional and post-translational regulation by GnRH-induced signaling cascades. These results suggest that Pin1 functions as a novel player in GnRH-induced signal pathways and is involved in gonadotropin β subunit gene transcription by modulating the activity of various specific transcription factors. In addition, in this study it has been shown that Pin1 can complex with the αGSU gene transcription factors, c-Jun and ATF3, and increase the protein level of ATF3 in gonadotropes. These findings would lay the foundation for investigating whether Pin1 plays a role in transcriptional regulation of αGSU gene.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

αACT	α activating element
Aβ	Amyloid-β peptide
ACTH	Adrenocortico-tropic hormone
AD	Alzheimer's disease
AF	Activation function domain
αGSU	α glycoprotein hormone subunit
Aos	Activator of SUMO
AP-1	Activator protein-1
APC	Adenomatous polyposis coli protein
APP	Amyloid precursor protein
ATF	Activating transcription factor
ATM kinase	Ataxia telangiectasia mutated kinase
Bax	BCL2-associated X protein
BCL	B-cell lymphoma
BCL2L11	BCL2 like 11
BDNF	Brain derived neurotrophic factor
BMK	Big MAPK
BrdU	5-bromodeoxyuridine
BRG-1	Brahma-related gene 1
bZIP	Basic leucine zipper
CAK	CDK-activating kinase
cAMP	Cyclic AMP
CBP	CREB binding protein
CDC25	Cell division cycle 25
CDK7	Cyclin dependent kinase 7
C/EBP	CCAAT/enhancer binding protein
ChIP	Chromatin immunoprecipitation

CnA	Calcineurin catalytic subunit A
CRE	cAMP response element
CREB	CRE binding protein
Cyp	Cyclophilin
CYP	Cytochrome P450 steroid hydroxylase
DAX-1	DSS-AHC critical region on the X chromosome protein 1
DBD	DNA binding domain
DINE	Damage-induced neuronal endopeptidase
DMEM	Dulbeco's modified Eagle's medium
DN	Dominant negative
DP103	DEAD-box protein 103
dpc	Days post coitum
DUB	Deubiquitination enzyme
EGF	Epidermal growth factor
EGFR	EGF receptor
Egr-1	Early growth response factor 1
EMSA	Electrophoretic mobility shift analysis
ERα	Estrogen receptor α
ERE	Estrogen responsive element
ERK	Extracellular signal related kinase
FBS	Fetal bovine serum
FBXW7	F-box and WD-repeat domain containing 7
FKBP	FK506-binding protein
FN-1	Fibronectin-1
FSH	Follicle stimulating hormone
FSK	Forskolin
Ftz-F1	Fushi-tarazu factor-1
GADD153	Growth arrest and DNA damage inducible protein 153
GATA	GATA binding protein
GCN5	General control nonderepressed 5

GnRH	Gonadotropin releasing hormone
GnRH RE	GnRH responsive element
GnRHR	GnRH receptor
GSE	Gonadotrope specific element
HBS	HEPES buffered saline
HBV	Hepatitis B virus
HDAC	Histone deacetylase
HECT	Homologous to E6-AP carboxyl terminus
HSF1	Heat shock factor 1
HSP27	Heat shock protein 27
Httex1p	Huntingtin exon 1 protein
Id1	Inhibitor of differentiation 1
IκB	Inhibitor of NFκB
IKK	IκB kinase
IL	Interleukin
INFβ	Interferon β
IRF3	Interferon-regulatory factor 3
IRS2	Insulin receptor substrate 2
ITCH	Itchy E3 ubiquitin protein ligase homolog
JNK	Jun N-terminal kinase
LBD	Ligand binding domain
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
LRF-1	Liver regenerating factor 1
M-2-H	Mammalian two-hybrid
MAPK	Mitogen activated protein kinase
MAT1	Ménage à trois 1
Mc2R	Melanocortin 2 receptor
Mdm2	Murine double minute 2
MEF2A	Myocyte specific enhancer factor 2A

MIS	Müllerian inhibitory substance
MKK	MAPK kinase
MMS	Methyl methanesulphonate
MPM-2	Mitotic phosphoprotein monoclonal 2
NCoR	Nuclear receptor corepressor
NDSM	Negatively charged amino acid dependent SUMOylation motif
NEK6	Never in mitosis gene α-related kinase 6
NEM	N-ethylmaleimide
NFκB	Nuclear factor κ B
NFY	Nuclear transcription factor Y
NIMA	Never in mitosis A
NLS	Nuclear localization signal
NMDAR	N-methyl-D-aspartate receptor
nNOS	Neuronal nitric oxide synthase
NSAID	Nonsteroidal anti-inflammatory drug
OTU	Ovarian tumour deubiquitinase
p53AIP1	p53-regulated apoptosis-inducing protein 1
PACAP	Pituitary adenylate cyclase activating polypeptide
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PC2	Polycomb 2 protein
PCAF	p300/CBP-associated factor
PCNA	Proliferating cell nuclear antigen
PDSM	Phosphorylation dependent SUMOylation motif
PGBE	Pituitary glycoprotein hormone basal element
PGC	Primordial germ cell
PIAS	Protein inhibitor of activated STAT
PIG3	p53-induced gene 3
Pin1	Protein interacting with NIMA-1
Pitx1	Pituitary homeobox 1

PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PLK1	Polo-like kinase 1
PMA	Phorbol 12-myristate 13-acetate
PML	Promyelocytic leukemia
PP2A	Protein Ser/Thr phosphatase 2A
PP2B	Protein Ser/Thr phosphatase 2B
PPIase	Peptidyl-prolyl *cis/trans* isomerase
pSer/Thr-Pro	Phosphorylated Ser/Thr-Pro
PTPA	Protein Ser/Thr phosphatase 2A activator
PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR
RanBP2	Ran binding protein 2
RanGAP1	Ran GTPase activating protein 1
RARα	Retinoic acid receptor α
RING	Really interesting new gene
RIP1	Receptor interacting protein 1
ROS	Roscovitine
RT	Reverse transcriptase
SAPK	Stress activated protein kinase
SBE	Smad binding elements
SCP-2	Sterol carrier protein 2
SDS PAGE	SDS-polyacrylamide gel
SENP	Sentrin specific protease
Ser/Thr-Pro	Serine/threonine residues preceding a proline
SF-1	Steroidogenic factor-1
SIL	SCL interrupting locus
SIM	SUMO interaction motif
siRNA	Small interfering RNA

Smad	Sma and Mad related protein
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
SNIP1	Smad nuclear interacting protein 1
SNURF/RNF4	Small nuclear ring finger protein/ring finger protein 4
SP1	Selective promoter factor 1
SP100	SP100 nuclear antigen
SP-RING	Siz-PIAS RING
SRC	Steroid receptor coactivator
SRY	Sex determining Region Y
StAR	Steroidogenic acute regulatory protein
SUMO	Small ubiquitin related modifier
SV40	Simian virus 40
TAX1BP1	Human T-cell leukemia virus type I transactivator binding protein 1
TFIIH	Transcription factor IIH
TGFβ	Transforming growth factor β
TLR4/IL-1R	Toll-like receptor 4 and interleukin-1 receptor
TNFR	Tumor necrosis factor receptor
TRAF6	TNF receptor associated factor 6
TRF1	Telomeric repeat binding factor 1
TSH	Thyroid stimulating hormone
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
Ub	Ubiquitin
Uba	Ubiquitin-like modifier activating enzyme
Ubc	Ubiquitin conjugating enzyme
uPA	Urokinase-type plasminogen activator
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

CHAPTER 1   INTRODUCTION

1.1 Gonadotropins

1.1.1 Pituitary gland and gonadotropes

The pituitary gland is functionally connected to the hypothalamus via the pituitary stalk, through which the release of pituitary hormones is regulated by hypothalamic hormones. The pituitary is composed of two anatomically and functionally distinct lobes: anterior pituitary and posterior pituitary. The anterior pituitary gland consists of five distinct cell types that synthesize and secrete a variety of peptide hormones regulating stress response, sex organ function, thyroid gland function, milk production, and growth. One of these specialized cell types, the gonadotropes, synthesizes and secretes two distinct hormones (gonadotropins): luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Nussey and Whitehead, 1999).

1.1.2 Gonadotropins and their biological functions

LH and FSH are heterodimeric glycoproteins comprising two noncovalently associated subunits: a common α glycoprotein hormone subunit (αGSU) and a hormone specific β subunit (LHβ or FSHβ) (Pierce and Parsons, 1981). Although it is the unique β subunit that confers the biological specificity of each hormone, full biological function is conferred only by the heterodimers.

LH and FSH are integral components of the mammalian reproductive axis in both male and female, binding to their specific receptors on the testes and ovaries to stimulate steroidogenesis and gametogenesis. In males, LH stimulates Leydig cells in the testes to synthesize and secret steroid hormones, especially androgens, while FSH acts on the Sertoli cells to stimulate spermatogenesis. In females, LH is required in the process of ovulation, luteinization, and the synthesis of progesterone and estrogen by the ovaries, while FSH stimulates the growth and development of ovarian follicles as well as promotes estrogen production by the ovaries.

1.1.3 Regulation of gonadotropin synthesis and secretion

The synthesis and secretion of gonadotropins are regulated by a number of factors at various sites along the hypothalamic pituitary gonadal axis, including gonadotropin releasing hormone (GnRH), steroid hormones (testosterone, estrogen and progesterone), and gonadal peptides (activin, inhbin and follistatin) (Pawson and McNeilly, 2005; Fig 1.1). The main regulator is GnRH, also known as luteinizing hormone releasing hormone (LHRH), which is a decapeptide neurohormone released by neurons within the hypothalamus in a pulsatile manner. The portal blood carries GnRH to the pituitary gland, which contains the gonadotrope cells. GnRH binds the GnRH receptor (GnRHR), a G-protein coupled seven transmembrane receptor on the gonadotrope cell surface, and stimulates the β isoform of phospholipase C (PLC). This results in the activation of a cascade of proteins involved in gonadotropin gene expression. GnRH differentially regulates gonadotropin gene expression and release

through varying pulses of different frequency and amplitude. High frequency GnRH pulses lead to LH release, while low frequency GnRH pulses stimulate FSH release (Dalkin et al., 1989; Haisenleder et al., 1988; Kirk et al., 1994). Androgens and estrogens act on the hypothalamus to alter GnRH pulsatility and also directly on the pituitary to either positively or negatively regulate LH and FSH synthesis and secretion (Burger et al., 2004; Luo et al., 2005).
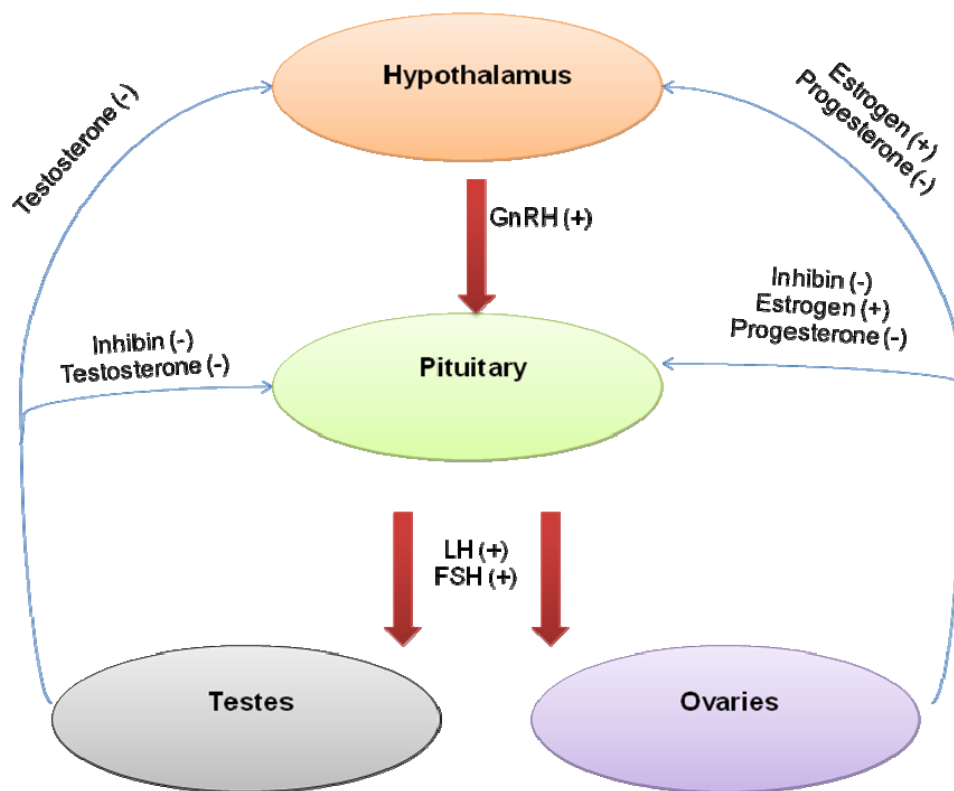
Fig 1.1: Schematic overview of the reproductive axis in male and female mammals.

GnRH, secreted from hypothalamus, binds to GnRH receptors on the surface of the gonadotrope in the anterior pituitary. GnRH acts on the gonadotrope to stimulate the synthesis and secretion of LH and FSH, which are responsible for the synthesis of testosterone, progesterone, estrogen and inhibin by the testes or ovaries. High levels of testosterone, progesterone and inhibin, inhibit GnRH, LH or FSH secretion via a negative feedback. High levels of estrogen exert a positive feedback on GnRH at the time of proestrus, subsequently leading to the LH surge and ovulation. (Adapted from Brown and McNeilly, 1999 with modification).

1.1.4 Immortalized murine gonadotrope cell lines

In the gonadotrope cell lineage, expression of αGSU is initiated at approximately embryonic day 11.5 (e11.5) in the mouse, and LHβ and FSHβ are expressed at e16.5 and e17.5, respectively (Japon et al., 1994). In order to generate immortalized gonadotrope cell lines representing gonadotropes at these different developmental stages, the αGSU and LHβ gene promoters were utilized to direct expression of the oncogenic SV40 T antigen in gonadotrope cells from e11.5 and e16.5 mouse pituitary, respectively (Windle et al., 1990; Alarid et al., 1996). The αT3-1 cell line represents the early gonadotrope (e11.5) that is not fully differentiated and expresses αGSU, GnRH receptor and transcription factors steroidogenic factor-1 (SF-1), pituitary homeobox 1 (Pitx1) and early growth response factor 1 (Egr-1), but does not express either the LHβ or the FSHβ gene (Windle et al., 1990). The LβT2 cell line represents the mature gonadotrope (e16.5) that is fully differentiated and expresses the GnRH receptor, SF-1, Pitx-1, Egr-1, and all of the three gonadotropin subunit genes (Turgeon et al., 1996). These two immortalized cell lines provide useful model systems to investigate gonadotrope development at different stages or transcriptional regulation of gonadotropin subunit genes at both the basal and GnRH-stimulated levels.

1.2 Basal and GnRH-induced gonadotropin subunit gene transcription

Transcriptional regulation of the gonadotropin subunits involves two key mechanisms, namely basal and GnRH-induced gene expression (Fig 1.2). Basal gene expression is maintained throughout development, whereas the second mechanism is activated during the reproductive years. GnRH-induced signaling to the LH and FSH subunit genes is predominantly via the protein kinase C (PKC) pathway and downstream mitogen activated protein kinase (MAPK) cascades, including extracellular signal related kinase 1/2 (ERK 1/2), Jun N-terminal kinase (JNK), p38 MAPK and big MAPK (BMK or ERK5), while protein kinase A (PKA) is also activated following GnRH-induced increase in cyclic AMP (cAMP) (Naor, 2009; Lim et al., 2009). Activated MAPKs and PKA phosphorylate downstream cytoplasmic protein kinases and ultimately target transcription factors to upregulate gonadotropin subunit gene expression. GnRH signaling also induces the release of calcium from intracellular stores which activates calmodulin, stimulating downstream calmodulin kinases and the phosphatase calcineuin, which play a crucial role in the derepression of the FSHβ gene (Lim et al., 2007).

Gonadotropin subunit gene transcription
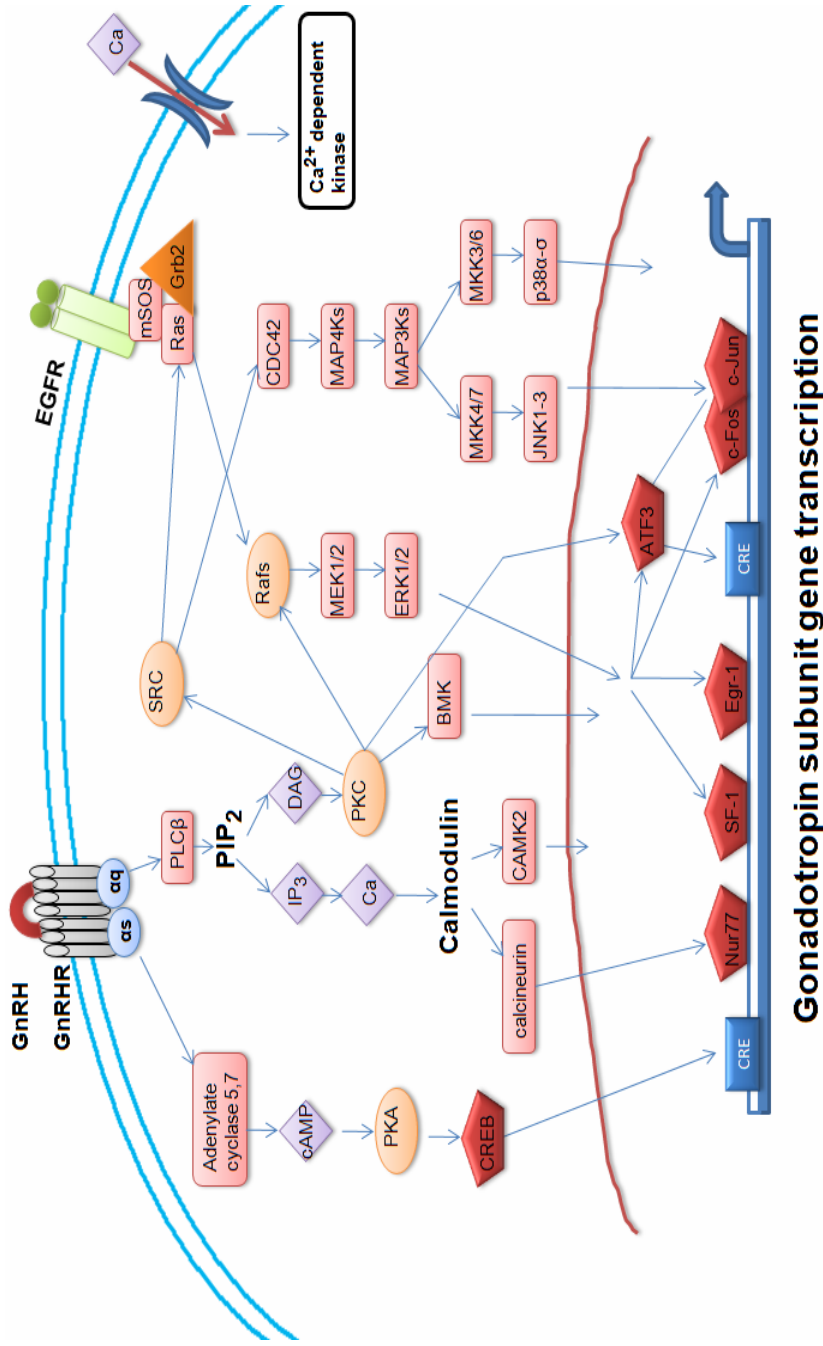
Fig 1.2: Schematic diagram of GnRH-induced signaling cascades.

The PKC pathway and four distinct downstream MAPK signaling cascades, including ERK 1/2, JNK, p38 MAPK and BMK (or ERK5) are activated following GnRH stimulation. PKA is also activated following GnRH-induced increase in cAMP. Calcium influx induced by GnRH which activates calmodulin, also increases levels of CaMKI and CaMKII, and leads to activation of the phosphatase calcineuin. A number of transcription factors, including CREB, ATF3, SF-1, Egr-1, c-Jun and c-Fos, are activated through phosphorylation by these kinases. These cascades may be involved in transcriptional regulation of gonadotropin subunit genes. Abbreviations are given in the text. (Adapted from Naor, 2009 with modification).

1.2.1 Transcriptional regulation of the αGSU subunit

The αGSU, the common subunit of the glycoprotein hormones (LH, FSH and thyroid stimulating hormone (TSH)), is expressed in the gonadotropes and thyrotropes of the pituitary and in the trophoblasts of the placenta. Cell or tissue-specific expression of αGSU is determined by distinct sets of *cis*-acting elements along with their cognate binding factors (Maurer et al., 1999). *Cis*-elements residing in the promoter that contribute to gonadotrope-specific αGSU gene transcription include the E boxes, Pitx1 binding element, gonadotrope specific element (GSE), cAMP response element (CRE) and pituitary glycoprotein hormone basal element (PGBE) (Jorgensen et al., 2004; Fig 1.3).
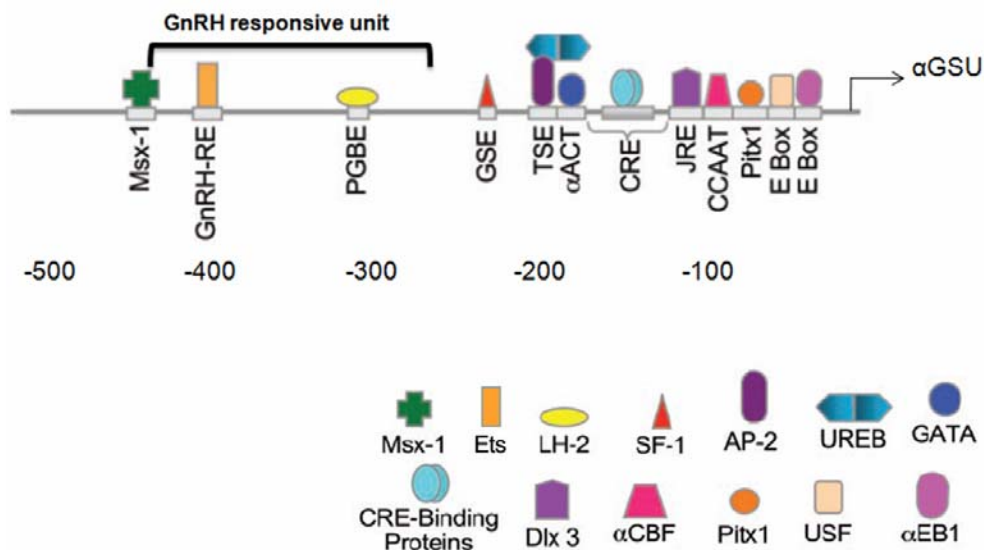
Fig 1.3: Schematic model of several elements defining the αGSU expression.

In the gonadotropes, specific promoter elements and their cognate binding factors are responsible for αGSU gene expression. The transcription of the αGSU gene is stimulated by synergistic participation from several transcription factors, including SF-1, Pitx1 and CREB, which bind the tandem *cis*-elements. The numbers indicate bp distance upstream of transcriptional start site. Abbreviations are given in the text. (Adapted from Jorgensen et al., 2004 with modification).

Among these *cis*-elements, the CRE site (5'-TGACGT(C/A)(G/A)-3') plays an indispensable role in both the basal and GnRH-induced αGSU expression. It has been reported that transcription factors binding to the CRE on the αGSU promoter include CRE binding protein (CREB) and activating transcription factors (ATF) 1, 2 and 3, which all belong to ATF/CREB family (Drust et al., 1991; Heckert et al., 1995; Heckert et al., 1996; Xie et al., 2005). ATF3, which is markedly induced by GnRH *in vivo* and in the αT3-1 gonadotrope cells due to a combined role of PKC, ERK and JNK, was reported to activate the human αGSU promoter (Xie et al., 2005). Supershift assay showed that recombinant ATF3 alone or in combination with c-Jun is sufficient to bind to the CRE on the αGSU promoter, which provides an explanation for CRE-dependent GnRH-induced αGSU promoter activity, since both *Atf3* and *c-Jun* have been identified as immediate early genes in response to GnRH (Xie et al., 2005).

Within 60 bp of the CRE are two other important regulatory elements, GSE and α activating element (αACT). The GSE, which resides between -220 and -202 bp of the mouse αGSU promoter, is a specific binding site of the orphan nuclear receptor SF-1 and confers a minor role for GnRH- or pituitary adenylate cyclase activating polypeptide (PACAP)-stimulated αGSU expression (Jorgensen et al., 2004; Fowkes and Burrin, 2002; Fowkes et al., 2002; Fowkes et al., 2003). Over-expression of SF-1 increases both the basal and PACAP-stimulated αGSU transcription (Fowkes et al., 2003). GATA binding protein (GATA)-2, which binds the neighboring αACT element, has been shown to interact physically with SF-1, transactivate the αGSU

gene alone or in synergy with SF-1, and mediate ERK-activation of human αGSU

gene transcription in LβT2 cells (Fowkes et al., 2002; Steger et al., 1994).

There are some additional fundamental elements and factors on the promoter

responsible for αGSU gene transcription, such as PGBE, binding the member of the

LIM homeodomain family, LH-2 or Lim3/Lhx3, which is required for the basal

αGSU promoter activity (Schoderbek et al., 1992; Brinkmeier et al., 1998; Roberson

et al., 1994; Bach et al., 1995). Furthermore, in αT3-1 cells, knock-down of Pitx1

results in suppression of both Lim3/Lhx3 and αGSU gene expression, suggesting

Pitx1 as an earlier regulator in promoting αGSU expression through both its action on

Lim3/Lhx3 and directly binding the Pitx1 responsive element located in the αGSU

gene promoter (Sheng et al., 1996; Tremblay et al., 1998).

The GnRH responsive element (GnRH RE), which is located in -416 to -385 bp,

confers robust GnRH-induced activity to the mouse αGSU gene promoter

(Schoderbek et al., 1993). A member of the Ets family is a likely *trans*-acting partner

that binds this site because over-expression of a dominant-negative form blocks the

GnRH response (Roberson et al., 1995). Furthermore, as mentioned above, CRE, GSE

and PGBE have also been found to provide supportive contribution to GnRH

responsiveness. The precise control of the basal and GnRH-stimulated αGSU gene

expression requires synergistic participation from those various *cis*-elements and their

trans-acting partners and is not dependent on a single regulator.

1.2.2 Transcriptional regulation of the gonadotropin β subunits

As synthesis of the β subunit is the rate limiting step in hormone synthesis, regulation of the gonadotropin β subunit genes is crucial in the production of the physiologically active hormone. Similar to the αGSU gene, gonadotropin β subunit genes, LHβ and FSHβ, are also regulated by interplay between extrinsic signals and intrinsic regulatory elements (Fig 1.4).
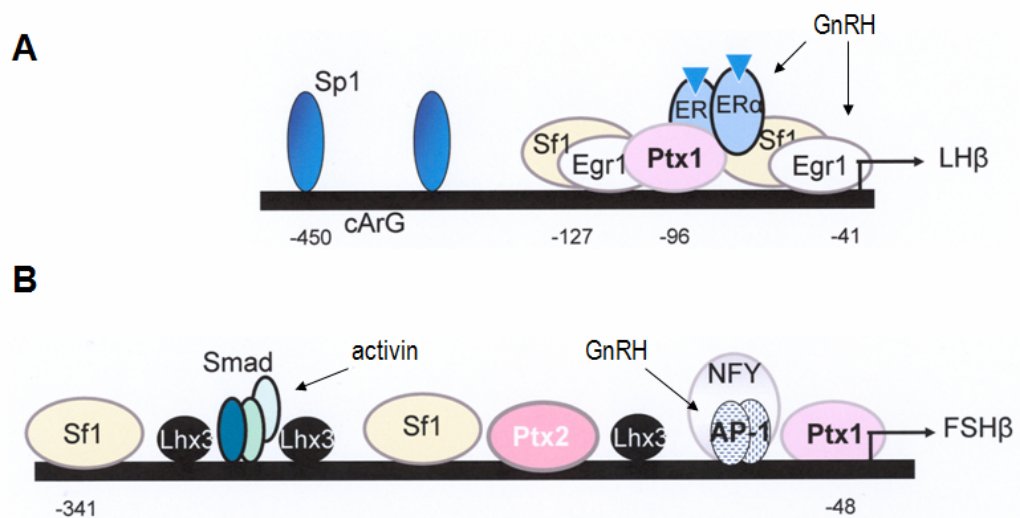
Fig 1.4: Schematic models of transcription factors activating rodent gonadotropin β subunit gene expression.

(A) The proximal region in the LHβ gene promoter binds SF-1 and Egr-1 at two sites, and also Pitx1; whereas ERα is recruited via protein–protein interactions with Pitx1 and SF-1. SP1 binds further upstream on the promoter and is also required for the GnRH response. (B) Conserved response elements for AP-1 are present on the proximal promoter of FSHβ. AP-1 response elements might mediate GnRH stimulatory effects to the FSHβ subunit. Pitx1 has also been shown to regulate the basal and GnRH-induced FSHβ gene expression. Other transcription factors that have been shown to activate the FSHβ promoter include NFY, Lhx3 and the Smad family. The numbers indicate bp distance upstream of transcriptional start site. Abbreviations are given in the text. (Adapted from Melamed et al., 2006 with modification).

The proximal 140 bp region in the mammalian LHβ gene promoters from several species contains a highly conserved tripartite element that binds SF-1, Egr-1 and Pitx1, which activate transcription individually and synergistically to mediate the basal and GnRH-stimulated promoter activity (Dorn et al., 1999; Halvorson et al., 1998; Quirk et al., 2001; Tremblay and Drouin, 1999).

SF-1, a member of the orphan nuclear receptor superfamily, is a key regulator of steroidogenic and gonadotropic gene expression (Parker and Schimmer, 1997; Luo et al., 1994). It is expressed predominantly in steroidogenic tissues including adrenals, gonads, ventromedial hypothalamus, anterior pituitary, and nonsteroidogenic organ---spleen (Ramayya et al., 1997). In the pituitary it is only expressed in gonadotropes (Ingraham et al., 1994; Asa et al., 1996). SF-1 has a predominant role in LHβ expression. The tandem SF-1 binding sites in the LHβ gene promoter are essential for the promoter activity (Keri and Nilson, 1996; Halvorson et al., 1996). Moreover, in the pituitaries of SF-1 knockout mice, the LHβ transcript is undetectable, while expression of αGSU and the FSHβ subunit are only significantly decreased (Ingraham et al., 1994; Ikeda et al., 1995; Shinoda et al., 1995).

Egr-1, a zinc finger transcription factor which is also known as NGFI-A, Zif268, or Krox-24, binds a pair of GC-rich regions closely associated with the tandem SF-1 binding sites in the LHβ gene promoter (Halvorson et al., 1996; Wolfe and Call, 1999; Tremblay and Drouin, 1999). Egr-1 is rapidly upregulated following GnRH treatment and has been shown to mediate much of the promoter response to GnRH, indicating

that it is a downstream effector of GnRH (Tremblay and Drouin, 1999). It has been demonstrated that the induction of Egr-1 by GnRH, which can be mimicked by the PKC activator phorbol 12-myristate 13-acetate (PMA), occurs through activation of the PKC and MAPK pathway (Dorn et al., 1999; Khokhlatchev et al., 1998).

Pitx1 is expressed in all pituitary cell types and is essential for both anterior pituitary development and the synthesis of most of the pituitary hormones including the gonadotropins and TSH (Tremblay et al., 1998; Melamed et al., 2002). Both the number of gonadotropes and thyrotropes, as well as the levels of αGSU, LHβ, FSHβ and TSHβ transcript and protein within the individual cells, are substantially reduced in Pitx1-null mice (Szeto et al., 1999; Tremblay and Drouin, 1999). The first characterized Pitx1 binding site in the LHβ promoter is nested between the pairs of Egr-1 and SF-1 binding sites (Tremblay et al., 1998, Tremblay & Drouin 1999, Quirk et al., 2001; Melamed et al., 2006). A second functional Pitx1 region spanning positions −73 to −52 in the promoter was identified several years later, and Pitx1 was shown to stimulate LHβ gene expression via these two functional DNA-regulatory regions (Jiang et al., 2005). Pitx1 also plays an important role in activating SF-1, in which the C-teminus of Pitx1 interacts with the putative ligand binding domain (LBD) of SF-1 to enhance transcriptional activity of SF-1 through an apparent unmasking of SF-1 (Tremblay et al., 1999). The SF-1 binding site in the promoter is strictly required for the transcriptional synergism between Pitx1 and SF-1 on LHβ gene (Tremblay et al., 1998; Tremblay et al., 1999). It should be noted here that both SF-1 and Pitx1 may

be phosphorylated possibly through GnRH-stimulated kinases (Hammer et al., 1999; Melamed et al., 2002).

Our group has demonstrated that the liganded estrogen receptor α (ERα) also regulates LHβ gene transcription, through protein–protein interactions with SF-1 and Pitx1 in an estrogen responsive element (ERE)-independent manner; these allow association of the activated ERα to the proximal promoter of mouse LHβ gene (Luo et al., 2005).

In rodents, selective promoter factor 1 (SP1) binds further upstream on the promoter and is also required for the GnRH response (Kaiser et al., 1998), but the ways in which SP1 and other factors binding the distal elements of the LHβ gene promoter activate LHβ transcription and mediate the GnRH response remain unclear. The suggested mechanism involves interaction of these factors with the proximal promoter binding proteins through a coactivator complex. One possible such coactivator appears to be the small nuclear ring finger protein (SNURF), also known as ring finger protein 4 (RNF4), which was shown to associate specifically with the LHβ promoter in chromatin immunoprecipitation (ChIP) assays and interacts with both SF-1 and SP1. Over-expression of SNURF increases both the basal and GnRH stimulated LHβ gene expression (Curtin et al., 2004).

Compared to the LHβ, regulation of the FSHβ subunit gene transcription at the molecular level is not well understood which might be due to that FSHβ is expressed

at very low level in LβT2 cells. Numerous studies have revealed that conserved response elements for activator protein-1 (AP-1) factors, c-Jun and c-Fos, are present on the proximal promoter of FSHβ. It was thought that AP-1 response elements might mediate GnRH stimulatory effects to the FSHβ subunit as both c-Jun and c-Fos are rapidly upregulated after GnRH stimulation (Strahl et al., 1998; Liu et al., 2002). However, these AP-1 elements may be not sufficient to mediate GnRH responsiveness to the FSHβ subunit gene in gonadotropes (Huang et al., 2001; Vasilyev et al., 2002). In the mouse FSHβ gene promoter, a response element localizing primarily to an AP-1 half site (-72/-69) and an adjacent CCAAT box which binds the basal transcription factor nuclear factor Y (NFY) are required for AP-1 binding and are critical for full activation of FSHβ by GnRH. NFY and AP-1 physically interact and co-occupy this site in gonadotropes after GnRH stimulation (Coss et al., 2004). Furthermore, it has been demonstrated that NFY also interacts functionally and physically with SF-1 via two SF-1 binding sites on the mouse FSHβ promoter to regulate basal FSHβ gene expression (Jacobs et al., 2003). Pitx1 has also been shown to regulate the basal and GnRH-induced FSHβ gene expression through both direct and indirect interactions with the promoter in LβT2 cells (Zakaria et al., 2002). Other transcription factors that have been shown to activate the FSHβ promoter include Lhx3 (West et al., 2004) and the Smad family (Suszko et al., 2003). Activin, a member of the transforming growth factor β (TGFβ) superfamily of ligands, induces phosphorylation of several Smad proteins allowing their translocation into the

nucleus and binding to Smad binding elements (SBE) on the FSHβ promoter, where

the Smads help recruit other activators and coactivators (Suszko et al., 2003).

1.2.3 Phosphorylation of transcription factors by MAPKs

It has been well accepted that serine/threonine residues preceding a proline

(Ser/Thr-Pro) form one of the major regulatory phosphorylation motifs. Protein or

peptide containing proline is able to form both the *cis* and *trans* isomers because the

unique five-membered ring structure of the proline side chain has the ability to adopt

either the *cis* or *trans* state of the backbone torsion angle. Most of the isomerization

occurs in a surface-accessible bend, coil or turn of the target protein, inducing protein

conformational change, therefore modulating the stability and/or activity of the

protein. Conformational change mediated by *cis/trans* isomerization of proline after

phosphorylation of Ser/Thr-Pro motif has been identified as an intrinsic regulatory

switch to modulate the stability and activity of proteins involved in various cellular

processes (Lu et al., 2007; Lu et al., 2002; Stukenberg and Kirschner, 2001).

Many of the transcription factors involved in the transcriptional regulation of

gonadotropin subunit genes have been shown to be activated through phosphorylation

at the Ser/Thr-Pro motif by Pro-directed kinases including the MAPKs. Studies have

demonstrated that SF-1 phosphorylation by MAPK at the Ser 203-Pro motif located in

the hinge region of the protein is required for recruitment of SF-1 cofactors and

maximal SF-1 target gene transcription (Hammer et al., 1999; Fowkes et al., 2003).

SP1 has been reported to be directly phosphorylated by p42/p44 MAPK at Thr 453 and Thr 739-Pro motifs both *in vitro* and *in vivo*, and this is implicated in the upregulation of vascular endothelial growth factor (VEGF) gene transcription (Milanini-Mongiat et al., 2002). Moreover, phosphorylation of SP1 at Ser 59-Pro by senescence-associated phosphorylated ERK 1/2 enhances p21 transcription (Kim et al., 2009). In LβT2 cells, ERα is phosphorylated at Ser 118-Pro in the nucleus after GnRH treatment, leading to its rapid association with the p300/CBP-associated factor (PCAF) and the transcriptional activation of fosB (Chen et al., 2009). Phosphorylation of c-Jun at Ser 63/73-Pro motifs by activated JNK or oncogenic Ras has been shown to enhance its transcriptional activity towards target genes such as cyclin D1 (Lu, 2004). c-Fos is phosphorylated by ERKs at multiple threonine residues within its C-terminal transactivation domain, resulting in increased transcriptional activity (Monje et al., 2005). Phosphorylation of Smad4 at Thr 276-Pro in the linker region by MAPK can result in enhanced TGF-β-induced nuclear accumulation and, as a consequence, enhanced transcriptional activity of Smad4 (Roelen et al., 2003). Collectively, phosphorylation of Ser/Thr-Pro motif is one of the most common means to transmit the GnRH signal from the membrane-bound GnRH receptor into the nucleus and stimulate gonadotropin subunit gene transcription in a precise and targeted manner (Naor, 2009).

