Pin1 Facilitates the Phosphorylation-Dependent Ubiquitination of SF-1 To Regulate Gonadotropin β -Subunit Gene Transcription[∇]

Zhuojuan Luo,¹ Andrea Wijeweera,² Yingzi Oh,¹ Yih-Cherng Liou,^{1*} and Philippa Melamed^{1,2*}

Department of Biological Sciences, 14 Science Drive 4, National University of Singapore, Singapore 117543, Singapore,¹ and Faculty of Biology, Technion-Israel Institute of Technology, Haifa, 32000, Israel²

Received 22 June 2009/Returned for modification 10 September 2009/Accepted 23 November 2009

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. Herein, we demonstrate that Pin1 is required for both basal and GnRH-induced gonadotropin β -subunit gene transcription, through 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, we provide evidence that Pin1 is required for the Ser203 phosphorylation-dependent ubiquitination of SF-1, which facilitates SF-1–Pitx1 interactions and therefore results in an enhancement of SF-1 transcriptional activity. Furthermore, we demonstrate that in gonadotrope cells, sufficient levels of activated Pin1 are maintained through transcriptional and posttranslational regulation by GnRH-induced signaling cascades. Our 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.

Pin1 is a unique peptidyl-prolyl isomerase (PPIase) that recognizes a phosphorylated serine or threonine residue preceding a proline (pSer/Thr-Pro) and isomerizes the *cis-trans* conformation of the prolyl-peptidyl bond. This sequence can be phosphorylated by various kinases, including the mitogen-activated protein kinases (MAPKs), which form an integral part of many signal transduction pathways. Phosphorylation is a common modification involved in transmission of intracellular signals, and recognition of this motif by Pin1 and the subsequent conformational change in the protein provide a partial explanation for one mechanism of phosphorylation-dependent protein regulation (34). The effects of the Pin1-induced isomerization on its target proteins are diverse and include altering the stability and localization of the target proteins, as well as modifying their interaction with other proteins (14, 27, 33, 41, 53). In this way, Pin1 regulates various cellular processes, including differentiation, progression through the cell cycle, gene transcription, and mRNA splicing, and its functions in cancer and Alzheimer's disease have been well established (32, 34, 53, 57, 64).

Pin1 is expressed ubiquitously and its transcription is stimulated by various growth factors through activation of E2F, and also by Notch1 in a positive feedback loop (48, 51, 65). Its activity is also regulated by phosphorylation of Ser16 in the center of the pSer/Thr-Pro binding pocket, which abolishes Pin1's capability to interact with its substrates and may facili-

tate its nuclear export (35). The kinases involved in this regulation have not been fully investigated, although protein kinase A (PKA) was implicated (35).

Some of the most marked abnormalities in knockout mice are in their reproductive development and function. The phenotype includes decreased fertility, testicular atrophy, reduced testis size, seminiferous tubule degeneration, and spermatogonial depletion, while the gradual degeneration of spermatogonia up to the age of 14 months suggests that Pin1 has multiple roles in regulating fertility in adults as well as during early stages of development (1). Notably, female Pin1 knockout mice exhibit severe reduction in mammary epithelial duct development (31). The molecular mechanisms underlying these reproductive defects have yet to be elucidated. However, given that spermatogonia are regulated by the gonadotropin hormones, it is likely that some of the noted effects of Pin1 knockout might involve the gonadotropins.

Reproduction is regulated by the hypothalamic hormone, gonadotropin-releasing hormone (GnRH), which binds a G-protein-coupled receptor on the pituitary gonadotrope to induce transcription of the three gonadotropin genes, a common α -subunit (α GSU) and the hormone-specific β -subunits (LH β and FSH β); synthesis of the β -subunit is the rate-limiting step in hormone synthesis (42). The gonadotropic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are then secreted into the circulation and reach the gonads to activate reproductive development and function.

GnRH-induced signaling to the gonadotropin genes is predominantly via the PKC pathway and downstream MAPK cascades, including extracellular signal-related kinase 1/2 (ERK1/2), Jun N-terminal kinase (JNK), p38 MAPK, and big MAPK (BMK or ERK5), while PKA is also activated following GnRH-induced increases in cyclic AMP (29, 42). Many of the transcription factors that regulate expression of the gonado-

* Corresponding author. Mailing address for Philippa Melamed: Faculty of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel. Phone: 972 4 8293760. Fax: 972 4 8225153. E-mail: philippa@tx.technion.ac.il. Mailing address for Yih-Cherng Liou: Department of Biological Sciences, 14 Science Drive 4, National University of Singapore, Singapore 117543, Singapore. Phone: 65 68747711. Fax: 65 6779 2486. E-mail: dbslyc@nus.edu.sg.

[∇] Published ahead of print on 7 December 2009.

FIG. 1. Pin1 induces gonadotropin β -subunit gene transcription. (A) LH β or FSH β promoter luciferase constructs were transfected into L β T2 cells with pCS2+ or pCS2+Pin1 vectors, and some of the cells were exposed to 100 nM GnRH for 4 h. Firefly luciferase levels were measured and normalized to those of *Renilla*; results are expressed as normalized levels over those in control cells. (B to D) pCS2+ or pCS2+Pin1 alone

troponin genes are activated through phosphorylation by these kinases and thus contain pSer/Thr-Pro motifs. The LH β gene promoter is activated at a basal level by the orphan receptor steroidogenic factor 1 (SF-1) and the paired-like homeodomain transcription factor 1 (Pitx1) (19, 39); both factors are expressed in a cell-restricted manner (18, 24, 25, 44, 55). Also crucial in LH β gene expression is the early growth factor 1 (Egr-1), whose expression and phosphorylation are induced by GnRH, allowing its synergistic interaction with SF-1 and Pitx1 and mediation of the GnRH effect (10, 15, 19, 21, 39, 59). The molecular mechanisms of the basal and GnRH-stimulated effects on the FSH β gene promoter have been less well elucidated, but they appear to involve SF-1 and various Pitx proteins, including Pitx1, as well as the activator protein-1 (AP-1) transcriptional complex, comprising c-Jun and c-Fos, both of which are transcriptionally regulated and phosphorylated by GnRH (7, 23, 37, 39, 62, 70).

SF-1 and Pitx1 act synergistically on both the LH β and Mullerian-inhibiting-substance gene promoters, and this was shown to be a result of Pitx1 binding directly to SF-1. Pitx1 enhances the transcriptional activity of SF-1 through an unmasking effect, in which the Pitx1 C terminus interacts with a region of SF-1 N-terminal to amino acid 279, possibly involving an activation domain located between amino acids 187 and 245, to activate SF-1 to a degree similar to that achieved by deletion of the ligand-binding domain (LBD) (58).

Both SF-1 and Pitx1 appear to be phosphorylated, either constitutively or possibly through GnRH-stimulated kinases (16, 38). Phosphorylation of SF-1 at Ser203, by ERK1/2 or CDK7, was shown to be required for maximal SF-1 target gene transactivation and recruitment of various cofactors (12, 16, 28). However, SF-1 is subject to additional posttranslational modifications which alter its activity, and it can be SUMOylated at two conserved lysines, Lys119 and Lys194, which are adjacent to the DNA-binding domain (DBD) and LBD, respectively. SUMOylation at Lys194 was seen to inhibit Ser203 phosphorylation and was also associated with reduced transactivation of various SF-1 target genes (5, 67). In gonadotropes, SF-1 was seen also to be ubiquitinated, and this form was associated with the LH β gene promoter (60). While clearly affecting SF-1 transactivation, the relationship between these various modifications and their roles are not yet clear.

In this study we hypothesized that the MAPK-directed phosphorylation of some of these gene-specific transcription factors might cause them to be targeted by Pin1. We have demonstrated a role for Pin1 in transcription of the gonadotropin β -subunit genes and elucidated its function in the activation of SF-1, via a phosphorylation-regulated pathway which facilitates SF-1–Pitx1 interactions as a result of promoting SF-1 ubiquitination. We also show that Pin1 activity is regulated by

GnRH, indicating that it constitutes a novel player in the GnRH-signaling pathway to gonadotropin gene expression.

MATERIALS AND METHODS

Cell culture and transfection. The murine gonadotrope α T3-1 and L β T2 cells (gifts from P. Mellon, University of California, San Diego) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal bovine serum (Gibco-Invitrogen), 10 mM HEPES [pH 7.4], 4 mM L-glutamine (HyClone), 100 U/ml penicillin (Gibco-Invitrogen), and 100 μ g/ml streptomycin (Gibco-Invitrogen). COS-1 cells, HEK 293T control small interfering RNA (siRNA) cells, HEK 293T Pin1 siRNA cells (50), MEF wild-type (WT) cells, and MEF Pin1^{-/-} cells (gifts from K. P. Lu, Harvard Medical School) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% certified fetal bovine serum (HyClone) and the same antibiotics. As appropriate, cells were exposed to 10 to 100 nM concentrations of the GnRH agonist buserelin (Sigma), 100 nM phorbol myristate acetate (PMA) (Sigma), 1 μ M forskolin (Sigma), 5 μ M cyclosporine (Sigma), 10 μ M MG132 (Sigma), 15 μ M roscovitine (Sigma), or 1 μ M U0126 (Promega), as indicated in the figure legends. All cells were maintained at 37°C under 5% CO₂. Transfection was carried out at 50 to 60% confluence. L β T2 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 to 48 h before harvest.

