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ORIGINAL PAPER

IRE1α controls cyclin A1 expression and promotes cell proliferation through XBP-1

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Abstract IRE1 is a conserved dual endoribonuclease/protein kinase that is indispensable for directing the endoplasmic reticulum (ER) stress response in yeast, flies, and worms. In mammalian systems, however, the precise biological activities carried out by IRE1α are unclear. Here, molecular and chemical genetic approaches were used to control IRE1 activity in a number of prostate cancer cell lines and the resulting impact on gene transcription, cell survival, and proliferation was examined. Modulating IRE1α activity had no transcriptional effect on the induction of genes classically associated with the ER stress response (*Grp78* and *CHOP*) or cell survival when confronted with ER stress agents. Rather, IRE1α activity was positively correlated to proliferation. Since *Xbp-1* mRNA is the sole known substrate for IRE1 endoribonuclease activity, the role of this transcription factor in mediating proliferation was examined. Repressing total *Xbp-1* levels by siRNA techniques effectively slowed proliferation. In an effort to identify IRE1/XBP-1 targets responsible for the cell cycle response, genome-wide differential mRNA expression analysis was performed. Consistent with its ability to sense ER stress, IRE1α induction led to an enrichment of ER-Golgi, plasma membrane, and secretory gene products. An increase in *cyclin A1* expression was the only differentially expressed cell cycle regulatory gene found. Greater cyclin A protein levels were consistently observed in cells with active IRE1α and were dependent on XBP-1. We conclude that IRE1α activity controls a subset of the ER stress response and mediates proliferation through tight control of *Xbp-1* splicing.

Keywords ER stress · Proliferation · Cyclin A · IRE1 · XBP-1

Introduction

The endoplasmic reticulum (ER) possesses a unique capacity to sense and respond to various stress stimuli. The ER stress response (ESR) can be triggered by the accumulation of excessive or mis-folded protein, deprivation of glucose, calcium fluctuations, and numerous other cellular imbalances (Kaufman 1999; Mori 2000; Schroder and Kaufman 2005). The ESR attempts to restore homeostasis through a set of three primary mechanisms, together termed the unfolded protein response (UPR). First, the UPR can call for the production of additional chaperone proteins to accelerate protein folding (Rutkowski and Kaufman 2004). Second, activating proteasome-mediated degradation through a reverse-translocation process, referred to as ER-associated degradation, serves to reduce the protein load (Hampton 2002; Jarosch et al. 2003; Plemper and Wolf 1999). The third strategy is to reduce protein translation (Brostrom and Brostrom 1998; Harding et al. 2002). These UPR activities normally succeed in restoring ER homeostasis and ensure cellular survival. However, if the stress burden becomes great enough, then cell death can result (Breckenridge et al. 2003; Ferri and Kroemer 2001; Rao et al. 2004).

The genetic elements controlling the ESR have been best characterized in lower eukaryotes and, more recently, the

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ER-specific pathways responsible for metazoan stress responses have been investigated. In *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans*, the ESR is mediated exclusively by IRE1 (Cox et al. 1993; Hollien and Weissman 2006; Mori et al. 1993; Shen et al. 2005), an ER-resident transmembrane dual endoribonuclease/kinase. The amino-terminal luminal region of IRE1 is responsible for sensing various ER stress stimuli. When properly induced, this signal causes dimerization, a subsequent conformational shift, autophosphorylation, and activation of the endoribonuclease activity (Shamu and Walter 1996; Welihinda and Kaufman 1996). The mRNA of the transcription factor *Xbp-1* is the only known substrate for mammalian IRE1 activity (Niwa et al. 2005; Yoshida et al. 2001). *Xbp-1* is processed through a unique unconventional endoribonuclease activity to remove 26 nucleotides within the coding region. This cleavage generates an alternative reading frame to be utilized. Translation of unspliced *Xbp-1* results in a protein (XBP-1^{U}) that contains a DNA-binding domain, while translation of spliced *Xbp-1* (XBP-1^{S}) generates a C-terminal transcriptional activation domain coded in the alternate reading frame adjacent to the DNA-binding region. XBP-1^{S} has been shown to activate transcription (Yoshida et al. 2001). Although XBP-1^{U} does not possess a functional transcriptional activation domain, this form has been proposed to regulate some biological activities (Tirosh et al. 2006; Yoshida et al. 2006).

An additional level of complexity arises in metazoan systems, as two other ER stress-sensing proteins are present that include the kinase PERK and transcription factor ATF6. Conflicting studies exist regarding how these players interact to regulate the mammalian ESR. A recent study found that IRE1 could modulate cell survival induced by the ER stress agents thapsigargin and tunicamycin (Lin et al. 2007). In contrast, using ATF6α null mouse embryonic fibroblasts (MEFs), ATF6α transcriptional induction was required for survival and for the expression of ER chaperones when confronted with ER stress (Wu et al. 2007; Yamamoto et al. 2007). Here, we engineered human epithelial cells to express human IRE1α constructs that either interfere with or induce the endoribonuclease activity in an effort to understand the role of IRE1 activity in mediating various facets of the ESR. We could find no role for this gene in mediating cell survival or initiating the ESR, but found that IRE1 and XBP-1 could regulate cell proliferation. Furthermore, by activating IRE1 it was possible to reproduce a physiologic change in *Xbp-1* splicing and globally assess the resulting differentially expressed genes using human exon microarrays. These results identified *cyclin A1* as an IRE1-dependent target. Together, these data suggest that IRE1 alone does not initiate a full ESR or confer survival in response to ER stress stimuli, but rather controls cyclin A levels and a subset of ER-resident gene products responsible for protein chaperone activity, detoxification, lipid synthesis, transcription, and anti-viral defenses.

Materials and methods

Cell culture

Human prostate cancer cell lines (LNCaP, DU145, and PC-3) and the Phoenix Amphotrophic retroviral packaging cell line were obtained from ATCC (Manassas, VA, USA). Prostate cancer cells were grown in low glucose (1.0 g/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Phoenix cells were grown in DMEM containing non-essential amino acids and 10% FBS. Velcade (Millenium Pharmaceuticals, Cambridge, MA, USA) was provided by the University of Kentucky Markey Cancer Center Pharmacy. Tunicamycin and thapsigargin were purchased from Sigma (St. Louis, MO, USA). MG132 and 1NM-PP1 were from EMD Biosciences (San Diego, CA, USA).

Cloning, mutagenesis, and mammalian protein expression

IRE1α (GenBank accession number NM_001433) was polymerase chain reaction (PCR) amplified from normal human prostate cDNA. Site-directed point mutations to IRE1α (K599A and I642G) were created by an overlapping PCR-based method and verified by sequence analysis. Stable cell lines were created by retroviral transduction as described (Schwarze et al. 2003).