1.3 Pin1

As reviewed above, the role of phosphorylation at Ser/Thr-Pro motif in modulating

protein function through inducing conformational change has long been considered to

act as a signal for recruitment of other proteins and/or substrates to signaling networks

(Lu and Zhou, 2007). However, *cis/trans* proline isomerization is a rather slow

conversion. A ubiquitous mechanism explaining how phosphorylation could induce

conformational change of protein was not elucidated in detail until the identification

of the unique and conserved peptidyl-prolyl *cis/trans* isomerase (PPIase) protein

interacting with NIMA (never in mitosis A)-1 (Pin1), which specifically isomerizes

phosphorylated Ser/Thr-Pro (pSer/Thr-Pro) motifs in a subset of proteins (Lu, 2004;

Fig 1.5).
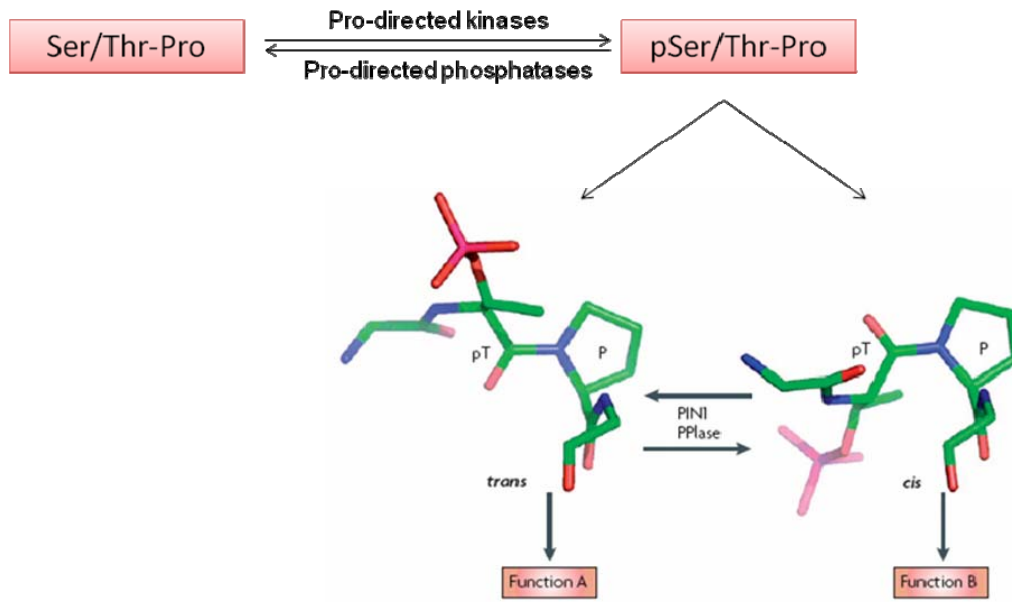
Fig 1.5: Schematic model of the action of Pin1 in catalyzing *cis-trans* isomerization of pSer/Thr-Pro motif.

Reversible phosphorylation of proteins on Ser/Thr-Pro motifs by Pro-directed kinases produces the substrate for Pin1, which alters the conformation of target proteins by catalyzing the *trans* to *cis* or the *cis* to *trans* isomerization, depending on specific target sites. The resulting functional changes of the target proteins include catalytic activity, protein dephosphorylation, protein–protein interaction, subcellular localization and/or turnover. (Adapted from Lu and Zhou, 2007 with modification).

1.3.1 The identification of Pin1

Pin1 was first identified as a suppressor of NIMA in *Aspergillus nidulans* through yeast two-hybrid screening (Lu et al., 1996). NIMA is an essential mitotic kinase which is phosphorylated at multiple Ser/Thr-Pro motifs during mitosis. The over-expression of NIMA in *A. nidulans* induces premature chromatin condensation and disrupts cytoplasmic microtubules, followed by cell death (Osmani et al., 1988). As its over-expression prevents mitotic catastrophe induced by NIMA over-expression, Pin1 was initially considered as a negative regulator of mitosis (Lu et al., 1996). Further studies revealed that Pin1 functions as a PPIase regulating diverse cellular processes besides cell division *per se*, including growth signal responses, cellular stress responses, cell proliferation and immune responses (Ranganathan et al., 1997; Stukenberg et al., 2001; Liou et al., 2002; Monje et al., 2005; Yeh et al., 2006; Saitoh et al., 2006).

1.3.2 The uniqueness of Pin1 amongst peptidyl prolyl isomerases

PPIases are an evolutionarily-conserved group of enzymes that catalyze the *cis/trans* isomerization of peptidyl-prolyl peptide bonds (Göthel and Marahiel, 1999). PPIases are categorized into four structurally distinct subfamilies: cyclophilins (Cyps), FK506-binding proteins (FKBPs), parvulins and protein Ser/Thr phosphatase 2A (PP2A) activator PTPA (Lu et al., 2007; Yeh and Means, 2007). Pin1 belongs to parvulin subfamily, and it is the only phosphorylation-dependent PPIase known so far

that specifically recognizes and isomerizes the Ser/Thr-Pro motifs after their phosphorylation. In addition, the specificity of Pin1 is accentuated by the finding that phosphorylation further restrains the already slow intrinsic isomerization rate of Ser/Thr-Pro bonds and also renders the phsophorylated motif resistant to the isomeric catalysis action of conventional PPIases (Yaffe et al., 1997).

The unique feature of Pin1 is determined by its structure. Pin1 is an approximately 18 kDa protein, with an N-terminal WW protein binding domain (amino acids 1-39) which is named after two highly conserved tryptophan residues spaced 20 to 22 amino acids apart, followed by a C-terminal PPIase catalytic domain (amino acids 45-163); these two domains are connected by a short flexible linker (Macias et al., 2002; Lu et al., 2007). X-ray structure of Pin1-phosphopeptide complex reveals that Ser 16, Arg 17 and Tyr 23 in the WW domain are responsible for binding the phosphate of pSer/Thr-Pro, and the aromatic rings of Tyr 23 and Trp 34 form an aromatic clamp to accommodate the ring atoms of proline in pSer/Thr-Pro (Verdecia et al., 2000). Structural analysis also shows that the conserved catalytic residues Lys 63, Arg 68 and Arg 69 in the PPIase domain sequester the proline and the peptide bond undergoing *cis/trans* isomerization in the phosphorylated Ser/Thr-Pro motif, and are involved in the catalysis (Ranganathan et al., 1997). Thus, the N-terminal WW domain and C-terminal PPIase domain together form a double-check mechanism conferring the unique substrate specificity of Pin1, reducing the energy barrier between *cis* and *trans* conformations, thus promoting conformational change in the substrates.

1.3.3 The involvement of Pin1 in diverse biological and pathological processes

Recent studies have uncovered that Pin1-catalyzed post-phosphorylation isomerization participates in various important physiological and pathological processes such as cell cycle control, cell signaling transduction, gene transcription, immune response and oncogenesis, through regulating its target protein activities including transcriptional activity, enzymatic activity, protein stability, subcellular localization, and protein–protein interaction (Lu et al., 2007; Lu and Zhou, 2007; Yeh and Means, 2007; Fig 1.6).

Furthermore, Pin1 often employs multiple mechanisms to regulate its target protein, and/or act on multiple factors at different levels of a specific biological process under certain conditions (Lu and Zhou, 2007). In the following sections, the role of Pin1 in oncogenesis and degenerative diseases will be elaborated based on the regulation of distinct targets by Pin1; the reproductive abnormalities in Pin1 knockout mice will also be highlighted.
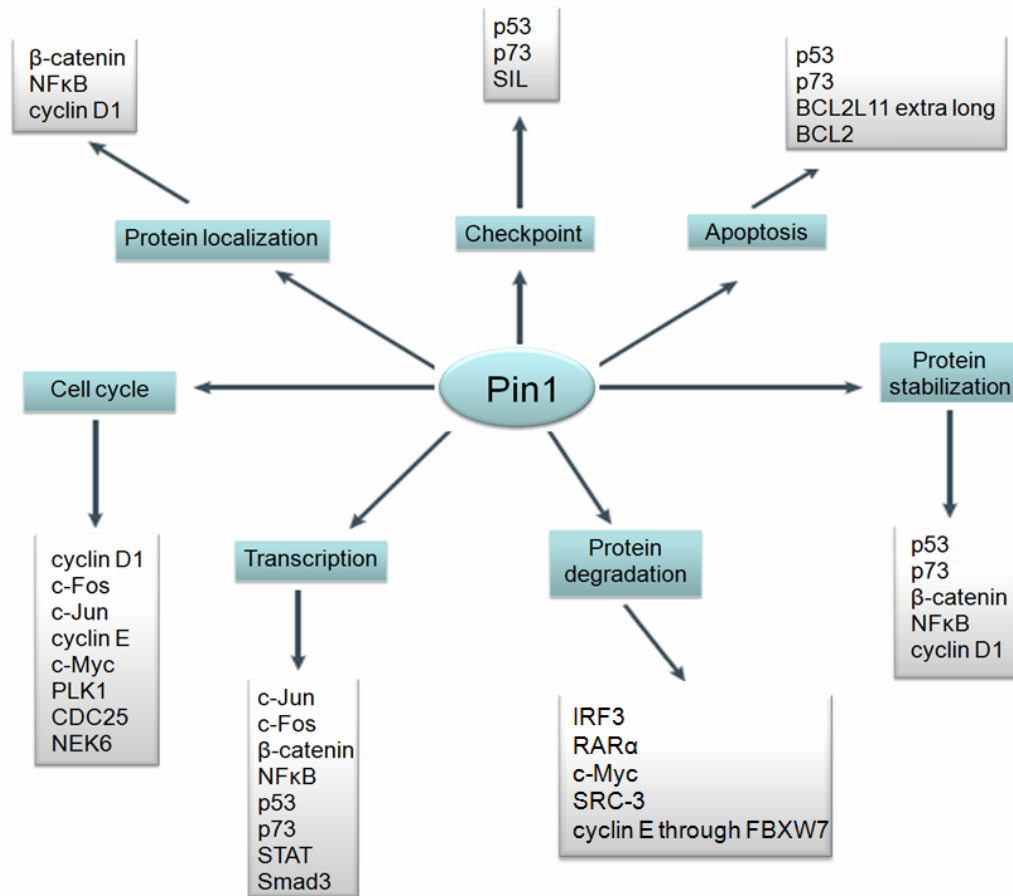
Fig 1.6: The involvement of Pin1 in various cellualr processes and functional targets of Pin1.

Pin1 regulates cell cycle progression through its interactions with proteins such as cyclin D1 (Liou et al., 2002; Wulf et al., 2001), cyclin E (Yeh et al., 2006; van Drogen et al., 2006), PLK1 (Shen et al., 1998; Eckerdt et al., 2005), c-Fos (Monje et al., 2005), c-Jun (Wulf et al., 2001), CDC25 (Crenshaw et al., 1998; Stukenberg and Kirschner, 2001; Zhou et al., 2000), and NEK6 (Chen et al., 2006); and influences checkpoint through p53 (Zacchi et al., 2002; Zheng et al., 2002; Wulf et al., 2002; Berger et al., 2005; Mantovani et al., 2007), p73 (Mantovani et al., 2004) and SIL (Campaner et al., 2005). Pin1 also is involved in apoptosis through p53, p73, BCL2 (Basu et al., 2002), and the extra long isoform of BCL2 like 11 (Becker and Bonni, 2006). Some of these effects are results of changing in protein localization, as shown for β-catenin (Ryo et al., 2001), NFκB (Ryo et al., 2003), and cyclin D1; protein stability, as shown for SRC-3 (Yi et al., 2005; Wu et al., 2007), β-catenin, cyclin D1, and cyclin E; or transcriptional activity, as shown for c-Jun, c-Fos, p53, β-catenin, and Smad2/3 (Nakano et al., 2009). Abbreviations are given in the text. (Adapted from Yeh and Means, 2007 with modification).

1.3.3.1 Pin1 and oncogenesis

An increasing number of studies suggest that Pin1 is involved in cell growth and oncogenesis, and that Pin1 is prevalently over-expressed in some human cancers including breast, prostate and lung cancers (Liou et al., 2002; Ryo et al., 2002; Yeh et al., 2006; Wulf et al., 2001; Yeh et al., 2004; Ayala et al., 2003; Zheng et al., 2009; Fan et al., 2009). In most of these cancer tissues, Pin1 expression level closely correlates with cyclin D1 and β-catenin levels and also with poor clinical outcome (Ryo et al., 2001; Wulf et al., 2001; Ayala et al., 2003). Cyclin D1 is both transcriptionally upregulated and post-translationally stabilized by Pin1 (Wulf et al., 2001; Ryo et al., 2001; Liou et al., 2002; Wulf et al., 2004). Pin1 cooperates with the Ras-JNK pathway to bind c-Jun phosphorylated at Ser 63/73-Pro motifs and increase the transcriptional activity of c-Jun towards the cyclin D1 promoter (Wulf et al., 2001). Pin1 also binds phosphorylated Ser 246-Pro motif in β-catenin, inhibits its interaction with the tumor suppressor adenomatous polyposis coli protein (APC) and promotes its translocation into the nucleus, thus also elevating cyclin D1 gene expression (Ryo et al., 2001). Furthermore, Pin1 directly binds the phosphorylated Thr 286-Pro motif in cyclin D1 and inhibits the export of nuclear cyclin D1 into the cytoplasm, where it is normally degraded by ubiquitin-proteasome pathway (Diehl and Sherr, 1997; Diehl et al., 1997; Liou et al., 2002). Depletion of Pin1 in mice also leads to a phenotype bearing a resemblance to cyclin D1 knockout mice in mammary gland and some other tissues (Liou et al., 2002; Wulf et al., 2004). Pin1 is also essential for Neu/Ras-induced transformed phenotypes of mammary epithelial cells;

Pin1 itself is an E2F downstream gene and its expression is enhanced by Neu/Ras signaling through a postitive feedback loop (Ryo et al., 2002).

Although above mentioned evidence from cell culture and animal models support that Pin1 is a key player for oncogenesis, many other studies have also revealed a contradictory role of Pin1 in cancer as a tumor suppressor. For example, Pin1 regulates the tumor suppressor p53 in response to DNA damage, by increasing its stability and transcriptional activity, stimulating p53 acetylation by p300 acetyltransferase and promoting the efficient binding of p53 to target promoters (Wulf et al., 2002; Mantovani et al., 2007). Pin1 also disrupts the association between p53 and the apoptosis inhibitor iASPP, activating the apoptotic function of p53 (Mantovani et al., 2007). In addition, Pin1 has been found to bind the p53 family member, p73, on its Ser 412/Thr 442/Thr 482-Pro motifs, increasing the accumulation of p73 in cells under stress; Pin1 is required for p73 acetylation by p300 (Mantovani et al., 2004). Other studies indicating Pin1 as a tumour suppressor include the finding that loss of Pin1 increases the stability of two oncoproteins, cyclin E and c-Myc, and arrests the cells in G1/S phase (Yeh et al., 2004; Yeh et al., 2006), and that Pin1 promotes the degradation of oncoprotein B-cell lymphoma (BCL) 6, which is involved in chromosomal translocations in many types of lymphomas (Phan et al., 2007).

Therefore, to date the contribution of Pin1 in tumorigenesis is still controversial and enigmatic. Possible explanations could be that Pin1 plays a positive or negative role in cancer depending on different cell type or genetic background.

1.3.3.2 Pin1 and degenerative disease

Pin1 knockout mice display several phenotypes including body weight loss, testicular and breast atrophy, retina degeneration, neuron degeneration, decreased fertility and decreased bone radiodensity (Zhou et al., 2000; Liou et al., 2002; Liou et al., 2003; Atchison et al., 2003; Lee et al., 2009), most of which are premature ageing phenotypes. Pin1 has been proposed to protect against the progressive neurodegenerative disorder Alzheimer's disease (AD), which is characterized by the formation of filamentous inclusions by hyper-phosphorylated microtubule associated protein tau and the aggregation of the amyloid-β peptide (Aβ). Pin1 binds to tau, promotes the dephosphorylation of tau, and restores its ability to associate with microtubule and accelerate microtubule assembly (Zhou et al., 2000; Lu et al., 1999; Liou et al., 2003). Pin1 also regulates amyloid precursor protein (APP) processing, thus reducing neurotoxic Aβ synthesis (Pastorino et al., 2006). A more recent study has demonstrated that Pin1 is involved in telomere maintenance and ageing through interacting with telomeric repeat binding factor 1 (TRF1), reducing the stability of TRF1 and its ability to bind to telomeres, resulting in gradual telomere loss (Lee et al., 2009). In addition, the interaction between Pin1 and the growth factor adapter p66Shc, after the phosphorylation of p66Shc by oxidative conditions, induced PKCβ, triggers

p66Shc mitochondrial accumulation and subsequent apoptosis, implicating that Pin1 plays an important role in oxidative stress and ageing (Pinton et al., 2007). Taken together, these findings clearly indicate that Pin1-catalyzed protein isomerization is involved in protecting against ageing-related degenerative diseases.

1.3.3.3 Pin1 and reproduction

Studies on Pin1 knockout mice showed that these mice also exhibit a range of reproductive abnormalities, including decreased fertility, testicular atrophy and reduced testes size, seminiferous tubule degeneration and spermatogonial depletion (Liou et al., 2002; Atchison et al., 2003; Atchison and Means, 2003). Furthermore, female Pin1 knockout mice have a phenotype showing severe reduction in mammary epithelial duct development during pregnancy (Liou et al, 2002). These marked reproductive defects in Pin1 knockout mice, together with the known phosphorylation events required for gonadotropin subunit gene transcription, suggest that Pin1 might have a role in mammalian reproductive system through mediating gonadotropin gene transcription.

1.3.4 The regulation of Pin1 activity

As Pin1 has been shown to be involved in numerous cellular processes, Pin1 activity has to be tightly regulated at multiple levels under physiological conditions to ensure proper cell function. Firstly, Pin1 expression is mediated by the transcription factor E2F and also by Notch1 in a positive feedback loop (Ryo et al., 2002; Rustighi et al.,

2009). Secondly, the subcellular localization and function of Pin1 depend on substrate interaction between its WW domain and the pSer/Thr-Pro motifs of target proteins (Lu et al., 2002). In addition, Pin1 itself can also be regulated by phosphorylation. Phosphorylation at Ser 16 in the centre of the pSer/Thr-Pro binding pocket of the WW domain abolishes the ability of Pin1 to interact with its substrates (Lu et al., 2002). Pin1 has also been demonstrated to be phosphorylated at Ser 65 by Polo-like kinase 1 (PLK1), which increases the stability of Pin1 by reducing its ubiquitination (Eckerdt et al., 2005). Furthermore, preliminary studies carried out by our group indicate that Pin1 may be both transcriptionally and post-transcripitionally regulated by GnRH in pituitary gonadotropes (Oh, 2007).

1.4 SF-1

The orphan nuclear receptor SF-1, also known as Ad4BP or NR5A1, was first identified as a key regulator of steroidogenic enzymes such as cytochrome P450 steroid hydroxylases (CYPs), from where it is named, steroidogenic factor 1 (Morohashi et al., 1992; Lala et al., 1992). Later studies have shown that SF-1 acts along the hypothalamic pituitary gonadal axis at multiple sites to regulate transcription of a set of genes related to steroidogenesis, reproduction, and sexual differentiation (Hoivik et al., 2010; Table 1.1). As noted above, SF-1 is one of the major transcription factors of αGSU, LHβ and FSHβ genes; SF-1 knockout mice have greatly impaired expression of the three gonadotropin subunit genes in the anterior pituitary (Ingraham et al., 1994; Shinoda et al., 1995).

Tissue	Target genes
Adrenal cortex	*CYP11A1*, *CYP11B1*, *CYP11B2*, *CYP21*, *DAX-1*, *CYP17*, *StAR*, *Mc2R*, *SCP-2* (sterol carrier protein 2)
Ovary	*Inhibin α*, *DAX-1*, *CYP11A1*, *CYP17*, *StAR*, *CYP19*, *MIS*, *Oxytocin*, *SCP-2*
Testis	*CYP19*, *DAX*-1, *SRY* (sex determining region Y), *Vanin-1*, *CYP11A1*,*CYP17*, *StAR*, *SCP-2*, *MIS*, *FSHR*, *Sox-9*, *MIS*
Pituitary (Gonadotropes)	*LHβ*, *FSHβ*, *GnRHR*, *αGSU*, *nNOS* (neuronal nitric oxide synthase), *Inhibin α*
Ventromedial hypothalamus	*BDNF* (brain derived neurotrophic factor), *NMDAR* (N-methyl-D-aspartate receptor)

Table 1.1: Overview of selected SF-1 target genes

1.4.1 Structure of SF-1

SF-1 is highly conserved among species from invertebrates to vertebrates. It shares structural homology with other members of nuclear receptor family and contains the classic domains of a nuclear receptor: an N-terminal DNA binding domain (DBD) including two zinc fingers, a hinge region with an activation function domain (AF)-1 in its distal part, and a ligand binding domain with a conserved C-terminal AF-2 hexamer domain (LLIEML) (Hoivik et al., 2010; Fig 1.7).
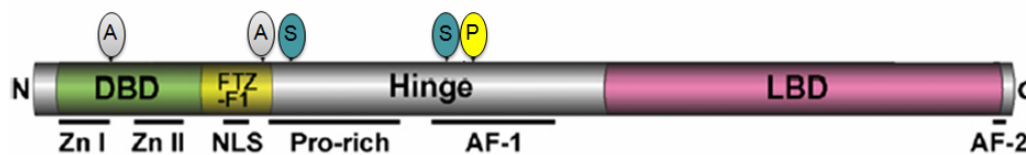
Fig 1.7: Schematic model of key structural domains in SF-1.

SF-1 possesses an N-terminal DNA binding domain which contains two zinc finger motifs (Zn I and Zn II) and is extended by a FTZ-F1 box, a flexible hinge region, and a C-terminal ligand binding domain. A nuclear localization signal is present downstream of the DBD. SF-1 is post-translationally modified by acetylation (A) in the DBD and Ftz-F1 box, SUMOylation (S) at Lys 119 and Lys 194, and phosphorylation (P) at Ser 203. Abbreviations are given in the text. (Adapted from Hoivik et al., 2010 with modification).

Different from most of other members in nuclear receptor superfamily, SF-1 binds

DNA as a monomer rather than as a homodimer or hetrodimer. The first zinc finger

motif and Fushi-tarazu factor-1 (FTZ-F1) box in DBD confer this specificity to SF-1

and are involved in the recognition of the consensus 5'-(T/C)CAAGG(T/C)C(G/A)-3'

in the promoters of SF-1 target genes (Ueda and Hirose, 1991; Wilson et al., 1993).

Similar to other nuclear receptors, the LBD of SF-1 is composed of 12 helices

(H1-H12) (Desclozeaux et al., 2002). However, SF-1 is considered to be an orphan

nuclear receptor because to date no evidence has proven that SF-1 binds to a naturally

occurring ligand to reach its maximum activity (Hammer et al., 1999). Although

several phospholipids (e.g., phosphatidyl glycerol and phosphatidyl ethanolamine)

were discovered in the large hydrophobic ligand binding pocket of SF-1 by

co-crystallographic analyses, biochemical analyses and an LBD helix assembly have

demonstrated that helices 1 and 12 pack against the α-helical bundle in response to

phosphorylation of Ser 203, indicating that the SF-1 LBD adopts an active

conformation in a ligand independent manner (Li et al., 2005; Wang et al., 2005;

Krylova et al., 2005; Desclozeaux et al., 2002).

The hinge region located between DBD and LBD is important for transcriptional

activity of SF-1 as it is targeted by post-translational modifications such as

phosphorylation and SUMOylation (Hammer et al., 1999; Komatsu et al., 2004;

Lewis et al., 2008; Yang et al., 2009; Fig 1.7). Furthermore, the AF-1 in the hinge

region cooperates with the AF-2 in the LBD to activate transcription of SF-1 target genes (Crawford et al., 1997; Hammer et al., 1999).

1.4.2 Regulation of SF-1 transcriptional activity

1.4.2.1 Binding with cofactors

Although an obligatory biological SF-1 ligand has not been identified, deletion of the C-terminal LBD of SF-1 unmasks transcriptional activity of SF-1, suggesting that mimicked-effect of ligand-receptor binding could be involved in the regulation of SF-1 activity (Shen et al., 1994; Crawford et al., 1997; Tremblay et al., 1999). In addition, this C-terminal LBD in SF-1 possesses a short α-helical AF-2 domain (LLIEML) which is a conserved cofactor binding surface (Yussa et al., 2001). To date, a number of cofactors have been reported to interact with SF-1 and modulate its transcriptional activity (Fig 1.8). For example, the interaction between SF-1 and p300/CBP potentiates the transcriptional activity of SF-1 towards the CYP11A and LHβ genes (Monte et al., 1998; Mouillet et al., 2004). Steroid receptor coactivator (SRC)-1 binding to AF-2 domain in SF-1 is required for the transactivation of several SF-1-target steroidogenic enzyme-coding genes and the SF-1-mediated steroidogenic phenotype in embryonic stem cells (Crawford et al., 1997). Temporal ChIP and mammalian two-hybrid (M-2-H) assays have demonstrated that, in response to cAMP stimulation, a ternary GCN5/SRC-1/SF-1 complex forms on the CYP17 gene promoter in a cyclic manner to regulate CYP17 gene transcription (Dammer et al.,

2007). On the other hand, some corepressors interact with SF-1 and inhibit its transcriptional activity. The atypical nuclear receptor DSS-AHC critical region on the X chromosome protein 1 (DAX-1) binds to the hydrophobic LLIEML motif of SF-1 and inhibits SF-1 transactivity to its target genes, such as StAR, MIS and some CYP genes, through recruiting corepressors like nuclear receptor corepressor (NCoR) (Suzuki et al., 2003; Lalli et al., 1998; Nachtigal et al., 1998; Wang et al., 2001; Hanley et al., 2001; Murayama et al., 2008). The physical interaction between SF-1 and the DEAD-box protein DP103 has also been shown to direct the relocalization of SF-1 to discrete nuclear bodies and repress the transcriptional activity of SF-1 (Ou et al., 2001; Yan et al., 2003; Lee et al., 2005).
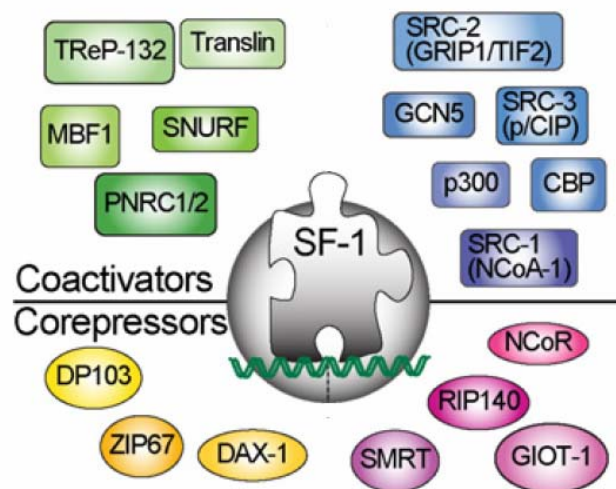
Fig 1.8: Overview of SF-1 interaction partners.

The activity of SF-1 is regulated through its interactions with coactivators such as CBP/p300 (Monte et al., 1998; Mouillet et al., 2004), GCN5 (Dammer et al., 2007; Jacob et al., 2001), SRC-1 (Winnay et al., 2006; Ito et al., 1998; Crawford et al., 1997), SRC-2 (Winnay et al., 2006), SRC-3 (Borud et al., 2002) and SNURF (Curtin et al., 2004); or corepressors such as SMRT (Kelly et al., 2005), Dax-1 (Suzuki et al., 2003; Babu et al., 2002) and NCoR (Crawford et al., 1998). Coactivators of SF-1 are represented as rectangles, and corespressors as ovals. Abbreviations are given in the text. (Adapted from Hoivik et al., 2010 with modification).

1.4.2.2 Post-translational modification of SF-1

Transcriptional activity of SF-1 is also regulated by various post-translational modifications. Phosphorylation of SF-1 at Ser 203 was discovered more than a decade ago (Hammer et al., 1999). Ser 203 is located within the AF-1 domain (PYASP) of the hinge region, and the mutation of this serine to alanine (S203A) greatly attenuates the cofactor recruitment capacity of SF-1 (Hammer et al., 1999). Both cyclin dependent kinase 7 (CDK7) and ERK 1/2 have been reported to be the kinase responsible for Ser 203 phosphorylation (Hammer et al., 1999; Lewis et al., 2008; Yang et al., 2009). On the one hand, CDK7 together with cyclin H and ménage à trois 1 (MAT1) form the CDK-activating kinase (CAK) complex, which is a component of basal transcription factor TFIIH, thus SF-1 may be anchored to the basal transcriptional machinery through its binding with CDK7 (Fisher, 2005). Notably, the transcriptional activity of SF-1 is hampered in HD2 human cells in which the interaction between CAK and TFIIH is destabilized (Lewis et al., 2008). On the other hand, SF-1 may integrate with the ERK signal to transmit extracellular stimulation to downstream genes, as many of the SF-1 target genes can be induced by hormones, such as GnRH and adrenocorticotropic hormone (ACTH) that also activate ERK 1/2. For example, in LβT2 gonadotrope cells, either deletion of the SF-1 binding site in the αGSU promoter or pharmacological blockage of the ERK 1/2 pathway by its specific inhibitor U0126, significantly attenuates PMA-stimulated transcription (Fowkes et al., 2002). It has also been reported that in Y1 adrenocortical cells, U0126 treatment abolishes the ACTH-dependent association of SF-1 with the melanocortin 2 receptor

33

(Mc2R) gene promoter (Winnay and Hammer, 2006). Collectively, these data have demonstrated the functional importance of the phosphorylation status of SF-1 to the expression of its downstream genes.

SF-1 can also be SUMOylated at two conserved lysines, Lys 119 and Lys 194, which are adjacent to the DNA binding domain and ligand binding domain, respectively, by the SUMO E2 conjugating enzyme, Ubc9, and E3 ligases of the PIAS family (Yang et al., 2009; Chen et al., 2004; Lee et al., 2005; Komatsu et al., 2004). As observed in most other nuclear receptors, SUMOylation of SF-1 represses its transcriptional activity. SUMOylation at Lys 119 results in a marked and selective loss of DNA binding to non-canonical SF-1 targets, as exemplified by the inhibin α gene promoter (Campbell et al., 2008; Yang et al., 2009). SUMOylation at Lys 194 is also associated with reduced transactivation of various SF-1 target genes and has been reported to inhibit Ser 203 phosphorylation (Campbell et al., 2008; Yang et al., 2009), which indicates a link between SUMOylation and phosphorylation.

In addition, it has been demonstrated that SF-1 is acetylated at two conserved sites. One of these, located in the middle of the DNA binding domain is targeted by GCN5 and the other, localized at the KQQKK motif in the Ftz-F1 box, is targeted by p300/CBP. Both GCN5 and p300/CBP act as coactivators of SF-1 by affecting its stability, localization and/or DNA binding activity (Chen et al., 2005; Jacob et al., 2001). It has also been shown that p300 coordinates the functional synergy between SF-1 and Egr-1 to activate LHβ gene expression, providing another explanation to

stimulation of SF-1 transcriptional activity by acetylation (Mouillet et al., 2004). Furthermore, SF-1 can be ubiquitinated, and the ubiquitinated form is associated with the LHβ gene promoter in gonadotropes (Chen et al., 2007; Walsh and Shupnik, 2009). It has become increasingly apparent that the transcriptional activity of SF-1 is modulated by various post-translational modifications, while the relationship between those modifications remains unclear.

1.5 ATF3

ATF3, also known as LRG-21, CRG-5, or TI-241 in mouse and liver regenerating factor 1 (LRF-1) in rat, is highly conserved in mammals from mouse to human. It is an approximately 22 kDa protein belonging to the ATF/CREB transcription factor family (Drysdale et al., 1996). Emerging evidence indicates that ATF3 is an immediate early gene participating in signaling cascades to convert an extracellular stimulus into intracellular changes through activating or repressing gene expression (Chen et al., 1994; Hai et al., 1999).

1.5.1 Induction of ATF3 by multiple signals

Under normal culture conditions the expression level of ATF3 is relatively low, but it can be rapidly induced by many physiological and pathological stimuli including toxic chemicals such as alcohol and carbon tetrachloride (Chen et al., 1996), genotoxic agents such as ionizing radiation and ultraviolet (UV) light (Amundson et al., 1999), anti-cancer drugs such as taxol and colchicine (Shtil et al., 1999) and

hormones such as GnRH (Xie et al., 2005). Furthermore, in animal models its expression is also increased in mechanically or chemically injured liver (Chen et al., 1996), in postseizure brain (Chen et al., 1996), in ischemic/reperfused heart, kidney and pancreas (Chen et al., 1996; Yin et al., 1997; Okamoto et al., 2001; Allen-Jennings et al., 2001), in wounded skin (Harper et al., 2005), and in all dorsal root ganglia neurons and motoneurons after the peripheral nerve is cut (Tsujino et al., 2000).

Studies have shown that multiple signaling pathways including p38 MAPK, ERK, and JNK/stress activated protein kinase (SAPK) are involved in the induction of ATF3 in a cell type and signal-specific manner (Cai et al., 2000; Inoue et al., 2004; Lu et al., 2007). In COS-1, HeLa, MEF and HEK 293 cells, activation of the p38 pathway by stress signal plays an important role in inducing the expression of the ATF3 gene, while ERK and JNK/SAPK are neither necessary nor sufficient (Lu et al., 2007). However, in human endothelial cells, inhibition of the JNK/SAPK signaling pathway by dominant negative MAPK kinase 4 (MKK4) or MKK7 has been demonstrated to reduce the ability of homocysteine to activate expression of the ATF3 gene (Cai et al., 2000). Furthermore, induction of ATF3 by ionizing radiation is through p53 dependent pathway, while UV or methyl methanesulphonate (MMS) induction of ATF3 does not require normal cellular p53 function (Fan et al., 2002).

In most cases, ATF3 induction is rapid but transient. Its mRNA level is increased shortly after exposure to the stress or other signals, and progressively decreased to the

basal level after the peak increase at around 2 h (Chen et al., 1996; Wolfgang et al., 2000; Xie et al., 2005). One of the mechanisms for the transient nature of ATF3 induction is that ATF3 auto-represses its own gene expression through binding to an ATF3 binding site (5'-TGATGCAAC-3') in its own promoter. This site, located immediately after the TATA box, is different from the consensus ATF/CRE binding site in the central residues but is required for the efficient auto-repression (Wolfgang et al., 2000; Mayer et al., 2008). Furthermore, ATF3 mRNA contains several AU-rich RNA destabilizing elements in its 3'-untranslated region and thus is unstable, with a 30 min half life in HeLa cells (Chen et al., 1994; Beelman and Parker, 1995; Liang et al., 1996). Therefore, its mRNA is not able to remain at a high level after the induction is turned off.

1.5.2 Binding partners of ATF3

ATF3 bears a basic leucine zipper (bZIP) motif, which is shared by all the members of the ATF/CREB family. The basic region in this motif is responsible for binding to the ATF/CRE site in various gene promoters, while the leucine zipper is required for forming homodimer or selective heterodimers with other bZIP-containing proteins such as c-Jun and c-Fos (Hsu et al., 1992; Chen et al., 1994). The ATF3 homodimer represses transcription of promoters with ATF binding sites, whereas heterodimers with c-Jun, c-Fos or JunB activate transcription (Hsu et al., 1992; Chen et al., 1994; Wolfgang et al., 1997). For example, ATF3 in complex with Jun binds to and transactivates the IFNγ gene promoter, inducing the differentiation of naive CD4+ T

helper lymphocytes into IFNγ-, IL-2-, and TNFβ-secreting T helper 1 cells (Filén et al., 2010). In neuron-like rat pheochromocytoma PC12 cell line and superior cervical ganglion neurons, the combination of ATF3 and c-Jun activates the expression of the anti-apoptotic factor, heat shock protein 27 (HSP27), thus leading to suppression of JNK-induced apoptosis (Nakagomi et al., 2003).

Besides the bZIP family, ATF3 also cooperates with other proteins to transduce environmental signals, regulate gene expression and change cellular function. The hepatitis B virus (HBV) X protein induces the expression of ATF3 and also directly interacts with ATF3, enhancing DNA binding potential of ATF3 for the CRE site *in vitro* and augmenting the transcriptional repression mediated by ATF3 *in vivo*, suggesting that ATF3 is involved in HBV-mediated hepatocarcinogenesis (Barnabas et al., 1997; Tarn et al., 1999; Barnabas and Andrisani, 2000). Following PMA stimulation, ATF3 and JunB direct the loading of the chromatin remodeling SWI/SNF complex to the AP-1 binding site on HIV-1 promoter, through their interaction with the ATPase subunit of the SWI/SNF complex, brahma-related gene 1 (BRG-1) protein (Henderson et al., 2004). After nerve injury the transcription factor SP1 interacts with the injury inducible transcription factors, ATF3, c-Jun and STAT3, and recruits them to the promoter of damage-induced neuronal endopeptidase (DINE) gene to activate its transcription synergistically (Kiryu-Seo et al., 2008). Other reported ATF3 binding partners include NFκB (Kaszubska et al., 1993), growth arrest and DNA damage inducible protein 153 (GADD153) (Chen et al., 1996), p53 (Yan et al., 2002; Yan et al., 2005; Yan and Boyd, 2006) and Smad3 (Kang et al., 2003).

