YB-1: A NEW PLAYER IN FSH REGULATION OF
GENE EXPRESSION IN GRANULOSA CELLS

By

ELYSE MARIE DONAUBAUER

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the requirements for the degree of

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School of Molecular Biosciences

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of ELYSE MARIE DONAUBAUER find it satisfactory and recommend that it be accepted.

________________________________________
Mary Hunzicker-Dunn, Ph.D., Chair

________________________________________
John Nilson, Ph.D.

________________________________________
Joseph Harding, Ph.D.

________________________________________
Michael Konkel, Ph.D.

________________________________________
Eric Shelden, Ph.D.
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Within the ovarian follicle, immature oocytes are surrounded and supported by granulosa cells (GCs). Stimulation of GCs by follicle stimulating hormone (FSH) promotes their proliferation and differentiation, events that are necessary for fertility. FSH activates multiple signaling pathways to regulate genes necessary for follicular maturation. This research focuses on the regulation by FSH of extracellular signal-regulated kinase (ERK) and identifies Y-box binding protein-1 (YB-1) as a downstream target of ERK signaling. FSH-dependent ERK(Thr^{202}/Tyr^{204}) phosphorylation is protein kinase A (PKA) dependent, yet requires the constitutively active upstream ERK signaling pathway proteins. Treatment with EGFR inhibitor AG1478, a dominant-negative RAS, and the MEK inhibitor PD98059 all blocked FSH-dependent ERK(Thr^{202}/Tyr^{204}) phosphorylation. We hypothesized that FSH via PKA regulates ERK phosphorylation by inhibiting the activity of a protein phosphatase that dephosphorylates ERK in the absence of FSH. Results show that treatment with MAP kinase phosphatase 3 (dual specificity phosphatase 6) (MKP3(DUSP6)) inhibitors increase ERK(Thr^{202}/Tyr^{204}) phosphorylation in the absence of FSH to levels comparable to ERK phosphorylated in the presence of FSH. Further, ERK coimmunoprecipitated with MKP3(DUSP6), and treatment with MKP3(DUSP6)
inhibitors blocked dephosphorylation of recombinant phospho-ERK2-GST. Together these results indicate that MKP3(DUSP6) is the phosphatase that dephosphorylates ERK in the absence of FSH. We further identified YB-1 as a downstream target of ERK. 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\textsuperscript{102} within 15 min that is maintained until ~8 h post treatment. FSH-stimulated phosphorylation of YB-1(Ser\textsuperscript{102}) is prevented by pretreatment of GCs with the PKA-selective inhibitor PKI, the MEK inhibitor PD98059, and the ribosomal S6 kinase-2 (RSK-2) inhibitor BI-D1870. Transduction of GCs with the dephospho-adenoviral-YB-1(S102A) mutant prevented the FSH-dependent induction of Egfr, Cyp19a1, and Inha mRNAs. Collectively, these results demonstrate novel regulation of ERK(Thr\textsuperscript{202}/Tyr\textsuperscript{204}) phosphorylation by FSH and identify ERK-dependent gene targets that are required for follicular maturation. These results reveal that the ERK signaling pathways contributes to GC maturation by promoting the phosphorylation of the transcriptional activator YB-1 that is required for expression of at least three crucial FSH target genes.
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### LIST OF COMMONLY ABBREVIATED WORDS

Common abbreviations used are:

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>GCs</td>
<td>granulosa cells</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKI</td>
<td>PKA inhibitor</td>
</tr>
<tr>
<td>RSK-2</td>
<td>ribosomal S6 kinase-2</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3- kinase</td>
</tr>
<tr>
<td>YB-1</td>
<td>Y-box binding protein-1</td>
</tr>
<tr>
<td>IGF1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>Myr</td>
<td>myristoylated</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>E2</td>
<td>estradiol-17β</td>
</tr>
<tr>
<td>P</td>
<td>penicillin</td>
</tr>
<tr>
<td>S</td>
<td>streptomycin</td>
</tr>
<tr>
<td>PP2</td>
<td>protein phosphatase 2</td>
</tr>
<tr>
<td>mRNP</td>
<td>mRNA-protein complex</td>
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<tr>
<td>SHP2</td>
<td>SRC homology-2 (SH2) domain-containing tyrosine phosphatase</td>
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Other abbreviations include:

- FOXO1, forkhead box- containing protein O1
- cAMP, cyclic adenosine monophosphate
- CREB, cAMP-response binding element protein
- EGFR, epidermal growth factor receptor
- MEK, mitogen-activated protein kinase kinase
- Egr1, early growth response factor – 1
- RTK, receptor tyrosine kinase
- JNK, c-Jun N-terminal kinase
- PTP, protein tyrosine phosphatase
- eIF4E, eukaryotic initiation factor 4E
- CDC25, cell division cycle 25
- MAPK, mitogen activated protein kinase
- RTK, receptor tyrosine kinases
- MKP, MAP kinase phosphatase
- PTP, protein tyrosine phosphatase
- ERK, extracellular signal-regulated kinase
- DUSP, dual specificity phosphatase
DEDICATION

This dissertation is dedicated to my parents who provided unlimited and much needed emotional support. They will have no idea what this dissertation is about, but will still attempt to read it.

That is unconditional love and support.
CHAPTER ONE
INTRODUCTION

FOLLICULAR MATURATION

In females, the ovarian follicle has a distinct role in fertility. The ovarian follicle serves to produce a mature oocyte that can be fertilized, to prepare the uterus to accept the fertilized oocyte, and to sustain the development of the embryo (reviewed in (1)). These processes are accomplished through response to and regulation by multiple endocrine and paracrine hormones. Endocrine hormones follicle stimulating hormone (FSH) and luteinizing hormone (LH) are required for follicular maturation and ovulation of the oocyte (2,3). Granulosa cells (GCs) within the follicle produce and secrete estradiol (4). The resulting estradiol circulating in the blood will act on the pituitary to regulate LH production, on GCs to stimulate growth, and on the uterus to prepare for and sustain the embryo after implantation (reviewed in (5) and (6)). Following ovulation, terminally differentiated GCs produce and secrete progesterone (4), which is also essential for the uterus to support the embryo and maintain pregnancy. Thus, the ovarian follicle has a central role in regulation of all these events.

Within the ovary, the follicle surrounds and supports the female gamete and is comprised of an oocyte surrounded by support GCs, basal lamina, and an external layer of theca cells that contains the vascular supply for the follicle. Follicular maturation occurs within the follicle in two distinct stages, the gonadotropin – independent stage and the gonadotropin – dependent stage (reviewed in (1)). During each reproductive cycle, a cohort of follicles undergoes maturation from immature primordial follicles to preantral follicles in the gonadotropin – independent stage.

Primordial follicles are formed prenatally following germ cell cyst breakdown. Germ cell cysts consist of clusters of dividing oogonia, and these oogonia become oocytes when they enter mitosis. As the germ cell cysts breakdown, there is programmed cell death of some of the oocytes, and the bordering GCs will surround single oocytes, forming primordial follicles (reviewed in (7)). Primordial follicles
develop into primary, then secondary follicles, which are marked by the growth of the oocyte, addition of multiple layers of GCs, and formation of the theca cell layer. Although the regulation of germ cell cyst breakdown and primordial follicle formation to primary and secondary follicle formation is still being elucidated, mouse knockout models have identified many essential regulators in the pathway (reviewed in (7)). For example, growth differentiation factor 9 (Gdf9)-knockout mice have ovaries that only contain primary follicles, indicating that GDF-9 is necessary for primary follicles to develop into secondary follicles (8). Anti-Müllerian hormone (Amh)-knockout mice have fewer primordial follicles, leading to premature follicle depletion (9). As AMH treatment caused a decrease in primordial follicle assembly, it is thought that the Amh-knockout mice have fewer primordial follicles to start with (10). These results indicate that both GDF-9 and AMH are a few of the important regulators of the maturation of primordial follicles to primary and secondary follicles.

During the gonadotropin-dependent stage the follicle responds to the gonadotropin hormones FSH and LH. Maturation of preantral follicles occurs in response to FSH and only a few follicles mature completely while most of the cohort undergoes apoptosis and atresia (11-13). Response to FSH promotes production and secretion of estrogen, proliferation and differentiation of GCs, and expansion of the follicle in size. Response to LH promotes ovulation of the oocyte and initiation of meiosis and formation of the corpus luteum that serves to support the oocyte upon fertilization. Only GCs express the FSH receptor, and in response to FSH, GCs undergo proliferation and differentiation, causing the follicle to increase in size. A fluid filled antrum forms within the center of the follicle, and two distinct populations of GCs develop. The cumulus cells surround the oocyte and provide it with nutrients, metabolites, and essential regulatory signals (14). The mural GCs line the interior adjacent to the basal lamina, and produce essential steroids (15,16) and express LH receptors (17,18). Interestingly, the cumulus GCs are distinguished from mural GCs by the low expression of Lhcgr mRNA (19). FSH initiates this transition through regulation of hundreds of genes within GCs (20,21), leading to changes in many cellular processes such as intracellular signaling, steroid production, and cell cycle regulation.
FSH facilitates regulation of the transcription of target genes through a variety of mechanisms, such as an increase in the expression of the transcription factors early growth response factor – 1 (Egr1) (22) and c-Fos (23). FSH promotes posttranslational modification of common transcription factors like cAMP response element binding protein (CREB) (24), FOXO1 (25), and Smad2/3 (26), as well as promotes an increase in phosphorylated and acetylated histone H3 bound to FSH target genes (27). These modifications, and others similar, lead to an FSH-dependent increase in transcription of genes that encode, for example, epiregulin (28), LH receptor (29), regulatory (R) IIβ subunit of protein kinase A (PKA) (30), and serum glucocorticoid kinase (SGK) (31).

FSH also promotes an increase in the paracrine and autocrine hormones produced by the GCs through activating gene targets (32,33). Examples include: increases in production of inhibin-α, the hormone necessary to negatively feedback and repress production of FSH at the pituitary and hypothalamus (27,34); increases in progesterone due to increased production of P-450 cholesterol side chain cleavage (SCC) necessary for progesterone production (35,36); and importantly increases in estrogen due to increased production of P-450 aromatase (aromatase) necessary in estrogen biosynthesis (37,38).

FSH triggers proliferation of the GCs. One way this is achieved is through regulation of the expression of the cell cycle protein cyclin D2. Cyclin D2 is necessary for follicular maturation, as demonstrated by the infertility of the cyclin D2-null mouse model (39). Interestingly, FSH alone is not sufficient to promote cyclin D2 expression in rat GCs in culture; a TGFβ agonist, such as activin, is required in addition to FSH (40,41). FSH and activin regulates the transcription factors FOXO1 and Smad2/3, respectively, to activate transcription of cyclin D2 (26), and to promote granulosa cell proliferation.

Collectively, these data indicate that the action of FSH on GCs promotes coordinated regulation of hundreds of genes. This regulation requires released repression of negative regulators, as well as
activation of multiple signaling pathways, and is essential to maturation of the follicle resulting in ovulation of the oocyte.

**SIGNALING IN GCs**

In order to regulate expression of essential target genes, FSH activates a variety of signaling cascades such as the phosphoinosidade 3-kinase (PI3K)/ protein kinase B (AKT) pathway and mitogen-activated protein kinases (MAPK)/ extracellular signal-regulated kinase (ERK) pathway (see Fig 1). FSH accomplishes these activations mainly through an increase in cAMP levels and activation of PKA. FSH binds to its G-protein coupled receptor, a seven transmembrane receptor protein that undergoes a conformational change after binding to FSH. The FSH receptor (FSHR) is only expressed in GCs, is coupled to the stimulatory G protein Gs that in turn activates adenylyl cyclase. Activation of adenylyl cyclase promotes an increase in intracellular cAMP levels. The role of cAMP in GCs is demonstrated by treatment with forskolin, an adenylyl cyclase activator. Forskolin treatment increases cellular levels of cAMP, mimicking the response in GCs to FSH, including activation of PKA and phosphorylation of CREB (42,43). PKA is a tetrameric holoenzyme that has two regulatory and two catalytic subunits. The regulatory subunits contain a pseudosubstrate motif that binds the substrate-binding site of the PKA catalytic domain, blocking its activity. cAMP binds to the regulatory subunit of PKA, inducing a conformational change and releasing the active catalytic subunits (44). PKA activates multiple signaling cascades, as well as stimulates phosphorylation of CREB, histone H3, and β – catenin (25,27) (Fig 1). Treatment with either the adenoviral – PKA inhibitor (PKI), a pseudosubstrate for the catalytic subunit of PKA, or the myristoylated (Mry) – PKI peptide blocks FSH mediated phosphorylation of direct PKA targets CREB(Ser^{133}) (25,43) and β – catenin(Ser^{552}) (25), blocks activation of both the PI3K/AKT and MAPK/ERK pathways (43,45), and blocks induction of FSH-dependent gene targets Map2D and Inha (27,43) as well as Lhcgr and Cyp11a1 (46). Additionally, expression of a constitutively active PKA (PKA-CQR) in GCs effectively mimics FSH treatment, including regulation of FSH target genes and
activation of estrogen and progesterone biosynthetic pathways necessary for follicular maturation (20), indicating that PKA is both necessary and sufficient to regulate target gene expression. Taken together, these results demonstrate that FSH acts via PKA to regulate targets and promote maturation of the follicle.

FSH promotes activation of PI3K/AKT (27,47,48) primarily through regulation of insulin receptor substrate 1 (IRS1) (45). PI3K is canonically activated with ligand binding to either the insulin receptor or insulin–like growth factor 1 receptor (IGF1R) receptor tyrosine kinases (RTKs) (reviewed in (49)). After ligand binding, the receptor dimerizes and autophosphorylates tyrosine residues within NPXY motif. These pTyr binding domains on the RTKs allow for binding of adaptor proteins (IRS1/2 or and GRB2 (growth factor receptor binding protein 2) associated binding protein (GAB)GAB1/2) and, in turn, the adaptor proteins are phosphorylated by RTKs on specific tyrosine resides in the carboxy terminus (50). The pTyr residues on either IRS1/2 or GAB1/2 create SRC homology-2 (SH2) binding domains (dual phosphorylated TyrMetXMet motifs) for PI3K (51). Dual SH2 domains on the 85 kDa regulatory subunit of PI3K (p85) bind dual phospho-YMXM motifs on IRS1/2 or GAB1/2, resulting in activation of the 100 kD catalytic subunit (p100) of PI3K (52). In GCs, FSH promotes phosphorylation of IRS1 on Tyr^989 (a phospho-YMXM motif) (45) that activates PI3K.

Active PI3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP3) through addition of a phosphate to phosphatidylinositol-4,5-bisphosphate (PIP2). Increased levels of PIP3 in the plasma membrane facilitate the binding of both phosphoinositide-dependent kinase 1 (PDK1) and AKT via their pleckstrin homology domains (53,54). With PDK1 in proximity to AKT, PDK1 will phosphorylate AKT on Thr^{308} (55,56). Full activation of AKT occurs after mammalian target of rapamycin (mTOR)/Rictor complex (mTORC2) phosphorylates AKT on Ser^{473} (57), and in GCs, is PKA dependent (45). Active AKT will translocate to other cellular locations, including the nucleus, to promote cellular proliferation, enhance translation and initiate differentiation (58).
Phosphorylated/active AKT facilitates GC proliferation and differentiation through regulation of a variety of substrates. In response to FSH, AKT inhibits tuberin, also called tuberous sclerosis complex 2 (TSC2), a negative regulator of cell growth. In the absence of stimuli, tuberin acts as a GTPase activating protein (GAP) for ras-homologue enriched in brain (RHEB), keeping inactive RHEB\textsuperscript{GDP} levels high (59). In response to FSH, AKT inhibits tuberin, allowing active Rheb\textsuperscript{GTP} levels to increase and to activate mTOR (60). mTOR will then phosphorylate p70 S6 kinase (p70S6K) and 4E-binding protein (BP)1 (4EBP1) (60), promoting an increase in translation. AKT also phosphorylates the transcription factor FOXO1 in response to FSH (25,26,61,62). FOXO1 is a member of the forkhead box-containing O subfamily and binds DNA in an unphosphorylated state. FOXO transcription factors have been shown to both activate and repress transcription in a gene dependent manner and FOXO1 is no exception. FOXO1 has been shown to activate the cell cycle inhibitor p27\textsuperscript{Kip1} (63), glucose-6-phosphatase (64), as well as to repress cyclin D1 and D2 (65). In GCs, in the absence of FSH, FOXO1 has been shown to be bound to the cyclin D2 promoter, and treatment with FSH promoted its dissociation (26). Additionally, treatment of GCs with a constitutively active adenoviral-FOXO1 resulted in the inhibition of the induction of the necessary follicular maturation gene targets \textit{Cyp19a1} and \textit{Cyp11a1}, \textit{Inha}, and \textit{Ereg} (26). Additional genes were identified as FOXO1 targets including the LH receptor (\textit{Lhcgr}) and genes required for cholesterol biosynthesis and steroidogenesis (66).

