Extracellular Signal-regulated Kinase (ERK)-dependent Phosphorylation of Y-Box-binding Protein 1 (YB-1) Enhances Gene Expression in Granulosa Cells in Response to Follicle-stimulating Hormone (FSH)*

Elyse M. Donaubauer and Mary E. Hunzicker-Dunn[1]

From the School of Molecular Biosciences, Washington State University, Pullman, Washington 99164

Within the ovarian follicle, immature oocytes are surrounded and supported by granulosa cells (GCs). Stimulation of GCs by FSH leads to their proliferation and differentiation, events that are necessary for fertility. FSH activates multiple signaling pathways to regulate genes necessary for follicular maturation. Herein, we investigated the role of Y-box-binding protein-1 (YB-1) within GCs. YB-1 is a nucleic acid binding protein that regulates transcription and translation. Our results show that FSH promotes an increase in the phosphorylation of YB-1 on Ser^{102} within 15 min that is maintained at significantly increased levels until ~8 h post treatment. FSH-stimulated phosphorylation of YB-1(Ser^{102}) is prevented by pretreatment of GCs with the PKA-selective inhibitor PKA inhibitor (PKI), the MEK inhibitor PD98059, or the ribosomal S6 kinase-2 (RSK-2) inhibitor BI-D1870. Thus, phosphorylation of YB-1 on Ser^{102} is PKA-, ERK-, and RSK-2-dependent. However, pretreatment of GCs with the protein phosphatase 1 (PP1) inhibitor tautomycin increased phosphorylation of YB-1(Ser^{102}) in the absence of FSH; FSH did not further increase YB-1(Ser^{102}) phosphorylation. This result suggests that the major effect of RSK-2 is to inhibit PP1 rather than to directly phosphorylate YB-1 on Ser^{102}. YB-1 coimmunoprecipitated with PP1β catalytic subunit and RSK-2. Transduction of GCs with the dephospho-adenoviral-YB-1(S102A) mutant prevented the induction by FSH of *Egfr*, *Cyp19a1*, *Inha*, *Lhcgr*, *Cyp11a1*, *Hsd17b1*, and *Pappa* mRNAs and estradiol-17β production. Collectively, our results reveal that phosphorylation of YB-1 on Ser^{102} via the ERK/RSK-2 signaling pathway is necessary for FSH-mediated expression of target genes required for maturation of follicles to a preovulatory phenotype.

In the female, fertility requires maturation of the ovarian follicle that contains the oocyte surrounded by granulosa cells (GCs)[2] and theca cells. Follicular maturation is a tightly regu-

lated process that is initiated by FSH. FSH regulates at least 500 target genes within GCs whose expression drives development of the follicle, allowing it to respond to the surge of luteinizing hormone (LH) that promotes ovulation, oocyte maturation, and formation of the corpus luteum that serves to support the developing embryo after fertilization and implantation (for review, see Refs. 1 and 2).

The mechanism by which FSH signals to regulate gene and protein expression in GCs has been extensively investigated. FSH binds to its G protein-coupled receptor (GPCR) expressed exclusively on GCs to activate adenylyl cyclase, raise intracellular cAMP levels, and activate PKA (3–6). PKA then either directly phosphorylates proteins that regulate transcription or indirectly activates signaling cascades whose targets regulate primarily transcription and translation. Direct PKA targets in GCs include cAMP-response element binding protein (CREB) (Ser^{133}) (7), histone H3 (Ser^{10}) (8), and β-catenin (Ser^{552} and Ser^{675}) (9). Upon phosphorylation, these direct PKA target proteins participate in the regulation of gene expression though interactions with DNA and additional transcription factors.

One of the signaling cascades activated by FSH is the MAPK/ERK pathway (8, 10), evidenced by the phosphorylation of ERK (Thr^{202}/Tyr^{204}) within 10 min of FSH treatment (11), and phosphorylation of its downstream target RSK-2 (Ser^{380}) (8), shown schematically in Fig. 1. FSH also activates the PI3K/protein kinase B (AKT) pathway (12, 13). In a PKA-dependent manner, FSH enhances phosphorylation of the pivotal kinase AKT on both the proximal Thr^{308} and the distal Ser^{473} sites within 5 min after FSH treatment (12, 14) followed by the rapid phosphorylation of downstream AKT targets, tuberin and forkhead box-containing protein O1 (FOXO1) on Thr^{1462} and Ser^{256}, respectively (12, 15, 16).

However, the mechanisms by which these signaling pathways regulate gene expression are less well understood. One of the more important pathways activated by FSH is the PI3K/AKT pathway, based on evidence that this pathway is necessary for GC differentiation (9, 15, 17, 18). Yet the only transcriptional factor regulated by this pathway that has been shown to

[1] To whom correspondence should be addressed: Biotechnology Life Sciences Bldg., Rm. 202, Washington State University, Pullman WA 99164. Tel.: 509-335-5614; E-mail: mehd@vetmed.wsu.edu.

[2] The abbreviations used are: GC, granulosa cell; YB-1, Y-box-binding protein-1; FSH, follicle stimulating hormone; PKI, PKA inhibitor; RSK-2, ribo-

somal S6 kinase-2; PP1 and PP2, protein phosphatase 1 or 2; LH, luteinizing hormone; CREB, cAMP-response element-binding protein; AKT, protein kinase B; FOXO1, forkhead box-containing protein O1; SHP2, SRC homology-2 (SH2) domain-containing tyrosine phosphatase; E2, estradiol-17β; P, penicillin; S, streptomycin; mRNP, mRNA-protein complex; RIA, radioimmunoassay; EGFR, epidermal growth factor receptor; CPT, 8-chlorophenyl-thiol; ANOVA, analysis of variance; Ad-E, adenoviral empty.
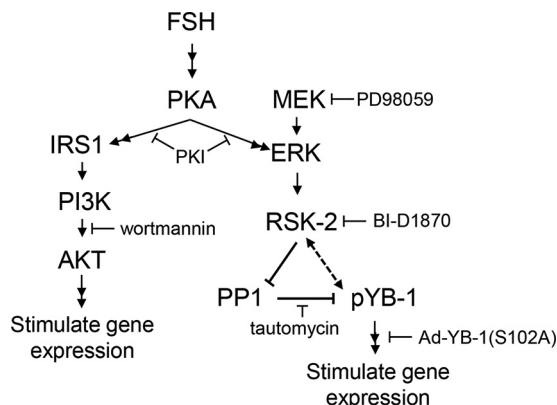
FIGURE 1. **Proposed signaling pathway by which FSH promotes the PKA-dependent phosphorylation of YB-1 on Ser^{102} to enhance expression of select FSH target genes.** Results support the schematic model in which FSH via PKA and ERK leads to an increase in phosphorylation of YB-1 on Ser^{102}. This increase in YB-1 phosphorylation on Ser^{102} is dependent on ERK activation of RSK-2. RSK-2 appears to enhance phosphorylation of YB-1 on Ser^{102} primarily by inactivating PP1. YB-1 phosphorylated on Ser^{102} is necessary for FSH-dependent induction of target genes. *IRS1* refers to insulin receptor substrate 1. Sites of actions of various inhibitors are indicated.

modulate gene expression in GCs is FOXO1 (15, 18). Our recent results, however, suggest the PI3K/AKT pathway is likely regulating additional transcriptional modulators, based on the ability of exogenous insulin-like growth factor 1 (IGF$_1$) at concentrations >1 ng/ml to synergize with FSH to enhance gene expression without further increasing the phosphorylation of FOXO1 (Ser^{256}) (19). Strong evidence in cancer cells identified YB-1 as an AKT target (20–23) that, upon phosphorylation on Ser^{102}, acts as a transcription factor to promote expression of genes such as *Egfr* (21, 24–26), a recognized FSH gene target (27).

YB-1 was first described as a major component of the inactive mRNA-protein complex (mRNP) (28, 29). Further studies demonstrated that YB-1 interacts with both RNA and DNA specifically through its cold shock domain (30, 31) and nonspecifically via basic and acidic clusters in its C terminus (for review, see Ref. 32). YB-1 functions in the cytosol to regulate mRNA translation and to stabilize mRNA; in the nucleus, YB-1 can regulate DNA repair, pre-mRNA splicing, and transcription (for review, see Ref. 32). Transcriptional targets for YB-1, identified mostly using cancer cell lines, in addition to *Egfr*, include multidrug resistance gene 1 (*Mdr1*) (33), protein-tyrosine phosphatase 1B (*PTP1B*) (34), cyclin A (*Ccna*) (35), and vascular endothelial growth factor (*Vegf*) genes (36).

Herein, we show that FSH promotes the protracted phosphorylation of YB-1 on Ser^{102}. This phosphorylation is mimicked by forskolin and inhibited by expression of the selective PKA inhibitor (PKI), indicating its dependence on PKA activity. Phosphorylation of YB-1 on Ser^{102} is unaffected by the PI3K inhibitor wortmannin but is blocked by both the MEK/ERK inhibitor PD98059 and the RSK-2 inhibitor BI-1870, showing dependence on ERK and downstream RSK-2 signaling (Fig. 1). Our results surprisingly suggest that the major RSK-2 target is not YB-1; rather, RSK-2 promotes inactivation of protein phosphatase 1 (PP1) to enhance YB-1(Ser^{102}) phosphorylation. Using adenoviral YB-1 phosphorylation mutants, we show that phosphorylation of YB-1 on Ser^{102} is necessary for the expres-

sion of multiple gene targets, including *Cyp19a1*, *Lhcgr*, *Cyp11a1*, *Egfr*, *Hsd17b1*, *Pappa*, and *Inha*. Together these results suggest that the PKA-dependent activation of ERK/RSK-2 pathways in GCs in response to FSH, by promoting phosphorylation of YB-1 on Ser^{102}, contributes to the expression of multiple FSH-dependent gene targets that are necessary for follicular maturation. These results also suggest a previously underappreciated role for the ERK signaling pathway in immature GCs.

Experimental Procedures

Materials—The following were purchased: ovine FSH (oFSH-19) was from the National Hormone and Pituitary Agency of the NIDDK, National Institutes of Health (Torrance, CA); recombinant human IGF$_1$ was from Atlanta Biologicals; purified mouse EGF, anti-FLAG (F3165), and testosterone propionate were from Sigma; human fibronectin was from BD Biosciences; antiphospho-YB-1 (Ser^{102}; CST 2900), anti-YB-1 (CST 4202), anti-phospho-AKT (Ser^{473}; CST 9271), anti-phospho-ERK (Thr^{202}/Tyr^{204}; CST 9107), anti-phospho-RSK-2 (Ser^{380}; CST 9335), and anti-GST (CST 2622) were from Cell Signaling Technologies; anti-SRC homology-2 (SH2) domain-containing tyrosine phosphatase (SHP2) (SC 7384) and Protein A/G PLUS-agarose (SC 2003) were from Santa Cruz Biotechnology; wortmannin, PD98059, tautomycin, okadaic acid, forskolin, BI-D1870, 8-chlorophenylthiol (CPT)-cAMP, and myristoylated PKI were from Calbiochem/EMD. 8-CPT-2′-O-methyl-cAMP was purchased from Life Sciences Institute; TRIzol-RNA Lysis Reagent and Fast SYBR Green Master Mix were from Life Technologies; qScript was from VWR.