1.5.3 Alternative splicing isoforms of ATF3

In addition to the diversity of cofactors, alternative splicing of pre-mRNAs encoding ATF3 also leads to its functional complexity in coordinating gene expression. Multiple ATF3 isoforms have been identified. ATF3ΔZIP from serum-stimulated HeLa cells lacks the leucine zipper dimerization domain and thus fails to bind to CRE, but activates transcription possibly by sequestering inhibitory cofactors away from the promoter (Chen et al., 1994). ATF3ΔZIP2a and ΔZIP2b from cells treated with various stimuli, such as calcium ionophore A23187, TNFα, endoplasmic reticulum stress, or oxidative stress, have been shown to counteract the transcription repression by full-length ATF3 (Hashimoto et al., 2002). ATF3ΔZIP2c and ΔZIP3 have been isolated from amino acid-deprived cells, and ATF3ΔZIP3 stimulates starvation-induced asparagine synthetase gene transcription mediated by ATF4 in a concentration-dependent manner (Pan et al., 2003). ATF3b, lacking the first 57 amino acids of ATF3 but preserving the intact bZIP domain, has been implicated in mediating cAMP signaling of proglucagon gene transcription in pancreatic α cells (Wang et al., 2003).

1.5.4 Dichotomous role of ATF3 in oncogenesis

The process of cancer initiation and development in different tissues is controlled by actions of a diverse set of oncogenic proteins and tumor suppressors that sense various signals and relay them to proliferative or apoptotic machinery (Hanahan and

Weinberg, 2000). Increasing evidence indicates that some proteins have dichotomous roles in oncogenesis. For example, it has been demonstrated that TGFβ induces apoptosis and growth arrest in normal or less transformed cells, but facilitates metastatic progression in advanced tumors (Lehmann et al., 2000; Massague, 2000; Derynck et al., 2001; Roberts and Wakefield, 2003). Maf bZIP transcription factors play a dual role in both terminal differentiation and oncogenesis, depending on the cellular context (Pouponnot et al., 2006). Similarly, ATF3 has also been shown to have a dichotomous function in cancer development, and ATF3 can promote either apoptosis or proliferation in specific cell lines (Ishiguro and Nagawa, 2000; Bottone et al., 2005; Lu et al., 2006; Bandyopadhyay et al., 2006; Fig 1.10).
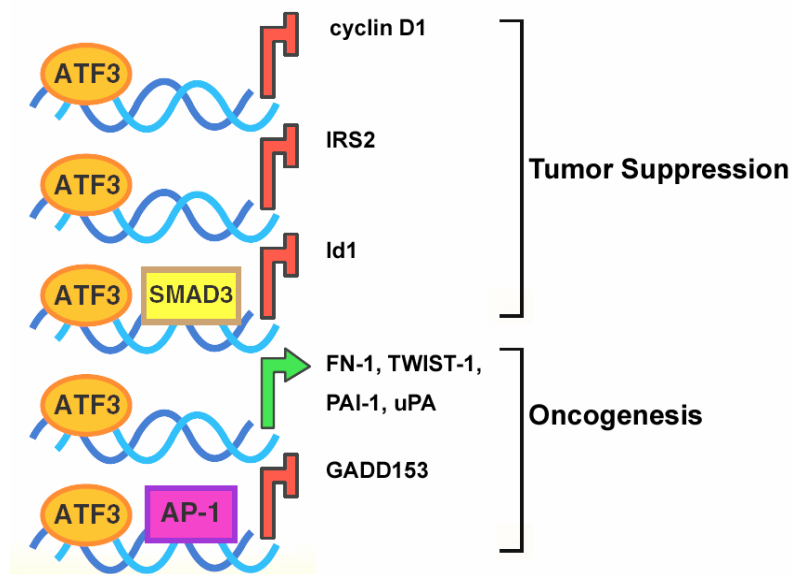
Fig 1.9: ATF3 is able to function as both a tumour suppressor and oncogenic protein.

ATF3 could prevent tumour growth by repressing transcription of cell cycle genes like cyclin D1 (James et al., 2006; Lu et al., 2006) and Id1 (Kang et al., 2003; Ling et al., 2006) and cell survival genes like IRS2 (Dearth et al., 2007; Li et al., 2008). On the contrary, induction of metastatic mediators FN-1, TWIST-1, PAI-1 and uPA (Yin et al., 2008), or suppression of GADD153 (Wolfgang et al., 1997; Fawcett et al., 1999; Maytin et al., 2001), may implicate ATF3 as an oncogenic protein. Abbreviations are given in the text. (Adapted from Thompson et al., 2009 with modification).

ATF3 was suggested to be an oncogenic protein because it is over-expressed in some types of human cancers like breast cancer, prostate cancer, and Hodgkin lymphoma (Yin et al., 2008; Pelzer et al., 2006; Janz et al., 2006). Over-expression of ATF3 leads to increased proliferation in DU 145 human prostate cancer cells and enhanced motility and invasiveness of PC-3MM and ALVA human prostate cancer cells, while knock-down of ATF3 expression in HT29 human colon cancer cells inhibits cell adhesion and reduces tumor growth rate of HT29 cell xenografts (Ishiguro et al., 2000; Bandyopadhyay et al., 2006; Pelzer et al., 2006). However, the mechanisms through which ATF3 promotes oncogenesis in these cells remain somewhat obscure; its repression of GADD153, a member of the CCAAT/enhancer binding protein (C/EBP) transcription factor family known to induce apoptosis in response to stress, could be one of the factors allowing the survival and proliferation of cancer cells (Wolfgang et al., 1997; Fawcett et al., 1999; Maytin et al., 2001).

In contrast to the above studies, some findings indicate that ATF3 can suppress oncogenesis. For example, ATF3 over-expression promotes apoptosis of PC3 human prostate cancer cells and human ovarian cancer cells (Syed et al., 2005; Huang et al., 2008). Nonsteroidal anti-inflammatory drugs (NSAIDs) exert their anti-invasive activity through upregulating the expression of ATF3, while over-expression of ATF3 reduces the size of subcutaneous HCT-116 human colorectal cancer cell xenograft tumors (Bottone et al., 2003; Bottone et al., 2005). In some studies the mechanisms behind the function of ATF3 in tumor suppression are disclosed. Induction of the ATF3 gene during mouse chondrocyte differentiation leads to suppression of cyclin

D1 and cyclin A transcription, accelerating cell cycle exit and terminal differentiation of the chondrocytes (James et al., 2006; Lu et al., 2006). ATF3 is upregulated by TGFβ signaling via Smad3 activation in epithelial cells, and then complexes with Smad3 to repress the expression of inhibitor of differentiation 1 (Id1), which is over-expressed in many different cancers and plays an important role in cancer cell survival and growth (Kang et al., 2003; Ling et al., 2006). Islets from insulin promoter-ATF3 transgenic mice exhibit enhanced rates of apoptosis, due to the ability of ATF3 to inhibit the transcription of the oncogenic protein, insulin receptor substrate 2 (IRS2) (Dearth et al., 2007; Li et al., 2008). Furthermore, distinct from its function as a transcription factor, a novel role of ATF3 in stabilizing the tumor suppressor p53 and p73 through inhibiting their ubiquitination was uncovered recently; this mechanism also explains the ability of ATF3 to suppress tumor growth (Yan et al., 2005; Yan et al., 2006; Oh et al., 2008).

The opposing roles of ATF3 in both tumour suppression and oncogenesis have been further confirmed in breast cancer cell lines that share almost the same genetic background but have different degrees of malignancy due to genetic and/or epigenetic alterations. Specifically, ATF3 induces apoptosis in the untransformed MCF10A mammary epithelial cell line through upregulating the transcription of several genes in the TNF pathway, but protects the malignant MCF10CA1a breast cancer cells from apoptosis and promotes their motility through increasing the expression of some key regulators of tumor metastasis such as TWIST-1, fibronectin-1 (FN-1), plasminogen activator inhibitor-1 (PAI-1), and urokinase-type plasminogen activator (uPA) (Yin et

al., 2008). Future studies to clarify the cellular and molecular context that determines whether ATF3 is an oncogenic protein or a tumor suppressor could provide insights into the design of anti-tumor agents.

1.6 Ubiquitination and SUMOylation pathways

Reversible post-translational modifications by ubiquitin (Ub) and small ubiquitin related modifier (SUMO) are efficient ways to dynamically regulate stability, subcellular localization and activity of target proteins functioning in various cellular events such as signal transduction, DNA repair, immune response, transcription regulation, and chromatin remodeling (Gill, 2004; Johnson, 2004; Sun and Chen, 2004; Ulrich, 2005; Chen and Sun, 2009). Unlike phosphate, acetyl groups and other small modifiers, ubiquitin and SUMO are entire protein moieties covalently attached to target proteins. Ubiquitin is a small protein (76 amino acids), highly conserved among different eukaryotic species (Hershko and Ciechanover, 1998). SUMO proteins are larger, composed of approximately 108 amino acids. Although SUMO proteins share very little sequence identity with ubiquitin, the structures of SUMO and ubiquitin are virtually super-imposable, in particular, containing a C-terminal di-glycine motif which is required for conjugation to substrates (Bayer et al., 1998; Bernier-Villamor et al., 2002; Schwartz and Hochstrasser, 2003). Therefore, ubiquitin and SUMO proteins share similar substrate selection and conjugation mechanisms. SUMO and ubiquitin are conjugated to target proteins by a hierarchical cascade of enzymes,

consisting of an energy-dependent activating enzyme (E1), a conjugating enzyme (E2)

and usually a ligase (E3), which confers substrate selectivity (Ulrich, 2005; Fig 1.9).
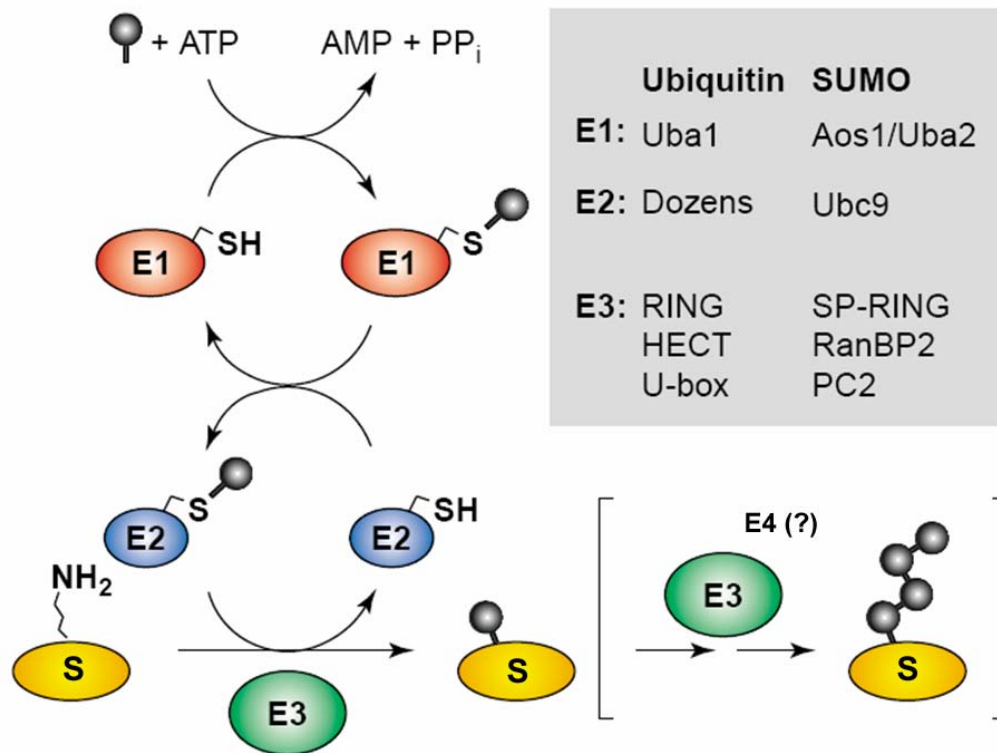
Fig 1.10: Overview of ubiquitination and SUMOylation conjugation pathway.

In an ATP-dependent reaction, the modifier (shown in gray), ubiquitin or SUMO, is covalently conjugated to a substrate protein (shown in yellow) via a lysine residue through the sequential actions of the E1 activating enzyme, E2 conjugating enzyme and E3 ligase. The attachment of multiple ubiquitin or SUMO by E3 to the substrate, perhaps with the help of an E4, results in polymeric chain formation. The specific enzymes for ubiquitin and SUMO attachment are shown in the box. Dozens of E2 enzymes and hundreds of E3 enzymes have been discovered for ubiquitin conjugation and are responsible for substrate selection. In many cases, Ubc9 can SUMOylate a substrate in an E3 independent manner; E3 is thought to increase the efficiency of the transfer of SUMO to the substrate. Abbreviations are given in the text. (Adapted from Ulrich, 2005 with modification).

Despite a similar conjugation mechanism, ubiquitination and SUMOylation differ widely with respect to their biological roles as the surface charge of SUMO is distinct from that of ubiquitin, and SUMO proteins, having a flexible extended N-terminus, are around 20 residues longer than ubiquitin (Bayer et al., 1998; Bylebyl et al., 2003). Polymeric chain formation through linking one modifier to an internal lysine of another is largely observed for ubiquitin, but happens to SUMO as well (Knipscheer et al., 2007; Windecker and Ulrich, 2008; Tatham et al., 2008; Ulrich, 2008). In the following section, the functions of ubiquitination and SUMOylation, and crosstalk between the two modifications will be discussed in detail.

1.6.1 Protein regulation by ubiquitination

The well-known function of the poly-ubiquitin chain is to target its substrates for proteasome-mediated degradation. However, in the past few years it has been uncovered that seven lysines (Lys 6, Lys 11, Lys 27, Lys 29, Lys 33, Lys 48, and Lys 63) in ubiquitin can be conjugated by another ubiquitin to form poly-ubiquitin chains (Ikeda and Dikic, 2008). The various Lys-linked poly-ubiquitin chains are structurally and functionally distinct. Poly-ubiquitin chains, at least four subunits long, linked through Lys 48 in ubiquitin, normally target proteins for proteasome degradation, whereas mono-ubiquitin or poly-ubiquitin chains linked through other lysines usually perform non-proteolytic functions including chromatin remodeling, protein kinase activation, endocytosis, and DNA repair (Chen and Sun, 2008). For example, mono-ubiquitination of histone H2B at Lys 123 promotes methylation of histone H3

at Lys 4 and Lys 79, and is associated with active chromatin state and gene expression (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002; Sun and Allis, 2002; Muratani and Tansey, 2003). Poly-ubiquitin chains linked through Lys 63, presumably acting as scaffolds to facilitate the assembly of protein kinase complexes, are required to activate NFκB pathway through attaching to the subunits of transforming growth factor β activated kinase-1 (TAK1) complex and inhibitor of NFκB kinase (IKK) complex (Adhikari et al, 2007). Upon stimulation with epidermal growth factor (EGF), the EGF receptor (EGFR) is multiply mono-ubiquitinated and poly-ubiquitinated through Lys 63 in ubiquitin, which generates internalization and sorting signals to lysosomes, culminating in endocytosis (Huang et al., 2006).

It should be noted that ubiquitination is a dynamic modification which can be reversed by deubiquitinating enzymes (DUBs) and can undergo ubiquitin editing. The ubiquitin-editing enzyme, A20, which contains both N-terminal ovarian tumour deubiquitinase (OTU) domain and C-terminal E3 ligase domain, cooperates with the regulatory protein human T-cell leukemia virus type I transactivator binding protein 1 (TAX1BP1) and the HECT domain Itchy E3 ubiquitin protein ligase homolog (ITCH) to attenuate the NFκB pathway. This is through removing signal-enhancing Lys 63-linked poly-ubiquitin chains from the critical NFκB signaling molecules, including receptor interacting protein 1 (RIP1) and TNF receptor associated factor 6 (TRAF6), and facilitating the formation of signal-extinguishing Lys 48-linked poly-ubiquitin chains that lead to proteasomal degradation (Wertz et al., 2004; Newton et al., 2008; Shembade et al., 2010).

1.6.2 Protein regulation by SUMOylation

Similar to the non-proteolytic functions of ubiquitination, SUMOylation regulates protein–protein interaction, subcellular localization and/or activity of a target protein, and is thereby involved in various cellular processes, including transcription regulation, cell cycle control and DNA damage response (Gill, 2004; Hay, 2005; Muller et al., 2004; Geiss-Friedlander and Melchior, 2007). SUMOylation modifies the surface of a given protein or induces its conformational change, which usually alters its intermolecular or intramolecular interaction specificity (Gill, 2004; Hay, 2005; Baba et al., 2006). It is believed that most of the effects of SUMOylation on proteins result from its modulation of protein–protein interactions (Geiss-Friedlander and Melchior, 2007). For example, un-SUMOylated Ran GTPase activating protein 1 (RanGAP1) is localized in the cytosol; SUMOylation-dependent interaction between RanGAP1 and the nucleoporin RanBP2, targets SUMOylated RanGAP1 to the nuclear pore (Mahajan et al., 1997; Mahajan et al., 1998; Matunis et al., 1998). SUMOylation of p300 leads to its interaction with histone deacetylase (HDAC) 6 and subsequent transcriptional repression (Girdwood et al., 2003). The yeast DNA helicase, SRS2, has a preference for interacting directly with the SUMOylated proliferating cell nuclear antigen (PCNA), and therefore is recruited to replication forks to prevent unwanted recombination of replicating chromosomes (Papouli et al., 2005; Pfander et al., 2005).

A distinctive feature of SUMOyaltion is that SUMO is often conjugated to the lysine within ψKXE (ψ denoting a hydrophobic residue) consensus motif in a target protein (Rodriguez et al., 2001; Bernier-Villamor et al., 2002). Some non-consensus SUMOylation sequences including TKET, TKED, AKCP, VKYP, VKFP and GKVEKVD, in which both lysines can be attached by SUMO, have also been reported (Johnson, 2004). Furthermore, two extended SUMOylation motifs, which contain regulatory information from the substrate itself, have also recently been identified. The first one is phosphorylation-dependent SUMOylation motif (PDSM), which consists of the conventional SUMOylation motif and a phosphorylated serine residue followed by a proline residue (ψKXEXXpSP). PDSM has been found in several proteins including Smad nuclear interacting protein 1 (SNIP1), heat shock factor 1 (HSF1), and myocyte specific enhancer factor 2A (MEF2A) (Hong et al., 2001; Hietakangas et al., 2003; Hietakangas et al., 2006). The second one is negatively charged amino acid dependent SUMOylation motif (NDSM). It has been shown that SUMOylation of Elk-1 is inhibited by mutation of the negatively charged residues C-terminal to the consensus sequence (Yang et al., 2006). The identification of these two extended motifs highlights that negative charge next to ψKXE motif is functionally important to enhance SUMOylation.

Like ubiquitination, SUMOylation is a reversible modification. SUMO can be removed from its substrates by sentrin specific proteases (SENPs). Only a small percentage of a target protein tends to be SUMOylated *in vivo* at a certain time point, but this can have dramatic effects, since SUMOylation is a highly dynamic process

(Geiss-Friedlander and Melchior, 2007). SUMO can also form polymeric chains on some of its target proteins such as HDAC4, SP100 nuclear antigen (SP100), and promyelocytic leukemia (PML) to regulate their functions (Tatham et al., 2001; Mukhopadhyay et al., 2006; Knipscheer et al., 2007; Lallemand-Breitenbach et al., 2008; Tatham et al., 2008).

1.6.3 Crosstalk between ubiquitination and SUMOylation

It has become clear that ubiquitination and SUMOylation often communicate with each other to jointly regulate a common target protein (Gill, 2004; Ulrich, 2005; Geiss-Friedlander and Melchior, 2007). In some cases SUMO antagonizes ubiquitin at the same lysine residue of a target, thereby protecting the target from proteasome-mediated degradation. It has been demonstrated that ubiquitination of inhibitor of NFκB (IκB) α at Lys 21 and Lys 22, dependent on its phosphorylation at Ser 32 and Ser 36, targets IκBα to degradation by the 26S proteasome, thus allowing the translocation of NFκB to the nucleus. SUMO can also target IκBα at Lys 21, blocking its ubiquitination and protecting it from the degradation induced by inflammatory stimuli (Desterro et al., 1998; Karin and Ben-Neriah, 2000). Huntingtin exon 1 protein (Httex1p), a pathogenic fragment of Huntingtin, can be modified by either SUMO or ubiquitin at identical lysines. SUMOylation has a stabilizing effect on Httex1p (Steffan et al., 2004). Examples of target proteins having a common lysine residue targeted by both SUMO and ubiquitin also include Smad4 and PCNA (Lin et al., 2003; Ulrich et al., 2005; Papouli et al., 2005). However, PCNA is an exception;

ubiquitin and SUMO do not compete with each other in this context. Mono-ubiquitination of PCNA at Lys 164 upon DNA damage in yeast does not target this protein for degradation but is critical for translesion synthesis repair through promoting its interaction with the translesion polymerase η (Hoege et al., 2002; Kannouche et al., 2004). SUMOylation of PCNA at Lys 164, and to a minor extent at Lys 127, has been shown to enhance DNA damage tolerance through recruiting the helicase SRS2 to the site of replication and inhibit unscheduled recombination, rather than prevent ubiquitin dependent activities (Stelter and Ulrich, 2003; Haracska et al., 2004).

Sequential actions of SUMO and ubiquitin on NFκB essential modifier (NEMO), the regulatory component IKK complex, also suggest that SUMO and ubiquitin can act cooperatively. SUMOylation of NEMO at Lys 277 and Lys 309 after exposure to DNA damaging agents leads to its retention in the nucleus, where the checkpoint kinase ATM dependent signaling induces its phosphorylation and subsequent ubiquitination. The ubiquitinated NEMO can then translocate back to the cytosol and associate with IKKα/β to form an active IKK kinase (Huang et al., 2003; Hay, 2004). Furthermore, it has been revealed recently that poly-SUMO chain on a target such as PML can serve as a binding site for the RING finger E3 ubiquitin ligase RNF4/SNURF, which bears four closely spaced SUMO interaction motifs (SIMs) at its N-terminus, to promote the ubiquitination and subsequent degradation of the target by the 26S proteasome (Sun et al., 2007; Lallemand-Breitenbach et al., 2008; Tatham

et al., 2008). In brief, the two modification systems, ubiquitination and SUMOylation, can cooperate in diverse ways to regulate a target protein.

1.7 Hypothesis and aims

1.7.1 Hypothesis

Due to the fact that loss of Pin1 in mice causes severe abnormalities in their reproductive development and function, and that many gonadotropin subunit gene transcription factors are phosphorylated at Ser/Thr-Pro motifs, it is hypothesized that Pin1 is involved in the transcriptional regulation of the gonadotropin subunit genes through acting on their upstream transcription factors, and that GnRH-induced pathway facilitates Pin1 activity to increase gonadotropin transcription.

1.7.2 Aims

The specific aims of the present study are:

1) to assess the roles of Pin1 in regulating gonadotropin β subunit gene transcription through examining the effects of Pin1 over-expression or knock-down on LHβ and FSHβ transcripts and their promoter activity in gonadotrope cells;

2) to uncover the mechanism of Pin1 actions towards gonadotropin β subunit gene transcription through testing Pin1 interactions with various gonadotropin

gene-specific transcription factors and its effects on their activity, stability and ability

to associate with other factors;

3) to verify the role of GnRH in regulating Pin1 activity.

CHAPTER 2   MATERIALS AND METHODS

2.1 Cell culture

2.1.1 Growth conditions

The murine gonadotrope αT3-1 and LβT2 cells (gifts from Dr. P. Mellon, UCSD) were cultured in Dulbeco's modified Eagle's medium (DMEM) supplemented with 10% dialyzed fetal bovine serum (FBS) (Gibco-Invitrogen), 10 mM HEPES [pH 7.4], 4 mM L-glutamine (Hyclone), 100 U/mL penicillin (Gibco-Invitrogen) and 100 µg/mL streptomycin (Gibco-Invitrogen). COS-1 cells, HEK 293T control siRNA cells, HEK 293T Pin1 siRNA cells (generated by Ms. Q. Y. Yang, NUS), MEF wild type (WT) cells, and MEF Pin1 –/– cells (gifts from Dr. K. P. Lu, Harvard Medical School) were cultured in Dulbeco's modified Eagle's medium supplemented with 10% certified FBS (Hyclone), and the same antibiotics. All cells were maintained at 37 °C under 5% CO_2.

2.1.2 Storing and recovery of cells

The culture medium was removed. The cells were rinsed once with 1×phosphate buffered saline (PBS) and detached from dish by trypsinization. 10 mL medium was then added into the dish to transfer the cells to a 50 mL falcon tube. After spinning down at 800 rpm for 5 minutes, the medium was discarded. The cell pellet was resuspended with 1 mL freezing medium (90% culture medium + 10% DMSO) and

the 1 mL suspension was then transferred to a 2 mL cryogenic microfuge tube (NUNC). The tube was incubated immediately at –20 °C for 1 h, followed by –80 °C overnight before being stored in liquid nitrogen.

The tube of frozen cells was placed in 37 °C water bath to thaw. The suspension in the tube was then directly added into 10 mL of culture medium in a 100 mm culture dish. The dish was swirled gently and incubated at 37 °C under 5% CO_2 for 24 h before the cells were rinsed once with 1×PBS and fresh medium replaced. The cells were then grown according to the required confluency before splitting the cells to be used for experiments.

2.1.3 Treatment of cells

Some of the cells were exposed to 100 nM of the GnRH agonist, buserelin (Sigma; dissolved in H_2O); 100 nM PMA (Sigma; dissolved in DMSO); 1 μM forskolin (Sigma; dissolved in H_2O); 10 μM MG132 (Sigma; dissolved in DMSO); 15 μM roscovitine (Sigma; dissolved in DMSO); 1 μM U0126 (Promega; dissolved in DMSO) or 100 μg/mL cycloheximide (Sigma; dissolved in DMSO), as indicated.

2.1.4 Transient transfection of cells

Transfection was carried out at 50 to 60% confluence. LβT2 cells were transfected using GenePORTER 2 (Gene Therapy Systems), HEK 293T cells were transfected using the calcium phosphate method, and the other cells were transfected using

Lipofectamine 2000 (Invitrogen). After transfection, cells were incubated for a further

24-48 h before harvest.

Transfection using GenePORTER 2 or Lipofectamine 2000 was performed according

to the manufacturers' instructions. The optimized transfection reagent to DNA ratios

are shown in Tables 2.1 and 2.2.

Cell type	Lipofectamine 2000 (µL) : DNA (µg)
COS-1	5 : 3
MEF WT	5 : 2
MEF Pin1 –/–	5 : 2

Table 2.1: Optimized Lipofectamine 2000 (µL) : DNA (µg)

Cell type	GenePORTER 2 (µL) : DNA (µg)
LβT2	7 : 2
αT3-1	5 : 1

Table 2.2: Optimized GenePORTER 2 (µL) : DNA (µg)

Calcium phosphate transfection method (for 60 mm dish)

The HEK 293T control siRNA cells or Pin1 siRNA cells were seeded into 60 mm culture dishes the day before transfection to reach about 50-60% confluence at the day of transfection. Before transfection the medium was removed, the cells were rinsed once with 1×PBS, and 3 mL fresh medium was added into each dish. DNA (4 μg) was added to ddH$_2$O to a total volume of 219 μL in a 1.5 mL sterile tube, and then 31 μL 2 M CaCl$_2$ was added to a final volume of 250 μL. After that, 250 μL of 2×HEPES buffered saline (2×HBS, the recipe for which as shown below) [pH 7.05] was added drop-wise to the DNA-CaCl$_2$ complex, and the final mixture was then swirled gently to form the DNA-CaPO$_4$ precipitate. Within 2 minutes after the addition of 2×HBS, the 500 μL final mixture was added directly to the cells drop-wise through the medium, the droplets were sprinkled evenly over the surface of the dish. The dishes were put back into the incubator at 37 °C under 5% CO$_2$ for 8-10 h and then replaced with fresh medium. The cells were harvested 24-48 h after transfection.

2×HBS [pH 7.05]	100 mL
NaCl	1.6 g
HEPES	1.0 g
Dextrose	0.2 g
KCl	74 mg
Na$_2$HPO$_4$ (anhydrous)	21.3 mg

The above mixture was dissolved in 90 mL ddH$_2$O, and then pH was adjusted to 7.05 with NaOH. The final volume was brought to 100 mL by adding ddH$_2$O. The solution was then filtered through 0.22 μM filter before being aliquoted into sterile tubes and kept frozen at –20 °C.

2.2 Plasmid constructs

2.2.1 Gonadotropin subunit gene promoter construct for luciferase assays

The 1300 bp of the mouse proximal LHβ gene promoter, or 600 bp of the mouse proximal FSHβ gene promoter in pGL2 Basic (Promega) were reported previously (Melamed et al., 2006; Feng et al., 2008).

2.2.2 Expression vectors

The expression vectors for Pitx1, Egr-1, and SF-1 were gifts from J. Drouin (Montreal, Canada), J. Milbrandt (St. Louis, MO), and K. Parker (Durham, NC), respectively. pEGFP Pin1 and pEGFP Pin1 S16A were gifts from Dr. K. P. Lu (Harvard Medical School). The coding sequences of Pin1 and ATF3 were PCR-amplified from LβT2 cell cDNA, and were ligated into pCS2+ expression vector. The primers used for the construction of pCS2+ Pin1 and pCS2+ ATF3 are shown in Table 2.3. The constructs were verified by sequencing (See Section 2.2.4).

Vector	Primer	Oligonucleotide sequence (5'-3')	Restriction Enzyme Site
pCS2+	Mouse Pin1 F	GCGCGAATTCATGGCGGACGAGG AGAAG	EcoRI
	Mouse Pin1 R	GCGCTCTAGATCATTCTGTGCGCA GGAT	XbaI
pCS2+	Mouse ATF3 F	GCGCGGATCCATGATGCTTCAACA TCCAG	BamHI
	Mouse ATF3 R	GCGCGAATTCTTAGCTCTGCAATG TTCCT	EcoRI

Table 2.3: Primer sequences for pCS2+ Pin1 and pCS2+ ATF3 constructs

For tagged constructs, sequence-specific primers containing restriction enzyme sites at the 5' ends were used to PCR-amplify the DNA fragments from the above expression vectors. The Pin1 coding sequence was inserted into p3×Flag-CMV 10 expression vector. Pitx1, SF-1, Egr-1 and ATF3 coding sequences were inserted into pxj40-HA, pxj40-Flag or pxj40-GFP (gifts from Dr. B. C. Low, NUS), as shown in Table 2.4. The primers used for the construction of tagged expression vectors are shown in Table 2.4. The pxj40-Myc SUMO-1, pxj40-Myc SUMO-2, pxj40-Myc SUMO-3, pxj40-Myc ubiquitin, pcDNA 3.1 HA ubiquitin K48R and pcDNA 3.1 HA ubiquitin K63R were constructed by Ms. Q. Y. Yang (NUS). The pcDNA3 CDK7 HA (P#633) and pcDNA3 CDK7 D155A HA (P#812) were purchased from Addgene. Site-directed mutations were carried out using the QuikChange site-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions, in order to introduce the specific amino acid substitutions. The primers used for site-directed

mutations are shown in Table 2.5. The constructs were verified by sequencing (See

Section 2.2.4).

Vector	Primer	Oligonucleotide sequence (5'-3')	Restriction Enzyme Site
p3×Flag-CMV 10	Pin1 F	GCGCAAGCTTGCGGACGAGGAGAAG	HindIII
	Pin1 R	GCGCGGATCCTCATTCTGTGCGCAGGAT	BamHI
pxj40-HA pxj40-Flag pxj40-GFP	SF-1 F	GCGCGGATCCGACTACTCGTACGACGAGGAC	BamHI
	SF-1 R	GCGCAAGCTTTCAAGTCTGCTTGGCCTGC	HindIII
pxj40-HA	Egr-1 F	GCGCGGATCCGCAGCGGCCAAGGCCGAGATG	BamHI
	Egr-1 R	GCGCAAGCTTTTAGCAAATTTCAATTGTCCTGG	HindIII
pxj40-HA pxj40-Flag	Pitx1 F	GCGCGGATCCGACGCCTTCAAGGGAGGCAT	BamHI
	Pitx1 R	GCGCAAGCTTTCAGCTGTTGTACTGGCAAGCG	HindIII
pxj40-HA pxj40-Flag	ATF3 F	GCGCAAGCTTATGCTTCAACATCCAGGCC	HindIII
	ATF3 R	GCGCCTCGAGTTAGCTCTGCAATGTTCCT	XhoI

Table 2.4: Primer sequences for tagged expression vectors

Amino acid substitution	Oligonucleotide sequence (5'-3') Forward
	Oligonucleotide sequence (5'-3') Reverse
SF-1 S203A	CAGAGCCCTATGCCGCCCCCCCACAACAGC
	GCTGTTGTGGGGGGGCGGCATAGGGCTCTG
SF-1 K119R	GGCCAATGGCTTCAGGCTGGAGACCGGAC
	GTCCGGTCTCCAGCCTGAAGCCATTGGCC
SF-1 K194R	TAACCGCACCATCAGGTCTGAGTATCCAG
	CTGGATACTCAGACCTGATGGTGCGGTTA
Pitx1 S207A	CCTTCTTCAACTCCATGGCCCCGCTCTCCTCTC
	GAGAGGAGAGCGGGGCCATGGAGTTGAAGAAGG
Pitx1 S259A	TCAACTCGGCCATGGCGCCGGGCGCCTGC
	GCAGGCGCCCGGCGCCATGGCCGAGTTGA
Pitx1 T267A S270A	GCCCCTACGGCGCCCCAGCCGCGCCCTACAGCGTCTA
	TAGACGCTGTAGGGCGCGGCTGGGGCGCCGTAGGGGC
ATF3 K42R	TAACCTGACACCCTTTGTCAGGGAAGAGCTGAGATTC
	GAATCTCAGCTCTTCCCTGACAAAGGGTGTCAGGTTA
ATF3 K78R	GATGTCAGTCACCAGGTCTGAGGCGGCC
	GGCCGCCTCAGACCTGGTGACTGACATC
ATF3 K107R	GTGTCGAAACAAGAGAAAGGAGAAGACAGAGTGC
	GCACTCTGTCTTCTCCTTTCTCTTGTTTCGACAC
ATF3 K110R	CGAAACAAGAAAAAGGAGAGGACAGAGTGCCTGC
	GCAGGCACTCTGTCCTCTCCTTTTTCTTGTTTCG
ATF3 K120R	GCAGAAAGAGTCAGAGAGACTGGAGAGTGTGAATGC
	GCATTCACACTCTCCAGTCTCTCTGACTCTTTCTGC
ATF3 K136R	CCAGATCGAGGAGCTGAGGAATGAGAAACAGC
	GCTGTTTCTCATTCCTCAGCTCCTCGATCTGG

Table 2.5: Primer sequences for site-directed mutations

2.2.3 Constructs for the two-hybrid assays

For the two-hybrid assays, the reporter gene construct was created as described previously (Luo et al., 2005). Other constructs were prepared using Gal4 DBD or Gal4 activation domain (AD) vectors, pM or pVP16 (Clontech). The pM Pin1 was constructed by A. Wijeweera (NUS); pVP ATF3 was created by inserting the ATF3 coding sequence into pVP16; pVP SF-1, pVP Egr-1, and pVP Pitx1 were created previously (Luo et al., 2005; Melamed et al., 2006). Site-directed mutation was carried out to create pVP SF-1 S203A using the QuikChange site-directed mutagenesis kit and pVP SF-1 as template. The constructs were verified by sequencing (See Section 2.2.4).

2.2.4 Verification by DNA sequencing

Automated DNA sequencing (ABI Prism® 3100) was carried out to verify the correct sequence of all of the constructs. The primers used are listed in Table 2.6. Sequencing PCR reactions were assembled as stated in Table 2.7. Cycling parameters are shown in Table 2.8.

Constructs	Sequencing primer	Oligonucleotide sequence (5'-3')
pCS2+	SP6	ATTTAGGTGACACTATAG
	T3	GCAATTAACCCTCACTAAAGG
pxj40	T7	TAATACGACTCACTATAGGG
pM/pVP16	pM Forward	TCATCGGAAGAGAGTAG
	pVP Forward	GCCGACTTCGAGTTTGAG
	pM/ pVP Reverse	GGTTCAGGGGGAGGTGTGGG

Table 2.6: Primers used for DNA sequencing

Reagent	Volume (µL)
DNA template (0.2-0.5 µg/µL)	1
Primer (5 pM)	1
Big dye version 3 (Applied Biosystems)	2
5×Sequencing Buffer	2
ddH$_2$O	4
Total reaction volume	10

Table 2.7: Sequencing PCR reaction mixture

Segment	Cycle	Temperature (°C)	Time
1	25	96	30 s
		50	15 s
		60	4 min
2	1	16	∞

Table 2.8: Sequencing PCR parameters

The 10 µL of the sequencing PCR reaction containing the amplified DNA was mixed with 20 µL of 3M NaOAc [pH 4.6] and 50 µL of 95% ethanol before precipitating at –20 °C for 15 min. The sample was then centrifuged at 14,000 rpm at room temperature for 30 min. The supernatant was removed and the pellet was washed twice with 500 µL of 75% ethanol, centrifuging at top speed for 10 min after each wash. The DNA pellet was dried at 90 °C before automated sequencing was carried out on the ABI Prism 377 (Perkin Elmer) according to the manufacturer's instructions.

