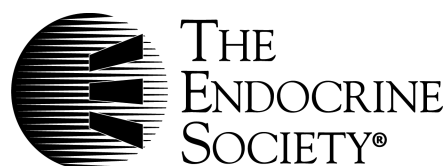# Endocrinology

Modulation of Gonadotropin-Releasing Hormone-Induced Extracellular Signal-Regulated Kinase Activation by Dual-Specificity Protein Phosphatase 1 in L²T2 Gonadotropes

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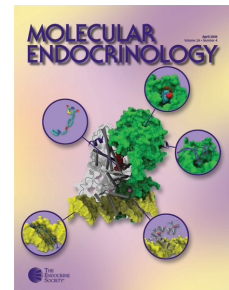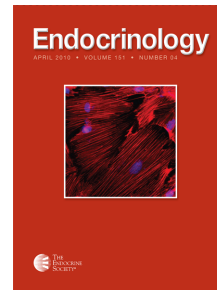
Modulation of Gonadotropin-Releasing Hormone-Induced Extracellular Signal-Regulated Kinase Activation by Dual-Specificity Protein Phosphatase 1 in LβT2 Gonadotropes

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As the regulator of pituitary reproductive hormone synthesis, the hypothalamic neuropeptide GnRH is the central regulator of reproduction. A hallmark of GnRH action is the differential control of gene expression in pituitary gonadotropes through varied pulsatile stimulation. Among other signaling events, GnRH activation of the ERK family of MAPKs plays a significant role in the transcriptional regulation of the luteinizing hormone β-subunit gene and regulation of cap-dependent translation. We evaluated the ERK response to different GnRH pulse amplitudes in the gonadotrope cell line LβT2. We found that low-amplitude stimulation with GnRH invokes a rapid and transient ERK activation, whereas high-amplitude stimulation invokes a prolonged activation specifically in the cytoplasm fraction of LβT2 cells. Nuclear and cytoplasmic targets of ERK, Ets-like gene 1, and eukaryotic initiation factor 4E, respectively, are similarly activated. Feedback control of ERK activation occurs mainly through the dual-specificity protein phosphatases (DUSPs). DUSP1 is localized to the nucleus in LβT2 cells but DUSP4, another member implicated in GnRH feedback, exists in both the nucleus and cytoplasm. Manipulation of nuclear DUSP activity through overexpression or knockdown of *Dusp1* modulates the ERK response to low and high GnRH pulse amplitudes and activation of the *Lhb* promoter. *Dusp1* overexpression abolishes sustained ERK activation and inhibits *Lhb* promoter activity induced by high amplitude pulses. Conversely, *Dusp1* knockdown enhances ERK activation by low-amplitude stimulation and increases stimulation of *Lhb* promoter activity. We conclude that DUSP1 feedback activity modulates ERK activation and the transcriptional response to GnRH. (*Endocrinology* 151: 4882–4893, 2010)

Pulsatile release of the hypothalamic decapeptide, GnRH, controls the expression and release of the gonadotropins, LH and FSH, from anterior pituitary gonadotropes (1, 2). The marked increase in GnRH pulse frequency and amplitude beginning in the late follicular phase is essential for generation of the preovulatory LH surge in females (3). In contrast, chronic stimulation with GnRH suppresses gonadotropin production (4, 5). Thus, the interpretation of GnRH pulse frequency and amplitude by gonadotropes represents a critical component of

reproductive competency. To maintain sensitivity to repeated stimuli, it is essential that negative feedback mechanisms modulate signaling responses to preserve sensitivity to subsequent GnRH stimulation.

The GnRH receptor (GnRHR) couples primarily to Gαq/11, leading to stimulation of phospholipase C and formation of inositol 1,4,5-trisphosphate and diacylglycerol, causing the elevation of intracellular free calcium and activation of protein kinase C. These early events underlie GnRH activation of multiple MAPKs including p38

Abbreviations: CMV, Cytomegalovirus; DUSP, dual-specificity protein phosphatase; EIF4E, eukaryotic initiation factor 4E; ELK, Ets-like gene; GnRHR, GnRH receptor; JNK, c-Jun N terminal kinase; MKP, MAPK phosphatase; MNK, MAPK-interacting kinase; sh, short hairpin; TBS, Tris and NaCl.

MAPK, c-Jun N terminal kinase (JNK), and ERK (5). Activation of MAPK signaling cascades is essential for targeted activation of *Lhb* and *Fshb* gene expression, but many details of the role of pulsatile stimulation of gene expression remain unclear.

The involvement of the MAPK phosphatase (MKP) family of dual-specificity protein phosphatases (DUSPs) in the regulation of MAPK activity is well established, although uncertainty remains over the functional specificity and localization of individual DUSP family members (6). The MKP subfamily specifically inactivates MAPKs. MAPK activation induces expression and activity of DUSPs, and this serves as a negative-feedback arm of the signaling regulatory loop (7, 8). Thus, the status of MAPKs is dependent on the balance of positive input and negative feedback. In the pituitary and immortalized gonadotropes, tonic, high-frequency, or high-amplitude GnRH treatment increases both DUSP1 and DUSP4 mRNA, but this differs among model systems used (9–12). A significant increase in *Dusp1* but not *Dusp4* gene expression occurs after tonic GnRH treatment (10). A corresponding regulation of DUSP1 and DUSP4 with JNK and ERK is also reported in GnRH-suppressed mice (12). DUSP feedback control of GnRHR signaling has been examined in HeLa cells, and multiple DUSPs were identified as influencing ERK phosphorylation and nuclear to cytoplasmic ratio (13). Thus, DUSPs generally influence the intensity and localization of ERK activity. Interestingly, neither DUSP1 nor DUSP4, those regulated by GnRH in gonadotropes and implicated in feedback control, was among those showing effect. In contrast, DUSP1 is responsible for negative-feedback regulation of ERK in fibroblasts and osteoblasts (7, 14, 15). Consistent with the observed effect of pulse amplitude on gene expression in LβT2 cells, we hypothesize that different levels of stimulation may distinctly alter ERK signaling pathways, and amplitude sensitivity may be partly regulated at the level of DUSP feedback.

In the present study, we report a differential activation of ERK in response to GnRH pulses. This difference is most significant in the resolution of ERK phosphorylation. We also demonstrate a specific role of DUSP1 in the feedback regulation of differential ERK and regulation of *Lhb* promoter activity, thus demonstrating a role for DUSP-negative feedback activity generally and DUSP1 specifically in regulating ERK activity and gonadotropin gene expression.

Materials and Methods

Cell culture

The pituitary gonadotrope cell line, LβT2 (16), was maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 4.5 mg/ml glucose, 10% fetal bovine serum, and 5% penicillin/streptomycin and incubated in a humidified atmosphere of 5% carbon dioxide. Generally, 1×10^7 cells were plated in 10-cm dishes and incubated for 24 h and then placed in serum-free medium 16–18 h. Cells were treated for 5 min with GnRH, washed, and provided fresh serum-free DMEM. After treatment, cells were washed with ice-cold PBS and harvested with 400 μl of Laemmli sample buffer and sonicated.

