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IRE1 REGULATES CYP11A1 TRANSCRIPTION TO MEDIATE STEROIDOGENESIS IN THE OVARY

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IRE1 REGULATES CYP11A1 TRANSCRIPTION TO MEDIATE STEROIDOGENESIS IN THE OVARY

by

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Dedication

To my husband and children, for their endless support, love and encouragement.

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IRE1 REGULATES CYP11A1 TRANSCRIPTION TO MEDIATE STEROIDOGENESIS IN THE OVARY

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CYP11A1 transcriptional activation in response to gonadotropin hormones is critical to maintain fertility and support pregnancy. Therefore, we are interested in identifying factors that regulate CYP11A1 transcription. Our laboratory has identified several signaling proteins that activate CYP11A1 transcription. In order to identify novel factors that mediate CYP11A1 transcriptional activation, we performed a kinase screen and identified inositol-requiring enzyme 1 alpha (IRE1) as a mediator CYP11A1 transcription. IRE1 is an ER stress signal transducer, however an ER stress response is not induced in KGN cells by forskolin treatment. We hypothesize that IRE1 is necessary to mediate CYP11A1 transcription in response to forskolin, a gonadotropin mimetic, in a mechanism independent to its role in ER stress. In the human KGN granulosa cell line, IRE1 is not predominantly localized to the endoplasmic reticulum. IRE1 colocalizes with Nup98, a nuclear localized marker, in the nuclear membrane in large, oligomeric punctate structures. Since IRE is necessary to mediate CYP11A1 expression, we sought to determine if IRE1 endoribonuclease activity is required for CYP11A1 expression. KGN cells were treated with and without forskolin, or tunicamycin and co-treated with the IRE1 endoribonuclease inhibitors MKC-4485 and MKC-3946. MKC-4485 and MKC-

3946 potently inhibited XBP1 splicing but had no effect CYP11A1 up-regulation in response to forskolin stimulation. Additionally, we sought to determine if XBP1u expression is necessary for up-regulating CYP11A1 in response to forskolin. Using RNA interference, XBP1 knockdown does not effect forskolin stimulated CYP11A1 expression. These data show that IRE1 mediates CYP11A1 in response to forskolin independent of its known mechanism in ER stress. In Y1 adrenal cells and JEG3 placental cells, IRE1 is required for forskolin stimulated CYP11A1 up-regulation and pregnenolone biosynthesis suggesting a general mechanism. In conclusion, IRE1 is critically important in activating CYP11A1 transcription. With global concerns increasing over declining fertility in humans, IRE1 represents a novel mechanism regulating steroidogenesis.

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Chapter 1 Steroidogenesis in the Ovary

Infertility affects approximately 15% of couples with ovulatory dysfunction as an underlying cause in 8% of women with impaired fertility [1, 2]. The main cause of ovulatory dysfunction is polycystic ovarian syndrome (PCOS) and effects 6%-10% of women of reproductive age making it the most prevalent endocrine disorder in young women [3-6]. Advanced maternal age due to cultural shifts has resulted in an increase in the number couples seeking assisted reproduction technology and a rise in the number of women experiencing infertility or reduced fertility.

HORMONAL REGULATION OF FEMALE REPRODUCTION

In normal regulation of the female reproductive axis, the hypothalamus produces gonadotropin-releasing hormone (GnRH) that signals the pituitary gland to produce follicle stimulating hormone (FSH) and luteinizing hormone (LH) in a cyclic manner. FSH and LH signal the granulosa cells (GC) and theca cells (TC) to produce estrogen and progesterone [7]. These hormones are required in different phases of follicular development to form a mature follicle and oocyte, as well as prepare the endometrium of the uterus for implantation. Estrogen and progesterone in the serum feedback to both the hypothalamus and the pituitary gland to maintain the reproductive cycle. In the first half of the cycle termed the follicular phase, the TCs and GCs synthesize estradiol by a paracrine mechanism. Increasing levels of LH signal the TCs to produce androstenedione, an androgen and precursor to both testosterone and estrone [8, 9]. Androstenedione diffuses to the GCs and is converted to estradiol. After ovulation, termed the luteal phase, LH stimulates the luteal cells (LCs) of the corpus luteum to synthesize progesterone. The biosynthetic pathway has been studied in many animal models but is not well studied in humans. Additionally, most of the steroid biosynthesis

has been studied in the TCs not the GCs. However, steroidogenesis in the ovaries of PCOS patients is dysregulated in both the TCs and the GCs; excessive androgens produced by the ovaries feed back to the pituitary and stimulate continued secretion of gonadotropins [1, 3, 4, 10-12]. Thus, the ovaries and the pituitary are involved in a cycle that results in a positive feedback loop.

The Menstrual Cycle and Follicular Development

In adult women, the average menstrual cycle is 28 days in length and is separated into two phases: the follicular phase and the luteal phase. During the follicular phase, the first 14 days of the cycle, a cohort of follicles is recruited into the gonadotropin dependent growth phase and by day 8 a single follicle is selected as the ovulatory follicle. LH and estrogen levels in the serum increase and estrogen levels peak around day 13 [7]. By day 8, a single follicle is selected as the ovulatory follicle and on day 14 there is a rapid surge in LH and minor peak in FSH that results in ovulation, the release of the oocyte from the follicle. The follicle involutes and enters the luteal phase. Progesterone production rapidly increases, promoting the proliferation of the uterine endometrium to prepare for implantation. If pregnancy occurs, human chorionic gonadotropin secreted by the embryo will act as a luteotropic hormone and sustain the corpus luteum for 12 weeks until the placenta takes over control of pregnancy. If pregnancy does not occur, the corpus luteum will enter apoptosis and progesterone levels will return to basal levels by day 28, and the cycle repeats. There is a late luteal increase in FSH that is necessary to rescue a cohort of follicles from atresia, and recruit those follicles for the next cycle.

The follicles recruited at the beginning of the estrus cycle are class 5 tertiary follicles. It takes approximately 85 days for a primary follicle to reach pre-ovulatory status. The majority of that time a small cohort of follicles mature in a gonadotropin independent manner, or tonic growth phase [7]. At the time of recruitment, most of the

follicles enter atresia except for a small pool of follicles measuring 2-5mm. At selection (day 8), a single follicle becomes dominant and the remaining follicles enter atresia.

Gonadotropin Signaling in the Ovary

The granulosa cells and the theca cells in the ovary work together in a paracrine interaction to produce the female sex steroids required to maintain fertility and to support pregnancy. There are two types of granulosa cells in an ovarian follicle: the cumulus GCs that surround the oocyte and secrete factors that aid in oocyte development, and the mural granulosa cells that line the follicle and produce estrogen and progesterone. The mural granulosa cells and the theca cells are separated from each other by a basal lamina. The granulosa cells express a FSH receptor (FSHR), a G-protein coupled receptor, during the follicular phase. Following the LH surge, GCs co-express the LH receptor (LHR). The follicular phase of the menstrual cycle occurs when developing secondary follicles become responsive to the gonadotropins, FSH and LH, secreted by the anterior pituitary in the brain. FSH signals to both the GCs and the theca cells to promote proliferation and growth of the follicle to a mature Graafian, or tertiary, follicle. FSH binds to the FSHR of TCs and GCs causing the G-protein to dissociate and activate adenylyl cyclase. Adenylyl cyclase increases the production of cAMP and induces a variety of signaling cascades such as the protein kinase A (PKA), protein kinase C (PKC) and the mitogen activated protein kinases (MAPK) [13]. These kinases pathways activate transcription factors that up-regulate genes involved in sex steroid biosynthesis, cell proliferation and differentiation, factors that promote oocyte development and competence, and atresia/apoptosis.

STEROIDOGENESIS IN THE FOLLICLES OF THE OVARY

The precursor to all ovarian steroid hormones including progestens, androgens and estrogens is cholesterol. Cholesterol enters the cell through the low density lipoprotein (LDL) receptor [8]. Cholesterol binds to the steroidogenic acute regulatory (StAR) protein that is bound to the outer membrane of mitochondria. StAR imports cholesterol into the lumen of the mitochondria where it is oxidized by cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1), also known as cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc), to form pregnenolone. CYP11A1 is considered to be the first step and the rate-limiting enzyme of steroidogenesis [14-16]. Cells that express CYP11A1 are characterized as steroidogenic. In the ovaries, the granulosa cells and the theca cells are the steroidogenic cells. Pregnenolone is transported out of the mitochondria and into the endoplasmic reticulum where the remainder of the steroidogenic enzymes reside such as CYP17A1, 3β-HSD-II and CYP19A1 [17, 18]. Pregnenolone is then oxidized by CYP17A1 and forms progesterone [19]. Progesterone may exit the cell either by diffusion or secretion, which is not well defined. In the GCs and TCs of the ovary, progesterone can be converted to dehydroepiandrosterone (DHEA), androstenedione, and estradiol. Androstenedione is exclusively produced by the theca cells and can be converted to testosterone and dihydrotestosterone in peripheral cells. Both granulosa cells and theca cells have been implicated to contribute to the hormonal dysregulation associated with PCOS**.**

The Two-Cell Theory

The menstrual cycle can be divided into two phases: the follicular phase and the luteal phase. During the follicular phase, the theca cells in the pre-ovulatory follicles express the LH receptor. Increasing levels of LH bind and activate the LH receptor

which signals to the transcription factor steroidogenic factor 1 (SF-1). SF-1 binds to the promoters of the genes CYP11A1, 3-beta hydroxysteroid dehydrogenase 2 (3β-HSD-II), CYP17A1. Activation of these genes enables the theca cells to convert cholesterol to androstenedione. Androstenedione diffuses through the basal lamina to the GCs. The GCs express the FSHR and its signaling activates the transcription factor liver receptor homolog 1 (LRH-1). LRH-1 binds the promoters of CYP19A1, or aromatase. CYP19A1 is able to convert androstenedione to estradiol. During the luteal phase, the ovarian follicle has involuted, vascularized, and produces large amounts of progesterone. The theca cells continue to express the LHR and produce large amounts of progesterone and androstenedione. However the granulosa cells express both the LHR and FSHR. LHR signaling activates SF-1 increasing the expression CYP11A1 and 3β-HSD-II and producing large amounts of progesterone that enters the blood stream. The coordinated actions of the TCs and GCs are tightly regulated and are necessary for fertility and pregnancy [20].