2.3 Antibodies

Antibodies used in this study were as follows: anti-Pin1 (Santa Cruz; sc-15340); anti-HA (Santa Cruz; sc-805 and sc-7392); anti-c-Myc (Santa Cruz; sc-40) (Sigma; C3956); anti-Flag (Santa Cruz; sc-807) (Sigma; F3165); anti-GAPDH (Santa Cruz; sc-47724); anti-ATF3 (Santa Cruz; sc-188); anti-c-Jun (Santa Cruz; sc-1694); anti-Pitx1 (Santa Cruz; sc-18922); anti-Egr-1 (Santa Cruz; sc-110); anti-β-Catenin (Santa Cruz; sc-7199); anti-Ubiquitin (P4D1; Santa Cruz; sc-8017); anti-ERK1 (Santa

Cruz; sc-94); anti-ERK2 (Santa Cruz; sc-154); anti-phospho ERK (Tyr 204; Santa

Cruz; sc-7976); anti-Calcineurin (PP2B-Aa (D-9); Santa Cruz; sc-17808); anti-green

fluorescent protein (GFP) (Santa Cruz; sc-81045 and sc-8334); anti-CDK7 (Santa

Cruz; sc-7344); anti-phospho CDK7 (T170; Abcam; ab59987); anti-SF-1 (Upstate

Biotechnology; 07-618); anti-phospho Pin1 (Ser 16; Cell Signalling; 3721);

anti-α-tubulin (Sigma; T9026).

2.4 RNA-mediated knock-down of Pin1 expression

Stealth small interfering RNA (siRNA) specific for mouse Pin1 (sense,

5'-GCCGGGUGUACUACUUCAA[dT][dT]-3'), and scrambled control (sense,

5'-GUGUUACAGCUCCAGAUGC[dT][dT]-3') were purchased from Invitrogen

(Ryo et al., 2005). The siRNA duplexes were dissolved in RNase-free ddH$_2$O to make

a 20 μM stock solution, aliquoted and stored at –20 °C before use. The siRNAs were

transfected into LβT2 cells using Oligofectamine (Invitrogen).

Transfection LβT2 cells with siRNA using Oligofectamine (for 60 mm dish)

The gonadotrope LβT2 cells were seeded into 60 mm culture dishes in 5 mL growth

medium containing 10% FBS without antibiotics the day before transfection to reach

about 40-50% confluence at the day of transfection. Before transfection the medium

was removed, the cells were rinsed once with 1×PBS, and 2.0 mL DMEM without

antibiotics was added into each dish. 10 μL of 20 μM siRNA was gently mixed with

200 μL Opti-MEM I medium (Invitrogen), and 10 μL of Oligofectamine was gently

mixed with 40 μL Opti-MEM I medium. After 5 min incubation at room temperature, the diluted siRNA and diluted Oligofectamine were combined (total volume 260 μL), mixed gently and incubated another 20 min at room temperature. The 260 μL final mixture was added directly to the cells drop-wise through the medium, the droplets were sprinkled evenly over the surface of the dish. The dishes were put back into the incubator at 37 °C under 5% CO_2 for 4-6 h and then 2.26 mL of growth medium containing 20% FBS without antibiotics was added. The cells were harvested 48-72 h after transfection.

2.5 Reverse transcriptase (RT)-PCR analysis

2.5.1 RNA isolation

Total RNA from LβT2 cells was isolated using 1 mL TRIZOL reagent (Invitrogen) per well for 6-well plate according to the manufacturer's instructions. The RNA was used immediately or stored at −80 °C for less than one month before analysis.

2.5.2 First strand cDNA synthesis

First strand cDNA was synthesized from 2 μg of the isolated RNA. Annealing of Oligo dT_{12-18} (New England Biolabs) to the poly-A tail of mRNA was carried out by heating the reaction mixture, shown in Table 2.9, to 65 °C for 5 min and then quickly chilling it on ice.

Reagent	Volume
Oligo dT$_{12-18}$ (500 μg/mL)	1 μL
RNA (2 μg)	2 μg
dNTP (10 mM)	1 μL
ddH$_2$O	Top up to final volume
Total volume	13.5 μL

Table 2.9: First strand cDNA synthesis reaction mixure

Subsequently, 4 μL of 5×first strand buffer, 2 μL of 0.1 M DTT and 0.25 μL RNase out^{TM} (Invitrogen) was added to the chilled reaction mixture and reheated to 42 °C for 2 min before the addition of 0.25 μL of Superscript III Reverse Transcriptase (Invitrogen). The mix was then incubated at 42 °C for 50 min before heat inactivation at 70 °C for 15 min. The cDNA was used immediately or stored at −80 °C.

2.5.3 PCR and gel electrophoresis

Of the 20 μL cDNA obtained, 1 μL was used for a semi quantitative PCR reaction with specific primers. Primers used to amplify the fragments of mouse SF-1, Pitx1 and ATF3 cDNA with β-actin as an internal control are listed in Table 2.10. The PCR reactions were assembled as stated in Table 2.11. Cycling parameters are shown in Table 2.12. Analysis of amplicons was performed using 1% agarose gel.

Primer	Oligonucleotide sequence (5'-3')
SF-1 F	ATGGACTATTCGTACGAC
SF-1 R	TCAAGTCTGCTTGGCCTG
Pitx1 F	ATGGACGCCTTCAAGGGAGGC
Pitx1 R	TCAGCTGTTGTACTGGCAAG
ATF3 F	ATGATGCTTCAACATCCAG
ATF3 R	TTAGCTCTGCAATGTTCCT
β-actin F	GCCATGTACGTAGCCATCCA
β-actin R	ACGCTCGGTCAGGATCTTCA

Table 2.10: Primers used for RT-PCR analysis

Reagent	Volume (μL)
cDNA template	1
dNTP (10 mM)	1
FP (10 μM)	1
RP (10 μM)	1
Taq polymerase	0.2
10× Taq polymerase buffer	2.5
ddH$_2$O	18.3
Total Volume	25

Table 2.11: PCR reaction mixure

Segment	Cycle	Temperature (°C)	Time
1	1	95	5 min
2	30	95	20 s
		60	30 s
		72	1 min
3	1	72	5 min
4	1	16	∞

Table 2.12: PCR parameters

2.5.4 Real-time PCR quantification analysis

Real-time PCR was performed using the SYBR green I dye with the ABI Prism 7900 sequence detector (Perkin-Elmer Applied Biosystems). Primers used in real-time PCR to amplify the fragments of mouse LHβ and FSHβ cDNA, with β-actin as an internal control, are listed in Table 2.13. The real-time PCR reactions were assembled as stated in Table 2.14. Cycling parameters are shown in Table 2.15. The comparative CT method was used to compare mRNA levels in the various samples which were assayed in triplicate.

Primer	Oligonucleotide sequence (5'-3')
LHβ F	CAGTCTGCATCACCTTCACC
LHβ R	GCAGTACTCGGACCATGCTA
FSHβ F	TGCACAGGACGTAGCTGTTT
FSHβ R	TGAGATGGTGATGTTGGTCA
β-actin F	CCTTCCTTCTTGGGTATGGA
β-actin R	ACGGATGTCAACGTCACACT

Table 2.13: Primers used for real-time PCR analysis

Reagent	Volume
2×SYBR green mix (Applied Biosystems)	2.5 μL
FP (10 nM)	1 μL
RP (10 nM)	1 μL
cDNA template	0.1 μg
ddH$_2$O	Top up to final volume
Total Volume	5 μL

Table 2.14: Real-time PCR reaction mixure

Segment	Cycle	Temperature (°C)	Time
1	1	50	2 min
2	1	95	10 min
3	40	95 60	15 s 1 min

Table 2.15: Real-time PCR parameters

2.6 Luciferase assay

2.6.1 Promoter study

LβT2 cells were plated in 96-well white plates. At 12-24 h after plating, expression vectors (50 ng for Pin1; 80 ng for SF-1 and Egr-1; 10 ng for Pitx1), the luciferase reporter vector (100 ng), and simian virus 40 (SV40) Renilla luciferase (2 ng) were transfected after equilibrating the total amount of DNA with pCS2+ empty vector or pWhitescript. The growth medium was removed 24-48 h post transfection and 20 μL 1×PBS and 10 μL Dual-Glo Luciferase reagent (Promega) were added into each well. The cells were incubated for 10 min on a gently rotating platform at room temperature. Firefly luciferase reading was then measured using the Veritas Microplate luminometer (Turner Biosystems) accompanied by the Veritas Microplated Luminometer software (Turner Biosystems), according to the manufacturer's instructions. After three rounds of readings, 10 μL Stop & Glo reagent (prepared freshly each time just before use by diluting Dual-Glo Stop & Glo Substrate with Dual-Glo Stop & Glo Buffer in 1:100; Promega) was added and after incubation for

10 min on a rotating platform at room temperature, the Renilla level was measured similarly. Luciferase activity was calculated by normalizing Firefly luciferase reading to Renilla luciferase reading. Reporter gene activity was calculated as activity over basal levels (n-fold) generated from transfection of the luciferase reporter vector and pCS2+ empty vector or pWhitescript.

2.6.2 Mammalian two-hybrid assay

For the two-hybrid assays, COS-1 or LβT2 cells were plated in 96-well plates before transfection using 150 ng of the pM and pVP16 fusion constructs, 50 ng of the reporter gene (Luo et al.2005), and 2 ng of simian virus 40-Renilla luciferase as internal control. Cells were incubated for 24-48 h before harvest, and luciferase activity was measured as above. Reporter gene activity was calculated as activity over basal levels (n-fold) generated from transfection of the empty pM and pVP16 constructs, after normalization with Renilla luciferase levels. Statistical analysis to determine protein interaction compared the additive effect of the pM fusion and pVP fusion expression vectors transfected separately with their effect when transfected together, as above.

2.7 Statistical analysis

Statistical analysis was performed using a simple unpaired t-test to determine means that were statistically different. Differences were considered significant when $P<0.05$. Synergy was determined by comparing the additive effects of expression vectors

transfected separately (using SE(x+y) = √[SE(x)2 + SE(y)2]) with the effect when both were transfected together, and was defined as a significantly (P<0.05) greater than additive effect.

2.8 Chromatin immunoprecipitation (ChIP)

LβT2 cells were grown to approximately 80% confluence in 100 mm dishes. The proteins were cross-linked to DNA with 1% formaldehyde (270 μL of 37% formaldehyde in 10 mL of culture medium) and incubated with gentle rotation for 10 min at room temperature. Cross-linking was arrested by addition of 0.125 M glycine (1250 μL of 1 M glycine in 10 mL of growth medium) and incubated with gentle rotation for 5 min at room temperature. The cells were then rinsed three times with cold 1×PBS, collected and resuspended in 1000 μL ChIP lysis buffer, which was prepared as shown in Table 2.16. The samples were sonicated on ice with a MISONIX XL2020 sonifier to obtain DNA fragments of ~300 to 600 bp, at setting 3 for 10 s for 6 times, with a 10 s rest in between, and then pelleted by centrifuging at 13,000 rpm at 4 °C for 10 min. From the supernatant 50 μL was saved to quantify the input DNA in the samples and was stored at –20 °C until reverse cross-linking of the DNA together with that from the precipitated samples. Antibody [1 μg of anti-Pin1 (Santa Cruz; sc-15340) or anti-HA (Santa Cruz; sc-805)] and 20 μL Protein A Sepharose CL-4B (GE Health) were added into 450 μL lysate and subsequently incubated overnight at 4 °C with gentle rotation. The antibody-bound complexes were washed extensively with each of the following buffers: low-salt wash buffer, high-salt

wash buffer, and LiCl wash buffer (see Table 2.16). This was followed by two washes

with Tris-EDTA (see Table 2.16). The pellet was resuspended in 100 µL of elution

buffer (see Table 2.16) by gentle flicking, and incubated at room temperature for 20

min. The eluant was collected and the elution was repeated. Proteinase K (2 µL of 10

mg/mL) was added to each input sample and these were incubated in 65 °C water bath

for 4 h to reverse formaldehyde cross-links. The protein-bound DNA precipitated

from the ChIP assay and input DNA were then purified with phenol-chloroform and

dissolved in 25 µL H_2O. Precipitated and input DNA served as template for

promoter-specific PCR. Primers used to amplify the FSHβ or LHβ gene proximal

promoter are listed in Table 2.17.

Buffer	Components
ChIP lysis buffer	50 mM Tris-Cl [pH 7.5], 1% NP-40, 0.5% Na deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl, protease inhibitors
low-salt wash buffer	0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl [pH 8.0], 150 mM NaCl
high-salt wash buffer	0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl [pH 8.0], 500 mM NaCl
LiCl wash buffer	0.25 M LiCl, 1% NP-40, 1% Na deoxycholate, 1 mM EDTA, 10 mM Tris-Cl [pH 8.0]
Tris-EDTA	10 mM Tris-Cl [pH 8.0], 1 mM EDTA
elution buffer	0.1 M NaHCO$_3$, 1% SDS

Table 2.16: Buffers used in ChIP analysis

Primer	Oligonucleotide sequence (5'-3')
Mouse LHβ promoter F	CAATCTGGGGGTTCAGCGAG
Mouse LHβ promoter R	CCTTGGGCACCTGGCTTTAT
Mouse FSHβ promoter F	CACAGCCCATAGGAACAAGA
Mouse FSHβ promoter R	CCAAAGCAGTCTAAATGCC

Table 2.17: Primers used for amplification of the mouse LHβ and FSHβ

promoters in ChIP

2.9 Immunoprecipitation (IP)

For endogenous IP, LβT2 cells were washed with cold 1×PBS and lysed in RIPA buffer (20 mM Tris-Cl [pH 8.0], 125 mM NaCl, 0.5% NP-40, 5% glycerol, 20 mM NaF, 0.2 mM Na_3VO_4, 2 mM EDTA, and protease inhibitors) for 30 min. After centrifugation at 13,400×g for 40 min, 1 mg of total cell lysate was incubated with 1 μg of antibody and 20 μl protein A or G Sepharose CL-4B (GE Health) overnight at 4 °C with gentle rotation. The protein-antibody-bead complexes were washed four times with the lysis buffer. For Flag IP, 1 μg of Flag-tagged construct and 1 μg of HA-tagged construct were co-transfected into cells in 60 mm dishes. At 24 h after transfection, cells were washed with cold 1×PBS and lysed in RIPA buffer. After centrifugation at 13,400×g for 40 min, 100 μg of total cell lysate was incubated with 4 μl of EZview Red ANTI-Flag M2 Affinity Gel (Sigma) for 2 h at 4 °C with gentle rotation. The protein-antibody-bead complexes were washed at least four times with the lysis buffer to reduce non-specific binding. SDS-PAGE coupled with Western blot analysis was used to resolve the complexes (See Section 2.12).

2.10 *In vivo* ubiquitination assay

Flag- or HA-tagged WT or mutant SF-1 and Myc- or HA-tagged WT or mutant (K48R and K63R) ubiquitin constructs were co-transfected into the cells grown in 60 mm dish, and after 24 h, the cells were washed with cold 1×PBS and lysed for 30 min in RIPA buffer at 4 °C. After centrifugation at 13,400×g for 40 min at 4 °C, the

supernatants of the cell lysates were incubated with EZview Red ANTI-Flag M2 Affinity Gel or mouse HA antibody-conjugated protein G beads for 2 h at 4 °C with gentle rotation, followed by four washes with lysis buffer, after which Western blot analysis was performed (See Section 2.12).

The HA antibody was conjugated to the beads by incubating 1 µg of mouse HA antibody (Santa Cruz; sc-7392) with 20 µl protein G Sepharose CL-4B in RIPA buffer for 1 h at 4 °C, followed by extensive washes.

2.11 *In vivo* SUMOylation assay

Flag- or HA-tagged WT or mutant SF-1 and Myc-SUMO-1 constructs were co-transfected into the cells grown in 60 mm dish, and after 24 h, cells were washed with cold 1×PBS and lysed for 30 min in RIPA buffer containing 10 mM N-ethylmaleimide (NEM) at 4 °C. After centrifugation at 13,400×g for 40 min at 4 °C, the supernatants of the cell lysates were incubated with mouse HA antibody-conjugated protein G beads for 2 h at 4 °C with gentle rotation, followed by four washes with lysis buffer containing 10 mM NEM, after which Western blot analysis was performed (See Section 2.12).

2.12 Western blot

Cells were lysed in 1×lysis buffer, which was prepared as shown in Table 2.18. After centrifugation at 13,400×g for 40 min at 4 °C, the supernatant was collected and

protein concentration was measured using Bradford Protein Assay reagent (Biorad) according to manufacturer's instructions. For each sample, an equal volume of 2×SDS loading dye (Table 2.18) was added, and the mix was heated to 95 °C for 10 min before resolving on SDS-polyacrylamide gel (SDS PAGE) in SDS running buffer (Table 2.18) at 100 V for around 2 h at room temperature. The proteins were subsequently transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) in transfer buffer (Table 2.18) at 100 V for 1 h at 4 °C. The membrane was then blocked with blocking buffer (Table 2.18) on a gently rotating platform for 1 h at room temperature, or overnight at 4 °C. After washing with 1×TBST (Table 2.18), the membrane was incubated with primary antibody diluted in dilution buffer (Table 2.18) for 2 h at room temperature, or overnight at 4 °C. Subsequently, washing was carried out three times, for 10 min each with 1×TBST on a gently rotating platform at room temperature. After washing with 1×TBST for 30 min, the membrane was then incubated with goat or bovine HRP-conjugated secondary antibody to mouse, rabbit or goat IgG (Santa Cruz) in dilution buffer for 1 h at room temperature, followed by extensive washes with 1×TBST as above. The immunoreactive proteins were detected using the Super Signal Pico West chemiluminescent system (Pierce Chemical), followed by exposure to FUJI medical X-Ray film (FUJIFILM).

Buffer	Components
1×lysis buffer	50 mM Tris-Cl [pH 8.0], 100 mM NaCl, 1% SDS, 0.5% NP-40, 5% glycerol, 2 mM DTT, protease inhibitors
2×SDS loading dye	100 mM Tris-Cl [pH 6.8], 200 mM β-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol
Running buffer	12.5 mM Tris-Cl [pH 7.4], 96 mM glycine, 1.7 mM SDS
Transfer buffer	58 mM Tris-Cl [pH 8.3], 40 mM glycine, 1.6 mM SDS, 20% methanol
1×TBST	20 mM Tris-Cl [pH 7.4], 137 mM NaCl, 0.1% Tween-20 (add freshly)
Blocking buffer	5% BSA in 1×TBST
Dilution buffer	1% BSA in 1×TBST

Table 2.18: Buffers used in Western blot

2.13 Fluorescence imaging

LβT2 cells were seeded on ethanol-sterilized glass coverslips in 12-well plates, and after 24 h, transfected with pxj40-GFP empty vector, pxj40-GFP SF-1, or pxj40-GFP SF-1 K119R. Some of the cells were treated with 5 μM MG132 and/or 100 nM GnRH for 6 h, as indicated. The cells were fixed with cold-methanol at –20 °C for 10 min

and then permeabilized with 1×PBS containing 0.1% Triton X-100 for 20 min at room temperature. The fixed cells were washed twice with 1×PBS and then incubated with DAPI for 20 min, followed by washing with 1×PBS twice. The coverslips were mounted on glass slides using FluorSaveTM reagent (Calbiochem) and examined by LSM510 confocal fluorescence microscopy (Carl Zeiss).

CHAPTER 3   RESULTS

3.1 Pin1 induces gonadotropin β subunit gene transcription

3.1.1 Pin1 over-expression increases gonadotropin β subunit gene transcription

In order to test whether Pin1 has a role in transcription of the gonadotropin β subunit genes, luciferase assays were carried out to assess the effect of Pin1 over-expression on their promoter activity in mouse gonadotrope LβT2 cells. LHβ or FSHβ gene promoter construct was transfected into LβT2 cells together with pCS2+ empty vector or pCS2+ Pin1 expression vector. At 24 h after transfection, these cells were either left untreated or treated with 100 nM GnRH for 4 h, and were analyzed for luciferase activity. As shown in Fig 3.1 Pin1 over-expression increased both the basal and GnRH-induced murine LHβ and FSHβ promoter activity.
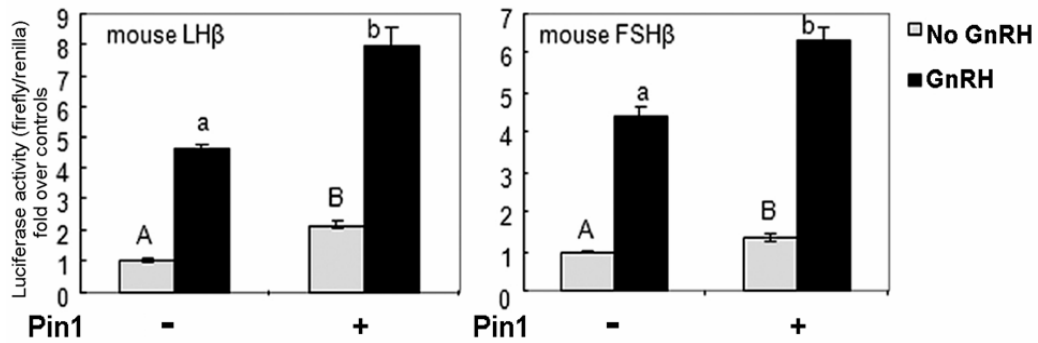
Fig 3.1: Pin1 over-expression increases promoter activity of gonadotropin β subunit genes.

LHβ or FSHβ promoter luciferase construct was transfected into LβT2 cells with pCS2+ empty vector or pCS2+ Pin1 expression vector, and some of the cells were exposed to 100 nM GnRH for 4 h before harvest. Luciferase assays were carried out and the levels of firefly luciferase were normalized to levels of Renilla; the results are expressed as normalized levels (n-fold) over those in control cells. Simple t-test compared mean values between groups with or without Pin1 over-expression, and was done separately for untreated and GnRH-treated groups; significantly different values (P<0.05) are designated with different letters, in upper case for untreated cells and in lower case for GnRH-treated cells. Results are shown as mean ± SEM; n=6.

The ability of Pin1 to increase transcription of the endogenous LHβ and FSHβ genes was next examined by real-time quantitative PCR (qPCR) using primers spanning an intron/exon border. As in the luciferase assays, Pin1 over-expression also increased the endogenous mRNA levels of LHβ and FSHβ in both untreated and GnRH treated cells (Fig 3.2A; over-expression of Pin1 was validated by Western blot as shown in Fig 3.2B).
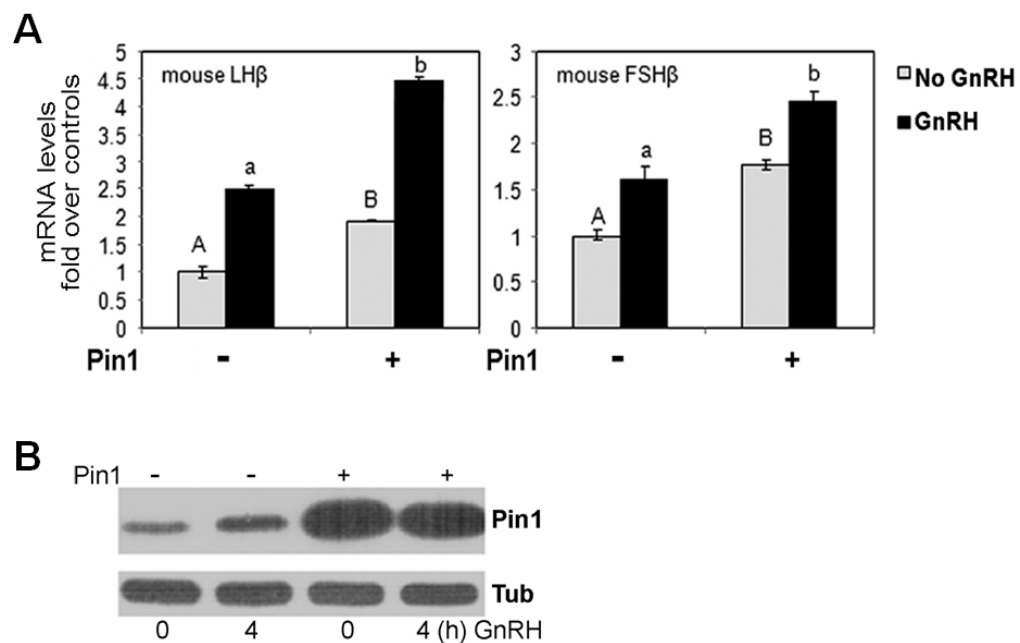
Fig 3.2: Pin1 over-expression increases endogenous mRNA levels of gonadotropin β subunit genes.

(A) pCS2+ empty vector or pCS2+ Pin1 expression vector was transfected into LβT2 cells, and some of the cells were exposed to 100 nM GnRH for 4 h before harvest. Total mRNA was extracted and endogenous LHβ or FSHβ mRNA levels were measured by real-time PCR, using β-actin as an internal control, and are expressed as the levels (n-fold) over those in control cells. Simple t-test compared mean values between groups with or without Pin1 over-expression, and was done separately for untreated and GnRH-treated groups; significantly different values (P<0.05) are designated with different letters, in upper case for untreated cells and in lower case for GnRH-treated cells. Results are shown as mean ± SEM; n=3. (B) Western blot was carried out to determine the protein levels of Pin1 in LβT2 cells following its over-expression, with or without 100 nM GnRH treatment for 4 h; α-tubulin (Tub) is shown as a loading control.

To confirm the role of Pin1 in increasing gonadotropin β subunit gene transcription, Pin1 WW domain mutant (Pin1 W34A) and PPIase domain mutant (Pin1 K63A) were over-expressed to test the requirement of the WW and PPIase domains. Real-time PCR showed that, in contrast to wild type (WT) Pin1, neither Pin1 W34A nor Pin1 K63A was able to increase transcript levels of either the LHβ or the FSHβ genes, and that the W34A mutant even reduced LHβ transcript levels to below those of the control (Fig 3.3A; levels of over-expression were validated by Western blot as shown in Fig 3.3B).
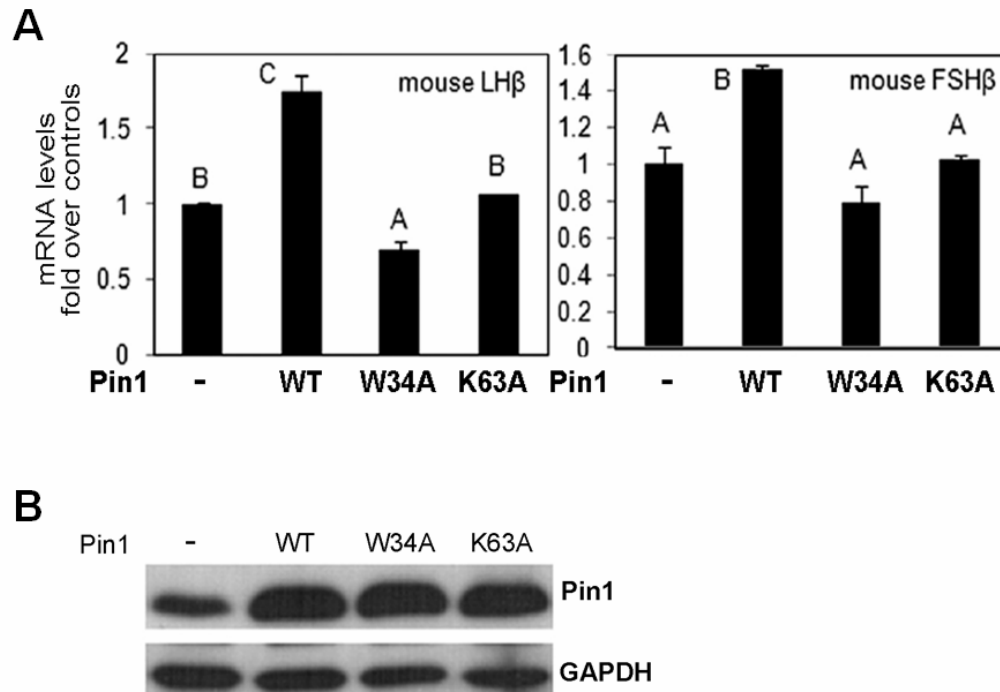
Fig 3.3: Wild type Pin1, but not its WW domain and PPIase domain mutants, increases endogenous mRNA levels of gonadotropin β subunit genes.

(A) pCS2+ empty vector, pCS2+ Pin1, pCS2+ Pin1 W34A or pCS2+ Pin1 K63A expression vector was transfected into LβT2 cells. Total mRNA was extracted 24 h after transfection, and endogenous LHβ or FSHβ mRNA levels were measured by real-time PCR, using β-actin as an internal control, and are expressed as the levels (n-fold) over those in control cells. ANOVA followed by a Bonferroni t-test compared all means; significantly different values ($P<0.05$) are designated with different letters. Results are shown as mean ± SEM; n=3. (B) Western blot was carried out to determine the protein levels of Pin1 and its mutants in LβT2 cells following their over-expression; GAPDH is shown as a loading control.

3.1.2 Pin1 knock-down decreases gonadotropin β subunit gene transcription

The actual role of Pin1 in regulating expression of these genes was then verified by the knock-down of Pin1 expression with specific siRNA. Transfection of the Pin1 targetting siRNA, but not the scrambled control siRNA, decreased the basal and, to a greater extent, the GnRH increased LHβ and FSHβ mRNA levels (Fig 3.4A; knock-down of Pin1 was validated by Western blot as shown in Fig 3.4B). This demonstrates that Pin1 plays a crucial role in GnRH-induced signaling pathways to induce mouse gonadotropin β subunit gene expression.

A

B
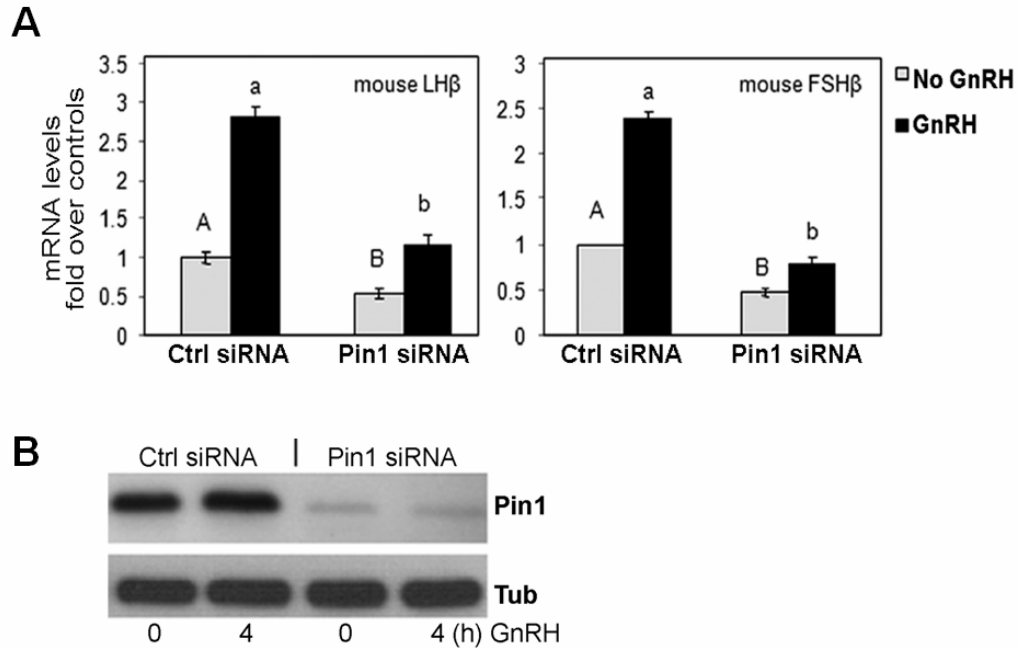
Fig 3.4: Pin1 knock-down decreases endogenous mRNA levels of gonadotropin β subunit genes.

(A) Control siRNA or Pin1 siRNA was transfected into LβT2 cells, and some of the cells were exposed to 100 nM GnRH for 4 h before harvest. Total mRNA was extracted and endogenous LHβ or FSHβ mRNA levels were measured by real-time PCR, using β-actin as an internal control, and are expressed as the levels (n-fold) over those in control cells. Simple t-test compared mean values between groups with or without Pin1 siRNA, and was done separately for untreated and GnRH treated groups; significantly different values (P<0.05) are designated with different letters, in upper case for untreated cells and in lower case for GnRH-treated cells. Results are shown as mean ± SEM; n=3. (B) Western blot was carried out to determine the protein levels of Pin1 in LβT2 cells following its knock-down, with or without 100 nM GnRH treatment for 4 h; α-tubulin (Tub) is shown as a loading control.

3.2 Pin1 is both transcriptionally and post-translationally regulated by GnRH

3.2.1 GnRH increases Pin1 protein levels

Previous studies by Wijeweera and Oh in our lab have demonstrated that GnRH upregulates Pin1 mRNA levels (Luo et al., 2010). Western blot on LβT2 whole cell lysate was performed to further study the effect of GnRH on Pin1 protein levels. Pin1 protein was increased progressively with lengthening GnRH treatment, in a rapid but more moderate response than seen for calcineurin catalytic subunit A (CnA) and the immediate early protein ATF3, while GAPDH remained virtually unchanged (Fig 3.5A). Fig 3.5B shows cell specificity of the GnRH effect on Pin1 by repeating the 2 h GnRH treatment in gonadotrope (αT3-1 and LβT2) and non-gonadotrope (MEF and COS-1) cell lines; the increase in Pin1 protein levels was seen only in the gonadotrope cells.
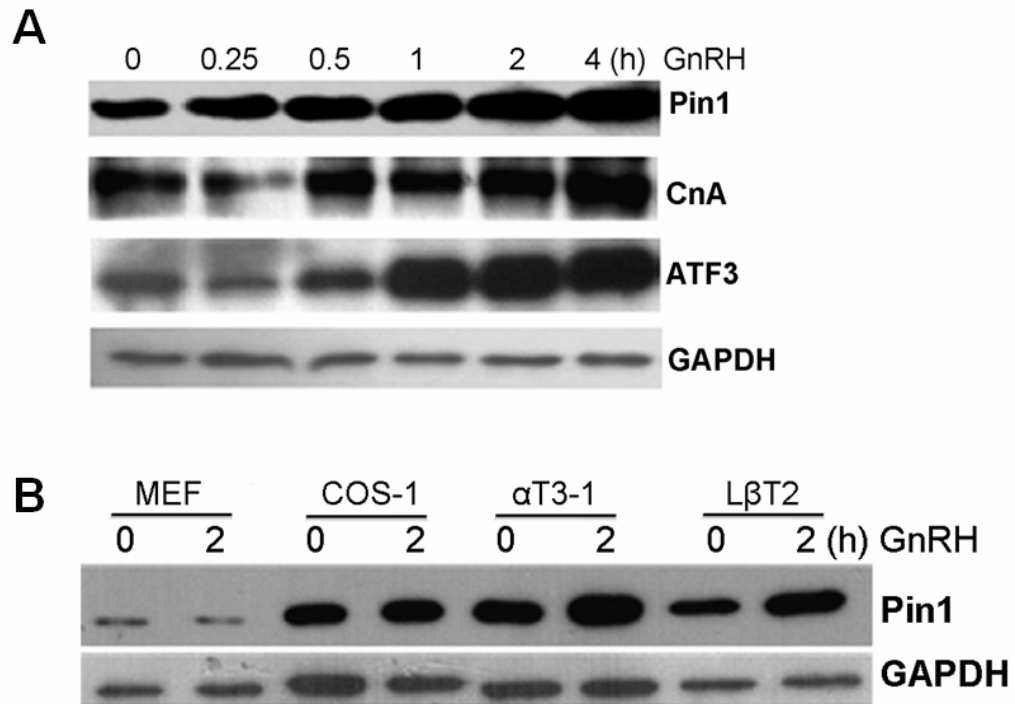
Fig 3.5: GnRH increases Pin1 protein levels.

(A) Western blot was carried out to determine the protein levels of Pin1, CnA and ATF3 in LβT2 cells following 100 nM GnRH treatment for 0-4 h; GAPDH is shown as a loading control. (B) Western blot was carried out to determine the protein levels of Pin1 in MEF, COS-1, αT3-1 and LβT2 cells following GnRH treatment for 0 or 2 h; GAPDH is shown as a loading control.