Western blotting and immunofluorescence

All samples were separated by SDS-PAGE on 10% gels and transferred onto polyvinyl difluoride. Membranes were blocked in 2× casein for 1 h (Vector Laboratories, Burlingame, CA) and incubated with anti phospho-ERK(1/2) primary antibody (Millipore, Billerica, MA) for 1 h at room temperature. After imaging, blots were stripped in 2% sodium dodecyl sulfate, 62.5 mM Tris-HCl, and 100 nM β-mercaptoethanol at 65 C for 30–45 min and reblotted for 1 h with anti-ERK(1/2) rabbit primary antibody to control for loading. Blots were visualized by chemiluminescence using a 1:5000 dilution of biotinylated secondary antibody and horseradish peroxidase-conjugated avidin-biotin complex (Vector Laboratories), and imaged using GeneSnap Bio imaging system (Syngene, Frederick, MD). Quantification of total ERK1/2 phosphorylation was performed relative to total ERK1/2 in the same lane for consistency. For analysis of immunoprecipitated protein, only immunoprecipitated product was quantified. For DUSP1 immunoprecipitation, cells were serum starved for 4 h before a 5-min exposure to GnRH at 1 or 100 nM. Cells were lysed in radioimmunoprecipitation assay buffer with phosphatase inhibitors. Protein was immunoprecipitated with anti-MKP1 antibody, (no. 07–535; Millipore) and protein A agarose according to manufacturer's instructions. Recovered protein was visualized by blotting with mouse monoclonal antiphosphoserine (ab6639; Abcam, Cambridge, MA), and goat antimouse IgG-horseradish peroxidase (SC-2004; Santa Cruz Biotechnologies, Santa Cruz, CA) in Starting Block buffer (37542; Pierce Thermo Fisher Scientific, Rockford, IL) followed by chemiluminescent visualization. Blots were stripped and incubated goat (ab-1351; Abcam) anti-MKP1 and goat antirabbit or rabbit antigoat IgG-horseradish peroxidase (SC-2005 or SC-2004; Santa Cruz) for detection of total V5-DUSP1 protein.

For immunofluorescence, LβT2 cells were seeded on poly lysine-coated chamber slides at a density of 50,000 cells/cm^2 and incubated 24 h in DMEM supplemented with 10% fetal bovine serum. After incubation overnight in serum-free DMEM, cells were treated with GnRH for 5 min and washed twice with PBS. Fresh serum-free DMEM was added and cells were incubated for a total of 60 min. Media was removed and cells fixed for 5 min in ice-cold methanol, rehydrated with a brief wash of 20 nM Tris (pH 7.4) and 150 mM NaCl (TBS), and permeabilized with TBS supplemented with 0.5% Triton X-100 for 10 min. After changing into TBS/0.1% Triton X-100, cell were treated with avidin-biotin blocking solutions (Vector Laboratories) and blocked with 3% normal goat serum. Phospho-ERK was detected using mouse monoclonal antibody SC-7383 (Santa Cruz Biotechnologies), and V5-DUSP1 was detected using rabbit polyclonal antibody ab9116 (Abcam). Secondary staining was accomplished using biotinylated goat antimouse IgG or goat antirabbit IgG and avidin-FITC conjugates (Vector Laboratories).

Perifusion

For perifusion studies, LβT2 cells were prepared as previously described (17). For ERK analysis, cells were given four 1- or

100-nM GnRH pulses at a frequency of one pulse per hour, and cells were harvested 5 min or 1 h after the final pulse. Alternatively, cells received three hourly vehicle pulses followed by a single 1- or 100 nM GnRH pulse and harvested 5 min afterward. For LHβ reporter analysis, transfected cells were perifused at a frequency of one pulse per hour for 6 h and harvested. Absorbance trace data were collected every 30 sec by monitoring of phenol-red dye in a GnRH-containing medium. Absorbance profiles were normalized, fitted, and plotted with PeakFit and Sigma Plot (both from Systat Software, San Jose, CA), respectively. Regressions of calibration data were performed using SigmaPlot.

Transfections

All transfections were performed with Fugene 6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Cells were changed into fresh DMEM medium before all transfections. For static cultures, LβT2 cells were plated at a density of 2.75×10^5 cells/cm^2 and incubated for 24 h before transfection. For perifusion experiments, cells were plated at a density of 1.5×10^7 cells/ml bed volume on Cytodex 3 microcarrier beads (GE Healthcare Life Sciences, Piscataway, NJ) and incubated for 5 d before transfection. The 1.8-kb rat *Lhβ* promoter-driven reporter plasmid pGL3-1.8 rLHβ-luc and the internal control pGL3-CMV-βGal were previously described (18). For perifusion, cells were transfected as previously described (17). Transfected cells were serum starved for 12–16 h before treatment with GnRH. Cells were harvested by lysis in 0.5 ml and 100 mM PBS with 0.1% Triton X-100, vortexed, and clarified by centrifugation. Lysates were assayed using the luciferase assay kit (Promega, Madison, WI) and Galacto-Light Plus kit (Tropix, Bedford, MA), respectively. Luminescence was measured in a Veritas microplate luminometer (Turner BioSystems, Sunnyvale, CA).

DUSP1 overexpression and knockdown

The pcDNA3.1D/ERKV5-His-TOPO plasmid was constructed by PCR cloning mouse MAPK1 (forward: 5′-CACCAACATG-GCGGCGGCG-3′ and reverse: 5′-AGATCTGTATCCTGGCT-GGAATCT-3′) from the pCMV·Sport 6 ERK plasmid (Open Biosystems, Huntsville, AL) into the pcDNA3.1D/V5-His-TOPO plasmid (Invitrogen, Carlsbad, CA). The resultant plasmid pcDNA3.1D/ERKV5-His-TOPO encodes MAPK1 with the C-terminal addition of the V5 epitope for immunoprecipitation. The clone was sequenced to confirm identity to *mus musculus* MKP1 (GenBank accession no. BC058258). For static culture treatments, cells were transfected with pcDNA3.1D/ERKV5-His-TOPO and the pCMV·Sport 6 DUSP-1 or pCMV·Sport 6 plasmids. After 36 h of transfection, cells were serum starved for 16–18 h before a 5-min, 100-nM treatment with GnRH. Cells were harvested at 60 min in 500–750 μl of 50 nM Tris, pH 8.0; 150 nM NaCl; 1% Triton X-100; complete protease inhibitor cocktail (Roche, Indianapolis, IN); 50 nM NaF; 50 nM β-glycerol phosphate; 0.1 nM Na$_3$VO$_4$; 1 mM phenylmethylsulfonyl fluoride; DUSP-1 regulation of ERK in LβT2 cells 11 and 10 nM Na$_4$P$_2$O$_7$·H$_2$O.

Protein was immunoprecipitated with Protein G-agarose (Roche, Mannheim, Germany) and goat anti-V5 antibody (Abcam) for 16–24 h. Immunoprecipitated proteins were blotted for phosphor- and total ERK1/2 as above with the exception that membranes were incubated overnight at 4 C. For DUSP1 overexpression, cells were transfected with pCMV·Sport 6 DUSP-1 or pCMV·Sport 6, pGL3–1.8 rLHβ-luc and pGL3-CMV-βGal. Transfected cells were perifused for 6 h and stimulated with GnRH

at one pulse per hour. Lysates were harvested 1 h after the last pulse and assayed. For *Dusp1* knockdown, cells were transfected with the pcDNA3.1D/ERKV5-His-TOPO and the pKo1shDUSP1 (RMM3981-9596427 or RMM3981-9596428; Open Biosystems) or the Mission nontarget plko1 short hairpin (sh) RNA control vector (Sigma, St. Louis, MO) plasmids. For verification of knockdown, cells were cotransfected with a green fluorescent protein expression vector for 24 h and then cultured in puromycin selection media for 48 h. Transfected cells were collected by fluorescence-activated cell sorting based on positive selection for green fluorescent protein fluorescence and negative selection for propidium iodide staining to eliminate nonviable cells. Selected cells were collected into ice-cold PBS, pelleted by centrifugation at $2000 \times g$ for 5′, and lysed in Trizol (Invitrogen) for mRNA purification according to the manufacturer's instructions. Alternatively, transfected cells were Western blotted for DUSP1 protein and β-actin for loading control. For perfusion experiments, cells were transfected with the rat 1.8-kb LHβ promoter-luciferase reporter plasmid pGL3-r1.8LH, the cytomegalovirus (CMV) promoter-based internal control plasmid pGL3-CMV-βGal, and cotransfected with plKo1shDUSP-1 or control. After 48 h, cells were incubated in serum-free DMEM for 12–16 h and perifused for a total of 6 h with 1 nM GnRH stimulation at a 1-h frequency. Cell lysates were assayed for luciferase activity to assess *Lhb* promoter reporter activity and normalized to β-galactosidase activity.