FSH also regulates mitogen-activated protein kinase kinase (MEK)/ERK signaling in GCs through regulation of ERK phosphorylation (43,67,68). As presented in Chapter 3, my recent results show that ERK activation is necessary for the induction of genes that are required for follicular maturation, including \textit{Inha} (one subunit of the hormone inhibin that functions to inhibit FSH expression by the pituitary as follicles mature), \textit{Egfr} (the epidermal growth factor receptor, that is required for LH-induced oocyte maturation), and \textit{Cyp19a1}(that encodes aromatase, the rate limiting enzyme in estrogen biosynthesis) (Fig 1) (69). Canonical MEK/ERK signaling originates with activation of RTKs at the plasma membrane (reviewed in (70)). After ligand binding, autophosphorylation of the RTKs creates
docking sites for Src homology 2 (SH2) containing proteins such as GRB2 (71). GRB2 is in complex with the guanine nucleotide exchange factor SOS (72), and after binding to the RTK, SOS activates RAS through release of GDP (73), triggering the MAPK signaling cascade. The tyrosine phosphatase SRC homology-2 (SH2) domain-containing tyrosine phosphatase (SHP2) is also recognized to contribute to the activation of RAS, although the mechanism by which SHP2 enhances RAS is poorly understood (74-76). Active RAS activates the Ser/Thr kinase RAF-1 (77), that subsequently phosphorylates the dual specificity kinase MEK (78). MEK will phosphorylate ERK on Thr\(^{202}\) and Tyr\(^{204}\), and ERK will phosphorylate the downstream kinase ribosomal S6 kinase (RSK-2) (79). Additionally, ERK activity has been shown necessary for many cellular processes, such as cell cycle regulation, transcription, and translation.

ERK activity is generally necessary for progression of cells through G1 into the S phase of the cell cycle. This is accomplished in part through regulation of the expression of cyclin D, part of the cyclin D-cyclin-dependent kinase (CDK) 4/6 complex, which is necessary for cell entry into S phase (80,81). Additionally, ERK activity promotes downregulation of p27\(^{kip1}\), a CDK inhibitor (82), required for the late G1 phase activation of cyclin E-CDK2 (83). Activation of cyclin E promotes induction of cyclin A and formation of active cyclin A-CDK2 complex. Both cyclin E and cyclin A are required for S phase entry and cyclin A is required for progression through S phase (reviewed in (81)). Thus, cyclin D is an integral player in this sequence of events, and the regulation of its expression by ERK demonstrates one way in which ERK contributes to cell cycle regulation.

ERK regulates transcription through altering the activities of transcription factors in many ways, including altering their cellular location, their expression or stability, and/or their ability to bind to other transcription factors or DNA (reviewed in (84,85)). For example, ERK phosphorylates the transcription factors JunD (86), c-Fos (87), GATA4 (88), Elk-1 (89), and c-Myc (90), enhancing their activity as transcriptional activators. Additionally phosphorylation of SF-1 (\(Nr5a1\) gene product) by ERK promotes recruitment of coactivators and ultimately results in an active conformation (91). Alternatively,
phosphorylation by ERK of the progesterone receptor (92) and GATA-1 (93) promotes their ubiquitination and subsequent degradation. Through these mechanisms, and others, ERK is able to regulate transcription of target genes.

Regulation of translation in response to ERK activity is accomplished through regulation of a few essential translational regulator proteins by either ERK itself or, more commonly, by its downstream kinase RSK. First, both ERK and RSK have been shown to phosphorylate and inactivate the translational inhibitor protein TSC1/2 (94). Additionally, both kinases phosphorylate mTORC1 protein Raptor, leading to increased mTORC1 activity (95, 96). Phosphorylation of the ribosomal protein S6 by RSK has been well established, and this phosphorylation correlates with eIF4F complex assembly and cap-dependent translation (97-99). RSK phosphorylates the additional components of the translational machinery, such as the initiation factor protein eIF4B (100) and the elongation factor 2 kinase (eEF2) (101). Thus, through regulation of multiple components necessary for translation, ERK is able to effect translational activity.

In response to FSH, there is a rapid, transient increase in the phosphorylation of ERK on Thr<sup>202</sup> and Tyr<sup>204</sup> and its downstream kinase RSK2 on Ser<sup>380</sup> (27, 43, 69). ERK activation is required for the induction of MAP2D (43), Egr-1 (22) and cyclin D2 (102) in GCs, based on the use of MEK inhibitors. Additionally, the importance of ERK signaling in GCs is demonstrated by the expression of a constitutively active RAS (KRAS-Gly12Asp) in preantral GCs that resulted in subfertility and the formation of abnormal follicles and blocked induction of the Lhcgr (103).

Previous results from our lab have shown that FSH-stimulated ERK phosphorylation is both PKA and MEK dependent, as demonstrated using the inhibitors Myr-PKI and PD98059, respectively (43). However, MEK is phosphorylated in untreated GCs, and the phosphorylation level remains constant in response to FSH. RAF-1 and RAS were also found to be active, and unresponsive to FSH stimulation (43). Additionally, both RAS and EGFR activity are necessary for FSH-dependent phosphorylation of ERK on Thr<sup>202</sup> and Tyr<sup>204</sup>, based on experiments using a dominant negative adenoviral-RAS and the EGFR inhibitor AG1487, respectively (see Chapter 2, (43). These results demonstrate that while the
upstream components of the MEK/ERK signaling cascade are necessary for FSH-dependent ERK phosphorylation, the activity of the upstream pathway is not regulated by FSH directly, and is most likely activated in an FSH independent manner.

Because MEK is still necessary for ERK(Thr\textsuperscript{202} and Tyr\textsuperscript{204}) phosphorylation, the FSH-dependent regulation of ERK(Thr\textsuperscript{202} and Tyr\textsuperscript{204}) phosphorylation is most likely mediated through inhibition of a phosphatase. In the absence of FSH, the phosphatase would be active and dephosphorylating ERK, but following FSH treatment the phosphatase would be inactivated, allowing for MEK dependent ERK(Thr\textsuperscript{202} and Tyr\textsuperscript{204}) phosphorylation to proceed and accumulate. Although a 100 kD protein tyrosine phosphatase (PTP), detected using a PTP-SL antibody (104), was identified to interact with ERK in GCs (43), further experiments utilizing a pan-PTP inhibitor Na\textsubscript{2}VO\textsubscript{3} failed to raise ERK(Thr\textsuperscript{202} and Tyr\textsuperscript{204}) phosphorylation in untreated cells (see Chapter 2). Additionally, different antibodies against PTP-SL failed to detect the 100 kD protein (see Chapter 2), indicating that the signal detected at 100 kD might not be the phosphatase that dephosphorylates ERK in the absence of FSH. My preliminary studies indicated that FSH-dependent ERK phosphorylation could be regulated by a member of the dual specificity phosphatases (DUSP) family (see Chapter 2).

**DUSPs**

DUSPs are members of the PTP family, and are grouped together because they are able to dephosphorylate both tyrosine and serine/threonine residues on the same substrate. There are different subgroups of DUSPs; those DUSPs that act on MAPK proteins (also called mitogen-activated protein kinase phosphatase [MKPs]) comprise one subfamily. Individual MKPs show substrate specificity preference among ERK, JNK (c-Jun N-terminal kinase), and p38 MAPK in response to stimuli, as well as differential cellular location (reviewed in (105)). Additionally, individual DUSPs are regulated differently, and can be induced or phosphorylated or degraded to alter their phosphatase activity (reviewed in (106)). While DUSPs in GCs are rather understudied, previous results can be used to focus
on the best candidates for examination. Out of the 61 DUSPs identified (reviewed in (105)), 10 are MKPs, and of those 10, GCs only express MKP1(DUSP1), MKP3(DUSP6), MKP/PyST2(DUSP7), MKP5(DUSP10), MKP7(DUSP16), in addition to cell cycle dual specificity phosphatases cell division cycle 25A (CDC25A), CDC25B, and CDC25C (20) (Herndon et al., manuscript in preparation). MKP5 and MKP7 are selective for p38 MAPK and JNK over ERK, and were eliminated from the investigation list. Unfortunately, at the time of publishing, MKPX did not have readily available inhibitors, and could not be easily investigated.

MKP1(DUSP1) was first isolated from skin cells (107) and was first shown to preferentially dephosphorylates p38 and JNK over ERK (108,109). However, MKP1(DUSP1) has since been shown to dephosphorylate ERK as well (110,111). MKP1(DUSP1) is generally localized to the nucleus in cells, and is regulated through induction in response to a variety of different stimuli (reviewed in (112)). MKP3(DUSP6) shows substrate preference for ERK over either p38 or JNK (113,114) and is localized to the cytoplasm. MKP3(DUSP6) is most commonly regulated through phosphorylations on Ser159 and Ser197 downstream of ERK that cause MKP3(DUSP6) degradation through the proteasome (115). CDC25A-C are cell cycle regulators that mainly dephosphorylated and activate cyclin-CDK complexes. CDK complexes are held inactive through inhibitory Thr and Tyr phosphorylations on the ATP binding loop, and will become active after dephosphorylation by CDC25. Thus, expression and activity of CDC25 is highly regulated and dependent on multiple mechanisms. Interestingly, CDC25 inhibitors have been shown to promote increased ERK(Thr202 and Tyr204) phosphorylation (116,117), and CDC25 has been shown to interact and dephosphorylate ERK directly (118). For these reasons, investigations focused on MKP1(DUSP1), MKP3(DUSP6), and CDC25A-C.

**YB-1**

Y-box-binding protein 1 (YB-1) is a multifunctional vertebrate protein that is a member of the cold shock domain (CDS) family. YB-1 is a nucleic acid chaperone protein, binding both DNA and RNA
Because of this trait, YB-1 is involved with most DNA- and mRNA-dependent processes, such as DNA replication, transcription, pre-mRNA processing, and translation (reviewed in (121,122)). Found in both the cytosolic and nuclear compartments of the cell, YB-1 interacts with an assortment of diverse proteins and is involved with many cellular processes including differentiation and proliferation (123-125).

YB-1 was described as a major component of the mRNA-protein complex (mRNP) first in birds (119,126), followed by mammals (rabbits) (127). When YB-1 binds to RNA, it melts the secondary structure (128), and acts as an mRNA chaperone protein. At a high YB-1:mRNA ratio, YB-1 binds along the whole mRNA strand and stabilizes the mRNA, protects mRNA from degradation by exonucleases, and blocks association with the translation initiation factors (129-131). However, at low YB-1:mRNA ratios, YB-1 stimulates the initiation of translation (132), possibly through promoting the movement of the small ribosomal subunit along the mRNA to the initiation codon (133).

YB-1 was first identified binding to DNA via a ‘Y-box-binding site’ (5’-CTGATTGG\textsubscript{C/C}T/TAA–3’) (134), but it was later shown that YB-1 could interact with a variety of sequences (135,136). YB-1 activates $Egfr$ (137), $Mdr1$ (multidrug resistance gene 1) (138), $Ptpn1$ (protein tyrosine phosphatase 1B) (139), and $Smad7$ (140) genes and inactivates $p53$ (141), $Vegf$ (142), and other genes (143,144) in response to various stimuli in multiple cancer cell lines. YB-1 has been shown to be misregulated in many oncogenic studies (145-150), and depending on the cancer cell line, is identified as either an oncoprotein or a tumor suppressor. Overexpression of YB-1 in cancer cells increases induction of MMP2 (matrix metalloproteinase 2) and is thought to contribute to the increase in capability for cancer cell migration and invasion (151,152). Overexpression of YB-1 in breast epithelial cells results in epithelial-mesenchymal transition (EMT) (153), possibly through an increase in cap-dependent translation of EMT regulators (154). The EMT is a significant stage in tumor metastasis, characterized by a loss of cellular cuboidal shape, apical basal polarity, and a near complete loss of E-cadherin and other epithelial cell markers (155).
YB-1 can regulate the transcription of genes through binding with other transcription factors. For example, in the promoter region for Mmp2, YB-1 is found in complex with AP-2 and p53, and this complex activates induction of the gene (156). YB-1 is often found in complex with purine-rich element binding protein A (PurA) and PurB, with the complex binding many targets genes, acting as both activator and repressor complexes (157-159).

YB-1 has been shown to be posttranscriptionally modified in a variety of different ways, most commonly through phosphorylation. YB-1 can be phosphorylated both in vitro and in vivo on Ser\textsuperscript{102} by the kinase Akt (160,161) and by the kinase RSK (162). Also, both ERK2 and GSK3β are able to phosphorylate YB-1 on Thr\textsuperscript{38} and/or Ser\textsuperscript{41} and/or Ser\textsuperscript{45} (142). These phosphorylations have been shown to alter the binding of YB-1 to both DNA and RNA (142,160), providing one possible mechanism for YB-1 participation in both transcription and translation. Additionally, mass – spectrometric studies have identified that YB-1 has multiple phosphorylation sites in addition to those previously mentioned, including Tyr\textsuperscript{162}, Ser\textsuperscript{165} and/or Ser\textsuperscript{167}, Ser\textsuperscript{174} and/or Ser\textsuperscript{176}, and Ser\textsuperscript{313} and/or Ser\textsuperscript{314} (163).

As described in Chapter 3, my results show that YB-1 is phosphorylated on Ser\textsuperscript{102} in response to FSH stimulation beginning approximately 15 min and lasting until approximately 8 hours following treatment. Phosphorylation of YB-1 on Ser\textsuperscript{102} is PKA-, ERK-, and RSK-dependent. Additionally, use of a dephospho-adenoviral-YB-1(S102A) mutant blocked induction by FSH of Egfr, Inha, and Cyp19a1 mRNAs (69). These results expand on the current knowledge of the role of YB-1 as a transcriptional regulator, reveal that phosph-YB-1(Ser\textsuperscript{102}) is crucial regulator of genes in GCs that define the preovulatory phenotype, and verify the regulation of YB-1 phosphorylated on Ser\textsuperscript{102} by RSK.

**TRANSLATION**

Regulation of translation occurs through regulation of the different protein components that make up the translational machinery. Translation occurs in three stages: initiation, elongation, and termination.
Although all three stages are regulated, most of the regulation occurs during initiation and the assembly of the different protein components (164).

Before initiation, pre-mRNA is processed before leaving the nucleus. The poly-(A) tail and the 5’-7-methyl guanine (m7G)-cap are added to the pre-mRNA to protect the free 5’ and 3’ ends (reviewed in (165)). Introns are removed from the pre-mRNA by the spliceosome (166), and following removal, mRNA is shuttled to mRNA export proteins for movement out of the nucleus (167). As mRNA leaves the nucleus, mRNA binding proteins, such as poly(A)-binding protein (PABP), will bind mRNA as it moves to different compartments of the cell (168).

Initiation begins with eIF4E binding to the 5’-m7G cap of mRNA, and recruitment of eIF4G and eIF4A, forming eIF4F (Fig. 2). Simultaneously, the 40S ribosome binds eIF1, eIF3 and eIF2-GTP-Met-tRNA\text{Met}, forming the 43S pre-initiation complex (reviewed in (169)). eIF4F promotes binding of the pre-initiation complex to the 5’-end of mRNA. eIF4F will also interact with PABP proteins binding to the poly-(A) tail, forming a circular ring structure that promotes continuous and efficient translation (170). The 43S pre-initiation complex will scan along the mRNA in the 5’ to 3’ direction until the start AUG codon is located (171). Upon recognition of the start AUG sequence, the 43S pre-initiation complex stalls, and eIF2-GTP is hydrolyzed to eIF2-GDP, promoting recruitment to the 60S ribosomal subunit (169). eIF5B facilitates the joining of the 60S ribosomal subunit, and eIF1, eIF2-GDP and eIF5B are released (172). The translational machinery is ready to continue down the mRNA strand, adding amino acids to the growing polypeptide chain.

Elongation occurs as tRNA-aminoacyl (AA) move through three sites on the ribosome, resulting in transfer of the AA from the tRNA to the growing polypeptide chain. tRNA-\text{AA} is bound to elongation factor 1A (eEF1A)-GTP and will bind the A site on the ribosome (173). Hydrolysis of GTP to GDP facilitates the release of eEF1A-GDP from the ribosome, leaving the tRNA-\text{AA} in the A site. Elongation factor eEF1B recycles eEF1A, through hydrolysis of GTP to GDP. Elongation factor 2 facilitates movement of the tRNAs into the next site on the ribosome (174). eIF5A is required to correctly position
the initiator tRNA-Met in the P-active site of the ribosomal complex (175). Addition of AA to the polypeptide chain will continue until the ribosomal complex encounters a stop codon.

Termination occurs upon recognition by the ribosome of one of three termination codons and binding of two eukaryotic release factors (eRFs) (176). eRF1 binds to the ribosome, and this binding promotes the ribosome to release the polypeptide chain. eRF3-GTP binds to the ribosome and hydrolysis of GTP to GDP releases eRF1 (177). A second hydrolysis of GTP to GDP releases eRF3 from the ribosome. The remaining components are disassembled in a virtually unknown process, freeing the small 40S ribosomal subunit to reassemble and start translation again.

The different ways in which FSH promotes translation were covered briefly in previous sections. FSH regulates translation through regulation of specific components of the translational complex, most notability through regulation of the phosphorylation of 4EBP1 and p70S6K via mTORC1 (Fig.1). FSH stimulates inhibition of TSC2, allowing for an increase in mTORC1 activity (178). Following FSH stimulation, mTORC1 stimulates phosphorylation of p70S6K and the 4EBP1 (60). FSH regulation of translation could thus promote a rapid increase in protein expression, allowing for rapid cellular responses.