3.2.2 GnRH alters phosphorylation status of Pin1

Since PKA and PKC which are known to be involved in the GnRH signaling pathway have been reported to phosphorylate Pin1 at Ser 16, Western blot was performed to examine whether GnRH also alters the phosphorylation status of Pin1. First, the specificity of the antibody to phosphorylated Pin1 at Ser 16 (p-Pin1) was confirmed by over-expressing GFP-Pin1 or GFP-Pin1 S16A in LβT2 cells treated with 100 nM GnRH for 0.5 h; immunoprecipitation of GFP-tagged proteins was followed by Western blot analysis using antibody to p-Pin1, Pin1 and GFP. Fig 3.6A shows that no immunoreactive band was seen for p-Pin1 antibody in either the input or GFP antibody immunoprecipitated sample in which GFP-Pin1 S16A was transfected. The p-Pin1 was easily detected after 15 min GnRH treatment and reached at a peak level after 30 min treatment, after which it gradually decreased (Fig 3.6B). In order to determine whether PKA and/or PKC is responsible for GnRH-induced Pin1 phosphorylation, LβT2 cells were treated with PKC activator, PMA, or PKA activator, forskolin, for 1 h. The Western analysis showed that both treatments strongly induced p-Pin1 levels, indicating an involvement of PKA and/or PKC pathways in phosphorylating Pin1 at Ser 16 (Fig 3.6B).
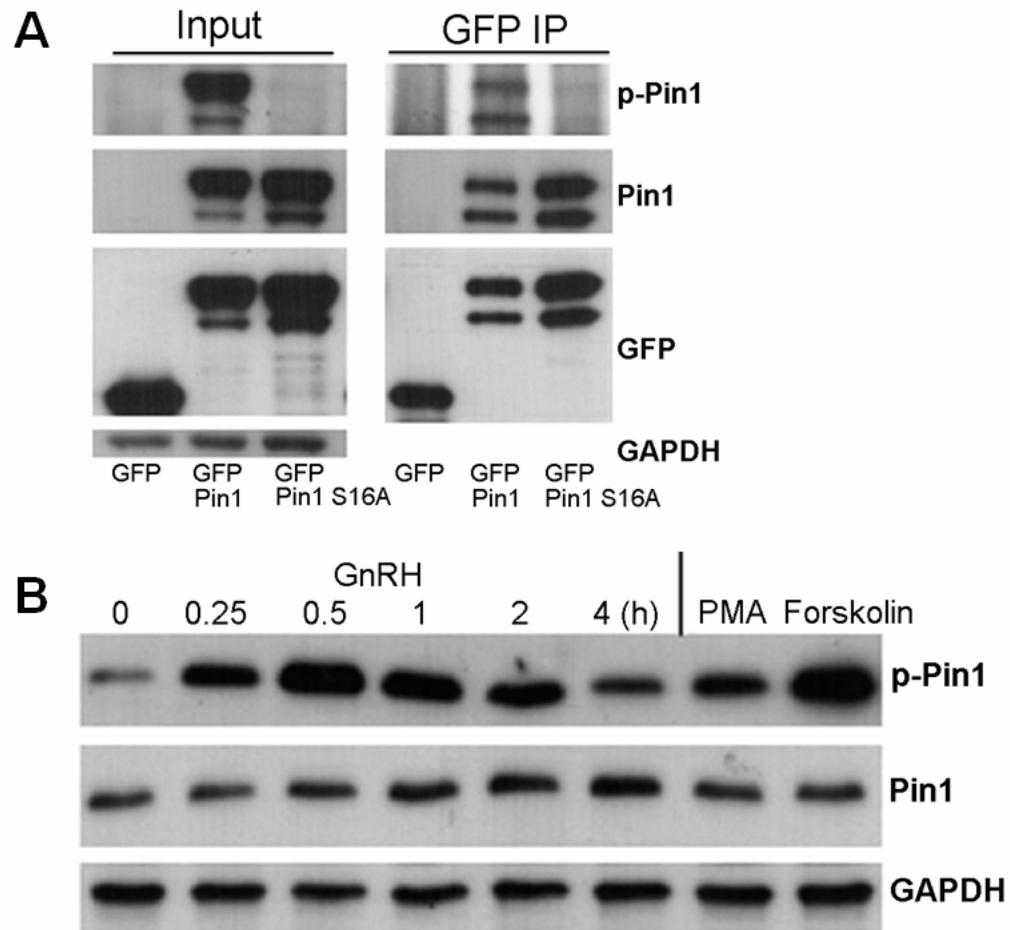
Fig 3.6: GnRH alters the phosphorylation status of Pin1.

(A) Specificity of the antibody to p-Pin1 was confirmed by immunoprecipitating GFP-tagged proteins from LβT2 cell lysates after transfection of pEGFP empty vector, pEGFP Pin1, and pEGFP Pin1 S16A; Western blot was carried out to determine levels of p-Pin1, total Pin1 and GFP-tagged proteins in both the input and immunoprecipitated samples; GAPDH is shown as a loading control. (B) Western blot was carried out to determine the protein levels of p-Pin1 and Pin1 in LβT2 cells following 0 to 4 h 100 nM GnRH treatment or exposure to 100 nM PMA or 1 μM forskolin for 1 h; GAPDH is shown as a loading control.

Phosphorylation of Pin1 at Ser 16 has been reported to hinder its activity by preventing its interaction with the substrates and its nuclear localization (Lu et al., 2002), and yet Pin1, although being temporarily phosphorylated by GnRH, appears required for LHβ and FSHβ gene transcription. It has been demonstrated that GnRH induces expression of the phosphatase calcineurin (Lim et al., 2007; Fig 3.5A), and initial studies by Oh (2009) in our lab have demonstrated that knock-down of calcineurin expression by specific siRNA, or inhibition of calcineurin activity by cyclosporin A increases p-Pin1 levels in LβT2 cells, indicating that GnRH-activated calcineurin might dephosphorylate p-Pin1 to allow its activity (Luo et al., 2010). To further confirm the actions of calcineurin on Pin1, co-immunoprecipitation was performed to examine whether calcineurin interacts with p-Pin1. The pCS2+ Pin1 was transfected into LβT2 cells and, 24 h after transfection, some of the cells were exposed to 100 nM GnRH for 0.5 h. The cells were lysed, and immunoprecipitated with anti-CnA antibody or IgG, followed by Western analysis using anti-p-Pin1, Pin1 or CnA antibody. The p-Pin1 was co-precipitated by the CnA antibody, but not rabbit IgG, and the co-immunoprecipitation was more significant after GnRH treatment (Fig 3.7). Taken together, these results clearly demonstrate a role for GnRH-activated calcineurin in regulating Pin1 activity.
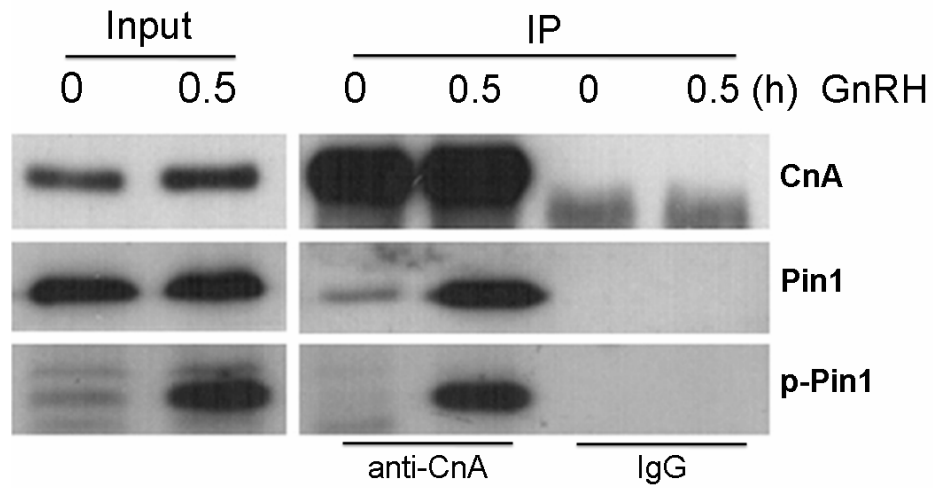
Fig 3.7: p-Pin1 is associated with calcineurin catalytic subunit A.

Cell lysates from LβT2 cells following Pin1 over-expression and exposure to 100 nM GnRH for 0 or 0.5 h, as marked, were immunoprecipitated using antibody to CnA or normal rabbit IgG; Western blot was carried out to determine levels of p-Pin1, total Pin1 and CnA in the input and precipitated samples.

3.3 Pin1 is present on the promoters of the LHβ and FSHβ genes and interacts with various gene-specific transcription factors

3.3.1 Pin1 is present on the promoters of the LHβ and FSHβ genes

In order to elucidate the mechanisms through which Pin1 activates LHβ and FSHβ gene transcription, chromatin immunoprecipitation was carried out to examine whether Pin1 is present on the promoters of these genes. After exposure to 100 nM GnRH for 0 or 4 h and cross-linking, the LβT2 cells were lysed and immunoprecipitation with Pin1 or HA antibody was performed to pull down the Pin1 associated DNA. Association of Pin1 with the promoters of LHβ and FSHβ genes was detected in untreated LβT2 cells and after GnRH exposure for 4 h through PCR using primers on the promoters of LHβ and FSHβ genes (Fig 3.8).
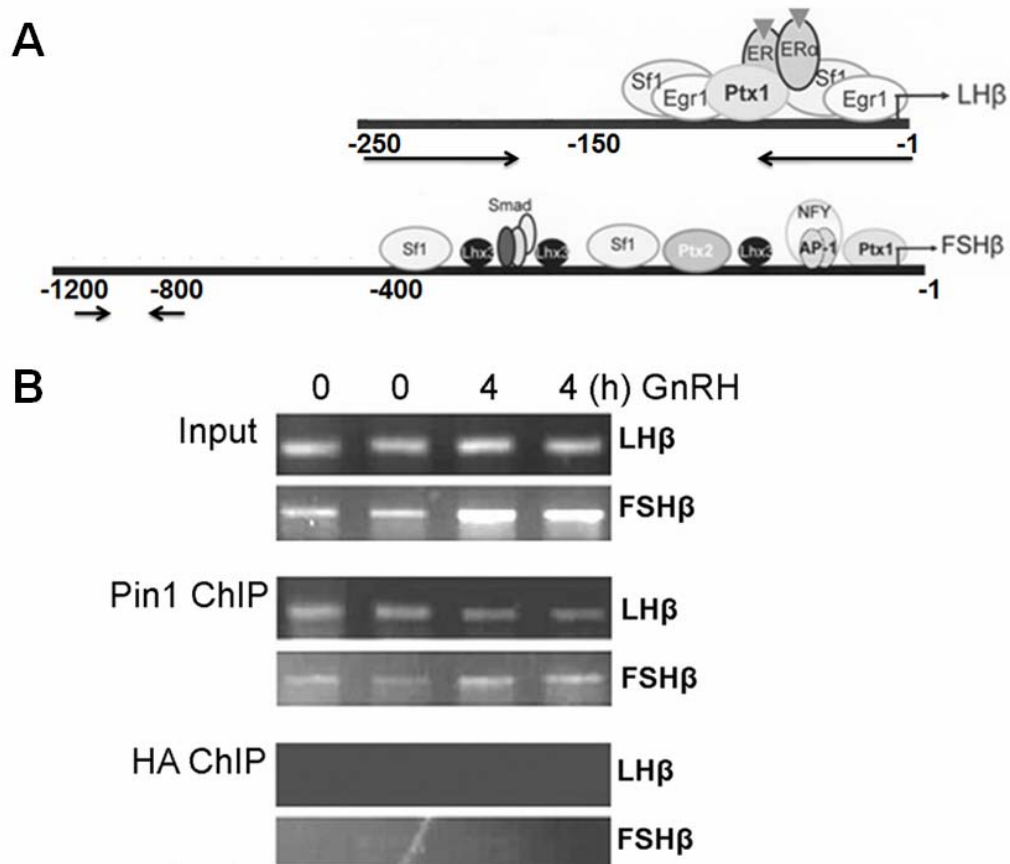
Fig 3.8: Pin1 is present on the proximal promoters of the LHβ and FSHβ genes.

(A) Schemes indicating the positions of PCR primers in the LHβ and FSHβ gene promoters. (B) ChIP was carried out using antibody to Pin1, or HA as control, in LβT2 cells with or without exposure to 100 nM GnRH for 4 h, and PCR was performed to detect the endogenous mouse LHβ and FSHβ gene promoters from the input and immunoprecipitated samples. All treatments were carried out in duplicate.

3.3.2 Pin1 interacts with various gene-specific transcription factors

Since Pin1 itself is not a DNA binding protein, it was hypothesized that Pin1 is recruited to LHβ and FSHβ gene promoters through its interaction with specific transcription factors known to regulate expression of these genes. Therefore, co-immunoprecipitation assays were carried out using LβT2 cell lysates, and Pitx1, Egr-1 and β-catenin were detected in the precipitates using the Pin1 antibody but not in those using the control IgG (Fig 3.9A). Because the mobility of SF-1 is similar to that of the IgG heavy chain, co-immunoprecipitation using antibody to SF-1 for the precipitation was performed, and Pin1 was precipitated by the SF-1 antibody from both untreated and GnRH treated cell lysates, but not by the control IgG (Fig 3.9B).

In order to confirm some of these interactions, mammalian two-hybrid assays were carried out in COS-1 cells using Pin1 fused to the Gal4 DBD (pM Pin1) and Pitx1, Egr-1, or SF-1 fused to VP16 AD (pVP Pitx1, pVP Egr-1, or pVP SF-1), together with Gal4-responsive luciferase reporter gene and pRL-SV40 Renilla as internal control. COS-1 cells were used to minimize the interference from endogenous expression of these transcription factors. Co-transfection of both pM Pin1, but not the empty pM vector, with pVP Pitx1, pVP Egr-1, or pVP SF-1 resulted in significant induction of the luciferase reporter gene activity (Figs 3.10 and 3.11A). This indicates that Pin1 interacts with Pitx1, Egr-1 and SF-1.
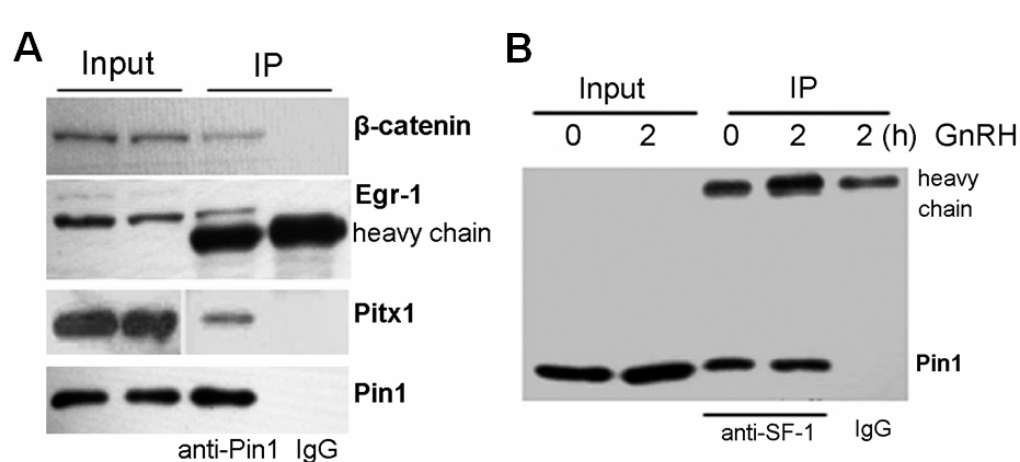
Fig 3.9: Co-immunoprecipitation of Pin1 and various gonadotropin β subunit gene-specific transcription factors.

(A) Cell lysates from LβT2 cells were immunoprecipitated using antibody to Pin1 or using normal IgG as control; Western blot was carried out to determine levels of β-catenin, Egr-1, Pitx1 and Pin1 in the input and precipitated samples. (B) Cell lysates from LβT2 cells following Pin1 over-expression and exposure to 100 nM GnRH, as marked, were immunoprecipitated using antibody to SF-1 or normal IgG; Western blot was carried out to determine levels of Pin1 in the input and precipitated samples.
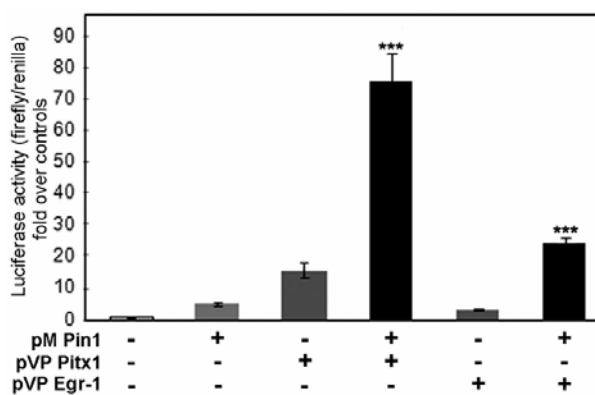
Fig 3.10: Interaction between Pin1 and various gonadotropin β subunit gene-specific transcription factors shown by mammalian two-hybrid assays.

Mammalian two-hybrid assays were carried out in COS-1 cells using a Gal4-responsive reporter gene, and a Renilla luciferase as internal control, together with a Gal4 DBD empty vector (pM) or Gal4 DBD-Pin1 fusion construct (pM Pin1) and VP16 AD empty vector (pVP) or VP16 AD fused to Pitx1 (pVP Pitx1), Egr-1 (pVP Egr-1). Luciferase assays were carried out and the levels of firefly luciferase normalized to those of Renilla; results are expressed as the normalized levels (n-fold) over those in control cells, in which unfused Gal4 DBD and VP16 AD constructs were transfected together. Statistical analysis (t-test) to assess interaction, compared means for the groups transfected with both fusion constructs, with the combined effects of each fusion construct alone (*** $P<0.001$). Results are shown as mean ± SEM; n=6.

The two-hybrid assay was also carried out using an expression vector for SF-1 in which the only potential Pin1 binding site at serine 203 was mutated to alanine. The interaction of this mutated protein (SF-1 S203A) with Pin1 was markedly reduced in comparison to that of the WT SF-1 (Fig 3.11A). This reduced interaction of SF-1 S203A with Pin1 was also demonstrated by co-immunoprecipitation assays in which the Flag-tagged SF-1 WT or S203A mutant protein was expressed in LβT2 cells and precipitated by Flag beads before immunoblotting with Pin1 antibody (Fig 3.11B).

Mutagenesis of various Ser/Thr-Pro motifs in Pitx1 was also carried out to determine which residues are involved in the interaction with Pin1. Precipitation of the Flag-tagged Pin1 was performed after co-over-expression of Flag-Pin1 and HA-tagged Pitx1 WT or mutant proteins, followed by immunoblotting with antibody to the HA tag on the Pitx1 proteins. The binding of Flag-Pin1 with the Pitx1 mutants, HA-Pitx1 S207A, HA-Pitx1 S259A, or HA-Pitx1 T267A S270A, was clearly reduced in comparison with the wild type HA-Pitx1 (Fig 3.11C).

These results indicate that Pin1 interacts directly with Pitx1, Egr-1 and SF-1, and demonstrate a crucial role for Ser 203 of SF-1 and for several residues of Pitx1 in these interactions.
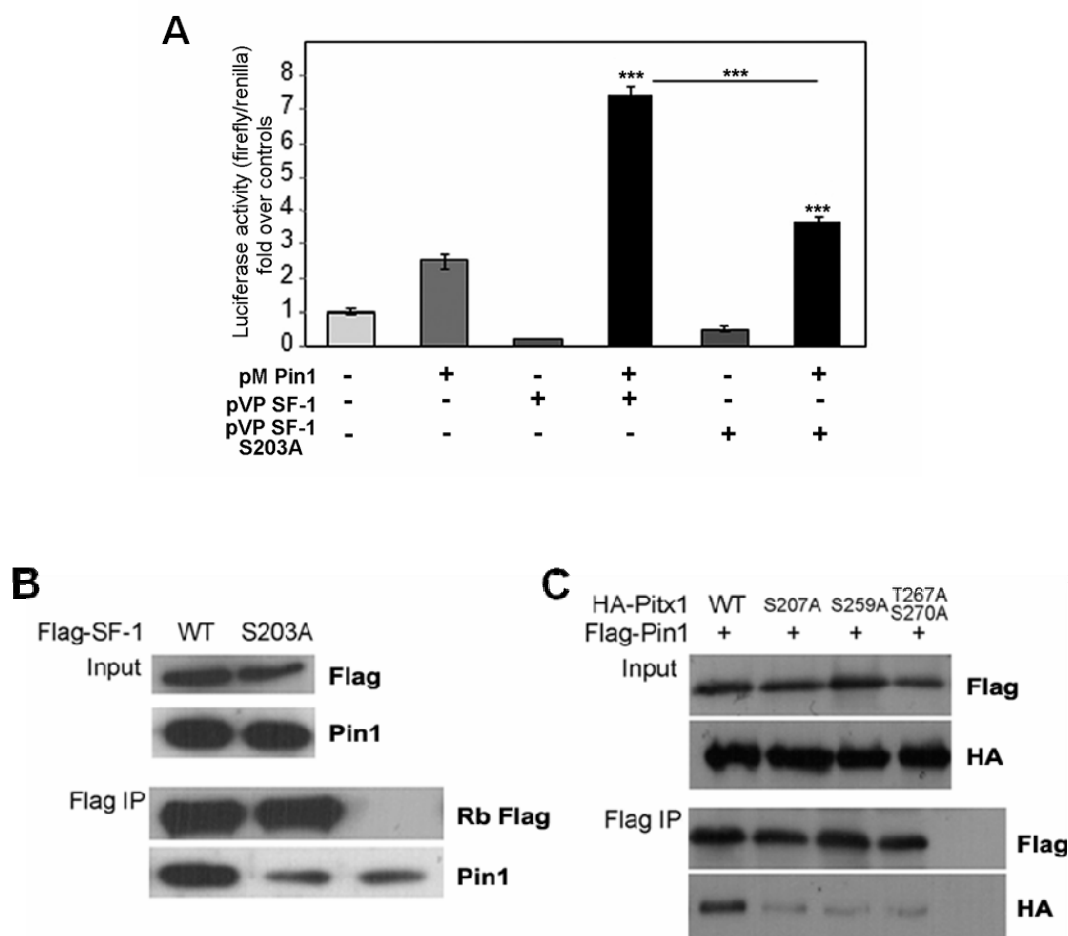
Fig 3.11: Mutagenesis of Ser/Thr-Pro motifs in SF-1 and Pitx1 reduces the interaction between Pin1 and SF-1 or Pitx1.

(A) Mammalian two-hybrid assays were carried out in COS-1 cells using a Gal4-responsive reporter gene, and a Renilla luciferase as internal control, together with a Gal4 DBD empty vector (pM) or Gal4 DBD-Pin1 fusion construct (pM Pin1) and VP16 AD empty vector (pVP) or VP16 AD fused to SF-1 (pVP SF-1), SF-1 S203A (pVP SF-1 S203A). Luciferase assays were carried out and the levels of firefly luciferase normalized to those of Renilla; results are expressed as the normalized levels (n-fold) over those in control cells, in which unfused Gal4 DBD and VP16 AD constructs were transfected together. Statistical analysis (t-test) to assess interaction, compared means for the groups transfected with both fusion constructs, with the combined effects of each fusion construct alone. In addition, the interactions of pM Pin1 with the WT and SF-1 S203A pVP fusion proteins were also compared (*** $P<0.001$). Results are shown as mean ± SEM; n=6. (B) Cell lysates from LβT2 cells following co-transfection of Pin1 and pxj40-Flag empty vector, Flag-SF-1 or Flag-SF-1 S203A were immunoprecipitated using anti-Flag M2 beads; Western blot was carried out to determine levels of Pin1, Flag-SF-1 and Flag-SF-1 S203A in the input and precipitated samples. (C) Cell lysates from LβT2 cells following co-transfection of Flag-Pin1 and pxj40-HA empty vector, HA-Pitx1 WT or mutant constructs were immunoprecipitated using anti-Flag M2 beads; Western blot was carried out to determine levels of Flag-Pin1, HA-Pitx1 WT and mutants in the input and precipitated samples.

3.4 Pin1 increases transcriptional activity of SF-1, Pitx1 and Egr-1

In order to examine whether the interaction between Pin1 and these transcription factors plays a functional role in transcription of the gonadotropin β subunit genes, the effects of Pin1 on transactivation of these genes by Pitx1, Egr-1 or SF-1 were examined using luciferase assays. LHβ or FSHβ promoter luciferase construct was transfected into LβT2 cells alone or together with the expression vectors for Pin1 and/or the transcription factors, and luciferase assays were carried out to assess whether they acted synergistically. The results showed that Pin1 and SF-1 had a synergistic effect on the LHβ but not on the FSHβ gene promoter (Fig 3.12), while Pin1 and Pitx1 had a synergistic effect on the FSHβ but not the LHβ gene promoter (Fig 3.13). Egr1, which targets only the LHβ gene, also had a synergistic effect with Pin1 on LHβ promoter activity (Fig 3.14).
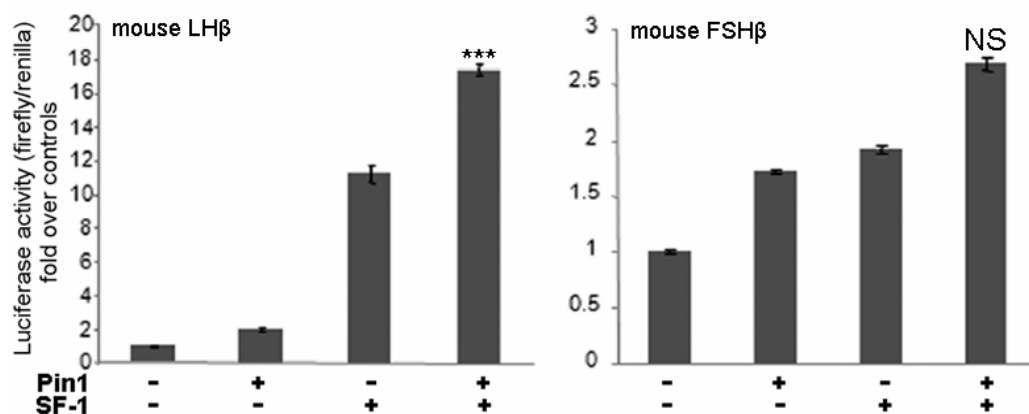
Fig 3.12: Pin1 and SF-1 have a synergistic effect on the LHβ but not on the FSHβ gene promoter.

LHβ or FSHβ promoter luciferase construct was transfected into LβT2 cells alone or together with Pin1 and/or SF-1 expression vectors. After 24 h, cells were harvested. Luciferase assays were carried out and the levels of firefly luciferase were normalized to levels of Renilla; the results are expressed as normalized levels (n-fold) over those in control cells. Simple t-test compared mean values between groups transfected with Pin1 and SF-1 expression vector, with the combined effects of each construct alone, where ***, $P<0.001$ and NS, not synergistic. Results are shown as mean ± SEM; n=6.
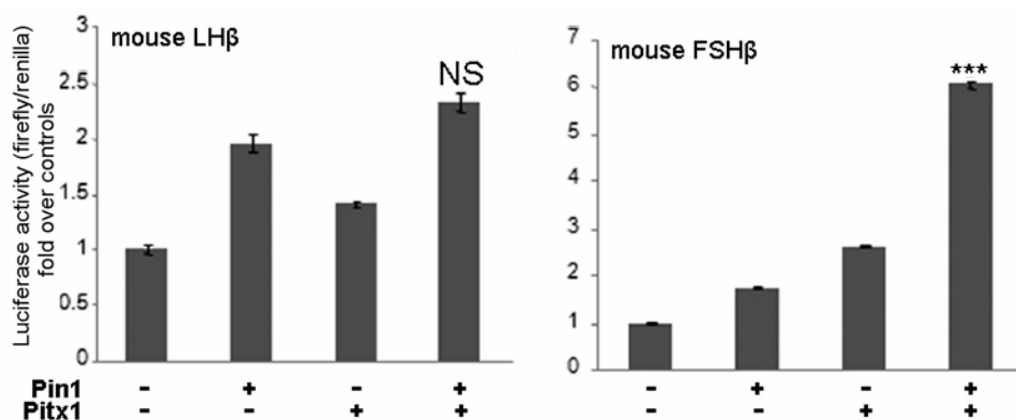
Fig 3.13: Pin1 and Pitx1 have a synergistic effect on the FSHβ but not on the LHβ gene promoter.

LHβ or FSHβ promoter luciferase construct was transfected into LβT2 cells alone or together with Pin1 and/or Pitx1 expression vectors. After 24 h, cells were harvested. Luciferase assays were carried out and the levels of firefly luciferase were normalized to levels of Renilla; the results are expressed as normalized levels (n-fold) over those in control cells. Simple t-test compared mean values between groups transfected with Pin1 and Pitx1 expression vector, with the combined effects of each construct alone, where ***, $P<0.001$ and NS, not synergistic. Results are shown as mean ± SEM; n=6.
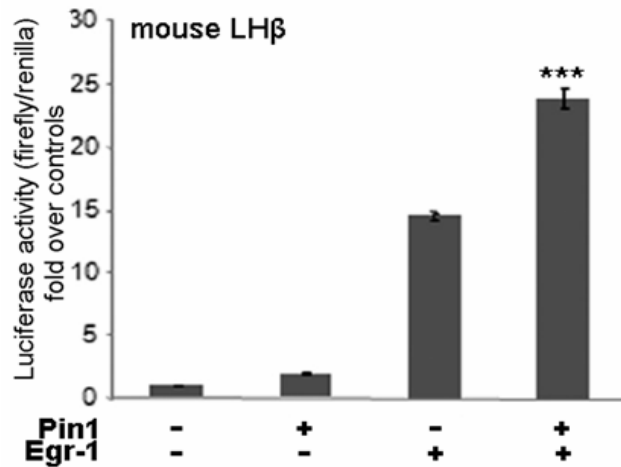
Fig 3.14: Pin1 and Egr-1 have a synergistic effect on the LHβ gene promoter.

LHβ promoter luciferase construct was transfected into LβT2 cells alone or together with Pin1 and/or Egr-1 expression vectors. After 24 h, cells were harvested. Luciferase assays were carried out and the levels of firefly luciferase were normalized to levels of Renilla; the results are expressed as normalized levels (n-fold) over those in control cells. Simple t-test compared mean values between groups transfected with Pin1 and Egr-1 expression vector, with the combined effects of each construct alone, where ***, $P < 0.001$. Results are shown as mean ± SEM; n=6.

3.5 Effects of Pin1 on the levels of these transcription factors

Since Pin1 binds directly to Pitx1, Egr-1 and SF-1 and enhances their transactivation, it was speculated that Pin1 might affect the stability of these transcription factors. To test this hypothesis, Pin1 expression vector was transfected into LβT2 cells and the cells were harvested 0, 24, 36 and 48 h after transfection. Western blot showed that Pin1 over-expression led to an increase in Pitx1 but a decrease in SF-1 levels, while Egr-1 and GAPDH protein levels remained unchanged over the 48 h (Fig 3.15A). Some of this could be due to Pin1 regulating transcription of these factors, as RT-PCR after over-expression of Pin1 showed a clear increase in Pitx1 mRNA levels, while SF-1 mRNA levels decreased marginally (Fig 3.15B). In order to investigate the post-transcriptional effects of Pin1 on these proteins, constructs encoding HA-tagged Pitx-1 or SF-1 were transfected with or without Pin1 expression vector. Western blot of the HA-tagged proteins showed the same trend of an increase in Pitx1 and a decrease in SF-1 protein levels, both of which were more pronounced when more of the Pin1 expression vector was transfected (Fig 3.15C).
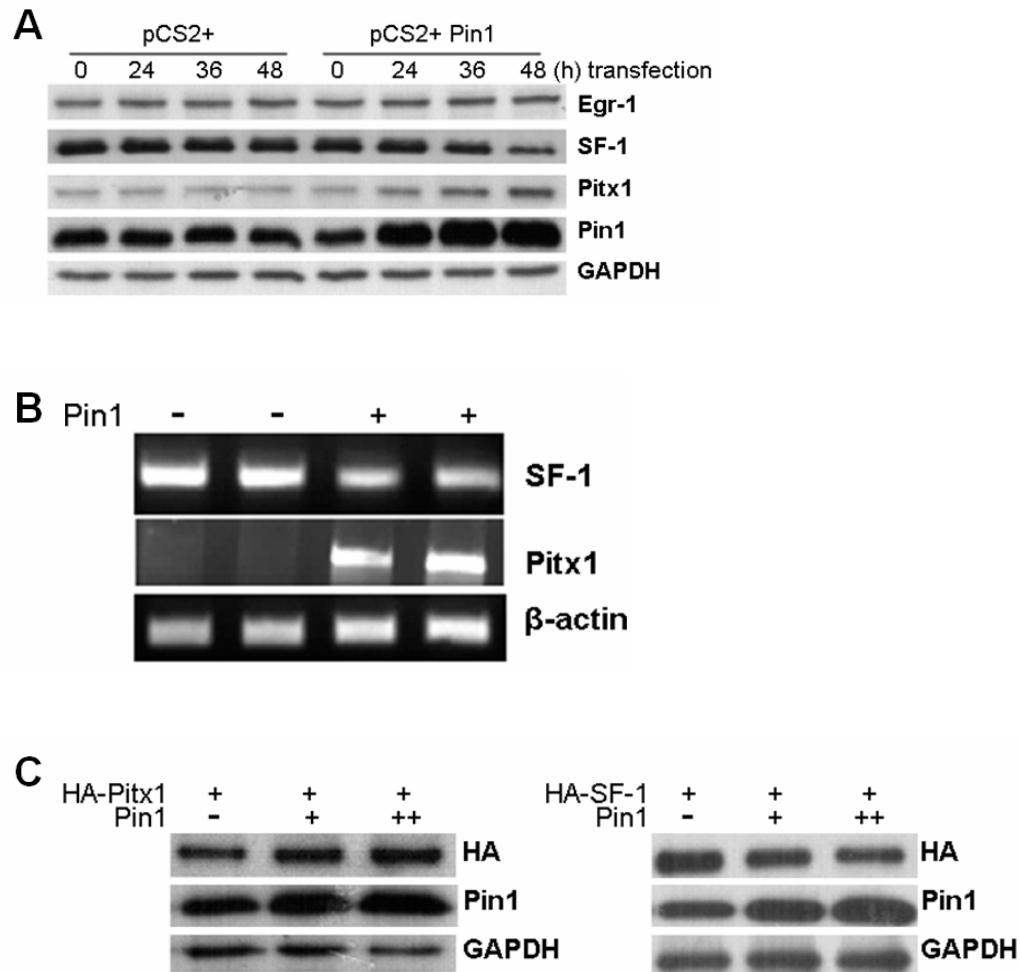
Fig 3.15: Effects of Pin1 on the levels of these transcription factors.

(A) Western blot was carried out to determine the protein levels of Egr-1, SF-1, Pitx1 and Pin1 in LβT2 cells transfected with pCS2+ empty vector or pCS2+ Pin1 expression vector for 0, 24, 36 and 48 h; GAPDH is shown as a loading control. (B) RT-PCR analysis of SF-1 and Pitx1 mRNA levels was carried out in LβT2 cells transfected with pCS2+ empty vector or pCS2+ Pin1 expression vector; β-actin was used as an internal control. All transfections were carried out in duplicate. (C) Western blot was carried out to detect the protein levels of HA-Pitx1 and HA-SF-1 in LβT2 cells transfected with pCS2+ empty vector or different amount of pCS2+ Pin1 expression vector; GAPDH is shown as a loading control.

3.6 Regulation of SF-1 ubiquitination

3.6.1 Ser 203 is required for SF-1 ubiquitination

To investigate the molecular mechanism underlying the Pin1-induced decrease in SF-1 protein levels, *in vivo* ubiquitination assays were carried out to examine whether Pin1 is involved in ubiquitination of SF-1. Ubiquitination of WT HA-SF-1 and HA-SF-1 S203A, in which the only Pin1 binding site was mutated, was analyzed by immunoprecipitation of the tagged protein followed by Western blot using an antibody against ubiquitin. In the input and HA-immunoprecipitated samples a mono-ubiquitinated form of SF-1 was evident (between the 50 and 75 kDa markers) following transfection of the WT but not the S203A mutant construct, while the SF-1 ubiquitination was reduced to an undetectable level when serine 203 in SF-1 was mutated to alanine (Fig 3.16A).

Similarly, the SF-1 WT or S203A construct was transfected with an expression vector for Myc-tagged ubiquitin, and immunoprecipitation was carried out using rabbit antibdoy to the Myc tag. The input and precipitated proteins were blotted with mouse antibody to HA. Again, in this reciprocal immunoprecipitation the mono- and poly-ubiquitinated SF-1 was visible only in the WT SF-1 transfected samples (Fig 3.16B).
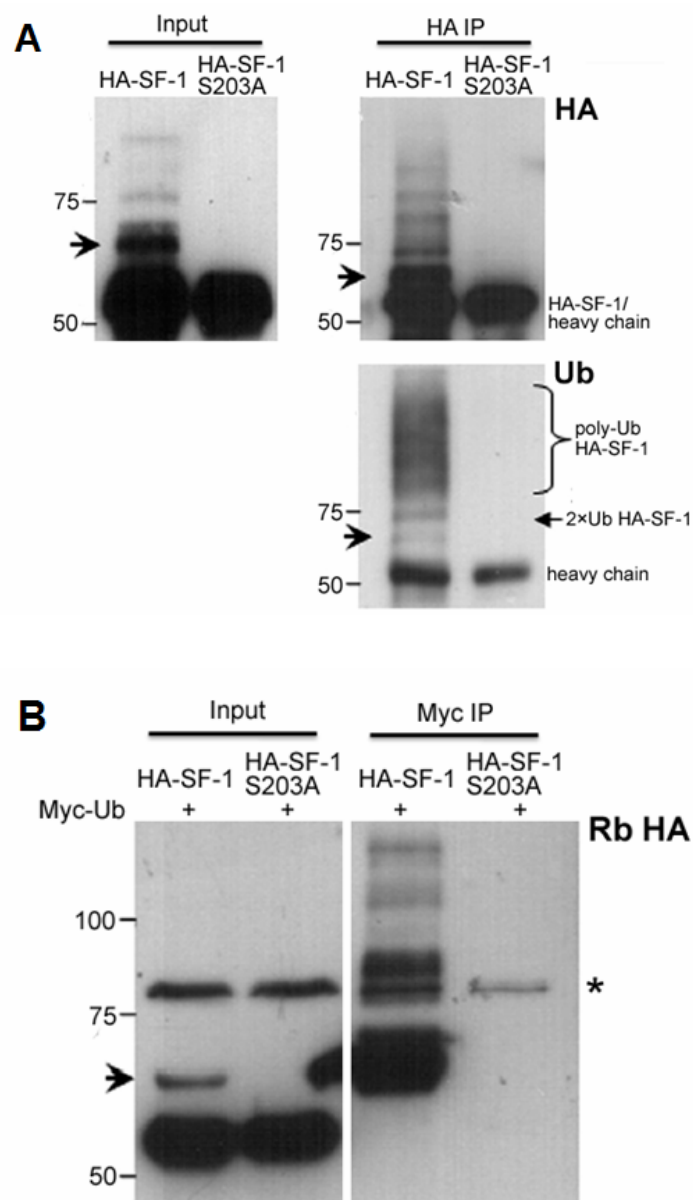
Fig 3.16: Ubiquitination of SF-1 requires Ser 203.