Statistical analysis

Statistical analysis was performed using JMP (SAS Institute, Cary, NC). Data are expressed as mean ± SEM of at least three samples per group. Results were analyzed for significant differences by ANOVA using data untransformed or optimally transformed by the method of Box and Cox (19) as implemented in JMP to correct for nonnormal distribution of variance. *Post hoc* paired or multiple group comparisons were made using Student's *t* or Tukey's honestly significant difference multiple comparison test. Significance of individual fold changes from control were made using Student's *t* test. A significant difference between groups was declared at $P \leq 0.05$ in all analyses.

Results

Differential activation of ERK, Ets-like gene (ELK)-1, and eukaryotic initiation factor 4E (EIF4E) by GnRH

Studies showing GnRH-stimulated phosphorylation of ERK in the pituitary and in gonadotrope-derived cell models have mainly used tonic treatments of GnRH and GnRH-agonists lasting from 15 min to hours (11, 20–23). Binding assays using rat and sheep GnRHR show a dissociation constant of 3–4 nM, and measured GnRH pulse values measured in portal sampling of surging and nonsurging ewes can range from 2 pg/min to excess of 20 pg/min over a 10-min sampling period (24, 25). To determine the corresponding activation of ERK in this dose range, we examined ERK activation in response to a single 5-min stimulation of increasing GnRH doses (0.01–100 nM). ERK activation was examined by Western blotting and quan-

titative chemiluminescent analysis (Fig. 1). A significant increase over unstimulated levels was observed between 1 and 100 nM. Stimulation with 1 nM GnRH is sufficient to
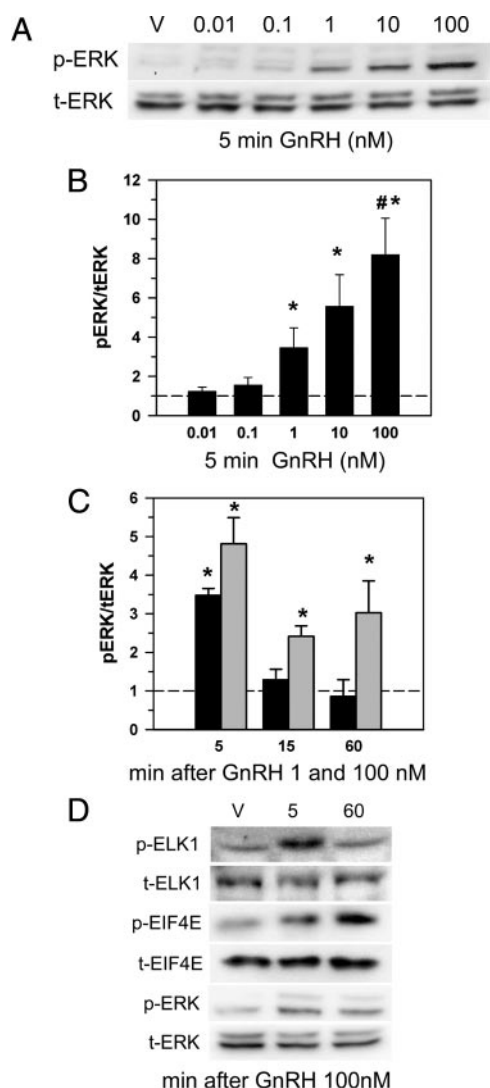

FIG. 1. Sustained ERK activation in LβT2 cells. LβT2 cells were treated with a 5-min pulse of GnRH or vehicle at indicated concentrations. A, Extracts were harvested and subjected to SDS-PAGE followed by Western blotting with phosphorylated ERK1/2 antibody. Blots were then stripped and reblotted with total ERK1/2 antibody for loading control. Phosphorylation levels were normalized to untreated control. V, Vehicle; p-ERK, phosphorylated ERK; t-ERK, total ERK. The results of three independent determinations are plotted in the histogram ± SEM (B). Significant difference from vehicle as determined by Student's *t* test is indicated by an *asterisk*. The 100-nM treatment group was also significantly greater than the 1-nM treatment group (#). C, The histogram represents the mean ratio of phospho-ERK1/2 to total ERK1/2 in LβT2 cells treated for 5 min with GnRH and harvested immediately at 5, at 15, or at 60 min after treatment with 1 nM (*black bars*) or 100 nM (*gray bars*) GnRH. Ratios were normalized to vehicle-treated control from quantitative chemiluminescent image analysis of three independent experiments ± SEM The *asterisk* indicates a significant difference from vehicle control designated as V. D, Differential activation of ERK and ERK targets ELK1 and EIF4E. Extracts harvested immediately and 60 min after a 5-min GnRH treatment were examined for activation of ERK, ELK1, and EIF4E. p-ELK1, Phosphorylated ELK1; t-EIF4E, total EIF4E.

cause a significant activation of ERK, and 100 nM stimulation causes a magnitude of activation significantly higher than 1 nM but not proportional to the increase in dose. Therefore, 1 and 100 nM GnRH pulse amplitudes were chosen as low and high doses for the remaining studies.

Both GnRH pulse amplitudes strongly activated ERK at 5 min. Activation by 1 nM GnRH resolved to control levels within 15 min, but activation by 100 nM GnRH was sustained at approximately 60% of the 5-min level up to 60 min after stimulation (Fig. 1B), similar to the sustained activation observed by others (26). These experiments demonstrate a concentration sensitivity that results in a difference in the kinetic profile of signal resolution. The immediate early gene *Egr*-1, a transcription factor targeted by GnRH signaling, exhibits similar behavior under high- and low-pulse amplitude stimulation, suggesting that amplitude sensitivity is a general feature of the signaling response to GnRH pulses (17).

The dose-dependent differences observed in ERK activation 60 min after GnRH stimulation suggests that downstream targets of ERK may also be differentially affected. The transcription factor ELK1 is an immediate, nuclear target of ERK that is rapidly activated by phosphorylation upon stimulation of the ERK signaling cascade. The mRNA cap-binding protein EIF4E is essential for initiation of translation of 5′capped mRNAs and is a cytoplasmic target of the ERK signaling cascade via the ERK-interacting kinase MAPK-interacting kinase (MNK)-1 (18). We examined the phosphorylation of ELK1 and EIF4E 5 or 60 min after stimulation with GnRH at 100 nM. (Fig. 1C). EIF4E exhibited a sustained activation at 60 min but ELK phosphorylation returned to control levels. Thus targets of ERK are not equivalently stimulated by sustained activity.