Regulation of CYP11A1

CYP11A1 is expressed in steroidogenic cells such as the adrenal cells in the adrenal gland, Leydig and Sertoli cells in the testes, granulosa, theca, luteal cells in the ovary, and in placenta cells [18, 21]. CYP11A1 is also expressed, albeit to a much lesser extent, in the brain, skin, and intestines [22]. The steroids produced by these tissues are classified as glucocorticoids, mineralocorticoids and sex hormones. Many laboratories have identified cis-regulatory elements and trans-regulatory elements involved in the tissue-specific activation of CYP11A1. In the ovary, the main cis-elements involved in CYP11A1 activation are SF-1 (steroidogenic factor 1) in the theca cells and LRH-1 (liver receptor homolog 1) in the granulosa cells [23-25]. These two transcription factors bind proximal (SF1RE) and upstream (U-CRS) response elements to activate CYP11A1

promoter in response to gonadotropins. Additionally, the CYP11A1 promoter also contains a response element that is responsive to insulin-like growth factor 1 and is termed the IGFRE. This response element was identified in adrenal cells, mouse fibroblast cells and porcine granulosa cells [26-28]. Two transcription factors bind the IGFRE, ptb-associated splicing factor (PSF) and Sp1, a ubiquitous transcription factor [26, 29]. PSF potently inhibits CYP11A1 and Sp1 activates transcription.

Although many studies have identified the regulatory elements and their transcription factors, very few studies have evaluated the kinases that signal to these transcription factors. One such study found that PKCι modulates the IGFRE response to IGF1 in porcine granulosa cell lines [30]. Additionally, the kinase ERK, and its signaling pathway, responds to insulin-like growth factor to activate CYP11A1 [31].

Chapter 2 Materials and Methods

REAGENTS

MKC-3946 and MKC-4485 are potent IRE1 endoribonuclease inhibitors and were provided by MannKind Corp. Forskolin and tunicamycin were obtained from Sigma Aldrich. Human ovarian tumor-derived KGN cells were obtained from RIKEN BRC Cell Bank (Ibaraki, Japan). Human choriocarcinoma JEG-3 Cells were obtained from ATCC (ATCC® HTB-36™). Mouse adrenal Y1 cells were from UTMB (Galveston, TX).

CELL CULTURE

KGN and Y1 cells were cultured in DMEM/F12 (Life Technologies) media with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Gibco). JEG-3 cells were cultured in Eagle's MEM (Life Technologies) and supplemented with 10% fetal bovine serum (Sigma Aldrich) and 1% penicillin/streptomycin (Gibco). Cells were plated at 80% confluence for 24 hours prior to treatment. Cells were treated with forskolin (1 μM) for 24 hours or Tunicamycin (1 μg/mL stock solution was diluted 1:1000 in media to a final concentration 1ng/ml) for 4 hours and co-treated with and without MKC-3946 (10 μM) or MKC-4485 (10 μM). 20mM stock solution in DMSO of MKC-3946 and MKC-4485 was diluted 1:20,000 in media for a final concentration of 10 μM. Cells were incubated at 37°C with 5% CO_2.

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

KGN cells were grown on glass coverslips. Cells were washed with PBS and fixed and permeabilized with 70% ethanol for 5 minutes on ice. Cells were washed with

0.25% Triton X-100 in PBS and blocked with 0.25% Triton X-100 and 1% BSA in PBS for 30 minutes. Blocking solution was removed and cells were incubated with specific antibodies against IRE1 (1:100, Santa Cruz Biotechnologies), Nup98 (1:100, Sigma), and Grp94 (1:100, Abcam) for one hour. Primary antibodies were removed and cells were washed four times with 0.25% Triton X-100 in PBS for 5 minutes each. Species-specific secondary antibodies AlexaFluor 488 and AlexaFluor594 (1/200, Invitrogen) were added and incubated for one hour. Cells were washed four times and mounted on glass slides with Vectashield Hard-Set for fluorescence mounting media (Vector Labs) and allowed to set overnight. Images were obtained using the Zeiss LSM-510 META confocal microscope with a Plan-Apochromat 63x, 1.4 numerical aperature oil immersion objective for untreated cells, and for Tm, Tg and forskolin treated cells the images were obtained with a Plan-Apochromat 100x, 1.4 numerical aperature oil immersion objective (Optical Imaging Laboratory, UTMB). The images were collected using 2 lines of excitation (488, and 543 nm argon ion laser) and two different channels of emission. Images collected vertical z-stacks with a 0.5 μm steps for untreated cells and 0.4 μm steps for cells treated with Tm, Tg, and forskolin. Images were processed with Metamorph software (Molecular Devices, Downington, PA).

siRNA Transfection

Y1 and JEG-3 cells were forward transfected with reagents and siRNA using 33 μM each of three Stealth Select siRNAs for IRE1 using Lipofectomine™ RNAiMAX (Invitrogen). Cells were plated at 50% confluence in normal growth media for 24 hours prior to transfection. Cells were incubated for 1 hour in normal growth media without antibiotics. Cells were transfected according to manufacturers instructions. Cells were incubated for 5 hours with transfection reagents and then media was changed to complete media for 48 hours. After 48 hours, cells were treated with 1uM forskolin for an

additional 24 hours. Conditioned media was collected and RNA harvested for downstream applications. KGN cells were reversed transfected with reagents and siRNA using 33 μM each of three Stealth Select siRNAs for XBP1 using Lipofectomine™ RNAiMAX (Invitrogen). Cells were plated at 80% confluence in normal growth media for 24 hours prior to transfection. Cells were incubated for 1 hour in normal growth media without antibiotics. Cells were transfected according to manufacturers instructions. Cells were incubated for 5 hours with transfection reagents and then media was changed to complete media for 48 hours. After 48 hours, cells were treated with 1 μM forskolin for an additional 24 hours. RNA was harvested for downstream applications.

RNA EXTRACTION AND QPCR

Total RNA was extracted using TRIzol. Two μg RNA (A260/280> 1.8) was reversed transcribed into cDNA using Superscript III First-Strand Synthesis System Kit for RT-PCR (Invitrogen). To analyze expression levels of target genes, RT-PCR was analyzed using DreamTaq Green PCR master mix (Fermentas). See primer sequences below. Thermal cycling conditions were 35 cycles of heating at 94°C for 30sec, 58°C for 30sec, and 72°C for 30sec. PCR products were resolved on a 2.5% agarose gel and visualized with ethidium bromide staining and UV excitation.

Table 1: Target Gene Sequences (5'-3')

Human Ire1	AGGCAAGAACAAGCTCAACTA
*Human Ire1*_rev	TCTCCAGCATCTTGGTAGAC
Human Cyclophilin	TTTCATCTGCACTGCCAAGA
Human Cyclophilin_rev	TTGCCAAACACCACATGCT

Human Xbp1	CCTGGTTGCTGAAGAGGAGG
Human Xbp1_rev	CCATGGGGAGATGTTCTGGAG
Human CYP11A1	GAGATGGCACGCAACCTGAAG
Human CYP11A1_rev	CTTAGTGTCTCCTTGATGCTGGC
mouse Ire1	GTTTGACCCTGGACTCAAAGG
mouse Ire1_rev	AGCAGGAATCACATTTTCTCGA
mouse Actin	AGTGTGACGTTGACATCCGTA
mouse Actin_rev	GCCAGAGCAGTAATCTCCTTCT

PREGNENOLONE ELISA

Conditioned media was collected at different time points from cells in culture and stored at -80°C. Samples were thawed on ice and centrifuged briefly to pellet any cellular debris. Media (50 µl) was assayed using BioVendor Pregnenolone ELISA. Optical Density was determine on a microwell plate reader at 450 nm.

REAL-TIME QUANTITATIVE PCR

Real-time quantitative PCR was performed on a BioRad CFX96 Real-Time System C100 Thermal Cycler. Reactions were run in triplicate and normalized to cyclophilin. Relative mRNA expression was determined using primers for CYP11A1, XBP1, and cyclophilin. Thermal cycling conditions were 95°C for 3 min; 45 cycles of 95°C for 10 sec, 64°C for 30 sec; gradual heating from 65°C to 95°C to generate melting curves.

