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Signaling Mechanism of Mammalian Endoplasmic Reticulum Stress Sensor IRE1

by

Han Li

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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by

Han Li

This thesis is dedicated to my mother
Shuyun Han
for her sacrifice and love,
for being my inspiration, friend and role model

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I want to first thank Peter Walter for accepting me into the Walter lab. In my early graduate school years, Peter looked passed and beyond my naiveness and inexperience in science, put trust and faith in me and taught me how to become a scientist, a thinker and an experimentalist. His creativity and passion in science is truly infectious and admirable. I appreciate his trust, honesty, encouragement, intellectual stimulation and discussions. Moreover, I particularly appreciate Peter's effort of making the Walter lab one big vibrant family. After attending numerous Walter lab retreats and Christmas parties, I am proud to say that there is no lab like the Walter lab, a place that feels like home.

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Chapter 2 Figures 2-5 and 2-6 are reprints of the material as it appears in “IRE1 signaling affects cell fate during the unfolded protein response”. Jonathan Lin is the first author who conducted the research and wrote the manuscript. Han Li generated and characterized drug-sensitized cell lines, and contributed to manuscript writing. Peter Walter directed and supervised the research Chapter 3 is a reprint of the material as it appears in “Mammalian endoplasmic reticulum stress sensor signals by dynamic clustering”. Han Li conducted the research and wrote the manuscript, Alexei Korrennykh and Peter Walter directed and supervised the research.

Signaling mechanism of mammalian ER stress sensor IRE1

by

Han Li

Abstract

The endoplasmic reticulum (ER) responds to the accumulation of misfolded proteins in its lumen (termed ER stress) by activating collective signal transduction pathways, called unfolded protein response (UPR). The UPR alleviates ER stress through its translational and transcriptional outputs carried out by three distinct ER stress sensors: PERK, ATF6 and IRE1. IRE1 is the best characterized UPR signal transduction molecule, and it is conserved from yeast to mammals. UPR signaling promotes apoptotic cell death if ER stress is not alleviated. However, the exact mechanism of how ER stress induces apoptosis remains unclear. Particularly, each branch of the UPR exerts opposing effects on cell survival under ER stress. To distinguish the effect of each branch of the UPR, we took a chemical-genetic approach, where we artificially prolonged IRE1 signaling by a small molecule, and observed enhanced cell survival under ER stress. Our data suggests a causal link between the duration of UPR branch signaling and life or death cellular decision.

To further dissect IRE1 signaling upon prolonged ER stress, we developed a visual assay to assess IRE1 activation in cells. IRE1 is activated by misfolded

protein direct binding to its luminal domain, which then transduces the signal to its cytosolic kinase and endoribonuclease domains. Using the *in vivo* imaging approach and an *in vitro* biochemical assay, we demonstrated that IRE1 oligomerizes at the ER membrane upon misfolded protein accumulation, which in turn activates its kinase and RNase activities. IRE1 signaling attenuation correlates perfectly with its oligomers dissociation, suggesting a built-in timer within IRE1 that may be important in switching the UPR from the initially cytoprotective phase to the apoptotic mode. We next took an unbiased proteomic approach to address IRE1 signal attenuation mechanism using mass spectrometry analysis. We identified over 400 proteins and compiled a list of interesting and potential IRE1 interacting partners and modulator.

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Chapter 1:

Introduction

Cells communicate with each other and the entire organism through secreted proteins and plasma membrane signal receptors. In eukaryotic cells, most secreted and membrane proteins are being synthesized, modified and folded in the lumen of the endoplasmic reticulum (ER) before they are transported to their proper destinations (Ron and Walter 2007). The ER is a maze-like network of membranous tubules and sheets, inside of which, reside molecular chaperones and protein-modification enzymes that are crucial for protein folding and assembly.