Plasmid constructs. Luciferase assay constructs were created by ligating 1,300 bp of the proximal murine LH β gene promoter or 600 bp of the proximal FSH β gene promoter into pGL2 Basic (Promega), as reported previously (11, 39). 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. The coding sequence of Pin1 was synthesized by reverse transcriptase PCR (RT-PCR) from L β T2 cell mRNA and was ligated into the expression vector pCS2+. For FLAG- or hemagglutinin (HA)-tagged constructs, sequence-specific primers containing restriction enzyme recognition 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 the expression vector p3x-FLAG-CMV 10. Pitx1, SF-1, Egr-1, ubiquitin, and SUMO1 sequences were inserted into pxj40-HA, pxj40-FLAG, and pxj40-Myc (gifts from B. C. Low, National University of Singapore). pxj40-GFP was used to construct pxj40 GFP SF-1 and pxj40 GFP SF-1 K119R. pcDNA3 CDK7 HA (P#633) and pcDNA3 CDK7 D155A HA (P#812) were purchased from Addgene. For the two-hybrid assays, the reporter gene construct was created as described previously (36). Other constructs were prepared by inserting the sequences from Pin1, Pitx1, SF-1, and Egr-1 expression vectors into the Gal4 DBD or Gal4 activation domain (AD) vector pM or pVP16 (Clontech). Site-directed mutations were carried out using a QuikChange site-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions, in order to introduce the specific amino acid substitutions. All constructs were verified by sequencing.

Antibodies. Antibodies used 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), anti-GAPDH (Santa Cruz; sc-47724), anti-ATF3 (Santa Cruz; sc-188), anti-Pitx1 (Santa Cruz; sc-18922), anti-Egr-1 (Santa Cruz; sc-110), anti- β -catenin (Santa Cruz; sc-7199), anti-Ub (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), anticalcineurin (PP2B-Aa [D-9]; Santa Cruz; sc-17808), anti-green fluorescent protein (GFP) (Santa Cruz; sc-81045 and sc-8334), anti-SF-1 (Upstate Biotechnology; 07-618), anti-phospho-Pin1 (Ser 16) (Cell Signaling; 3721), anti- α -tubulin (Sigma; T9026), and anti-FLAG (Sigma; F3165).

RNA-mediated knockdown of gene expression. Stealth small interfering RNAs (siRNAs) specific for Pin1 (sense, 5'-GCCGGGUGUACUACUCAAAdTdT-3'

(B) or together with W34A WW domain binding mutant- or K63A PPIase mutant-Pin1 expression vector (C) or control or Pin1 siRNA (D) were transfected into L β T2 cells, and some of the cells were exposed to 100 nM GnRH for 4 h. LH β or FSH β mRNA levels were measured by real-time PCR, using β -actin as an internal control, and levels are expressed as fold over those in control cells. For panels A, B, and D, a *t* test compared means between groups with or without manipulation of Pin1 expression and was done separately for untreated and GnRH treated groups; for panel C, ANOVA followed by a Bonferroni *t* test compared all means; significantly different values (*P* < 0.05) are designated with different letters, in uppercase for untreated cells and in lowercase for GnRH-treated cells. Values are means $\pm$ standard errors of the means (SEM); *n* = 3 to 6. (E) Western blot to determine protein levels of Pin1 and its mutants in L β T2 cells following their overexpression or knockdown, with or without 100 nM GnRH treatment for 4 h; α -tubulin (Tub) or GAPDH was used as a loading control.

FIG. 2. Pin1 is both transcriptionally and posttranslationally regulated by GnRH. (A) RT-PCR of Pin1, LHβ, and FSHβ mRNA levels was carried out in LβT2 cells following 10 nM GnRH for 0 to 12 h; β-actin was used as an internal control. Normalized values are relative to values in untreated cells (means ± SEM, *n* = 3). (B to E) Western blot showing Pin1, calcineurin A (CnA), and ATF3 in LβT2 cells following GnRH treatment for 0 to 4 h (B); Pin1 in MEF, COS-1, αT3-1, and LβT2 cells, some of which were exposed to 100 nM GnRH for 2 h (C); and

[50]), and scrambled control (sense, 5'-GUGUUACAGCUCCAGAUGCdTdT-3') were purchased from Invitrogen. The siRNAs were transfected into L β T2 cells using Oligofectamine (Invitrogen) according to the manufacturer's protocols.

For construction of calcineurin A short hairpin RNA (shRNA)-encoding plasmids, a pair of oligonucleotides including the specific 19-bp target sequence for calcineurin A (5'-TTACAATCTTCTCGGCACC-3') was synthesized, annealed, and ligated into pSUPER Basic vector (Oligoengine, Seattle, WA). The construct was verified by sequencing. pSUPER Basic GFP shRNA was used as a control, as described previously (36). The vector-based shRNA constructs were transfected into L β T2 cells using Geneporter 2.

Luciferase assays. L β T2 cells were plated in 96-well white plates. At 12 to 24 h after plating, expression vectors (50 ng for Pin1, 80 ng for SF-1 and Egr-1, and 10 ng for Pitx1), the luciferase reporter vector (100 ng) and simian virus 40-Renilla luciferase (2 ng) were transfected after equilibration of the total amount of DNA with pCS2+ empty vector or pWhitescript. Cells were incubated for 24 to 48 h before harvest. Luciferase activity was measured using the Dual-Glo system (Promega) and a Veritas Microplate luminometer (Turner Biosystems) and normalized to levels of Renilla luciferase. 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. Statistical analysis was performed using a simple unpaired *t* test to determine means that were statistically different. Differences were considered significant when *P* was <0.05. Synergy was determined by comparing the additive effects of expression vectors transfected separately (using $SE(x+y) = \sqrt{SE(x)^2 + SE(y)^2}$) with the effect when both were transfected together and was defined as a significantly (*P* < 0.05) greater-than-additive effect.

For the two-hybrid assays, COS-1 or L β T2 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, and 2 ng of simian virus 40-Renilla luciferase as an internal control. Cells were incubated for 24 to 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.

Reverse transcriptase PCR and real-time PCR. RNA was extracted using TRIzol (Invitrogen), and the total RNA (2 μ g) was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and random oligo(dT) primers (New England Biolabs). Of the 20 μ l cDNA obtained, 1 μ l was used for a semiquantitative PCR with specific primers. Primers for the LH β RT-PCR were as follows: forward, 5'-GCCTGTCAACGCAACTCTGG-3'; reverse, 5'-CAGG CCAATTGGTTGAGTCCT-3'. Primers for the FSH β RT-PCR were as follows: forward, 5'-AGCACTGACTGCACCGTGAG-3'; reverse, 5'-CCTCAGCCAG CTTTCATCAGC-3'. Primers for the β -actin RT-PCR were as follows: forward, 5'-GCCATGTACGTAGCCATCCA-3'; reverse, 5'-ACGCTCGGTCCAGGATC TTCA-3'. Primers for the Pin1 RT-PCR were as follows: forward, 5'-CCGGA ATTCATGGCGGACGAGGAGAAG-3'; reverse, 5'-TGCTCTAGATCATTC TGTGCGCAGGAT-3'. Primers for the SF-1 RT-PCR were as follows: forward, 5'-ATGGACTATTCTGACGAC-3'; reverse, 5'-TCAAGTCTGCTTGGCCTG-3'. Primers for the Pitx1 RT-PCR were as follows: forward, 5'-ATGGACGCC TTCAAGGGAGGC-3'; reverse, 5'-TCAGCTGTTGTACTGGCAAG-3'.

Real-time PCR was carried out using SYBR green I dye with an ABI Prism 7900 sequence detector (Perkin-Elmer Applied Biosystems). PCRs were performed in a 5- μ l volume, containing PCR Master Mix, 0.1 μ g cDNA template, and forward and reverse primers. Primers for the LH β real-time PCR were as follows: forward, 5'-CAGTCTGCATCACCTTCACC-3'; reverse, 5'-GCAGTA CTCGGACCATGCTA-3'. Primers for the FSH β real-time PCR were as follows: forward, 5'-TGACACGAGCAGTAGCTGTTT-3'; reverse, 5'-TGAGATG GTGATGTTGGTCA-3'. Primers for the β -actin real-time PCR were as follows: forward, 5'-CCTTCTCTTCTGGGTATGGA-3'; reverse, 5'-ACGGATGTCAA

CGTCACACT-3'. The samples were heated to 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The comparative cycle time (CT) method was used to compare mRNA levels in the various samples which were assayed in duplicate.

Western blot. Cells were lysed in 1 $\times$ lysis buffer (50 mM Tris-Cl [pH 8.0], 100 mM NaCl, 1% sodium dodecyl sulfate [SDS], 0.5% NP-40, 5% glycerol, 2 mM dithiothreitol, and protease inhibitors). After centrifugation at 13,400 $\times$ g for 40 min at 4°C, the supernatant was collected and protein content was measured with the Bradford protein assay reagent (Bio-Rad). The proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and subsequently transferred to polyvinylidene difluoride membranes (Millipore). The membrane was blocked with 5% bovine serum albumin in TBST (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20). After washing with TBST, the membrane was incubated with primary antibody (Ab) diluted in TBST for 2 h at room temperature or overnight at 4°C. After washing with TBST for 30 min, the membrane was then incubated with goat or bovine horseradish peroxidase-conjugated secondary Ab to mouse, rabbit, or goat immunoglobulin G (IgG) (Santa Cruz) for 1 h at room temperature, followed by extensive washes with TBST. 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).

Chromatin immunoprecipitation (ChIP). L β T2 cells were grown to approximately 80% confluence in 100-mm plates. The proteins were cross-linked to DNA with 1% formaldehyde. Cross-linking was arrested by addition of 0.125 M glycine. The cells were then rinsed, collected, and resuspended in 1,000 μ l 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, and protease inhibitors). 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 six times, with a 10-s rest between, and then pelleted by centrifuging at 13,000 rpm at 4°C for 10 min. 1 μ g of Ab [anti-Pin1 (Santa Cruz; sc-15340) or anti-HA (Santa Cruz; sc-805)] and 20 μ l protein A Sepharose CL-4B (GE Health) were added into 450 μ l lysate and subsequently incubated overnight at 4°C with rotation. The Ab-bound complexes were washed extensively with each of the following buffers: low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], 500 mM NaCl), and LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.0]). This was followed by two washes with Tris-EDTA. The protein-bound DNA precipitated from the ChIP assay and input DNA were purified with phenol-chloroform and dissolved in 25 μ l H₂O. Precipitated and input DNA served as templates for promoter-specific PCR. Primers used to amplify the FSH β and LH β gene-proximal promoters were as described previously (11).