Activated ERK is localized to the cytoplasm

Differential phosphorylation of ELK1 and EIF4E by GnRH implies that ERK remains active in cytoplasmic rather than nuclear subcellular domains. Acute treatment with GnRH induces transient activation and translocation of phosphorylated ERK into the nucleus. In the immature gonadotrope precursor cell line αT3-1, stable interactions of the GnRHR with phosphorylated ERK are found in membrane-associated signaling complexes after treatment with GnRH analog (27). We examined the long-term activation of ERK in nuclear and cytoplasmic subdomains to determine the localization of phosphorylated ERK (Fig. 2A). After a 5-min treatment with 100 nM GnRH, phosphorylated ERK relative to total was found in both nuclear and cytosolic fractions of LβT2 cells (Fig. 2B). Phosphorylated ERK is found mainly in the cytosolic fraction 60 min after stimulation, confirming that stable phosphory-
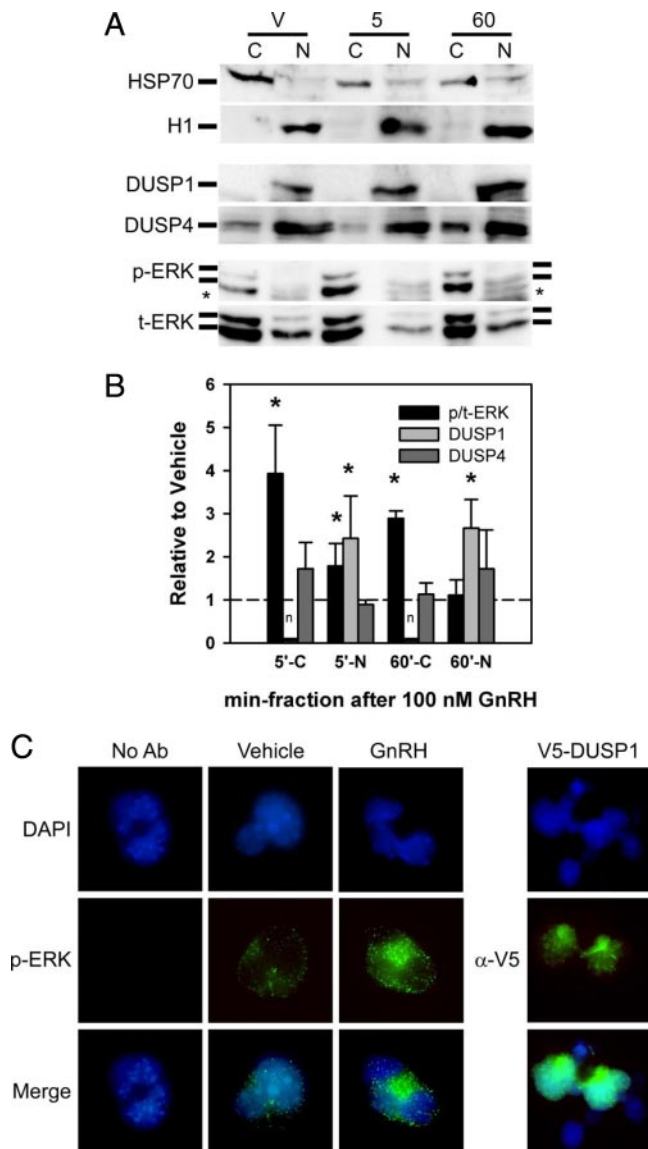
FIG. 2. Sustained ERK is localized to the cytosolic fraction of LβT2 cells. A, Cytosolic and nuclear fractions were prepared from LβT2 cells stimulated with vehicle or 100 nM GnRH for 5 min, washed, and harvested at the times indicated. Nuclear and cytosolic fractions were Western blotted for HSP70 and H1 to confirm separation. Blots were further probed with antibodies for DUSP1 and DUSP4 as well as phosphorylated and total ERK. The *asterisk* indicates nonspecific protein detected by the p-ERK antibody. C, Cytosolic fraction; N, nuclear fraction; V, vehicle; HSP70, heat shock protein 70; H1, histone H1; p-ERK, phosphorylated ERK; t-ERK, total ERK. B, Quantitative chemiluminescence was used to compare the levels of phospho-ERK to total-ERK ratio as well as DUSP1 and DUSP4 levels relative to untreated control. The histogram represents mean values ± SEM normalized to vehicle control (*broken line*) of four independent determinations. For ERK quantification, only bands superimposable in both p-ERK and t-ERK images were quantified to avoid inclusion of nonspecific proteins detected in nuclear fractions. The *asterisk* indicates a significant difference from vehicle control. n, Not detected. All values reported are means ± SEM of four independent determinations. C, Immunofluorescent labeling of phospho-ERK in LβT2 cells treated with GnRH for 5 min and fixed at 60 min after treatment and in LβT2 cells transfected with V5-DUSP1. *Green* indicates phosphor-ERK or V5-DUSP1. Nuclei are visualized in *blue* using 4′,6-diamidino-2-phenylindole (DAPI). Ab, Antibody; V5-DUSP1, V5 epitope-tagged DUSP1.

lation of ERK is localized mainly to the cytosolic domain. To confirm this, immunocytochemical analysis of LβT2 cells was carried out 60 min after stimulation with 100 nM GnRH for 5 min. Phosphorylated ERK was localized almost exclusively to the cytoplasmic domain (Fig. 2C).

Dephosphorylation of MAPKs after receptor-mediated activation is an important regulatory feedback mechanism necessary for the reestablishment of basal signaling cascade activity. In the case of ERK, dephosphorylation is mediated by the MKP subfamily of DUSPs. Although several family members are coexpressed, DUSPs exhibit individual substrate preferences for ERK, JNK, and p38 and are differentially localized to nuclear and cytosolic domains. DUSP1 and DUSP4 are induced in whole mouse pituitary by GnRH analog stimulation, and multiple genome-wide assessments of GnRH-induced gene transcription have shown that several family members are induced by GnRH (9, 10, 28, 29). We examined subcellular fractions of LβT2 cells stimulated with GnRH to assess the localization of both DUSP1 and DUSP4, both major feedback regulators of ERK activity (Fig. 2A). Although DUSP1 was found exclusively in the nuclear fraction and was undetectable in the cytosolic fraction, DUSP4 was found in both fractions (Fig. 2B). DUSP1 levels were significantly increased in the nuclear fraction within 5 min of GnRH stimulation and remained high 60 min after stimulation. This observation suggests that cytosolic DUSP4 is not sufficient to dephosphorylated ERK and that DUSP1 is not likely to play a role in cytosolic ERK dephosphorylation because it is localized almost exclusively to the nuclear fraction.

To confirm the exclusive localization of DUSP1 to the nuclear domain, LβT2 cells were transfected with a cDNA expression plasmid encoding a mouse DUSP1 cDNA bearing a C-terminal V5 epitope tag. Transfected cells were visualized by immunocytochemical staining for the V5 epitope (Fig. 2C). Overexpressed DUSP1-V5 is highly localized to the nucleus in LβT2 cells.