Viability and proliferation assays

Cell viability was determined with cells (70,000/well) plated in a 24-well dish using a standard colorimetric MTT assay as described (Thorpe et al. 2008). Cell proliferation was determined by 5-Bromo-2-deoxy-uridine (BrdU) labeling. Cells were plated (500,000) in 6-cm dishes. The following day, the media were changed to contain 2.5% serum. BrdU labeling and flow cytometric analysis was carried out as previously described (Schwarze et al. 2003). The percentage of BrdU positive cells (10,000 gated events) and the cell fraction in G0/1 and G2/M determined using CellQuest software (B-D). All assays were carried out in replicates of three. All statistical assessments were made using a Student's *t* test.

Xbp-1 splicing analysis

Total RNA was purified using the RNeasy purification kit (Qiagen). cDNA was generated with SuperScript II

Reverse Transcriptase (Invitrogen). A PCR-based method was employed to quantify the status of *Xbp-1* splicing (Shang 2005). The primers were designed to span the 26-nucleotide long excision characteristic of $Xbp\text{-}1^S$. The PCR products were resolved by electrophoresis either through a 2% agarose gel in a Tris–acetate–EDTA (TAE) buffer, or through a 4% polyacrylamide gel in TAE. DNA was visualized by ethidium bromide staining and a digital imaged captured (Ultra-Violet Products, Upland, CA, USA). The percent of unspliced and spliced *Xbp-1* transcript was quantified using ImageJ software.

siRNA-mediated Xbp-1 repression

An XBP-1 and non-targeting SMARTpool siRNA were purchased (Dharmacon, Lafayette, CO, USA). Cells were transfected with DharmaFECT (Dharmacon) in media containing 10% serum as described by the manufacturer. The next day, cells were fed with DMEM containing 10% serum or harvested to examine mRNA levels.

Real-time quantitative PCR

Total RNA was isolated using the RNeasy RNA isolation kit and cDNA was prepared. Quantitative RT-PCR was performed by monitoring the SYBR Green fluorescence using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as described (Morrison et al. 1998; Wittwer et al. 1997). Values were normalized to the relative amounts of 18S or cyclophilin B RNA. Primer sequences are available upon request.

Western blot analysis

Cell lysates were prepared and Western blot analysis carried out as described (Peterson et al. 2006). Antibodies were obtained from the following sources: IRE1a, 14C10, and cyclin A, BF683 (Cell Signaling Technology); GRP78, H-129 (Santa Cruz Biotechnology); GAPDH (Mab374), Millipore; and ß-actin, AC-74 (Sigma).

Microarray analysis

PC-3 cells (2.5×10^6) overexpressing wild-type IRE1 or I642G IRE1 were plated in quadruplicate and treated for 12 h with 5 μM 1NM-PP1. Total mRNA was isolated (Qiagen) and the mRNA labeled according to the manufacturer's directions (GeneChip® Whole Transcript (WT) Sense Target Labeling Assay, Affymetrix, Santa Clara, CA, USA). Labeled mRNA was hybridized to GeneChip® Human Exon 1.0 ST Arrays (Affymetrix). The University of Kentucky Microarray Core performed the labeling and hybridization. Statistical analysis of variance (ANOVA) between groups was carried out with Partek Genomic Suites 6.4 Software (St. Louis, MO, USA).

Results

ESR induction in prostate epithelial cells

Our overriding goal was to examine the effect of IRE1 activity on the ER stress response using prostate epithelial

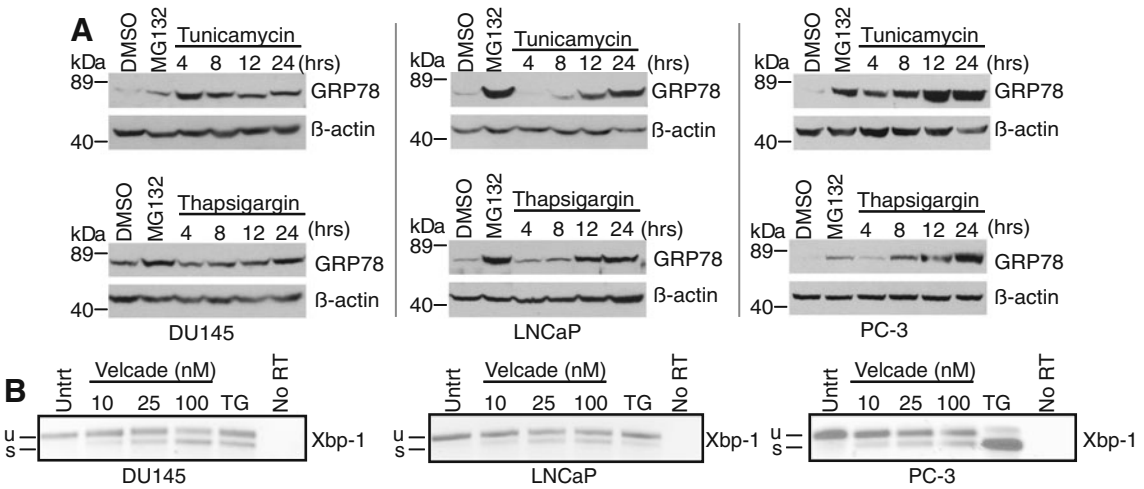


Fig. 1 Induction of the ER stress response in human prostate epithelial cells. **a** DU145, LNCaP, and PC-3 cells were exposed to MG132 (1 μM) for 24 h or tunicamycin (2 μg/ml) or thapsigargin (300 nM) for the indicated times and Western blot analysis was performed to detect GRP78 and ß-actin. **b** Cell lines were exposed to thapsigargin (300 nM) or the indicated concentrations of Velcade for 24 h. Total RNA was isolated and cDNA examined by RT-PCR and agarose gel electrophoresis to discern the *Xbp-1* splicing status. *u* unspliced, *s* spliced

cells as a model. First, the competency of several prostate cancer cell lines to engage the ER stress machinery was tested. LNCaP, DU145, and PC-3 cells were exposed to proteasome inhibitors (MG132 and Velcade), thapsigargin, and tunicamycin at concentrations known to induce the ESR. Cells were then examined for GRP78 induction, a hallmark of cells responding to ER stress, by Western blot analysis. All cell lines consistently increased steady-state GRP78 upon treatment with each agent (Fig. 1a). Cells were also examined for the induction of IRE1 activity by measuring the status of *Xbp-1* splicing. All cell lines demonstrated a dose-dependent increase in *Xbp-1*^{S} with Velcade and thapsigargin administration (Fig. 1b). These studies indicate that the LNCaP, DU145, and PC-3 cell lines possess the ability to mount an ESR.