Immunoprecipitation (IP). For endogenous IP, L β T2 cells were washed with cold 1 $\times$ phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-Cl [pH 8.0], 125 mM NaCl, 0.5% NP-40, 5% glycerol, 20 mM NaF, 0.2 mM Na₃VO₄, 2 mM EDTA, protease inhibitors) for 30 min. After centrifugation at 13,400 $\times$ g for 40 min, 1 mg of total cell lysate was incubated with 1 μ g of Ab and 20 μ l protein A or G Sepharose CL-4B (GE Health) overnight at 4°C with gentle rotation. The protein-Ab-bead complexes were washed four times with the lysis buffer. For FLAG IP, 1 μ g of FLAG-tagged construct and 1 μ g of HA-tagged construct were cotransfected into cells in 60-mm plates. At 24 h after transfection, cells were washed with cold 1 $\times$ PBS and lysed in RIPA buffer. After centrifugation at 13,400 $\times$ g for 40 min, 100 μ g of total cell lysate was incubated with 4 μ l of EZview red anti-flag M2 affinity gel (Sigma) for 2 h at 4°C with gentle rotation. The protein-Ab-bead complexes were washed at least four times with the lysis buffer to reduce nonspecific binding. SDS-PAGE coupled with Western blot analysis was used to resolve the complexes.

In vivo ubiquitination assay. FLAG- or HA-tagged WT or mutant SF-1 and ubiquitin constructs were cotransfected, and after 24 h, the cells were washed with cold 1 $\times$ PBS and lysed for 30 min in RIPA buffer. The supernatants of the

phosphorylated Pin1 at Ser 16 (pPin1) and total Pin1 levels in L β T2 cells following 0 to 4 h GnRH (100 nM) or exposure to PMA (100 nM) or forskolin (1 μ M) for 1 h (D, left). (D, right) Specificity of the Ab to pPin1 in L β T2 cells treated with 100 nM GnRH for 0.5 h. IP utilized Ab to GFP, and IP samples were blotted using Ab to the proteins shown. Notably, there are no immunoreactive bands for pPin1 Ab in samples in which the S16A was transfected. (E) pPin1 in L β T2 cells following treatment with CsA (20 μ M for 2.5 h) or CnA knockdown by siRNA (48 h), with or without GnRH treatment (100 nM for 2 h). GAPDH is a loading control. (F) Cell lysates from L β T2 cells following Pin1 overexpression and exposure to 100 nM GnRH, as marked, were precipitated using Ab to CnA or normal IgG and blotted to reveal total Pin1 and pPin1 levels in input and IP samples.

FIG. 3. Pin1 is present on the promoter region of the LH β and FSH β genes and interacts with various gene-specific transcription factors. (A) ChIP was carried out using Ab to Pin1, or HA as a control, in L β T2 cells with or without exposure to 100 nM GnRH for 4 h (in duplicate), and PCR was performed to detect the endogenous mouse LH β and FSH β gene promoters. (B) L β T2 cell lysates were precipitated with Ab to Pin1, or normal IgG as a control. Western blotting was carried out for β -catenin, Egr-1, Pitx1, and Pin1 levels in input and IP samples. (C) L β T2 cell lysates, following Pin1 overexpression and exposure to 100 nM GnRH, were precipitated with Ab to SF-1 or normal IgG, and Western blotting revealed Pin1 levels. (D and E) Mammalian two-hybrid assays were carried out in COS-1 cells using a Gal4-responsive luciferase reporter gene

cell lysates were incubated with EZview red anti-FLAG M2 affinity gel (Sigma) or mouse HA Ab (Santa Cruz; sc-7392)-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. The HA Ab was conjugated to the beads by incubating 1 µg of mouse HA Ab (Santa Cruz; sc-7392) with 20 µl protein G Sepharose CL-4B in RIPA buffer and for 1 h at 4°C, followed by extensive washes.

In vivo SUMOylation assay. HA-tagged WT or mutant SF-1 and Myc-SUMO1 constructs were cotransfected, and after 24 h, cells were washed with cold 1× PBS and lysed in RIPA buffer containing 10 mM *N*-ethylmaleimide (NEM). The supernatants of the cell lysates were then incubated with mouse HA Ab-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.

Fluorescence imaging. LβT2 cells were seeded on ethanol-sterilized glass coverslips in 12-well plates and after 24 h were transfected with the 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 in the figure legends. The cells were fixed with cold-methanol at −20°C for 10 min and then permeabilized with PBS containing 0.1% Triton X for 20 min at room temperature. The fixed cells were washed twice with PBS and then incubated with DAPI (4',6'-diamidino-2-phenylindole) for 20 min, followed by washing with PBS twice. The coverslips were mounted on glass slides using FluorSave™ reagent (Calbiochem) and examined by LSM510 confocal fluorescence microscopy (Carl Zeiss).

RESULTS

Pin1 induces gonadotropin β-subunit gene transcription.

We first assessed the effect of Pin1 overexpression on gonadotropin β-subunit gene promoter activity in mouse gonadotrope LβT2 cells using luciferase assays and saw that it increased both basal and GnRH-induced murine LHβ and FSHβ promoter activity (Fig. 1A; levels of overexpression are shown in Fig. 1E). The ability of Pin1 to increase expression of the endogenous genes was shown by quantitative real-time RT-PCR using primers spanning an intron/exon border. As in the luciferase assays, Pin1 overexpression increased the basal and GnRH-induced LHβ and FSHβ gene transcription (Fig. 1B). The finding that Pin1 increases LHβ and FSHβ gene transcription, as opposed to altering stability of the transcript, was verified by treating cells with actinomycin D and measuring rates of transcript degradation, which were similar for LHβ in cells with or without transfection of the Pin1 expression vector (data not shown).

To confirm the role of Pin1 in these effects, W34A and K63A mutants were overexpressed to test the requirement of the WW and PPIase domains. Real-time PCR confirmed that, while the WT Pin1 increases transcript levels of both genes, neither mutant was able to do the same, and the W34A mutant reduced LHβ transcript levels to below those of the control (Fig. 1C; levels of overexpression are shown in Fig. 1E).

The actual role of Pin1 in regulating expression of these

genes was verified by knocking down Pin1 expression with siRNA (Fig. 1D and E). Transfection of the Pin1-targeting siRNA decreased the basal and, to a greater degree, the GnRH-induced LHβ and FSHβ mRNA levels (Fig. 1D). This demonstrates that Pin1 plays a crucial role in GnRH-induced signaling pathways to induce mouse gonadotropin β-subunit expression.

Pin1 is both transcriptionally and posttranslationally regulated by GnRH. Given that GnRH appeared to increase Pin1 protein levels (Fig. 1E), we examined the ability of GnRH to affect Pin1 expression. LβT2 cells were exposed to 10 nM GnRH for 0, 6, 8, or 12 h, and RNA was extracted for RT-PCR. In addition to the Pin1 mRNA, LHβ and FSHβ mRNAs were also measured to confirm the effective GnRH treatment. Pin1 mRNA levels increased at 6 h but declined thereafter; a similar pattern was seen in the FSHβ mRNA levels, while those of LHβ remained elevated even after 12 h, and those of β-actin remained constant (Fig. 2A). Western blot confirmed that Pin1 protein levels increased progressively with lengthening GnRH treatments, in a rapid but more moderate response than seen for calcineurin (CnA) and ATF3, while GAPDH levels remained virtually unchanged (Fig. 2B). The cell specificity of the GnRH effect was shown 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. 2C).

To determine whether GnRH alters the phosphorylation status of Pin1, the level of Pin1 phosphorylated at Ser16 (pPin1) was examined by Western blotting. The specificity of the Ab was confirmed by overexpressing GFP-Pin1 or GFP-Pin1 S16A; IP of the GFP-tagged proteins was followed by Western analysis with the pPin1 Ab (Fig. 2D, right). In GnRH-treated cells, pPin1 was easily detected after 15 min GnRH and remained at a high level after 30 min, after which it gradually decreased (Fig. 2D). Both PKA and PKC are activated by the GnRH signaling cascade, and treatment of LβT2 cells with PMA or forskolin showed that both treatments strongly induced pPin1 levels, indicating involvement of either or both pathways in phosphorylating Pin1 (Fig. 2D).