Chapter 3 IRE1 is Necessary to Mediate CYP11A1 Transcription

INTRODUCTION

KGN Cells are a Model of Pre-Ovulatory Granulosa Cells

The KGN cell line is a human cell line developed from a granulosa tumor [17]. This cell line is unique from other cell lines because it retains a functional FSH receptor upon transformation, thus making it very useful for studies on steroidogenesis. The KGN cell line is capable of producing pregnenolone and progesterone and showed inducible aromatase activity when treated with androstenedione. Enzyme activity assays using radioimmunoassays showed that CYP11A1 and 3β-HSD were active in KGN cells, but the CYP17A1 enzyme activity was at the limits of detection. Many cell types can modify hormones but only certain cell types can synthesize steroids de novo. The presence of the CYP11A1 classifies the KGN cells as steroidogenic cells. Forskolin is used to stimulate cAMP and protein kinase A by activating adenylyl cyclase. Hedin and Rosberg [18] showed that forskolin treatment stimulated steroid production similar to LH treatment. Therefore forskolin is a gonadotropin mimic. Forskolin induced the production of progesterone in the media of cultured KGN cells in a dose dependent manner over 24 hours (**Figure 1A**, unpublished data). KGN cells are responsive to FSH and forskolin, but not LH (data not shown), establishing the KGN line as a model of pre-ovulatory granulosa cells. KGN cells release progesterone into the media in response to forskolin. In KGN cells, forskolin stimulation up-regulates CYP11A1 mRNA expression over 24 hours with maximal CYP11A1 expression after 24 hours (**Figure 1B**, unpublished data). Attenuation of CYP11A1 expression using specific siRNA reduces CYP11A1 mRNA (**Figure 1E,** unpublished data) and progesterone production in response to forskolin (data not shown).

Discovery of IRE1 as a Novel Mediator of Granulosa Cell Steroidogenesis

In order to identify factors involved in CYP11A1 gene regulation, we performed a kinase screen. The screen was designed to utilize KGN cells and forskolin stimulated progesterone secretion to identify kinases that potently inhibit progesterone production when interrupted with siRNA. We screened all 626 human kinases by transfection with siRNA targeting each kinase. The four kinases we discovered that had reduced progesterone production were further screened individually (**Table 2**, unpublished data). Conditioned media was collected from cells treated as described and progesterone measured. Cells were processed for measuring CYP11A1 mRNA by quantitative, real time RT-PCR. We discovered, using siRNA, that knockdown of one of the kinases, IRE1, potently inhibited progesterone synthesis (**Figure 1C,** unpublished data), as well as reduced CYP11A1 mRNA (**Figure 1D**, **E,** unpublished data). KGN cells treated with siRNA against IRE1 resulted in the loss of progesterone (**Figure 2A,** unpublished data), pregnenolone production (**Figure 2B,** unpublished data) and down-regulation of IRE1 at the RNA (**Figure 2C,** unpublished data) and protein level (**Figure 2D,** unpublished data).

IRE1 is an Endoplasmic Reticulum Stress Response Signal Transducer

Inositol-requiring enzyme 1 alpha (IRE1) is a resident endoplasmic reticulum transmembrane protein first identified in yeast [32, 33]. IRE1 is one component of the tripartite unfolded protein response (UPR) (reviewed in [34-36]). The IRE1 signaling pathway is the most highly conserved pathway of the UPR as well as the best characterized pathway. The ATF6 and PERK pathways are only found in the metazoans [37]. In mammals there are 2 homologs of IRE1: IRE1α which is expressed ubiquitously, and IRE1β which is expressed in gastrointestinal epithelium and mucous cells in the airway [38, 39]. IRE1 has an ER luminal domain that senses unfolded proteins, and

binds ER chaperones, a transmembrane domain and linker domain, a serine/threonine kinase domain, and an endoribonuclease domain. In homeostatic conditions, IRE1 can be found spanning the ER membrane in a complex with the ER chaperone BiP [34, 40-42]. During ER stress, IRE1 senses the presence of unfolded proteins in the lumen of the endoplasmic reticulum, BiP dissociates from the complex and enables IRE1 to oligomerize, trans-autophosphorylate, and splice the x-box binding protein, XBP1u, to its spliced active form, XBP1s [34]. XBP1 is transcription factor that binds ER stress response elements and activates genes that are important in re-establishing homeostatic conditions (reviewed in [34-36]). In chronic stress conditions, IRE1 is able to initiate apoptosis via regulated IRE1 dependent decay (RIDD) of select microRNAs [43].

RESULTS

In order to determine if IRE1 mediating steroid biosynthesis is general mechanism and is not specific to granulosa cells in the ovary, mouse Y1 adrenal cells and human JEG3 placental cells were screened. Y1 and JEG-3 cells were transfected with IRE1 specific siRNA to knockdown IRE1. After 48 hours, media was removed, and RNA was harvested to determine IRE1 knockdown, or fresh media added with and without 1 μM forskolin. Cells were treated for 24 hours and pregnenolone concentration was measured in the conditioned media by ELISA. Both JEG-3 and Y1 cells had a significant reduction in pregnenolone production in response to forskolin when IRE1 was knocked down (**Figure 3A, 3B**). As well, IRE1 mRNA expression was significantly knocked down (**Figure 3C, 3D**).

In order to determine the cellular localization of IRE1 in granulosa cells, KGN cells were grown on glass coverslips for 24 hours to attach. Immunocytochemistry was performed and cells were immunostained with anti-IRE1 antibody (red) and co-stained with a nuclear envelope protein, (Nup98) or an endoplasmic reticulum marker, Grp94

(green). Cells were imaged using a confocal microscope and optically sliced in 0.5 μm steps. Interestingly, IRE1 did not localize predominantly in the endoplasmic reticulum (**Figure 4B**). Instead, IRE1 co-localized with Nup98 (**Figure 4A**). When viewed from either a top view (**Figure 4C**) or from the z-direction in a side view (**Figure 4D**), IRE1 localizes constitutively on the nuclear membrane in large punctate oligomers. This was a surprising localization of IRE1. To test the specificity of the antibody, KGN cells were transfected with IRE1 siRNA or scrambled control siRNA, and immunostained with an anti-IRE1 antibody (red). Cells transfected with scrambled siRNA have IRE1 present on the nuclear membrane, however cells with IRE1 knocked down show very little IRE1 staining (**Figure 4E**).

CONCLUSIONS

CYP11A1 is the rate-limiting enzyme in steroidogenesis. Although the CYP11A1 promoter has been well studied to determine the cis- and trans-regulatory elements that are responsible for its activation or repression, very little data is available on kinases that signal to those elements, or directly to the CYP11A1 promoter. Through the loss-of-function kinase screen, IRE1 was identified as a potential critical kinase in the expression of CYP11A1 and steroid biosynthesis (**Figure 1**). IRE1 regulates CYP11A1 mRNA expression and pregnenolone and progesterone biosynthesis in granulosa cells (**Figure 2**). Pregnenolone is used as a read-out on CYP11A1 activity. Additionally, IRE1 is necessary for pregnenolone biosynthesis in mouse adrenal Y1 cells and human JEG-3 placental cells suggesting that this is a general mechanism in different steroidogenic tissues and across species.

IRE1 constitutively localizes to the nuclear membrane in large punctate oligomers, with very little IRE1 staining in the endoplasmic reticulum. This is a surprising finding, but other studies suggest that in the endoplasmic reticulum, IRE1 is a

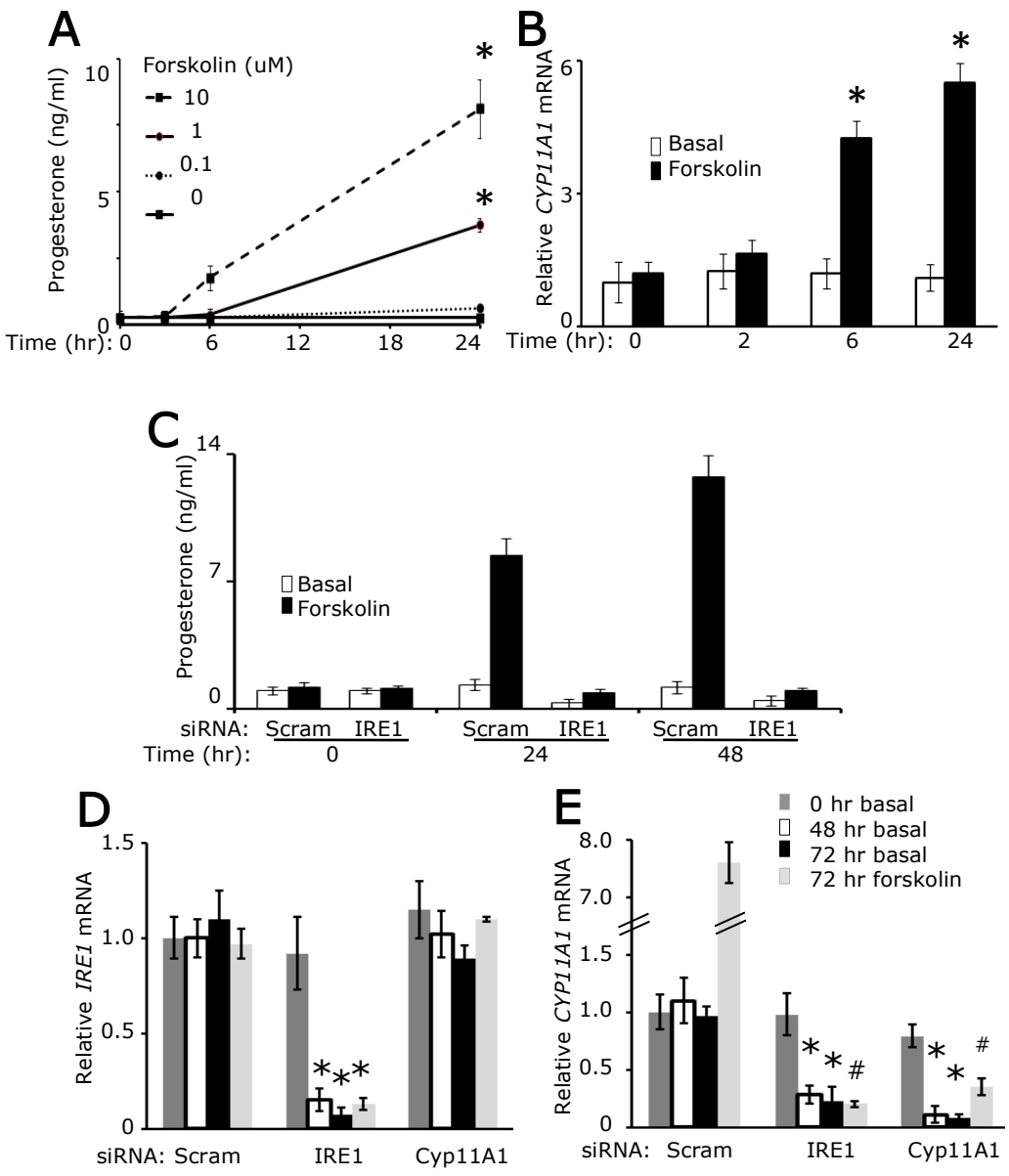
relatively low abundant protein which weakly stains under homeostatic conditions [44-46]. When cells are under ER stress IRE1 forms large, dynamic clusters [44]. Other groups have suggested that IRE1 localizes to the perinuclear region in COS1 cells [45, 46]. These data suggest that in granulosa cells IRE1 resides in, or is closely associated with the nuclear membrane.