Blocking IRE1 activity impairs proliferation

The introduction of a mutated (K599A) kinase-defective IRE1α (IRE1) construct (Tirasophon et al. 2000) has been frequently used as a tool to diminish IRE1 activity and to block *Xbp-1* splicing (Drogat et al. 2007; Hu et al. 2006; Kaneko et al. 2002). Here, K599A IRE1 was stably introduced into the PC-3 cell line by retroviral-mediated gene transduction. A polyclonal population was selected and found to readily overexpress the K599A IRE1 protein (Fig. 2a) and impair *Xbp-1* splicing in response to tunicamycin and thapsigargin (Fig. 2b). First, the ability of the kinase-defective IRE1 to modulate the expression of two UPR-dependent hallmark genes upon ER stress was examined. Although Velcade and thapsigargin elevated *CHOP* and *Grp78* expression, no significant change between the control PC-3 cells and the kinase-defective IRE1 was noted (Fig. 2c). The ability of IRE1-defective cells to modulate survival in response to ER stress agents was also studied. PC-3 cells were treated with Velcade or thapsigargin and cell viability was assayed. Both agents reduced viability, but no difference between PC-3 puro and the K599A IRE1 cells was noted (Fig. 2d). As a control, the effect of TRAIL, a cell death ligand that acts through a distinct receptor-mediated process, was also tested. Once again, no difference in cell viability between the two cell lines could be demonstrated (Fig. 2d).

Although no effect on the aforementioned ER stress-related parameters was noted, it was readily apparent that the K599A IRE1 overexpressing cells were dividing slower. Therefore, cell proliferation was assessed by quantifying BrdU incorporation into DNA. PC-3 cells expressing the kinase-defective IRE1 were found to proliferate 36% slower than the PC-3 puro control cells (Fig. 2e). LNCaP cells were also engineered to stably express K599A IRE1 (Supplementary material S1a) and also were observed to have a decrease in the rate of proliferation (Fig. 2e). We attempted to express K599A IRE1 in DU145 cells, but only obtained a twofold overexpression and noted no effect on *Xbp-1* splicing or BrdU labeling (Supplementary material S1b). These data suggest IRE1 activity, and possibly basal XBP-1^S expression, may contribute to normal cell growth.

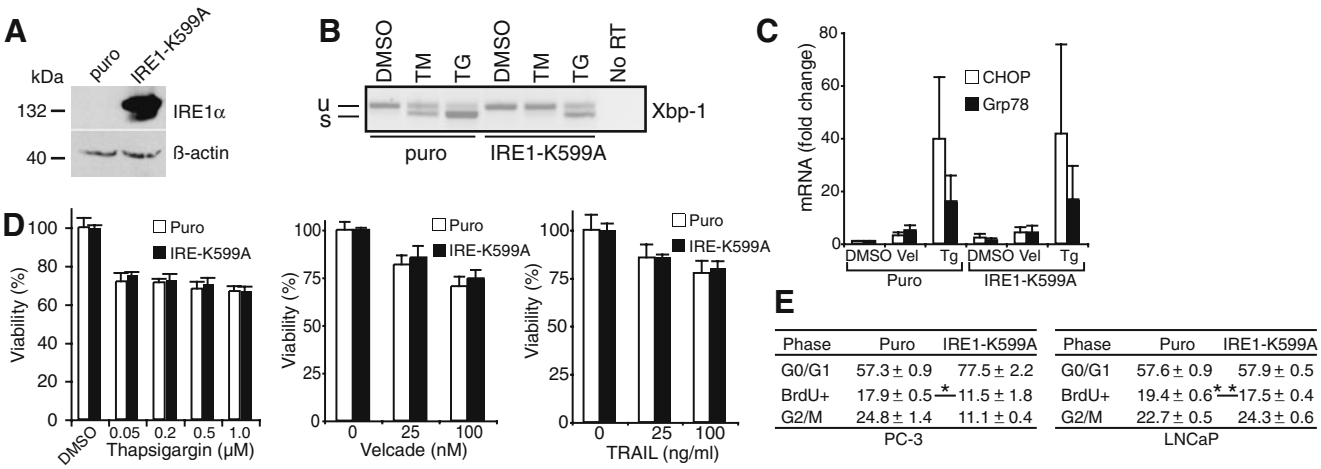


Fig. 2 Reduction in IRE1 activity slows cells proliferation. **a** PC-3 cells stably expressing kinase-defective IRE1 (K599A) or the control pBabe puro (Puro) vector were examined by Western blot analysis for the indicated proteins. **b** PC-3 puro and K599A IRE1 expressing cells were exposed to tunicamycin (2 μg/ml) and thapsigargin (300 nM) for 24 h. Cells were analyzed for the abundance of unspliced (*u*) and spliced (*s*) *Xbp-1*. **c** *CHOP* and *Grp78* mRNA expression was determined in the PC-3 cells assayed in **b** by qPCR following standardization to cyclophilin B or 18S rRNA. 18S standardization was used only for thapsigargin (Tg) as cyclophilin B was induced by this treatment. The average of three experiments±the standard deviation is shown. **d** PC-3 cells (Puro and K599A IRE1) were treated for 24 h with the indicated concentrations of thapsigargin (*left*), Velcade (*middle*), or TRAIL (*right*) and viability was determined by a colorimetric MTT assay. **e** BrdU labeling was carried out on PC-3 (*left*) and LNCaP (*right*) cells expressing the K599A IRE1 construct or vector only (Puro). The average of three replicates±the standard deviation is shown. *P=0.002; **P=0.01

IRE1 activity enhances Xbp-1 splicing and proliferation

IRE1 is a unique protein kinase as it changes conformation and becomes active upon nucleotide binding (Papa et al. 2003). By generating a point mutation (I642G) in the nucleotide binding cleft it is possible to induce the endoribonuclease activity with the addition of the bulky kinase inhibitor 1NM-PP1 (Lin et al. 2007). The 'inducible' I642G IRE1 construct was stably expressed in prostate cancer cells to examine the effect of IRE1 activation on cell survival, UPR gene induction, and proliferation. PC-3 cells readily overexpressed the I642G IRE1 construct and the addition of 1NM-PP1 conferred *Xbp-1* splicing (Fig. 3a), indicating robust endoribonuclease activity. The I642G IRE1 overexpressing cells were tested for survival in response to ER stress stimuli. PC-3 I642G IRE1 and the paired control PC-3 puro cell populations were treated with Velcade and thapsigargin in the presence or absence of 1NM-PP1. Velcade and thapsiargin treatment led to a reduction in cell viability; however, no difference between the I642G IRE1 expressing and control PC-3 puro cells was observed (Fig. 3b). The ability of IRE1 to regulate the expression of ER stress hallmark genes was also studied. IRE1 activation was unable to induce *CHOP* and *Grp78* mRNA levels (Fig. 3c), further suggesting that their expression is IRE1 independent. DU145 cells overexpressing the I642G IRE1 construct (Fig. 3d) also showed no difference in cell survival (Fig. 3e).