Animals—Sprague-Dawley, CD-outbred rats (breeders from Charles River Laboratories) were from a colony maintained by our laboratory in a pathogen-free facility at Washington State University. The facility is maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals using protocols approved by the Washington State University Animal Care and Use Committee.

GC Culture and Western Blotting—Immature female rats were primed with subcutaneous injections of 1.5 mg of estradiol-17β (E2) in propylene glycol on days 21–23 to promote growth of preantral follicles. GCs were collected from ovaries on day 24 by puncturing individual follicles using 27-gauge needles (37). Cells were plated on fibronectin-coated plates at a density of ~1 × 10^6 cells/ml of serum-free media supplemented with 100 units/ml penicillin (P), 100 μg/ml streptomycin (S), and 1 nM E2 (6). For the experiments measuring secreted estradiol-17β levels, 10 ng/ml androstenedione was added instead of E2. GC cultures are reported to be 88.6 ± 6.4% pure, based on the presence of the FSHR as detected by flow cytometry (38), are free of contaminating theca cells, based on the absence of response to LH (39), and initially contain ~0.02% oocytes (40) that are removed with the addition of fresh media. These GC cultures faithfully mimic differentiation responses to FSH (for review, see Ref. 3) but do not mimic proliferation response to FSH (15, 41, 42). Indicated treatments were added to cells ~20 h after plating and terminated by aspirating media and washing once with PBS. For Western blotting, total cell extracts were collected by scraping cells in SDS sample buffer

(43) followed by heat denaturation. Equal protein loading was accomplished by plating equal numbers of cells, collecting at 50 μl/1 × 10^6 cells in SDS sample buffer, and loading equal volumes of extract per gel lane. Equal loading was verified by probing for total SHP2 or total AKT, as indicated. Proteins were separated by SDS/PAGE and transferred onto either Hybond C-extra or Protran (Amersham Biosciences) nitrocellulose membrane (8). The membrane was incubated with primary antibody overnight at 4 °C, and antigen-antibody complexes were detected using enhanced chemiluminescence (Thermo-Fisher). Westerns were scanned using an Epson Perfection V500 scanner and Adobe Photoshop CS2 9.0 software with minimal processing and quantified using Quantity One software (Bio-Rad). Experimental densitometric values were divided by load control protein values and expressed relative to vehicle values. Results were analyzed using GraphPad Prism, and significance was determined using either a one way ANOVA with Tukey's Multiple Comparison Test or a one-tailed Student's *t* test.

Immunoprecipitation—Briefly, ~10^7 cells were scraped into 0.5 ml of immunoprecipitation lysis buffer (14), sonicated twice on ice for 45 s, and clarified by centrifugation at 10,000 × *g* for 5 min at 4 °C. After removal of a 0.03-ml sample for input, soluble GC extracts were precleared using a species-matched matrix (ExactaCruz Preclearing Matrix, Santa Cruz Biotechnology) for 60 min at 4 °C. Samples were then incubated by rotating at 4 °C overnight with the specified antibodies and Protein A/G PLUS-agarose (Santa Cruz Biotechnology; SC 2003) or antibody-agarose conjugate along with a species matched IgG antibody control (Santa Cruz Biotechnology). After centrifugation at 10,000 × *g* for 5 min at 4 °C, 0.4 ml of the supernatant fraction, representing the unbound protein fraction, was collected in SDS sample buffer. Agarose beads were washed by rotating 5 min at 4 °C with 1 ml of immunoprecipitation wash buffer (14) 4 times. Bound proteins were collected in SDS sample buffer and subjected to SDS/PAGE and Western blotting. Inputs represent ~2%, and bound sample was ~98% of total sample.

Transfection of GCs and Luciferase Assay—*Egfr* (−1109 to −16 bp) (44), *Inha* (−2021 to +68 bp) (45), and *Cyp19a1* (−294 to + 20 bp) (46) promoter-luciferase constructs were previously described. Cells were plated on fibronectin-coated plates at a density of 1 × 10^6 cells/ml in Opti-MEM + P/S + E2 with expression constructs (500 ng/1 × 10^6 cells) and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 6 h of incubation, media were removed, and fresh DMEM +P/S + E2 was added. Cells were treated as indicated after an ~16 h recovery period then lysed in 0.1 ml of luciferase assay buffer (25 mM Hepes, pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 1 mM DTT, 1% Triton X-100). Samples were analyzed for luciferase activity using a Burthold Technologies Lumat LB 9507 luminometer using the Promega Luciferase Assay System according to the manufacturer's instructions.

Adenoviral Production—Full-length *YB-1-FLAG* was purchased from OriGene (Rockville, MD). *YB-1-FLAG* mutants were obtained using QuikChange Lightning Site-Directed Mutagenesis kit from Agilent Technologies (Santa Clara, CA)

according to the manufacturer's instructions. The following primers were used: *YB-1(S102D)* forward (5′-GTC TCT CCA TCT CCT ACA TCG CGA AGG TAC TTC CTG GG-3′) and reverse (5′-CCC AGG AAG TAC CTT CGC GAT GTA GGA GAT GGA GAG AC-3′); *YB-1(S102A)* forward (5′-CCA GGA AGT ACC TTC GCG CTG TAG GAG ATG GAG AGA-3′) and reverse (5′-TCT CTC CAT CTC CTA CAG CGC GAA GGT ACT TCC TGG-3′). *YB-1-FLAG*, *YB-1 (S102D)-FLAG*, and *YB-1 (S102A)-FLAG* were cloned into pShuttleX and then cloned into Adeno-X by Vector Development Laboratory (Baylor College of Medicine, Houston TX). Adenoviral (Ad)-*YB-1(S102D)*- FLAG was used at 1.4 × 10^8 pfu/ml, and Ad-*YB-1(S102A)*-FLAG was used at 1 × 10^9 pfu/ml.

Cellular Fractionation—Fractionation was performed using a modified protocol previously described (47). GCs were treated as indicated, washed once with cold PBS, and collected in 0.5 ml of cytoplasmic lysis buffer (10 mM Hepes, pH 7.4, 10 mM KCl, 0.01 mM EDTA, 0.1 mM EGTA, 2 mM DTT, 5 mM Na₂VO₄, 10 mM NaF, 20 mM sodium β- glycerophosphate, 0.1% Nonidet P-40, Halt protease inhibitor mixture) and incubated on ice for 20 min. Nuclei were pelleted though centrifugation at 5000 × *g* 4 °C for 10 min, and the supernatant containing the cytoplasmic fraction was removed. The cytoplasmic fraction was collected in SDS sample buffer and heat-denatured. The nuclear pellet was washed with 1.0 ml of cytoplasmic lysis buffer and repelleted. The supernatant was removed, and the nuclear pellet was resuspended in 0.1 ml of SDS sample buffer and heat-denatured. Samples were subjected to SDS/PAGE and Western blotting with the indicated antibodies. Cytoplasmic fraction represents 9% of total sample, and the nuclear fraction represents 50% of total sample.

Adenoviral Transduction of GCs—GCs were plated on fibronectin-coated 6-well plates at a density of 1.5 × 10^6 cells/well in DMEM/F12 + P/S + E2. After allowing the cells to adhere to the plate for ~4 h, the indicated adenoviruses were added to the media (9). After an overnight treatment, cells were allowed ~1 h recovery and then treated as indicated.