Table 2: Results of the kinase screen in KGN cells

	% Inhibition	
Kinase	progesterone	p450scc mRNA
IRE1-α	93	70
ROR2	95	10
HIPK1	92	82
CDK5	80	8

Kinases were identified that reduced progesterone production when each kinase was knocked out. A second screen measured CYP11A1 mRNA and kinases were selected if relative mRNA was reduced in response to the siRNA.

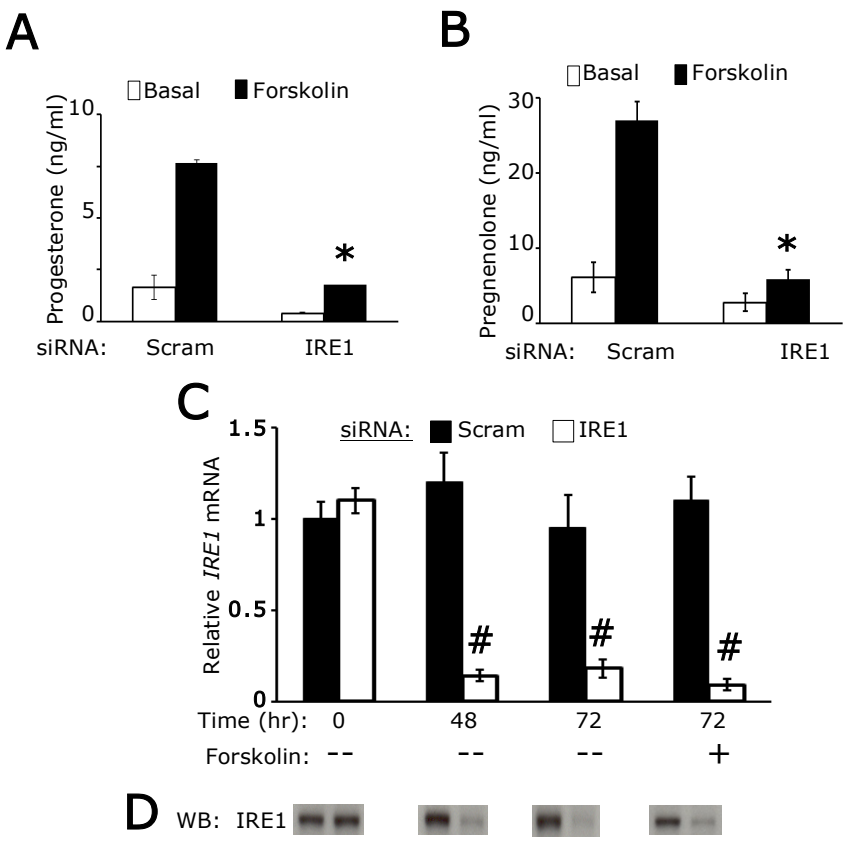
Figure 1: IRE1 is required for CYP11A1 expression and progesterone production



Forskolin treatment induces the production of progesterone in the conditioned media and up-regulates CYP11A1. **(A)** KGN cells were treated with 0, 0.1, 1.0, 10 μM forskolin for 0, 6, 12, 18, and 24 hours and progesterone was measured by electrochemiluminescence. **(B)** KGN cells were treated with and without 1.0 μM forskolin for 0, 2, 6, or 24 hours and CYP11A1 expression was determined using real-time PCR. IRE1 knockdown potently inhibits progesterone production and IRE1 and CYP11A1 mRNA expression. **(C)** KGN

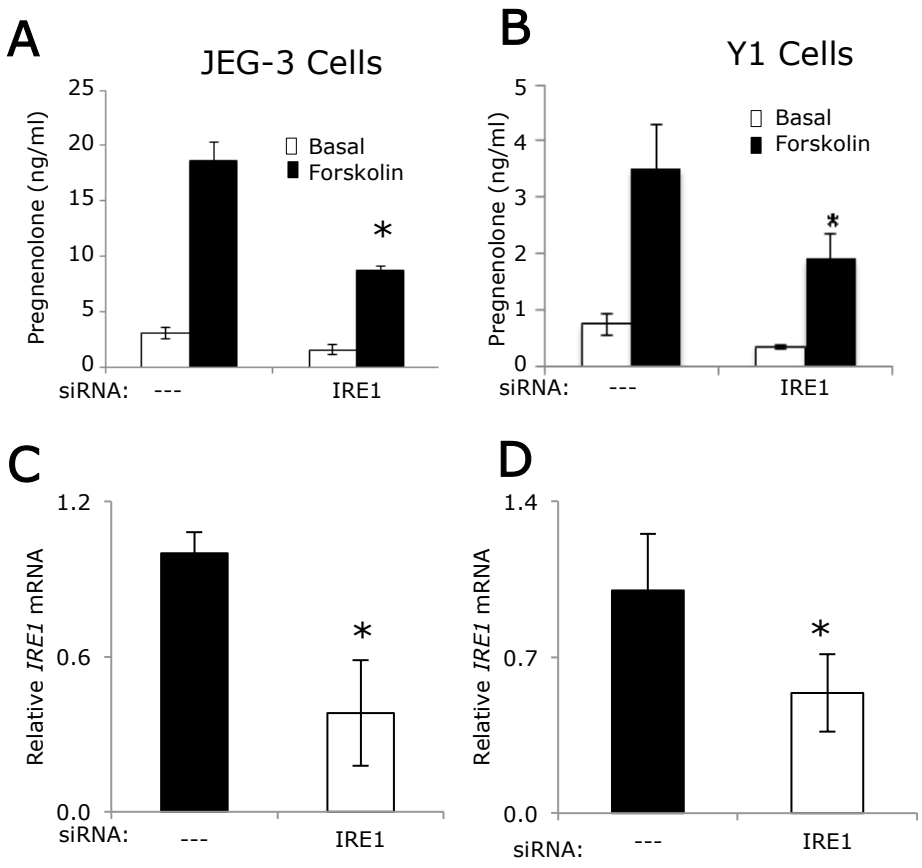
cells were transfected with IRE1 siRNA for 48 hours and treated with and without forskolin for 0, 24, and 48 hours and progesterone was measured by electrochemiluminescence. **(D)** Relative IRE1 and **(E)** CYP11A1 mRNA expression was measured by real-time PCR in basal media for 0 48, and 72 hours and with 1 μM forskolin for 72 hours. *p-value < 0.05 compared to scrambled control; #p-value < 0.01 compared to scrambled control with forskolin.