Proteins enter the ER as unfolded polypeptide chains. The influx of these nascent proteins depends on constantly changing cellular environment. Many physiological conditions, such as cell differentiation or antibody production during immune responses, increases the protein influx into the ER. It is extremely crucial for the cells to handle these dynamic situations to ensure proper cellular functions. To adjust protein-folding capacity of the ER according to need and to maintain the quality of cell surface and secreted proteins, cells turn on a homeostatic control response, called the unfolded protein response (UPR). UPR signaling can protect cells from ER stress by expanding the ER, enhancing the folding or degradation of misfolded proteins, and reducing the synthesis of new proteins. If homeostasis cannot be reestablished, however, UPR signaling induces cell death by apoptosis, as an effective means of protecting the organism from rogue cells expressing dysfunctional signaling molecules.

The oldest branch of the UPR, is mediated through the stress-regulated inositol-requiring enzyme 1 (Ire1), which is conserved from yeast to mammals (Patil and Walter 2001). Yeast UPR signaling solely relies on Ire1, an ER resident type I transmembrane serine/threonine kinase and endoribonuclease that responds to the imbalance between protein folding load and capacity (Cox, Shamu et al. 1993). The luminal domain of Ire1 senses protein-folding environment in the ER lumen (Credle, Finer-Moore et al. 2005). In response to misfolded proteins, yeast Ire1 oligomerizes in the ER membrane (Aragon, van Anken et al. 2009; Korennykh, Egea et al. 2009), allowing trans-auto-phosphorylation of its kinase domain, which in turn induces a conformational change that activates its endoribonuclease activity. Ire1 RNase initiates a non-conventional splicing of an mRNA substrate *HAC1*, encoding the transcription factor Hac1p. Hac1p targets UPR genes encoding proteins involved in protein folding, secretion/transport and degradation (Cox and Walter 1996; Travers, Patil et al. 2000).

In contrast to yeast, mammalian UPR consists a more elaborate network of signaling events. At least two outputs enhance the protein folding capacity of the ER under conditions of stress in mammals: one suppresses the influx of newly synthesized unfolded proteins through translational regulation, and the other one increases the protein folding or degradation ability within the ER at the transcriptional level. Three distinct mammalian UPR sensors together carry out the translational and transcriptional outputs, namely, PERK (PKR-like ER kinase),

ATF6 (activating transcription factor 6), and IRE1 (inositol requiring enzyme 1) (Ron and Walter 2007) (Figure 1-1).

PERK contains a luminal domain with a weak homology to its counterpart in IRE1, and a cytosolic portion possessing a kinase domain that phosphorylates eukaryotic initiation factor 2 α (eIF2 α), resulting in global translation attenuation, hence reduces the protein influx into the ER (Harding, Zhang et al. 1999).

ATF6, on the other hand, is a type II ER transmembrane protein containing an N-terminal region sensing misfolded proteins in the ER lumen and a cytosolic domain composed of a basic leucine zipper (bZIP) transcription factor (Haze, Okada et al. 2001). Upon ER stress, ATF6 translocates from the ER to the Golgi, where it undergoes site specific proteolysis by SIP (site-1 protease) and S2P (site-2 protease) (Ye, Rawson et al. 2000). The cleaved cytosolic portion of ATF6 is liberated from the membrane and translocated to the nucleus, subsequently, transcriptionally up-regulate UPR-related genes and ERAD components (Haze, Okada et al. 2001).

IRE1, the conserved stress sensor, site specifically cleaves *Xbp-1* (x-box binding protein-1) mRNA, a metazoan equivalent of *HAC1* mRNA. XBP-1 protein, alone or in conjunction with ATF6, launches a transcriptional program to produce chaperones (such as Grp78 (BiP)) and proteins involved in ER biogenesis, phospholipid synthesis, ER-associated protein degradation (ERAD) and secretion. In addition to executing the non-conventional splicing of *Xbp-1* mRNA,

mammalian IRE1 has acquired additional functions to cope with the complexity of mammalian protein folding environment. Recent work suggested that IRE1 has a surprising alternative function in regulating the degradation of mRNAs localized to the ER membrane, the process is named rapid IRE1-dependent decay (RIDD) (Han, Lerner et al. 2009; Hollien, Lin et al. 2009). However, whether RIDD mRNAs are direct substrates of IRE1 still remains unresolved.