RNA Isolation and Real-time PCR—GCs were treated as indicated, rinsed once with PBS, and collected in 1.0 ml of TRIzol-RNA lysis reagent. RNA isolation was carried out per the manufacturer's instructions. RNA concentration was determined using a NanoDrop 2000. cDNA was generated using qScript, and real time-PCR was performed using Fast SYBR Green Master Mix, both per the manufacturer's instructions. Primers used were: *Lhcgr* forward (5′-TCC AGA ACA CCA AAA ACC TGC-3′) and reverse (5′-AAG GGT TCG GAT GCC TGT G-3′); *Egfr* forward (5′-AGA TTG CAA AGG GCA TGA ACT AC-3′) and reverse (5′-ACA TTC CTG CTG CCA AGT C-3′); *Cyp19a1* forward (5′-CAC GGA TGT TTG ATG GTC TGA-3′) and reverse (5′-CTC GGC TTG CTG ACA AAC C-3′); *Cyp11a1* forward (5′-GGG TGG ACA CGA CCT CCA T-3′) and reverse (5′-ACC TTC AAG TTG TGT GCC ATT TC-3′); *Hsd17b1* forward (5′-CCT GCA CTT GGC TGT TCG T-3′) and reverse (5′-CGC AAT GTG GCA TAA ACT TTG A-3′); *Pappa* forward (5′-CCG TGA TCA CAG GGC TGT ATG-3′) and reverse (5′-CCC ATG ACC CAT CCT CGA T-3′); *Inha* forward (5′-TGG GAC CGC TGG ATC GTA-3′) and reverse (5′-GCA TCC CGC AGC TAC CAT-3′); *Star* forward (5′-AAG
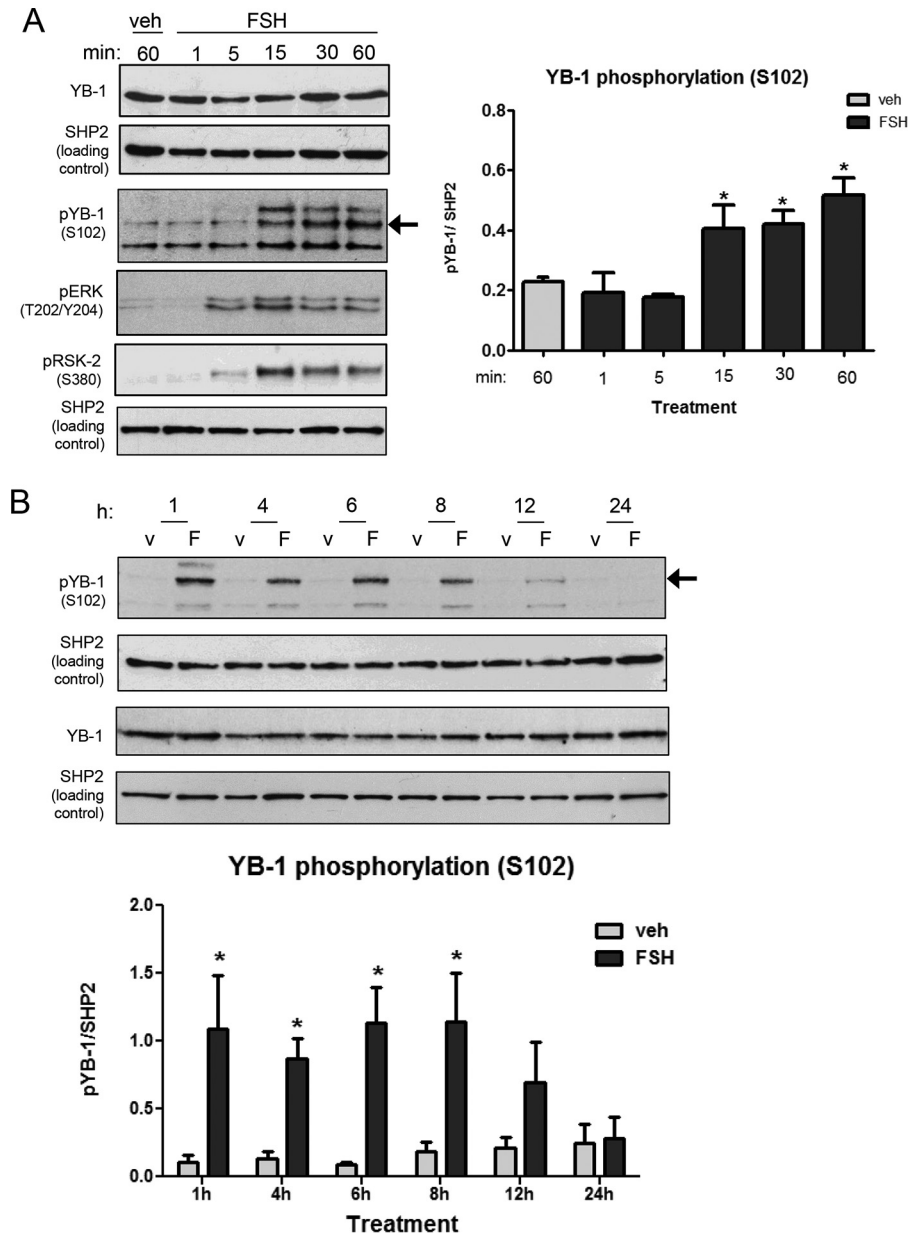
FIGURE 2. **Treatment of GCs with FSH leads to prolonged phosphorylation of YB-1 on Ser[102].** *A*, GCs were treated for the indicated times without (*veh*) or with 50 ng/ml FSH. Samples were heat-denatured after collection in SDS sample buffer, and proteins were separated by SDS/PAGE. A blot of whole cell extracts was probed with indicated antibodies. Results are representative of three independent experiments. Quantification of results represents the mean ± S.E. of three independent experiments, with the (*) representing $p < 0.05$, based on one-way ANOVA followed by Tukey's post test. *B*, GCs were treated without (*v*) or with FSH (*F*) for the indicated times, and samples were collected and analyzed as described in *A*, with (*) representing $p < 0.05$ based on a 1-way ANOVA followed by Tukey's post test. *Arrow* refers to phospho-YB1(Ser[102]).

GAC TGC CCA CCA CAT CTA C-3′) and reverse (5′-TCT CGT TGT CCT TGG CTG GAA-3′); *Egr1* forward (5′-CCT TTT GCC TGT GAC ATT TGT G-3′) and reverse (5′-TGG TAT GCC TCT TGC GTT CA-3′); *Ereg* forward (5′-GCT CTG ACA TGG ACG GCT ACT-3′) and reverse (5′-CTC GCT CAT GTC CAC CAG GTA-3′); *ribosome L19* forward (5′-GTG ACC TGG ATG CGA AGG A-3′) and reverse (5′-GCC TTG TCT GCC TTC AGT TTG-3′).

Estradiol-17β Radioimmunoassay (RIA)—GCs were treated as described above in the presence of 10 ng/ml androstenedione. After treatment, cellular media was collected. Estradiol-17β levels were measured by RIA at the

UVA Center for Research in Reproduction Ligand Assay and Analysis Core (48).

Results

FSH Signals to Stimulate Phosphorylation of YB-1(Ser[102]) through a PKA-dependent Pathway—We initially determined whether YB-1 was expressed in GCs and whether its expression at the protein level was regulated by FSH. Results (Fig. 2, *A* and *B*) show that YB-1 total protein, which migrates on SDS-PAGE at 50 kDa, is readily detected in GCs, and its expression is not acutely regulated by FSH. We then determined whether YB-1 was phosphorylated on Ser[102] in response to FSH. FSH pro-
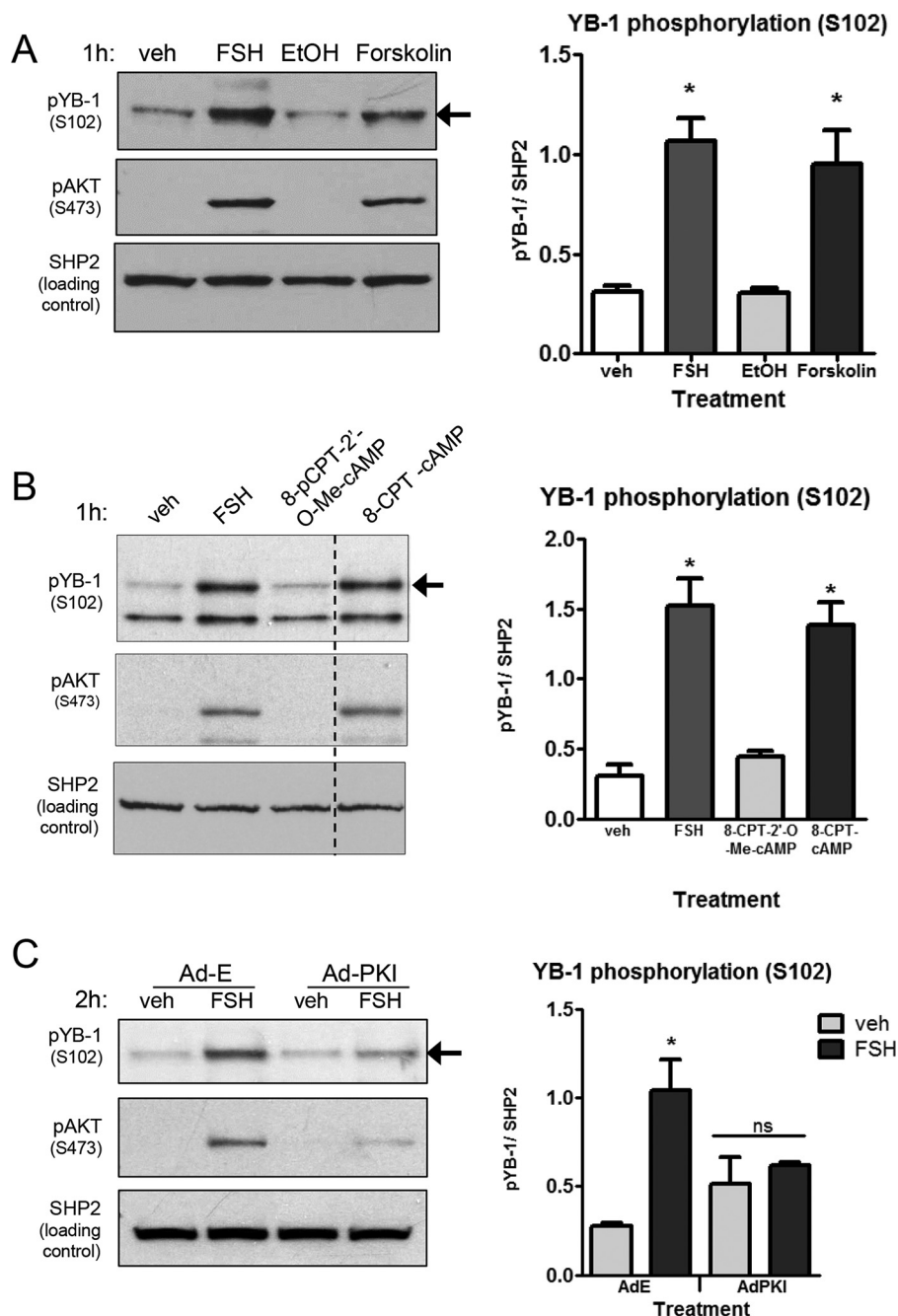
FIGURE 3. **Phosphorylation of YB-1 on Ser[102] is mediated by cAMP and PKA and independent of exchange proteins activated by cAMP.** *A*, GCs were treated for 1 h without (*veh*) or with FSH (*lanes 1* and *2*) or without (EtOH) or with 10 μM forskolin in 0.25% ethanol, DMEM (*lanes 3* and *4*). Samples were collected as described in Fig. 2A. Quantification of the results represents the mean $\pm$ S.E. of three independent experiments, with the (*) representing $p < 0.01$, based on one-way ANOVA followed by Tukey's post test. *B*, GCs were treated for 1 h without (*veh*) or with FSH or with 250 μM concentrations of either 8-pCPT-2'-O-Me-cAMP or 8-CPT-cAMP. Samples were collected as described in Fig. 2A. *Dotted lines between lanes* represent cropped images. Results are representative of three independent experiments. Quantification was as described in *A*, with (*) representing $p < 0.01$, based on one-way ANOVA followed by Tukey's post test. *C*, GCs were transduced overnight with Ad-Empty (E) (5.8×10^3 optical particles per unit (OPU)/cell) or Ad-PKI (1.8×10^4 optical particles per unit (OPU)/cell), which expresses the full-length PKI protein. After media replacement, cells were treated for 2 h without (*veh*) or with FSH. Samples were collected as described in Fig. 2A, and quantification was as described in *A*, with (*) representing $p < 0.01$, based on a one-way ANOVA followed by Tukey's post test. Results are representative of three independent experiments.

moted a relatively slow increase in YB-1(Ser[102]) phosphorylation first detected 15 min after treatment (Fig. 2A). YB-1(Ser[102]) phosphorylation peaked 1 h after FSH treatment; this peak level of phosphorylation was maintained until ~8 h and then declined to undetectable levels by 24 h (Fig. 2B) (the identities of the faster and slower migrating bands in phospho-YB-1 blots are not known). Increased phosphorylation of

YB-1(Ser[102]) was mimicked by the adenylyl cyclase activator forskolin (Fig. 3A). Phosphorylation of AKT(Ser[473]) served as a positive control. YB-1(Ser[102]) phosphorylation also increased in response to 8-CPT-cAMP (Fig. 3B), a cell-permeable cAMP analog that activates both exchange proteins activated by cAMP and PKA (49). But 8-CPT-2'-O-Me-cAMP, a cAMP analog that does not activate PKA (50), failed to increase

YB-1(Ser[102]) phosphorylation (Fig. 3*B*). Treatment of GCs with Ad-PKI, a pseudosubstrate for the catalytic subunit of PKA (51), led to a loss of FSH-dependent YB-1(Ser[102]) phosphorylation (82% ± 17%) (Fig. 3*C*). Similarly, treatment with myristoylated-PKI peptide resulted in a loss of YB-1 phosphorylation mediated by FSH (data not shown). Phosphorylation of AKT(Ser[473]) has been shown to be PKA-dependent in GCs (14) and serves as a positive control. Taken together, these data indicate that FSH stimulation results in phosphorylation of YB-1 on Ser[102] in a PKA-dependent manner in GCs.