Since the kinase-defective IRE1 construct reduced cell proliferation, we hypothesized that the 'inducible' I642G IRE1 would have the opposite effect. The proliferation of cells expressing I642G IRE1 was tested in the presence and absence of 1NM-PP1. Surprisingly, the I642G IRE1 overexpressing PC-3 cells proliferated significantly faster even in the absence of the ATP analog (Fig. 3f). DU145 cells overexpressing I642G IRE1 in the absence of 1NM-PP1 also were proliferating faster regardless of the serum concentration (Fig. 3f). LNCaP cells stably overexpressing I642G IRE1 further confirmed these findings (Supplementary material S2).

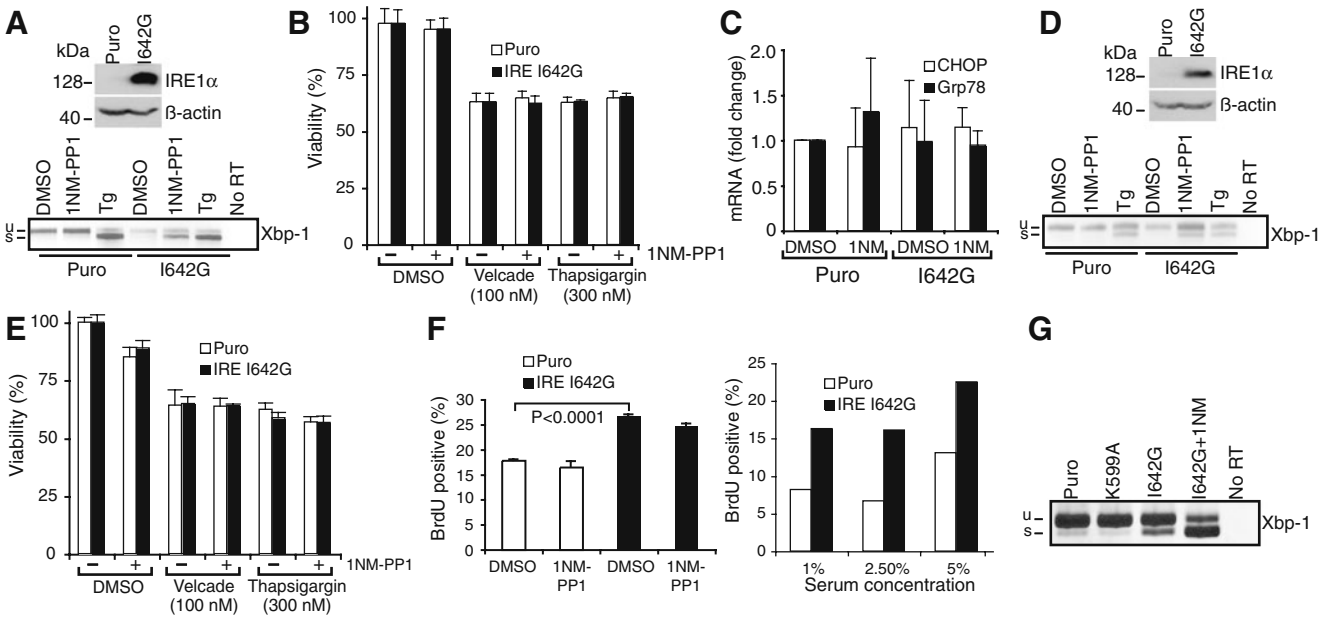


Fig. 3 IRE1 I642G confers endoribonuclease activity and induces proliferation. **a** (*top*) Control (Puro) and PC-3 cells stably expressing the I642G IRE1 construct were subjected to Western blot analysis to detect IRE1 expression. (*bottom*) Control (Puro) and PC-3 cells stably expressing the I642G IRE1 construct were treated with DMSO, 1NM-PP1 (5 μM), or thapsigargin (Tg, 300 nM) for 24 h and *Xbp-1* splicing examined. **b** PC-3 cells (Puro and IRE1-I642G) were treated with Velcade and thapsigargin in the presence or absence of 1NM-PP1 (5 μM) for 24 h and cell viability quantified with an MTT assay. Values depict the average ± the standard deviation. **c** Expression of *CHOP* and *Grp78* mRNA in PC-3 cells (Puro and IRE1-I642G) treated with DMSO or 1NM-PP1 (5 μM) for 24 h as determined by quantitative real-time PCR. Values were standardized and compared to Puro cells treated with DMSO only. *Error bars* reflect the standard deviation. **d** (*top*) Control (Puro) and DU145 cells stably expressing the I642G IRE1 construct were subjected to Western blot analysis to detect IRE1 expression. (*bottom*) Puro and DU145 cells stably expressing I642G IRE1 were treated with DMSO, 1NM-PP1 (5 μM), or thapsigargin (Tg, 300 nM) for 24 h and *Xbp-1* splicing examined. (**e**) DU145 cells (Puro and I642G IRE1) were treated and the results depicted as described in **b**. **f** BrdU labeling was carried out on PC-3 (*left*) and DU145 (*right*) cells expressing the I642G IRE1 construct or vector only (Puro). 1NM-PP1 (5 μM) was added to the PC-3 cells for 24 h. BrdU labeling in the DU145 cells as a function of serum concentration was conducted in the absence of 1NM-PP1. The average of three replicates ± the standard deviation is shown. **g** *Xbp-1* splicing status in PC-3 cells stably expressing the IRE1 K599A, or I642G, or empty pBabe Puro construct. PCR reactions were analyzed by polyacrylamide gel electrophoresis. Note, a reduction in *sXbp*-1 levels in the K599A cells and an induction of the spliced *Xbp-1* message in the I642 cells compared to the Puro control