It is clear that the UPR does not always result in successful alleviation of ER stress and reestablish ER homeostasis. Often driven by severe or prolonged protein misfolding signals, the UPR can induce cell death via apoptosis, as a means of eliminating the misfolded or malfunctioning proteins and to protect the organism from rogue cells that do not receive or relay signals properly (Rao, Ellerby et al. 2004). It has long been speculated how ER stress links to apoptosis. Genetic studies have begun to assign cytoprotective or proapoptotic functions to individual UPR branch and each target genes. For instance, expression of ER chaperone BiP protects cells from ER stress (Morris, Dorner et al. 1997), whereas CHOP, a B-ZIP transcription factor induced by PERK branch of the UPR, promotes cells death (Garrison, Kunkel et al. 2005). IRE1, on the other hand, had very undefined role in life-death decision until recently (Lin, Li et al. 2007; Lin, Li et al. 2009). Tumor necrosis factor receptor-associated factor 2 (TRAF2) associates with IRE1 at the ER membrane, which leads to activation of ASK1 that ultimately initiates a phosphorylation cascade resulting in cJUN NH₂-terminal kinases (JNK) activation (Nishitoh, Saitoh et al. 1998; Urano, Wang et al.

2000). Even though JNK activation has often linked to cell death, the precise physiological impact on IRE1-dependent-JNK activation is still awaiting to be answered. Furthermore, IRE1 is also found to interact with components of the cell-death machinery, such as Bax and Bak (Hetz, Bernasconi et al. 2006) and caspase-12 (Yoneda, Imaizumi et al. 2001). It is intriguing how these mitochondrial proteins sense signals from the ER upon misfolded proteins accumulation. Markedly, anti-apoptotic factor, Bax inhibitor-1 (BI-1) also associates with IRE1 and regulates its activity upon ER stress (Lisbona, Rojas-Rivera et al. 2009).

Interesting questions arise from collective works on mammalian IRE1. First of all, how does IRE1 make life-death cell fate decision in the face of misfolded proteins accumulation? It is rather confounding how and why both pro- and anti-apoptotic factors get recruited to the ER membrane, and associate with IRE1 under ER stress. Also, if IRE1 is regulated by apoptotic factors/machineries (such as Bax/Bak and BI-1) upon ER stress, then the timing of their recruitment to IRE1 is the a key to unlock the link between UPR and apoptosis since cell death do not occur until later phase of the ER stress. In order to answer how IRE1 is regulated, we need a better understanding of IRE1 signaling activation and regulation. Though the mechanism of yeast Ire1 activation has been well studied, little has done to address whether mammalian IRE1 undergoes similar activation mechanism, considering there are two other parallel UPR branches in mammals. Is IRE1 oligomerization a conserved signaling mechanism from yeast

to human? Furthermore, it is intriguing whether and how IRE1 activity is regulated. The only known kinase substrate of IRE1 is itself, and the only RNase substrate is *Xbp-1*/HAC1 mRNA. Most studies that identified IRE1 interacting partners have been biased toward the proteins of interest in an over-expression system, which can be misleading regarding the relevant physiological significance of such interactions. Unbiased approaches are essential to colligate regulation machinery of IRE1.

This thesis dissected IRE1 signaling with mechanistic details. It provided powerful cellular and biochemical tools to study human IRE1 signaling both *in vivo* and *in vitro*.

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Figure Legends

Figure 1-1. The mammalian unfolded protein response (UPR)

The unfolded protein response is comprised of three ER-transmembrane sensors, PERK, IRE1 and ATF6. Three pathways converge to turn on translational and transcriptional programs to alleviate ER stress. *Xbp-1* mRNA (u) denotes unspliced *Xbp-1*; *Xbp-1* mRNA (s) denotes spliced *Xbp-1*.