Figure 2: IRE1 is required for sex steroid biosynthesis in granulosa cells



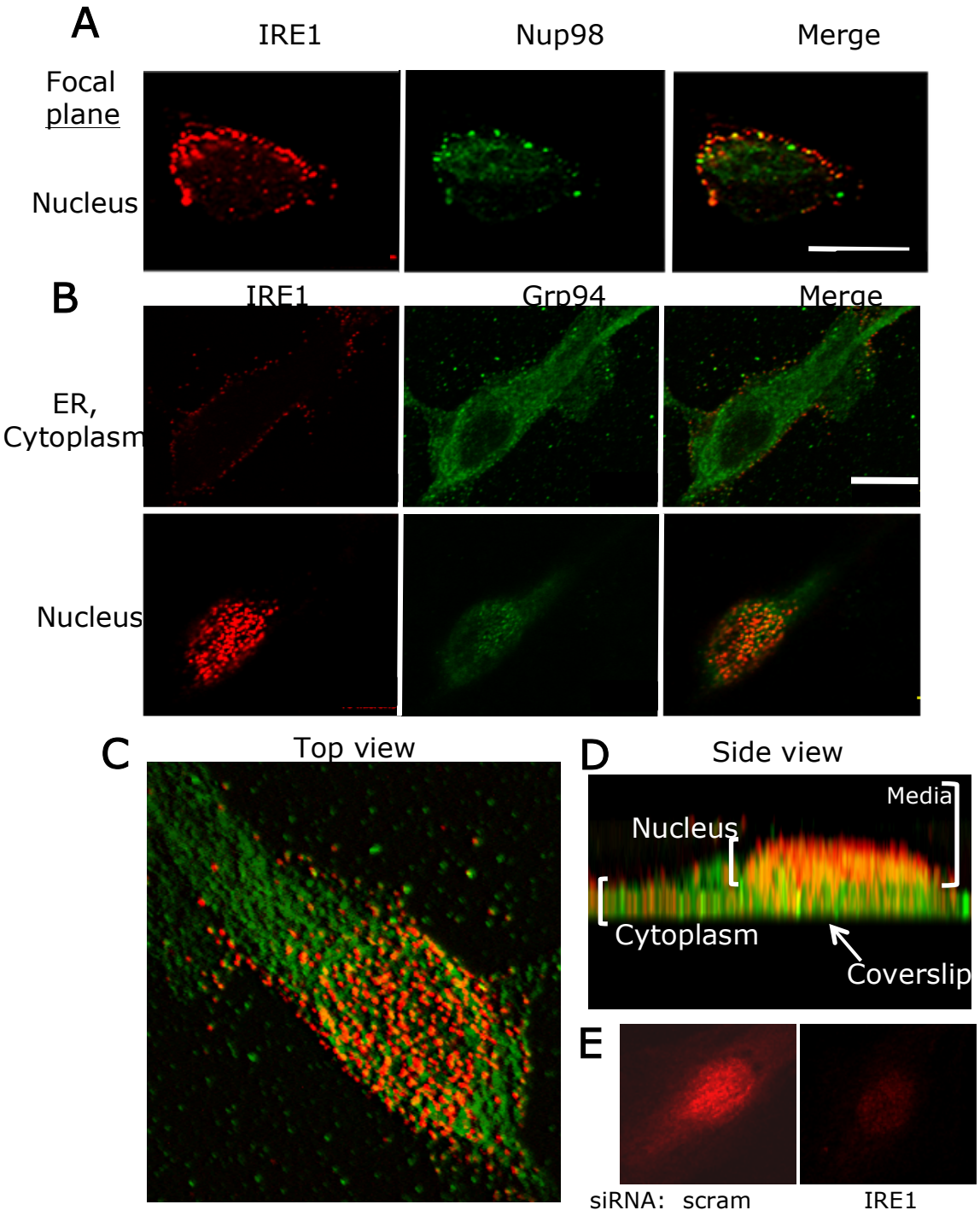
Knockdown of IRE1 potently inhibits both progesterone and pregnenolone biosynthesis. KGN cells were transfected with scrambled siRNA and IRE1 siRNA for 48 hours and treated with 1 μM forskolin for 24 hours. **(A)** Progesterone was measured by electrochemiluminescence and **(B)** Pregnenolone was measured by ELISA. **(C)** IRE1 mRNA expression and **(D)** protein is knocked down for 72 hours under both basal and forskolin treatment.

Figure 3: IRE1 is required for sex steroid biosynthesis in non-ovarian steroidogenic cells



IRE1 is necessary for CYP11A1 expression and pregnenolone biosynthesis in other steroidogenic tissues. **(A, C)** Human JEG-3 and **(B, D)** mouse Y1 cells were transfected with and without IRE1 siRNA for 48 hours and treated with 1 μM forskolin or basally for an additional 24 hours. **(A, B)** Pregnenolone was measured in the conditioned media by ELISA and **(C, D)** IRE1 expression was measured by real-time PCR.

Figure 4: IRE1 localizes to the nuclear membrane in KGN cell



IRE1 localizes to the nuclear membrane of KGN cells in large, punctate oligomers. (**A**) KGN cells were grown on glass coverslips and incubated 24 hours to attach. Cells were fixed with 70% cold ethanol for 5 minutes and washed with 0.25% Triton X-100 in PBS, and blocked with 1% BSA in PBS. Cells were stained with rabbit anti-IRE1 and goat anti-rabbit AlexaFluor 594 and co-stained with rat anti-Nup98 and goat anti-rat AlexaFluor 488, a marker for the nuclear membrane or (**B**) mouse anti-Grp94 and goat anti-mouse AlexaFluor 488, a marker for the endoplasmic reticulum. IRE1 is in a distinct location from the endoplasmic reticulum. (**C**) A three-dimensional z-stack reconstruction viewed from the top view or (**D**) from the side view in the z-dimension. IRE1 immunostaining is specific for IRE1. (**E**) KGN cells cultured on glass coverslips for 24 hours and transfected with scrambled siRNA or IRE1 siRNA. Cells were immunostained with rabbit anti-IRE1 and goat anti-rabbit AlexaFluor 594 Scale bar, 10 μm.

Chapter 4 IRE1 Mediates CYP11A1 Transcription in a Non-Canonical Signaling Pathway

INTRODUCTION

The Unfolded Protein Response

The endoplasmic reticulum is primarily responsible for protein synthesis and folding and plays a central in the formation of secretory proteins. It is essential that the endoplasmic reticulum coordinates a quality control system to maintain the fidelity of the many ER functions [34, 35, 47, 48]. The unfolded protein response (UPR) is a highly conserved mechanism in eukaryotes to sense and ameliorate the accumulation of unfolded proteins in the ER. The accumulation of misfolded proteins triggers the UPR through ER transmembrane sensors. The goal of the UPR is to maintain homeostasis, however the UPR can initiate apoptosis if homeostasis cannot be restored. IRE1 is the most highly conserved signal transducer of the UPR, and along with PERK and ATF6 comprise the UPR present in metazoans [36]. IRE1 and PERK exert translational control by targeted mRNA decay that results in a decrease in ER folding load. All three signal transducers initiate a transcriptional response that up-regulates genes to increase ER folding capacity and expand the ER via up-regulation of lipid biogenesis. Once ER stress reaches a threshold, IRE1 is able to directly induce apoptosis. IRE1 selectively cleaves anti-Casp2 pre-miRNAs and removes the repression of CASP2 expression. Caspase-2 (CASP2) is a pro-apoptotic protease and is essential in apoptosis [43]. Thus IRE1 controls cell fate determination [49].

CANONICAL IRE1 SIGNALING IN THE UPR

IRE1 is serine/threonine kinase and endoribonuclease that is localized in the endoplasmic reticulum [32, 33, 50, 51]. In unstressed conditions, monomeric IRE1 is constitutively bound to the ER chaperone binding immunoglobulin protein (BiP), also known as glucose-regulated protein 78 (GRP-78) or heat shock 70 kDa protein 5 (HSPA5) [52, 53]. In the presence of ER stress, BiP senses unfolded or misfolded proteins in the ER lumen, dissociates, and binds the unfolded proteins to initiate ER-associated degradation. Dissociation of BiP, enables IRE1 to undergo a conformational change and oligomerize [53, 54]. IRE1 activates itself through transautophosphorylation on serine-724. The conformational shift enables the kinase domain and endoribonuclease (RNase) domain to align and activate. IRE1 then unconventionally splices Xbp1u to its active form Xbp1s. by removing a 26-nucleotide intron in Xbp1. This results in frame shift and converts Xbp1 from a 267 amino acid long form to a spliced 371 amino acid long form that contains a DNA binding domain [55]. XBP1 targets a wide array of genes involved in protein folding, lipid biogenesis, ER associated degradation, and protein chaperones.

PERK AND ATF6 SIGNALING IN THE UPR

The second arm of the UPR involves double stranded RNA activated protein kinase (PKR)-like ER kinase (PERK), an ER resident protein kinase [37]. In unstressed conditions, PERK is monomeric in the ER [56]. In ER stress, PERK senses misfolded proteins oligomerizes and autophosphorylates itself. Phosphor-PERK phosphorylates the ubiquitous translation factor eukaryotic initiation factor 2 (eIF2) and inactivates it [57]. This inhibits protein translation, helps reduce the number of mRNA being translated and

reduces the protein-folding load in the ER [58]. eIF2 phosphorylation enables the up-regulation of activating transcription factor 4 (ATF4) and induces the expression of ER stress associated genes, such as CHOP, and XBP1.

The third arm of the UPR involves activating transcription factor 6 (ATF6), an ER-resident transmembrane protein with one large subunit in the ER lumen and a bZIP transcriptional domain in the cytosolic region [37]. Upon ER stress, ATF6 is transported to the Golgi apparatus where the cytosolic domain is cleaved to an active transcription factor [59-61]. Among its target genes is BiP, as well as many genes involved in protein folding, processing and ERAD.

Steroid Biosynthesis does not Induce ER Stress

The majority of steroidogenic enzymes reside in the endoplasmic reticulum, with the exception of CYP11A1. Gonadotropin stimulation requires a rapid increase in the expression and translation of these enzymes to produce the sex hormones. We hypothesized that the steroid biosynthetic program must induce ER stress and require the activation of IRE1 to ameliorate this stress. We treated KGN cells with forskolin for 24 hours, or the ER stress inducers tunicamycin (Tm) and thapsigargin (Tg) for 4 hours. In order to determine if forskolin treatment induced ER stress in KGN cells, we probed our western blots with antibodies targeting the three ER stress response pathways. KGN cells treated with Tm or Tg show an increase in phosphorylation of IRE1 (pIRE1), PERK (pPERK), and eIF2α (peIF2α) (**Figure 5A**, unpublished data). Additionally, BiP is up-regulated and ATF-6 cleavage is induced in response to Tm and Tg. XBP1 splicing is induced in response to Tm treatment. Interestingly, Forskolin treatment does not induce any canonical ER stress responses. Thus, forskolin treatment does not induce ER stress. IRE1-dependent regulation of CYP11A1 transcription is independent of its known role in ER stress response.