GnRH regulates DUSP1 protein and mRNA synthesis

Several DUSP family members are expressed in LβT2 cells including a number in the MKP subfamily (17). DUSP proteins are activated by a number of mechanisms including direct phosphorylation by their substrates, which occurs in the case of DUSP1 by ERK (30). Phosphorylation of DUSP1 leads to an increased half-life and stabilization of the protein. We examined DUSP1 activation at various times after a single 5-min stimulation with 100 nM GnRH by immunoprecipitation with DUSP1 antibody followed by Western blotting for phosphoserine and DUSP1 (Fig. 3A). An increase in serine-phosphorylated DUSP1 relative
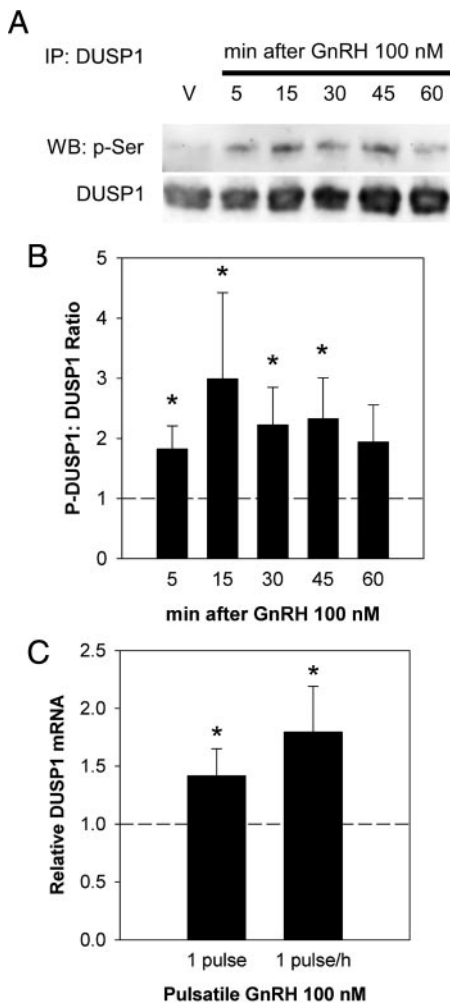
FIG. 3. DUSP1 is regulated by GnRH in LβT2 cells. A, Immunoprecipitation of DUSP1 from GnRH-treated LβT2 cells harvested at the times indicated after 5 min treatment with 100 nM GnRH. Whole-cell extract was immunoprecipitated with DUSP1 antibody and Western blotted with antiphosphoserine antibody, quantified by chemiluminescent imaging, stripped, and reblotted with a second DUSP1 antibody. IP, Immunoprecipitation; WB, Western blot; p-Ser, phosphoserine antibody. B, Quantization of chemiluminescence from immunoprecipitated DUSP1 in A showing an increase in serine phosphorylation after GnRH treatment. *Asterisk* indicates a significant difference from control vehicle-treated cells as represented by the reference line at 1 (*broken line*). C, Results from quantitative PCR of Dusp1 mRNA in LβT2 cells treated with a single pulse or one pulse per hour with GnRH at 100 nM peak amplitude in perifusion chambers and harvested after 4 h as detailed in the text. The *asterisk* indicates a significant difference from control vehicle-pulsed cells represented as the reference line at 1 (*broken line*). All values reported are means ± SEM of three independent determinations.

to total DUSP was detected within 5 min of GnRH administration and maintained up to 45 min afterward (Fig. 3B). An increase in DUSP1 mRNA levels relative to glyceraldehyde-3-phosphate dehydrogenase mRNA was measured by quantitative PCR 60 min after a single 5-min 100 nM GnRH treatment and in cells 60 min after the final of four consecutive pulses of GnRH at a 60-min pulse interval (Fig. 3C). These observations demonstrate that DUSP1

is a target of GnRH signaling at both the protein and the transcript level.

ERK phosphorylation by pulsatile GnRH

The studies above examined the ERK response to a single 5-min exposure to 1 and 100 nM GnRH stimulation. Regulation of gene expression in gonadotropes is controlled through altered regimens of pulse amplitude and frequency, and both parameters influence the magnitude of response to GnRH. Furthermore, repeated stimulation of a signaling cascade can have cumulative effects such as reduced sensitivity through receptor or signal cascade desensitization, increased sensitivity through sensitization via increased expression of receptors or signaling intermediates, or a neutral effect through cellular regulatory mechanisms maintaining signaling homeostasis. Thus, repeated pulses may induce different changes based on the overall response to repeated stimulation. To examine this, we subjected LβT2 cells cultured in a perifusion system to multiple pulses of 1 and 100 nM GnRH peak amplitude (Fig. 4A). We examined ERK phosphorylation 5 min after a single pulse to assess the maximum effect of stimulation of naïve cells. We also examined ERK phosphorylation 5 min after the final of four repeated pulses at a 1-h pulse frequency to assess any changes in signaling activation and 60 min after a final pulse to assess sustained effects of repeated stimulation (Fig. 4B). As in the single stimulation studies in static culture, repeated GnRH stimulation at 1 nM pulse amplitude did not affect the return of ERK phosphorylation levels to baseline (Fig. 4C). After repeated 1 nM GnRH stimulation, ERK phosphorylation was significantly reduced to approximately 70% of single pulse levels: 19.3 ± 3.8- *vs.* 13.4 ± 1.6-fold activation relative to the untreated controls. Repeated stimulation at 100 nM pulse amplitude caused a decline in ERK phosphorylation 5 min after the pulse to 55% of single pulse levels: 21.0 ± 5.3- *vs.* 11.5 ± 3.1-fold phosphorylation relative to the untreated controls. Furthermore, 100 nM pulse stimulation led to a sustained ERK phosphorylation 60 min after the final pulse: 7.7 ± 3.5-fold over untreated controls.

Differential activation of GnRH targets by pulsatile GnRH

Previous work demonstrated that cap-dependent translation is significantly activated by 1-h pulses (18). We examined the ERK signaling pathway targeting cap-dependent translation in cells pulsed at 1 and 100 nM peak amplitudes to assess the phosphorylation status (Fig. 5A). The downstream MNK1 kinase and its target, EIF4E cap-binding protein, were similarly activated by a single pulse of both 1 and 100 nM GnRH at a level that was significant but not proportional to the difference in GnRH concen-
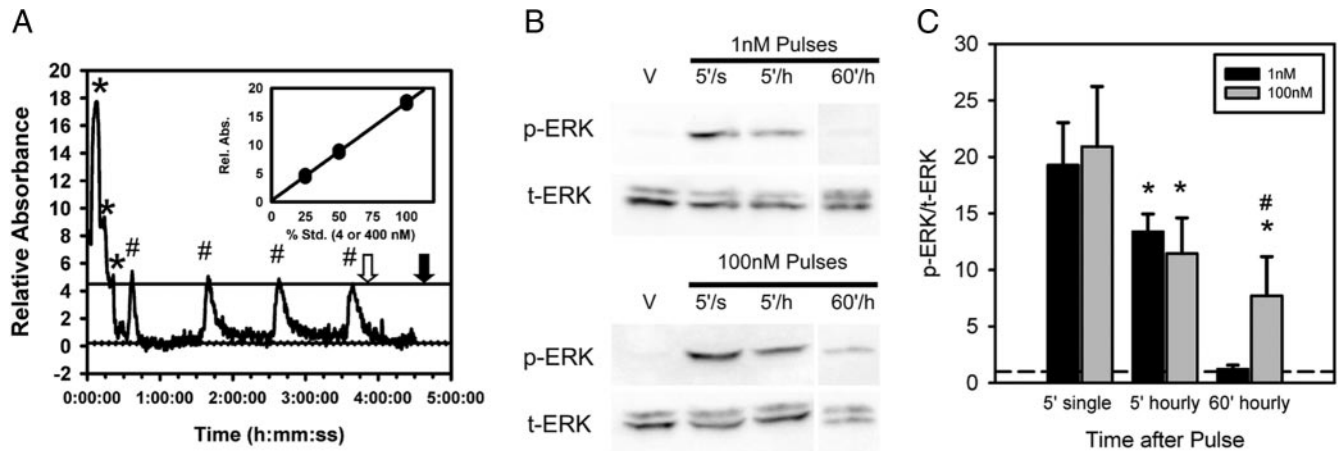
FIG. 4. High-amplitude but not low-amplitude GnRH pulses invoke sustained ERK activation. LβT2 cells cultured on microcarrier beads were placed into a perifusion column and subjected to four hourly pulses of GnRH at 1 or 100 nM peak pulse amplitude. A representative absorbance profile of a pulsed culture is presented in A. The *inset plot* is the linear fitting of calibration data of 100, 50, and 25% calibration dye peaks (*asterisk*) and calculated baseline. Individual pulse peaks are indicated (#). The *open arrow* indicates the point of harvest 5 min after the final pulse. The *solid arrow* indicates the time of harvest 60 min after the final pulse. B, Representative Western blot of GnRH pulsed LβT2 cells showing phosphorylation levels of ERK by 1 and 100 nM GnRH 5 min after a single pulse or 5 min after the fourth hourly pulse. The single pulse was administered coincident with the fourth pulse. The 5- and 60-min harvest points are indicated by *open arrow* and *solid arrow* on the trace in A, respectively. Cells were also harvested 60 min after the final hourly pulse and examined. V, Vehicle; p-ERK, phosphorylated ERK; t-ERK, total ERK; 5′/s, 5 min after single pulse; 5′/h, 5 min after the fourth hourly pulse; 60′/h, 60 min after the final hourly pulse. Quantization of chemiluminescence is represented in C as p-ERK to t-ERK ratio normalized to vehicle-treated controls shown as the reference line at 1 (*broken line*). The significant difference from the 5-min, single-pulse p-ERK to t-ERK ratio is indicated by an *asterisk*. The significant difference between 1 and 100 nM treatment is indicated (#). All except the 60-min hourly 1-nM GnRH pulse group were significantly increased compared with untreated values. All values reported are means ± SEM of four independent determinations.