Phosphorylation of Pin1 is reported to hinder its activity by preventing its interaction with the substrate and its nuclear localization (35), yet Pin1, although being temporarily phosphorylated by GnRH, appears required for LHβ and FSHβ gene transcription. We have shown that GnRH induces expression of the phosphatase calcineurin (30) (Fig. 2B), and we therefore hypothesized that GnRH-activated calcineurin might dephosphorylate pPin1 to allow its activity. To test this, LβT2 cells were treated with a calcineurin inhibitor, cyclosporine

and *Renilla* luciferase as an internal control, together with a Gal4 DBD empty vector (pM) or Gal4 DBD-Pin1 fusion construct (pM Pin1) with the VP16 AD fused to Pitx1 (pVP Pitx1) or Egr-1 (pVP Egr-1) (D) or SF-1 (pVP SF-1) or SF-1 S203A (pVP SF-1 S203A) (E). Luciferase assays were carried out as for Fig. 1A, and results were expressed as relative to those in control cells, in which unfused Gal4 DBD and VP16 AD constructs were transfected together. Statistical analysis (*t* test) compared means for the groups transfected with both fusion constructs with the combined effects of each fusion construct alone. The interactions of pM Pin1 with the WT and SF-1 S203A pVP fusion proteins were also compared (***, *P* < 0.001). Values are means ± SEM; *n* = 6. (E) LβT2 cell lysates after cotransfection of Pin1 and pxj40 FLAG empty vector, FLAG-SF-1, or FLAG-SF-1 S203A were precipitated with anti-FLAG M2 beads. Western blotting revealed Pin1, FLAG-SF-1, or FLAG-SF-1 S203A levels in input and IP samples. (F) LβT2 cell lysates after cotransfection of FLAG-Pin1 and WT HA-Pitx1 or HA-Pitx1 mutants were precipitated with anti-FLAG M2 beads, and Western blotting revealed FLAG-Pin1, WT HA-Pitx1, or HA-Pitx1 mutant levels in input and IP samples.

(CsA), which was added 30 min prior to GnRH treatment for 2 h. There was a clear increase in pPin1 levels in the CsA-treated cells, which was more obvious in the GnRH-treated cells. This finding was confirmed by transfection of calcineurin siRNA, which effectively blocked calcineurin expression in untreated cells but, not surprisingly, was less effective after GnRH treatment, which likely explains the less obvious effect of the siRNA in the GnRH-treated cells (Fig. 2E).

To further confirm the actions of calcineurin on Pin1, co-IP was carried out to examine whether calcineurin is associated with pPin1. Pin1 was overexpressed in L β T2 cells, some of which were treated with GnRH for 0.5 h. The cells were lysed and precipitated with anti-CnA or IgG, followed by blotting with anti-CnA or pPin1 Ab. The pPin1 was coprecipitated by the calcineurin Ab but not rabbit IgG, and significantly more so in the GnRH-treated cell lysate (Fig. 2F). Taken together, these results clearly demonstrate a role for GnRH-activated calcineurin in regulating Pin1 activity.

Pin1 is present on the promoters of the LH β and FSH β genes and interacts with various gene-specific transcription factors. In order to elucidate the mechanisms through which Pin1 activates LH β and FSH β gene transcription, ChIP was carried out to examine whether Pin1 is present on the promoters of these genes. Association of Pin1 with the proximal regions of the LH β and FSH β gene promoters was detected in untreated L β T2 cells and after GnRH exposure for 4 h (Fig. 3A).

We hypothesized that Pin1 is recruited to these promoters through its interaction with gene-specific transcription factors. Therefore, co-IP was carried out using L β T2 cell lysates, and Pitx1, Egr-1, and β -catenin were detected in precipitates using the anti-Pin1 Ab but not in those using the control IgG (Fig. 3B). Because the mobility of SF-1 is similar to that of the IgG heavy chain, we performed co-IP using Ab to SF-1 for the precipitation; Pin1 was detected in the lysates from untreated and GnRH-treated cells but not in those using the control IgG (Fig. 3C).

To confirm some of these interactions, mammalian two hybrid assays were carried out in COS-1 cells using Pin1 fused to the Gal4 DBD (in pM vector) and Pitx1, Egr-1, or SF-1 fused to VP16 AD (in pVP vector), together with Gal4-responsive luciferase reporter gene and pRL-SV40 *Renilla* luciferase as an internal control. Cotransfection of pM-Pin1, but not the empty pM vector, with pVP-Egr-1, pVP-Pitx1, or pVP-SF-1 revealed significant induction of the luciferase reporter gene activity (Fig. 3D and E), indicating that Pin1 interacts with Pitx1, Egr-1, and SF-1. The two-hybrid assay was also carried out using an expression vector for SF-1 with an S203A mutation. The interaction of this mutated protein with Pin1 was markedly reduced in comparison to that of the wild-type SF-1. The reduced interaction of SF-1 S203A with Pin1 was seen also in co-IP assays, in which the FLAG-tagged SF-1 proteins were expressed in L β T2 cells and precipitated before immunoblotting with Pin1 Ab (Fig. 3E).

Mutagenesis of the putative Pin1 target motifs in Pitx1 was also carried out to determine the residues involved in the interaction with Pin1. After expression of the FLAG-tagged Pin1 and HA-tagged Pitx1 proteins, the FLAG-tagged Pin1 was precipitated before immunoblotting with Ab to the HA tag on the Pitx1 protein or mutant proteins. The binding of FLAG-

Pin1 with the Pitx1 mutants, HA-Pitx1 S207A, HA-Pitx1 S259A, or HA-Pitx1 T267AS270A, was clearly reduced in comparison with the wild-type HA-Pitx1 (Fig. 3F).

These results indicate that Pin1 interacts directly with Pitx1, Egr-1, and SF-1 and demonstrate a crucial role for S203 of SF-1 and for several residues of Pitx1 in these interactions.

Pin1 increases transcription factor activity on the LH β and FSH β gene promoters, despite reducing SF-1 levels. In order to investigate whether Pin1 binding with these transcription factors plays a functional role in transcription of the gonadotropin β -subunit genes, the effect of Pin1 on transactivation of these genes by Pitx1, Egr-1, or SF-1 was examined in luciferase assays, in which their expression vectors were transfected alone or in combination. Pin1 and SF-1 had a synergistic effect on the LH β but not the FSH β gene promoter, while Pin1 interacted synergistically with Pitx1 on the FSH β but not the LH β gene promoter. Egr1, which targets only the LH β gene, also had a synergistic effect with Pin1 on LH β promoter activity (Fig. 4A).

Since we found that Pin1 binds directly to Pitx1, Egr-1, and SF-1 and enhances their transactivation, we speculated that Pin1 affects the stability of these factors. Western blotting showed that Pin1 overexpression lead 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 tested (Fig. 4B). Some of this could be due to Pin1 regulation of transcription of these factors, as RT-PCR after overexpression of Pin1 showed a clear increase in Pitx1 mRNA levels, while those of SF-1 decreased marginally (Fig. 4C). In order to test for posttranscriptional effects of Pin1 on these proteins, constructs encoding HA-tagged Pitx1 or SF-1 were transfected, with or without Pin1 overexpression. Western blotting 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 following transfection of more of the Pin1 expression vector (Fig. 4D).

Ubiquitination of SF-1 requires both Ser203 and Pin1. To explore the molecular mechanism underlying the Pin1-induced drop in SF-1 levels, we examined whether Pin1 is involved in ubiquitination of SF-1. Ubiquitination of wild-type HA-SF-1 and HA-SF-1 S203A was analyzed by immunoprecipitation of the tagged protein followed by Western blotting using Ab against ubiquitin. A monoubiquitinated form of SF-1 was evident (between the 50- and 75-kDa markers) following transfection of the WT but not the S203A mutant; in the latter samples, the SF-1 ubiquitination was reduced to an undetectable level (Fig. 5A). Similarly, the SF-1 WT and S203A constructs were transfected with an expression vector for Myc-tagged ubiquitin, and IP was carried out using rabbit Ab to the Myc tag. The precipitated proteins were blotted with mouse Ab to HA, and again the mono- and polyubiquitinated SF-1 was visible only in the WT SF-1-transfected samples (Fig. 5B).

In order to assess the role of Pin1 in the SF-1 ubiquitination, *in vivo* ubiquitination assays were carried out in L β T2 after overexpression of FLAG-tagged SF-1, with or without Pin1 siRNA. SF-1 was clearly polyubiquitinated in L β T2 control cells, but in the Pin1 knockdown cells, SF-1 ubiquitination was reduced (Fig. 5C). Further studies were carried out in HEK 293T control or Pin1 knockdown cells (expressing Pin1 but at lower levels) (Fig. 5D), after overexpression of HA-tagged WT SF-1 or the S203A mutant. SF-1 was seen to be polyubiquiti-

FIG. 4. Pin1 increases transcription factor activity on the LH β and FSH β gene promoters, despite reducing SF-1 levels. (A) FSH β (top) or LH β (bottom) promoter-luciferase constructs were transfected into L β T2 cells alone or together with Pin1, Pitx1, SF-1, and/or Egr-1 expression vectors. After 24 h, luciferase assays were carried out, and results are presented as in Fig. 1A, except that statistical analysis tested for a synergistic effect (***, $P < 0.001$; NS, not synergistic). Values are means $\pm$ SEM; $n = 6$. (B) Western blot detected Egr-1, Pitx1, SF-1, and Pin1 in L β T2 cells transfected with pCS2+ or pCS2+Pin1 for 24–48 h; GAPDH is a loading control. (C) RT-PCR of SF-1 and Pitx1 mRNA levels was carried out in L β T2 cells transfected, in duplicate, with pCS2+ or pCS2+Pin1; β -actin is an internal control. (D) Western blotting revealed HA-Pitx1 and HA-SF-1 in L β T2 cells transfected with pCS2+ or various amounts of pCS2+Pin1; GAPDH is a loading control.

nated in HEK 293T control cells, but in the Pin1 knockdown cells SF-1 ubiquitination was clearly reduced, while in neither cell line did the SF-1 S203A appear to be ubiquitinated (Fig. 5D). These results indicate that Pin1 increases SF-1 ubiquitination through a mechanism involving Ser203.

GnRH treatment stimulates SF-1 ubiquitination. Ubiquitinated SF-1 is detectable in untreated L β T2 cells; however, GnRH treatment for 6 h greatly enhanced its levels (Fig. 6A).