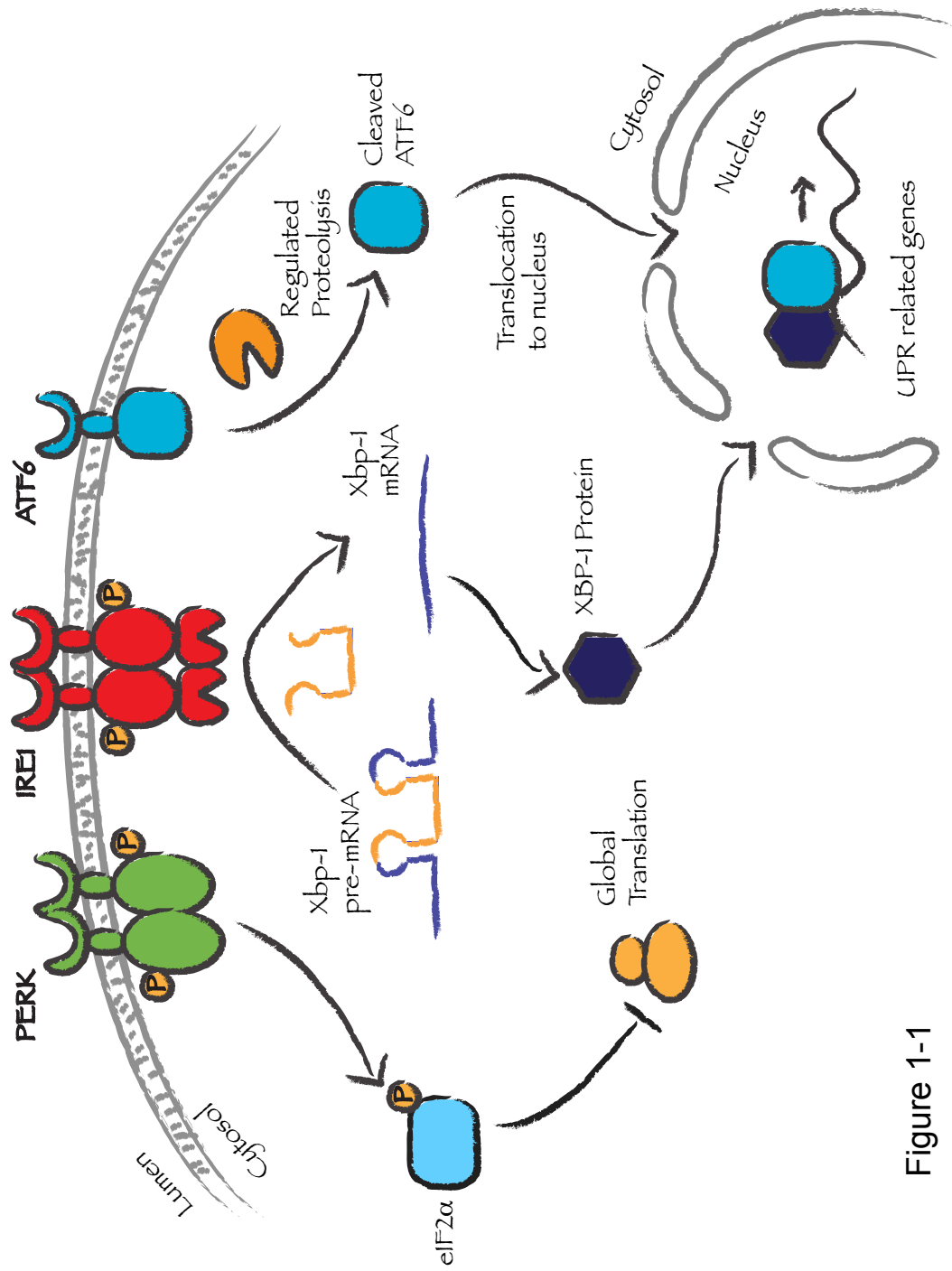


Figure 1-1

Chapter 2:
**Chemical-genetic manipulation of IRE1
signaling**

Background:

In mammalian cells, ER stress activates all three branches of the UPR concurrently. Unexpectedly, the dynamic of each individual signaling pathway varied markedly with time after the onset of ER stress (Lin, Li et al. 2007). IRE1 signaling attenuated within 8 hours despite the persistence of the stress and unresolved ER homeostasis. ATF6 branch shut off followed IRE1 attenuation with delayed timing. On the contrary, the responses mediated by PERK signaling persisted under prolonged ER stress (Figure 2-1). These observations suggest that the kinetics of activation and deactivation of each individual ER-signaling branch may promote time-regulated specific outputs influencing the cell's ultimate fate in response to ER stress. To dissect the significance of such dynamic UPR signaling, means of differentiating each branch become invaluable. However, such task is not trivial due to the complexity of the mammalian UPR network.