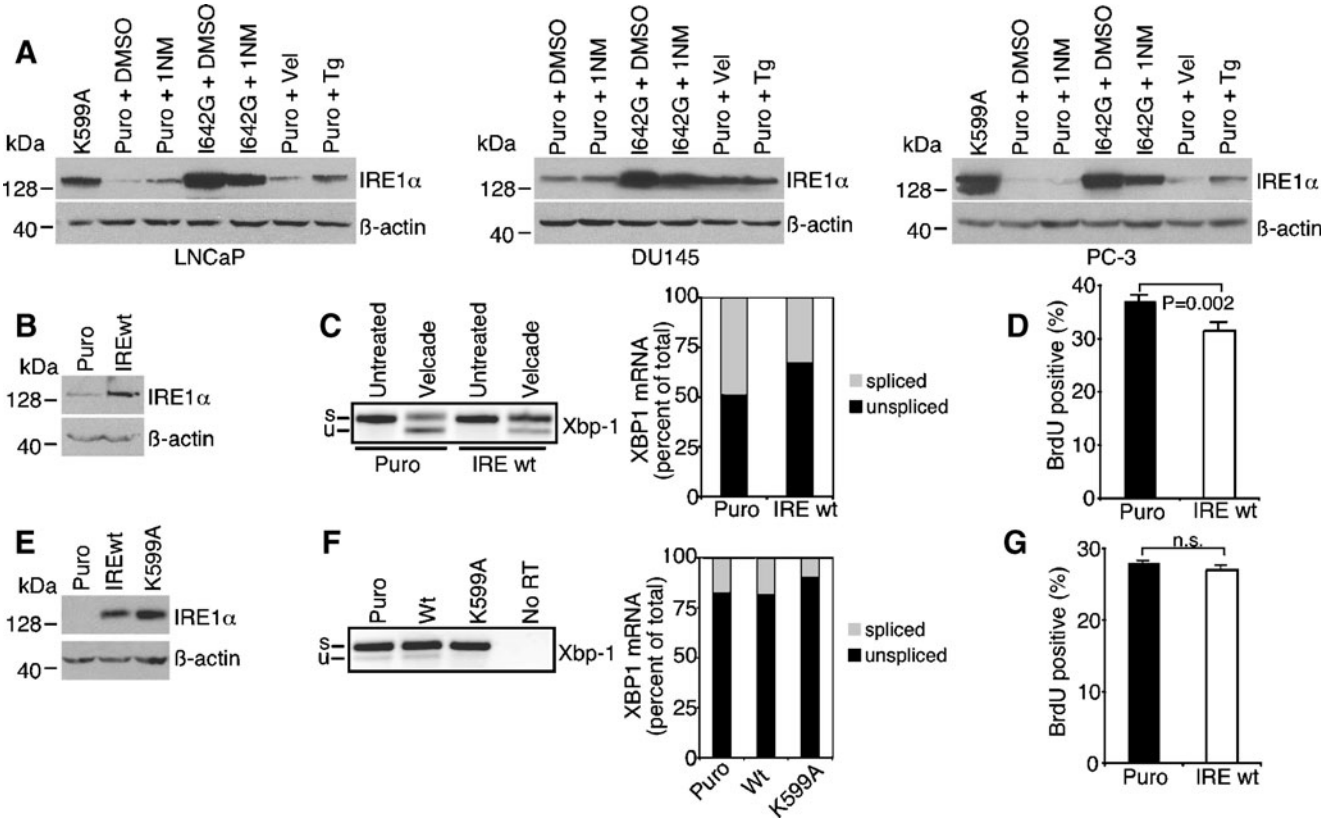
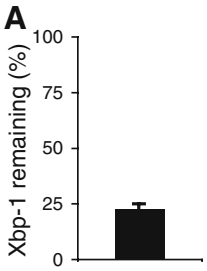


Fig. 4 Effect of wild-type IRE1 overexpression on Xbp-1 splicing and proliferation. **a** Western blot analysis was carried out on the three cell lines stably expressing the indicated constructs to detect IRE1 and ß-actin expression. Cells were treated for 24 h with 1NM-PP1 (5 μM), Velcade (100 nM) or thapsigargin (Tg, 300 nM). **b** DU145 cells stably expressing wild-type IRE1 were examined by Western blot analysis to confirm protein overexpression. **c** DU145 cells overexpressing wild-type IRE1 were exposed to Velcade for 24 h and the *Xbp-1* splicing status revealed by agarose gel electrophoresis (*left*) and quantified by NIH ImageJ software (*right*). **d** BrdU incorporation analysis was carried out on DU145 cells overexpressing wild-type IRE1 and the Puro control cells. Values reflect the average ± the standard deviation. **e** PC-3 cells stably overexpressing wild-type IRE1 were generated and protein overexpression analyzed by Western blot analysis. **f** PC-3 cells overexpressing the IRE1 constructs and control cells were exposed to Velcade for 4 h and the *Xbp-1* splicing status revealed by agarose gel electrophoresis (*left*) and quantified by NIH ImageJ software (*right*). **g** BrdU incorporation analysis was carried out on control PC-3 cells (Puro) and those overexpressing wild-type IRE1. Values reflect the average±the standard deviation

Dominant negative and active IRE1 modulate basal endoribonuclease activity

The ability of the K599A and I642G IRE1 constructs to regulate proliferation suggested that IRE1 was active in the absence of exogenous ER stress stimuli. To detect endogenous IRE1 activity, *Xbp-1* splicing PCR reactions were electrophoresed through 4% polyacrylamide gels instead of agarose gels. This method allowed the relatively low percentage of *Xbp-1*^{S} (compared to *Xbp-1*^{U}) to be resolved



B

	Puro		I642G-IRE1	
	scrambled	XBP1 siRNA	scrambled	XBP1 siRNA
G0/G1	42.2 ± 0.6	45.0 ± 0.3	40.2 ± 0.2	43.3 ± 1.1
BrdU+	30.9 ± 0.9 *	23.8 ± 0.5	37.8 ± 0.9 **	32.5 ± 0.4
G2/M	28.7 ± 0.4	29.6 ± 0.2	21.5 ± 0.8	23.4 ± 0.7

Fig. 5 Regulation of cell proliferation by spliced XBP-1. **a** Percent *Xbp-1* mRNA following siRNA-mediated knockdown in DU145 cells compared to a non-targeting control. **b** DU145 cells were transfected with siRNA to target *Xbp-1* or a scrambled control. After 48 h, cells were analyzed by BrdU labeling. Values reflect the average±the standard deviation. *P=0.0002, **P=0.0003

and revealed that the K599A IRE1 construct reduced basal *Xbp-1* splicing, while the I642G mutation yielded a significantly higher basal $Xbp\text{-}1^S$ level compared to the control cell population (Fig. 3g). Therefore, IRE1 activity does exist in cell culture in the absence of ER stress agents, the I642G IRE1 construct is somewhat leaky, and the resulting $Xbp\text{-}1^S$ levels positively correlate with the proliferation rate.