(A) Cell lysates from LβT2 cells following transfection of HA-SF-1 or HA-SF-1 S203A expression vector were immunoprecipitated using mouse anti-HA antibody; the input and precipitated samples were analyzed by Western blot using rabbit anti-HA and mouse anti-ubiquitin (Ub) antibodies. (B) Cell lysates from LβT2 cells following co-transfection of Myc-ubiquitin (Myc-Ub) and HA-SF-1 or HA-SF-1 S203A expression vector were immunoprecipitated using mouse anti-Myc antibody, and input and precipitated samples were analyzed by Western blot using mouse anti-HA antibody. The mono-ubiquitinated SF-1 is marked with arrows on the left. An apparently non-specific immunoreactive protein is marked with an asterisk.

3.6.2 Pin1 is required for SF-1 ubiquitination

In order to assess the role of Pin1 in the SF-1 ubiquitination, *in vivo* ubiquitination

assays were carried out in LβT2 cells after over-expression of Flag-tagged SF-1, with

control siRNA or Pin1 specific siRNA transfection. SF-1 was clearly

poly-ubiquitinated in LβT2 control cells, but in the Pin1 knock-down cells SF-1

ubiquitination was reduced (Fig 3.17A). To confirm this, further studies were carried

out in stably transfected HEK 293T control or Pin1 knock-down cells (expressing

Pin1 but at lower levels) (Fig 3.17B) after over-expression of HA-tagged WT SF-1 or

the S203A mutant. From the blot of the immunoprecipitated samples with ubiquitin

antibody (Fig 3.17B, bottom panel on the right), it is evident that SF-1 was also

poly-ubiquitinated in HEK 293T control cells, but in the Pin1 knock-down cells, SF-1

ubiquitination was clearly reduced, while in neither cell line did the SF-1 S203A

appear to be ubiquitinated. The apparently mono-ubiquitinated band again can be seen

in the input and HA-immunoprecipitated samples when the WT SF-1 was transfected,

but it was barely detectable in the Pin1 knock-down cells (Fig 3.17B, top left and top

right panels). These results indicate that Pin1 increases SF-1 ubiquitination through a

mechanism involving Ser 203.
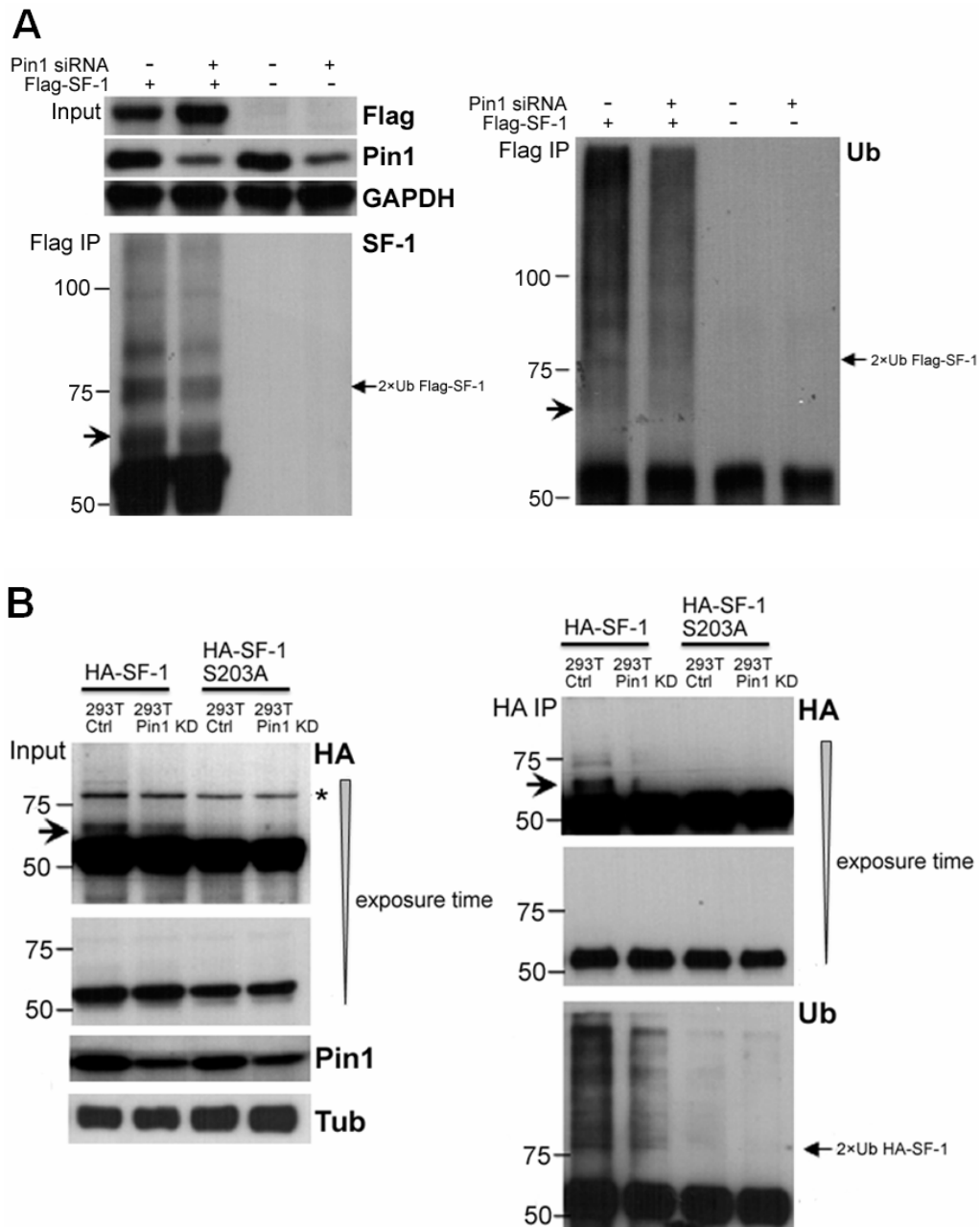
Fig 3.17: Ubiquitination of SF-1 requires Pin1.

(A) Cell lysates from LβT2 cells transfected with Flag-SF-1, 12 h after transfection of control or Pin1 siRNA, were immunoprecipitated using anti-Flag M2 beads; the input and precipitated samples were analyzed by Western blot using antibodies to Flag, SF-1, Ub, and Pin1, with GAPDH as a loading control. (B) Cell lysates from 293T control (293T Ctrl) and Pin1 siRNA (293T Pin1 KD) cells transfected with HA-SF-1 or HA-SF-1 S203A expression vectors were immunoprecipitated using mouse anti-HA antibody; the input and precipitated samples were analyzed by Western blot using antibodies to HA, Ub, and Pin1, with α-tubulin (Tub) as a loading control. Longer exposure time also showed the ubiquitinated SF-1 in HA antibody blotting. The mono-ubiquitinated SF-1 is marked with arrows on the left. An apparently non-specific immunoreactive protein is marked with an asterisk.

3.7 GnRH treatment stimulates SF-1 ubiquitination

It has already been shown that SF-1 is ubiquitinated in untreated LβT2 cells. Next,

GnRH treatment was performed to examine the effect of GnRH on SF-1

ubiquitination. Fig 3.18 shows that ubiquitinated SF-1 was detectable in untreated

LβT2 cells and was greatly enhanced after GnRH treatment for 6 h.
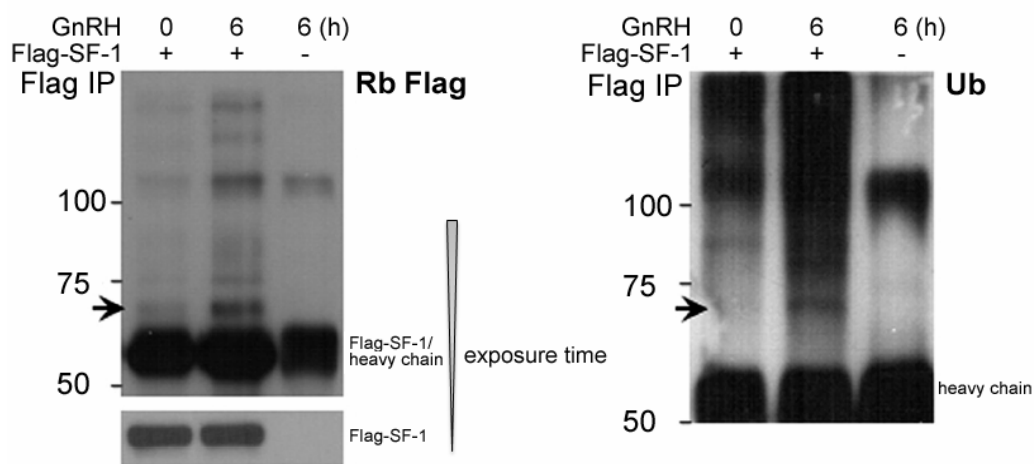
Fig 3.18: GnRH treatment increases ubiquitination of SF-1.

Cell lysates from LβT2 cells following transfection of Flag-SF-1 expression vector or pxj40-Flag empty vector and exposure to 100 nM GnRH for 0 or 6 h, as marked, were immunoprecipitated using anti-Flag M2 beads; the precipitated samples were analyzed by Western blot using rabbit anti-Flag and mouse anti-ubiquitin antibodies. Longer exposure time also showed the ubiquitinated SF-1 in Flag antibody blotting. The mono-ubiquitinated SF-1 is marked with arrows on the left.

The fact that GnRH induces SF-1 ubiquitination led to the speculation that phosphorylation of SF-1 at Ser 203 by GnRH-activated ERK 1/2 might be the cause of the increase in SF-1 ubiquitination. GnRH-induced activation of ERK within 30 min, and an effect was still seen after 2 and 4 h, but treatment with the specific inhibitor, U0126, virtually abolished this GnRH-induced effect (Fig 3.19A). Next, the effect of this U0126-induced ERK inhibition on SF-1 ubiquitination was examined by *in vivo* ubiquitination assay. In unstimulated cells, U0126 treatment did not change the levels of poly-Ub SF-1, however, in GnRH treated cells U0126 treatment reduced SF-1 poly-ubiquitination to a modest level as compared to that in cells treated with GnRH alone (Fig 3.19B). These results suggest that phosphorylation of SF-1 at Ser 203 by GnRH-activated ERK 1/2 contributes to SF-1 ubiquitination.
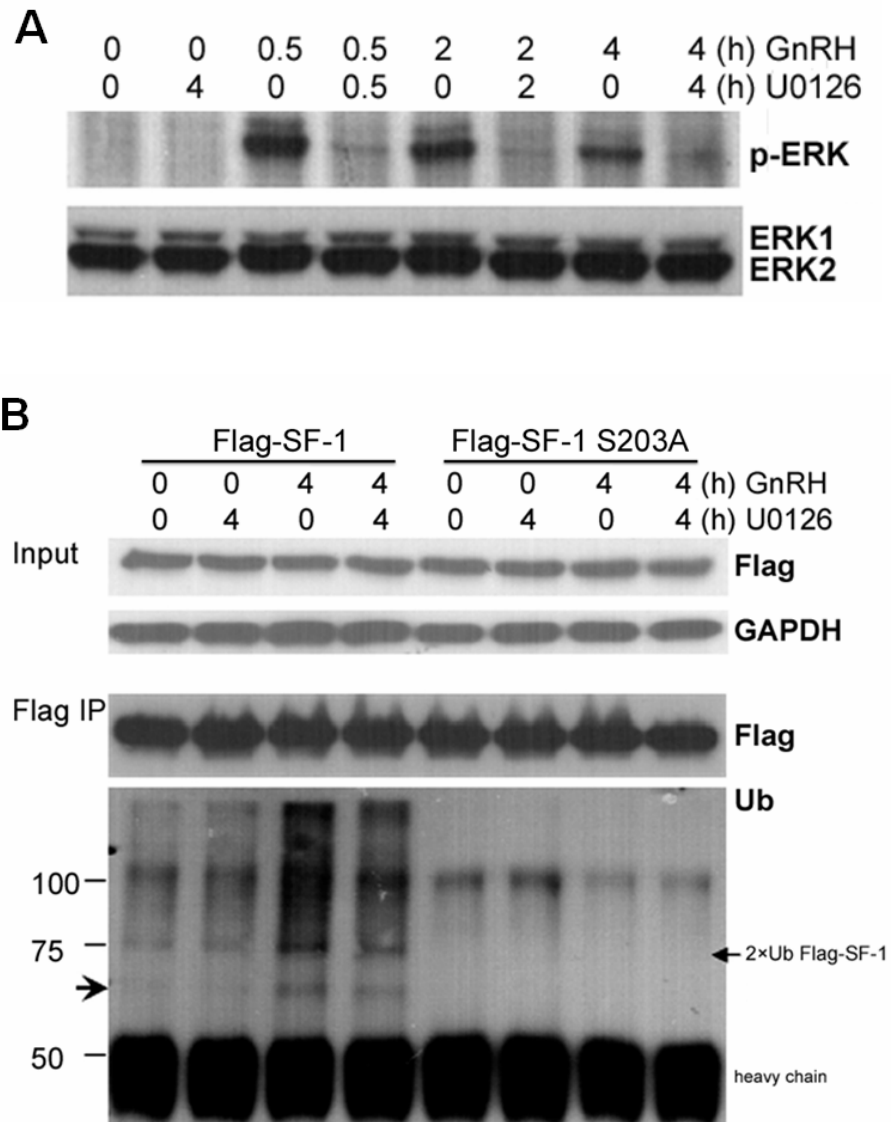
Fig 3.19: U0126 treatment inhibits GnRH-induced SF-1 ubiquitination.

(A) Western blot was carried out to determine the protein levels of phosphorylated ERK (p-ERK) in LβT2 cells after exposure to 100 nM GnRH for 0 to 4 h and/or 1 μM U0126, as indicated; total ERK is shown as a loading control. (B) Cell lysates from LβT2 cells following transfection of Flag-SF-1 or Flag-SF-1 S203A expression vector and exposure to 100 nM GnRH and/or 1 μM U0126 for 0 or 4 h, as marked, were immunoprecipitated using anti-Flag M2 beads; the input and precipitated samples were analyzed by Western blot using rabbit anti-Flag and mouse anti-ubiquitin antibodies, with GAPDH as a loading control. The mono-ubiquitinated SF-1 is marked with arrows on the left.

It has been reported that SF-1 is constitutively phosphorylated under normal cell culture condition without any treatment (Hammer et al., 1999), and CDK7, a component of the general transcription factor IIH (TFIIH), has been shown recently to phosphorylate SF-1 at Ser 203 (Lewis et al., 2008). Therefore, it was hypothesized that SF-1 ubiquitination is also affected by CDK7- mediated phosphorylation of SF-1. Since activation of CDK7 is dependent on its phosphorylation at Thr 170, Western blot was carried out to determine the level of phosphorylated CDK7 in LβT2 cells treated with GnRH for different times. Phosphorylated CDK7 at Thr 170 was detected in unstimulated cells, and the level was not obviously affected by GnRH treatment (Fig 3.20A). Subsequently, *in vivo* ubiquitination assays of WT and S203A SF-1 were performed in the absence or presence of the CDK7 inhibitor, Roscovitine (ROS), with or without GnRH. The treatment with ROS clearly inhibited SF-1 ubiquitination in both untreated and GnRH treated cells (Fig 3.20B).
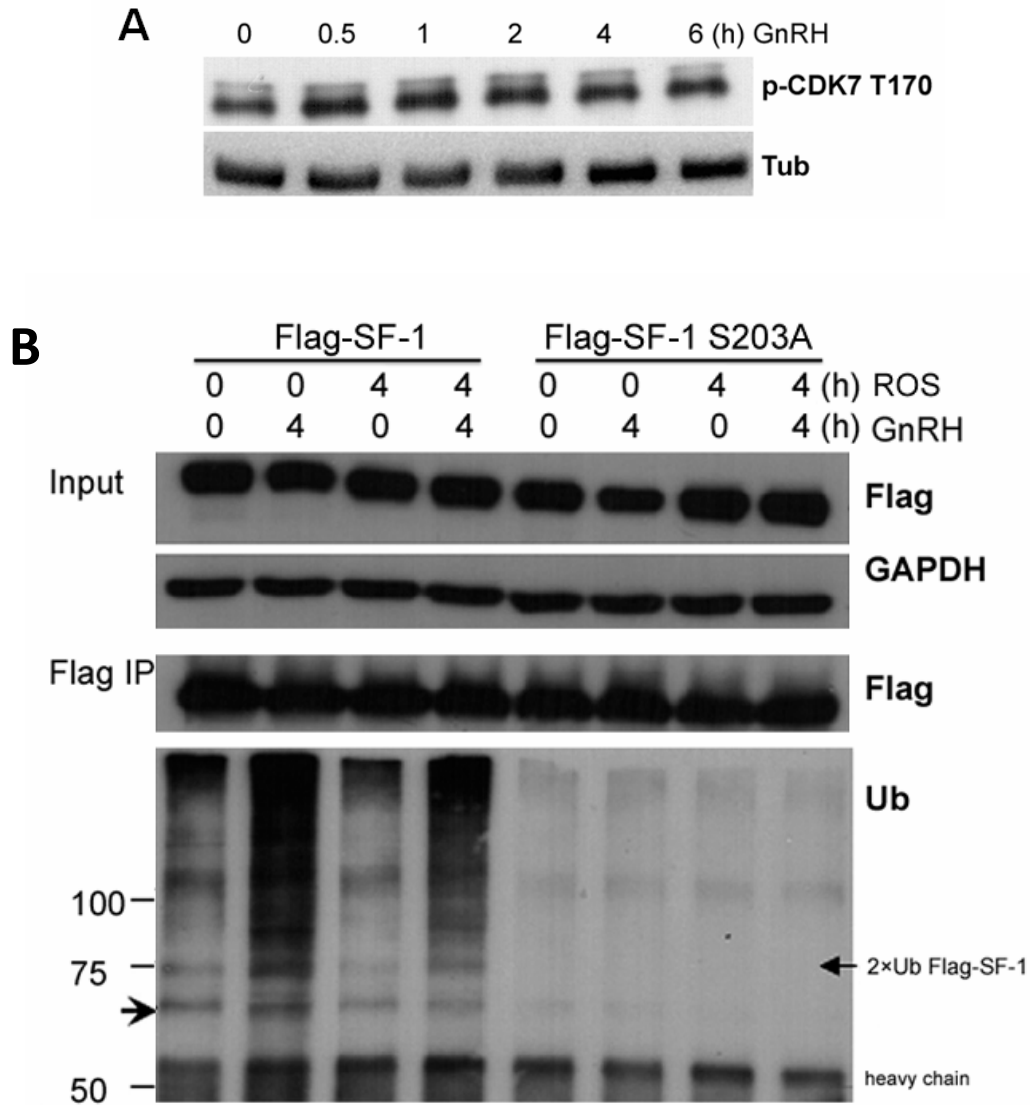
Fig 3.20: Roscovitine (ROS) treatment inhibits SF-1 ubiquitination.

(A) Western blot was carried out to determine the protein levels of phosphorylated CDK7 on Thr 170 (p-CDK7 T170) in LβT2 cells after exposure to 100 nM GnRH for 0 to 6 h, as indicated; α-tubulin (Tub) is shown as a loading control. (B) Cell lysates from LβT2 cells following transfection of Flag-SF-1 or Flag-SF-1 S203A expression vector and exposure to 100 nM GnRH and/or 15 μM ROS for 0 or 4 h, as marked, were immunoprecipitated using anti-Flag M2 beads; the input and precipitated samples were analyzed by Western blot using rabbit anti-Flag and mouse anti-ubiquitin antibodies, with GAPDH as a loading control. The mono-ubiquitinated SF-1 is marked with arrows on the left.

To exclude the possible indirect effects caused by ROS-induced delay in cell cycle progression, HA-tagged WT or dominant negative (DN) CDK7 were over-expressed to confirm the specificity of the CDK7 effect on SF-1 ubiquitination. Over-expression of the WT protein clearly increased levels of SF-1 ubiquitination, while the DN form failed to do so and caused a minor decrease compared to levels in cells transfected only with Flag-tagged SF-1 expression vector (Fig 3.21).

Taken together, these results demonstrate that ubiquitination of SF-1 is Ser 203 phosphorylation dependent, and that GnRH treatment increases SF-1 ubiquitination at least partially via the activation of ERK1/2, although in unstimulated conditions CDK7 also plays a role.
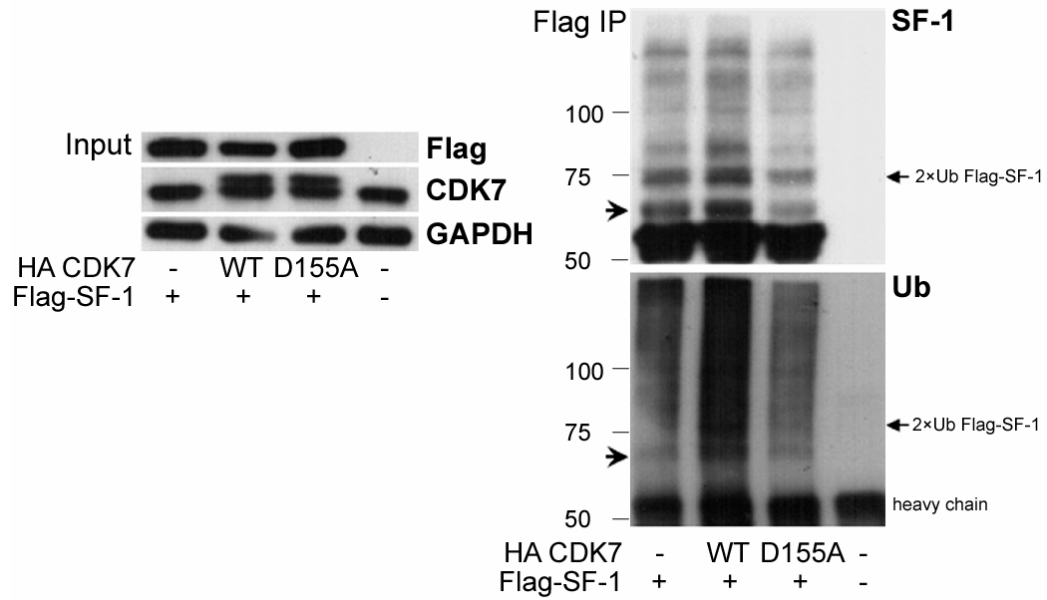
Fig 3.21: Dominant negative mutant CDK7 inhibits SF-1 ubiquitination.

Cell lysates from LβT2 cells following transfection of Flag-SF-1 alone or together with wild type HA-CDK7 or dominant negative HA-CDK7 D155A expression vector, as marked, were immunoprecipitated using anti-Flag M2 beads; the input and precipitated samples were analyzed by Western blot using anti-CDK7, anti-Flag, rabbit anti-SF-1 and mouse anti-ubiquitin antibodies, with GAPDH as a loading control. The mono-ubiquitinated SF-1 is marked with arrows on the left.

3.8 SF-1 can be ubiquitinated or SUMOylated at Lys 119

Considering that SF-1 is SUMOylated at both Lys 119 and Lys 194, and that a similar conjugation mechanism is shared by SUMOylation and ubiquitination, *in vivo* ubiquitination assays were performed to examine whether SF-1 is ubiquitinated at Lys 119 and/or Lys 194. The ubiquitination of WT HA-SF-1, and a series of mutants: HA-SF-1 S203A, HA-SF-1 K119R, HA-SF-1 K194R, and HA-SF-1 K119R & K194R (2KR) were examined in LβT2 cells. The *in vivo* ubiquitination assay showed that both WT HA-SF-1 and HA-SF-1 K194R are efficiently poly-ubiquitinated in LβT2 cells, while mutation of Lys 119 to Arg or mutation of Ser 203 to Ala prevented SF-1 from being poly-ubiquitinated (Fig 3.22). Thus, the ubiquitination site in SF-1 is Lys 119.
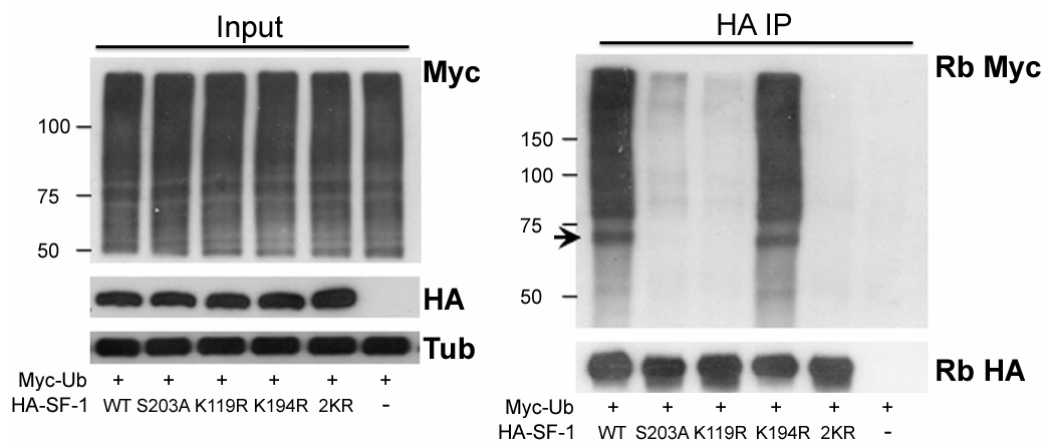
Fig 3.22: SF-1 is ubiquitinated at Lys 119.

Cell lysates from LβT2 cells following transfection of Myc-tagged ubiquitin and WT HA-SF-1, HA-SF-1 S203A, K119R, K194R, 2KR (K119R & K194R), or pxj40-HA empty vector, as marked, were immunoprecipitated using mouse anti-HA Ab; the input and precipitated samples were analyzed by Western blot using rabbit or mouse anti-Myc and anti-HA antibodies, with α-tubulin (Tub) as a loading control. The mono-ubiquitinated SF-1 is marked with arrows on the left.

Since it has been shown that ubiquitination of SF-1 is dependent on phosphorylation

of SF-1 at Ser 203, and that both ubiquitin and SUMO target the same lysine residue

in SF-1, it was speculated that SUMOylation of SF-1 might be also affected by its

phosphorylation. Using an *in vivo* SUMOylation assay in which wild type HA-SF-1

and the same SF-1 mutant constructs used above were transfected, it was found that

SUMOylation of SF-1 was not affected by mutation of Ser 203 to Ala (Fig 3.23).

However, SUMOylation at Lys 119 does not occur when Lys 194 is mutated to Arg

(Fig 3.23), indicating that SUMOylation at Lys 194 is a prerequisite of Lys 119

SUMOylation.
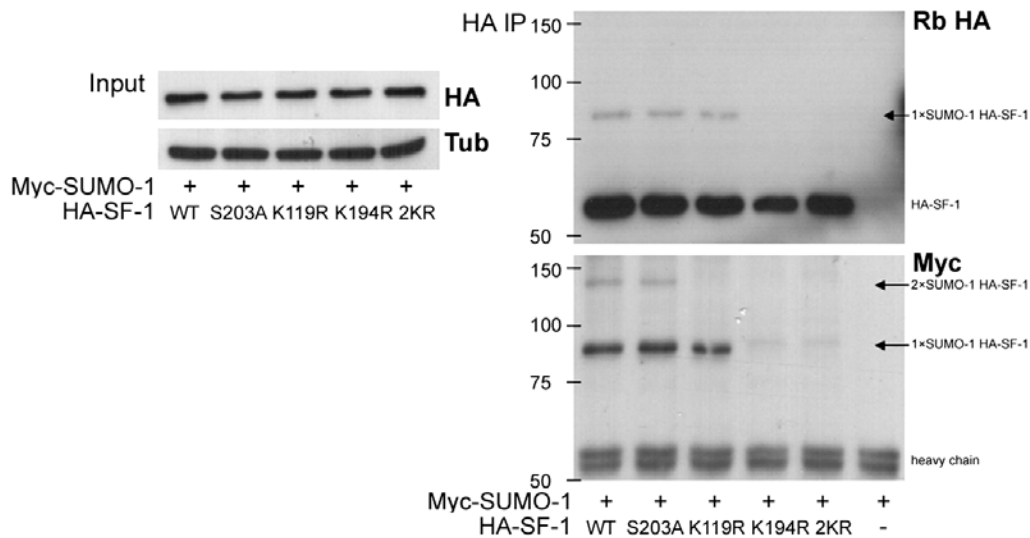
Fig 3.23: SF-1 is SUMOylated at Lys 119 and Lys 194.

LβT2 cells were transfected with Myc-tagged SUMO-1 and WT HA-SF-1, HA-SF-1 S203A, K119R, K194R, 2KR (K119R & K194R), or pxj40-HA empty vector, as marked. After 24 h, the cells were lysed in NEM-RIPA buffer and lysates were immunoprecipitated with mouse anti-HA antibody; the input and precipitated samples were analyzed by Western blot using anti-Myc and rabbit or mouse anti-HA antibodies, with α-tubulin (Tub) as a loading control. It should be noted that the SUMOylated proteins are expected to run more slowly than predicted by their size, and the labelled proteins correspond to the previously reported mobility of SUMOylated SF-1 (Yang et al., 2009).

Together, these results show that both ubiquitination and SUMOylation of SF-1 can occur at the same site, Lys 119, and that this ubiquitination is Ser 203 phosphorylation dependent while SUMOylation of Lys 119 is not, but requires Lys 194 SUMOylation.

3.9 Poly-ubiquitin chains on SF-1 are assembled through either Lys 48 or Lys 63 of ubiquitin

As reviewed in Section 1.6.1, poly-ubiquitin chains linked through Lys 48 in ubiquitin reportedly target a protein for proteasomal degradation, while attachment through Lys 63 is thought to indicate non-proteolytic functions including transcriptional regulation and immune response (Pickart and Fushman, 2004; Sun and Chen, 2004). Therefore, *in vivo* ubiquitination assays were carried out to examine whether Lys 48 or Lys 63 in ubiquitin is involved in the formation of poly-ubiquitin chains on SF-1. Lysate of LβT2 cells transfected with Flag-SF-1 and HA-Ub K48R or HA-Ub K63R were precipitated by Flag beads, and the precipitates were subject to Western analysis by HA antibody. The results revealed that mutation of either residue failed to prevent SF-1 ubiquitination, however mutation of Lys 63 reduced poly-ubiquitin chain formation on SF-1 more than mutation of Lys 48 (Fig 3.24). This demonstrates that SF-1 can be ubiquitinated via Lys 48- and Lys 63-linked poly-ubiquitin chains, and indicates the possibility that this modification is likely involved in several mechanisms of moderating SF-1 function, not all of which are proteosome dependent.
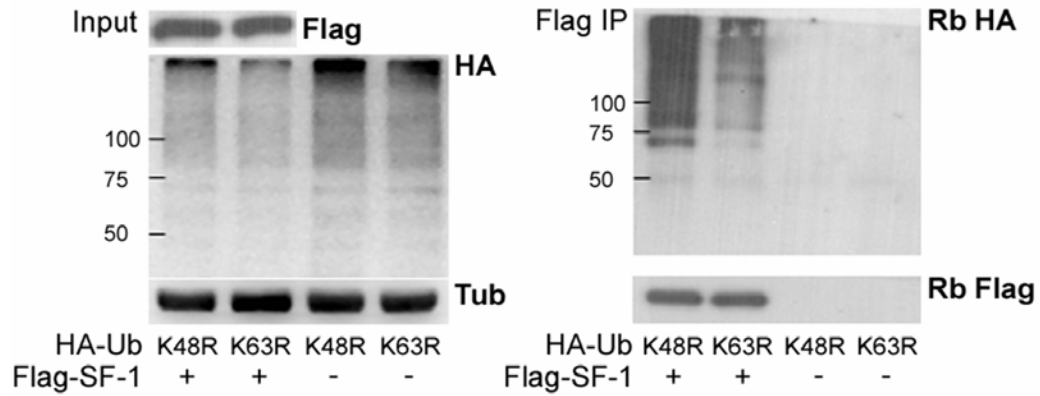
Fig 3.24: SF-1 is ubiquitinated via Lys 48- and Lys 63-linked poly-ubiquitin chains.

Cell lysates from LβT2 cells following transfection of HA-Ub K48R or HA-Ub K63R alone or together with Flag-SF-1 expression vector, as marked, were immunoprecipitated using anti-Flag M2 beads; the input and precipitated samples were analyzed by Western blot using rabbit or mouse anti-Flag and anti-HA antibodies, with α-tubulin (Tub) as a loading control.

3.10 SF-1 ubiquitination, but not SUMOylation, facilitates its interaction with Pitx1

Previous studies by others have already shown that SUMOylation of SF-1 suppresses transcription activity of SF-1 by inhibiting the interaction between SF-1 and cofactors, and that phosphorylation of SF-1 at Ser 203 is critical for the recruitment of cofactors (Hammer et al., 1999; Campbell et al., 2008; Yang et al., 2009). Since ubiquitination and SUMOylation of SF-1 can occur at the same site, Lys 119, and poly-ubiquitin chains targeting on SF-1 can be linked in ubiquitin through either Lys 48 or Lys 63, next it was asked whether the Ser 203 phosphorylation-dependent ubiquitination of SF-1 promotes gonadotropin β subunit gene transcription by increasing SF-1–Pitx1 interactions, in a way that SUMOylation does not, and whether this is proteosome-dependent. Initially, Western analysis was performed using Flag-immunoprecipitated samples from LβT2 cell lysates after transfection with Flag-SF-1 and HA-Pitx1 (Fig 3.25A), or on the endogenous proteins (Fig 3.25B), in the absence or presence of MG132 for 6 h. In both cases, treatment with the proteasome inhibitor, MG132, increased the binding between SF-1 and Pitx1 (Fig 3.25A and B). Both SF-1 and Pitx1 are poly-ubiquitinated in LβT2 cells and it was found that treatment of cells with MG132 substantially increased the levels of both poly-ubiquitinated proteins (Fig 3.25C). Therefore, to exclude the possibility that their enhanced binding results from an effect of MG132 on Pitx1, LβT2 cells were transfected with Flag-SF-1 and Myc-Ub, or Flag-SF-1 and pxj40-Myc empty vector. The Flag-SF-1 and associated proteins were precipitated and incubated with LβT2 cell

lysates containing HA-Pitx1. Western blot showed that Myc-Ub over-expression led to an increase in Flag-SF-1 poly-ubiquitination, while the Flag-SF-1-Ub-Myc bound more HA-Pitx1 than did the Flag-SF-1 when transfected alone (Fig 3.25D). Similarly, Myc-SUMO-1 was transfected with Flag-SF-1, which increased levels of SUMOylated Flag-SF-1, but led to a decrease in the interaction between Flag-SF-1 and HA-Pitx1 (Fig 3.25D). Together, these results suggest that ubiquitination of SF-1 increases, and SUMOylation decreases, the binding between SF-1 and Pitx1, and that the transcriptional activity of SF-1 results from the balance between these two modifications that compete for the same Lys 119.
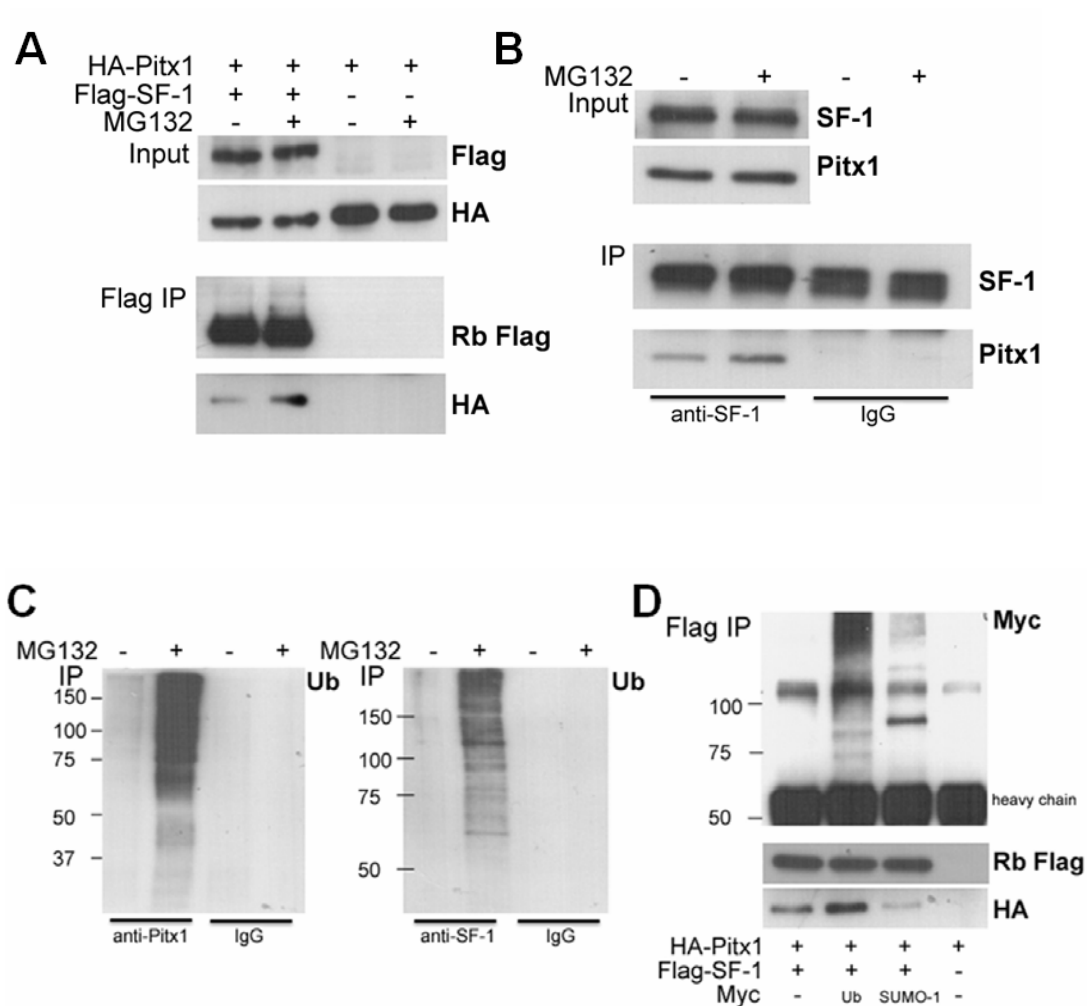
Fig 3.25: SF-1 ubiquitination facilitates its interaction with Pitx1.