Phosphorylation of YB-1(Ser[102]) Is Dependent on the ERK Pathway and Not the PI3K/AKT Pathway—The phosphorylation of YB-1(Ser[102]), investigated in various cancer cell lines, is most commonly mediated by AKT (20–23), although there is evidence that this site can be phosphorylated by RSK-2 downstream of ERK (26, 52). Because FSH in a PKA-dependent manner activates both the PI3K/AKT (14) and ERK signaling pathways (8, 10, 11, 53) in GCs, we investigated both pathways as possible regulators of YB-1 phosphorylation. Pretreatment of GCs with the PI3K inhibitor wortmannin (54) did not decrease FSH-stimulated YB-1(Ser[102]) phosphorylation, whereas AKT(Ser[473]) phosphorylation was inhibited significantly (74.2% ± 13.0%) (Fig. 4*A*). However, pretreatment with the MEK inhibitor PD98059 (54) promoted a near complete inhibition of YB-1(Ser[102]) phosphorylation (94.2% ± 3.6%) in the presence of FSH (Fig. 4*B*), similar to the inhibition of ERK(Thr[202]/Tyr[204]) phosphorylation (98.1% ±5.9%) that is used as the positive control. Equivalent results were obtained with a different MEK inhibitor U0126 (54) (data not shown). Consistent with these results, treatment of GCs with EGF, a known activator of the MEK/ERK pathway, promoted increased levels of YB-1(Ser[102]) phosphorylation as well as RSK-2(Ser[380]) phosphorylation (Fig. 4*C*). When GCs were treated with the PI3K/AKT pathway activator IGF$_1$ or FSH, only cells treated with FSH showed enhanced YB-1(Ser[102]) phosphorylation (Fig. 4*C*). Unlike many other cell models, IGF$_1$ does not activate the ERK pathway in GCs (55). Additionally, GCs treated with the RSK-2 inhibitor BI-D1870 (56) exhibited a marked inhibition (89.4% ± 5.3%) of YB-1(Ser[102]) phosphorylation in the presence of FSH (Fig. 5). CREB(Ser[133]) phosphorylation served as a negative control. Taken together, these results indicate that FSH-stimulated phosphorylation of YB-1(Ser[102]) is mediated by RSK-2 downstream of ERK.

Phosphorylation of YB-1(Ser[102]) Is Dependent on PP1 Inhibition—Enhanced phosphorylation of YB-1(Ser[102]) is relatively slow compared with the phosphorylation of CREB, which is detected within 1 min (11) or of ERK, which is detected within 10 min (8, 11) of FSH treatment. Moreover, YB-1(Ser[102]) phosphorylation is detected up to 8–12 h (Fig. 2*B*), whereas phosphorylations of both ERK (11) and RSK-2 (8) are undetectable by 2 h post treatment with FSH. We, therefore, sought to determine if the inhibition of a Ser/Thr phosphatase contributed to the phosphorylation of YB-1(Ser[102]).

Pretreatment of GCs with the protein phosphatase 2 (PP2) inhibitor okadaic acid (at 200 nM; Refs. 57 and 58) resulted in an increase in YB-1(Ser[102]) phosphorylation in both vehicle- and FSH-treated GCs (Fig. 6*A*). Phosphorylation of RSK-2(Ser[380]) is indicative of RSK-2 activity (59) and provides insight into the

regulation of YB-1(Ser[102]) phosphorylation in the presence of okadaic acid. RSK-2(Ser[380]) phosphorylation was enhanced both in the absence and presence of FSH in response to okadaic acid and appears to account for the increase in YB-1(Ser[102]) phosphorylation. Fold activation by FSH relative to EtOH vehicle in the absence and presence of okadaic acid for pYB-1(Ser[102]) was 6.2 and 16.0 and for pRSK-2(Ser[380]) was 7.2 and 21.2, respectively. These results suggest that okadaic acid inhibits a phosphatase that acts upstream of YB-1 rather than at the level of YB-1.

Pretreatment of GCs with the PP1 inhibitor tautomycin (at 1 μM; Refs. 57 and 58) resulted in an increase in YB-1(Ser[102]) phosphorylation in vehicle-treated cells to levels equivalent to those seen in FSH-treated cells in the absence of tautomycin, whereas FSH did not further enhance YB-1(Ser[102]) phosphorylation (Fig. 6*B*). Tautomycin pretreatment did not enhance FSH-stimulated RSK-2(Ser[380]) phosphorylation. Fold activation by FSH relative to EtOH vehicle in the absence and presence of tautomycin for pYB-1(Ser[102]) was 3.4 and 4.6 and for pRSK-2(Ser[380]) was 3.7 and 3.2, respectively. These results suggest that PP1 promotes the dephosphorylation of YB-1 on Ser[102] in the absence of FSH.

If PP1 maintains the dephosphorylation of YB-1(Ser[102]) in the absence of FSH, then a PP1 catalytic subunit should co-immunoprecipitate with YB-1. As total YB-1 antibody was not immunoprecipitating, we transiently transfected GCs with a full-length *FLAG*-tagged *YB-1* plasmid, allowed ~16 h for cells to recover, treated GCs with vehicle or FSH, and then immunoprecipitated FLAG-tagged YB-1 with anti-FLAG antibody. Results (Fig. 6*C*) show that anti-FLAG antibody selectively pulls down comparable levels of the catalytic subunit PP1β (PP1cβ) in both vehicle- and FSH-treated samples. Additionally, when the same experiment was performed and the presence of RSK-2 was investigated, results show that RSK-2 also interacted (directly or indirectly) with FLAG-tagged YB-1 in a FSH-independent manner (Fig. 6*C*). These results suggest that both catalytic subunit PP1β (PP1cβ) and RSK-2 are in a complex with YB-1 and that these associations are independent of FSH.

Taken together, these results suggest (i) that PP2 inhibits YB-1 phosphorylation on Ser[102] indirectly through regulation of RSK-2 itself or at a site upstream of RSK-2 and (ii) that PP2 activity is not regulated by FSH. Additionally, these results suggest (iii) that PP1 maintains YB-1(Ser[102]) in a dephosphorylated state in the absence of FSH and (iv) that FSH signals to inhibit the activity of PP1, preventing the dephosphorylation of YB-1 at Ser[102]. The inability of FSH to significantly enhance YB-1(Ser[102]) phosphorylation in the presence of tautomycin suggests that the major effect of PKA via ERK/RSK-2 is to promote inactivation of PP1.

YB-1 Phosphorylated on Ser[102] Is Readily Detected in the Nuclear Fraction—As the phosphorylation of YB-1 on Ser[102] in breast cancer cells is most often linked to its function as a transcriptional activator (Refs. 21, 23, 24, 26, and 33; for review, see Ref. 60), we asked if phospho-YB-1 was preferentially localized to the nuclear fraction of GCs.