Having demonstrated that Ser203 is crucial for Pin1-induced SF-1 ubiquitination, we hypothesized that phosphorylation of SF-1 by GnRH-activated ERK1/2 might lead to 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 effect (Fig. 6B). We went on to test the effect of ERK inhibition on SF-1 ubiquitination. In unstimulated cells, U0126

did not change the levels of poly-Ub SF-1; however, in GnRH-treated cells, U0126 reduced SF-1 polyubiquitination to a modest level compared to that in cells treated with GnRH alone (Fig. 6C). These results suggest that phosphorylation of SF-1 at S203 by GnRH-activated ERK2 contributes to SF-1 ubiquitination.

SF-1 was reported to be constitutively phosphorylated under normal culture conditions (16), and CDK7, a component of the general transcription factor IIH, was shown to phosphorylate SF-1 on Ser203 (28). Therefore, we asked whether SF-1 ubiquitination is also affected by CDK7-mediated phosphorylation of SF-1. *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. 6D). The specificity of CDK7 effect was shown by overexpressing a WT or dominant negative (DN) CDK7. Overexpression 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 (seen in the SF-1 blot [Fig. 6E]). These results demonstrate that ubiquitination of SF-1 is Ser203 phosphorylation dependent and that GnRH treatment increases SF-1 ubiquitination at least partially via the activation of ERK1/2, although CDK7 likely also plays a role.

SF-1 can be ubiquitinated or SUMOylated at Lys119. Given that SF-1 is SUMOylated at Lys119 and Lys194, and that a similar conjugation mechanism is shared for SUMOylation and ubiquitination, we examined whether SF-1 is ubiquitinated at these residues. 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 and K194R (2KR) was examined. The *in vivo* ubiquitination assay showed that WT HA-SF-1 and HA-SF-1 K194R were efficiently polyubiquitinated in L β T2 cells, while mutation of Lys119 to Arg prevented SF-1 from being polyubiquitinated (Fig. 7A). Thus, the ubiquitination site in SF-1 is Lys119.

Using an *in vivo* SUMOylation assay in which the same SF-1 mutant constructs were transfected, we found that SUMOylation of SF-1 was not affected by mutation of Ser203 to Ala (Fig. 7B). However, SUMOylation at Lys119 does not occur when Lys194 is mutated to Arg (Fig. 7B), indicating that SUMOylation at Lys194 is a prerequisite for Lys119 SUMOylation. Together, these results show that both ubiquitination and SUMOylation of SF-1 can occur at the same site, Lys119, and that this ubiquitination is Ser203 phosphorylation dependent, while SUMOylation of Lys119 is not, but requires Lys194 SUMOylation.

SF-1 ubiquitination, but not SUMOylation, facilitates interaction of SF-1 with Pitx1. Since ubiquitination and SUMOylation of SF-1 can occur at the same Lys119, we sought to determine whether the Ser203 phosphorylation-dependent ubiquitination of SF-1 promotes gonadotropin β -subunit gene transcription by increasing SF-1-Pitx1 interactions and whether this is proteasome dependent. Initially, Western analysis was performed using FLAG-precipitated samples from L β T2 cell lysates after transfection with FLAG-SF-1 and HA-Pitx1 (Fig. 8A), or on the endogenous proteins (Fig. 8B), in the absence or presence of MG132 for 6 h. In both cases, treatment with the proteasome inhibitor increased the binding between SF-1 and Pitx1 (Fig. 8A and B).

Both SF-1 and Pitx1 are polyubiquitinated in L β T2 cells, and treatment of cells with MG132 substantially increased the levels of both polyubiquitinated proteins (Fig. 8C). To exclude the possibility that their enhanced binding results from an effect of MG132 on Pitx1, we transfected L β T2 cells with FLAG-SF-1 and Myc-Ub or with FLAG-SF-1 and p β 40 Myc empty vector. The FLAG-SF-1 and associated proteins were precipitated and incubated with L β T2 cell lysates containing HA-Pitx1. Western blotting showed that Myc-Ub overexpression led to FLAG-SF-1 polyubiquitination, while the FLAG-SF-1-Ub-Myc bound more HA-Pitx1 than did the FLAG-SF-1 when transfected alone (Fig. 8D). Similarly, Myc-SUMO1 was transfected with FLAG-SF-1, which revealed SUMOylated FLAG-SF-1 but led to a decrease in the interaction between FLAG-SF-1 and HA-Pitx1 (Fig. 8D). 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 Lys119.

Polyubiquitin chains linked through Lys48 of ubiquitin reportedly target a protein for proteasomal degradation, while attachment through Lys63 is thought to indicate nonproteolytic functions, including transcriptional regulation (46, 56). Therefore, we performed ubiquitination assays to examine whether Lys48 or Lys63 is involved in the formation of polyubiquitin chains on SF-1. Lysates of L β T2 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 Ab. Mutation of either residue failed to prevent SF-1 ubiquitination; however, mutation of Lys63 reduced polyubiquitin chain formation on SF-1 more than mutation of Lys48 (Fig. 8E). This demonstrates that SF-1 can be ubiquitinated via Lys48- and Lys63-linked polyubiquitin chains and confirms the possibility that this modification is likely involved in several mechanisms of modulating SF-1 function.

FIG. 5. Ubiquitination of SF-1 requires both Ser203 and Pin1. (A) L β T2 cell lysates, after transfection with HA-SF-1 or HA-SF-1 S203A expression vector, were precipitated with mouse anti-HA Ab. The input and IP samples were analyzed by Western blotting using rabbit (Rb) anti-HA Ab and mouse antiubiquitin (Ub) Ab. (B) L β T2 cell lysates after transfection with HA-SF-1 or HA-SF-1 S203A and Myc-tagged ubiquitin expression vectors, were precipitated with mouse anti-Myc Ab, and samples analyzed using rabbit (Rb) anti-HA Ab. (C and D) Cell lysates from L β T2 cells transfected with FLAG-SF-1, 12 h after transfection of control or Pin1 siRNA, were precipitated with anti-FLAG M2 beads (C), or 293T control and Pin1 siRNA cells, transfected with HA-SF-1 or HA-SF-1 S203A expression vector, were precipitated with mouse anti-HA Ab (D). The samples were analyzed using antibodies to HA, FLAG, SF-1, Ub, and Pin1 with α -tubulin or GAPDH as a loading control. The monoubiquitinated SF-1 is marked with arrows on the left. An apparently nonspecific immunoreactive protein is marked with an asterisk.

FIG. 6. GnRH treatment stimulates SF-1 ubiquitination. (A) L β T2 cell lysates after transfection with FLAG-SF-1 and exposure of some cells to 100 nM GnRH for 6 h were precipitated with anti-FLAG M2 beads. The IP samples were analyzed using anti-FLAG, Rb anti-FLAG, and anti-Ub Ab. (B) Western blotting detected phosphorylated ERK (pERK) in L β T2 cells after exposure to 100 nM GnRH for 0 to 4 h and/or 1 μ M U0126; total ERK is shown as a loading control. (C and D) L β T2 cell lysates after transfection with WT FLAG-SF-1 or SF-1 S203A, some of which were exposed to 100 nM GnRH and 1 μ M U0126 (C) or 15 μ M roscovitine (ROS) (D) for 0 or 4 h, were precipitated with anti-FLAG M2 beads. The input and IP samples were analyzed using anti-FLAG, anti-Ub, and anti-GAPDH Ab. (E) L β T2 cell lysates after transfection with FLAG-SF-1 together with pcDNA3, pcDNA3 CDK7 HA, or pcDNA3 CDK7 D155A HA were precipitated with anti-FLAG M2 beads. The input and IP samples were analyzed by Western blotting. Monoubiquitinated SF-1 is marked with arrows on the left.

FIG. 8. SF-1 ubiquitination, but not SUMOylation, facilitates its interaction with Pitx1. (A) LBT2 cell lysates, after cotransfection with HA-Pitx1 and FLAG-SF-1 or ptx40 FLAG empty vector and exposure of some cells to 5 μ M MG132 for 6 h, were precipitated with anti-FLAG M2 beads. The samples were analyzed using anti-FLAG, Rb anti-FLAG, and anti-HA Ab. (B and C) Cell lysates from LBT2 cells, some of which had been exposed to 5 μ M MG132 for 6 h, were precipitated with normal IgG or anti-SF-1 Ab (B and C) and also anti-Pitx1 Ab (C), and samples

Finally, to test whether the GnRH-induced ubiquitination of SF-1 causes a change in its localization, we examined the localization of GFP-SF-1 and GFP-SF-1 K119R in L β T2 cells treated with GnRH and/or MG132. In nontreated 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. 8F). It appears, therefore, that nuclear export of SF-1 by GnRH is followed by either proteasome degradation or deubiquitination in the cytosol.

Pin1 targets SF-1 to increase its interaction with Pitx1. We have 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, HA-Pitx1, and/or HA-Egr-1 before carrying out co-IP using the cell lysates. Western blotting showed that the interaction between SF-1 and Pitx1 was markedly reduced in MEF Pin1^{-/-} cells compared to that in MEF WT cells (Fig. 9A). This contrasted with the interaction of SF-1 with Egr-1, which did not differ between the cell lines, while the interaction of Pitx1 with Egr-1 was only marginally reduced in the Pin1 knockout cells (Fig. 9A). Notably, 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. 9B).

In order to confirm the factor targeted by Pin1 that is responsible for the increase in Pitx1-SF-1 interaction, we examined 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. 3E), mutation in Pitx1 of Ser207, Ser259, or Thr267 and Ser270 did not lead to significant alteration of its interaction with SF-1 (Fig. 9C). However, mutation of SF-1 at Ser203 markedly reduced the interaction with Pitx1 (Fig. 9D). These results indicate that Pin1 increases the interaction of SF-1 with Pitx1 through targeting SF-1.