Each of the UPR pathway alleviates misfolded protein induced ER stress by turning on their perspective downstream effectors to restore ER homeostasis via translational and transcriptional programs. Even though each of the UPR branch has its specific and distinct function and targets, the three branches, to certain extends, crosstalk with one another under ER stress. For example, IRE1 downstream effector, XBP-1, is transcriptionally targeted by ATF6 upon UPR activation (Yoshida, Matsui et al. 2001). CHOP, downstream of PERK signaling, is a transcriptional target of ATF4 as well as XBP-1 and ATF6 (Ron and Walter 2007). Such crosstalk among the sensors present a great challenge to dissect

each individual branch of the UPR upon ER stress. In particular, each branch of the UPR elicits either protective or apoptotic response or both depends on the duration of the stress. No trigger for ER stress has been identified that selectively elicits only one branch of the UPR but not others. ER stress activates all UPR signaling pathways, thereby simultaneously producing antagonistic outputs. To address this challenge, we developed means to examine the molecular behavior of the UPR, in particular, IRE1 signaling, in human cells exposed to persistent and pharmacologically induced ER stress.

Genetic approaches by knocking out one UPR sensor have been the standard methodology to uncouple one branch of the UPR from the other two and to dissect the effect of the deficient branch. However, over time these knockout cells become adapted to two-branched UPR, and as a consequence, the result from these studies can misrepresent the true significance of the deficient branch. Recent years, genetic methods influenced by chemical biology have emerged to manipulate specific protein's enzymatic domains by addition of small molecules. This chemical-genetic strategy allows "chemical-sensitive" mutant proteins to be uniquely inhibited by rationally designed small-molecule inhibitors.

A functional kinase domain precedes IRE1's RNase domain on the cytosolic side of the ER membrane. Activation of IRE1 leads to dimerization of its luminal domain in the ER membrane, followed by trans-autophosphorylation (Liu, Schroder et al. 2000; Liu, Wong et al. 2002; Liu, Xu et al. 2003; Zhou, Liu et al.

2006). Mutations of catalytically essential kinase active-site residue or mutations of residues that are known to be phosphorylated prevent *HAC1/Xbp-1* mRNA splicing and abrogate UPR signaling, demonstrating that IRE1's kinase phospho-transfer function is essential for its RNase activity.

To dissect the role of IRE1 kinase function, the Walter lab utilized the novel chemical and genetics strategy that allows the sensitization of IRE1 to specific kinase inhibitors. The method involves a mutation of a conserved bulky residue in the adenosine 5'-triphosphate (ATP)-binding pocket to a glycine or alanine. The resulting analogue-sensitive kinase is able to bind bulky ATP analogues that fit into the mutant-binding pocket but cannot bind to wild-type kinase (Bishop, Shah et al. 1998). Importantly, the mutation is buried deeply inside ATP-binding pocket, away from protein substrate binding site, hence, it is not expected to alter substrate specificity of the kinase (Witucki, Huang et al. 2002). The Shokat lab synthesized derivatives of PP1, a potent Src kinase inhibitor (Hanke, Gardner et al. 1996), among which is 4-amino-1-tert-butyl-3-(1-naphthylmethyl)pyrazolo[3,4-d]pyrimidine (1NM-PP1). By enlarging a kinase's ATP binding pocket through mutagenesis, 1NM-PP1 binds to the ATP binding pocket with a higher affinity and competes with ATP binding, thus reduces the kinase activity (Bishop, Shah et al. 1998; Bishop, Ubersax et al. 2000). Taking advantage of this strategy, Papa et al., mutated the conserved kinase gatekeeper residue of yeast Ire1 Leu 745 to Ala or Gly to sensitize Ire1 to 1NM-PP1 by creating enlarged active-site pocket (Papa, Zhang et al. 2003). Most kinases are not affected by these mutations, but

others are partially or severely impaired. To everyone's surprise, not only the mutation has no inhibitory effect on yeast Ire1 kinase activity, the addition of 1NM-PP1 unexpectedly restored sensitized Ire1 kinase function monitored by UPR reporters (Papa, Zhang et al. 2003). 1NM-PP1-sensitized mutant bypassed Ire1 kinase activity, responded to DTT induced ER stress in combination with 1NM-PP1, spliced *HAC1* mRNA to a level comparable to wildtype Ire1. We took hints from our yeast work, demonstrated that we could manipulate and prolong IRE1 branch signaling using the same chemical-genetic approach. Additionally, such manipulation of IRE1 by 1NM-PP1 alone acts as a molecular switch of its activity, which is different from our yeast work.