SUMMARY

Herein, we show in Chapter 2 that while ERK signaling upstream of MEK is constitutively active, it is necessary for FSH-dependent ERK(Thr\textsuperscript{202} and Tyr\textsuperscript{204}) phosphorylation. Results show that ERK(Thr\textsuperscript{202} and Tyr\textsuperscript{204}) phosphorylation is selectively inhibited by DUSP inhibitors. Inhibitors of MKP3(DUSP6) increase ERK(Thr\textsuperscript{202} and Tyr\textsuperscript{204}) phosphorylation in the absence of FSH to levels comparable to FSH-stimulated ERK(Thr\textsuperscript{202} and Tyr\textsuperscript{204}) phosphorylation. ERK co-immunoprecipitates with MKP3(DUSP6), and MKP3(DUSP6) inhibitors block dephosphorylation of ERK2-GST. These results indicated that in the absence of FSH in GCs, ERK is dephosphorylated by MKP3(DUSP6).
Results presented in Chapter 3 show that YB-1 is phosphorylated on Ser^{102} in response to FSH-stimulation. YB-1 phosphorylation on Ser^{102} is PKA-, ERK-, and RSK-2-dependent. Additionally, YB-1(Ser^{102}) phosphorylation is dependent on protein phosphatase 1 (PP1), and YB-1 interacts with both PP1βc and RSK-2. Expression of a dephospho-adenoviral-YB-1(S102A) mutant prevented the FSH-stimulated induction of *Egfr*, *Cyp19a1*, and *Inha* mRNA. Together these results reveal an unexpected role for the MEK/ERK/RSK pathway and for YB-1 in target gene induction by FSH in GCs.

In Chapter 4, we demonstrate that YB-1 interacts with select mRNA binding proteins and initiation factor proteins. As a result of the isolation of proteins binding the 5’ cap using an m7GTP-Sepharose bead, we detect total YB-1 protein, and YB-1 increases binding following FSH stimulation. Using a Flag-tagged YB-1 in immunoprecipitation studies, we detected PABP1 binding to YB-1, and this binding is FSH-independent. In an investigation of mRNAs bound to YB-1, we observe an increase in *Hif1α* and *Egr1* mRNAs bound to YB-1 over IgG control. We also observe an increase *Egr1* mRNA bound to YB-1 in response to FSH stimulation. Together these results suggest that in response to FSH, YB-1 increases binding to mRNAs and the initiation factor complex.

Overall, the research presented in this document focuses on FSH-stimulated ERK signaling and the role of its downstream target YB-1. We present results that expand the current knowledge of ERK-dependent gene targets in GCs and demonstrate that their expression is also YB-1-dependent. These gene targets, *Egfr*, *Cyp19a1*, and *Inha*, are all necessary for FSH-dependent follicular maturation. These results demonstrate the critical role that both ERK and YB-1 play in GC maturation in response to FSH stimulation.
FIGURE LEGENDS AND FIGURES

FIGURE 1. Overview of FSH-regulated signaling in GCs. This schematic represents our understanding of FSH-dependent signaling via PKA to direct gene targets and to the PI3K/AKT and ERK signaling cascades.
Fig. 1
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CHAPTER TWO

FSH DEPENDENT REGULATION OF ERK PHOSPHORYLATION BY MKP3(DUSP6)

E. M. Donaubauer, N.C. Law, B. Kyriss, T. Woodiwiss and M. E. Hunzicker – Dunn

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ABSTRACT

Within the ovarian follicle, granulosa cells (GCs) surround and support immature oocytes. FSH promotes differentiation and proliferation of GCs and is essential for fertility; FSH receptor knockout mice are infertile. This research focuses on the function and phospho-regulation by FSH of ERK within GCs. Interestingly, in GCs, only ERK phosphorylation is increased in response to FSH. The upstream components are constitutively active and their activity is unaffected following FSH treatment. ERK(Thr\textsuperscript{202}/Tyr\textsuperscript{204}) phosphorylation is PKA dependent, however it requires activity of the upstream pathway proteins. Treatment of GCs with EGFR inhibitor AG1478, a dominant-negative RAS, and the MEK inhibitor PD98059 all blocked FSH-dependent ERK(Thr\textsuperscript{202}/Tyr\textsuperscript{204}) phosphorylation. We hypothesize that FSH via PKA is regulating ERK phosphorylation by inhibiting the activity of a protein phosphatase that is continually dephosphorylating ERK. Treatment with FSH would inactivate the phosphatase, and allow for ERK to be phosphorylated, activated and to accumulate within GCs. Treatment of GCs with different phosphatase inhibitors allowed for elimination of both Ser/Thr phosphatases and protein tyrosine phosphatases (PTPs), and implicated dual specificity phosphatases (DUSPs). Our results show that treatment with MKP3(DUSP6) inhibitors increase ERK(Thr\textsuperscript{202}/Tyr\textsuperscript{204}) phosphorylation in the absence of FSH to levels comparable to ERK phosphorylated in the presence of FSH. ERK coimmunoprecipitated with MKP3(DUSP6) and treatment of GCs with MKP3(DUSP6) inhibitors block dephosphorylation of recombinant ERK2-GST. This research aims to better comprehend
the regulation of ERK signaling in GCs in response to FSH to gain further understanding of follicular maturation.

**INTRODUCTION**

FSH acts selectively on granulosa cells (GCs) contained within ovarian follicles to promote proliferation and differentiation of GCs to a preovulatory phenotype (reviewed in (1)) as well as meiotic competency of the enclosed oocyte (2,3). Luteinizing hormone (LH) then initiates meiosis and promotes ovulation and differentiation of remaining follicular cells into luteal cells. While it is well recognized that the ERK signaling pathway plays a predominant role in the ovulatory response to LH in preovulatory GCs (4), the functional significance of the ERK signaling pathway in immature GCs is less well understood. We recently showed that the ERK signaling pathway in immature GCs is required for the induction of at least a subset of FSH gene targets that define the mature preovulatory GC, including Inha (that encodes the α subunit of the hormone inhibin), Egfr (that encodes the epidermal growth factor receptor [EGFR] required for ovulation), and Cyp19a1 (that encodes the rate limiting enzyme in estrogen biosynthesis) (5). ERK-dependent gene expression in immature GCs is mediated in part by phosphorylation of the transcriptional activator Y-box binding protein-1 (YB-1) on Ser102 (5). Based on the relevance of the ERK signaling pathway to immature GC maturation, we sought to better understand the mechanism by which FSH activates ERK.

ERK is canonically activated by receptor tyrosine kinases (RTKs). Ligand-dependent activation of RTKs recruit the RAS guanine exchange factor SOS to the plasma membrane, resulting in RAS and subsequently RAF activation (reviewed in (6)). The Ser/Thr kinase RAF then phosphorylates/activates the dual specificity kinase MEK that phosphorylates ERK. ERK then phosphorylates downstream kinases like ribosomal S6 kinase (RSK-2) (7). Phosphorylation of ERK by MEK on both Thr202 and Tyr204 is required for ERK activation; hence, inhibition of either phosphorylation by protein tyrosine phosphatases
(PTPs), Ser/Thr phosphatases, or dual specificity phosphatases (DUSPs) blocks ERK activation (reviewed (8)).

In GCs, FSH activates ERK in a PKA-dependent manner (9-12). We previously reported that the canonical pathway upstream of ERK is constitutively active in immature GCs but that ERK phosphorylation/activity is restrained by a PTP (10). We hypothesized that FSH inactivated this phosphatase, thereby permitting dual phosphorylation of ERK on Tyr<sup>202</sup>/Thr<sup>204</sup> by MEK. We identified the PTP as a 100 kDa putative member of the PTP-SL (STEP-like) family (13,14) based on the following criteria: (a) a western blot of GC extracts using rabbit polyclonal anti-PTP-SL antibody (15) revealed a signal at 100 kDa; (b) an in gel PTP assay detected a signal at 100 kDa in concentrated ovarian extracts; (c) an anti- PTP-SL reactive signal at 100 kDa was selectively immunoprecipitated with ERK-conjugated agarose from GC extracts; (d) in GCs loaded with <sup>32</sup>Pi, FSH stimulated the phosphorylation of a protein at 100 kDa immunoprecipitated with anti-PTP-SL antibody that was inhibited by the ACG kinase inhibitor H89 (16); (e) in an ERK-agarose immunoprecipitation, the anti-PTL-SL reactive band at 100 kDa was reduced by ~ 50% in GCs treated with FSH. Previous results by the Pulido laboratory showed that PTP-SL was phosphorylated on Ser<sup>231</sup> in a PKA-dependent manner, relieving inhibition of ERK and its restriction to the cytoplasm and allowing ERK to translocate to the nucleus (17,18).

However, our recent results question our interpretation of our previous results. (a) As we show below, the pan-PTP inhibitor Na<sub>2</sub>VO<sub>3</sub>, which should inhibit PTP-SL (15), does not block FSH-stimulated ERK phosphorylation. (b) PTP-SL has a MW of ~ 55 kDa, not 100 kDa (19). (c) A second antibody directed against PTPBR7, which is identical to PTP-SL except for a 127 amino acid insertion in the N-terminus (14), does not detect a signal on western blots of GC extracts at 100 kDa (10). While the gene that encodes PTP-SL *Ptprr* is expressed in GCs (based on RNA seq results; Herndon et al., manuscript in preparation), we thus question whether the PTP activity at 100 kDa that is detected by an anti-PTL-SL antibody is the phosphatase that constitutively dephosphorylates ERK in the absence of FSH.
Results below confirm that the ERK signaling pathway upstream of MEK is constitutively active in GCs, that ERK but not MEK phosphorylation is PKA-dependent, and that ERK phosphorylation is prevented in the absence of FSH. FSH-stimulated ERK activation is not inhibited either by inhibitors of Ser/Thr protein phosphatase PP1 or PP2 or by the pan-PTP inhibitor Na$_2$VO$_3$. Rather, using a panel of DUSP inhibitors, our results show that inhibitors of DUSP6/MAP kinase phosphatase 3 (MKP3) selectively enhance phosphorylation of ERK in the absence of FSH equivalent to levels of ERK phosphorylated in the presence of FSH. ERK co-immunoprecipitates with MKP3(DUSP6); MKP3(DUSP6) inhibitors block MKP3(DUSP6) phosphatase activity in GC extracts in the absence of FSH. Together, these results suggest that ERK in GCs is maintained in a dephosphorylated state in the absence of FSH by MKP3(DUSP6).

**EXPERIMENTAL PROCEDURES**

*Materials* - The following were purchased: ovine FSH (oFSH-19) from the National Hormone and Pituitary Agency of the National Institute of Diabetes and Digestive and Kidney Diseases (Torrence, CA); human fibronectin from BD Biosciences; anti-phospho–AKT (Ser$^{473}$; CST 9271), anti-AKT (CST 9272), anti-phospho–ERK (Thr$^{202}$/Tyr$^{204}$; CST 9107), anti-ERK (CST 9107), anti-phospho–MEK (Ser$^{217}$/Ser$^{220}$; CST 9121), anti-S6 (CST 2217), anti-phospho-CREB (Ser$^{133}$; CST 9191), anti-phospho-GAB (Ser$^{159}$; CST 3844) and (Tyr$^{452}$; CST 3881), anti-phospho-GSK3β (Ser$^{9}$; CST 9336), anti-phospho-myosin light chain (MLC) (Ser$^{19}$; CST 3671), anti-GAPDH (CST 5144) from Cell Signaling Technologies; anti-SRC homology-2 (SH2) domain-containing tyrosine phosphatase (SHP2) (SC 7384), anti-MKP1 (SC 10769), anti-MKP3 (SC 28902), and anti-phospho-IRS1 (Tyr$^{989}$; SC 17200) and Protein A/G PLUS Agarose (SC 2003) from Santa Cruz Biotechnology; anti-RAS (cat. number R02120) from Transduction Laboratories; tautomycin, okadaic acid, BCI, NSC 663284, active phospho-ERK2-GST (14-173) from Calbiochem/EMD Millipore; NSC 87877 from ACROS Organics; Na$_2$VO$_3$ from Fukma.
Animals - Sprague-Dawley, CD-outbred rats (breeders from Charles River Laboratories) were from a breeder 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 Cultures – Immature female rats were primed with subcutaneous injections of 1.5 mg estradiol-17β (E2) in propylene glycol on days 21-23. Ovaries were collected following three days of injections. GCs were collected by puncturing individual follicles using 27-gauge needles (20). Cells were plated on fibronectin-coated plates at a density of ~1x10⁶ cells/mL of serum–free media supplemented with 1 nM E2, 100 U/mL penicillin (P), and 100 µg/mL streptomycin (S). Indicated treatments were added to cells ~20 h following plating, and terminated by aspirating media and washing once with PBS followed by sample collection.

Western Blotting - Total cell extracts were collected by scraping cells in a SDS sample buffer (21) at 50 µL/1x10⁶ cells, followed by heat denaturation. Equal protein loading was accomplished by plating equal numbers of cells and collecting in a standardized SDS collection volume. Equal volumes of protein extract were loaded per gel lane, and 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 (22). The membrane was incubated with primary antibody overnight at 4°C, and antigen–antibody complexes were detected using enhanced chemiluminescence (ThermoFisher). 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 (BioRad Laboratories). 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 a one way ANOVA with Tukey’s Multiple Comparison Test.
Adenoviral Transductions of GCs – Transduction with adenoviruses was done as previously described (23). Briefly, GCs were plated in 35-mm plates at 1.5 x 10^6 cells/2 mL in DMEM/F12 +E/PS. Four h after plating, the indicated adenoviruses were added to the cells. The next morning, the adenovirus was removed, the cells were washed with PBS, and fresh DMEM/F12 + E/PS was added. The cells were treated as indicated. Adenoviral optical particle unit (OPU) concentration per milliliter viral stock was calculated from the A260 OD as described previously (24). Results are expressed as OPU per cell and based on the number of GCs plated and the volume of virus added. Ad-PKI was kindly provided by Marco Conti (University of California, San Francisco, CA) (25). Ad-(S17N)-RAS was kindly provided by Valina Dawson (John Hopkins University School of Medicine, Baltimore, MD) (26).

Immunoprecipitation – Briefly, ~10^7 cells were scraped into 0.5 mL Immunoprecipitation Lysis buffer [50 mM Hepes, pH 7.0, 100 mM NaCl, 0.5% Nonidet P-40, 20 mM NaF, 2 mM NaVO_3, 2 mM Na_4P_2O_7, 5 mM EGTA, 5 mM EDTA, 20 mM benzamidine, 10 μg/mL calpain inhibitor III, 50 μg/mL antipain, 50 μg/mL soybean trypsin inhibitor, 10 μM isomethylbutylxanthine, Halt protease inhibitor mixture [1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.8 μM aprotinin, 50 μM bestatin, 15 μM E-64, 20 μM leupeptin, 10 μM pepstatin; (Thermo Fischer Scientific)], sonicated and clarified by centrifugation at 10,000 x g for 5 min at 4°C. Following removal of 0.03 mL sample for input, soluble cell extracts were precleared using species matched preclearing 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). Following centrifugation, 0.4 mL of the supernatant fraction, representing the unbound protein fraction, was collected in SDS sample buffer. Agarose beads were washed 4 times by rotating 1 mL Immunoprecipitation Wash Buffer (20 mM Hepes, pH 7.0, 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100) followed by centrifugation at 10,000 x g at 4°C. Bound
proteins were collected in SDS sample buffer and subjected to SDS/PAGE and western blotting. Inputs represent ~2%, and bound sample ~98% of total sample.

**Whole Cell Lysate Phosphatase Assay** – Protocol was adapted from a previous report (27), as detailed below. Cells were treated as indicated, then collected in 0.2 mL Phosphatase Assay Buffer [10 mM EDTA, 10 mM EGTA, 50 mM HEPES (pH 7.6), 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml leupeptin, 1 mg/ml aprotinin]. Lysates were subjected to five rounds of freeze/thaw, followed aspiration through a 25-guage needle. Lysates were clarified by centrifugation at 13,000 x g for 30 min at 4°C. To assess phosphatase activity, 50 ng of active ERK2-GST (EMD Millipore # 14-173) was added to each sample, and samples were incubated by rotating for 30 min at 30°C. The reaction was halted by adding 100 µL of 3X STOP, and samples were subjected to SDS/PAGE and western blotting.

**Statistics** – 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 Students t test.

**RESULTS**

*Both canonical signaling to MEK and PKA activity are required for FSH stimulated phosphorylation of ERK(Thr<sup>202</sup>/Tyr<sup>204</sup>).* We initially sought to confirm that the canonical ERK signaling pathway in GCs upstream of MEK is tonically active and independent of FSH. Our previous results demonstrated an apparent requirement for the tyrosine kinase activity of the EGFR, based on results showing that the selective EGFR inhibitor AG1478 (28) blocked FSH-stimulated ERK(Thr<sup>202</sup>/Tyr<sup>204</sup>) phosphorylation. However, we did not evaluate the effect of AG1478 on MEK(Ser<sup>217</sup>/Ser<sup>219</sup>) phosphorylation (10). To this end, GCs were pretreated with AG1478 followed by treatment without or with FSH. Results (Fig. 1A) show (a) that MEK(Ser<sup>217</sup>/Ser<sup>219</sup>) phosphorylation is readily detected in vehicle-treated GCs and is independent of FSH while ERK(Thr<sup>202</sup>/Tyr<sup>204</sup>) phosphorylation depends on FSH, and (b) that AG1478 not only abolishes ERK(Thr<sup>202</sup>/Tyr<sup>204</sup>) phosphorylation but also abolishes MEK(Ser<sup>217</sup>/Ser<sup>219</sup>) phosphorylation. AKT(Ser<sup>473</sup>) phosphorylation serves as a negative control. These
results indicated that the signaling pathway that promotes MEK phosphorylation is active in the absence of FSH and that this pathway requires the tyrosine kinase activity of the ERGR.