(A) Cell lysates from LβT2 cells following transfection of HA-Pitx1 alone or together with Flag-SF-1 expression vectors, and exposure to 5 µM MG132 for 0 or 6 h, as marked, were immunoprecipitated using anti-Flag M2 beads; the input and precipitated samples were analyzed by Western blot using rabbit or mouse anti-Flag and anti-HA antibodies. (B) Cell lysates from LβT2 cells following exposure to 5 µM MG132 for 0 or 6 h, as marked, were immunoprecipitated with anti-SF-1 antibody or normal IgG; the input and precipitated samples were analyzed by Western blot using anti-SF-1 and anti-Pitx1 antibodies. (C) Cell lysates from LβT2 cells following exposure to 5 µM MG132 for 0 or 6 h, as marked, were immunoprecipitated with anti-SF-1 antibody, anti-Pitx1 antibody, or normal IgG; the precipitated samples were analyzed by Western blot using anti-Ub antibody. (D) LβT2 cells were co-transfected with Flag-SF-1or pxj40-Flag empty vector and Myc-Ub, Myc-SUMO-1 or pxj40-Myc empty vector, as indicated. After 24 h, the cells were lysed in NEM-RIPA buffer and lysates were immunoprecipitated with anti-Flag M2 beads. After extensive washes, the Flag beads were incubated with HA-Pitx1-containing cell lysates for 3 h. The precipitated samples were analyzed by Western blot using rabbit anti-Flag, anti-Myc and anti-HA antibodies.

3.11 Ubiquitination of SF-1 leads to its nuclear export

Finally, in order to test whether the GnRH-induced ubiquitination of SF-1 causes a change in its localization, the localization of GFP-SF-1 and GFP-SF-1 K119R was examined in LβT2 cells treated with GnRH and/or MG132. In non-treated cells, GFP-SF-1 was exclusively localized in the nucleus; however, exposure to GnRH and/or MG132 resulted in both nuclear and cytoplasmic distribution of GFP-SF-1. GFP-SF-1 K119R was localized only in the nucleus, even after GnRH and/or MG132 treatment (Fig 3.26). Therefore, it is speculated that nuclear export of SF-1 by GnRH is followed by either proteasome degradation or deubiquitination in the cytosol.
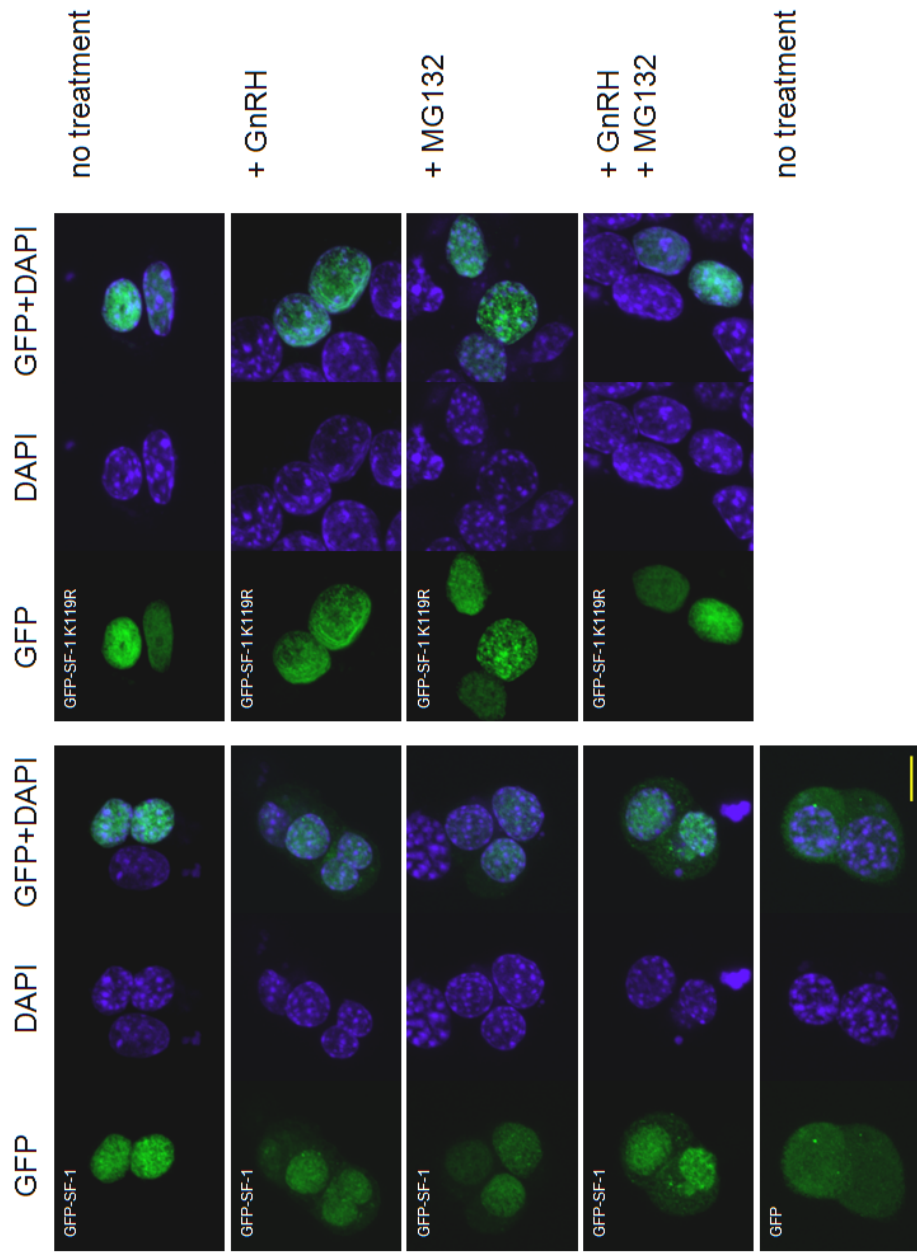
Fig 3.26: Ubiquitination of SF-1 induces its nuclear export.

LβT2 cells were transfected with pxj40-GFP, pxj40-GFP SF-1, or pxj40-GFP SF-1 K119R, and some were exposed to 5 µM MG132 and/or 100 nM GnRH for 0 or 6 h. The cells were fixed, stained with DAPI, and analyzed by LSM510 confocal fluorescence microscopy. Bar, 5 µm.

3.12 Pin1 targets SF-1 to increase its interaction with Pitx1

It has been demonstrated that ubiquitination of SF-1 increases the binding between SF-1 and Pitx1, and that Pin1 upregulates this ubiquitination, indicating that Pin1 likely increases interaction of the two transcription factors. To test this possibility, MEF WT and Pin1 –/– cells were transfected with Flag-SF-1 and HA-Pitx1 or HA-Egr-1 before carrying out co-immunoprecipitations using the cell lysates. Western blot showed that the interaction between SF-1 and Pitx1 was markedly reduced in MEF Pin1 –/– cells compared to that in MEF WT cells (Fig 3.27A). This contrasted with the interaction of SF-1 with Egr-1 which did not differ between the two cell lines (Fig 3.27B), while the interaction of Pitx-1 with Egr-1 was only marginally reduced in the Pin1 knockout cells (Fig 3.27C).
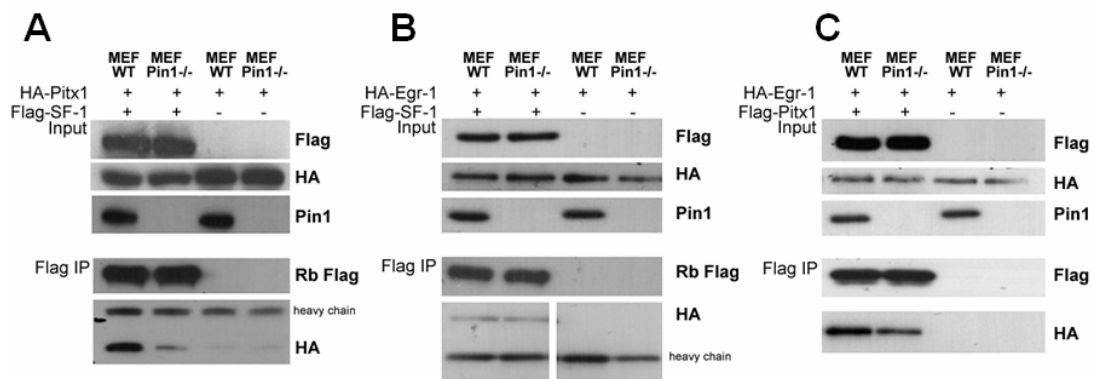
Fig 3.27: Interaction between SF-1 and Pitx1 decreases in the absence of Pin1.

(A) Cell lysates from MEF WT and MEF Pin1 –/– cells following transfection of Flag-SF-1 and HA-Pitx1 were immunoprecipitated using anti-Flag M2 beads; the input and precipitated samples were analyzed by Western blot using rabbit or mouse anti-Flag, anti-HA, and anti-Pin1 antibodies. (B) Cell lysates from MEF WT and MEF Pin1 –/– cells following transfection of Flag-SF-1 and HA-Egr-1 were immunoprecipitated using anti-Flag M2 beads; the input and precipitated samples were analyzed by Western blot using rabbit or mouse anti-Flag, anti-HA, and anti-Pin1 antibodies. (C) Cell lysates from MEF WT and MEF Pin1 –/– cells following transfection of Flag-Pitx1 and HA-Egr-1 were immunoprecipitated using anti-Flag M2 beads; the input and precipitated samples were analyzed by Western blot using anti-Flag, anti-HA, and anti-Pin1 antibodies.

In order to confirm that the decreased interaction between SF-1 and Pitx1, or Pitx1 and Egr-1 in MEF Pin1 –/– cells is the direct effect of deficiency of Pin1 in these cells, MEF Pin1 –/– cells, which were reconstituted with wild type Pin1, Pin1 W34A, or Pin1 K63A mutant, were transfected with Flag-SF-1 and HA-Pitx1, or Flag-Pitx1 and HA-Egr-1, followed by co-immunoprecipitations using the cell lysates. Western blot showed that the reduced interaction of Pitx1 with SF-1 or Egr-1 was rescued by exogenous Pin1, but not to the same degree by the WW or PPIase mutants (Fig 3.28A and B).
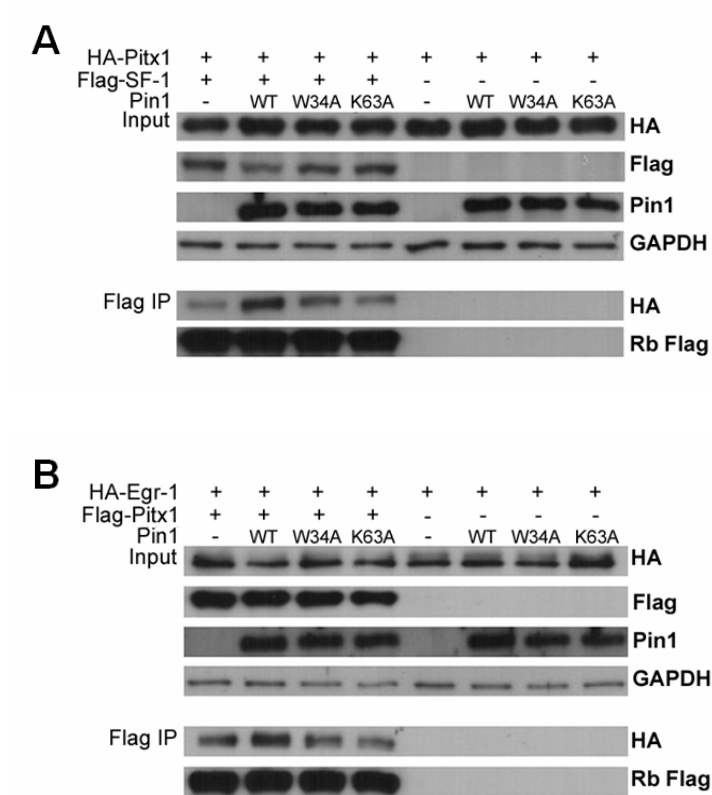
Fig 3.28: Exogenous Pin1 rescues the interaction between SF-1 and Pitx1 in MEF Pin1 –/– cells.

(A) Cell lysates from MEF Pin1 –/– cells following transfection of Flag-SF-1 and HA-Pitx1 or pxj40-HA empty vector, together with pCS2+ empty vector, pCS2+ Pin1, pCS2+ Pin1 W34A, or pCS2+ Pin1 K63A, as marked, were immunoprecipitated using anti-Flag M2 beads; the input and precipitated samples were analyzed by Western blot using rabbit or mouse anti-Flag, anti-HA, and anti-Pin1 antibodies, with GAPDH as a loading control. (B) Cell lysates from MEF Pin1 –/– cells following transfection of Flag-Pitx1 and HA-Egr-1 or pxj40-HA empty vector, together with pCS2+ empty vector, pCS2+ Pin1, pCS2+ Pin1 W34A, or pCS2+ Pin1 K63A, as marked, were immunoprecipitated using anti-Flag M2 beads; the input and precipitated samples were analyzed by Western blot using rabbit or mouse anti-Flag, anti-HA, and anti-Pin1 antibodies, with GAPDH as a loading control.

In order to examine which factor targeted by Pin1 is responsible for the increase in Pitx-1-SF-1 interaction, co-immunoprecipitations and Western blot were performed to examine the binding between Flag-SF-1 and wild type HA-Pitx1 or mutant HA-Pitx1, in which various Pin1 binding sites were mutated. Although these residues were shown to bind Pin1 (Fig 3.11C), mutation in Pitx1 of Ser 207, Ser 259, or Thr 267 and Ser 270 did not lead to significant alteration of its interaction with SF-1 (Fig 3.29A). However, SF-1 mutation of Ser 203 to Ala markedly reduced the interaction with Pitx1 (Fig 3.29B). These results indicate that Pin1 increases the interaction of SF-1 with Pitx1 through targeting SF-1.
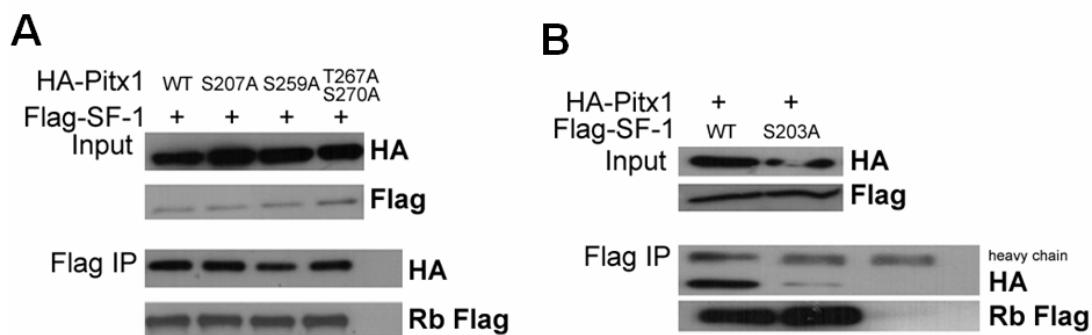
Fig 3.29: Mutation of Pin1 binding site in SF-1 reduces its interaction with Pitx1.

(A) Cell lysates from LβT2 cells following co-transfection of Flag-SF-1 and pxj40-HA empty vector, HA-Pitx1 WT or mutant constructs were immunoprecipitated using anti-Flag M2 beads; the input and precipitated samples were analyzed by Western blot using rabbit or mouse anti-Flag and anti-HA antibodies. (B) Cell lysates from LβT2 cells following co-transfection of HA-Pitx1 and Flag-SF-1 or Flag-SF-1 S203A were immunoprecipitated using anti-Flag M2 beads; the input and precipitated samples were analyzed by Western blot using rabbit or mouse anti-Flag and anti-HA antibodies.

3.13 Pin1 interacts with ATF3

While searching for gonadotropin gene-specific transcription factors regulated by Pin1, co-immunoprecipitation assays revealed that Pin1 can also bind to ATF3 (Fig 3.30A and B), which was shown previously to mediate activation of human αGSU promoter by GnRH (Xie et al., 2005). In order to confirm the interaction, mammalian two-hybrid assays were performed in COS-1 cells using pM Pin1 and pVP ATF3, together with Gal4-responsive luciferase reporter gene and pRL-SV40 Renilla as internal control. Co-transfection of both pM Pin1, but not the empty pM vector, with pVP ATF3, resulted in significant induction of the luciferase reporter gene activity (Figs 3.30 C). Taken together, these results clearly indicate that Pin1 interacts with ATF3.
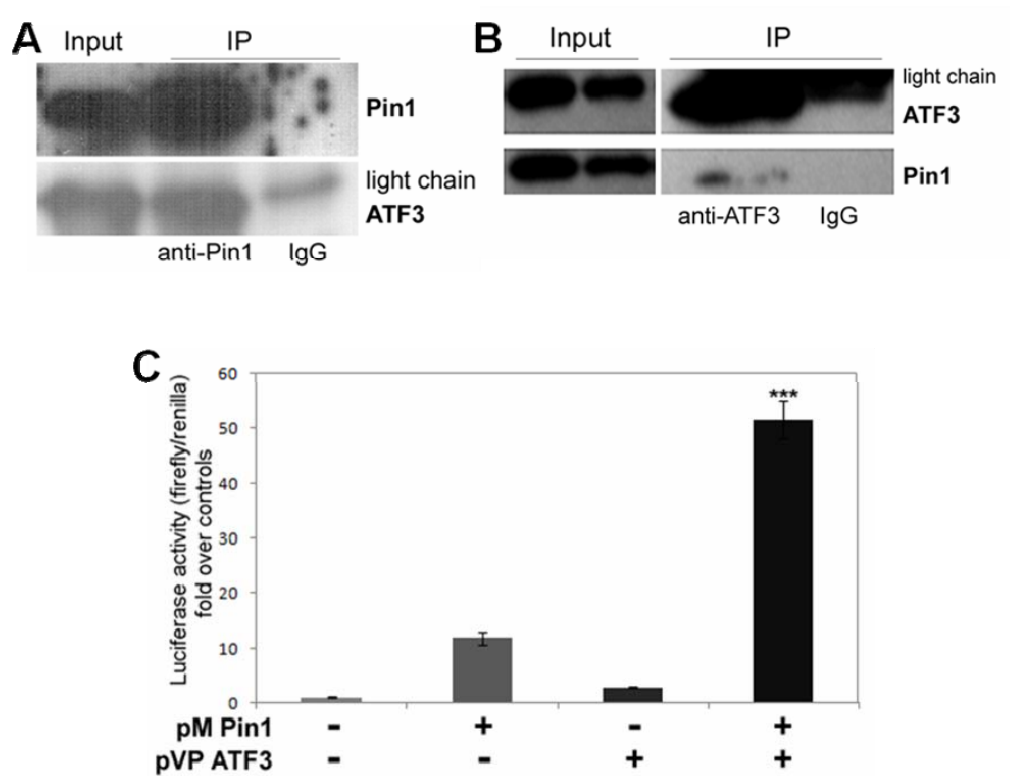
Fig 3.30: Interaction between Pin1 and ATF3.

(A) Cell lysates from LβT2 cells were immunoprecipitated using antibody to Pin1 or using normal IgG as control; Western blot was carried out to determine levels of ATF3 and Pin1 in the input and precipitated samples. (B) Cell lysates from COS-1 cells co-transfected with Pin1 and ATF3 expression vectors were immunoprecipitated using antibody to ATF3 or using normal IgG as control; Western blot was carried out to determine levels of ATF3 and Pin1 in the input and precipitated samples. (C) Mammalian two-hybrid assays were carried out in COS-1 cells using a Gal4-responsive reporter gene, and a Renilla luciferase as internal control, together with a Gal4 DBD empty vector (pM) or Gal4 DBD-Pin1 fusion construct (pM Pin1) and VP16 AD empty vector (pVP) or VP16 AD fused to ATF3 (pVP ATF3). Luciferase assays were carried out and the levels of firefly luciferase normalized to those of Renilla; results are expressed as the normalized levels (n-fold) over those in control cells, in which unfused Gal4 DBD and VP16 AD constructs were transfected together. Statistical analysis (t-test) to assess interaction, compared means for the groups transfected with both fusion constructs, with the combined effects of each fusion construct alone (*** $P<0.001$). Results are shown as mean ± SEM; n=6.

3.14 Pin1 increases the stability of ATF3

Since Pin1 interacts with ATF3, Western blot was carried out to examine whether Pin1 over-expression affects the level of ATF3 in gonadotropes. ATF3 protein level was seen to increase after over-expressing Pin1 (Fig 3.31A); however, its mRNA level was not obviously affected by Pin1 (Fig 3.31B). Therefore, it was speculated that Pin1 might be able to stabilize the ATF3 protein. In order to test this hypothesize, the half life of HA-tagged ATF3 in both MEF WT and Pin1 –/– cells was compared by exposing those cells to the protein synthesis inhibitor cycloheximide for various times. HA-ATF3 in Pin1 deficient MEF cells was clearly less stable than in MEF WT cells; after 2 h cycloheximide treatment, the HA-ATF3 level was significantly reduced in the absence of Pin1, while only a slight reduction was observed in MEF WT cells (Fig 3.31C). This suggests that Pin1 increases the stability of ATF3 protein.
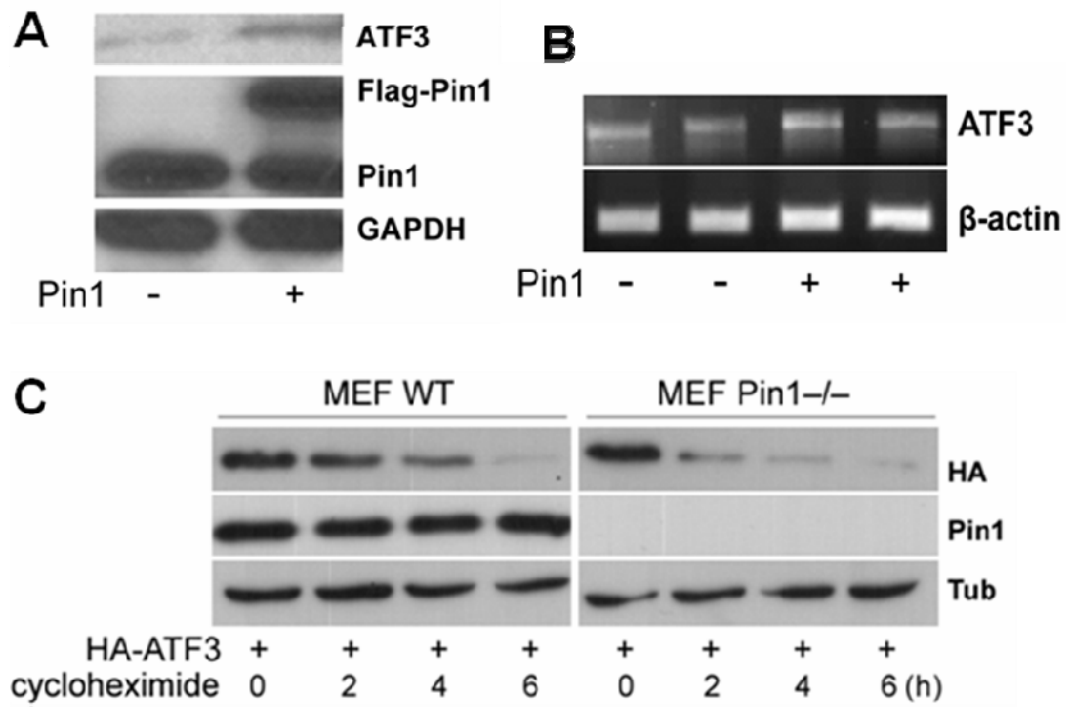
Fig 3.31: Pin1 stabilizes ATF3.

(A) Western blot was carried out to determine the protein levels of ATF3 and Pin1 in LβT2 cells transfected with pCS2+ empty vector or pCS2+ Pin1 expression vector; GAPDH is shown as a loading control. (B) RT-PCR analysis of ATF3 mRNA levels was carried out in LβT2 cells transfected with pCS2+ empty vector or pCS2+ Pin1 expression vector; β-actin was used as an internal control. All transfections were carried out in duplicate. (C) Western blot was carried out to determine the protein levels of HA-ATF3 and Pin1 in MEF WT or Pin1 –/– cells transfected with HA-ATF3 expression vector after exposure to 100 μg/mL cycloheximide for 0-6 h, as indicated; α-tubulin (Tub) is shown as a loading control.

3.15 GnRH stimulates ATF3 transcription

Previous reports by others have shown that GnRH induces ATF3 expression in the immature gonadotrope αT3-1 cells (Xie et al., 2005; Mayer et al., 2008). The upregulation of ATF3 by GnRH was also observed in the mature gonadotrope LβT2 cells (Fig 3.32A, B and C). As in αT3-1 cells, ATF3 mRNA and protein were easily detected in LβT2 cells after 30 min GnRH treatment and both peaked at 1 h (Fig 3.32B and C). The increase in ATF3 mRNA diminished rapidly after the peak, while its protein had a sustained high level for several hours (Fig 3.32B and C). However, the induction of ATF3 by GnRH in LβT2 cells was significantly higher than that in αT3-1 cells (Fig 3.32A). In order to examine whether GnRH also has a protective effect on ATF3 protein, HA-tagged ATF3, which is not under the control of native ATF3 promoter was transfected into LβT2 cells in order to exclude the effect of GnRH on its gene transcription, and the cells were then treated with GnRH for various times. Western blotting showed that the exogenous ATF3 protein remained virtually unchanged in GnRH-treated LβT2 cells (Fig 3.32D), which indicates that GnRH may not stabilize ATF3 protein. Taken together, these results suggest that the upregulation of ATF3 by GnRH is mainly through its action on ATF3 transcription.
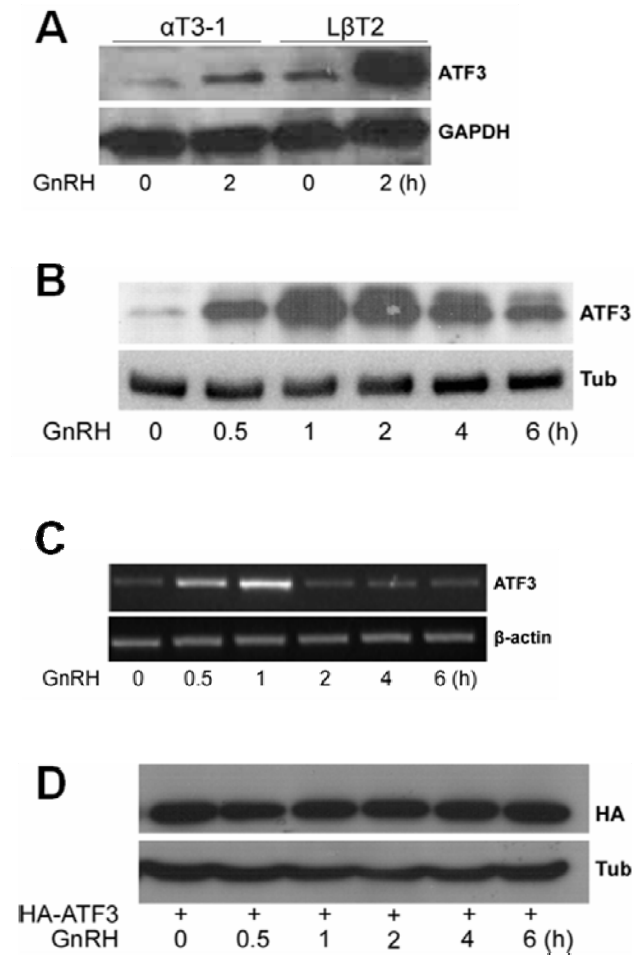
Fig 3.32: GnRH upregulates ATF3 transcriptionally.

(A) Western blot was carried out to determine the protein level of ATF3 in αT3-1 and LβT2 cells following 100 nM GnRH treatment for 0 or 2 h; GAPDH is shown as a loading control. (B) Western blot was carried out to determine the protein level of ATF3 in LβT2 cells following 100 nM GnRH treatment for 0-6 h; α-tubulin (Tub) is shown as a loading control. (C) RT-PCR analysis of ATF3 mRNA levels was carried out in LβT2 cells following 100 nM GnRH treatment for 0-6 h; β-actin was used as an internal control. (D) Western blot was carried out to determine the protein level of HA-ATF3 in LβT2 cells transfected with the same amount of HA-ATF3 expression vector after exposure to 100 nM GnRH for 0-6 h, as indicated; α-tubulin (Tub) is shown as a loading control.

3.16 Pin1, c-Jun and ATF3 form a complex in gonadotropes

As reviewed in Section 1.5.2, ATF3 and c-Jun can form a heterodimer to activate

gene transcription. Moreover, c-Jun itself is also a GnRH-responsive immediate early

gene, and GnRH activates its expression in a trend similar to ATF3 induction in both

the immature and mature gonadotropes (Wurmbach et al., 2001; Fig 3.33A and B).

GnRH treatment was seen to lead to the recruitment of ATF3 and c-Jun to the dual

CRE on the human αGSU promoter (Xie et al., 2005). Since c-Jun is an established

Pin1 binding partner (Wulf et al., 2001) and in this study it has been found that ATF3

also interacts with Pin1, it was speculated that Pin1, c-Jun and ATF3 might form a

tripartite complex in gonadotropes. Co-immunoprecipitation assays showed that both

Pin1 and ATF3 are present in the c-Jun antibody precipitates from untreated or

GnRH-treated LβT2 cell lysates, and that GnRH enhances the interaction between

c-Jun and ATF3, presumably through increasing their protein levels (Fig 3.33C). In

order to test whether the three proteins exist in the same complex,

co-immunoprecipitation using ATF3 antibody was performed and Pin1 and c-Jun

were found in the precipitates (Fig 3.33D). These results indicate that Pin1, ATF3 and

c-Jun could complex together in gonadotropes.
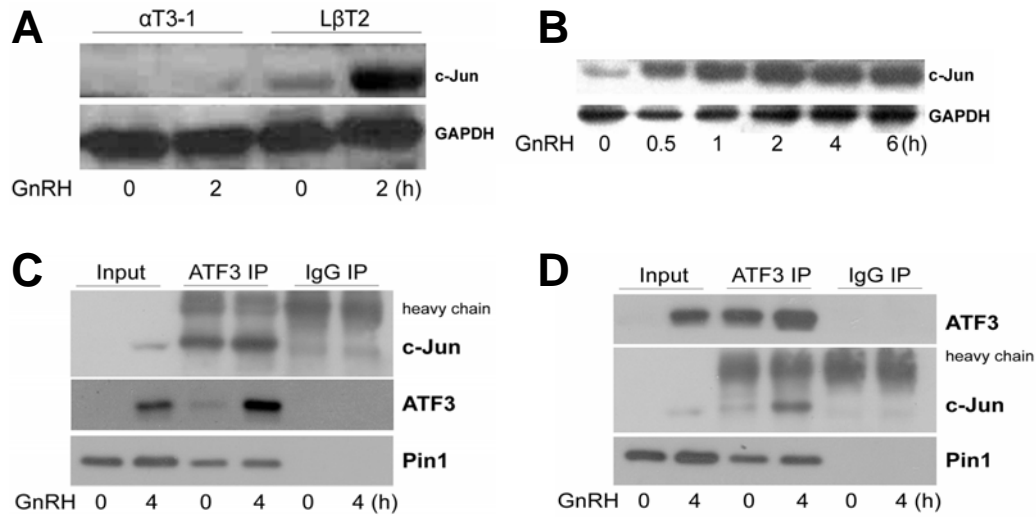
Fig 3.33: Pin1, c-Jun and ATF3 form a complex in gonadotropes.

(A) Western blot was carried out to determine the protein levels of ATF3 and c-Jun in αT3-1 and LβT2 cells following 100 nM GnRH treatment for 0 or 2 h; GAPDH is shown as a loading control. (B) Western blot was carried out to determine the protein level of c-Jun in LβT2 cells following 100 nM GnRH treatment for 0-6 h; α-tubulin (Tub) is shown as a loading control. (C) Cell lysates from LβT2 cells treated with 100 nM GnRH for 0 or 4 h were immunoprecipitated using antibody to c-Jun or using normal IgG as control; Western blot was carried out to determine levels of c-Jun, ATF3 and Pin1 in the input and precipitated samples. (D) Cell lysates from LβT2 cells treated with 100 nM GnRH for 0 or 4 h were immunoprecipitated using antibody to ATF3 or using normal IgG as control; Western blot was carried out to determine levels of c-Jun, ATF3 and Pin1 in the input and precipitated samples.

3.17 ATF3 is SUMOylated

Besides its role in activating ATF3 transcription, GnRH was also able to increase the SUMOylation of ATF3 in LβT2 cells (Fig 3.34A). As reviewed in Section 1.6.2, most proteins are conjugated to SUMOs via lysines within ψKXE (ψ denoting a hydrophobic residue) consensus motifs (Rodriguez et al., 2001; Bernier-Villamor et al., 2002). Therefore, in order to identify the SUMO acceptor sites in ATF3, a series of ATF3 mutants were created in which lysines within the ψKXE motif were mutated to a non-SUMOylatable arginine, K42R, K78R, K107R, K110R, K120R and K136R. The ATF3 wild type and these mutant expression vectors, together with Myc-SUMO-1, were transfected into LβT2 cells and the lysates were immunoprecipitated using HA antibody. Analysis of the precipitates by Western blot showed that the upper band between the 50 and 37 kDa markers disappeared when Lys 78 was mutated to Arg, whereas the K42R mutation eliminated the lower band between 50 kDa and 37 kDa (Fig 3.34B). Thus, the substitutions of Lys to Arg indicate that K42 and K78 are two acceptor sites in ATF3 for SUMO modification. Moreover, it was found that ATF3 was specifically targeted by SUMO-1, while SF-1 can be conjugated to either SUMO-1 or SUMO-2/3 as reported (Fig 3.34C; Komatsu et al., 2004; Yang et al., 2009).
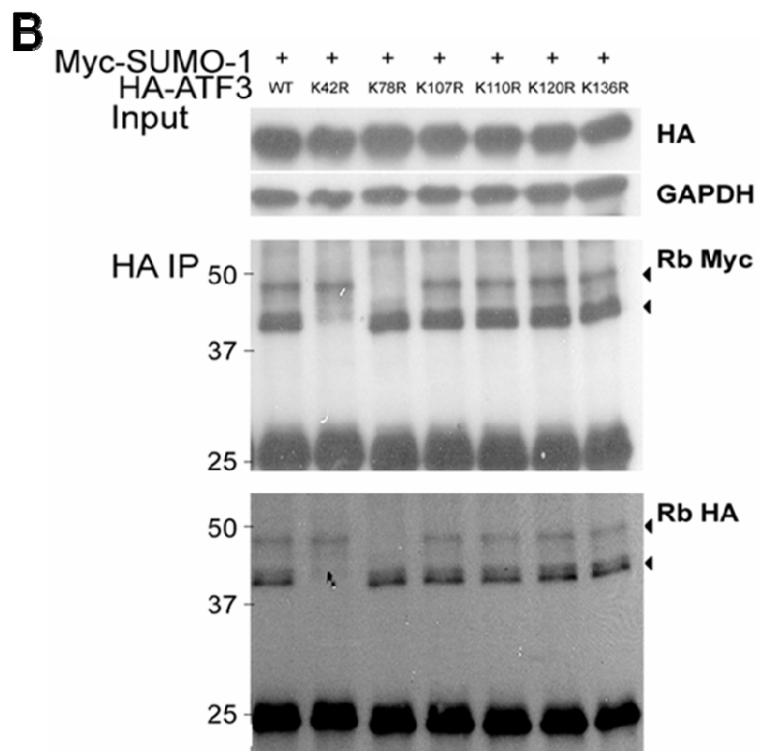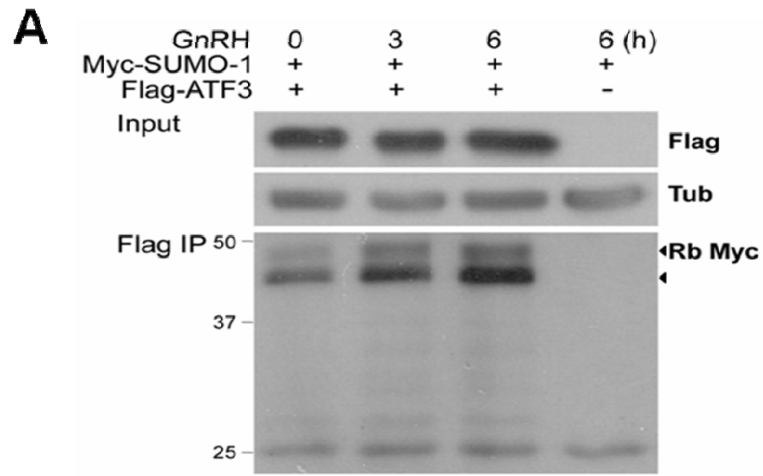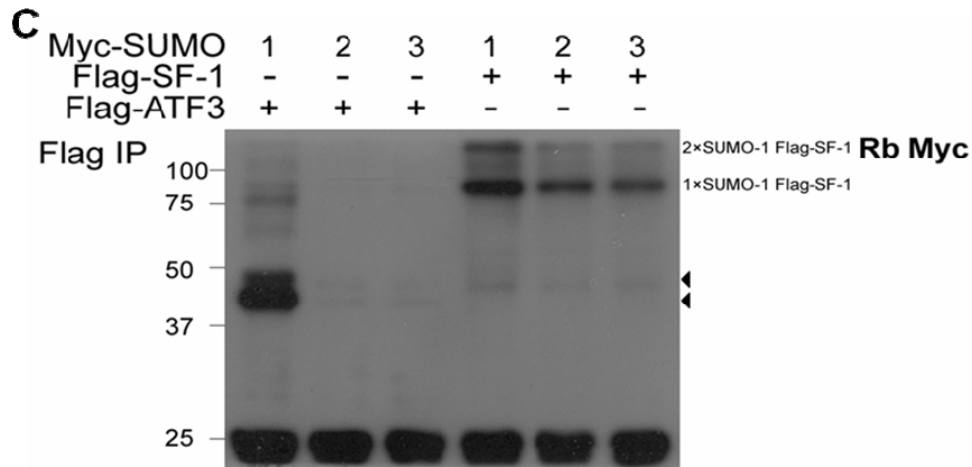
Fig 3.34: ATF3 is SUMOylated in LβT2 cells.