To address this question, GCs were transfected with *FLAG*-tagged *YB-1* plasmids that express wild type (WT) YB-1, the phospho- YB-1(S102D) mimic, and dephospho-YB-1(S102A)
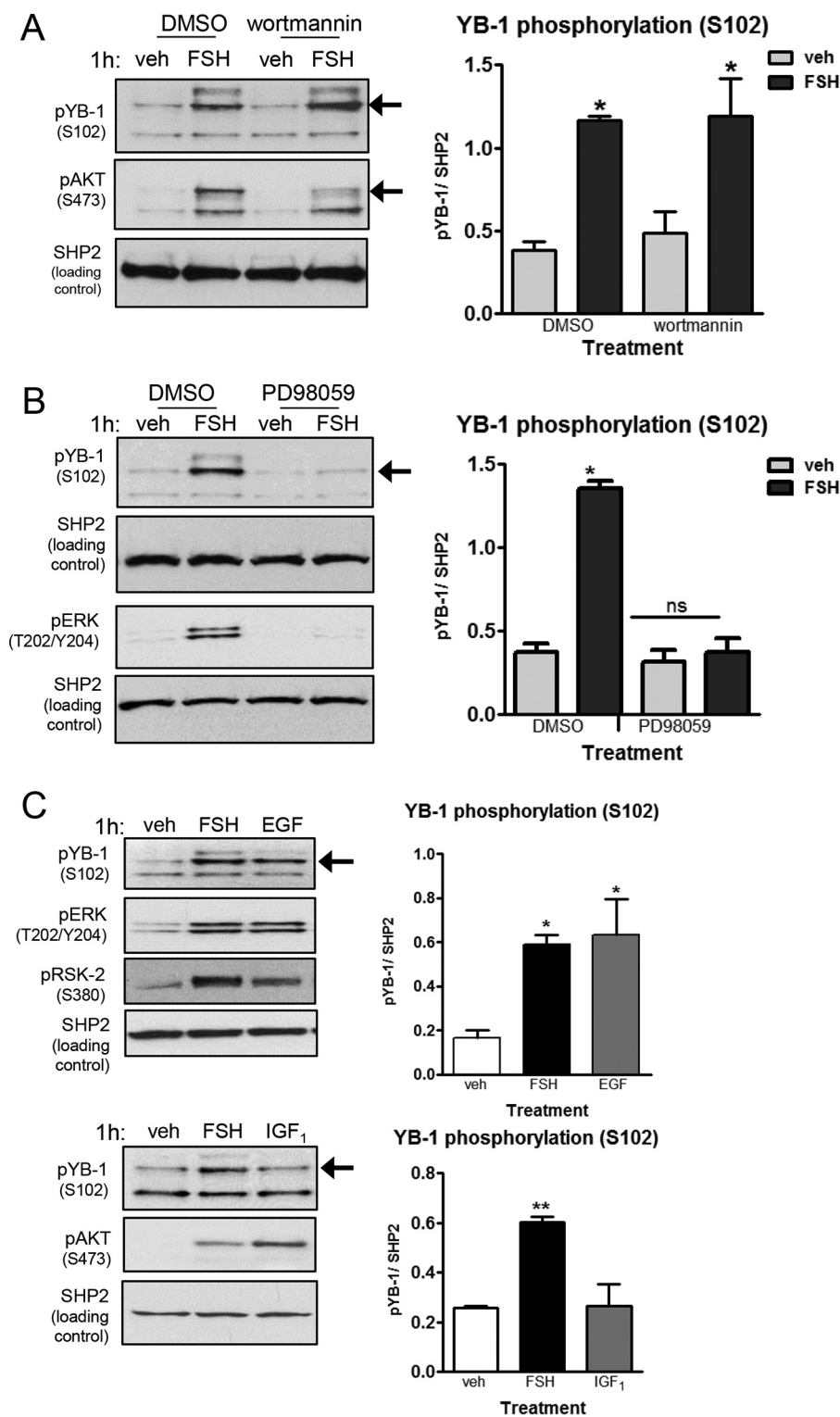
FIGURE 4. **FSH-stimulated phosphorylation of YB-1 on Ser[102] is via ERK signaling, not PI3K/AKT.** *A*, GCs were pretreated without (DMSO) or with 100 nM wortmannin for 1 h followed by treatment without (*veh*) or with FSH for 1 h. Results are representative of three independent experiments. Samples were collected and quantified as described in Fig. 2A with (*) representing $p < 0.05$, based on a one-way ANOVA followed by Tukey's post test. *B*, GCs were pretreated without (DMSO) or with 50 μM PD98059 for 1 h followed by treatment without (*veh*) or with FSH for 1 h. Samples were collected, and quantification was as described in Fig. 2A, with (*) representing $p < 0.001$, based on a one-way ANOVA followed by Tukey's post test. Results are representative of three independent experiments. *ns*, not significant. *C*, GCs were treated without (*veh*) or with FSH, 50 ng/ml EGF, or 50 ng/ml IGF$_1$ as indicated. Samples were collected and quantified as described in Fig. 2A with * representing $p < 0.05$ and ** representing $p < 0.01$, based on a one-way ANOVA followed by Tukey's post test. Results are representative of three independent experiments.
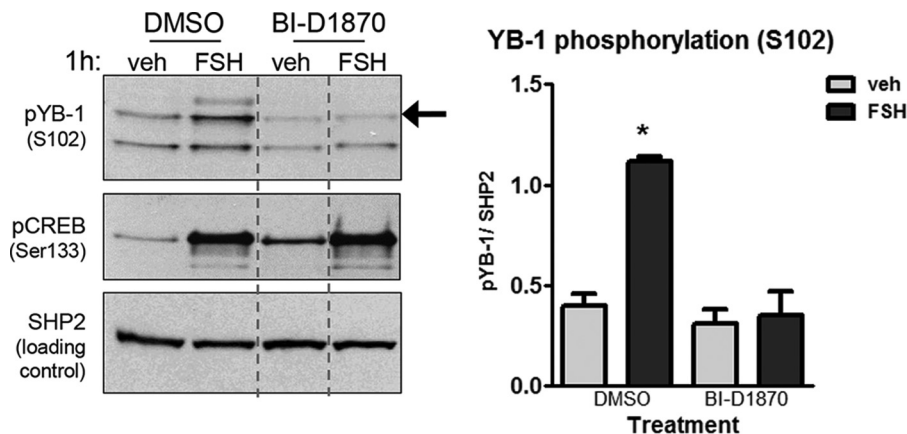
FIGURE 5. **FSH-stimulated phosphorylation of YB-1 on Ser[102] is RSK-2-dependent.** GCs were pretreated without (DMSO) or with 10 μM BI-D1870 for 1 h followed by treatment without (*veh*) or with FSH for 1 h. Samples were collected and quantified as described in Fig. 2*A*, with (*) representing $p < 0.001$, based on a one-way ANOVA followed by Tukey's post test. Results are representative of three independent experiments. *Dotted lines between lanes* represent cropped images.

mutant. After a 16-h recovery period, GCs were treated with vehicle or FSH and then fractionated into cytosolic and nuclear fractions; GAPDH and histone H3 serve as positive controls for the purity of the cytoplasmic and nuclear fractions, respectively (cytoplasmic and nuclear fractions represent 9% and 50% of total sample, respectively). All of the FLAG-tagged constructs were readily detected in the cytoplasmic fraction (Fig. 7*A*), consistent with the presence of both phosphorylated YB-1(Ser[102]) and non-phosphorylated YB-1(Ser[102]) in this fraction (as shown below). However, the phospho-YB-1(S102D) mimic was readily detected in the nuclear fraction at higher levels compared with both YB-1-WT and the dephospho-YB-1(S102A) mutant.

Phosphorylated YB-1(Ser[102]) was also readily detected using the phospho-YB-1(Ser[102]) antibody in both the nuclear and cytosolic fractions of non-transfected GCs (Fig. 7*B*, *left panel*). However, FSH did not promote a change in total levels of YB-1 within the nuclear or cytoplasmic fractions (Fig. 7*B*, *right panel*), suggesting either that the phosphorylation of YB-1 on Ser[102] occurs within the nuclear fraction or that a relatively small fraction of total YB-1 phosphorylated in the cytoplasmic fraction translocates into the nuclear fraction. Although the majority (~90%) of total and phosphorylated YB-1 in non-transfected GCs is localized to the cytoplasmic fraction, the presence of phosphorylated YB-1(Ser[102]) in the nuclear fraction suggests a potential role for this phosphoprotein in the regulation of gene expression.

Phospho-YB-1(Ser[102]) Is Necessary for Expression of Select FSH Target Genes—Based on the increased levels of the phospho-YB-1(S102D) mutant detected in the nuclear fraction compared with WT and dephospho-YB-1(S102A) mutant, we hypothesized that YB-1 could participate in the transcriptional regulation of FSH target genes. Although ERK signaling is critical to LH-induced ovulation and luteinization in preovulatory GCs (61), the importance of the ERK signaling pathway to FSH-stimulated differentiation of GCs is less well understood. We, therefore, initially sought to identify FSH target genes that were regulated by the ERK signaling pathway. Pretreatment of GCs with the MEK inhibitor U0126 resulted in inhibition of FSH-induced *Egfr* (85.7% $\pm$ 6.2%), *Inha* (82.5% $\pm$ 3.2%), *Cyp19a1*

(96.1% $\pm$ 2.65%), and *Ereg* (67.7% $\pm$ 4.4%) mRNA levels measured at 48 h post FSH treatment, whereas *Star* mRNA levels were not affected (Fig. 8). Treatment with the MEK inhibitor PD98059 showed significant inhibition of FSH-induced expression of the immediate early gene *Egr1* (86.4% $\pm$ 3.2%) 1 h post FSH treatment (Fig. 8).

We next transduced GCs with adenoviral phospho- and dephospho-mutants of YB-1 to determine if select ERK-regulated gene targets were activated by the phosphorylation of YB-1 on Ser[102]. We initially focused on the three ERK-regulated target genes that showed the greatest inhibition by the MEK inhibitor at the 48 h time point; *Star* expression was included as a negative control. Transduction of GCs with Ad-YB-1(S102D) phospho-mutant resulted in a statistically significant FSH-independent increase in *Egfr* and *Cyp19a1* mRNAs compared with Ad-Empty (E), which was not significantly enhanced further by FSH (Fig. 9*A*). In contrast, *Inha* mRNA levels were not different between Ad-E and Ad-YB-1(S102D) treatments. The ability of the Ad-YB-1(S102D) phospho-mutant to enhance *Egfr* and *Cyp19a1* mRNA levels in the absence of FSH suggests that phosphorylated YB-1(Ser[102]) is a major contributor to the induction of these genes.