Effect of wild-type IRE1 overexpression on proliferation

It remained possible that IRE1 overexpression contributed, at least in part, to the observed proliferative changes. Furthermore, Western blot examination of PC-3, DU145, and LNCaP cells revealed an increase in steady-state IRE1 protein levels with the addition of ER stress-inducing agents (Fig. 4a), suggesting a potential feedback mechanism may exist that controls biological activities mediated by IRE1. Therefore, cell populations overexpressing wild-type IRE1 were generated (Fig. 4b, e). The wild-type IRE1 overexpressing DU145 cells showed a minor impairment of basal *Xbp-1* splicing (Fig. 4c) with a concomitant (15%) reduction in BrdU labeling (Fig. 4d). In contrast, IRE1 overexpression in PC-3 cells did not impair basal *Xbp-1* splicing or proliferation (Fig. 4f, g). These data suggest that the ability of wild-type IRE1 overexpression to alter the endoribonuclease activity is minor and cell-type specific. Furthermore, the impairment of *Xbp-1* splicing in the DU145 cells with a reduction in proliferation remains consistent with ability of IRE1 activity to regulate proliferation.

XBP-1 can regulate cell proliferation

Since *Xbp-1* is the only known ribonuclease substrate for IRE1, this transcription factor was examined for its ability to regulate proliferation. First, XBP-1 expression was targeted by siRNA transfection. DU145 cells were chosen as a cell model to carry out these studies, as they were consistently the most sensitive to changes in IRE1 activity. DU145 cells were transfected with a siRNA pool to target total (spliced and unspliced) *Xbp-1*. This reagent reduced *Xbp-1* expression by 77% (Fig. 5a). Cell proliferation examined 48-h post-transfection showed that targeting *Xbp-1* reduced proliferation by 23% in the control Puro cells, but was less effective (14% decrease) with I642G IRE1 overexpression (Fig. 5b).

Genome-wide mRNA expression analysis upon IRE1 induction

To identify IRE1/XBP-1-dependent target genes associated with proliferation and other facets of the ESR, I642G IRE1 overexpressing PC-3 cells were compared to wild-type IRE1 overexpressing cells following 12 h of 1NM-PP1 treatment. The comparison between wild-type and the inducible I642G was utilized to obtain the greatest difference in *Xbp-1* splicing (K599A IRE1 expressing cells spliced *Xbp-1* following 1NM-PP1 addition; Supplementary material S3a). The 12-h time point was selected to provide an early assessment of XBP-1-dependent gene changes based on *DNAJB9* levels (Supplementary material S3b).

Table 1 Categorical list of genes downregulated greater than 1.5-fold as determined by microarray analysis in I642G IRE1 PC-3 cells compared to wild-type IRE1 PC-3 cells in the presence of 1NM-PP1

Symbol	Genbank accession	Gene name	Cellular component	Fold change	*P* value
Enzymatic					
DGAT2	NM_032564	Acyl CoA diacylglycerol acetyl transferase	ER	−1.9	<0.001
SPTLC3	NM_018327	Serine palmitoyl transferase	Golgi mem	−1.5	0.03
Signaling					
TAS2R49	NM_176889	Taste receptor type 2, 49	Plasma mem	−2.4	0.002
LGR5	NM_003667	GPR49	Plasma mem	−1.8	0.001
OR51L1	NM_001004755	Olfactory receptor, family 51, subfamily L, 1	Plasma mem	−1.7	<0.001
CDH6	NM_004932	K-cadherin	Plasma mem	−1.6	<0.001
ODZ1	NM_014253	Odd Oz/ten-m homolog 1	Plasma mem	−1.6	<0.001
SPARC	NM_003118	Osteonectin	Secreted	−1.5	<0.001
Uncategorized					
SCN3A	NM_006922	Voltage gated Na + channel, type IIIa	Plasma mem	−2.1	<0.001
SERPINB2	NM_002575	Plasminogen activator inhibitor 2	Secreted	−1.7	0.002
SYT11	NM_152280	Synaptotagmin	Plasma mem	−1.5	<0.001
PROS1	NM_000313	Protein S	Secreted	−1.6	<0.001

Mem membrane

Hybridizations to Affymetrix Exon Arrays were carried out. After ANOVA analyses, 105 genes were found to be upregulated and 82 downregulated at $P<0.01$ following IRE1 activation. The entire datasets are available online. Those genes with expression changes greater than 1.5-fold are shown (Tables 1 and 2). The gene ontology demonstrated a strong enrichment of ER/Golgi, plasma membrane, and secreted gene products (Fig. 6a). Of note, IRE1-dependent *cyclin A1* induction was the only cell cycle regulatory gene significantly altered. Quantitative RT-PCR analysis confirmed this finding in separate samples (Fig. 6b). To further test if *cyclin A1* expression could be induced by a

Table 2 Categorical list of genes upregulated greater than 1.5-fold as determined by microarray analysis in I642G IRE1 PC-3 cells compared to wild-type IRE1 PC-3 cells in the presence of 1NM-PP1