tration (Fig. 5B). Both MNK1 and EIF4E remained activated 60 min after the final 100-nM pulse, consistent with the concurrent stable activation of ERK. Neither protein was persistently activated by the 1-nM pulse amplitude, although some activation of EIF4E was observed. We also determined changes in DUSP1 expression in pulsed LβT2 cells and found that protein expression is increased approximately 5.4-fold over untreated controls.

DUSP1 overexpression inhibits ERK and *Lhb* activation by high-amplitude pulses

Excess DUSP1 expression present at the time of ERK stimulation will lead to suppression of ERK activity through rapid dephosphorylation. In the case of GnRH stimulation, increased DUSP activity should lead to a suppression of GnRH-induced ERK phosphorylation at high-pulse amplitude. To test this, LβT2 cells cotransfected with the DUSP1 expression vector pCMV-SPORT6-DUSP1 and pcDNA3.1-ERK-V5-His expression vector were stimulated with 100 nM GnRH for 5 min. Cells were harvested 60 min after the treatment and ERK1-V5 was immunoprecipitated using V5 epitope-specific antibody. Immunoprecipitates were Western blotted for phosphorylated ERK and total ERK (Fig. 6A). In control transfected cells, ERK phosphorylation was increased 11.3 ± 4.0-fold over untreated controls. In DUSP1 cotransfected cells, ERK phosphorylation was limited to 4.0 ± 2.3-fold over untreated control levels (Fig. 6B). This result is consistent with the interpretation that excess DUSP activity limits

ERK activation, resulting in a reduction in sustained ERK phosphorylation. The apparent decrease in ERK-V5 expression in DUSP1-expressing lanes was not consistent between experiments. It should be noted that both homo- and heterodimeric ERK complexes have been reported by others and are detected in these extracts (31).

DUSP1 is largely restricted to the nuclear compartment in LβT2 cells (Fig. 2). Therefore, overexpression of DUSP1 should lead to suppression of GnRH-stimulated *Lhb* promoter activity. This was tested by cotransfection of pCMV-SPORT6-DUSP1 with the 1.8-kb rat *Lhb* promoter-luciferase reporter plasmid pLHβ1.8-Luc and the pGL3-CMV-β-Gal internal control plasmid to assess the specific effects of DUSP1 overexpression on GnRH activation of the *Lhb* promoter. Transfected cells were subjected to a 6-h, 100-nM amplitude pulse paradigm described in Fig. 4. Overexpression of DUSP1 abolished the activation of the transfected *Lhb* promoter by GnRH (Fig. 6C). Taken together, these data show that DUSP1 overexpression inhibits both ERK phosphorylation and *Lhb* promoter activation by GnRH. This activity may not be restricted to DUSP1 because there are wide substrate preferences for many DUSP species.

DUSP knockdown promotes activation of ERK and *Lhb* transcription by GnRH

The wide substrate specificity and expression of multiple DUSP species may provide redundant-negative feedback pathways. To test whether DUSP1 specifically participates in modulation of GnRH-stimulated ERK
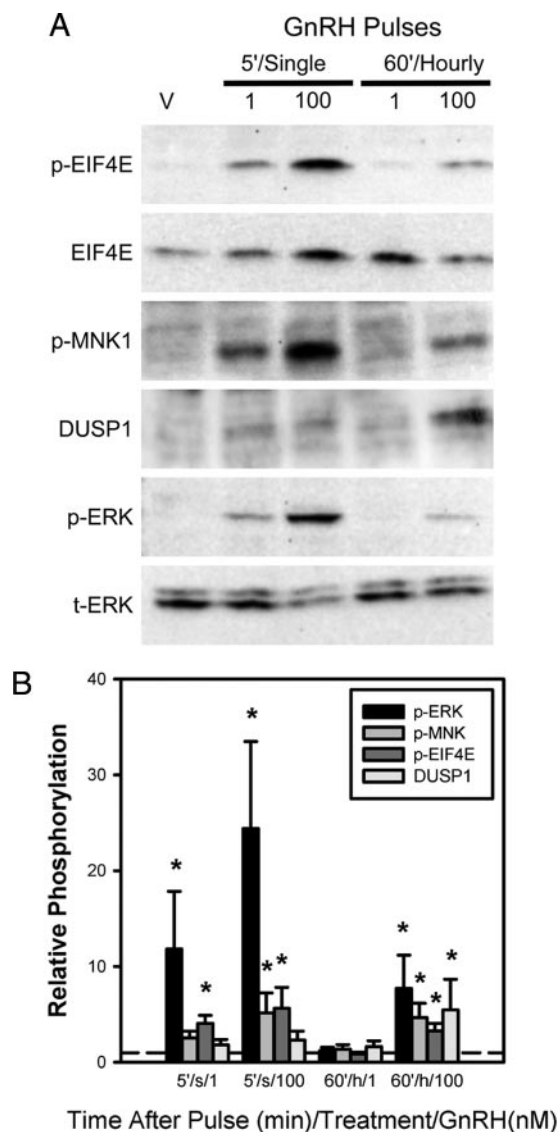
FIG. 5. High-amplitude but not low-amplitude GnRH pulses cause sustained activation of cytoplasmic ERK targets. Perifused LβT2 cells pulsed with 1 and 100 nM GnRH were harvested 5min after a single pulse and 60 min after the final of four hourly pulses and phosphorylation levels of pEIF4E (p-EIF4E) and its upstream activating kinase MNK1 (p-MNK1) were determined by Western blot (A). Activation of DUSP1 protein expression and p-ERK to t-ERK ratio (p-ERK) were also determined. V, Vehicle; p-ERK, phosphorylated ERK; t-ERK, total ERK. Quantization of chemiluminescence from multiple trials is represented in B normalized to untreated levels represented by the reference line at 1 (*broken line*). The significant increase over untreated levels is indicated by an *asterisk*. All values reported are means ± SEM of three independent determinations.