The cellular localization studies indicated that IRE1 was present on, or in close association with, the nuclear membrane with little to no ER expression. We hypothesized that IRE1 expression in the ER was at very low expression levels or in a monomeric form and therefore unable to be clearly visualized. In order to show that IRE1 expression is on the nuclear membrane, and possibly in the ER as well, we performed subcellular fractionation. KGN cells were transfected with IRE1 siRNA or scrambled control siRNA, and differentially centrifuged to obtain a cytoplasmic fraction (Cyto), a nucleoplasmic fraction (Nuc) and a nuclear membrane fraction (Memb). IRE1 was found in both the cytoplasmic fraction, which contains the ER, and the nuclear membrane fraction (**Figure 5B**). RNA interference of IRE1 reduced IRE1 presence in all fractions. Nup98, which traverses the nuclear membrane was found in both the nucleoplasm and the nuclear membrane fractions. Glucose-regulated protein 94 (GRP94), an ER resident protein, was exclusively found in the cytoplasmic fraction indicating that this fraction contains the ER. Beta tubulin is a cytosolic protein and is also only found in the cytoplasmic fraction. Lamin-B lines the inner nuclear membrane and was exclusively present in the nuclear membrane fraction. This indicates that IRE1 is present in both the endoplasmic reticulum and the nuclear membrane.

Non-Canonical IRE1 signaling

New data is emerging that suggests that IRE1 may have roles outside of its canonical role as an ER stress signal transducer. A recent review by Hetz and Glimcher [34] proposed that IRE1 forms a complex that mediates the cellular response to ER stress. There are many groups that have reported the direct association of many proteins with IRE1 to facilitate activation of signaling networks that regulate the cellular response to ER stress (**Table 3**). These IRE1-associated proteins, termed the 'IRE1 interactome,' have the ability to modulate the function of IRE1 in a cell-type specific level [34-36, 42].

Increasing evidence suggest that IRE1 may have a variety of functions outside of canonical IRE1 signaling in ER stress. Two particular instances of IRE1 non-canonical signaling are inflammation and glucose homeostasis. IRE1 is able to cross talk with the c-Jun n-terminal kinase (JNK) pathway and nuclear factor kappa B (NF-κB) signaling cascades via the tumor necrosis factor (TNF)-associated factor 2 (TRAF2) [62, 63]. TRAF2 is recruited to IRE1 after it has been activated by ER stress and is responsible for phosphorylating and activating JNK and associated protein 1 (AP1). AP1 is able to turn on inflammatory genes. Additionally, TRAF2 interacts with and phosphorylates inhibitor of kappa B (IκB) kinase (IKK) enabling it to phosphorylate IκB and lead to its degradation [64, 65]. This relieves repression of NF-κB and enables it to enter the nucleus and activate its target genes, such as TNFα.

Non-canonical IRE1 signaling has been reported to be involved in glucose homeostasis. Glucose homeostasis, insulin signaling and obesity are linked through IRE1-JNK signaling by phosphorylating the insulin receptor substrate-1 (IRS-1) and Xbp1 haploinsufficiency leads to an increase in insulin resistance [62, 66, 67]. Additionally, XBP1 interacts with FoxO1 by delivering it to the proteasome for degradation and leading to the activation of gluconeogenic genes [68]. Another interesting study found that glucagon signaling phosphorylated IRE1 at serine-724. Attenuation of IRE1 reduced glucagon-induced expression of gluconeogenic genes thereby reducing hyperglycemia and glucose intolerance [69]. This provides evidence that IRE1 is able to be phosphorylated by a kinase not just autophosphorylation of itself. Lastly, IRE1 has been implicated to play a role in lipid metabolism. Knockdown of IRE1 in hepatocytes have down-regulation of many genes involve in lipid metabolism [70]

RESULTS

KGN cells were grown in glass coverslips and treated with tunicamycin (Tm) for 4 hours or forskolin for 24 hours and immunocytochemistry was performed. Cells were immunostained with anti-IRE1 (red) and co-stained with anti-Nup98 antibodies (green). In cells treated with Tm, IRE1 forms large oligomeric punctae in the extranuclear space (**Figure 6A**). These punctae are in similar size and appearance to the punctae that are constitutively present on the nuclear membrane (**Figure 4**). On the other hand, when cells are treated with forskolin, IRE1 does not form punctae in the extranuclear space and instead only retain the nuclear associated IRE1 oligomers (**Figure 6B**). This data provides visual evidence that the ER stress response is intact and inducible in KGN cells, however steroidogenesis does not activate a stress response.

IRE1 endoribonuclease activity is responsible for splicing XBP1u to the active form, XBP1s in response to ER stress [42, 51, 55]. This mechanism produces a functional transcription factor in XBP1s that is responsible for up-regulating many genes involved in the amelioration of ER stress. In addition to this canonical mechanism for the endoribonuclease domain, new evidence shows that the IRE1 RNase activity is involved in non-ER stress associated functions. IRE1 RNase activity has been shown to cleave select mRNAs including degradation of insulin mRNA [71, 72], and microRNA [43]. Recent work by MannKind Corporation has developed two specific IRE1 endoribonuclease inhibitors, MKC-3946 and MKC-4485 [73]. Their work shows that these inhibitors effectively block XBP1 splicing in response to ER stress. In order to determine if IRE1 endoribonuclease activity is required for CYP11A1 expression, KGN cells were treated either basally, or with forskolin for 24 hours or tunicamycin for 4 hours. Cells were co-treated with the IRE1 endoribonuclease specific inhibitors, MKC-3946 and MKC-4485. RNA was harvested and semi-quantitative PCR and real-time PCR were performed. Tunicamycin treatment induced XBP1 splicing, but in cells co-

treated with either inhibitor XBP1 splicing was blocked (**Figure 7A**). CYP11A1 expression was quantified using real-time PCR. Treatment with either inhibitor did not alter CYP11A1 up-regulation in response to forskolin (**Figure 7B**).

IRE1 is constitutively bound to Xbp1u and splices Xbp1u to produce the active transcription factor Xbp1s. Several reports have shown that Xbp1u is involved in non-UPR associated functions. Xbp1u has been shown to suppress autophagy by binding FoxO and delivering it to the proteasome [68, 74] and thereby improves glucose homeostasis independent of ER stress. Since knockdown of IRE1 may result in the loss of XBP1u due to the lack of its primary binding partner, Xbp1u may be involved in the regulation of CYP11A1. In order to determine if Xbp1 plays an important role in CYP11A1 expression, KGN cells were transfected with Xbp1 siRNA and treated with forskolin, tunicamycin or basally. Knock-down of Xbp1 (**Figure 7C**) did not alter CYP11A1 up-regulation in response to forskolin (**Figure 7D**). Thus, neither Xbp1u, Xbp1s, or IRE1 endoribonuclease activity are important components in CYP11A1 expression in response to forskolin. Therefore, IRE1 is mediating CYP11A1 transcription independent of its known function in ER stress.

CONCLUSIONS

Steroid biosynthesis requires a large amount of ER resident enzymes to be quickly up-regulated in response to gonadotropin stimulation. Thus the ER must be able to sense these signals, and be able to rapidly prep the ER for this rapid increase in enzymatic activity. Gonadotropin stimulation with forskolin treatment for 24 hours does not induce an overt ER stress response, although the ER stress machinery is present and inducible with ER stress inducers tunicamycin and thapsigargin (**Figure 5A**). IRE1 is constitutively present on the nuclear membrane in large punctate oligomers (**Figure 4A**) and oligomerization is induced in the ER with tunicamycin treatment (**Figure 6A**) but not

forskolin treatment (**Figure 6B**). This interesting cellular localization pattern was corroborated by a distinct biochemical method, subcellular fractionation, where IRE1 was localized in both the cytoplasmic and nuclear membrane fractions (**Figure 5B**). The differential localization pattern observed may provide evidence that IRE1 functions in distinct pathways, either as an ER stress inducer in the ER, and possible as a kinase or kinase intermediary for up-regulation of CYP11A1.