Symbol	Genbank accession	Gene name	Cellular component	Fold change	*P* value
Adhesion					
CLDN7	NM_001307	Claudin 7	Plasma mem	2.7	<0.001
MPZL2	NM_144765	Myelin protein zero-like 2	Plasma mem	2.6	<0.001
CDH1	NM_004360	E-cadherin	Plasma mem	2.2	<0.001
ITGB6	NM_000888	Integrin ß6	Plasma mem	2.2	<0.001
LAD1	NM_005558	Ladinin-1	Secreted	2.0	<0.001
CNTN1	NM_001843	Contactin 1	Plasma mem	1.9	<0.001
TNS4	NM_032865	Tensin 4	Cytoplasm	1.6	0.004
Cytoskeleton					
MYO5B	NM_001080467	Myosin Vß	Cytoskeleton	1.9	<0.001
ACTG2	NM_001615	Actin, gamma 2	Cytoskeleton	1.5	0.009
Signaling					
TACSTD1	NM_002354	Tumor-associated calcium signal transducer 1	Plasma mem	1.8	<0.001
TSPAN2	NM_005725	Tetraspanin 2	Plasma mem	1.5	0.008
Transcription factor					
GRHL2	BC069633	Grainyhead-like 2	Nucleus	2.7	<0.001
HOXB9	NM_024017	Homeobox B9	Nucleus	1.6	<0.001
CREB3L2	NM_194071	cAMP responsive element binding protein 3-like 2	ER/Nucleus	1.5	0.004
Transport/carrier					
AP1M2	NM_005498	Adaptor related protein, complex 1, mu 2	Golgi	2.5	<0.001
CALB1	NM_004929	Calbindin	Cytosol	1.9	<0.001
TCN1	NM_001062	Transcobalamin I	Secreted	1.7	0.003
SLC9A2	NM_003048	Sodium/hydrogen exchanger 2	Membrane	1.5	0.002
Enzymatic					
CYP1B1	NM_000104	Cytochrome P450 B1	ER	3.7	<0.001
BCAT1	NM_005504	Branched chain aminotransferase 1	Cytosol	3.3	<0.001
LPCAT2	NM_017839	Lysophosphatidylcholine acyltransferase 2	ER/Golgi	2.6	<0.001
ST14	NM_021978	Membrane-type serine protease 1	Plasma mem	2.4	<0.001
TMPRSS2	NM_005656	Transmembrane protease, serine 2	Plasma mem	1.5	0.003
TINAGL1	BC064633	Tubulointerstitial nephritis antigen-like 1	Secreted	1.5	<0.001
Interferon inducible					
IFITM1	NM_003641	Interferon-induced transmembrane protein 1	Plasma mem	2.0	0.006
IFI6	NM_002038	Interferon, α-inducible protein 6	Membrane	1.8	0.002
MX1	NM_002462	Myxovirus resistance 1	Cytoplasm	1.8	<0.001
IRF6	NM_006147	Interferon regulatory factor 6	Nucleus	1.6	<0.001
OAS2	NM_002535	2'-5'-oligoadenylate synthetase 2	ER/mem	1.5	0.008
Uncategorized					
CCNA1	NM_003914	Cyclin A1	Nucleus	2.1	0.007
DNAJB9	NM_012328	Heat shock protein 40	ER	1.7	0.006

Mem membrane

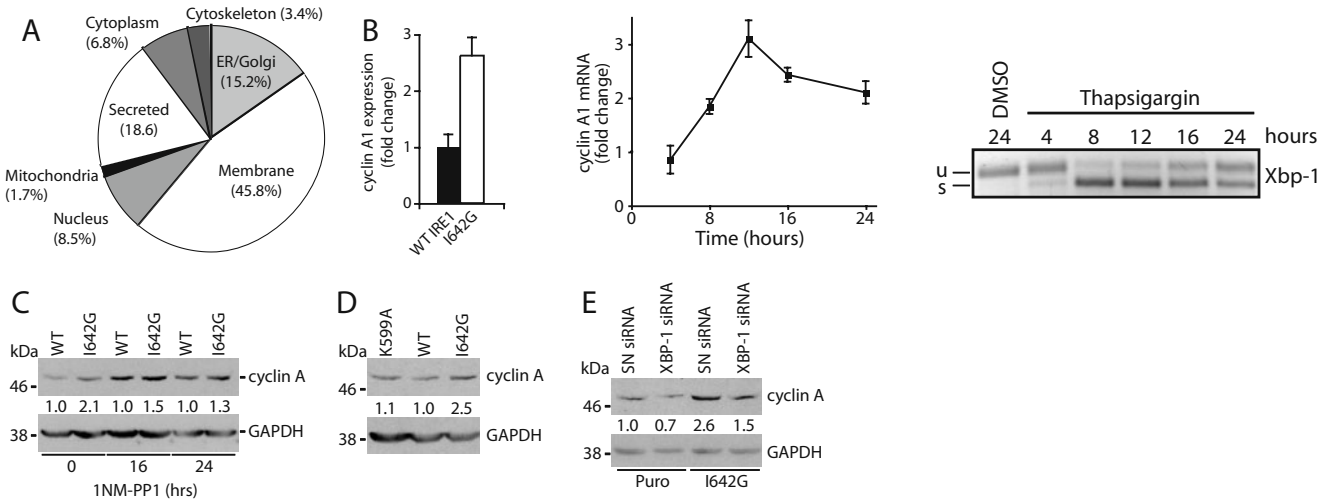


Fig. 6 Identification of *cyclin A1* as an IRE1/XBP-1-dependent gene. **a** Gene ontology demonstrating the localization of genes found to be differentially expressed in IRE1 I642G PC-3 cells compared to IRE1 wild-type cells. **b** Quantitative PCR assessment of *cyclin A1* gene expression in (*left*) I642G IRE1 PC-3 cells compared to wild-type IRE1 and (*middle*) in PC-3 cells following thapsigargin treatment compared to DMSO treated cells. *Error bars* reflect the fold change ± the standard deviation. (*right*) The Xbp-1 splicing status of the thapsigargin treated cells was assessed by PCR. **c** Cyclin A protein expression in wild-type and I642G IRE1 PC-3 cells as determined by Western blot analysis in the presence or absence of 1NM-PP1 as indicated. The *numbers* reflect the fold change in cyclin A expression compared to wild-type IRE1. **d** Cyclin A protein expression in wild-type, K599A, and I642G IRE1 PC-3 cells as determined by Western blot analysis in the absence of 1NM-PP1. The *numbers* reflect the fold change in cyclin A expression compared to wild-type IRE1. **e** DU145 cells expressing I642G IRE1 and Puro control cells were transfected for 48 h with a scrambled negative (SN) or XBP-1 siRNA and examined for cyclin A expression by Western blot analysis. The *numbers* reflect the fold change in cyclin A expression compared to the SN transfected PC-3 puro cells

second IRE1 stimulus, cells were treated with thapsigargin up to 24 h. XBP-1 splicing was observed upon thapsigargin treatment and preceded cyclin A1 induction (Fig. 6b). Cyclin A protein expression was subsequently probed in the wild-type and I642G IRE1 PC-3 cells in the presence and absence of 1NM-PP1. Consistent with the proliferative response, cyclin A protein expression was elevated in the I642G cell line, even in the absence of 1NM-PP1 (Fig. 6c). Cyclin A was also examined in cells expressing the K599A IRE1 construct; however, no difference in protein expression was observed in comparison to wild-type IRE1 (Fig. 6d). Finally, the dependence of XBP-1 expression on cyclin A levels was tested in the DU145 cell line. Consistent with the proliferative response (Fig. 5b), I642G expressing cells demonstrated higher cyclin A levels over control cells (Fig. 6e). Furthermore, *Xbp-1* repression abrogated the cyclin A induction in both control and I642G IRE1 cell lines.