phosphorylation and *Lhb* promoter activity, we examined the effect of DUSP1 knockdown on GnRH-induced ERK phosphorylation and *Lhb* promoter activity. Specifically, if DUSP1 participates in the suppression of GnRH-induced activation of either ERK phosphorylation or *Lhb* promoter activation, then reduction of DUSP1 activity will permit greater activation by low-amplitude GnRH stimulation. To test this, we examined the effect of DUSP1 knockdown on ERK phosphorylation and *Lhb* promoter
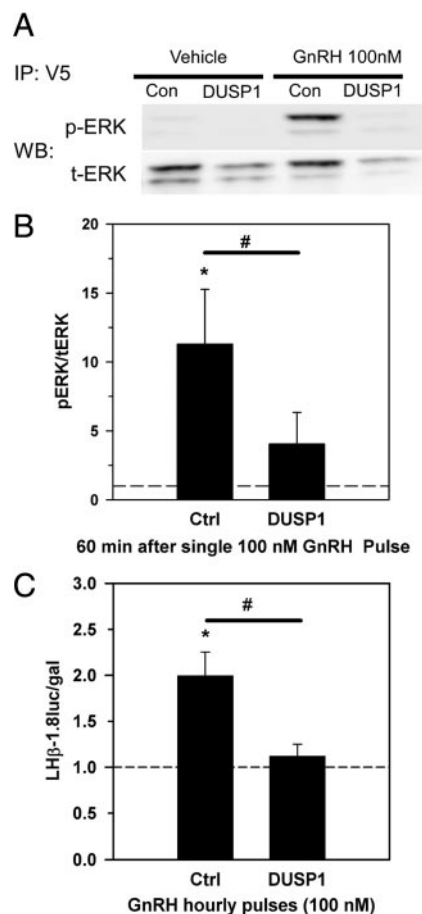

FIG. 6. DUSP1 overexpression inhibits ERK phosphorylation and *Lhb* promoter activation by GnRH. LβT2 cells were cotransfected with a null or DUSP1 expression vector and a V5-epitope-tagged ERK1 cDNA expression plasmid for 48 h before stimulation with 100 nM GnRH for 5 min, washed, and incubated until 60 min after stimulation. Extracts were prepared and immunoprecipitated with anti-V5 antibody. Precipitates were Western blotted for p-ERK and t-ERK levels (A). IP, Immunoprecipitation; WB, Western blot; Con, control; p-ERK, phosphorylated ERK; t-ERK, total ERK. Quantization of phospho-ERK and ERK chemiluminescence is illustrated in B, showing significant suppression of ERK phosphorylation by 100 nM GnRH in the presence of excess DUSP1 (#). A significant difference from control p-ERK to t-ERK ratio marked by the reference line at 1 (*broken line*) is indicated by an *asterisk*. LβT2 cells cultured on microcarrier beads were cotransfected with either a null or DUSP1 expression plasmid, a firefly luciferase reporter plasmid under control of the rat 1.8 kb *Lhb* promoter, and a control β-galactosidase reporter plasmid under the control of the CMV promoter. Cells were placed in a perifusion column and pulsed hourly with 100 nM GnRH for 6 h. Ctrl, Control. Extracts of harvested cells were measured for luciferase and β-galactosidase activity and results are plotted in C. A significant difference in *Lhb* promoter activity between control (Ctrl) and DUSP1 transfected cells is indicated (#). A significant difference from control luciferase to β-galactosidase ratio marked by the reference line at 1 (*broken line*) is indicated by an *asterisk*. All values reported are means ± SEM of three independent determinations.

activation by a 5-min, 1-nM GnRH treatment in static culture and 1 nM peak pulsatile treatment in perifusion culture, respectively. We confirmed a reduction in DUSP1 (Fig. 7A) and mRNA (Fig. 7B) in cells transfected with the *Dusp1*-specific shRNA vector pKo1shDUSP1. We then
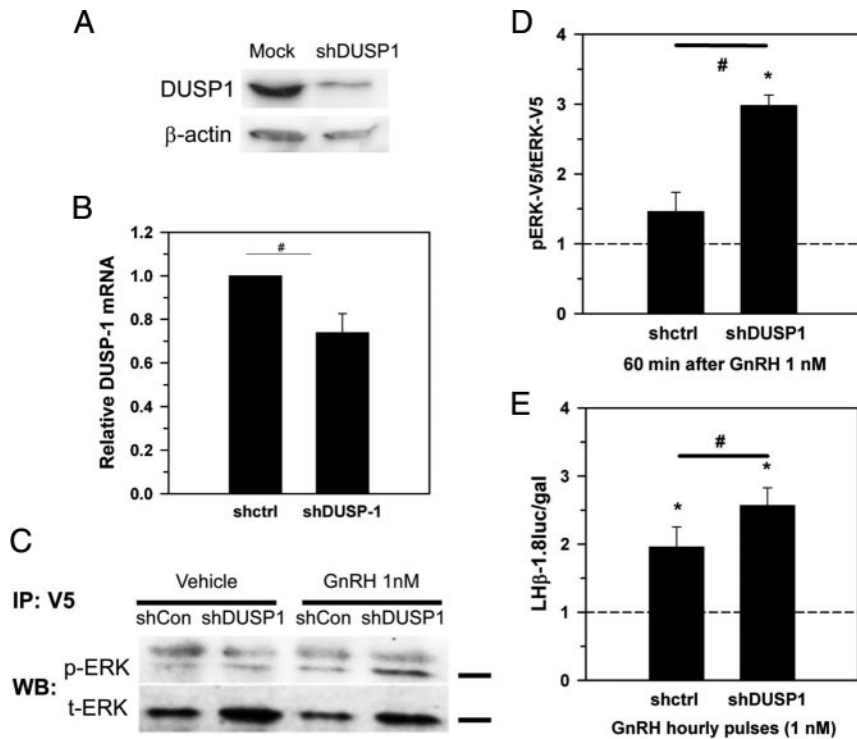
FIG. 7. shRNA targeting DUSP1 increases ERK phosphorylation and *Lhb* promoter activation by GnRH. Knockdown of DUSP1 protein (A) and mRNA (B) in LβT2 cells transfected with a shDUSP1 expression plasmid. LβT2 cells were cotransfected with a null or shDUSP1 expression plasmid and a V5-epitope-tagged ERK1 cDNA expression plasmid for 48 h before stimulation with 1 nM GnRH for 5 min, washed, and incubated until 60 min after stimulation. Extracts were prepared and immunoprecipitated with anti-V5 antibody. Precipitates were Western blotted for p-ERK and t-ERK levels as shown in a representative blot (C). *Bars* indicate bands identified by both p-ERK and t-ERK antibodies and used for quantification. IP, Immunoprecipitation; WB, Western blot; Con, control; p-ERK, phosphorylated ERK; t-ERK, total ERK. The GnRH-induced phospho-ERK and total ERK chemiluminescence ratio relative to vehicle treatment is illustrated in D, showing a significant increase in ERK phosphorylation by 1 nM GnRH in the presence of shDUSP1 but not shControl (#). A significant difference from the respective vehicle-treated p-ERK to t-ERK ratio, marked by the reference line at 1 (*broken line*), is indicated by an *asterisk*. The difference between shControl and shDUSP1 fold change is also indicated (#). LβT2 cells cultured on microcarrier beads were cotransfected with a null or shDUSP1 expression plasmid, a firefly luciferase reporter plasmid under control of the rat 1.8 kb *Lhb* promoter, and a control β-galactosidase reporter plasmid under the control of the CMV promoter. Cells were placed in a perifusion column and pulsed hourly with 1 nM GnRH for 6 h. Extracts of harvested cells were measured for luciferase and β-galactosidase activity and results are plotted in E. A significant difference in the *Lhb* promoter activity between control (Ctrl) and shDUSP1 transfected cells is indicated (#). A significant difference from the control luciferase to β-galactosidase ratio marked by the reference line at 1 (*broken line*) is indicated by an *asterisk*. All values reported are means ± SEM of four independent determinations.

cotransfected cells with the ERK-V5 expression plasmid and the shControl or shDUSP1 plasmids. In shRNA control-transfected cells, ERK-V5 was activated 1.5 ± 0.3-fold over control levels as determined by the phosphorylated ERK to total ERK ratio. In cells transfected with shDUSP1 ERK-V5 phosphorylation was increased to 3.0 ± 0.1-fold over untreated control levels as determined by the phosphorylated ERK to total ERK ratio (Fig. 7C, quantified in Fig. 7D). Thus, decreased DUSP1 resulted in increased ERK-V5 phosphorylation by GnRH, demonstrating a direct role of DUSP1 in modulation of GnRH-induced phosphorylation.