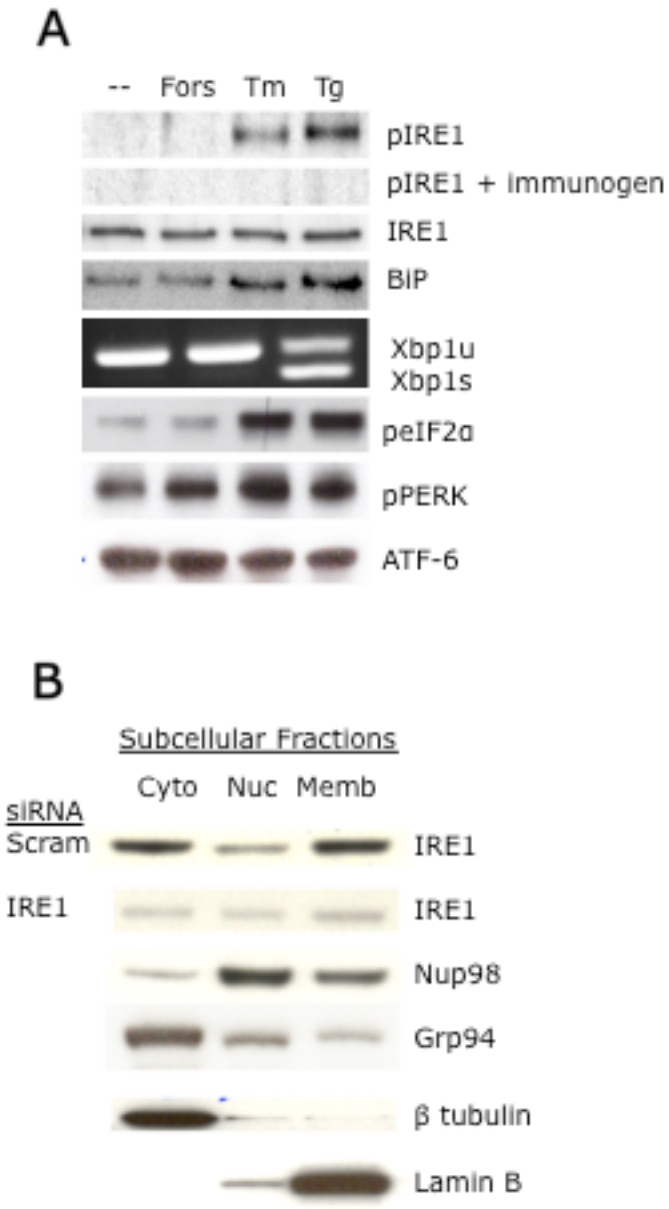
IRE1 endoribonuclease activity is responsible for the unconventional mRNA splicing of Xbp1u to Xbp1s, a potent transcription factor that up-regulates UPR genes. Additionally, IRE1 is able to recognize a stem loop consensus element in other mRNAs and miRNAs [75, 76] and cleave these mRNAs in a mechanism termed regulated IRE1-dependent decay (RIDD) [72]. In order to determine whether IRE1 RNase activity is necessary for CYP11A1 up-regulation in response to forskolin, we utilized an IRE1 RNase specific inhibitors to show that inhibition of IRE1 RNase activity (**Figure 7A**) does not alter CYP11A1 up-regulation in response to forskolin stimulation (**Figure 7B**). Recent studies have shown that XBP1 is able to activate some genes outside of UPR genes in a cell type specific manner even in unstressed or basal level conditions. Knock-down of XBP1 by RNA interference (**Figure 7C**) did not alter CYP11A1 up-regulation in response to forskolin (**Figure 7D**). These data show that gonadotropin stimulation in granulosa cells does not induce an ER stress response, although the ER stress machinery is inducible in KGN cells. IRE1 mediates CYP11A1 transcription in a pathway that is distinct from its canonical signaling pathway in the unfolded protein response.

Table 3: Proteins reported to associate with IRE1

Associated Protein	Function	Reference
BiP	Binds inactive IRE1 and dissociates upon ER stress; protein chaperone	[35, 36, 77]
TRAF-2	Binds active Ire1 with prolonged ER stress	[62, 65]
AIP1	Binds active Ire1; pro-apoptotic	[41, 47]
Bax/Bak	Binds active Ire1; pro-apoptotic	[47, 78]
BI-1	Binds inactive IRE1 and inhibits activation and XBP1 splicing	[79]
Nck	ERK activated adaptor protein	[55, 80]
DERLIN-1,-3 SEL-1, HRD-1	ERAD components bound to inactive IRE1	[71]
USP-14	Ubiquitin specific protease; binds inactive IRE1	[71]
HSP90	Binds to IRE1 and protects it from degradation	[81]
ASK1		[63]
RACK1	Recruits PP2Ato dephosphorylate IRE1 to reduce glucose activated IRE1	[82]
PSF, P54nrb	Binds IRE1, inhibits CYP11A1 transcription	Unpublished data

Proteins that have been reported to associate with IRE1 and their reported function.

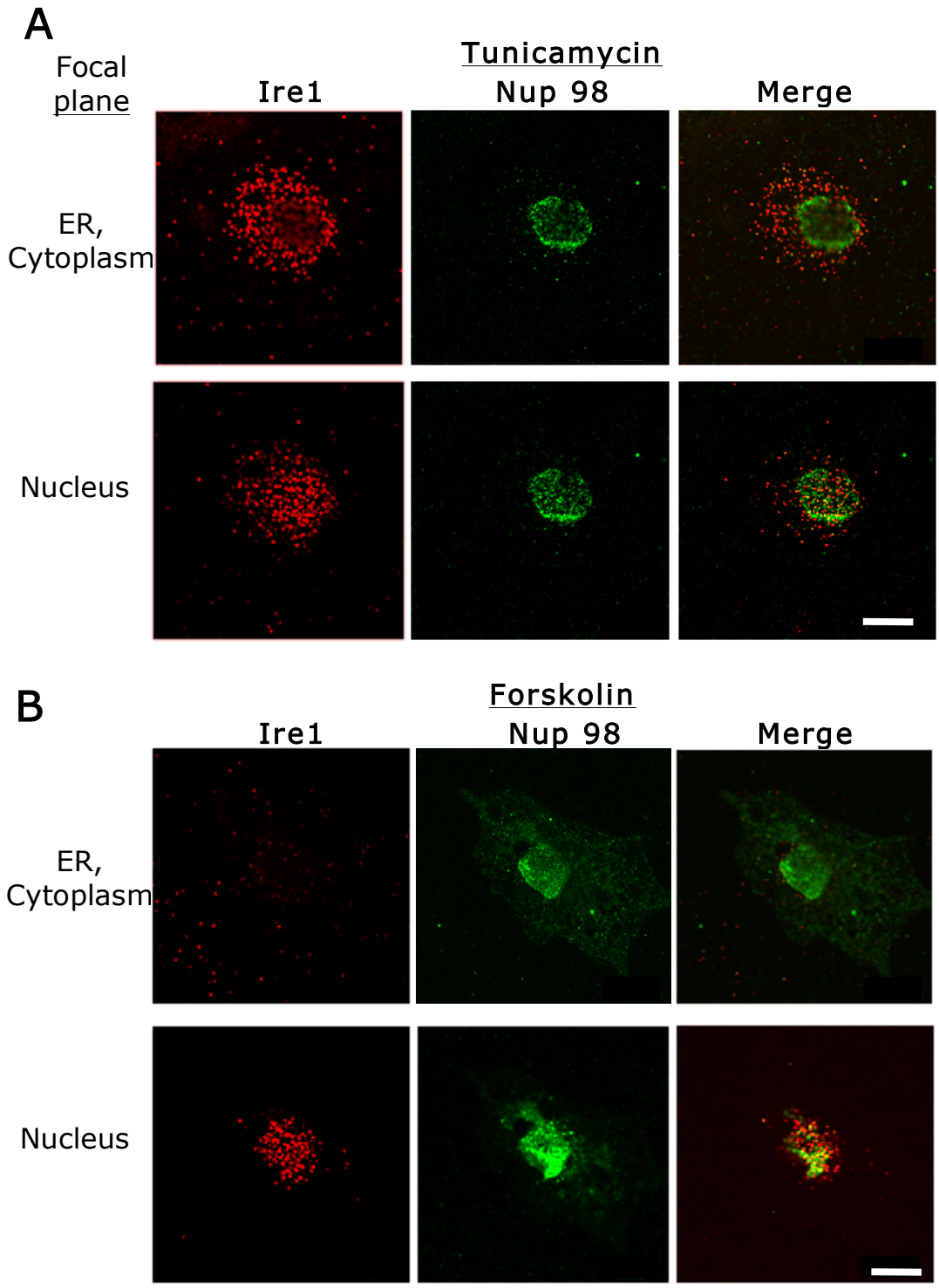
Figure 5: IRE1 is involved in steroid biosynthesis independent of its role in ER stress



KGN cells were treated with forskolin (1 μM for 24 hours) or ER stress inducers tunicamycin (Tm, 1 ng/ml for 4 hr) or thapsigargin (Tg, 1 μM for 4 hours). (**A**) Immunoblots were probed with antibodies for phospho-IRE1, total IRE1, BiP, phospho-eIF2α, phospho-PERK, and ATF-6. XBP1 splicing was determined by semi-quantitative PCR. IRE1 is localized in both the nuclear membrane and the endoplasmic reticulum. (**B**)