Transduction of GCs with the dephospho-Ad-YB-1(S102A) mutant prevented the induction by FSH of *Egfr* (82.0% $\pm$ 9.5%) and *Cyp19a1* (88.6% $\pm$ 5.5%) mRNAs (Fig. 9*B*), consistent with results in Fig. 9*A*. FSH-stimulated induction of *Lhcgr* (98.6 $\pm$ 0.75%), *Inha* (90.9% $\pm$ 2.9%), *Cyp11a1* (85.3 $\pm$ 3.2%), *Hsd17b1* (94.5 $\pm$ 3.5%), and *Pappa* (92.7 $\pm$ 6.3%) mRNAs were also abrogated by the dephospho-Ad-YB-1(S102A) mutant. In contrast, FSH-stimulated *Star* mRNA expression was not reduced by the dephospho-Ad-YB-1(S102A) mutant. The unexpected ability of the dephospho-YB-1(S102A) mutant to induce expression of *Egfr* in the absence of FSH may be a consequence of YB-1 over-expression, such that the phosphorylation requirement for YB-1 to promote expression of this gene was negated (62). Taken together, we interpret these results to suggest that YB-1 phosphorylated on Ser[102] is necessary for the induction of *Lhcgr*, *Cyp19a1*, *Cyp11a1*, *Inha*, *Egfr*, *Hsd17b1*, and *Pappa* mRNA by FSH but does not participate in the induction of *Star* mRNA by FSH.
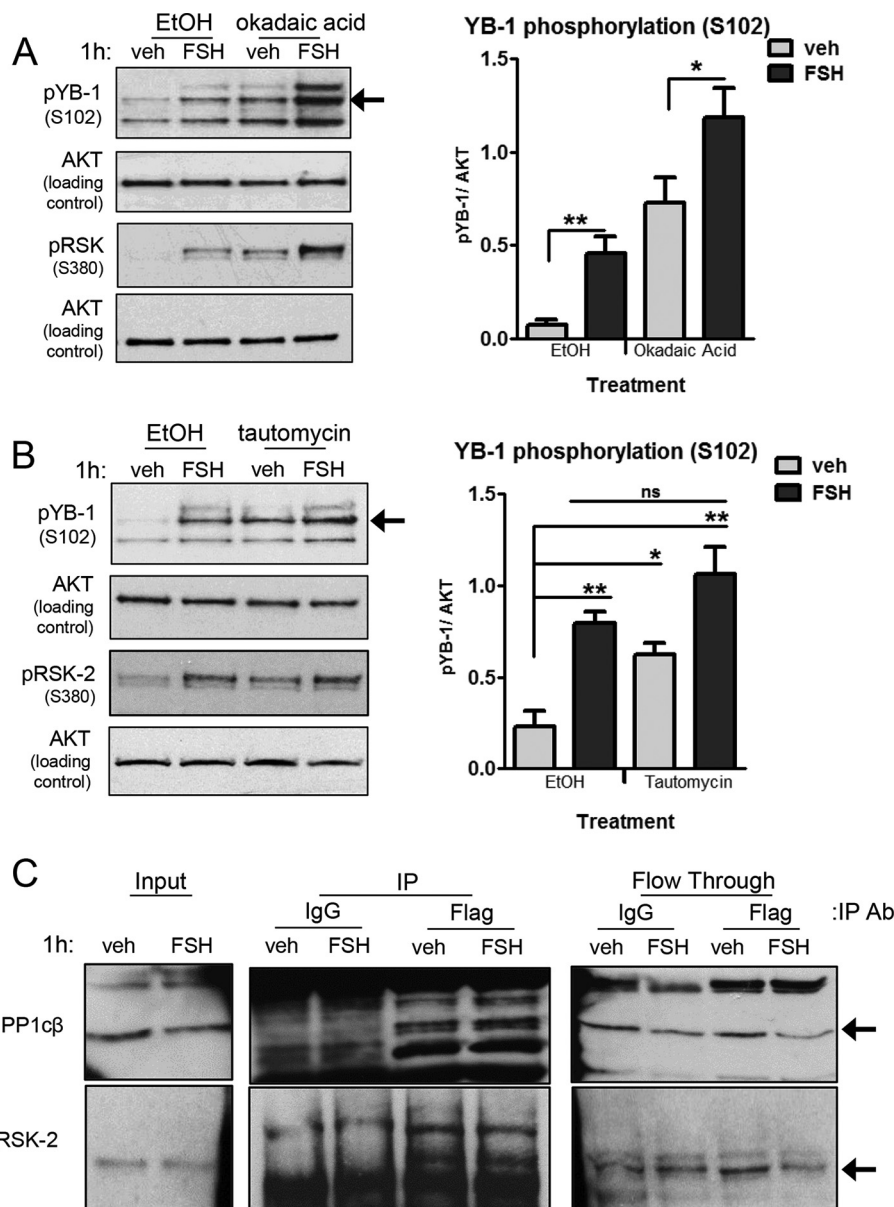
FIGURE 6. **Prolonged FSH-stimulated phosphorylation of YB-1 on Ser^{102} is dependent on inhibition of the Ser/Thr phosphatase PP1.** *A*, GCs were pretreated without (EtOH) or with 200 nM okadaic acid for 1 h followed by treatment without (*veh*) or with FSH for 1 h. Samples were collected as described in Fig. 2*A*, and quantification represents the mean ± S.E. of three independent experiments, with (**) representing $p < 0.01$ and (*) representing $p < 0.05$ based on a one-tailed Student's *t* test. *B*, GCs were pretreated without (EtOH) or with 1 μM tautomycin for 5.5 h followed by treatment without (*veh*) or with FSH for 1 h. Samples were collected, and quantification was as described in Fig. 2*A*, with (**) representing $p < 0.01$ and (*) representing $p < 0.05$, based on a one-tailed Student's *t* test. EtOH FSH-, tautomycin vehicle-, and tautomycin FSH-treated samples were not statistically different from each other (*ns*). Results are representative of three independent experiments. *C*, GCs were transfected with 500 ng of *YB-1-FLAG* plasmid for 6 h as described under "Experimental Procedures." After an overnight recovery, GCs were treated without (*veh*) or with FSH for 1 h and then collected in immunoprecipitation lysis buffer and sonicated. After removal of the insoluble particles, the soluble fraction was rotated overnight with 20 μg of anti-FLAG (Sigma *F3165*) as described under "Experimental Procedures." Inputs, bound (*IP*), and unbound (flow-though) samples were separated by SDS/PAGE, and the Western blot was probed with the indicated antibodies. *Arrows* indicate the protein band of interest. Results are representative of two independent experiments.

GCs transfected with the indicated promoter-luciferase constructs followed by transduction with Ad-YB-1(S102A) mutant promoted a significant ($p < 0.05$) inhibition of *Egfr* (64.9% ± 13.2%), *Cyp19a1* (59.9% ± 10.0%), and *Inha* (72.5% ± 5.0%) promoter-luciferase activities (Fig. 10). These promoter-luciferase results suggest that phosphorylated YB-1 acts primarily at the level of the gene promoters and not at the level of mRNA stability. The lack of complete inhibition of FSH-stimulated signal by the dephospho-YB-1(S102A) mutant of *Cyp19a1* and *Inha* promoter-luciferase constructs as opposed to the ~90%

inhibition of mRNA expression shown in Fig. 9 either reflects the nature of the promoter-luciferase constructs that may be lacking important regulatory elements and/or provides indirect evidence that phospho-YB-1 not only regulates gene transcription but also mRNA stability.

Finally, estradiol-17β levels were measured in culture media of GCs that were transduced with Ad-E *versus* Ad-YB-1(S102A) and treated with vehicle or FSH for 48 h. Consistent with the ability of the dephospho-Ad-YB-1(S102A) mutant to inhibit the induction by FSH of *Cyp19a1*, Ad-YB-1(S102A)
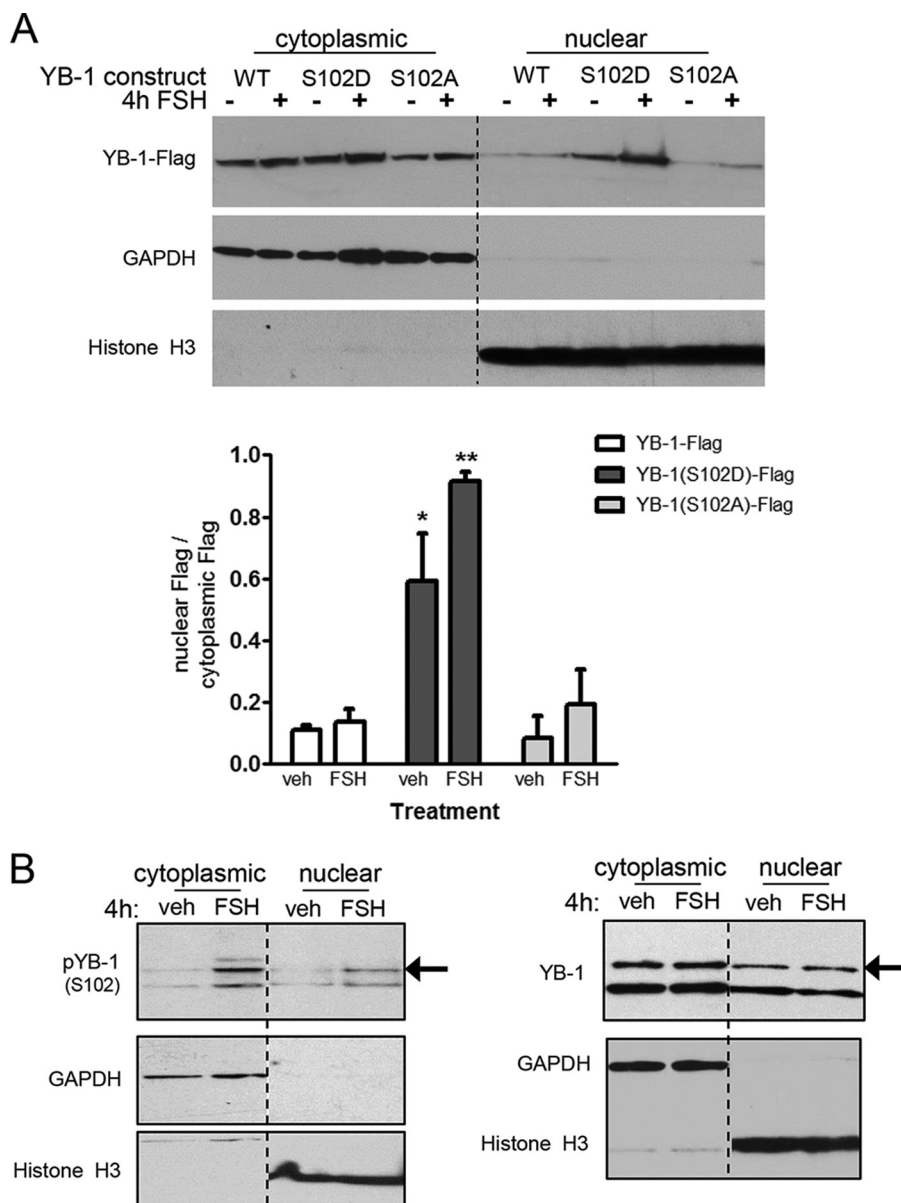
FIGURE 7. **YB-1 and phospho-YB-1(Ser[102]) are detected in the both the nuclear and cytosolic fractions.** *A*, GCs were transfected with 500 ng of the indicated plasmid for 6 h as described under "Experimental Procedures." After recovery, GCs were treated without (*veh*) or with FSH for 4 h, then collected and separated into cytoplasmic and nuclear fractions as described under "Experimental Procedures." Quantification of results represents the mean ± S.E. of two independent experiments, with (*) representing $p < 0.05$ and (**) representing $p < 0.001$, based on a one-way ANOVA followed by Tukey's post test. *B*, GCs were treated without (*veh*) or with FSH for 4 h, then collected and separated into cytoplasmic and nuclear fractions as described under "Experimental Procedures." Western blots were probed with the indicated antibodies. Results are representative of three independent experiments.

blocked the ability of FSH to enhance estradiol-17β biosynthesis in the presence of exogenous androstenedione (95.3 ± 2.9%) (Fig. 11). Collectively, these results indicate that YB-1(Ser[102]) phosphorylation is necessary for FSH-mediated induction of *Egfr*, *Cyp19a1*, *Inha*, *Lhcgr*, *Cyp11a1*, *Hsd17b1*, and *Pappa* genes that characterize the preovulatory GC as well as for estradiol-17β biosynthesis.

Discussion

Our results show that YB-1 is phosphorylated on Ser[102] in response to FSH. However, in contrast to our original premise, YB-1 in GCs is not an AKT target. Rather, our results reveal that YB-1 is phosphorylated on Ser[102] in response to FSH in a PKA- and ERK/RSK-2-dependent manner, consistent with previous

reports that YB-1(Ser[102]) is a RSK-2 target (26, 52). Unexpectedly, our results suggest that the major effect of RSK-2 on YB-1(Ser[102]) phosphorylation is to inactivate PP1 rather than to promote the direct phosphorylation of YB-1, as discussed below. YB-1 phosphorylated on Ser[102] is readily detected in the nuclear fraction and is required for the ability of FSH to induce key target genes required for follicular maturation. Together, our results also reveal a previously unidentified role for YB-1 and an unappreciated role for the ERK/RSK-2 signaling pathway in FSH-dependent GC maturation.