While AG1478 primarily inhibits the kinase activity of the EGFR, it is also reported to inhibit non-kinase targets (29). As the EGFR canonically activates RAS, we utilized two additional approaches to confirm that the signaling pathway downstream of the EGFR was required for FSH to activate ERK and was tonically active in the absence of FSH. We transduced GCs with a dominant negative adenoviral (Ad)-(S17N)-RAS or control Ad-GFP, then treated GCs with vehicle or FSH. Results (Fig. 1B) show that the dominant negative RAS blocked MEK (Ser^{217}/Ser^{219}) phosphorylation both in the absence and presence of FSH as well as FSH-stimulated ERK(Thr^{202}/Tyr^{204}) phosphorylation. CREB(Ser^{133}) phosphorylation serves as a negative control that is independent of EGFR/RAS signaling. The tyrosine phosphatase SRC homology-2 (SH2) domain-containing tyrosine phosphatase (SHP2) is also required for RTK signaling into MEK/ERK and its generally believed to contribute to the activation of RAS (30-32). We determined whether SHP2 activity was required for the phosphorylation of MEK(Ser^{217}/Ser^{219}) by pretreating GCs without or with NSC 87877, a compound that selectively inhibits SHP1 (which is not expressed in rat GCs, based on RNA-seq results; Herndon et al., manuscript in preparation) and SHP2 (33). NSC 87877 markedly attenuated MEK(Ser^{217}/Ser^{219}) phosphorylation in vehicle- and FSH-treated GCs and hence similarly reduced FSH-stimulated ERK(Thr^{202}/Tyr^{204}) phosphorylation (Fig. 1C). Taken together, these results show that the signaling pathway that promotes MEK(Ser^{217}/Ser^{219}) phosphorylation is tonically active in the absence of FSH and requires the EGFR, RAS and SHP2.

FSH stimulated ERK(Thr^{202}/Tyr^{204}) phosphorylation is also recognized to be dependent on PKA (9,10). In the following experiment, we sought to confirm that while the PKA inhibitor PKI, that functions as a PKA catalytic subunit pseudosubstrate (34), blocks FSH-stimulated ERK(Thr^{202}/Tyr^{204}) phosphorylation, it does not affect MEK(Ser^{217}/Ser^{219}) phosphorylation. As shown in Fig. 2, while transduction of GCs with Ad-PKI blocked FSH-stimulated ERK(Thr^{202}/Tyr^{204}) phosphorylation, MEK(Ser^{217}/Ser^{219}) phosphorylation was unaffected. Taken together these results demonstrated that the
effect of PKA must be downstream of MEK at the level of ERK phosphorylation. As MEK should readily phosphorylate ERK yet ERK is not phosphorylated in the absence of FSH, a phosphatase must actively dephosphorylate ERK in the absence of FSH. It then follows that FSH via PKA must inactivate the ERK phosphatase.

ERK phosphorylation is not directly regulated by a Ser/Thr phosphatase or PTP. In the following experiments, we determined whether the phosphatase that dephosphorylates ERK on Thr^{202} and/or Tyr^{204} is a Ser/Thr protein phosphatase (PP1 or PP2) or a Tyr phosphatase. Pretreatment of GCs with PP2 inhibitor okadaic acid (at 200 nM (35-38)) resulted in no change in phosphorylation of ERK(Thr^{202}/Tyr^{204}) (Fig. 3A). Phosphorylation of GSK3β(Ser^{9}) increased in the okadaic acid alone treatment, and is used as a positive control. Pretreatment of GCs with the PP1 inhibitor tautomycin (at 1 µM (35-38)) promoted a decrease in both ERK(Thr^{202}/Tyr^{204}) and MEK(Ser^{217}/Ser^{219}) phosphorylations (Fig. 3B). Blockade of the dephosphorylation of MLC(Ser^{19}) serves as a positive control for tautomycin. These results indicate that PP1 is necessary for signaling into MEK and acts upstream of MEK, not on ERK. Pretreatment of GCs with the pan-PTP inhibitor Na_{2}VO_{3} (39) resulted in an increase in both basal and FSH-stimulated ERK(Thr^{202}/Tyr^{204}) phosphorylation (Fig. 3C). Concomitantly, MEK(Ser^{217}/Ser^{219}) phosphorylation also increased with Na_{2}VO_{3} treatment. The target(s) of the PTP is thus upstream of MEK, likely at the level of the EGFR, and not at the level of ERK itself. Taken together, these results suggest that the phosphatase that dephosphorylates ERK in the absence of FSH is neither a Ser/Thr phosphatase nor a PTP.

ERK phosphorylation is regulated by a DUSP. The inability of selective inhibitors of PP1, PP2, or PTPs to preferentially enhance ERK phosphorylation in the absence of FSH suggested that the relevant phosphatase must be a member of the family of DUSPs. Indeed, in a recent report, DUSP27 blocked ERK, but not MEK phosphorylation in a rat luteal cell line in response to prolactin treatment (40). However, DUSP27 is not expressed in rat GCs, based on both microarray (41) and RNA sequencing (Herndon et al., manuscript in preparation) results. While 61 DUSPs have been identified (as reviewed in
preantral GCs only express DUSPs 1, 3, 6, 7, 10, 11, 12, 16, 18, 19, and 22, as well as cell division cycle (CDC) dual specificity phosphatase CDC2A, 25B, and 25C, based on RNA sequencing results (Herndon et al., manuscript in preparation). Only a subgroup of the DUSPs are classified as MKPs, based on the presence of a CDC25-like domain (CD2) which confers specificity towards the ThrXTyr motif in MAPKs (as reviewed in (42)). These include: MKP1(DUSP1), MKP3(DUSP6), MKPX/PYST2(DUSP7), MKP5(DUSP10), MKP7(DUSP16) and CDC25A-C. Of those, MKP5 and MKP7 are selective for c-Jun N-terminal kinase and p38 MAPK over ERK (as reviewed in (42)) and were not investigated. Based on the availability of commercially available selective inhibitors and protein detection by Western blotting in GCs (not shown), in the following experiments we tested the contributions of MKP1(DUSP1), MKP3(DUSP6) and CDC24A-C to the level of ERK phosphorylation in GCs.

GCs were pretreated without or with NSC 663284, a selective CDC26A, 25B, and 25C inhibitor that does inhibit MKP1(DUSP1) (43,44). Results (Fig. 4A) show that ERK(Thr202/Tyr204) phosphorylation in the absence or presence of FSH is not affected (lanes 3 and 4 versus 1 and 2) by this competitive CDC25 inhibitor. CDC25A-C are thus not functioning as ERK phosphatases in GCs.

GCs were next pretreated without or with BCI, a selective inhibitor of MKP1(DUSP1) and MKP3(DUSP6) (45). Results show that BCI increased ERK(Thr202/Tyr204) phosphorylation in vehicle-treated GCs (lane 3) to levels equivalent to those of FSH-treated cells (lane 2) (Fig. 4B). MEK(Ser217/Ser219) phosphorylation in the presence of BCI (lanes 3 and 4) was equivalent to that of FSH treated GCs in the absence of DMSO (lane 2). These results suggest that either MKP1(DUSP1) or MKP3(DUSP6) may be the relevant ERK DUSP in GCs.

We next sought to verify expression of both MKP1(DUSP1) and MKP3(DUSP6) proteins within GCs, especially since MKP1(DUSP1) is most commonly induced as an immediate early gene upon RTK activation of ERK (as reviewed in (42). GCs were treated with vehicle or FSH for the indicated times. Results show that both MKP1(DUSP1) and MKP3(DUSP6) are readily detected in GCs and total protein levels do not change within 60 min of FSH treatment (Fig. 4C).
To distinguish between MKP1(DUSP1) and MKP3(DUSP6) as regulators of ERK(Thr²⁰²/Tyr²⁰⁴) phosphorylation, GCs were pretreated with the selective MKP1(DUSP1) inhibitor triptolide (46,47). Treatment with triptolide had no effect on ERK(Thr²⁰²/Tyr²⁰⁴) phosphorylation in the absence or presence of FSH (Fig. 4D, compare lanes 3 and 4 with 1 and 2). Similarly, treatment of GCs with the MKP1(DUSP1) selective inhibitor chelerythrine did not affect ERK(Thr²⁰²/Tyr²⁰⁴) phosphorylation (data not shown). These results suggest that the relevant ERK DUSP in GCs is likely MKP3(DUSP6) and not MKP1(DUSP1).

As we were unable to identify a selective MKP3(DUSP6) inhibitor, we pretreated GCs with NSC 295642, a partial MKP3(DUSP6), CDC 25A and 25B inhibitor (48). Results (Fig. 4E) show that NSC 295642 promoted an increase in ERK(Thr²⁰²/Tyr²⁰⁴) phosphorylation in vehicle-treated GCs (lane 3) nearly equivalent to that of FSH-treated cells (lane 2); FSH treatment did not further enhance ERK phosphorylation in the presence of NSC 295642 (lanes 3 and 4). Because the results from NSC 663284 treatments (see Fig. 4A) allowed us to rule out contributions from CDC25A-C, we can conclude from these inhibitor studies that MKP3(DUSP6) is likely the DUSP that dephosphorylates ERK(Thr²⁰²/Tyr²⁰⁴) in the absence of FSH. Collectively, these results indicate that MKP3(DUSP6) is dephosphorylating ERK in untreated GCs, and that FSH promotes inactivation of MKP3(DUSP6) to increase ERK(Thr²⁰²/Tyr²⁰⁴) phosphorylation.

**ERK and MKP3(DUSP6) interact within GCs.** If MKP3(DUSP6) is dephosphorylating ERK in the absence of FSH, then MKP3(DUSP6) should bind to ERK in GCs. To determine if MKP3(DUSP6) and ERK interact, ERK2 was immunoprecipitated from vehicle- and FSH treated GCs. Results (Fig. 5) show the presence of MKP3(DUSP6) in ERK2 immunoprecipitated samples from both vehicle- and FSH-treated GCs. The identity of the doublet detected by the MKP3 antibody that is most prominent in the ERK2 immunoprecipitation but is also faintly detectable on the long exposure in input lanes is not known but might correspond to upshifted bands on MKP3. Unfortunately, the MKP3(DUSP6) antibody does not
readily immunoprecipitate MKP3(DUSP6) (not shown), so we could not perform the reverse immunoprecipitation experiment.

**ERK(Thr^{202}/Tyr^{204}) dephosphorylation is dependent on MKP3(DUSP6) activity.** In the following experiments, we sought to demonstrate that MKP3(DUSP6) but not MKP1(DUSP1) is able to dephosphorylate ERK in vitro. Using the MKP1 and MKP3 inhibitors discussed above, whole GC lysate phosphatase assays were performed using GST-tagged phospho-ERK2. GCs were pretreated without or with indicated inhibitors, followed by treatment with vehicle or FSH. Lysates were then assayed for phosphatase activity, evidenced by their ability to dephosphorylated GST-tagged phospho-ERK2, as detected by western blotting with phospho-ERK(Thr^{202}/Tyr^{204}) antibody. Pretreatment of GCs with BCI, the dual MKP1(DUSP1) and MKP3(DUSP6) inhibitor, blocked the DUSP that dephosphorylated ERK(Thr^{202}/Tyr^{204}) in the absence of FSH (Fig. 6A, compare lanes 3 and 1). Equivalent results were obtained upon pretreatment of GCs with NSC 295642, the MKP3(DUSP6) inhibitor (Fig. 6B). Taken together, these results support the conclusion that MKP3(DUSP6) is the DUSP that dephosphorylates ERK in the absence of FSH.

**DISCUSSION**

Our results confirm that canonical MEK/ERK signaling upstream of ERK is constitutively active, and activity of these upstream components is necessary for FSH-dependent ERK(Thr^{202} and Tyr^{204}) phosphorylation. FSH-dependent ERK(Thr^{202} and Tyr^{204}) phosphorylation is dependent on inhibition of a DUSP, most likely MKP3(DUSP6), based the ability of MKP3(DUSP6) inhibitors to raise ERK(Thr^{202} and Tyr^{204}) phosphorylation in the absence of FSH to levels comparable to FSH-stimulated ERK(Thr^{202} and Tyr^{204}) phosphorylation. Additionally, ERK co-immunoprecipitates with MKP3(DUSP6), and MKP3(DUSP6) inhibitors block dephosphorylation of ERK2-GST. These results indicated that in the absence of FSH in GCs, ERK is dephosphorylated by MKP3(DUSP6). Our final studies, which are in progress, are investigating the effect of adenoviral- MKP3(DUSP6) shRNA on the ability of FSH to
enhance ERK phosphorylation. The expectation is that successful down-regulation of MKP3(DUPS6) will mimic the effect of the MKP3(DUPS6) inhibitors and raise ERK phosphorylation in vehicle-treated cells to levels in cells treated with FSH.

FSH-dependent PKA signaling in GCs has been shown to activate multiple signaling cascades, but until recently, the mechanisms by which PKA accomplished this regulation of canonical signaling pathways remained elusive. Our results demonstrate that through regulation of MKP3(DUPS6), FSH is able regulate ERK(Thr^{202} and Tyr^{204}) phosphorylation, while the upstream components of the MEK/ERK signaling cascade remain active, unregulated by FSH treatment (10). Interestingly, our lab recently published results demonstrating FSH- and PKA-dependent regulation of PP1 to activate PI3K/AKT signaling in GCs (49). Collectively, these results indicate that FSH via PKA regulates at least two signaling cascades through regulation of phosphatases, seemingly hijacking these signaling cascades.

MKP3(DUPS6) is a dual specificity phosphatase, and member of the MKP subfamily, whose substrates are the MAPKs (ERK, p38 MAPK and JNK) (reviewed in (50)). MKP3(DUPS6) has been shown to have substrate preference for ERK over p38 MAPK and JNK (51). Regulation of MKP3(DUPS6) is most often placed downstream of ERK signaling (52,53), and is thought to be a negative feedback mechanism for down-regulating mitogenic signaling. Regulation has also been reported through induction of Mkp3/Dusp6 mRNA or additional posttranslational modifications of the MKP3(DUPS6) protein.

Mkp3/Dusp6 is often induced downstream of ERK signaling following ERK stimulation (54), and requires regulation by the transcription factor Ets1 (53,54). We investigated total MKP3(DUPS6) protein levels immediately following FSH treatment, and showed that MKP3(DUPS6) protein levels remain relatively constant, giving no indication of a large change in total protein expression (Fig. 4C). These results indicate that, in GCs at the time points checked, initial regulation of MKP3(DUPS6) is not at the transcription level, as protein levels remain stable up to an hour following treatment with FSH.
Active ERK phosphorylates MKP3(DUPS6) on Ser^{159} and Ser^{197}, and these phosphorylations are reported to promote degradation of MKP3(DUPS6) by the proteosome (55). However, MKP3(DUPS6) can interact with other proteins, such as casein kinase 2 α (CK2α) and Ca^{2+}/calmodulin-dependent protein kinase 1 (CAMK1) (56). Additionally, MKP3(DUPS6) can be phosphorylated by CK2α, and CK2α is reported to inhibit MKP3(DUPS6) phosphatase activity (56). So although it is not common, regulation of MKP3(DUPS6) activity by phosphorylation in an ERK-independent manner is not unprecedented. It is also noteworthy that that rat MKP3(DUSP6) also contains one potential PKA phosphorylation motif (RR/XX/S/T): RSVThr^{302}V. Consistent with our results, we hypothesize that MKP3(DUSP6) is phosphorylated by PKA on Thr^{302}, resulting in an inhibition of its phosphatase activity.

In conclusion, we have shown that FSH-dependent ERK(Thr^{202} and Tyr^{204}) phosphorylation is PKA dependent, as well as being dependent on the constitutively active canonical upstream MEK/ERK signaling components. In the absence of FSH, ERK is maintained in a dephosphorylated state by a DUSP, most likely MKP3(DUPS6). ERK2 co-immunoprecipitates with MKP3(DUPS6), and MKP3(DUPS6) inhibitors are able to raise ERK(Thr^{202} and Tyr^{204}) phosphorylation in the absence of FSH. These results indicate that MKP3(DUPS6) dephosphorylates ERK in untreated GCs, and FSH treatment inhibits its activity.
ACKNOWLEDGMENTS: We thank Drs. John H. Nilson and Maria Herndon and Nate Law for many helpful discussions. This work was supported by National Institutes of Health Grants R01HD065859 and R01HD62503 (to MEH-D) and National Institute of General Medical Science Training Grant T32GM083864 (to John H Nilson).

CONFLICT OF INTEREST: The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS: EMD and MEH-D designed the experiments. EMD, NL, BK, and TW performed the experiments. EMD and MEH-D analyzed the data and wrote the manuscript.