DISCUSSION

We hypothesized in this study that Pin1 plays a role in activating GnRH-stimulated gonadotropin subunit gene expression through interactions with phosphorylated transcription factors, involving changes in stability and/or protein-protein interactions. We further hypothesized that Pin1 activity might be regulated by GnRH. The data presented support this role for Pin. We show that Pin1 is required for the full GnRH response of the gonadotropin β -subunit genes, while its overexpression increased basal and GnRH-activated transcription,

as well as that specifically activated by various transcription factors. One of the mechanisms of this Pin1 action is through targeting SF-1, a key factor regulating all three gonadotropin subunit genes (18, 71). We show that Pin1-mediated SF-1 isomerization coordinates phosphorylation and ubiquitination, which facilitates Pitx1 binding, to increase SF-1 transcriptional activity. Moreover, GnRH increases Pin1 expression levels and regulates its activity through both phosphorylation and dephosphorylation, indicating that Pin1 is an integral part of GnRH signaling (Fig. 10).

Pin1 expression is reportedly mediated through the activation of E2F by various oncogenes and Ras-activated signaling, possibly involving Sp1, which can interact with E2F; notably, the Pin1 promoter has two GC boxes that might confer Sp1 binding (51). In the gonadotrope, the Ras-MEK-pathway is activated by GnRH, which stimulates E2F (4, 42), and Sp1 mediates some of the GnRH responsiveness of the LH β gene (20), suggesting a possible mechanism of GnRH activation of Pin1 expression. However, GnRH also rapidly increased Pin1 phosphorylation on S16, which was shown to inactivate Pin1 by preventing its interaction with the substrate and its translocation into the nucleus (35). This apparent paradox is reconciled by the discovery that GnRH dephosphorylates Pin1 through calcineurin, which presumably reactivates Pin1, providing an additional regulatable pathway to ensure sufficient levels of active nuclear Pin1. To our knowledge, this is the first report that links these two proteins and indicates that Pin1 activity can be regulated by the same extracellular signals that activate the phosphorylation cascade targeted by Pin1.

Pin1 was found at the promoters of both gonadotropin β -subunit genes, presumably recruited through SF-1, Pitx1, and/or Egr-1, with which it interacts and in some cases shows functional synergy, although this appears to be promoter context specific. Most striking was the functional interaction with SF-1 on the LH β gene, despite a clear reduction by Pin1 of SF-1 protein levels, which contrasted with the opposite effect on Pitx1. SF-1 is ubiquitinated (6, 60), and we have shown that this is dependent on Pin1 and the phosphorylation of Ser203. Ser203, which is recognized and bound by Pin1, is found in the hinge region adjacent to helix 1, and its phosphorylation was shown previously to induce major conformational change in the protein, as indicated by a change in protease sensitivity. That study also showed that the activity of this hinge/helix 1 region was enhanced by phosphorylation and that the S203 phosphorylation increased cofactor recruitment, likely as a result of the change in conformation (9). It is quite possible that this conformational change is the Pin1-induced isomerization, which exposes SF-1 to the ubiquitin-conjugation machinery,

were analyzed by Western blotting using anti-SF-1 and anti-Pitx1 Ab (B) or anti-Ub Ab (C). (D) L β T2 cells were cotransfected with FLAG-SF-1 or ptx40 FLAG empty vector and Myc-Ub, Myc-SUMO1, or ptx 40 Myc empty vector. After 24 h, the cells were lysed in NEM-RIPA buffer, and lysates were precipitated with anti-FLAG M2 beads. After washing, the FLAG beads were incubated with HA-Pitx1-containing cell lysates for 3 h. The IP samples were analyzed using Rb anti-FLAG, anti-Myc, and anti-HA Ab. (E) L β T2 cell lysates, after cotransfection with ptx40 FLAG or FLAG-SF-1 and HA-Ub K48R or HA-Ub K63R, were precipitated with anti-FLAG M2 beads. The samples were analyzed using Rb anti-FLAG, anti-FLAG, anti-HA, and anti- α -tubulin Ab. (F) L β T2 cells were transfected with ptx40 GFP, ptx40 GFP SF-1, or ptx40 GFP SF-1 K119R, and some were exposed to 5 μ M MG132 and/or 100 nM GnRH for 6 h. The cells were fixed, stained with DAPI, and analyzed by LSM510 confocal fluorescence microscopy. Bar, 5 μ m.

FIG. 10. 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. DeSUMOylation of SF-1 at K194, which occurs through an unknown mechanism, results in enhanced phosphorylation of SF-1 at Ser203 by CDK7 (67). DeSUMOylation at K119 and 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 K119, 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, for which we do not yet have direct proof. Dashed lines represent pathways that have or may have (for PKA and PKC phosphorylation of Pin1) intermediate elements that are not shown.

polyubiquitination and eventual promoter clearance of SF-1 are likely important for the next round of transcription initiation, as shown for other transcriptional activators (47, 63). This appears to be similar to the GnRH-induced ubiquitination of estrogen receptor α (ER α) in activation of the LH β gene promoter, although it involves a very different mechanism, in which GnRH upregulates the ubiquitin-conjugating enzyme, Ubc4, that targets ER α (36). This pathway of SF-1–Pitx1 interaction, clearance, and subsequent degradation via Pin1-stimulated ubiquitination would explain the requirement for Pin1 and yet its reduction in SF-1 protein levels. However, this is clearly not a universal action of Pin1, as the same drop in protein levels of Pitx1 and Egr-1 were not noted, despite its interaction with both proteins at the LH β promoter.

This model is consistent with ubiquitination of SRC-3, which is dependent on GSK3-induced phosphorylation and controls both its transactivation and its clearance from the promoter. It was proposed that the transition from mono- to polyubiquitination, which occurs during transcription, serves as a clock to regulate its lifetime (63). In a different study, Pin1 was seen to interact with phosphorylated SRC-3, regulate its cellular turn-

over, and enhance its functional interaction with CBP/p300 (69). These observations 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, such as Bcl2, c-Myc, and cyclin E (2, 45, 52, 68). Negative regulation by Pin1-induced ubiquitination was also shown for Daxx, which inhibits the apoptotic response as a result of its proteasomal degradation (49). A recent report showed 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 (41). A change in protein-protein interactions in this pathway, as a result of Pin1 binding, was also 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 (8). However, in these reports, the outcome of the Pin1-mediated ubiquitination is negative regulation. Our results, as well as those pertaining to the activity of SRC-3, indicate that the Pin1-induced isomerization allows access to E3 ligases which promote assorted ubiquitin linkages with diverse outcomes.

We have demonstrated that ubiquitin targets SF-1 on Lys119, which is also targeted by SUMO. SF-1 is SUMOylated at two conserved lysines, Lys119 and Lys194, which reside adjacent to the DBD and LBD, respectively, and this modification clearly represses SF-1 transactivation (5, 22, 26, 67). This was attributed to the SUMOylation on Lys194, which was shown to reduce Ser203 phosphorylation, decrease coregulator binding, and cause a selective loss of binding to certain target genes (5, 67). In contrast, we found that SF-1 SUMOylation was not affected by S203 phosphorylation and that SUMOylation on Lys194 is a prerequisite for SUMOylation on Lys119, which has been confirmed in other reports (22, 69). Our results indicate that ubiquitination of SF-1 on Lys119 enhances, while SUMOylation inhibits, its interaction with Pitx1, which functions as a crucial coactivator in gonadotropin gene expression (58).

The transcriptional activity of SF-1 is thus dynamically modulated by a pathway of posttranslational modifications. The deSUMOylation on Lys194 which facilitates Ser203 phosphorylation allows Pin1-mediated isomerization, enabling the subsequent Lys119 ubiquitination, to facilitate the interaction with its specific coactivator, Pitx1, and promoter activation. We have demonstrated a central role for Pin1 in controlling the activity of SF-1, a key factor regulating all three subunit genes. Given that Pin1 expression and activity are also regulated by GnRH, we propose Pin1 as a novel mediator in GnRH-activated signal transduction pathways. Transcription is often stimulated by extracellular signaling involving activation of MAPK kinases which phosphorylate gene-specific transcription factors, but this is just one of many types of posttranslational modifications that determine the rapid activation and tight regulation of gene-specific transcription factors (3). It seems clear that in many cases Pin1 may have a crucial role in recognizing this phosphorylation signal and in coordinating additional modifications through isomerization to facilitate access to various modifying enzymes, which lead to diverse outcomes.

ACKNOWLEDGMENTS

This research was supported by funding from the Ministry of Education Academic Research Fund, grant number R-154-000-410-112.

We thank Lilach Pnueli and Ye Fan for technical help. Thanks go to P. Mellon for the α T3-1 and L β T2 cells, K. P. Lu for MEF WT and MEF Pin1^{-/-} cells, and Q. Y. Yang for HEK 293T Ctrl siRNA and Pin1 siRNA cells. We also thank J. Drouin, J. Milbrandt, and K. Parker for Pitx1, SF-1, and Egr-1 expression vectors and B. C. Low for ptx40 HA, FLAG, and Myc vectors.