YB-1 is a multifunctional DNA and RNA-binding protein and consequently participates in most DNA- and RNA-dependent cellular functions (30, 31). In the cytoplasm YB-1 is one of two primary proteins (along with poly-A-binding protein) that
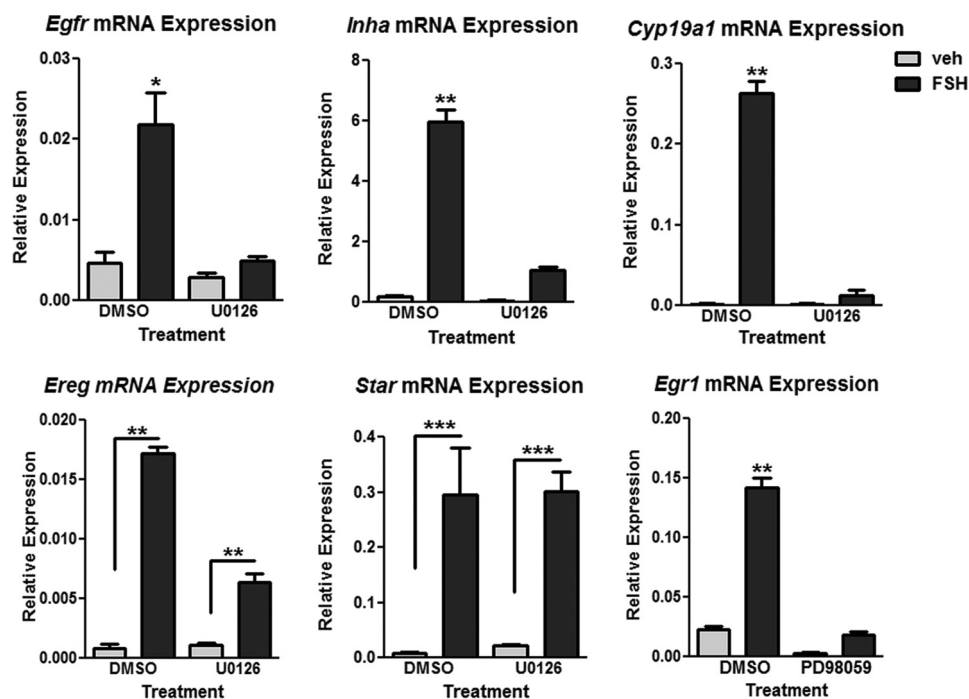
FIGURE 8. ERK signaling is necessary for FSH-stimulated target gene mRNA expression. For *Egfr*, *Ereg*, *Cyp19a1*, *Inha*, and *Star*, GCs were pretreated without (DMSO) or with 10 μM U0126 for 1 h, then treated without (*veh*) or with FSH for 48 h. For *Egr1*, GCs were pretreated without (DMSO) or with 50 μM PD98059 for 1 h, then treated without (*veh*) or with FSH for 1 h. Cells were collected for RNA isolation, and real-time PCR was performed for the genes indicated as described under "Experimental Procedures." Quantification of results represents the mean ± S.E. of three independent experiments for *Egfr* and *Ereg*, and two independent experiments for *Cyp19a1*, *Inha*, *Egr1*, and *Star* with (*) representing $p < 0.05$, (**) representing $p < 0.01$, and (***) representing $p < 0.001$, based on a one-way ANOVA followed by Tukey's post test.

compose the mRNP complex (for review, see Ref. 32). Hence, YB-1 is associated with both free nontranslated and polysomal mRNA and is a major regulator of translation (63–65). YB-1 also functions as a transcriptional factor to both repress and activate transcription by binding directly to gene promoters or in complex with other transcriptional factors (for review, see Refs. 22 and 32). YB-1 is generally viewed as an "oncogenic" transcription factor based on its overexpression in breast and lung cancer cells (21, 66) and on its gene targets, which include *Egfr*, *Her2*, *Pi3kca*, and *Mdr1* (for review, see Ref. 32). Although YB-1 can be phosphorylated on a number of Ser and Tyr residues, phosphorylation of Ser[102] within the cold shock domain is most commonly associated with the regulation of translation and transcription (for review, see Ref. 32). Phosphorylation of YB-1 on Ser[102] promotes its nuclear localization in ovarian and breast cancer cells (20, 22, 26) and is required for *Egfr* expression in breast cancer cells (21, 23, 24, 26).

Our results show that YB-1 is also phosphorylated on Ser[102] in ovarian GCs and that a phospho-YB-1(S102D) mutant is readily detected in the nuclear fraction. Unexpectedly, our results also reveal that YB-1 phosphorylated on Ser[102] is necessary for the expression of select FSH gene targets that define a mature, preovulatory GC, such as *Lhcgr*, *Cyp11a1*, *Egfr*, *Hsd17b1*, *Pappa*, *Cyp19a1*, and *Inha*.

Lhcgr encodes the LH receptor that is required for ovulation, cumulus cell expansion, and the resumption of meiosis (67); *Cyp11a1* encodes cytochrome P450 side chain cleavage, the rate-limiting enzyme for progesterone biosynthesis (18, 68); the EGFR contributes to LH-stimulated expansion of cumulus granulosa cells, resumption of meiosis, and ovulation (27, 69,

70); *Cyp19a1* encodes aromatase, the rate-limiting enzyme in estrogen biosynthesis (71); *Inha* encodes for the inhibin hormone subunit inhibin-α that inhibits FSH expression by the anterior pituitary (72); *Hsd17b1* encodes the enzyme that converts androstenedione and estrone to testosterone and estradiol-17β, respectively, and is required for normal ovulation and corpus luteum formation (73); and *Pappa* encodes a secreted metalloproteinase that cleaves IGF-binding proteins and contributes to estrogen and progesterone biosynthesis and to ovulation (74).

FSH-stimulated gene expression in GCs is complex and requires activation of a number of transcriptional factors coupled with inhibition of transcriptional repressors. These events are mediated by multiple signaling pathways activated by FSH and PKA (for review, see Ref. 2). CREB, SF-1(*Nr5a1*)/LRH-1(*Nr5a2*), and GATA-4/6 enhance expression of both *Cyp19a1* and *Inha* (46, 75–77), and FOXO1 (15) and T-cell factor 3 (9) repress expression of both genes. Many transcriptional proteins that regulate expression of the *Lhcgr* (9, 78, 79) and *Cyp11a1* (76, 80, 81) have been elucidated, although the mechanisms by which FSH enhances *Egfr* and *Pappa* expression have not been reported. However, a role for YB-1 and indeed for the ERK signaling pathway in the transcriptional activation of FSH target genes is novel.

FSH-dependent phosphorylation of YB-1 on Ser[102] is mimicked by treatment of GCs with EGF. Although ERK phosphorylation is FSH-dependent, we previously reported that the canonical pathway upstream of ERK is constitutively active, yet necessary for ERK phosphorylation in immature GCs (8, 11). Consistent with this premise, FSH-stimulated ERK phosphor-
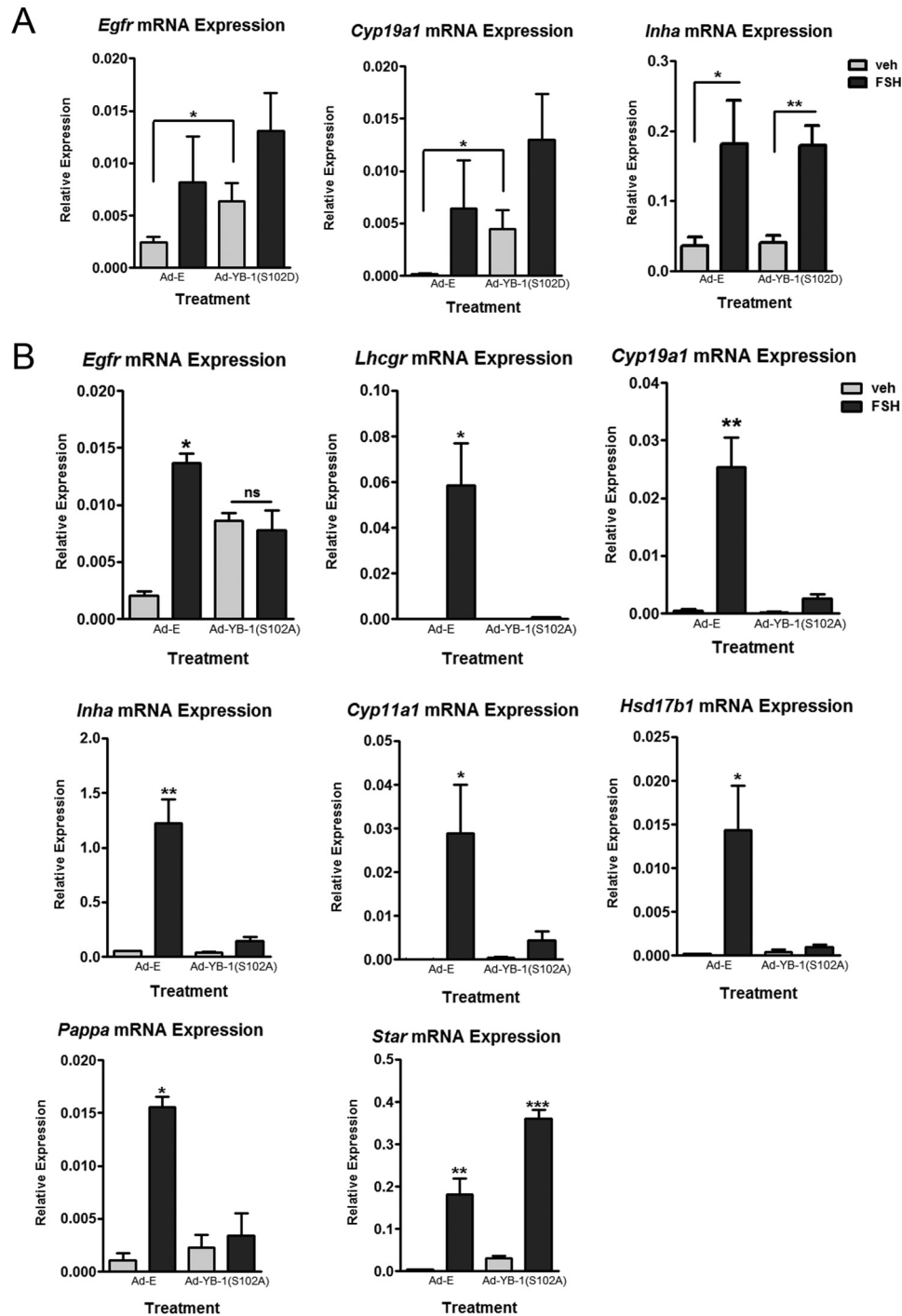
FIGURE 9. **Phospho-YB-1 regulates FSH- and ERK-dependent target gene mRNA expression.** *A*, GCs were transduced with Ad-*YB-1(S102D)*-FLAG or Ad-Empty (E) overnight. After media replacement and >1 h of recovery, cells were treated without (*veh*) or with FSH for 48 h. GCs were collected and analyzed as described in Fig. 8. Quantification of results represents the mean ± S.E. of three independent experiments with (*) representing $p < 0.05$ and (**) representing $p < 0.01$, based on a one-tailed Student's *t* test. *B*, GCs were transduced with Ad-*YB-1(S102A)*-FLAG or Ad-Empty overnight. After media replacement and >1 h recovery, cells were treated without (*veh*) or with FSH for 48 h. Cells were collected and analyzed as described in *A*. Quantification of results represents the mean ± S.E. of three independent experiments for *Egfr, Cyp19a1, Inha Cyp11a1*, and *Hsd17b1* and the mean ± S.E. of two independent experiments for *Lhcgr* and *Pappa*, with (**) representing $p < 0.01$ and (***) representing $p < 0.001$, based on a one-tailed Student's *t* test.