FOOTNOTES

1To whom correspondence should be addressed: Dr. Mary E. Hunzicker-Dunn, Biotechnology Life Sciences Building, Room 202, Washington State University, Pullman WA 99164, Telephone: 509-335-5614; Email: mehd@vetmed.wsu.edu

2Commerially available inhibitors for the DUSPs were determined according to Jeffery, K.L., et. al. (57), and at time of publication, there are no commerically available inhibitors for MKPX/PYST2(DUSP7).
REFERENCES


FIGURE LEGENDS AND FIGURES

FIGURE 1. Canonical MAPK signaling pathway is active in GCs independent of FSH stimulation and is necessary for phosphorylation of ERK1/2(Thr\textsuperscript{202}/Tyr\textsuperscript{204}). (A) GCs were treated for 15 min with 250 nM AG1478, a potent EGFR inhibitor, followed by treatment without (veh) or with 50 ng/mL FSH for 15 min. 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 six independent experiments. (B) GCs were transduced with Ad-GFP or Ad-(S17N)RAS overnight, followed by treatment without (veh) or with FSH 15 min. Samples were collected as described in A. Results are representative of four independent experiments. (C) GCs were treated without (DMSO) or with 20 mM NSC 87877, a SHP2 inhibitor, for 3 h, followed by treatment without (veh) or with FSH for 15 min. Samples were collected as described in A. Results are representative of five independent experiments.
Fig. 1

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250 nM, 15 min

15 min

- pERK1/2 (T202/Y204)
- pMEK (S217/S220)
- AKT (loading control)
- pAKT (T308)
- GAPDH (loading control)

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15 min

- pERK1/2 (T202/Y204)
- RAS
- SHP2 (loading control)
- ERK
- pMEK (S217/S220)
- SHP2 (loading control)
- pCREB (S133)
- AKT (loading control)

C  
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20 mM, 3 h

15 min

- pERK1/2 (T202/Y204)
- S6 (loading control)
- pMEK (S217/S220)
- pGAB2 (Y452)
- pGAB2 (S169)
- SHP2 (loading control)
- ERK
- AKT (loading control)
FIGURE 2. Phosphorylation of ERK1/2 on Thr\(^{202}\)/Tyr\(^{204}\) is dependent on PKA activity. GCs were transduced overnight with Ad-GFP or Ad-PKI, an inhibitor of PKA. Cells were then treated without (veh) or with FSH for 30 min. Samples were collected as described in IA. Results are representative of three independent experiments.
Fig. 2

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30 min

- **pERK1/2** (T202/Y204)
- **SHF2** (loading control)
- **pMEK** (S217/S220)
- **SHF2** (loading control)
**FIGURE 3.** Effect of Ser/Thr and Tyr phosphatase inhibitors on ERK1/2(Thr\(^{202}\)/Tyr\(^{204}\)) phosphorylation.

*(A)* GCs were pretreated without (EtOH) or with 0.2 µM okadaic acid, a PP2 inhibitor, for 1 h, followed by treatment without (veh) and with FSH for 15 min. Samples were collected as described in *IA*. Results are representative of three independent experiments. *(B)* GCs were pretreated without (EtOH) or with 1 µM tautomycin, a PP1 inhibitor, for 5.5 h, followed by treatment without (veh) and with FSH for 15 min. Samples were collected as described in *IA*. Results are representative of six independent experiments. *(C)* GCs were pretreated without (H\(_2\)O) or with 50 µM Na\(_2\)VO\(_3\), a tyrosine phosphatase inhibitor, for 12 h, followed by treatment without (veh) and with FSH for 30 min. Samples were collected as described in *IA*. Results are representative of three independent experiments.
Fig. 3

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0.2 μM, 1 h

15 min

pERK1/2 (T202/Y204)

S6 (loading control)

pGSK3β (S9)

S6 (loading control)

B

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1 μM, 5.5 h

15 min

pERK1/2 (T202/Y204)

SHP2 (loading control)

pMLC (S19)

ERK (loading control)

pMEK (S217/S220)

MEK

SHP2 (loading control)

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50 μM, 12 h

30 min

pERK1/2 (T202/Y204)

SHP2 (loading control)

pIRS1 (Y989)

pMFK (S217/S220)

SHP2 (loading control)
FIGURE 4. Effect of DUSP inhibitors on ERK1/2(Thr202/Tyr204) phosphorylation. (A) GCs were pretreated without (DMSO) or with 5 µM NSC 663284, a selective CDC25A, 25B and 25C inhibitor (that does not inhibit MKP1(DUSP1)), for 30 min, followed by treatment without (veh) and with FSH for 30 min. Samples were collected as described in IA. Results are representative of three independent experiments. (B) GCs were pretreated without (DMSO) or with 5 µM BCI, a selective MKP1(DUSP1) and MKP3(DUSP6) inhibitor, for 30 min, followed by treatment without (veh) and with FSH for 30 min. Samples were collected as described in IA. Results are representative of three independent experiments. (C) GCs were treated without (veh) and with FSH for the indicated times. Samples were collected as described in IA. (D) GCs were pretreated without (DMSO) or with 10 µM triptolide, a selective MKP1(DUSP1) inhibitor, for 1 h, followed by treatment without (veh) and with FSH for 30 min. Samples were collected as described in IA. Results are representative of three independent experiments. (E) GCs were pretreated without (DMSO) or with 5 µM NSC 295642, a selective MKP3(DUSP6) and CDC25A and 25B inhibitor, for 30 min, followed by treatment without (veh) and with FSH for 30 min. Samples were collected as described in IA. Results are representative of three independent experiments.
FIGURE 5. MKP3(DUSP6) interacts with ERK1/2 in GCs. GCs were treated without (veh) or with FSH for 15 min, and then collected in Immunoprecipitation Lysis buffer and sonicated. After removal of the insoluble particles, the soluble fraction was rotated overnight with 20 µg Anti–ERK2 (SC 1647) as described in 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.
Fig. 5

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Short exposure

Long exposure

MKP3
FIGURE 6. Active ERK2-GST dephosphorylation is blocked by MKP3(DUSP6) inhibitors. (A) GCs were pretreated without (DMSO) or with 5 µM BCI for 60 min, followed by treatment without (veh) and with FSH for 30 min. Samples were collected in Phosphatase Assay Buffer as described in Experimental Procedures. Active ERK2-GST was added to samples and incubated for 60 min. Samples were separated by SDS/PAGE and the western blot was probed with the indicated antibodies. Results are representative of two independent experiments. (B) GCs were pretreated without (DMSO) or with 5 µM NSC 295642 for 30 min, followed by treatment without (veh) and with FSH for 30 min. Samples were collected as described in 6A. Results are representative of two independent experiments.
Fig. 6

A  + ERK-GST

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5 µM, 60 min
15 min

pERK1/2 (T202/Y204)


B  + ERK-GST

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5 µM, 30 min
15 min

pERK1/2 (T202/Y204)
CHAPTER THREE

ERK–DEPENDENT PHOSPHORYLATION OF Y-BOX BINDING PROTEIN-1 (YB-1) ENHANCES GENE EXPRESSION IN GRANULOSA CELLS IN RESPONSE TO FSH

E. M. Donaubauer and M. E. Hunzicker – Dunn

Running Title: Function of phosphorylated YB-1 in ovarian granulosa cells

Submitted to the Journal of Biological Chemistry (JBC) November 2015, currently in revision

ABSTRACT

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 both transcription and translation. Our results show that FSH promotes an increase in the phosphorylation of YB-1 on Ser102 within 15 min that is maintained at significantly increased levels until ~8 h post treatment. FSH-stimulated phosphorylation of YB-1(Ser102) is prevented by pretreatment of GCs with the PKA-selective inhibitor PKI, the MEK inhibitor PD98059, or the ribosomal S6 kinase-2 (RSK-2) inhibitor BI-D1870. Phosphorylation of YB-1 on Ser102 is thus PKA, ERK, and RSK-2 dependent. However, pretreatment of GCs with the protein phosphatase 1 (PP1) inhibitor tautomycin increased phosphorylation of YB-1(Ser102) in the absence of FSH; FSH did not further increase YB-1(Ser102) phosphorylation. This result suggests that the major effect of RSK-2 is to inhibit PP1 rather than to directly phosphorylate YB-1 on Ser102. 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, and Inha mRNAs. 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.

INTRODUCTION

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 regulated process that is initiated by FSH. FSH regulates at least 4000 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 (reviewed in (1,2)).

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 binding element 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). 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 following 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 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 greater than 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 though its cold shock domain (30,31), and nonspecifically via basic and acidic clusters in its C-terminus (reviewed in (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 (reviewed in (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. Our results surprisingly suggest that the major RSK-2 target is not YB-1, rather RSK-2 promotes inactivation of PP1 to enhance YB-1(Ser$^{102}$) phosphorylation. FSH leads to an increase of phospho-YB-1 in the nucleus. Using
adenoviral YB-1 phosphorylation mutants, we show that phosphorylation of YB-1 on Ser\textsuperscript{102} is necessary for the expression of Cyp\textit{19a1}, Inha, and Egfr. 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\textsuperscript{102}, contributes to the expression of at least three target genes, Cyp\textit{19a1}, Inha, and Egfr, that are necessary for follicular maturation. These results also suggest a previously underappreciated role for the ERK signaling pathway in immature GCs.

EXPERIMENTAL PROCEDURES

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

\textit{Animals} - Sprague-Dawley, CD-outbred rats (breeders from Charles River Laboratories) were from a breeder colony maintained by our laboratory in a pathogen-free facility at Washington State University. The facility is maintained in accordance with the \textit{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 estradiol-17β (E2) in propylene glycol on days 21-23 to promote growth of preantral follicles. Ovaries were collected following three days of injections. GCs were collected by puncturing individual follicles using 27-gauge needles (37). Cells were plated on fibronectin-coated plates at a density of ~1x10^6 cells/mL of serum–free media supplemented with 1 nM E2, 100 U/mL penicillin (P), and 100 µg/mL streptomycin (S) (38). Indicated treatments were added to cells ~20 h following plating, and terminated by aspirating media and washing once with PBS. For western blotting, total cell extracts were collected by scraping cells in a SDS sample buffer (5) at 50 µL/1x10^6 cells, followed by heat denaturation. Equal protein loading was accomplished by plating equal numbers of cells and collecting in a standardized SDS collection volume (5). Equal volumes of protein extract were loaded per gel lane, and 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 (ThermoFisher). 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 (BioRad Laboratories). 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 Students t test.

Immunoprecipitation- Immunoprecipitations were performed using a modified protocol previously described (14). Briefly, ~10^7 cells were scraped into 0.5 mL Immunoprecipitation Lysis buffer [50 mM Hepes, pH 7.0, 100 mM NaCl, 0.5% Nonidet P-40, 20 mM NaF, 2 mM NaVO₃, 2 mM Na₂P₂O₇, 5 mM EGTA, 5 mM EDTA, 20 mM benzamidine, 10 µg/mL calpain inhibitor III, 50 µg/mL antipain, 50 µg/mL soybean trypsin inhibitor, 10 µM isomethylbutylxanthine, Halt protease inhibitor mixture [1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.8 µM aprotinin, 50 µM bestatin, 15 µM E-64,
20 μM leupeptin, 10 μM pepstatin; (Thermo Fisher Scientific)], sonicated twice on ice for 45 s, and clarified by centrifugation at 10,000 x g for 5 min at 4°C. Following removal of 0.03 mL sample for input, soluble cell extracts were precleared using species matched preclearing 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). Following centrifugation at 10,000 x 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 Immunoprecipitation Wash Buffer (20 mM Hepes, pH 7.0, 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100) 4 times. Bound proteins were collected in SDS sample buffer and subjected to SDS/PAGE and western blotting. Inputs represent ~2%, and bound sample ~98% of total sample.

Transfection of GCs and Luciferase Assay- Cells were plated on fibronectin-coated plates at a density of 1x10⁶ cells/mL in OptiMEM + P/S + E2 with expression constructs (500 ng/ 1x10⁶ cells) and transfected using LipofectAMINE 2000 (Invitrogen) according to manufacturer’s instructions. After 6 h incubation, the media was removed and fresh DMEM +P/S +E2 was added. Cells were treated as indicated following ~16 h recovery period. Cells were lysed in 0.1 mL 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 manufacturer’s instructions.

DNA constructs and adenoviral production- The full length human Egfr-promoter luciferase was kindly provided by Dr. Wenlong Bai (University of South Florida, Tampa, FL) (39). Cyp19a1-promoter luciferase and Inha–promoter luciferase constructs were previously described (40,41). 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 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’ reverse 5’
ACC TTC GCG CTG TAG GAG ATG GAG AGA 3’;

**YB-1(S102A)** forward 5’
CCA GGA AGT ACC TTC GCG CTG TAG GAG ATG GAG AGA 3’ 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 x 10^8 pfu/mL) and Ad-

**Adenoviral transduction of GCs**- Transduction of GCs was performed as previously described (9). Briefly, GCs were plated on fibronectin-coated 6 well plates at a density of 1.5x10^6 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. Following an overnight treatment, cells were allowed ~1 h recovery and then treated as indicated.
RNA Isolation and Real–time PCR- RNA isolation was performed per manufactures instructions. Briefly, cells were treated as indicated, rinsed once with PBS and collected in 1.0 mL of TRIzol-RNA Lysis Reagent. After 10 min incubation at room temperature, 0.2 mL chloroform was added and the samples were gently mixed and incubated for 10 min at room temperature. The phases were resolved by centrifugation for 15 min at 12, 000 x g at 4°C. The upper aqueous phase was removed, 0.5 mL isopropanol was added to the lower phase and the sample was gently mixed. The sample was incubated on ice for >30 min, then the RNA was pelleted upon centrifugation for 15 min at 12, 000 x g at 4°C. The pellet was washed with 75% EtOH, and then allowed to air dry. RNA concentration was determined using a NanoDrop 2000. cDNA was generated using qScript per manufacturer’s instructions. Real time-PCR was performed using Fast SYBR Green Master Mix per manufacturer’s instructions. Primers used were: 

- **Egfr** forward 5' AGA TTG VCAA AGG GCA TGA ACT AC 3’ reverse 5’ ACA TTC CTG GCT GCC AAG TC 3’; 
- **Cyp19a1** forward 5’ CAC GGA TGT TTG ATG GTC TGA 3’ reverse 5’ CTC GGC TTG CTG ACA AAC C 3’; 
- **Inha** forward 5’ TGG GAC CGC TGG ATC GTA 3’ reverse 5’ GCA TCC CGC AGC TAC CAT 3’; 
- **Star** forward 5’ AAG GAC TGC CCA CCA CAT CTA C 3’ reverse 5’ TCT CGT TGT CCT TGG CTG GAA 3’; 
- **Egr1** forward 5’ CCT TTT GCC TGT GAC ATT TGT G 3’ reverse 5’ TGG TAT GCC TCT TGC GTT CA 3’; 
- **Ereg** forward 5’ GCT CTG ACA TGG ACG GCT ACT 3’ reverse 5’ CTC GCT CAT GTC CAC CAG GTA 3’; 
- **ribosome L19** forward 5’ GTG ACC TGG ATG CGA AGG A 3’ reverse 5’ GCC TTG TCT GCC TTC AGT TTG 3’.

Statistics – 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 Students t test.

RESULTS

**FSH signals to stimulate phosphorylation of YB-1(Ser°02) 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.1A and 1C) show that YB-1 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\textsuperscript{102} in response to FSH. FSH promoted a relatively slow increase in YB-1(Ser\textsuperscript{102}) phosphorylation first detected 15 min after treatment (Fig. 1B). YB-1(Ser\textsuperscript{102}) phosphorylation peaked 1 h following FSH treatment; this peak level of phosphorylation was maintained until about 8 h and then declined to undetectable levels by 24 h (Fig. 1C). (The identities of the faster and slower migrating bands in phospho-YB-1 blots are not known.) Increased phosphorylation of YB-1(Ser\textsuperscript{102}) was mimicked by the adenyl cyclase activator forskolin (Fig. 2A). Phosphorylation of AKT(Ser\textsuperscript{473}) serves as a positive control. YB-1(Ser\textsuperscript{102}) phosphorylation also increased in response to 8-CPT–cAMP (Fig. 2B), a cell permeable cAMP analog that activates both exchange proteins activated by cAMP and PKA (14,43). But 8-CPT-2'-O-Me-cAMP, a cAMP analog that does not activate PKA, (44), failed to increase YB-1(Ser\textsuperscript{102}) phosphorylation (Fig. 2B). Treatment of GCs with Ad–PKA inhibitor (PKI), a pseudosubstrate for the catalytic subunit of PKA, led to a loss of FSH-dependent YB-1(Ser\textsuperscript{102}) phosphorylation [82% ± 17%] (Fig. 2C). Similarly, treatment with Myr–PKI peptide resulted in a loss of YB-1 phosphorylation mediated by FSH (data not shown). Phosphorylation of AKT(Ser\textsuperscript{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\textsuperscript{102} in a PKA-dependent manner in GCs.