Discussion

Two recent studies defining the sensory machinery responsible for mediating the ER stress response in mammalian cells have provided varying accounts on the role of IRE1 (Lin et al. 2007; Yamamoto et al. 2007). Here, evidence that IRE1 activity directs cell survival or ESR hallmark genes was not found. Instead, IRE1 activity was identified as a cell proliferation regulator. In every circumstance in which basal IRE1 activity (in the absence of ER stress agents) was impaired, cell proliferation was slowed; while elevating the basal IRE1 activity was able to stimulate cell proliferation in all three cell lines examined. Using a high-resolution polyacrylamide gel electrophoresis method, we discovered that the I642G IRE1 mutation possessed enhanced *Xbp-1* splicing activity even in the absence of 1NM-PP1, while the K599A IRE1 mutation blocked the basal *Xbp-1* splicing. Therefore, IRE1 endoribonuclease activity is present in the absence of ER stress stimuli and can promote cell proliferation. Furthermore, the use of siRNA that targeted total *Xbp-1* provided a downstream mechanistic link to the basal IRE1 activity, as those cells with reduced Xbp-1 levels were impaired in their ability to proliferate. These data are consistent with a model in which IRE1 controls an arm of the ER stress response and highlight a biological function for IRE1 activity in cell proliferation control.

The independence of IRE1 activity on the expression of hallmark ESR genes in human cells was confirmed in this study. Recently, it has been demonstrated that the transcriptional induction of *Grp78* and *Grp94* was significantly impaired in ATF6α-deficient MEFs (Wu et al. 2007; Yamamoto et al. 2007). It appears, therefore, that ATF6 is necessary to drive the expression of these genes in the ESR. However, we cannot definitely exclude IRE1/XBP-1 involvement in their transcriptional control. Based on recent findings

(Yamamoto et al. 2007), which showed dimerization between XBP-1 and ATF6, it is possible that if both IRE1 and ATF6 become activated simultaneously, then XBP-1 may participate in the transcription of additional genes unable to be modulated by IRE1 activation alone. As IRE1 and ATF6 are normally simultaneously induced upon the ESR, transcription co-regulation remains possible.

Gene expression array methodology was employed to provide insight into the mechanisms behind both cell cycle control and other IRE1-dependent functions. This screen was designed to identify genes regulated by both the basal induction of IRE1 activity and those with high IRE1 activity similar to that observed under an ESR. *Cyclin A1* was the only gene differentially expressed with a direct role in cell division. Several pieces of additional data support a role for cyclin A in mediating proliferation by IRE1. First, elevated cyclin A protein expression was observed in both the PC-3 and DU145 I642G IRE1 cell lines. Second, *Xbp-1* silencing repressed cyclin A levels and correlated with the decreased BrdU labeling index. Third, active IRE1 was associated with a decrease in the percent of cells in the G1 and G2 phase and *Xbp-1* targeting increased G1 and G2 content. This cell cycle distribution is consistent with the characterized involvement of cyclin A in both G1/S and G2/M progression (Yang et al. 1999). Although it is impossible to know the function of several non-annotated transcripts and short-sighted to exclude the possibility of annotated genes in cell cycle control, *cyclin A1* induction provides one mechanism for the observed proliferation by the IRE1/XBP-1 pathway. Importantly, we were able to use a second means of IRE1 activation, that of thapsigargin treatment, to induce *cyclin A* expression. Clearly, thapsigargin is a well-documented cytotoxic agent and has a negative effect on cell growth. However, if a situation would arise, in tumor cells for example, that could activate IRE1 exclusively, then it may be possible to activate cell growth through this pathway.

Consistent with IRE1 acting as an ER sensory molecule, examination of the gene products differentially expressed upon IRE1 activation revealed a strong enrichment for those that enter the ER and reside in this location, localize to the plasma membrane, or are destined for secretion. The specific gene identities revealed several biological functions that could be considered in agreement with mediating a stress response. The gene most significantly upregulated is the ER-resident detoxifying monooxygenase cytochrome P450 B1. CYP1B1 metabolizes procarcinogens, such as polycyclic aromatic hydrocarbons, and steroids (Pottenger and Jefcoate 1990). One of the highest activities is toward the substrate 17ß-estradiol, which is converted to the 4-hydroxyestradiol (Lee et al. 2003a, b). The induction of the chaperone *DNAJB9* was observed in the microarray and confirmed the qPCR data. *DNAJB9* has been reported to be driven by XBP-1^S expression (Lee et al. 2003a, b), thus providing a link to the enhanced protein chaperone activity in the ESR. One entire class upregulated by IRE1 includes several genes classically induced by interferons. It is well established that numerous viruses including hepatitis, herpesvirus, and flavivirus induce an ESR (Bechill et al. 2006; Mulvey et al. 2007; Yu et al. 2006). Several of these studies have shown IRE1/XBP-1 is the sensor that recognizes the infection and participates in the biological response. Finally, a decrease in two rate-limiting enzymes involved in lipid synthesis, DGAT2 and SPTLC3, was observed. Pathological excesses of nutrients are known to stimulate an ESR (Gregor and Hotamisligil 2007) and mice haploinsufficient for *Xbp-1* have an increase in adipose tissue compared to wild-type mice (Ozcan et al. 2004). Since DGAT2 is the primary mechanism by which triglycerides are formed and is essential for adipose formation (Bluher et al. 2002), it is possible that the IRE1/XBP-1 pathway prevents fat deposition though transcriptional repression of DGAT2. SPTLC3 is the rate-limiting step in de novo sphingolipid synthesis. A relationship between sphingolipid levels and the ESR has not been reported to date.

The contribution of ER stress pathways to tumorigenesis has become an intense area of interest. The extent to which the IRE1/XBP-1 pathway is modulated in human cancer is only beginning to emerge. *Xbp-1* splicing has been observed both in hepatocellular carcinoma and in breast cancer (Lacroix and Leclercq 2004; Shuda et al. 2003). The mechanism behind the presumed induction of IRE1 activity is not clear, but a reduction in oxygen tension can increase *Xbp-1* splicing (Koong et al. 2006). Although one recent report has linked IRE1 activity and pro-angiogenic processes (Drogat et al. 2007), few studies have explored how IRE1 activation may lead to tumor promotion. The microarray study carried out here revealed a number of changes that could be pro or anti-cancer. For example, the down-regulation of proto-oncogenic K-cadherin and the induction of E-cadherin and several other genes with cell adhesion functions could point to a tumor or metastasis prevention function. However, the induction of a gene such as CYP1B1 could generate additional DNA damage. Functional studies will ultimately need to be performed to discern how each arm of the ESR alone and in combination modifies tumorigenesis.

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