ylation is blocked by the EGFR antagonist AG1478 (11). We expect that, in a similar manner, FSH-stimulated ERK/RSK-2-dependent YB-1 phosphorylation on Ser[102] is dependent on an active upstream EGFR/MEK pathway that would be blocked by inhibition of upstream signaling proteins, such as the EGFR, RAS, and RAF.

Although YB-1 phosphorylated on Ser[102] is readily detected in the nuclear fraction of GCs, the great majority of both phosphorylated YB-1(Ser[102]) and total YB-1 is present in the cytoplasmic fraction (see Fig. 7, where the cytoplasmic and nuclear fractions represent 9% and 50% of the total fraction). Cytoplasmic YB-1 has been linked with both translated polysomal
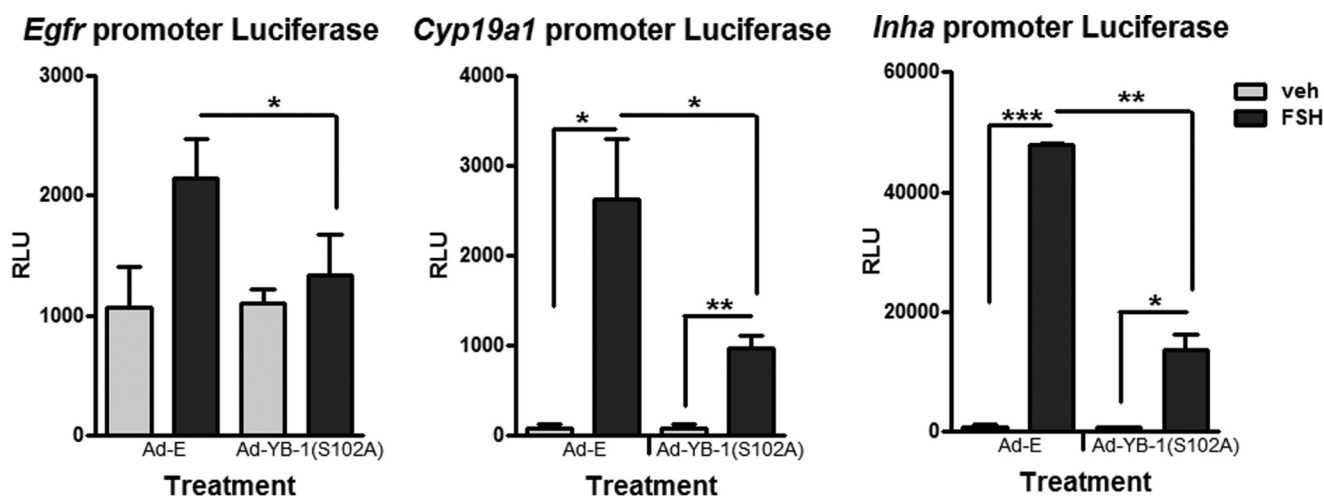
FIGURE 10. Phospho-YB-1 regulates FSH dependent activity of *Egfr*, *Cyp19a1*, and *Inha* promoter-reporter luciferase constructs. GCs were transfected with the indicated promoter-luciferase constructs for 6 h as described under "Experimental Procedures" followed by overnight transduction with Ad-*YB-1(S102A)*-FLAG or Ad-Empty (E). After media replacement and >1 h of recovery, cells were treated without (*veh*) or with FSH for 6 h then collected. Samples were analyzed (see "Experimental Procedures") and results are expressed as the relative luciferase units (RLU) mean ± S.E. of triplicates and are representative of three independent experiments. (*) represents $p < 0.05$, (**) represents $p < 0.01$, and (***) represents $p < 0.001$, based on a one-tailed Student's t test.

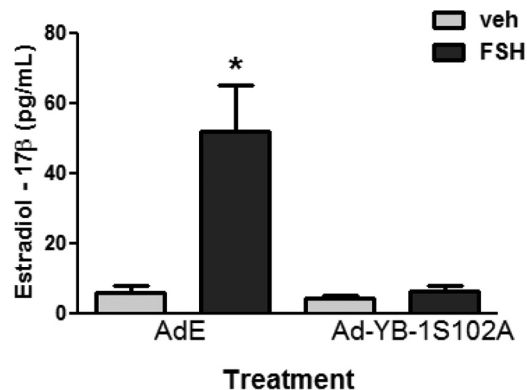**FIGURE 11. Phospho-YB-1 is necessary for FSH-dependent production of estradiol-17β by GCs.** GCs plated in the presence of 10 ng/ml androstenedione were transduced with Ad-*YB-1(S102A)*-FLAG or Ad-Empty (E) overnight. After media replacement and >1 h of recovery, cells were treated without (*veh*) or with FSH for 48 h. Media was collected, and estradiol-17β levels were measured by RIA. Results represent the mean ± S.E. of three independent experiments, with (*) representing $p < 0.05$ based on a one-way ANOVA followed by Tukey's post test.

mRNPs and free untranslated mRNPs (for review, see Ref. 32). YB-1 is recognized to stabilize both long- and short-lived mRNAs at high YB-1/mRNA ratios effectively by burying mRNAs within YB-1 protein complexes (for review, see Ref. 32). In NIH3T3 cells, AKT-dependent phosphorylation of YB-1 on Ser[102] stimulated Cap-dependent translation (64). It is thus likely that cytosolic YB-1 is playing a major role in both stabilizing GC mRNA and, upon phosphorylation, enhancing translation.

FSH-stimulated phosphorylation of YB-1 on Ser[102] in GCs is mediated by PKA, ERK, and its downstream kinase RSK-2. However, to our surprise, our results suggest that the major effect of RSK-2 is to inhibit the phosphatase activity of PP1. This conclusion is based largely on the inability of FSH to increase phospho-YB-1(Ser[102]) signal over that seen in vehicle-treated cells in the presence of the PP1 inhibitor tautomycin (see Fig. 6B). Moreover, the RSK-2 inhibitor BI-D1870 abrogates YB-1(Ser[102]) phosphorylation in vehicle- and FSH-

treated cells even though PKA remains active (evidenced by CREB phosphorylation on Ser[133]; see Fig. 5). Additionally, the PP1β catalytic subunit, RSK-2, and YB-1 co-immunoprecipitate in a complex. The presence of phosphorylated YB-1(Ser[102]) in GCs pretreated with tautomycin and treated with vehicle (*i.e.* in the absence of FSH) further suggests that although RSK-2 may also directly phosphorylate YB-1 in the presence of FSH, RSK-2 is not the kinase that phosphorylates YB-1 on Ser[102] in vehicle-treated cells. We hypothesize that Ser[102] may be phosphorylated by the constitutively active kinase casein kinase 2, as the phosphorylation motif on YB-1 (KYLR**S**VGD, where is S̲ is Ser[102]) partially aligns with that of casein kinase 2 (S(E/D)*X*(E/D)) (82).

We do not know the mechanism by which RSK-2 inactivates PP1 activity. The catalytic subunits of PP1 can associate with a large number of regulatory subunits (up to 200 have been identified), many of which inhibit the catalytic activity of PP1 (83). Unfortunately, we have not been able to identify the PP1 regulatory subunit inhibited by RSK-2. We hypothesize, however, that RSK-2-dependent phosphorylation of the PP1 regulatory subunit in GCs promotes its degradation, allowing for the prolonged phosphorylation of YB-1 that persists up to 8 h post FSH treatment despite the shorter-lived phosphorylation of RSK-2 that is undetectable within 2 h post FSH treatment (8).

The prolonged phosphorylation of YB-1 was not observed with other transcriptional targets in GCs, such as CREB and histone H3, whose phosphorylation has returned to the levels of untreated cells by 4 h post FSH treatment (8). We do not know the functional significance of this sustained phosphorylation of YB-1. It is unlikely to be required for transcriptional regulation. The prolonged YB-1 phosphorylation is more likely to participate either in mRNA stabilization and/or translation.

Although Ser[102] is the most studied phosphorylation site on YB-1, multiple kinases have been shown to phosphorylate YB-1. ERK and glycogen synthase kinase 3β are reported to phosphorylate YB-1 on Thr[38] and/or Ser[41] and/or Ser[45] (36).

Indeed, mass spectrometric analysis has identified additional phosphorylations on YB-1, including Tyr^{162}, Ser^{165} and/or Ser^{167}, Ser^{174} and/or Ser^{176}, and Ser^{313} and/or Ser^{314} (84, 85). Therefore, we cannot rule out the possibility that FSH promotes phosphorylation of YB-1 on additional sites by other kinases.

In conclusion, we have shown that FSH promotes YB-1(Ser^{102}) phosphorylation through a PKA- and ERK/RSK-2-dependent signaling pathway. The major effect of RSK-2 appears to be to inhibit the Ser/Thr phosphatase PP1 rather than to directly phosphorylate YB-1 on Ser^{102}. YB-1 phosphorylated on Ser^{102} is necessary for the expression of FSH-dependent gene targets that are essential for GC maturation. Taken together, our results not only establish a functional role for YB-1 as a transcriptional activator of FSH target genes in the GCs but also reveal an underappreciated role for ERK signaling pathway in the activation of gene expression by FSH in GCs.

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