**Phosphorylation of YB-1(Ser\textsuperscript{102}) is dependent on the ERK pathway and not PI3K/AKT pathway**

The phosphorylation of YB-1(Ser\textsuperscript{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 (26,45). Because FSH in a PKA-dependent manner activates both the PI3K/AKT (14) and ERK signaling pathways (8,10,11,46) in GCs, we investigated both pathways as possible regulators of YB-1 phosphorylation. Pretreatment of GCs with the PI3K inhibitor wortmannin (47), did not decrease FSH stimulated YB-1(Ser\textsuperscript{102}) phosphorylation, while AKT(Ser\textsuperscript{473}) phosphorylation was inhibited significantly [74.2% ± 13.0%] (Fig. 3A). However, pretreatment with the MEK inhibitor
PD98059 (47) promoted a near complete inhibition of YB-1(Ser\textsuperscript{102}) phosphorylation [94.2% ± 3.6%] in the presence of FSH (Fig. 3B), similar to the inhibition of ERK(Thr\textsuperscript{202}/Tyr\textsuperscript{204}) phosphorylation [98.1% ± 5.9%] that is used as the positive control. Equivalent results were obtained with a different MEK inhibitor U0126 (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\textsuperscript{102}) phosphorylation as well as RSK-2(Ser\textsuperscript{380}) phosphorylation (Fig. 3C). When GCs were treated with the PI3K/AKT pathway activator IGF\textsubscript{1} or FSH, only cells treated with FSH showed enhanced YB-1(Ser\textsuperscript{102}) phosphorylation (Fig. 3C). Unlike many other cell models, IGF\textsubscript{1} does not activate the ERK pathway in GCs (48). Additionally, GCs treated with the RSK-2 inhibitor BI-D1870 exhibited a marked inhibition [89.4% ± 5.3%] of YB-1(Ser\textsuperscript{102}) phosphorylation in the presence of FSH (Fig. 4). CREB(Ser\textsuperscript{133}) phosphorylation serves as a negative control. Taken together, these results indicate that FSH-stimulated phosphorylation of YB-1(Ser\textsuperscript{102}) is mediated by RSK-2 downstream of ERK.

Phosphorylation of YB-1(Ser\textsuperscript{102}) is dependent on protein phosphatase 1 (PP1) inhibition—Enhanced phosphorylation of YB-1(Ser\textsuperscript{102}) is relatively slow, compared to the phosphorylation of CREB which is detected within 1 min (7) or of ERK which is detected within 10 min (10) of FSH treatment. Moreover, YB-1(Ser\textsuperscript{102}) phosphorylation is detected up to 8-12 h (Fig. 1B) while 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\textsuperscript{102}).

Pretreatment of GCs with the protein phosphatase 2 (PP2) inhibitor okadaic acid (at 200 nM) resulted in an increase in YB-1(Ser\textsuperscript{102}) phosphorylation in both vehicle- and FSH-treated GCs (Fig. 5A). Phosphorylation of RSK-2(Ser\textsuperscript{380}) is indicative of RSK-2 activity (49) and provides insight into the regulation of YB-1(Ser\textsuperscript{102}) phosphorylation in the presence of okadaic acid. RSK-2(Ser\textsuperscript{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\textsuperscript{102}) phosphorylation. These results suggest that okadaic acid is inhibiting 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) resulted in an increase in YB-1(Ser\textsuperscript{102}) phosphorylation in vehicle-treated cells to levels equivalent to those seen in FSH-treated cells in the absence of tautomycin, while FSH did not further enhance YB-1(Ser\textsuperscript{102}) phosphorylation (Fig. 5B). Tautomycin pretreatment did not enhance FSH-stimulated RSK-2(Ser\textsuperscript{380}) phosphorylation. These results suggest that PP1 promotes the dephosphorylation of YB-1 on Ser\textsuperscript{102} in the absence of FSH.

If PP1 maintains the dephosphorylation of YB-1(Ser\textsuperscript{102}) in the absence of FSH, then a PP1 catalytic subunit should co-immunoprecipitate with YB-1. As total YB-1 antibody is 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, then immunoprecipitated Flag-tagged YB-1 with anti-Flag antibody. Results (Fig. 5C) show that anti-Flag antibody selectively pulls down comparable levels of the catalytic subunit PP1\textbeta (PP1c\textbeta) in both vehicle- and FSH-treated samples. (Longer exposures in right panel reveal input and flow through signals for PP1c\textbeta.) Additionally, when the same experiment was performed and the presence of RSK-2 was investigated, results show that RSK-2 also interacts (directly or indirectly) with Flag-tagged YB-1 in a FSH-independent manner (Fig. 5C). These results suggest that both PP1c\textbeta and RSK-2 are in a complex with YB-1 in an FSH-independent manner.

Taken together, these results suggest (i) that PP1 maintains YB-1(Ser\textsuperscript{102}) in a dephosphorylated state in the absence of FSH, (ii) that FSH signals to inhibit the activity of PP1, preventing the dephosphorylation of YB-1 at Ser\textsuperscript{102} and (iii) that RSK-2 inhibits PP1 activity while in complex with YB-1. The inability of FSH to significantly enhance YB-1(Ser\textsuperscript{102}) phosphorylation in the presence of tautomycin suggests that the major effect of PKA via ERK/RSK-2 is to promote inactivation of PP1.

*Phosphorylation of YB-1 on Ser\textsuperscript{102} promotes its nuclear localization* - As the phosphorylation of YB-1 on Ser\textsuperscript{102} in breast cancer cells is most often linked to its function as a transcriptional activator (21,23,24,26,33) (and as reviewed in (50)), 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 wildtype YB-1, the phospho- YB-1(S102D) mimic, and dephospho-YB-1(S102A) mutant. Following 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, respectively. All of the Flag-tagged constructs were readily detected in the cytoplasmic fraction (Fig. 6A), consistent with the presence of both phosphorylated YB-1(Ser102) and non-phosphorylated YB-1(Ser102) in this fraction. However, the phospho-YB-1(S102D) mimic strongly localized to the nuclear fraction compared to the dephospho-YB-1(S102A) mutant, consistent with reports in breast and ovarian cancer cells (20,22,26).

Phosphorylated YB-1(Ser102) was also readily detected using the phospho-YB-1(Ser102) antibody in both the nuclear and cytosolic fractions of non-transfected GCs (Fig. 6B, left panel). However, FSH did not promote a change in total levels of YB-1 within the nucleus (Fig. 6B, right panel), suggesting that YB-1 phosphorylated on Ser102 comprises a relatively small fraction of total YB-1.

These results suggest that while the majority (~90%) of total and phosphorylated YB-1 is localized to the cytoplasmic fraction, phosphorylated YB-1(Ser102) in GCs might have a distinctive function in the nucleus.

Phospho-YB-1(Ser102) is necessary for expression of select FSH target genes- Based on the localization of the YB-1(Ser102) phospho-mutant to the nuclear fraction, we hypothesized that YB-1 could participate in the transcriptional regulation of FSH target genes. While ERK signaling is critical to LH-induced ovulation and luteinization in preovulatory GCs (51), the importance of the ERK signaling pathway to FSH-stimulated differentiation of GCs is less well understood. We therefore sought to identify FSH target genes that were regulated by the ERK signaling pathway to identify possible YB-1 gene targets. Pretreatment of GCs with the MEK inhibitor U0126 resulted in inhibition of FSH-induced Egfr [85.7% ± 6.2%], Cyp19a1 [96.1% ± 2.65%], Inha [82.5% ± 3.2%] and Ereg [67.7% ± 4.4%] mRNA levels measured at 48 h post FSH treatment, while Star mRNA levels were not affected (Fig. 7).
Treatment with the MEK inhibitor PD98059 showed significant inhibition of FSH-induced expression of the immediate early gene \textit{Egr1} [86.4\% ± 3.2\%] 1 h post FSH treatment (Fig. 7).

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\textsuperscript{102}. We focused on the three ERK-regulated target genes that showed the greatest inhibition by the MEK inhibitor at the 48 h time point; \textit{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 \textit{Egfr} and \textit{Cyp19a1} mRNAs compared to Ad-Empty (E), that was not significantly enhanced further by FSH (Fig. 8A). In contrast, \textit{Inha} mRNA levels were not different between Ad-E and Ad-YB-1(S102D) treatments. Transduction of GCs with the dephospho-Ad-YB-1(S102A) mutant prevented the induction by FSH of \textit{Egfr} [82.0\% ± 9.5\%] and \textit{Cyp19a1} [88.6\% ± 5.5\%] mRNAs (Fig. 8B), consistent with results in Fig. 8A. FSH-stimulated induction of \textit{Inha} mRNA was also abrogated by the dephospho-Ad-YB-1(S102A) mutant [90.9\% ± 2.9\%]. In contrast, FSH-stimulated \textit{Star} mRNA expression was not reduced by the dephospho-Ad-YB-1(S102A) mutant. We interpret these results to suggest that YB-1 phosphorylated on Ser\textsuperscript{102} is necessary for the induction of \textit{Egfr}, \textit{Cyp19a1}, and \textit{Inha} mRNA by FSH, but does not participate in the induction of \textit{Star} mRNA by FSH. The ability of the Ad-YB-1(S102D) phospho-mutant to enhance \textit{Egfr} and \textit{Cyp19a1} mRNA levels in the absence of FSH suggests that phosphorylated YB-1(Ser\textsuperscript{102}) is a major contributor to the induction of these genes.

Finally, 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 \textit{Egfr} [64.9\% ± 13.2\%], \textit{Cyp19a1} [59.9\% ± 10.0\%], and \textit{Inha} promoter – luciferase [72.5\% ± 5.0\%] activities (Fig. 8C). These promoter-luciferase results suggest that phosphorylated YB-1 is acting at the level of the gene promoters and not at the level of mRNA stability. Collectively, these results indicate that YB-1(Ser\textsuperscript{102}) phosphorylation is necessary for FSH-mediated induction of \textit{Egfr}, \textit{Cyp19a1}, and \textit{Inha} gene expression.
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 (26,45). 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 localized to 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 comprise the mRNP complex (as reviewed in (32)). Hence, YB-1 is associated with both free nontranslated and polysomal mRNA, and is a major regulator of translation (52-54). 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 (as reviewed in (22,32)). YB-1 is generally viewed as an “oncogenic” transcription factor, based on its overexpression in breast and lung cancer cells (21,55) and on its gene targets, that include Egfr, Her2, Pi3kca, and Mdr1 (as reviewed in (32)). While 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 (as reviewed in (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 phosphorylated YB-1 or a phospho-YB-1(S102D) mutant localizes to the nuclear fraction.
our results also reveal that YB-1 phosphorylated on Ser^{102} is necessary for the expression of select FSH targets that define a mature, preovulatory GC, such as Egfr, Cyp19a1, and Inha. The EGFR is required for LH-induced ovulation (56); Cyp19a1 encodes aromatase, the rate limiting enzyme in estrogen biosynthesis (57); and Inha encodes for the inhibin hormone subunit inhibin-α that inhibits FSH expression by the anterior pituitary (58). While it is well-known that CREB, SF-1(Nr5a1)/LRH-1(Nr5a2), and GATA-4/6 enhance expression of both Cyp19a1 and Inha (40,59-61), and that FOXO1 (15) and T-cell factor 3 (9) repress expression of both genes, a role for YB-1 in the transcriptional activation of these genes is novel. To our knowledge, the mechanism by which FSH enhances Egfr expression has not been previously described.

While YB-1 phosphorylated on Ser^{102} localizes to the nucleus, the great majority of both phosphorylated YB-1(Ser^{102}) and total YB-1 is present in the cytoplasmic fraction of GCs (see Fig. 6, where the cytoplasmic and nuclear fractions represent 9% and 50% of the total fraction, respectively). Cytoplasmic YB-1 has been localized to both translated polysomal mRNPs and to free untranslated mRNPs (reviewed in (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 (reviewed in (32)). In NIH3T3 cells, AKT-dependent phosphorylation of YB-1 on Ser^{102} stimulated Cap-dependent translation (53). 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. 5B). 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.4). 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 while 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 (KYLRS^{102}VGD) partially aligns with that of casein kinase 2 (S*E/DXE/D) (62).

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 (63). 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 is 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.

While 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} (64,65). 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\textsuperscript{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\textsuperscript{102}. YB-1 phosphorylated on Ser\textsuperscript{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.
ACKNOWLEDGMENTS: We thank Dr. Wenlong Bai for the full length human Egfr-promoter luciferase. We thank Drs. John H. Nilson and Maria Herndon and Nate Law for many helpful discussions. This work was supported by National Institutes of Health Grants R01HD065859 and R01HD62503 (to MEH-D) and National Institute of General Medical Science Training Grant T32GM083864 (to John H Nilson).

CONFLICT OF INTEREST: The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS: EMD and MEH-D designed the experiments. EMD performed the experiments and analyzed the data. EMD and MEH-D wrote the manuscript.
References


endothelial growth factor in ovarian granulosa cells in response to follicle-stimulating hormone. Endocrinology 150, 915-928


FOOTNOTES

1To whom correspondence should be addressed: Dr. Mary E. Hunzicker-Dunn, Biotechnology Life Sciences Building, Room 202, Washington State University, Pullman WA 99164, Telephone: 509-335-5614; Email: mehd@vetmed.wsu.edu

2The abbreviations used are: GCs, granulosa cells; YB-1, Y-box binding protein-1; FSH, follicle stimulating hormone; PKA, protein kinase A; PKI, PKA inhibitor; RSK-2, ribosomal S6 kinase-2; PP1, protein phosphatase 1; LH, luteinizing hormone; GPCR, G protein-coupled receptor; CREB, cAMP-response binding element protein; AKT, protein kinase B; PI3K, phosphatidylinositol-3-kinase; FOXO1, forkhead box-containing protein O1; IGF1, insulin-like growth factor 1; SHP2, SRC homology-2 (SH2) domain-containing tyrosine phosphatase; Myr, myristoylated; E2, estradiol-17β; P, penicillin; S, streptomycin; PP2, protein phosphatase 2; PP1cβ, PP1β catalytic subunit; mRNP, mRNA-protein complex.

3The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
FIGURE LEGENDS AND FIGURES

FIGURE 1. Treatment of GCs with FSH leads to prolonged phosphorylation of YB-1 on Ser\(^{102}\). (A) GCs were treated for the indicated times without (v) or with 50 ng/mL FSH (F). 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. (B) GCs were treated with FSH for the indicated times and samples were collected as described in A. Blot is representative of three independent experiments. Quantification results represent the mean ± SEM of three independent experiments, with (*) representing p < 0.05, based on a 1–way ANOVA followed by Tukey’s posttest. (C) GCs were treated with FSH (F) for the indicated times, and samples were collected and analyzed as described in B, with (*) representing p < 0.05, based on a Students t-test of veh vs FSH.
Fig. 1

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YB1 phosphorylation (S102)

Treatment

pYB1 / SHP2

Treatment

*
FIGURE 2. Phosphorylation of YB-1 on Ser\textsuperscript{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 IA. Quantification of results represents the mean ± SEM of three independent experiments, with (*) representing p < 0.01, based on a 1–way ANOVA followed by Tukey’s posttest. (B) GCs were treated for 1 h without (veh) or with FSH or with 250 µM of either 8-pCPT-2’-O-Me-cAMP or 8-CPT-cAMP. Samples were collected as described in IA. Dotted lines between lanes represent cropped images. Results are representative of three independent experiments. Quantification is as described in 2A, with (*) representing p < 0.01, based on a 1–way ANOVA followed by Tukey’s posttest. (C) GCs were transduced overnight with Ad-Empty (E) (5.8 x 10\textsuperscript{5} OPU/cell) or Ad-PKI (1.8 x 10\textsuperscript{4} OPU/cell) that expresses the full length PKI protein. Following media replacement, cells were treated for 2 h without (veh) or with FSH. Samples were collected as described in IA, and quantification is as described in 2A, with (*) representing p < 0.01, based on a 1–way ANOVA followed by Tukey’s posttest. Results are representative of three independent experiments.
Fig. 2

A 1h: veh FSH EtOH Forskolin

- pYB1 (S102)
- pAKT (S473)
- SHP2 (loading control)

YB1 phosphorylation (S102)

B 1h: veh FSH 8-pCPT-2’-O-Me-cAMP 8-CPT-c-AMP

- pYB1 (S102)
- pAKT (S473)
- SHP2 (loading control)

YB1 phosphorylation (S102)

C 2h: veh FSH veh FSH

- pYB1 (S102)
- pAKT (S473)
- SHP2 (loading control)

YB1 phosphorylation (S102)
FIGURE 3. FSH- stimulated phosphorylation of YB-1 on Ser\textsuperscript{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 as described in 1A and quantification as described in 1B, with (*) representing p < 0.05, based on a 1–way ANOVA followed by Tukey’s posttest. (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 as described in 1A and quantification is as described in 1B, with (*) representing p < 0.001, based on a 1–way ANOVA followed by Tukey’s posttest. Results are representative of three independent experiments. (C) GCs were treated without (veh) or with FSH, 50 ng/mL EGF or 50 ng/mL IGF\textsubscript{1} as indicated. Samples were collected as described in 1A and quantification is as described in 1B, with (*) representing p < 0.05 and (**) representing p < 0.01, based on a 1–way ANOVA followed by Tukey’s posttest. Results are representative of three independent experiments.
FIGURE 4. FSH-stimulated phosphorylation of YB-1 on Ser$^{102}$ is RSK-2 dependent. GCs were pretreated without or with 10 µM BI–D1870 for 1 h followed by treatment without or with FSH for 1 h. Samples were collected as described in IA, and quantification is as described in IB, with (*) representing p < 0.001, based on a 1-way ANOVA followed by Tukey’s posttest. Results are representative of three independent experiments. Dotted lines between lanes represent cropped images.
Fig. 4

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YB1 phosphorylation (S102)