(A) Cell lysates from LβT2 cells following transfection of Myc-SUMO-1 expression vector alone or together with Flag-ATF3 expression vector and exposure to 100 nM GnRH for 0 to 6 h, as marked, were immunoprecipitated using anti-Flag M2 beads; the precipitated samples were analyzed by Western blot using rabbit anti-Myc antibody, and the input samples were analyzed by Western blot using Flag antibody with α-tubulin (Tub) as a loading control. (B) Cell lysates from LβT2 cells following transfection of Myc-SUMO-1 expression vector together with WT HA-ATF3, HA-ATF3 K42R, K78R, K107R, K110R, K120R, or K136R expression vector were immunoprecipitated using mouse anti-HA antibody; the precipitated samples were analyzed by Western blot using rabbit anti-Myc and anti-HA antibodies, and the input samples were analyzed by Western blot using HA antibody with GAPDH as a loading control. (C) Cell lysates from LβT2 cells following transfection of Myc-SUMO-1, Myc-SUMO-2 or Myc-SUMO-3 expression vector together with Flag-ATF3 or Flag SF-1 expression vector, as marked, were immunoprecipitated using anti-Flag M2 beads; the precipitated samples were analyzed by Western blot using rabbit anti-Myc antibody. The SUMOylated forms of ATF3 are marked with triangles on the left. It should be noted that SUMOylation at different lysines can cause slight difference in electrophoresis mobility (Tiefenbach et al., 2006).

CHAPTER 4   DISCUSSION AND CONCLUSIONS

4.1 Reproductive abnormalities in Pin1 knockout mice

In an early report, it was found that male and female Pin1 knockout mice are infertile when mated together, and that both Pin1-deficient males and females are subfertile when mated with wild type mice (Atchison et al., 2003). Further investigation of the reproductive organs has revealed that the number of germ cells in newborn Pin1 knockout mice testes and ovaries is significantly fewer than that in wild type or Pin1 –/+ mice (Atchison et al., 2003). Immunohistochemistry study and 5-bromodeoxyuridine (BrdU) incorporation assay suggested that the severe reduction in gamete number in the absence of Pin1 could result from the prolonged cell cycle and impaired cell proliferation of primordial germ cells (PGCs), which are precursors of gonocytes and oocytes undergoing proliferation and expansion during and after their migration to the gonads from 8.5 to 13.5 days post coitum (dpc) (Atchison et al., 2003). That report highlighted cell proliferation defects in the gonads of Pin1 deficient mice that occur in the very early developmental stage, before the establishment of hypothalamic pituitary gonadal axis. A later study carried out by the same group reported that Pin1 also plays an important role in maintaining spermatogonia in adult testes (Atchison and Means, 2003). Pin1-knockout mice exhibit a gradual depletion of spermatogonia, which leads to spermatocyte and spermatid degeneration, and eventually complete germ cell loss and empty seminiferous tubules by the age of 14 months, although the remaining gonocytes in

140

Pin1 –/– testis were observed to be able to initiate and complete the entire spermatogenic process in 1 week old mice (Atchison and Means, 2003). A study conducted by Liou et al. also revealed a series of very similar phenotypes in Pin –/– male mice, including testicular atrophy, seminiferous tubule degeneration, and complete loss of mature sperm in the lumen by the age of 15-18 month (Liou et al., 2002). These two reports indicate that Pin1 may have mutiple roles in regulating fertility in adults as well as during early stages of development.

Since in many different genetic mouse models a testis degeneration phenotype is correlated with increased germ cell apoptosis, TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assays in 3 and 7 month old wild type and Pin1 –/– mice were carried out by Atchison and Means to detect apoptotic cells. However, the results showed lack of increased germ cell apoptosis in the absence of Pin1 (Atchison and Means, 2003). Given the previous study performed by this group showing the role of Pin1 in regulating PGCs proliferation (Atchison et al., 2003), they speculated that Pin1 could also regulate spermatogonial proliferation in adult testis (Atchison and Means, 2003). In addition, Liou et al have demonstrated that this testis atrophy is not due to the smaller body weight of Pin1 –/– male adults because by 3-5 month of age their average body weight is not significantly less than that of wild type controls, but the average weight of Pin1 –/– testes is only 56% of that of wild type testes (Liou et al., 2002). Therefore, the mechanisms by which these phenotypes occurred in Pin1 –/– testis are less clear. However, given that during the mouse embryo development the expression of gonadotropin genes is initiated after GnRH

delivery to the pituitary, which starts around e16.5, and that the hypothalamic pituitary gonadal axis is active in both males and females during the midtrimester of the fetal period and the early postnatal period (Japon et al., 1994; Grumbach and Styne, 2003), it is reasonable to consider that the mechanism responsible for the adult phenotype involve Pin1 regulation of the gonads through its action on gonadotropins synthesis.

Interestingly, some of the reported Pin1 –/– testis phenotypes are largely mirrored in FSHβ knockout mice, such as the reduced testis size (beginning at 2 weeks of age and continually through the adult age), decreased fertility, decreased seminiferous tubule size (at 8-9 weeks), while germ cells in FSH–/– testis also maintain the ability to complete spermatogenesis (Kumar et al., 1997). It has also been reported that expression of transgenic FSH in gonadotropin-deficient hypogonadal mice stimulates the postnatal cellular development in seminiferous tubules and increases total spermatogonia proliferation (Haywood et al., 2003). Furthermore, reduced testis size was also seen in LHβ knockout male mice (Ma et al., 2004). Since the FSH and LH pathways are complexly interconnected *in vivo* and the two hormones act synergistically in regulating spermatogonia and preventing germ cell degeneration (Russell et al, 1993; Zirkin et al., 1994), it is very possible that some of the effects of Pin1 on testis are exerted through regulating LH and FSH production.

The present study has demonstrated that Pin1 is required for gonadotropin β subunit gene transcription. This finding, together with regulation of PGC proliferation by Pin1,

suggests that Pin1 is diversely involved in different aspects of reproductive control, which is entirely in accordance with the nature of its effect: a commonly used mechanism involved in signal transduction through converting a target into different structure after phosphorylation. Therefore, it is still highly possible that Pin1 could also have additional roles at other points along the reproductive axis.

4.2 Pin1 is involved in gonadotropin synthesis through its action on specific transcription factors

In present study it has been shown that in gonadotropes Pin1 over-expression increases the basal and GnRH-induced LHβ and FSHβ gene transcription, while knock-down of Pin1 by siRNA results in decreased expression of LHβ and FSHβ (Fig 3.1-3.4). Pin1 regulates gonadotropin β subunit gene transcription through targeting the specific transcription factors SF-1, Pitx1 and Egr-1, and modulating their stability or protein–protein interactions. These findings demonstrate for the first time an essential role of Pin1 in gonadotropin production and reveal the molecular mechanisms underlying some of the reproductive abnormalities in Pin1 knockout mice.

The activity of transcription factors is characteristically regulated by various post-translational modifications. One such modification is the *cis/trans* isomerization of phosphorylated Ser/Thr-Pro bonds carried out by Pin1 together with proline-directed serine and threonine kinases (e.g., CDKs or MAPKs), which together

lead to conformational change in target protein and alteration in the transactivation potential of the transcription factor via changing its stability, protein–protein interaction, protein-DNA interaction, or intracellular localization.

The known transcription factors targeted by Pin1 include c-Jun (Wulf et al., 2001), c-Fos (Monje et al., 2005), SRC-3/AIB1 (Yi et al., 2005), c-Myc (Yeh et al., 2004), β-catenin (Ryo et al., 2001), p53 (Zacchi et al., 2002; Zheng et al., 2002; Wulf et al., 2002), p73 (Mantovani et al., 2004), p65/Rel (Ryo et al., 2003) and interferon-regulatory factor 3 (IRF3) (Saitoh et al., 2006). In many cases, the involvement of Pin1 in various biological events is mediated through its actions on specific transcription factors. For example, Pin1 plays an important role in cell growth control and oncogenesis through augmenting transactivation by c-Jun and promoting the translocation of β-catenin from cytoplasm to nucleus to regulate the expression of cyclin D1 (Wulf et al., 2001; Liou et al., 2002; Miyashita et al., 2003; Ryo et al., 2001). Pin1 participates in cell cycle arrest and apoptosis through cooperating with genotoxic stresses to increase stability and nuclear accumulation of p53 and p73, ultimately upregulating their target genes including BCL2-associated X protein (Bax), p53-induced gene 3 (PIG3) and p53-regulated apoptosis-inducing protein 1 (p53AIP1) (Zacchi et al., 2002; Zheng et al.,2002; Mantovani et al., 2004). The regulatory function of Pin1 in innate antiviral response is achieved by altering the conformation of IRF3 so that IRF3 is more easily targeted by the ubiquitination machinery and finally degraded by the proteasome, leading to downregulation of its downstream interferon β (INFβ) (Saitoh et al., 2006). In this study it has been shown that Pin1 also

participates in gonadotropin β subunit gene transcription through its effects on the transcription factors SF-1, Pitx1 and Egr-1.

4.2.1 Pin1 is present on the promoters of both gonadotropin β subunit genes

In this study, ChIP experiments demonstrated that Pin1 is present on the promoters of both gonadotropin β subunit genes (Fig 3.8). Since Pin1 itself is not a DNA binding protein, it is presumably recruited to promoter region through specific transcription factors, such as c-Jun, SF-1, Pitx-1 and/or Egr-1, with which it interacts. Previous studies by others have revealed that, although barely detectable under non-stimulated condition, Pin1 is recruited to p53 binding element in the p21 and BAX promoters after UV or etoposide treatments, which are known to increase the interaction between Pin1 and p53 (Mantovani et al., 2007). However, the present study has shown that Pin1 binds to gonadotropin β subunit gene promoters without GnRH stimulation, which could be due to the fact that some of the specific transcription factors such as SF-1 are already phosphorylated at Ser/Thr-Pro motif under normal culture condition (Hammer et al., 1999). The possible reason that 4 h GnRH-treatment did not obviously increase the association of Pin1 with the DNA could be that the enhancement of Pin1/DNA binding occurs at earlier time points, since the downstream kinases are rapidly activated by GnRH. Alternatively, the increased interaction between Pin1 and the phosphorylated transcription factors after GnRH stimulation could lead to conformational change of the promoters (e.g., forming a loop structure) since Pin1 and these transcription factors already sit on, maybe

145

different regions of, the promoters without GnRH treatment, but the amount of Pin1

on the promoter is not changed.

4.2.2 Effect of Pin1 on the transactivation function of these transcription factors

The present study has shown that Pin1 acts synergistically with SF-1, Pitx1 and Egr-1

to activate downstream gene promoters (Fig 3.12-3.14). These results clearly suggest

that Pin1 is required for these gonadotropin β subunit gene transcription factors to

acquire their fully active conformation. Since the two promoters both contain SF-1

binding sites and Pitx1 bindig sites, it might be expected that Pin1/SF-1 or Pin1/Pitx1

should display positive transcriptional cooperation on both of the promoters. However,

the synergy appears to be promoter-context specific. Pin1 and SF-1 exhibit obvious

synergy on the LHβ promoter, but not the FSHβ promoter, while Pin1 and Pitx1

synergistically activate the FSHβ promoter, but not the LHβ promoter (Fig 3.12-3.14).

As reviewd in Section 1.2.2, SF-1 plays a predominant role in LHβ transcription, but

transcription regulation of FSHβ by SF-1 may be more indirect; while Pitx1 exerts its

effect on LHβ transcription partly through SF-1 (Ingraham et al., 1994; Ikeda et al.,

1995; Keri and Nilson, 1996; Halvorson et al., 1996; Tremblay et al., 1998; Tremblay

et al., 1999). The reason for this promoter-specific synergism could be that the mere

simultaneous recruitment of Pin1 and non-master-regulatory transcription factor may

not be sufficient for effective synergistic activation. In addition, overall topology

differences between the two promoters regarding the close proximity of SF-1 or Pitx1

binding elements to the transcription start site or other *cis*-acting elements, and

interplay with other protein cofactors could also influence the level of cooperativity on specific promoters.

4.2.3 Pin1 promotes ubiquitination of SF-1, which is required for SF-1 cofactor recruitment

Most strikingly, it was found in the present study that the SF-1 protein level is clearly reduced after Pin1 over-expression, which contrasts with the opposite effect on Pitx1 (Fig 3.15), even though SF-1 is functionally activated by Pin1 on the LHβ gene promoter (Fig 3.12). Previous studies by two groups have indicated that SF-1 is poly-ubiquitinated (Walsh and Shupnik, 2009; Chen et al., 2007) and in this study it has been further shown that SF-1 ubiquitination is dependent on its phosphorylation of Ser 203 and Pin1. Furthermore, ubiquitination of SF-1 facilitates the interaction between SF-1 and its cofactor Pitx1 (Fig 3.25). These findings not only uncover evidence for the existence of post-phosphorylation events on SF-1, but also reveal a novel mechanism by which post-translational modifications modulate transcriptional activity of SF-1.

The Ser 203 which is recognized and bound by Pin1, locates in the hinge region of SF-1, adjacent to helix 1 (Fig 1.7), and its phosphorylation has been shown previously to induce major conformational change in the protein, as indicated by change in protease sensitivity (Desclozeaux et al., 2002). That study also indicated that the Ser 203 phosphorylation enhances the activity of this hinge/helix 1 region and cofactor

recruitment, likely as a consequence of the change in conformation (Desclozeaux et al., 2002). It is conceivable that this conformational change is the Pin1-induced isomerization which increases the accessibility of SF-1 to the ubiquitin-conjugation machinery, leading to its interaction with Pitx1 and ultimately to proteasome mediated degradation.

The finding that GnRH increases SF-1 ubiquitination is consistent with a recent report by Walsh and Shupnik (2009). Suppression of GnRH-induced SF-1 ubiquitination by the ERK-specific inhibitor, U0126, suggests that the signal could stem from activation of ERK2, which has been shown to phosphorylate SF-1 both *in vivo* and *in vitro* (Fowkes et al., 2003; Hammer et al., 1999; Desclozeaux et al, 2002). However, SF-1 is also reportedly phosphorylated by CDK7 and TFIIH (Lewis et al., 2008). Activation of CDK7 relies on its phosphorylation at Thr 170 and association with cyclin H and MAT1 to form a ternary complex (Garrett et al., 2001). Although the association of these proteins in the gonadotropes has not been examined, CDK7 is phosphorylated at Thr 170 even without GnRH stimulation (Fig 3.20A), indicating that CDK7 may be constitutively active in the gonadotrope cells. This level of activation is likely responsible for the maintenance of phosphorylation of a small pool of SF-1 to ensure basal gene transcription, and would explain an earlier report that SF-1 is constitutively phosphorylated following its expression in MCF7 and COS cells (Hammer et al., 1999). These findings are in line with the results in the present study showing that without GnRH stimulation, ubiquitination of SF-1 is also dependent on phosphorylation by CDK7.

A number of recent works have highlighted the role for ubiquitination in transcriptional regulation, and this likely involves both mono-ubiquitination which increases certain protein–protein interactions, as well as poly-ubiquitination through various linkages, only some of which are recognized by the proteasome, and may facilitate promoter clearance (Sun and Chen, 2004; Hicke 2001; Muratani and Tansey, 2003; Pickart and Fushman, 2004; Wu et al., 2007; Sims and Cohen, 2009; Xu et al., 2009; Wang et al., 2008). In the present study it has been shown that SF-1 is modified by both mono-ubiquitination and poly-ubiquitination. The poly-ubiquitinated chains on SF-1 are linked through either Lys 48 or Lys 63 in ubiquitin, the Lys 63 linkage being apparently more abundant (Fig 3.24). It should be noted that poly-ubiquitination editing can occur, whereby ubiquitin is first Lys 63-linked and then undergoes a change to Lys 48 linkages that target its substrate protein for degradation by the proteasome, such that unconventional ubiquitin chains can also lead to proteasomal degradation (Newton et al., 2008; Xu et al., 2009). For example, the zinc finger protein A20 functions as an ubiquitin-editing enzyme, which first cleaves Lys 63-linked poly-ubiquitin chains and then conjugates Lys 48-linked poly-ubiquitin chains to enable proteasomal degradation, toward the downstream effector or adapter proteins like RIP1 and TRAF6 in the tumor necrosis factor receptor (TNFR) pathway or Toll-like receptor 4 and interleukin-1 receptor (TLR4/IL-1R) pathways to control the strength and duration of inflammatory signaling (Wertz et al., 2004; Shembade et al., 2010). Therefore, it is also reasonable to speculate that there is an ubiquitin-editing enzyme for SF-1, which can remove Lys 63-linked ubiquitin chains

from SF-1 after the cofactor recruitment, and add Lys 48-linked ubiquitin chains on SF-1, leading to its proteasome degradation and the eventual promoter clearance of the transcription machinery.

It has been demonstrated in this study that SF-1 ubiquitination increases SF-1 interaction with Pitx1 and requires phosphorylation of S203 (Fig 3.25 and 3.29), which was shown previously to increase SF-1 interaction with various cofactors, while others have shown that SF-1 at the LHβ promoter is indeed ubiquitinated (Hammer et al., 1999; Desclozeaux et al., 2002; Walsh and Shupnik, 2009). Given that relatively large amounts of mono-ubiquitinated SF-1 are detected, and that the mono-ubiquitination is also increased by GnRH, it is likely that this modification might signal the increased interaction with Pitx-1. The poly-ubiquitination and eventual clearance of SF-1 from promoter is likely important for the next round of transcription initiation, as shown for other regulated transcriptional activators (Reid et al., 2003; Wu et al., 2007). This is a similar mechanism to that which our group showed for GnRH-induced ubiquitination of ERα in the activation of the LHβ gene promoter, although it involves a very different mechanism, in which GnRH upregulates the ubiquitin conjugating enzyme, ubc4, which targets ERα (Luo et al., 2005). This pathway of SF-1-Pitx-1 interaction, clearance and subsequent degradation via Pin1-stimulated ubiquitination would explain the requirement for Pin1 and yet its reduction in SF-1 protein levels.

This model is consistent with reports in which ubiquitination of SRC-3 is dependent on GSK3-induced phosphorylation, and controls both its transactivation and its clearance from the promoter before the next round of transcriptional initiation. It has been proposed that the transition from mono-to poly-ubiquitination of this activator, which takes place during transcription, serves as a clock to regulate its lifetime (Wu et al., 2007). In a different study, it was shown that Pin1 interacts with phosphorylated SRC-3, regulates SRC-3 cellular turnover and enhances the functional interaction between SRC-3 and CBP/p300, while both proteins activate synergistically estrogen response element- and progesterone response element-driven luciferase reporter genes (Yi et al., 2005). These clearly suggest that the ubiquitin proteasome degradation of SRC-3 is phosphorylation- and Pin1-dependent.

Pin1 has been linked to promoting ubiquitin-proteasome-mediated degradation of other proteins, for example the turnover of BCL2, c-Myc and cyclin E are increased following Pin1 interaction (Phan et al., 2007; Basu and Haldar, 2002; Sears, 2004; Yeh et al., 2006). Negative regulation by Pin1-induced ubiquitination has also been shown for the death associated protein, Daxx, which inhibits the apoptotic response as a result of its proteasomal degradation (Ryo et al., 2007). A recent report has indicated that ubiquitination of Smad proteins occurs after binding by Pin1, as a result of the phosphorylated Smad's increased interaction with the E3 ligase Smurf, which is negatively regulates Smad activity (Nakano et al., 2009). A change in protein–protein interactions in this pathway, as a result of Pin1 binding, has also been shown for the RNA polymerase binding protein, Che-1, which is degraded in response to apoptotic

stimuli after Pin1-induced conformational changes, allow it to interact with the E3

ligase, HDM2 (De Nicola et al., 2007). However in all of these reports, the outcome

of the Pin1 mediated ubiquitination is negative regulation. The results shown in

present study, as well as those pertaining to the activity of SRC-3, indicate that the

Pin1-induced isomerization allows access to various E3 ligases which promote

assorted ubiquitin linkages with diverse outcomes.

4.2.4 Effect of Pin1 on protein stability of Pitx1

The current study has revealed that the action of Pin1 on SF-1 is not universal, as the

same drop in the protein levels of Pitx1 and Egr-1 were not observed, despite its

interaction with both proteins in the LHβ promoter. On the contrary, both endogenous

and exogenous tagged Pitx1 protein levels are increased after Pin1 over-expression

(Fig 3.15), indicating that Pin1 has a positive role in Pitx1 protein level. It is highly

possible that Pin1 stabilizes Pitx1 protein through protecting it from

ubiquitin-proteasome-mediated degradation machinery, since poly-ubiquitinated Pitx1

has been detected in gonadotropes after treatment with the proteasome inhibitor,

MG132(Fig 3.25C). The augmentation by Pin1 of Pitx1 transactivation towards the

FSHβ gene promoter could be a consequence of Pin1-induced Pitx1 stabilization (Fig

3.13). The enhancement of transcription factor activity by Pin1 through increasing its

stability has been exemplified by other transcription factors, such as β-catenin (Ryo et

al, 2001), p53 (Zacchi et al, 2002; Zheng et al, 2002), p73 (Mantovani et al., 2004)

and p65/Rel (Ryo et al., 2003). It has been shown that Pin1 functions as a positive

regulator of β-catenin by interfering with its interaction with the APC complex, resulting in its stabilization and thus upregulation of its target genes, such as c-myc and cyclin D1 (Ryo et al, 2001). Pin1 also targets p53 on phosphorylated Ser/Thr-Pro motifs, generated by stress-induced kinases, and protects it from the E3 ubiquitin ligase murine double minute 2 (Mdm2), which leads to p53 stabilization and upregulation of its downstream checkpoint control genes (Zacchi et al, 2002; Zheng et al, 2002). Thus, Pin1 may act as a coactivator during gene transcription, protecting transcription factors from degradation and allowing them to participate in promoter binding and gene activation.

4.3 Regulation of Pin1 by GnRH

A number of studies have demonstrated the function of Pin1 through its interaction partners in regulating various biological process and disease development (Lu, 2004; Lu and Zhou, 2007). Therefore, the investigation of the regulation of Pin1 activity could lay the foundation for exploring Pin1-specific small molecule inhibitors or activators for therapeutic purposes. This study has demonstrated that GnRH increases Pin1 expression levels and regulates its activity through both phosphorylation and dephosphorylation which, for the first time, indicates that Pin1 is a controllable mediator in signal transduction pathways.

4.3.1 Expression of Pin1 is induced by GnRH

In agreement with the results showing that Pin1 upregulates gonadotropin β subunit gene transcription, we have also shown that GnRH stimulates Pin1 expression (Fig 3.5; Luo et al., 2010). It has been reported that Pin1 expression is mediated through the activation of E2F by various oncoproteins including Ras, possibly involving also SP1 which can interact with E2F and might bind to the two GC boxes on Pin1 promoter (Ryo et al., 2002). In the gonadotrope, the Ras-MEK-pathway is activated by GnRH, which stimulates E2F (Naor, 2009; Berkovich and Ginsberg, 2001) and SP1 mediates some of the GnRH responsiveness of the LHβ gene (Kaiser et al., 1998), suggesting that this is a likely mechanism of elevation of Pin1 mRNA levels in gonadotrope cells by GnRH.

4.3.2 Phosphorylation of Pin1 is regulated by GnRH

We have also demonstrated that GnRH rapidly increases Pin1 phosphorylation at Ser 16, which was reported to inactivate Pin1 by preventing its interaction with the substrate and its translocation into the nucleus (Lu et al., 2002). These apparently conflicting actions of GnRH on Pin1 activity are reconciled by the discovery that GnRH also dephosphorylates Pin1 through calcineurin (Luo et al., 2010). Calcineurin is the calmodulin-activated protein Ser/Thr phosphatase 2B (PP2B), which is upregulated by GnRH (Fig 3.5; Lim et al., 2007). It is conceivable that the phosphorylation of Pin1 on Ser 16 residue results in the translocation of Pin1 out of

the nucleus (Lu et al., 2002), which would allow calcineurin, which is localized in the cytoplasm to interact with and dephosphorylate Pin1, thereby allowing the active Pin1 to return back into the nucleus. Therefore, the dephosphorylation of Pin1 by calcineurin presumably reactivates Pin1, so providing an additional distinct and regulatable pathway through which GnRH ensures sufficient levels of active nuclear Pin1. This finding is the first to link these two proteins, Pin1 and calcineurin, and indicates that Pin1 activity can be regulated by the same extracellular signals that activate the phosphorylation cascade targeted by Pin1.

4.4 Regulation of SF-1 transcriptional activity by the crosstalk between various post-translational modifications

Phosphorylation by the MAPK signal pathway of SF-1 at Ser 203, located in the AF-1 domain of the hinge region of this protein, was first identified in 1999, and notably, this modification is required for recruitment of cofactors to maximally activate SF-1 downstream target genes (Hammer et al., 1999; Fowkes et al., 2003; Lewis et al., 2008; Yang et al., 2009). Furthermore, another functional consequence of this phosphorylation is in decreasing the sensitivity of SF-1 to protease (Desclozeaux et al., 2002). However, the mechanism through which Ser 203 phosphorylation modulates SF-1 activity remained unclear until the present findings uncovering its additional post-phosphorylation modification. As discussed in Section 4.2.3, phosphorylation of SF-1 at Ser 203 is required for its interaction with Pin1 and its ubiquitination, which promote the recruitment of Pitx1 and upregulate downstream gene transcription.

We have also found that ubiquitin targets SF-1 at Lys 119, which is also targeted by SUMO (Fig 3.22). Previous studies by others revealed that SF-1 is SUMOylated at two conserved lysines (Lys 119 and Lys 194) that locate adjacent to the DBD and LBD respectively, and this modification repressesed SF-1 transactivation function (Komatsu et al., 2004; Lee et al., 2005; Campbell et al., 2008; Yang et al., 2009). This was attributed to the SUMOylation at Lys 194 which has been shown to reduce Ser 203 phosphorylation, decrease coregulator binding, and cause a selective loss of binding to certain target genes (Yang et al., 2009; Campbell et al., 2008). In contrast, it has been shown in present study that SF-1 SUMOylation is not affected by Ser 203 phosphorylation but SUMOylation at Lys 194 is a prerequisite for SUMOylation at Lys 119, which have been indicated also in other reports (Komatsu et al., 2004; Yang et al., 2009). Furthermore, this study also has demonstrated that ubiquitination of SF-1 at Lys 119 enhances, while SUMOylation inhibits, its interaction with Pitx1 which functions as a crucial coactivator in gonadotropin gene expression (Fig 3.25; Tremblay et al., 1999). These results reveal that the competition between SUMO and ubiquitin for the same Lys 119 in SF-1 affects SF-1 transcriptional activity in an opposite way. Thus, the transcriptional activity of SF-1 is dynamically and tightly modulated by a pathway of post-translation modifications. The de-SUMOylation on Lys 194 which facilitates Ser 203 phosphorylation allows Pin1-mediated isomerization, enabling the subsequent Lys 119 ubiquitination, to facilitate the interaction with its specific coactivator, Pitx1, and promoter activation.

4.5 Interaction between Pin1 and ATF3

In this study it has been shown that Pin1 can bind to ATF3 (Fig 3.30). Inspection of the amino acid sequence of ATF3 reveals that this protein contains three Ser/Thr-Pro motifs, Ser 24-Pro, Thr 38-Pro, and Thr 162-Pro, which are potential Pin1 binding sites if phosphorylated (Lu, 2004; Lu and Zhou, 2007). However, to date the only reported phosphorylation in ATF3 occurs at a tyrosine residue (Stearns et al., 2004). We have tried to use mitotic phosphoprotein monoclonal 2 (MPM-2) antibody which can recognize pSer/Thr-Pro epitope on about 50 mitotic proteins (Westendorf et al., 1994; Matsumoto-Taniura et al., 1996; Ding et al., 1997), to examine whether ATF3 is phosphorylated on these Ser/Thr-Pro motifs. However MPM-2 antibody fails to immunoreact with purified ATF3 from untreated or GnRH-treated LβT2 cell lysates (data not shown), likely because ATF3 does not belong to the group of proteins which are phosphorylated during G2 and dephosphorylated at the end of mitosis (Renzi et al., 1997; Shen et al., 1998). Therefore, we cannot exclude the possibility that ATF3 could be phoshorylated at potential Pin1 binding sites. However, it has been demonstrated in some recent publications that Pin1 is able to bind to non-canonical motifs in its target proteins including cyclin E (Yeh et al., 2006) and BNIP-H (Buschdorfv et al., 2008), BPGAP1 (Pan et al., 2010). This finding widens the spectrum of Pin1 targets and raises another possibility that Pin1 could directly interact with ATF3 even if ATF3 is not phosphorylated at Ser/Thr-Pro motifs. In this study the observation that Pin1, c-Jun and ATF3 form a complex in gonadotropes also

suggests that Pin1 and ATF3 might interact with each other in an indirect manner with c-Jun serving as a bridge protein between them.

4.6 The Effect of Pin1 on ATF3 protein level

In the present study it has been found that in gonadotropes, Pin1 increases the protein level of ATF3 (Fig 3.31A). Although RT-PCR showed that Pin1 over-expression does not lead to an elevation of ATF3 mRNA (Fig 3.31B), the possibility should not be ruled out that Pin1 could activate ATF3 transcription, as ATF3 is a target gene of Egr-1 (Mayer et al., 2008), whose transactivation capacity towards LHβ gene promoter is augmented by Pin1 (Fig 3.14). As reviewed in Section 1.5.1, ATF3 mRNA induction is usually transient, therefore changes in ATF3 mRNA levels, if occuring, could have gone undetected.

Since in MEF cells the half life of ATF3 protein is much shorter in the absence of Pin1 (Fig 3.31C), and in gonadotropes GnRH activates Pin1 expression (Fig 3.5; Luo et al., 2010), it might be expected that GnRH could play a protective role in ATF3 protein via Pin1. However, GnRH treatment does not affect ATF3 post-translationally in LβT2 cells (Fig 3.32D). This could be due to the fact that although Pin1 is required for maintaining a stable state of ATF3 protein, the amount of Pin1 is already sufficient in LβT2 cells. Moreover, based on the data presented in this study, we cannot exclude the possibility that Pin1 affects ATF3 protein stability indirectly; thus,

it is also likely that some ATF3 stabilizers or destabilizers regulated by Pin1 could be present in MEF cells but not gonadotropes.

4.7 SUMOylation of ATF3

SUMOylation has recently emerged as an important post-translational regulatory mechanism in controlling transcription factor activity (Gill, 2004; Geiss-Friedlander and Melchior, 2007). In this study it has been found that ATF3 is specifically targeted by SUMO-1 at Lys 42 and Lys 78 in gonadotropes (Fig 3.34). To date, three mammalian SUMO proteins SUMO-1, SUMO-2 and SUMO-3 have been detected (Schwarz et al., 1998; Azuma et al., 2003). Different SUMOs share redundant properties but also have some specific cellular functions (Gill, 2004; Hay, 2005; Geiss-Friedlander and Melchior, 2007). SUMO-1 shares 48% and 46% sequence identity with SUMO-2 and SUMO-3, respectively, while SUMO-2 and -3 are 96% identical to each other (Saitoh and Hinchey, 2000). The structural analyses have revealed that the three SUMO proteins adopt the compact and globular ubiquitin-like fold, and that the major difference between SUMO-1 and SUMO-2/3 are found in the second β-strand and the α-helix, which are key regions for binding to SUMO-interaction motifs on target proteins and mediating the transcriptional inhibitory properties of SUMOs (Bayer et al., 1998; Huang et al., 2004; Ding et al., 2005; Chupreta et al., 2005; Hecker et al., 2006). Apparently, future work should focus on how the modification of ATF3 by SUMO-1 is translated into biological effects.

4.8 Conclusions

The present study has demonstrated that Pin1 is required for the activation of basal and GnRH-stimulated gonadotropin β subunit gene transcription through interacting with the phosphorylated transcription factors SF-1, Pitx1 and Egr-1 and changing their stability, transcriptional activity, and/or protein–protein interactions. One of the mechanisms of this Pin1 action is through targeting SF-1, a key factor regulating all three gonadotropin subunit genes. Pin1-mediated SF-1 isomerization coordinates phosphorylation and ubiquitination, which facilitates Pitx1 binding, to increase SF-1 transcriptional activity. Furthermore, Pin1 expression and activity are also regulated by GnRH, indicating that Pin1 is an integral part of GnRH signaling (Fig 4.1).

This study has also shown that Pin1 forms a complex with the αGSU gene transcription factors, c-Jun and ATF3, and increases the protein level of ATF3 in gonadotropes, and that ATF3 is targeted by SUMO-1 at Lys 42 and Lys 78. These findings would lead to a better understanding of the transcriptional regulation of αGSU gene.

Given that the transcription factors targeting the gonadotropin genes are also involved in regulatory mechanisms in other physiological systems, the study of Pin1 function in gonadotropin subunit gene expression enhances our understanding of its role Pin1 in coordinating signaling by extracellular factors to gene transcription.
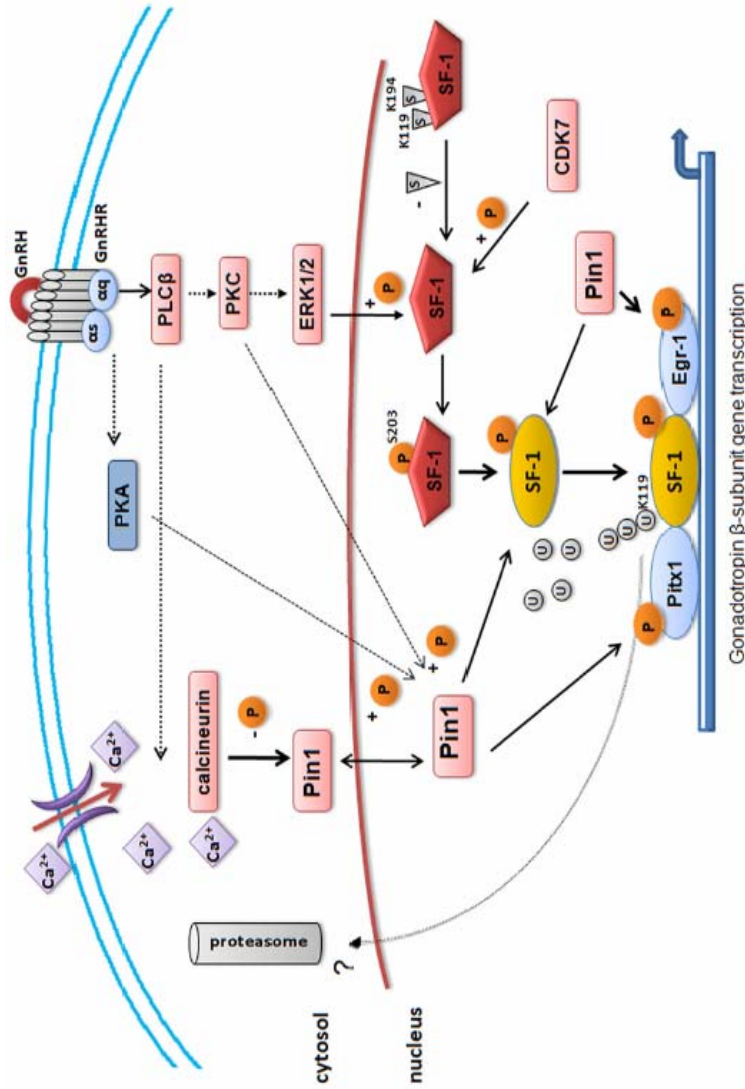
Fig 4.1: Model of regulation of gonadotropin β subunit gene transcription by Pin1.

GnRH activates PKC and PKA, either of which might phosphorylate Pin1 at Ser 16, causing its nuclear export. However, GnRH also elevates intracellular calcium levels and activates calcineurin, which dephosphorylates Pin1, allowing it to translocate back into the nucleus. Inside the nucleus Pin1, interacts with its target transcription factors, including SF-1, Pitx1, and Egr-1, enhancing their transcriptional activity toward the gonadotropin β subunit genes. De-SUMOylation of SF-1 at Lys 194, which occurs through an unknown mechanism, results in enhanced phosphorylation of SF-1 at Ser 203 by CDK7 (Yang et al., 2009) and de-SUMOylation at Lys 119. GnRH-activated ERK1/2 also increase SF-1 phosphorylation. Phosphorylation of SF-1 and its subsequent isomerization by Pin1 promote ubiquitination of SF-1 at Lys 119, facilitating the interaction between SF-1 and Pitx1 (and possibly other cofactors), to upregulate gonadotropin β subunit gene transcription. The proteasomal degradation of ubiquitinated SF-1 in the cytosol is a putative event. Dashed lines represent pathways that have or may have intermediate elements that are not shown. Abbreviations are given in the text. (From Luo et al., 2010)

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