REFERENCES

- Atchison, F. W., and A. R. Means. 2003. Spermatogonial depletion in adult Pin1-deficient mice. *Biol. Reprod.* **69**:1989–1997.
- Basu, A., and S. Haldar. 2002. Signal-induced site specific phosphorylation targets Bcl2 to the proteasome pathway. *Int. J. Oncol.* **21**:597–601.
- Benayoun, B. A., and R. A. Veitia. 2009. A post-translational modification code for transcription factors: sorting through a sea of signals. *Trends Cell Biol.* **19**:189–197.
- Berkovich, E., and D. Ginsberg. 2001. Ras induces elevation of E2F-1 mRNA levels. *J. Biol. Chem.* **276**:42851–42856.
- Campbell, L. A., E. J. Faivre, M. D. Show, J. G. Ingraham, J. Flinders, J. D. Gross, and H. A. Ingraham. 2008. Decreased recognition of SUMO-sensitive target genes following modification of SF-1 (NR5A1). *Mol. Cell. Biol.* **28**:7476–7486.
- Chen, W. Y., J. H. Weng, C. C. Huang, and B. C. Chung. 2007. Histone deacetylase inhibitors reduce steroidogenesis through SCF-mediated ubiquitination and degradation of steroidogenic factor 1 (NR5A1). *Mol. Cell. Biol.* **27**:7284–7290.
- Coss, D., S. B. Jacobs, C. E. Bender, and P. L. Mellon. 2004. A novel AP-1 site is critical for maximal induction of the follicle-stimulating hormone β gene by gonadotropin-releasing hormone. *J. Biol. Chem.* **279**:152–162.
- De Nicola, F., T. Bruno, S. Iezzi, M. Di Padova, A. Floridi, C. Passananti, G. Del Sal, and M. Fanciulli. 2007. The prolyl isomerase Pin1 affects Che-1 stability in response to apoptotic DNA damage. *J. Biol. Chem.* **282**:19685–19691.
- Desclozeaux, M., I. N. Krylova, F. Horn, R. J. Fletterick, and H. A. Ingraham. 2002. Phosphorylation and intramolecular stabilization of the ligand binding domain in the nuclear receptor steroidogenic factor 1. *Mol. Cell. Biol.* **22**:7193–7203.
- Dorn, C., Q. Ou, J. Svaren, P. A. Crawford, and Y. Sadovsky. 1999. Activation of Luteinizing hormone gene by gonadotropin-releasing hormone requires the synergy of early growth response-1 and steroidogenic factor-1. *J. Biol. Chem.* **274**:13870–13876.
- Feng, J., M. A. Lawson, and P. Melamed. 2008. A proteomic comparison of immature and mature mouse gonadotrophs reveals novel differentially expressed nuclear proteins that regulate gonadotropin gene transcription and RNA splicing. *Biol. Reprod.* **79**:546–561.
- Fowkes, R. C., M. Desclozeaux, M. V. Patel, S. J. B. Aylwin, P. King, H. A. Ingraham, and J. M. Burrin. 2003. Steroidogenic factor-1 and the gonadotropin-specific element enhance basal and pituitary adenylate cyclase-activating polypeptide-stimulated transcription of the human glycoprotein hormone α -subunit gene in gonadotropes. *Mol. Endocrinol.* **17**:2177–2188.
- Garrett, S., W. A. Barton, R. Knights, P. Jin, D. O. Morgan, and R. P. Fisher. 2001. Reciprocal activation by cyclin-dependent kinases 2 and 7 is directed by substrate specificity determinants outside the T loop. *Mol. Cell. Biol.* **21**:88–99.
- Gianni, M., A. Boldetti, V. Guarnaccia, A. Rambaldi, E. Parrella, I. Raska, Jr., C. Rochette-Egly, G. Del Sal, A. Rustighi, M. Terao, and E. Garattini. 2009. Inhibition of the peptidyl-prolyl-isomerase Pin1 enhances the responses of acute myeloid leukemia cells to retinoic acid via stabilization of RAR α and PML-RAR α . *Cancer Res.* **69**:1016–1026.
- Halvorson, L. M., U. B. Kaiser, and W. W. Chin. 1999. The protein kinase C system acts through the early growth response protein 1 to increase LH β gene expression in synergy with steroidogenic factor-1. *Mol. Endocrinol.* **13**:106–116.
- Hammer, G. D., I. Krylova, Y. Zhang, B. D. Darimont, K. Simpson, N. L. Weigel, and H. A. Ingraham. 1999. Phosphorylation of the nuclear receptor SF-1 modulates cofactor recruitment: Integration of hormone signaling in reproduction and stress. *Mol. Cell* **3**:521–526.
- Hicke, L. 2001. Protein regulation by monoubiquitin. *Nat. Rev. Mol. Cell Biol.* **2**:195–201.
- Ingraham, H. A., D. S. Lala, Y. Ikeda, X. Luo, W. H. Shen, M. W. Nachtigal, R. Abbud, J. H. Nilson, and K. L. Parker. 1994. The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. *Genes Dev.* **8**:2302–2312.
- Jorgensen, J. S., C. C. Quirk, and J. H. Nilson. 2004. Multiple and overlapping combinatorial codes orchestrate hormonal responsiveness and dictate cell-specific expression of the genes encoding luteinizing hormone. *Endocr. Rev.* **25**:521–542.
- Kaiser, U. B., E. Sabbagh, M. T. Chen, W. W. Chin, and B. D. Saunders. 1998. Sp1 binds to the rat luteinizing hormone β (LH β) gene promoter and mediates gonadotropin-releasing hormone-stimulated expression of the LH β subunit gene. *J. Biol. Chem.* **273**:12943–12951.
- Kaiser, U. B., L. M. Halvorson, and M. T. Chen. 2000. Sp1, steroidogenic factor 1 (SF-1), and early growth response protein 1 (egr-1) binding sites form a tripartite gonadotropin-releasing hormone response element in the rat luteinizing hormone- β gene promoter: an integral role for SF-1. *Mol. Endocrinol.* **14**:1235–1245.
- Komatsu, T., H. Mizusaki, T. Mukai, H. Ogawa, D. Baba, M. Shirakawa, S. Hatakeyama, K. I. Nakayama, H. Yamamoto, A. Kikuchi, and K. Morohashi. 2004. Small ubiquitin-like modifier 1 (SUMO-1) modification of the synergy control motif of Ad4 binding protein/steroidogenic factor 1 (Ad4BP/SF-1) regulates synergistic transcription between Ad4BP/SF-1 and Sox9. *Mol. Endocrinol.* **18**:2451–2462.
- Lamba, P., V. Khivansara, A. C. D'Alessio, M. M. Santos, and D. J. Bernard. 2008. Paired-like homeodomain transcription factors 1 and 2 regulate follicle-stimulating hormone β -subunit transcription through a conserved cis-element. *Endocrinology* **149**:3095–3108.
- Lancôt, C., A. Moreau, M. Chamberland, M. L. Tremblay, and J. Drouin. 1999. Hindlimb patterning and mandible development require the Ptx1 gene. *Development* **126**:1805–1810.
- Lancôt, C., B. Lamolet, and J. Drouin. 1997. The bicoid-related homeoprotein Ptx1 defines the most anterior domain of the embryo and differentiates posterior from anterior lateral mesoderm. *Development* **124**:2807–2817.
- Lee, M. B., L. A. Lebedeva, M. Suzawa, S. A. Wadekar, M. Desclozeaux, and H. A. Ingraham. 2005. The DEAD-box protein DP103 (Ddx20 or Gemin-3) represses orphan nuclear receptor activity via SUMO modification. *Mol. Cell. Biol.* **25**:1879–1890.
- Lee, T. H., A. Tun-Kyi, R. Shi, J. Lim, C. Soohoo, G. Finn, M. Balastik, L. Pastorino, G. Wulf, X. Z. Zhou, and K. P. Lu. 2009. Essential role of Pin1 in