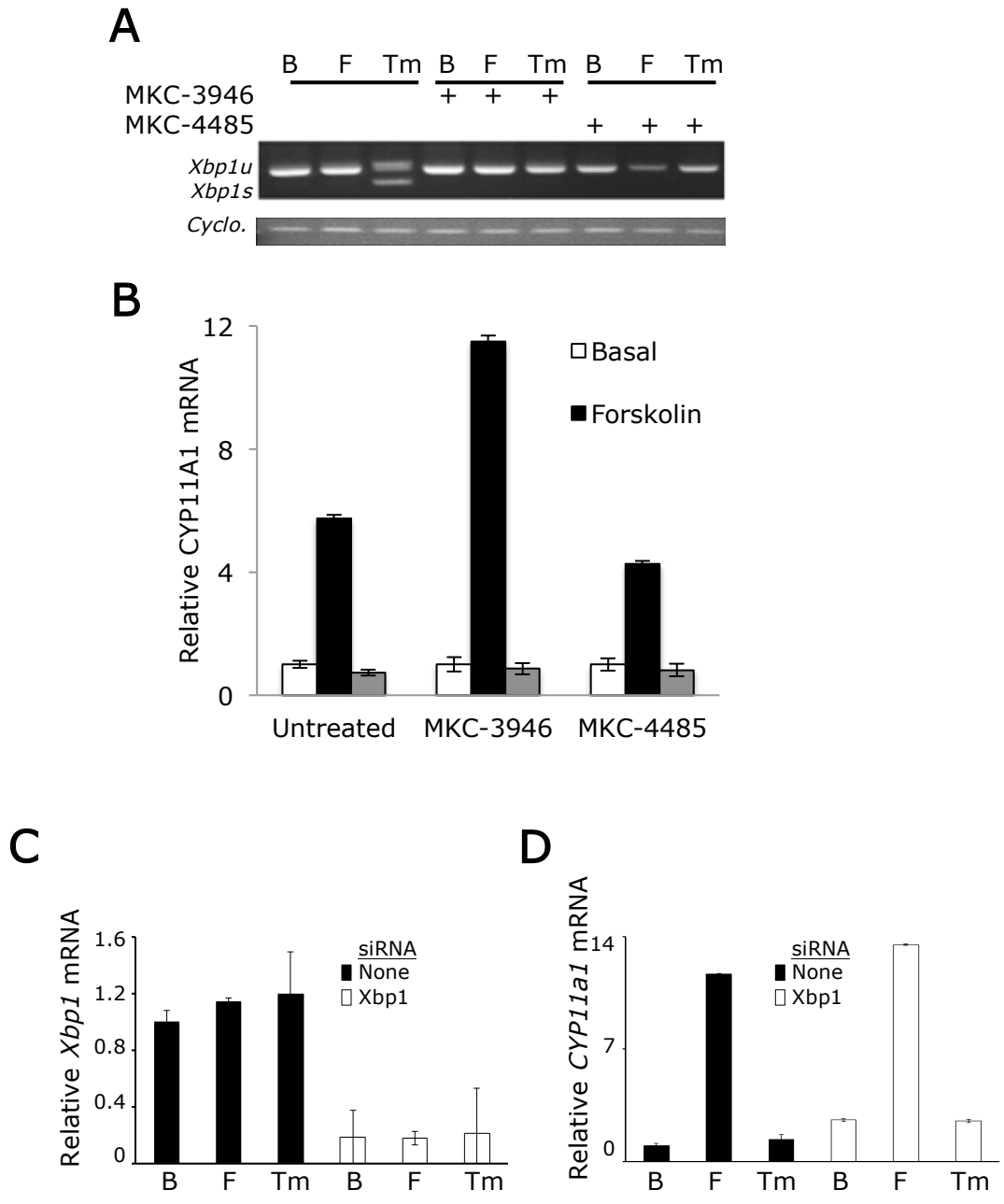
KGN cells were cultured under basal conditions or transfected with IRE1 siRNA and differential centrifugation performed.

Figure 6: IRE1 forms large punctate oligomers in the endoplasmic reticulum during ER stress conditions



ER stress but not steroid biosynthesis induces the formation of large, punctate oligomers in the cytoplasm and endoplasmic reticulum. KGN cells were cultured on glass coverslips for 24 hours and (**A**) treated with the ER stress inducer tunicamycin (Tm, 1 ng/ml for 4 hr) or (**B**) forskolin (1 μM for 24 hours). Cells were fixed, permeablized and immunostained with rabbit anti-IRE1 and goat anti-rabbit AlexaFluor 594 and co-stained with rat anti-Nup98 and goat anti-rat AlexaFluor 488. Scale bar, 10 μm.

Figure 7: IRE1 endoribonuclease activity and XBP1 are not necessary for steroid biosynthesis



IRE1 endoribonuclease activity and XBP1 are not necessary for CYP11A1 expression in KGN cells. (**A**) KGN cells were treated with forskolin (F, 1 μM for 24 hours), the ER

stress inducer tunicamycin (Tm, 1 ng/ml for 4 hr) or untreated (B for 24 hours) and co-treated with the IRE1 endoribonuclease specific inhibitors MKC-3946 or MKC-4485 (10 μM for 4 hours). XBP1 expression was determined by semi-quantitative PCR and (**B**) CYP11A1 expression was determined by real-time PCR. XBP1 expression is not required for steroid biosynthesis. (**C, D)** KGN cells were reverse transfected with XBP1 siRNA for 48 hours and treated with forskolin for 24 hours, tunicamycin for 4 hours or basally. (**C**) Xbp1 and (**D**) CYP11A1 mRNA expression was determined by real-time PCR.

Chapter 5 Concluding Remarks

IRE1 plays a critical role in the endoplasmic reticulum to respond to and ameliorate ER stress. Recent studies suggest that IRE1 is able to function well beyond its canonical ER stress signaling pathway, and take on more diverse functions in regulating metabolism and glucose homeostasis. Indeed, the proteins that interact with the IRE1 'interactome' may be cell-type specific enabling IRE1 to maintain balance in a cell-type specific manner. The results of this study suggest that IRE1 is able to play a key role in sex steroid biosynthesis, by a novel mechanism not previously attributed to IRE1.

IRE1: Master Regulator of Fertility

IRE1 is necessary to mediate CYP11A1 expression (**Figures 1 and 2**). Loss of IRE1 by RNA interference potently inhibits CYP11A1 in human granulosa cells, as well as in mouse adrenal cells and human placenta cells (**Figures 2 and 3**). Without CYP11A1, pregnenolone and progesterone production are inhibited, and cells are unable to respond to the external stimulus. One of the possible mechanisms is that IRE1 may be an important signaling intermediary between the gonadotropin signaling axis and the CYP11A1 promotor. Since the majority of steroidogenic enzymes are resident ER proteins, IRE1 may be able to act as an important checkpoint to determine if the endoplasmic reticulum is in a healthy state. This study shows that granulosa cells, under basal conditions or with forskolin treatment, are not undergoing ER stress (**Figure 5, 6, and 7**) although the ER stress program is inducible. IRE1 is able to relay an unknown signal to initiate the sex steroid program. The signal may be either to relieve repression of CYP11A1, or to activate a transcription factor. ER stress signals from within the ER induce IRE1 oligomerization and a conformational change to activate the RNase domain.

This conformational change may prevent IRE1 from acting as an independent kinase, as in unstressed conditions, or respond to signals from the cytoplasm. It will be important to determine how stress affects gonadotropin stimulation. Unstressed conditions may be able to mediate an alternative IRE1 transcriptional program such as the sex steroid program.

In PCOS, there is an underlying metabolic dysfunction. PCOS women have a high incidence of diabetes mellitus, metabolic syndrome, which includes dyslipidemia, obesity, hypertension, and cardiovascular disease. Many of these disorders involve chronic low-grade inflammation. Inflammation is able to activate IRE1 independent of ER stress, and activate an alternate inflammation transcriptional program. This alternate transcriptional program may interfere with normal physiological responses to gonadotropins.

An alternative mechanism may involve a divergent IRE1 mechanism dependent on its cellular localization. IRE1 is constitutively present on or in close association with the nuclear membrane in large, punctate oligomers (**Figures 4 and 6**). IRE1 has been shown in unpublished data in our lab to co-immunoprecipitate with a potent inhibitor of CYP11A1, ptb-associated splicing factor (PSF) and its binding partner p54nrb. PSF is a nuclear protein that has been shown to bind the IGFRE of the CYP11A1 promoter and repress CYP11A1 expression. IRE1 present on nuclear membrane may be able to associate directly with the CYP11A1 transcriptional machinery making IRE1 a critical scaffold for the rapid up-regulation of CYP11A1 in response to gonadotropins. In this scenario, only the IRE1 on the nuclear membrane is critical for CYP11A1 transcriptional activation, whereas IRE1 oligomers in the endoplasmic reticulum function only in response to ER stress and perturbations in ER homeostasis. It would be important to determine if the proteins that interact with IRE1 at the nuclear membrane are distinct from the proteins that interact with IRE1 in the endoplasmic reticulum.

In conclusion, IRE1 has been shown to play a critical role CYP11A1 transcription. The role of IRE1 in fertility provides a powerful new mechanism for the control and regulation of fertility, and a possible target for the study of disorders affecting female fertility, such as obesity, metabolic syndrome, diabetes and PCOS.

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Vita

Rebekah Viner was born on September 19th, 1980 in Texas City, TX. Her father is Leslie Nolan, Jr. and her mother is Janice Nolan. She attended Texas City High School and graduated in May 1999 with honors. She attended Sam Houston State University where she studied finance and biology. She was on both the Dean's List of Academic Honors, and President's List of Academic Honors, a member of several honor societies and the Chi Omega fraternity. She was lab instructor for both the department of geography and the department of biological sciences. She graduated in May 2002 with a Bachelor of Business Administration and again in December 2004 with a Bachelor of Science. She obtained a position with Sigma Aldrich in The Woodlands, Texas as a quality control chemist. She entered the Graduate School of Biomedical Sciences at the University of Texas Medical Branch in 2009, and joined the laboratory of Dr. Larry Denner. During her graduate career she served as secretary of the Society of Cell Biology and joined the Endocrine society as trainee member. She married Tom Viner in October 2011, and is a proud mother of three beautiful children, Drew, 9, Caleb, 7, and Evie, 1 year.

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