- veh
- FSH

* Indicates significant difference.
FIGURE 5. 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 1A, and quantification represents the mean ± SEM of three independent experiments, with (**) representing p < 0.01 and (*) representing p < 0.05, based on a one tailed Student 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 is as described in 1B, with (*) representing p < 0.05, based on a 1–way ANOVA followed by Tukey’s posttest. EthOH FSH, tautomycin veh, and tautomycin FSH treated samples are not statically different from each other (ns). Results are representative of three independent experiments. (C) GCs were transfected with 500 ng YB-1–Flag plasmid for 6 h as described in Experimental Procedures. Following 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 Anti–Flag (Sigma F3165) as described in Experimental Procedures. Inputs, bound (IP) and unbound (flow–through) samples were separated by SDS/PAGE and the western blot was probed with the indicated antibodies. Arrows indicate the protein band of interest. Dotted lines between lanes represent cropped images. Results are representative of two independent experiments.
FIGURE 6. YB-1 and phoso-YB-1(Ser^{102}) are detected in the both the nuclear and cytosolic fractions; phosphorylation of YB-1 on Ser^{102} promotes nuclear localization. (A) GCs were transfected with 500 ng of the indicated plasmid for 6 h as described in Experimental Procedures. Following recovery, GCs were treated without (veh) or with FSH for 4 h, then collected and separated into cytoplasmic and nuclear fractions as described in Experimental Procedures. Samples were collected as described in IA and are representative of two independent experiments. Quantification of results represents the mean ± SEM of two independent experiments, with (*) representing p < 0.05 and (**) representation p < 0.001, based on a 1–way ANOVA followed by Tukey’s posttest. (B) GCs were treated without (veh) or with FSH for 4 h, then collected and separated into cytoplasmic and nuclear fractions as described in Experimental Procedures. Samples were collected as described in IA, and western blots were probed with the indicated antibodies. Results are representative of three independent experiments.
Fig. 6

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YB1-Flag

GAPDH (loading Control)

Histone H3 (loading control)

B

| 4h: veh FSH | cytoplasmic | nuclear |

pYB1 (S102)

GAPDH (loading Control)

Histone H3 (loading control)
FIGURE 7. ERK signaling is necessary for FSH-stimulated target gene mRNA expression. For *Egfr*, *Ereg*, *Cyp19a1*, *Inha*, and *Star*, GCs were pretreated without (DMSO) and with 10 µM U0126 for 1 h, then treated without (veh) or with FSH for 48 h. For *Egr1*, GCs were pretreated without (DMSO) and 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 in Experimental Procedures. Quantification results represent the mean ± SEM of three independent experiments for *Egfr* and *Ereg*, and two independent experiments for *Cyp19a1*, *Inha*, *Egr1* and *Star* with (*) representing p < 0.01, (**) representing p < 0.001 and (***) representing p < 0.05, based on a 1–way ANOVA followed by Tukey’s posttest.
Fig. 7

- **Egfr mRNA Expression**
  - Relative Expression
  - Treatment: DMSO, U0126
  - Comparison: veh vs FSH

- **Inha mRNA Expression**
  - Relative Expression
  - Treatment: DMSO, U0126
  - Comparison: veh vs FSH

- **Cyp19a1 mRNA Expression**
  - Relative Expression
  - Treatment: DMSO, U0126
  - Comparison: veh vs FSH

- **Ereg mRNA Expression**
  - Relative Expression
  - Treatment: DMSO, U0126
  - Comparison: veh vs FSH

- **Star mRNA Expression**
  - Relative Expression
  - Treatment: DMSO, U0126
  - Comparison: veh vs FSH

- **Egr1 mRNA Expression**
  - Relative Expression
  - Treatment: DMSO, PD98059
  - Comparison: veh vs FSH
FIGURE 8. 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 overnight. Following media replacement and >1 h recovery, cells were treated without (veh) and with FSH for 48 h. GCs were collected and analysis as described in 7, and quantification results represent the mean ± SEM of three independent experiments with (*) representing p < 0.05 and (**) representing p < 0.01, based on a one tailed Students t test. (B) GCs were transduced with Ad- YB-1(S102A)-Flag or Ad-Empty overnight. Following media replacement and >1 h recovery, cells were treated without (veh) and with FSH for 48 h. Cells were collected, and analysis and quantification is as described in 8A, with (*) representing p < 0.05 and (**) representing p < 0.01, based on a one tailed Students t test. (C) GCs were transfected with the indicated promoter Luciferase constructs for 6 h as described in the Experimental Procedures, followed by overnight transduction with Ad- YB-1(S102A) - Flag or Ad- Empty. Following media replacement and >1 h recovery, cells were treated without (veh) and with FSH for 6 h, then collected. Samples were analyzed according to Experimental Procedures, and results are expressed as the RLU mean ± SEM of triplicates and are representative of three independent experiments. (*) represents p < 0.05 and (****) represents p < 0.01, based on a one tailed Students t test.
FIGURE 9. Proposed signaling pathway by which FSH promotes the PKA-dependent phosphorylation of YB-1 on Ser\textsuperscript{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\textsuperscript{102}. This increase in YB-1 phosphorylation on Ser\textsuperscript{102} is dependent on ERK-dependent activation of RSK-2. RSK-2 appears to enhance phosphorylation of YB-1 on Ser\textsuperscript{102} primarily by inactivating PP1. YB-1 phosphorylated on Ser\textsuperscript{102} is necessary for FSH dependent induction of target genes.
Fig. 9

FSH

↓

PKA

↓

ERK

↓

RSK-2

PP1  pY31

Stimulate gene expression
CHAPTER FOUR

YB-1 INTERACTS WITH INITIATION FACTOR COMPLEX PROTEINS AND mRNA

INTRODUCTION

Follicle stimulating hormone (FSH) stimulation results in maturation of the ovarian follicle from a preantral to a preovulatory state (1,2). This maturation is characterized by differentiation and proliferation of the granulosa cells (GCs). This is accomplished through regulation of over 500 target genes. Additionally, FSH promotes an increase in translation through activation of many essential translation proteins (reviewed in (3)).

FSH binds to its G protein coupled receptor FSHR that is only expressed on GCs. Activation of the FSHR results in stimulation of adenylyl cyclase and an increase in intracellular levels of cAMP. The primary role of cAMP within GCs is to bind the regulatory subunit of protein kinase A (PKA) (4), releasing the active catalytic subunits. Active PKA can directly phosphorylate multiple targets, such as cAMP-response binding element protein (CREB) and β-catenin that function as transcriptional activators, and can activate multiple signaling pathways, such as extracellular regulated kinase (ERK) signaling cascade (5,6) and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling cascade (7,8).

Recent studies from our lab have shown that FSH promotes the PKA-dependent phosphorylation of insulin receptor substrate 1 (IRS1) on select tyrosine residues to which PI3K binds (7). Binding of the regulatory subunit of the PI3K heterodimer to phosphorylated tyrosine residues on IRS1 is sufficient to activate the catalytic activity of PI3K (9). The resulting generation of phosphatidylinositol 3,4,5-tris phosphate (PIP3) brings AKT to the plasmatic membrane, where it is phosphorylated by proline dependent kinase 1 (PDK1) on Thr^{308} and by mTORC2(mTOR/Rictor) on Ser^{473}, resulting in full activation of AKT. Activated AKT then phosphorylates and inactivates the GTPase activating protein (GAP) protein tuberin. Active tuberin stimulates the conversion of active Ras homolog enriched in brain (RHEB)^{GTP} to inactive RHEB^{GDP} by enhancing the GTPase activity of RHEB (10). Inactivation of tuberin by AKT results in
accumulation of active RHEB$_{GTP}$ that in turn promotes the phosphorylation/activation of mTOR/Raptor (mTORC1). mTORC1 then stimulates phosphorylation of p70 S6 kinase (p70S6K) and the binding protein of eIF4E (4EBP1), both of which regulate translation (11). Active p70S6K promotes phosphorylation of the ribosomal protein S6, a member of the 40S ribosomal subunit complex (12). Phosphorylation of 4EBP1 results in release of eIF4E, allowing eIF4E to bind the 5’-methyl cap of mRNAs, stimulating assembly of the initiation factor complex and cap-dependent translation (13).

FSH stimulated phosphorylation of tuberin(Thr$_{1462}$) is blocked by the PI3K inhibitor wortmannin and is thus PI3K dependent, and occurs within 10 min of treatment. FSH stimulated phosphorylation of p70S6K(Thr$_{389}$) and its target S6(Ser$_{235}$/Ser$_{236}$) are also PI3K dependent as well as mTOR dependent based on the inhibition by wortmannin or rapamycin, respectively (14). Through regulation of at least these two proteins, FSH is able to stimulate translation of mRNAs.

Translation consists of three steps: initiation, elongation and termination. All three steps are tightly regulated, but initiation of translation is the rate-limiting step overall, and thus is more highly regulated than elongation and termination (15). Initiation is canonically activated through a cap-dependent mechanism following necessary processing of pre-mRNA such as removing introns and addition of the 5’ 7-methylguanylate (m7) cap and the poly-(A) tail. The rate-limiting step of initiation is eIF4E binding to the 5’ cap, due to limiting amounts of eIF4E protein (16). Following binding of the scaffolding protein eIF4G and the ATP-dependent RNA helicase eIF4A, the 40S ribosomal subunit is recruited to the complex and will scan the 5’ untranslated region until the initiation AUG codon is encountered (17). A stable circular mRNA ring is formed by eIF4G binding to poly(A) binding protein 1 (PABP1), linking together the 5’ cap and the 3’ poly(A) tail (18). Once the initiation AUG codon is recognized, scanning by the 40S ribosome is arrested, the 60S ribosomal subunit joins the complex and translation of mRNA begins (19).

In addition to the regulation of translation itself, the availability of mRNAs is also regulated through interactions with mRNA binding proteins. Y box binding protein-1 (YB-1) is a mRNA binding
protein that was first identified as a major component of the cytoplasmic messenger ribonuclear protein (mRNP) complex (20,21) and has since been shown to be able to both promote and inhibit translation of specific mRNAs depending on the YB-1/mRNA ratio (22,23). Additionally, YB-1 is able to melt mRNA secondary structure and to accelerate annealing (22,24).

Herein, we identify YB-1 as an mRNA binding protein in GCs. Using an m7GTP-Sepharose bead to isolate proteins bound to the 5’ cap, we detected increased YB-1 protein binding to the initiation factor complex following FSH stimulation. Utilizing Flag-tagged YB-1, we were able to detect an FSH-independent interaction between YB-1 and PABP1, but not between YB-1 and eIF4E. We detected increased levels of Egr1, but not of Hif1α mRNAs, bound to YB-1 in response to FSH. Together, these results indicate that in GCs, YB-1 is interacting with other mRNA binding proteins, and in response to FSH, increases binding to mRNA of at least one immediate early gene induced by FSH, Egr1.

EXPERIMENTAL PROCEDURES

Materials – The following were purchased: ovine FSH (oFSH-19) from the National Hormone and Pituitary Agency of the National Institute of Diabetes and Digestive and Kidney Diseases (Torrence, CA); anti–Flag (F3165) from Sigma–Aldrich; human fibronectin from BD Biosciences; antiphospho-YB-1 (Ser102; CST 2900), anti-YB-1 (CST 4202), anti-PABP1 (CST 4992), anti-eIF4E (CST 2067); Protein A/G PLUS Agarose (SC 2003) from Santa Cruz Biotechnology; m7GTP-4B Sepharose beads from GE Heathcare; TRIzol-RNA Lysis Reagent and Fast SYBR Green Master Mix from Life Technologies; qScript from VWR.

Animals - Sprague-Dawley, CD-outbred rats (breeders from Charles River Laboratories) were from a breeder 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 Cultures and Western blotting – Immature female rats were primed with subcutaneous injections of 1.5 mg estradiol-17β (E2) in propylene glycol on days 21-23. Ovaries were collected following three days of injections. GCs were collected by puncturing individual follicles using 27-gauge needles (25). Cells were plated on fibronectin-coated plates at a density of ~1x10^6 cells/mL of serum-free media supplemented with 1 nM E2, 100 U/mL penicillin (P), and 100 µg/mL streptomycin (S). Indicated treatments were added to cells ~20 h following plating, and terminated by aspirating media and washing once with PBS. Samples were collected by scraping cells in a SDS sample buffer (26) at 50 µL/1x10^6 cells, followed by heat denaturation. Proteins were separated by SDS/PAGE and transferred onto Protran (Amersham Biosciences) nitrocellulose membrane (27). The membrane was incubated overnight at 4°C with primary antibody, and enhanced chemiluminescence (ThermoFisher) was used to detect the antigen-antibody complexes. 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 (BioRad Laboratories).

Transfection of GCs - Cells were plated on fibronectin-coated plates at a density of 1x10^6 cells/mL in OptiMEM + P/S + E2 with expression constructs (250 ng/1x10^6 cells) and transfected using LipofectAMINE 2000 (Invitrogen) according to manufacturer’s instructions. After 6 h incubation, the media was removed and fresh DMEM +P/S+E2 was added. Cells were treated as indicated following ~16 h recovery period. Cells collected for immunoprecipitation or immunoprecipitation – RNA isolation.

Immunoprecipitation and Pulldowns – Immunoprecipitations (IP) and pulldowns were performed as previously described (7,28). Briefly, ~10^7 cells were scraped into 0.5 mL IP Lysis buffer [50 mM Hepes, pH 7.0, 100 mM NaCl, 0.5% Nonidet P-40, 20 mM NaF, 2 mM NaVO₃, 2 mM Na₄P₂O₇, 5 mM EGTA, 5 mM EDTA, 20 mM benzamidine, 10 µg/mL calpain inhibitor III, 50 µg/mL antipain, 50 µg/mL soybean trypsin inhibitor, 10 µM isomethylbutylxanthine, Halt protease inhibitor mixture [1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.8 µM aprotinin, 50 µM bestatin, 15 µM E-64, 20 µM leupeptin, 10 µM pepstatin; (Thermo Fischer Scientific)], sonicated twice on ice for 45 s, and
clarified by centrifugation at 10,000 x g for 5 min at 4°C. Following removal of 0.03 mL sample for input, soluble cell extracts were precleared using species matched preclearing matrix (ExactaCruz Preclearing Matrix, Santa Cruz Biotechnology) for >60 min at 4°C if necessary. Samples were incubated overnight by rotating at 4°C with either (i) the specified antibodies and Protein A/G PLUS Agarose (Santa Cruz Biotechnology; SC 2003), along with a species matched IgG antibody control (Santa Cruz Biotechnology) for immunoprecipitations or with (ii) 7-methyl-GTP-Sepharose 4B beads (GE Heathcare) for pull downs. Following centrifugation at 10,000 x 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. Beads were washed 4 times by rotating for 5 min at 4°C with 1 mL IP Wash Buffer (20 mM Hepes, pH 7.0, 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100). Bound proteins were collected in SDS sample buffer and subjected to SDS/PAGE and western blotting. Inputs represent ~2%, and bound sample ~98% of total sample.

**Immunoprecipitation-RNA Isolation and Real–time PCR -** Protocol was adapted from a previous report (29), as detailed below. GCs (~10^7) were scraped into 0.25 mL Polysome Lysis Buffer (50 mM Tris, pH 7.5, 100 mM KCl, 12 mM MgCl₂, 1% Nonidet P-40, 1 mM DTT, 200 U/mL Promega RNasin, 1 mg/mL heparin, 100 µg/mL cycloheximide, Sigma protease inhibitor mixture). Samples were subjected to six rounds of freeze/thaw to lyse cells. Lysates were clarified by centrifugation at 10,000 x g for 10 min at 4°C. Following removal of 0.03 mL for input, samples were incubated overnight by rotating at 4°C with 10 µg antibody and protein G magnetic beads (Dynabeads; Invitrogen). Following collection of the immunoprecipitated complexes using a magnetic stand, the beads were washed three times by rotating for 5 min at 4°C with 0.4 mL High Salt Wash (50 mM Tris, pH 7.5, 300 mM KCl, 12 mM MgCl₂, 1% Nonidet P-40, 1 mM DTT, 100 µg/mL cycloheximide). Input and bead (IP) samples were subject to DNase (Thermo Scientific) treatment per manufacturer’s instructions. Qiagen RLT Buffer (0.07 mL) was added to samples and RNA was isolated according to manufacturer’s instructions using the RNeasy MinElute Kit (Qiagen). RNA concentration was determined using a NanoDrop 2000. cDNA was
generated using qScript per manufacturer’s instructions. Real time-PCR was performed using Fast SYBR Green Master Mix per manufacturer’s instructions. Primers used were: *Hif1α* forward 5’ GAT CAG TTG TCA CCA TTA GAG AGC A 3’ reverse 5’ TGG GTC TGC TGC AAT CCT GTA 3’; *Egr1* forward 5’ CCT TTT GCC TGT GAC ATT TGT G 3’ reverse 5’ TGG TAT GCC TCT TGC GTT CA 3’.