One predicted effect of decreased DUSP1 is increased sensitivity of the *Lhb* promoter to low-amplitude pulse stimulation by GnRH. To test this, we cotransfected cells with the pLHβ1.8-Luc and the pGL3-CMV-β-Gal internal control plasmid as above to assess the specific effect of decreased DUSP1 on *Lhb* promoter activation by low-amplitude GnRH pulses. Transfected cells were subjected to the pulse regime in perifusion culture described above using 1-nM peak pulse amplitudes for 6 h. Stimulation with 1 nM GnRH pulses over 4 h is sufficient to significantly induce *Lhb* promoter activity to 2.0 ± 0.3-fold over control unstimulated levels. In the presence of the shDUSP1 expression plasmid, 1-nM GnRH pulses caused a significant increase to 2.5 ± 0.3-fold over unstimulated levels. Similar results were obtained using a second shDUSP1 expression plasmid targeting a different region of the DUSP1 mRNA sequence (not shown).

The demonstration of a specific effect of DUSP1 knockdown on ERK phosphorylation and *Lhb* promoter activation indicates that DUSP1 contributes specifically but not exclusively to the regulation of GnRH signaling and the relevant gonadotropin gene expression in LβT2 gonadotropes.

Discussion

GnRH activates ERK1/2, p38 MAPK, and JNK at varying levels and rates in pituitary gonadotropes (5). Pulsatile GnRH alters ERK responsiveness (32) and activation of the immediate early gene *Egr1* (17, 32, 33). Blockade of the ERK pathway by the MAPK kinase inhibitor PD098059 abolishes GnRH-induced *Lhb* promoter activity (20, 32) and protein synthesis (21). Dominant-negative forms of the rat sarcoma signaling system, Ras activated factor 1, and MAPK kinase significantly reduce ERK activation, protein synthesis, and *Lhb* promoter activity in αT3-1 and LβT2 cells (20, 34). In the mouse, female fertility requires the presence of ERK (35). These data suggest a significant role of ERK in GnRH-activated expression of the *Lhb* gene.

Some have demonstrated a link between GnRH pulse amplitude and gonadotropin mRNA synthesis: a high-amplitude pulse stimulates a greater increase in *Lhb* mRNA than a low-amplitude pulse (36). Furthermore, low and high GnRH concentrations have differential effects on EGR1 protein synthesis, an immediate-early gene responsive to GnRH stimulation (17, 37). GnRH pulse frequency also influences the resolution of ERK activation. A 30-min pulse frequency leads to transient ERK activation that resolves within 30 min, whereas a 2-h pulse frequency response resolves within 40 min (32). The rapid resolution of ERK activation under a high-pulse frequency is consistent with an increase in DUSP gene expression under high- but not low-pulse frequency (17). Together these results show that both GnRH pulse frequency and amplitude modify signaling responses. Some caution in the interpretation of feedback regulatory studies in LβT2 cells is warranted because these cells are transformed with and express the Simian virus 40 T antigens (16). The small T antigen potentially inhibits MAPK dephosphorylation through blockade of protein phosphatase 2A (38), which exhibits cross talk with DUSP1 in ERK inactivation (39). In such a scenario, ERK phosphorylation would not be resolved under any condition. Stimulation with 1 nM GnRH is rapidly resolved, indicating that broad inactivation of phosphatase activity by the small T antigen is not likely involved in the modulation of ERK activation by GnRH in LβT2 cells.

The mechanisms involved in pulse resolution are not understood. Some models of ERK activation provide insight into the potential role of feedback regulators. One model suggests that the ERK signaling network exhibits hysteresis and that a stable state of low or high activation is established by a previous stimulation. This priming then contributes to the level of sustained activation with multiple pulses and modulation of positive and negative feedback maintain activation. In the case of ERK, this has been proposed to be cytosolic phospholipase A$_2$ and DUSP1, respectively (7, 8). Here we confirm the existence of negative feedback through DUSP activity, but a thorough examination of this model has not been undertaken. An alternative model based on a threshold effect has also been proposed. In this model, saturation of parallel signaling networks that share a common early signaling component can lead to a threshold effect on full activation of the ERK cascade. Experimental evidence for this has been presented (40), and our observations are consistent with this. In this scheme, it is possible that negative feedback regulators such as DUSP family members fulfill the role of a noise suppressor that contributes to the establishment of the threshold level. Thus, elevated input through high-frequency or high-amplitude stimulation may provide the

necessary signal to induce a highly activated state. Spatiotemporal factors may also play a role. In αT3 cells, stable cytoplasmic ERK signaling complexes are observed in the presence of high levels of GnRH analog (27, 41). Preformed signaling complexes containing ERK 1/2 have been demonstrated in LβT2 cells, and these are responsible for cytoplasmic ERK signaling to paxillin in cytoplasmic focal adhesions (42). The threshold effect may involve establishment of these complexes in the cytoplasm, which are resistant to rapid resolution by nuclear DUSP activity.

DUSP1 was identified as a point at which flexible ERK responses occur in fibroblasts (7). Accordingly, *Dusp1* was identified as regulated by GnRH in LβT2 cells (10, 17, 28, 29). GnRH stimulation regulates *Dusp1* and *Dusp4* mRNA and protein expression in αT3-1 cells (11). DUSP1 protein expression is more responsive and transient than DUSP4 (20). Whereas our data define a role for DUSP1 in regulation of ERK and *Lhb* promoter activity in LβT2 cells, other evidence that multiple DUSPs contribute to ERK responses to GnRHR signaling has been presented in αT3-1 and HeLa cells (11, 13). Moreover, expression of *Dusp2, -4, -8, -11, -16,* and *-19* and *Rgs2,* a negative regulator of the major G protein used by GnRHR, is increased by 100 nM pulsatile GnRH at the mRNA level (17), suggesting a complex network supporting the contribution of many factors to the organization of a MAPK signaling. We examined the role of DUSP1 using 1 nM stimulation to avoid the activation of multiple DUSPs that occurs at 100 nM (17). Combined experimental and modeling studies of the contribution of multiple MAPK pathways in decoding GnRH pulses have concluded that DUSP1 feedback contributes to GnRH pulse interpretation (43). Our results conform to the predictions of this model in both static and perifusion culture. The LHβ promoter is stimulated similarly by both 1 and 100 nM GnRH (Figs. 6C and 7E, respectively). DUSP1 is restricted to the nucleus (Fig. 2), and alteration of DUSP1 levels alters promoter activation in response to GnRH. These observations indicate that DUSP1 participates in the modulation of ERK phosphorylation. Confirmation of these regulatory mechanisms will require study of feedback regulation in genetically modified *in vivo* models.

In summary, we demonstrate a role for DUSP1 in negative feedback of ERK activation by GnRHR. Alteration of *Dusp1* gene expression alters ERK activation and impacts *Lhb* gene promoter activity, linking DUSP activity to GnRH-mediated regulation of gonadotrope gene expression. Under low-pulse amplitude stimulation, ERK transiently activates both cytoplasmic and nuclear targets EIF4E and ELK1, respectively. Under high-pulse amplitude, increased ERK activation causes stable cytoplasmic signaling (27). In the nucleus, DUSP maintains rapid res-

olution of nuclear ERK activity and serves a mechanism to maintain acute sensitivity to GnRH stimulation.

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