- the regulation of TRF1 stability and telomere maintenance. *Nat. Cell Biol.* **11**:97–105.
28. Lewis, A. E., M. Rusten, E. A. Hoivik, E. L. Vikse, M. L. Hansson, A. E. Wallberg, and M. Bakke. 2008. Phosphorylation of steroidogenic factor 1 is mediated by cyclin-dependent kinase 7. *Mol. Endocrinol.* **22**:91–104.
 29. Lim, S., L. Pnueli, J. H. Tan, Z. Naor, G. Rajagopal, and P. Melamed. 2009. Negative feedback governs gonadotrope frequency-decoding of gonadotropin releasing hormone pulse-frequency. *PLoS One* **29**:e7244.
 30. Lim, S., M. Luo, M. Koh, M. Yang, M. N. bin Abdul Kadir, J. H. Tan, Z. Ye, W. Wang, and P. Melamed. 2007. Distinct mechanisms involving diverse histone deacetylases repress expression of the two gonadotropin β -subunit genes in immature gonadotropes, and their actions are overcome by gonadotropin-releasing hormone. *Mol. Cell. Biol.* **27**:4105–4120.
 31. Liou, Y. C., A. Ryo, H. K. Huang, P. J. Lu, R. Bronson, F. Fujimori, T. Uchida, T. Hunter, and K. P. Lu. 2002. Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. *Proc. Natl. Acad. Sci. U. S. A.* **99**:1335–1340.
 32. Liou, Y. C., A. Sun, A. Ryo, X. Z. Zhou, Z. X. Yu, H. K. Huang, T. Uchida, R. Bronson, G. Bing, X. Li, T. Hunter, and K. P. Lu. 2003. Role of the prolyl isomerase Pin1 in protecting against age-dependent neurodegeneration. *Nature* **424**:556–561.
 33. Lu, K. P., G. Finn, T. H. Lee, and L. K. Nicholson. 2007. Prolyl *cis-trans* isomerization as a molecular timer. *Nat. Chem. Biol.* **3**:619–629.
 34. Lu, K. P., and X. Z. Zhou. 2007. The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease. *Nat. Rev. Mol. Cell Biol.* **8**:904–916.
 35. Lu, P. J., X. Z. Zhou, Y. C. Liou, J. P. Noel, and K. P. Lu. 2002. Critical role of WW domain phosphorylation in regulating phosphoserine binding activity and Pin1 function. *J. Biol. Chem.* **277**:2381–2384.
 36. Luo, M., M. Koh, J. Feng, Q. Wu, and P. Melamed. 2005. Cross talk in hormonally regulated gene transcription through induction of estrogen receptor ubiquitylation. *Mol. Cell. Biol.* **25**:7386–7398.
 37. Melamed, P. 2010. Hormonal signaling to follicle stimulating hormone β -subunit gene expression. *Mol. Cell. Endocrinol.* **314**:204–212.
 38. Melamed, P., M. Koh, P. Preklathan, L. Bei, and C. Hew. 2002. Multiple mechanisms for Ptx-1 transactivation of a luteinizing hormone β subunit gene. *J. Biol. Chem.* **277**:26200–26207.
 39. Melamed, P., M. N. Abdul Kadir, A. Wijeweera, and S. Seah. 2006. Transcription of gonadotropin β subunit genes involves cross-talk between the transcription factors and co-regulators that mediate actions of the regulatory hormones. *Mol. Cell. Endocrinol.* **252**:167–183.
 40. Muratani, M., and W. P. Tansey. 2003. How the ubiquitin-proteasome system controls transcription. *Nat. Rev. Mol. Cell Biol.* **4**:192–201.
 41. Nakano, A., D. Koinuma, K. Miyazawa, T. Uchida, M. Saitoh, M. Kawabata, J. Hanai, H. Akiyama, M. Abe, K. Miyazono, T. Matsumoto, and T. Imamura. 2009. Pin1 down-regulates transforming growth factor- β (TGF- β) signaling by inducing degradation of smad proteins. *J. Biol. Chem.* **284**:6109–6115.
 42. Naor, Z. 2009. Signaling by G-protein-coupled receptor (GPCR): studies on the GnRH receptor. *Front. Neuroendocrinol.* **30**:10–29.
 43. Newton, K., M. L. Matsumoto, I. E. Wertz, D. S. Kirkpatrick, J. R. Lill, J. Tan, D. Dugger, N. Gordon, S. S. Sidhu, F. A. Fellouse, L. Komuves, D. M. French, R. E. Ferrando, C. Lam, D. Compagn, C. Yu, I. Bosanac, S. G. Hymowitz, R. F. Kelley, and V. M. Dixit. 2008. Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies. *Cell* **134**:668–678.
 44. Parker, K. L., and B. P. Schimmer. 1996. The roles of the nuclear receptor steroidogenic factor 1 in endocrine differentiation and development. *Trends Endocrinol. Metab.* **7**:203–207.
 45. Phan, R. T., M. Saito, Y. Kitagawa, A. R. Means, and R. Dalla-Favera. 2007. Genotoxic stress regulates expression of the proto-oncogene Bcl6 in germinal center B cells. *Nat. Immunol.* **8**:1132–1139.
 46. Pickart, C. M., and D. Fushman. 2004. Polyubiquitin chains: polymeric protein signals. *Curr. Opin. Chem. Biol.* **8**:610–616.
 47. Reid, G., M. R. Hübner, R. Métivier, H. Brand, S. Denger, D. Manu, J. Beaudouin, J. Ellenberg, and F. Gannon. 2003. Cyclic, proteasome-mediated turnover of unliganded and liganded ER α on responsive promoters is an integral feature of estrogen signaling. *Mol. Cell* **11**:695–707.
 48. Rustighi, A., L. Tiberi, A. Soldano, M. Napoli, P. Nuciforo, A. Rosato, F. Kaplan, A. Capobianco, S. Pece, P. P. Di Fiore, and G. Del Sal. 2009. The prolyl-isomerase Pin1 is a Notch1 target that enhances Notch1 activation in cancer. *Nat. Cell Biol.* **11**:133–142.
 49. Ryo, A., A. Hirai, M. Nishi, Y. C. Liou, K. Perrem, S. C. Lin, H. Hirano, S. W. Lee, and I. Aoki. 2007. A suppressive role of the prolyl isomerase Pin1 in cellular apoptosis mediated by the death-associated protein Daxx. *J. Biol. Chem.* **282**:36671–36681.
 50. Ryo, A., H. Uemura, H. Ishiguro, T. Saitoh, A. Yamaguchi, K. Perrem, Y. Kubota, K. P. Lu, and I. Aoki. 2005. Stable suppression of tumorigenicity by Pin1-targeted RNA interference in prostate cancer. *Clin. Cancer Res.* **11**:7523–7531.
 51. Ryo, A., Y. C. Liou, G. Wulf, M. Nakamura, S. W. Lee, and K. P. Lu. 2002. Pin1 is an E2F target gene essential for the Neu/Ras-induced transformation of mammary epithelial cells. *Mol. Cell. Biol.* **22**:5281–5295.
 52. Sears, R. C. 2004. The life cycle of C-myc: from synthesis to degradation. *Cell Cycle* **3**:1133–1137.
 53. Shaw, P. E. 2007. Peptidyl-prolyl *cis/trans* isomerases and transcription: is there a twist in the tail? *EMBO Rep.* **8**:40–45.
 54. Sims, J. J., and R. E. Cohen. 2009. Linkage-specific avidity defines the lysine 63-linked polyubiquitin-binding preference of rap80. *Mol. Cell* **33**:775–783.
 55. Stallings, N. R., N. A. Hanley, G. Majdic, L. Zhao, M. Bakke, and K. L. Parker. 2002. Development of a transgenic green fluorescent protein lineage marker for steroidogenic factor 1. *Mol. Endocrinol.* **16**:2360–2370.
 56. Sun, L., and Z. J. Chen. 2004. The novel functions of ubiquitination in signaling. *Curr. Opin. Cell Biol.* **16**:119–126.
 57. Takahashi, K., C. Uchida, R. W. Shin, K. Shimazaki, and T. Uchida. 2008. Prolyl isomerase, Pin1: new findings of post-translational modifications and physiological substrates in cancer, asthma and Alzheimer's disease. *Cell. Mol. Life Sci.* **65**:359–375.
 58. Tremblay, J. J., A. Marcil, Y. Gauthier, and J. Drouin. 1999. Ptx1 regulates SF-1 activity by an interaction that mimics the role of the ligand-binding domain. *EMBO J.* **18**:3431–3441.
 59. Tremblay, J. J., and J. Drouin. 1999. Egr-1 is a downstream effector of GnRH and synergizes by direct interaction with Ptx1 and SF-1 to enhance luteinizing hormone β gene transcription. *Mol. Cell. Biol.* **19**:2567–2576.
 60. Walsh, H. E., and M. A. Shupnik. 2009. Proteasome regulation of dynamic transcription factor occupancy on the GnRH-stimulated luteinizing hormone β -subunit promoter. *Mol. Endocrinol.* **23**:237–250.
 61. Wang, H., A. Matsuzawa, S. A. Brown, J. Zhou, C. S. Guy, P. H. Tseng, K. Forbes, T. P. Nicholson, P. W. Sheppard, H. Häcker, M. Karin, and D. A. Vignali. 2008. Analysis of nondegradative protein ubiquitylation with a monoclonal antibody specific for lysine-63-linked polyubiquitin. *Proc. Natl. Acad. Sci. U. S. A.* **105**:20197–20202.
 62. Wang, Y., J. Fortin, P. Lamba, M. Bonomi, L. Persani, M. S. Roberson, and D. J. Bernard. 2008. Activator protein-1 and smad proteins synergistically regulate human follicle-stimulating hormone β -promoter activity. *Endocrinology* **149**:5577–5591.
 63. Wu, R. C., Q. Feng, D. M. Lonard, and B. W. O'Malley. 2007. SRC-3 coactivator functional lifetime is regulated by a phospho-dependent ubiquitin time clock. *Cell* **129**:1125–1140.
 64. Wulf, G., A. Ryo, Y. C. Liou, and K. P. Lu. 2003. The prolyl isomerase Pin1 in breast development and cancer. *Breast Cancer Res.* **5**:76–82.
 65. Wulf, G., P. Garg, Y. C. Liou, D. Iglehart, and K. P. Lu. 2004. Modeling breast cancer in vivo and ex vivo reveals an essential role of Pin1 in tumorigenesis. *EMBO J.* **23**:3397–3407.
 66. Xu, K., H. Shimelis, D. E. Linn, R. Jiang, X. Yang, F. Sun, Z. Guo, H. Chen, W. Li, H. Chen, X. Kong, J. Melamed, S. Fang, Z. Xiao, T. D. Veenstra, and Y. Qiu. 2009. Regulation of androgen receptor transcriptional activity and specificity by RNF6-induced ubiquitination. *Cancer Cell* **15**:270–282.
 67. Yang, W. H., J. H. Heaton, H. Brevig, S. Mukherjee, J. A. Iñiguez-Lluhi, and G. D. Hammer. 2009. SUMOylation inhibits SF-1 activity by reducing CDK7-mediated serine 203 phosphorylation. *Mol. Cell. Biol.* **29**:613–625.
 68. Yeh, E. S., B. O. Lew, and A. R. Means. 2006. The loss of PIN1 deregulates cyclin E and sensitizes mouse embryo fibroblasts to genomic instability. *J. Biol. Chem.* **281**:241–251.
 69. Yi, P., R. C. Wu, J. Sandquist, J. Wong, S. Y. Tsai, M. J. Tsai, A. R. Means, and B. W. O'Malley. 2005. Peptidyl-prolyl isomerase 1 (Pin1) serves as a coactivator of steroid receptor by regulating the activity of phosphorylated steroid receptor coactivator 3 (SRC-3/AIB1). *Mol. Cell. Biol.* **25**:9687–9699.
 70. Zakaria, M. M., K. H. Jeong, C. Lacza, and U. B. Kaiser. 2002. Pituitary homeobox 1 activates the rat FSH β (rFSH β) gene through both direct and indirect interactions with the rFSH β gene promoter. *Mol. Endocrinol.* **16**:1840–1852.
 71. Zhao, L., M. Bakke, and K. L. Parker. 2001. Pituitary-specific knockout of steroidogenic factor 1. *Mol. Cell. Endocrinol.* **185**:27–32.