**RESULTS**

*YB-1 interacts with 5’ cap and initiation factor complex.* YB-1 has been described as an mRNA binding protein, and shown to be a regulator of translation (30-33). We sought to determine if total YB-1 is interacting with the translational protein complex in GCs. In these experiments, we utilized m7GTP-sepharose beads that mimic the m7GTP of the 5’ end of mRNA (34,35). The initiation factor proteins will assemble around m7GTP, allowing for isolation of the initiation factor complex and study of the initiation complex of translation under different treatment groups. GCs were treated without or with FSH for the indicated times, and the initiation factor complex was pulled down using m7GTP-Sepharose beads. At all timepoints checked, FSH promotes an increase in total YB-1 bound to m7GTP (Fig. 1A, right panel), while total YB-1 remained unchanged (Fig. 1A left panel). YB-1(Ser 102) phosphorylation has been shown to be important for translation in breast cancer cells (30). Although we observed an increase in YB-1(Ser 102) phosphorylation in total GC extracts at 1 and 4 h in response to FSH (Fig. 1A left panel), phosphorylated YB-1(Ser 102) bound to m7GTP and the initiation factor complex was barely detectable 1 h and undetectable 4 h post FSH treatment (Fig. 1A right panel). Because eIF4E is the first initiation factor to directly bind the 5’ cap, eIF4E is used as a positive control for m7GTP sepharose bead pull down. To verify these results, GCs were transfected with Flag-tagged YB-1, treated with FSH for 60 min, and the initiation factor complex was pulled down using m7GTP-Sepharose. Using an anti-Flag antibody to detect Flag-tagged YB-1, results (Fig. 1B) show that FSH promotes an increase in Flag-tagged YB-1 bound to m7GTP. eIF4E was used as a positive control for the m7GTP pull down. Taken together, these results indicate that FSH promotes an increase in YB-1 associated with m7GTP, the 5’ cap, and the initiation factor complex.
YB-1 binds to PABP1, but not eIF4E. YB-1 has been shown to interact with both the initiation factor complex proteins (31), as well as with the general mRNA binding proteins (36). Based on these results, we sought to determine if YB-1 interacts with any other mRNA binding proteins in GCs, and if binding is FSH dependent. GCs were transfected with Flag-tagged YB-1, then treated without and with FSH. YB-1 was immunoprecipitated from lysates using anti-Flag antibody. In contrast to the m7GTP-Sepharose pull down results, eIF4E was not detected bound to YB-1-Flag. Interestingly, PABP1 was found to selectively bind to YB-1-Flag in both vehicle- and FSH-stimulated samples. These results indicate that YB-1 and PABP1 interact within GCs independently of FSH.

YB-1 binding to mRNAs increases in response to FSH-stimulation. Because YB-1 has been identified as an mRNA binding protein (31,37,38), and we’ve shown it binding to PABP1 in GCs, we sought to determine if YB-1 binding to mRNA increases in response to FSH treatment. GCs were transfected with Flag-tagged YB-1 and treated with FSH. RNA was isolated from YB-1-Flag immunoprecipitates, and mRNAs were analyzed using real-time PCR. Protein samples were saved to verify immunoprecipitation of Flag-YB-1 (Fig. 3A). As shown in Fig. 3B, Egr1 mRNA levels from input samples increased in response to FSH while HIF1α mRNA levels remained constant, consistent with previous results (39) and (14), respectively. Results show that YB-1 binds HIF1α and Egr1 mRNAs in GCs at increased amounts relative to the IgG control. Additionally, FSH promotes an increase in Egr1 mRNA bound to YB-1. These results indicated that in addition to binding PABP1, YB-1 also binds mRNAs within GCs. In response to FSH, Egr1 mRNA is bound at higher amounts to YB-1.

DISCUSSION

Our results show that YB-1 interacts with components of the initiation factor complex, and exhibits binding to HIF1α and Egr1 mRNA. These results indicate a possible role of YB-1 in translational regulation in response to FSH in GCs.
YB-1 is a nucleic acid binding protein that has many functions within the cell due to its interactions with both DNA and RNA (reviewed in (40)). Through interactions with RNA, YB-1 is associated with both nontranslated (free) and translating (polysomal) mRNA, and is a component of the mRNP complex along with PABP (22,36,41).

Our results show that FSH promotes an increase in the binding of total YB-1 to the m7GTP-Sepharose 5’-cap and the initiation factor complex. Surprisingly, however, phosphorylated YB-1(Ser\textsuperscript{102}) is barely detectable in the m7GTP-Sepharose pull down. This result is contradictory to previous studies that have reported that YB-1(Ser\textsuperscript{102}) phosphorylation is necessary for YB-1 binding to mRNA (30,31). However, YB-1 can be phosphorylated on multiple Ser and Tyr residues (30,42), so it is possible that FSH is promoting phosphorylation of a different residue that increases its binding to the initiation factor complex.

While it is possible that FSH is promoting phosphorylation of additional sites on YB-1, previous fractionation results from our lab showed that the majority of YB-1 phosphorylated on Ser\textsuperscript{102} localizes to the cytoplasmic fraction (see Chapter 3, (39)). If YB-1 phosphorylated on Ser\textsuperscript{102} is not interacting with the initiation factor complex, it is possible that cytosolic YB-1(Ser\textsuperscript{102}) phosphorylation is important for mRNA stability. At high YB-1/mRNA ratios, YB-1 proteins cover the mRNA strand and block other proteins from binding, thus both protecting the mRNA from degradation and blocking translation (through obstructed binding of eIF4E). At low YB-1/mRNA ratios, YB-1 is thought to release portions of the mRNA, including the 5’cap, perhaps promoting binding of eIF4E (37,41). So it is likely that YB-1(Ser\textsuperscript{102}) may be participating in mRNA stability.

This hypothesis is further verified by our YB-1 immunoprecipitation results. We report that the interaction between YB-1 and PABP1 is FSH-independent, indicating a stable interaction. As the two main components of mRNPs, YB-1 and PABP interact with each other and with mRNA (36). Additionally, we do not detect YB-1 binding to eIF4E, indicating that the binding of YB-1 seen in the m7GTP-Sepharose pull down may be indirect. The initiation factor proteins and mRNA form a stable
circular complex (18), so it is possible that YB-1 binding to the mRNA or other proteins in the complex
would be detected with the m7GTP-Sepharose pull down. Perhaps YB-1 proteins bind to the 3’ UTR and
poly(A) tail of mRNA, as the YB-1 immunoprecipitation data suggests. The consistent interaction
between PABP1 and YB-1 indicates YB-1 may be acting on mRNA stability, rather than binding to
eIF4E and acting in the initiation of translation.

Our results also show that in GCs, YB-1 is bound to HIF1α and Egr1 mRNAs, and that FSH
enhances the binding of total YB-1 protein to Egr1 mRNA. As FSH promotes an increase in the
transcription of Egr1 mRNA (see Fig 3B, (43)), we cannot determine if the increased binding of YB-1 to
Egr1 mRNA is only due to the increase in Egr1 mRNA or if FSH is promoting YB-1 to bind more
mRNAs, including Egr1. However, based on the ability of YB-1 versus control IgG to bind increased
levels of HIF1α mRNA in an FSH-independent manner, results indicate that YB-1 selectively binds
mRNAs in GCs.

Cytoplasmic YB-1 has been localized to both nontranslated (free) mRNPs and translating
(polysomal) mRNPs (30). Bound to mRNAs and possibly part of the mRNP complex in GCs, YB-1 might
be facilitating stability of mRNAs and participating in the movement of mRNAs from nontranslated to
polysomal mRNPs. Following an increase in translation in response to FSH, YB-1 containing mRNPs
may move to the polysomal mRNPs and interact with the initiation factor complex proteins.

These preliminary results indicate that YB-1 interacts with the initiation factor complex, and its
binding increases with FSH treatment. Further studies are needed to determine the role that YB-1 plays
following mRNA binding in response to FSH. Many roles have been determined for YB-1 bound to
mRNA (reviewed in (40)), and it is likely that more than one apply to YB-1 binding mRNA in response
to FSH.
CONFLICT OF INTEREST: The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS: EMD designed and performed the experiments. EMD and MEH-D analyzed the data and wrote the manuscript.
REFERENCES


messenger ribonucleoprotein particles in somatic cells is a member of the Y-box binding transcription factor family. *J. Biol. Chem.* **270**, 3186-3192


FIGURE LEGENDS AND FIGURES

FIGURE 1. YB-1 interacts with 5’cap and initiation factor complex. (A) GCs were treated without (veh) or with FSH for the indicated times, and then collected in Immunoprecipitation Lysis buffer and sonicated. After removal of the insoluble particles, the soluble fraction was rotated overnight with 20 µg m7GTP-Sepharose 4B beads (GE Healthcare) as described in Experimental Procedures. Inputs, bound (pulldown) 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. Blots are representative of three independent experiments. (B) GCs were transfected with 500 ng YB-1 – Flag plasmid for 6 h as described in Experimental Procedures. Following an overnight recovery, GCs were treated without (veh) or with FSH for 60 min, then collected as described in IA. Blots are representative of two independent experiments. Dotted lines between lanes represent cropped images. Results are representative of two independent experiments.
FIGURE 2. YB-1 binds to PABP1, but not eIF4E. (C) GCs were transfected with 500 ng YB-1 – Flag plasmid for 6 h as described in Experimental Procedures. Following an overnight recovery, GCs were treated without (veh) or with FSH for 60 min, and then collected in Immunoprecipitation Lysis buffer and sonicated. After removal of the insoluble particles, the soluble fraction was rotated overnight with 20 µg Anti–Flag (Sigma F3165) as described in 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.
Fig. 2

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FIGURE 3. YB-1 binding to mRNAs increases in response to FSH-stimulation. GCs were transfected with 500 ng YB-1 – Flag plasmid for 6 h as described in Experimental Procedures. Following 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 Anti–Flag (Sigma F3165). Protein (A) and RNA (inputs are B and IP are C) were collected from the samples as described in Experimental Procedures. (A) Inputs, bound (IP) and unbound (flow – though) protein samples were separated by SDS/PAGE and the western blot was probed with the indicated antibodies. Blot is representative of three independent experiments. (B/C) Real–time PCR was performed on input (B) and IP (C) samples for the genes indicated as described in Experimental Procedures. Quantification results represent the mean ± SEM of three independent experiments with (*) representing p < 0.05 and (**) representing p < 0.01, based on a one tailed Students t test.
Fig. 3

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B

mRNA Expression

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C

Hif1α mRNA associated with YB1-Flag

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Egr1 mRNA associated with YB1-Flag

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CHAPTER FIVE
CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

In this document, we show that FSH dependent ERK/RSK signaling to downstream target YB-1 is necessary for induction of gene targets that include Egfr, Inha, and Cyp19a1 that are required for follicular maturation.

In GCs, ERK(Thr^{202}/Tyr^{204}) phosphorylation requires both the constitutively active upstream components of canonical MEK signaling and the FSH-dependent inhibition of a DUSP, most likely MKP3(DUSP6). FSH-stimulated ERK(Thr^{202}/Tyr^{204}) phosphorylation is PKA-dependent, indicating that FSH via PKA is inhibiting a phosphatase, allowing for MEK-dependent ERK(Thr^{202}/Tyr^{204}) MKP3(DUSP6) phosphorylation to accumulate. Using multiple inhibitors, our results suggest that MKP3(DUSP6) is dephosphorylating ERK in the absence of FSH. Following treatment with the MKP3(DUSP6) inhibitor, NSC 295624, ERK(Thr^{202}/Tyr^{204}) phosphorylation increased in the absence of FSH to levels comparable of ERK phosphorylated in the presence of FSH. ERK2 co-immunoprecipitates with MKP3(DUSP6), and treatment of GCs with MKP3(DUSP6) inhibitors block dephosphorylation of recombinant ERK2-GST. Current studies aim to show an increase in ERK(Thr^{202}/Tyr^{204}) phosphorylation in the absence of FSH due to selective knockdown of MKP3(DUSP6) using a MKP3(DUSP6) shRNA (1).

We next show that YB-1 is present in GCs and, in response to FSH stimulation, is phosphorylated on Ser^{102}. YB-1(Ser^{102}) phosphorylation is first detectable after approximately 15 min and lasts approximately 8 hr following FSH treatment. This phosphorylation is both PKA- and ERK-dependent, based on loss of FSH-dependent YB-1(Ser^{102}) phosphorylation following treatment with selective PKA and MEK inhibitors, respectively. Treatment with the RSK inhibitor BI-D1870 results in loss of FSH-
stimulated YB-1(Ser$^{102}$) phosphorylation, demonstrating RSK dependence. Thus, we place FSH-stimulated YB-1(Ser$^{102}$) phosphorylation downstream of PKA and ERK/RSK signaling in GCs.

In order to identify YB-1-dependent genes, we first identify $Egfr$, $Inha$, $Cyp19a1$, $Ereg$, and $Egr1$ as ERK gene targets using MEK inhibitors. We then identify $Egfr$, $Inha$, and $Cyp19a1$ as YB-1 gene targets using adenoviral YB-1(Ser$^{102}$) mutants. Transduction of GCs with the dephospho-mutant Ad-YB1(S102A) results in a loss of FSH-dependent induction of $Egfr$, $Inha$, and $Cyp19a1$ mRNA. Finally, GCs transfected with promoter-luciferase constructs, followed by transduction with Ad-YB1(S102A), show inhibition of $Egfr$, $Cyp19a1$, and $Inha$ promoter – luciferase activities. These results indicate that YB-1(Ser$^{102}$) interacts with these gene promoters directly. Collectively, these results demonstrate a FSH-dependent role for YB-1 phosphorylated on Ser$^{102}$ in regulating the expression of gene targets necessary for follicular maturation.

Lastly, we show YB-1 interacts with the initiation factor complex, as it pulls down with m7GTP-Sepharose beads in an FSH-dependent manner and with PABP in an FSH-independent manner. Additionally, YB-1 binds to $HIF1\alpha$ and $Egr1$ mRNA over IgG control, and YB-1 bound to $Egr1$ mRNA increases following FSH treatment. Taken together, these data place YB-1 binding to mRNAs and mRNA binding proteins, indicating a possible role for YB-1 regulating mRNA stability or interacting with initiation factor complex in GCs in response to FSH.

Collectively, these results demonstrate novel regulation of ERK(Thr$^{202}$/Tyr$^{204}$) phosphorylation and identify novel ERK-dependent gene targets in GCs. Additionally, we identify YB-1(Ser$^{102}$) phosphorylation as being FSH- and ERK-dependent, and necessary for induction of FSH-dependent gene expression that is crucial for follicular maturation.

**FUTURE DIRECTIONS**
For a multitude of reasons, there were questions we were unable to answer within the scope of this research. I would now like to address questions that I would like to answer on the research presented and additional questions I believe this research topic can still investigate.

I would like to further examine the PKA-dependence of MKP3(DUSP6) inhibition. Examination of the protein sequence of MKP3(DUSP6) reveals a potential site for PKA phosphorylation (Thr\textsuperscript{302}). First co-immunoprecipitation experiments can be performed between PKA and MKP3(DUSP6) to determine if the two proteins interact. I can also immunoprecipitate MKP3(DUSP6) from GCs treated with vehicle and FSH, and probe with phospho-PKA substrate antibody to indicate that MKP3(DUSP6) is a PKA target in GCs. Also, phosphorylation mimic (T302D) and dephospho (T302A)-mutations of this site can be created, and the ability of the mutant to (i) interact with ERK and (ii) dephosphorylate pERK-GST can be investigated. Finally, co-immunoprecipitation experiments could be performed between PKA and the MKP3(DUSP6) mutants to determine if the mutation affects binding.

For YB-1, I would like to determine the regulatory protein that interacts with PP1cβ in GCs in response to FSH and inhibits dephosphorylation of YB1(Ser\textsuperscript{102}). RNA-seq data from our lab (Herndon et al., MS in preparation) has identified 26 unique PP1 regulatory proteins that are expressed untreated GCs. Individually examining each regulatory protein on this list for interactions with PP1 in GCs using co-immunoprecipitation experiments is daunting, because (i) of the large number of candidates, and (ii) many of these regulatory proteins do not yet have readily available antibodies. I propose performing YB-1-Flag immunoprecipitation experiments, and analyzing the pull down sample using mass-spec (PNNL).

Additionally, I would like to further investigate the role of YB-1 as a transcription factor in GCs. Our results indicate YB-1 is binding to the promoters of \textit{Egfr}, \textit{Cyp19a1}, \textit{Inha} using promoter-luciferase constructs. I would like to confirm these results through ChIP experiments, and determine the specific region of DNA that YB-1 is interacting with in these promoters. Furthermore, because these genes have been shown to be regulated by many different transcription factors in GCs (2-6) and YB-1 has been
shown to bind other transcription factors (7,8), I would also like to investigate YB-1 binding to additional transcription factors.

Finally, I would like to investigate the role of YB-1 in GCs in the regulation of translation in response to FSH. We identified total YB-1 binding to the initiation factor complex, but not to eIF4E. I would like to determine if YB-1 could be interacting with any other members of the initiation factor complex, such as eIF4G. More likely, YB-1 is binding directly to the mRNA, as it is a mRNA stabilizing protein (9,10). I would like to use our adenoviral wild type, (S102A), and (S102D) YB-1 mutants to further investigate the mRNA’s bound to YB-1 in vehicle and FSH-treated GCs at 1 vs 24 h post treatment. Also, I would like to perform polyribosome isolations combined with YB-1-Flag, and investigate the possibility that YB-1 travels with mRNA as it moves from the monoribosomal (non-translating) to the polyribosomal (translating) fractions (9). Our preliminary results indicate that YB1(Ser102) phosphorylation is not important to YB-1 interacting with the translational machinery. I would like to confirm these results using our adenoviral YB-1 mutants. I would also like to investigate if FSH is causing the phosphorylation of YB-1 on one or more additional sites by submitting YB-1 from vehicle and FSH treated cells to phospho-mass spec (PNNL). If one or more additional sites are identified Ser to Ala mutations could be created, and the binding of these mutants to mRNA, the initiation factor complex, or the polyribosome could be investigated. There are many questions left to investigate with regards to the role of YB-1 in translation in GCs in response to FSH.

In summary, the results presented in this document comprise the first investigation of YB-1 in GCs, and there are still many questions left to be investigated. YB-1 has been shown to play a role in many different cellular functions (reviewed in (11)), and investigating its role in GCs in response to FSH open many opportunities for future investigations.
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