Results

Chemical-genetic control of IRE1 signaling in mammalian cells

We followed the strategy that permitted chemical regulation of IRE1's RNase activity using ATP analog 1NM-PP1 (Figure 2-2A). A leucine-to-glycine (Leu 745---> Gly 745 (L745)) mutation rendered yeast Ire1 sensitivity to 1NM-PP1. We constructed an allele of human IRE1 in which the orthologous amino acid (Isoleucine 642 (Ile642)) was mutated to glycine (I642G) (Figure 2-2B). We used flippase-mediated, site-specific DNA recombination to introduce the drug sensitized IRE1(I642G) mutant allele directly into a single locus in the genome of IRE1^{-/-} mouse embryonic fibroblast (MEF) cells bearing an *frt* site (Cohen, Royce-Tolland et al. 2005). No deleterious growth defects were observed in the transgenic cells expressing IRE1(I642G) allele. The UPR was not constitutively

induced in these cells, as indicated by the absence of spliced *Xbp-1* mRNA, unlike in IRE1^{-/-} MEFs reconstituted with wildtype IRE1 allele (Figure 2-3, compare panel A lane 1 with panel B lane1). 1NM-PP1 alone activated IRE1 signaling in a concentration dependent manner (Figure 2-3B, lanes 3, 5, 7, and 9). Addition of ER stress inducer tunicamycin (Tm), which blocks N-linked glycosylation of proteins, further increased *Xbp-1* mRNA splicing (Figure 2-3B, lanes, 4, 6, 8 and 10), suggesting that 1NM-PP1 sensitized IRE1 is still sensitive to ER stress. In contrast, the corresponding 1NM-PP1 sensitized allele in yeast required both misfolded protein and 1NM-PP1 to activate *HAC1* mRNA splicing and reporter activities (Papa, Zhang et al. 2003). Thus, the human drug sensitized IRE1 allele, provided a molecular switch regardless of ER protein folding status.

JNK activation requires IRE1 kinase activity

In response to ER stress, IRE1 initiates a signal transduction pathway that activates JNK (Urano, Wang et al. 2000). Deletion of IRE1 kinase domain diminishes JNK activity, which suggests three possibilities: 1) IRE1 kinase domain serves as a scaffold for signaling molecules required for JNK activation, 2) IRE1 kinase phosphorylation activity is required for JNK signal transduction, or 3) the combination of 1) and 2). Taking advantage of the new chemical-genetic manipulation of IRE1, we examined JNK activation upon ER stress. We first verified IRE1-dependent JNK activation in our cell culture system. Ultraviolet (UV) irradiation is known to induce JNK activation, which was used as a positive

control of JNK signaling (Urano, Wang et al. 2000). In IRE1^{-/-}-hIRE1 reconstituted and IRE1^{-/-} MEFs, we monitored phosphorylation of JNK using phospho-specific antibodies, which detected both 55 kDa and 46 kDa isoforms of JNK (Figure 5A). UV light induced a robust activation of JNK in IRE1^{-/-}-hIRE1 reconstituted cells, but only slightly activated JNK in IRE1^{-/-} cells (Figure 2-4A, compare lanes 2 and 7) . We then used both tunicamycin (TM) and thapsigargin (TG), which blocks ER calcium pumps, to induce ER stress, 4 hour of TG treatment induced the highest level of JNK activation in IRE1^{-/-}-hIRE1 reconstituted cells, evident by the amount of phospho-specific JNK species under this condition (Figure 2-4A, compare lanes 8, 9 and 10); however, IRE1^{-/-} cells showed no signs of JNK activation under the same experimental conditions (Figure 2-4A, lanes 3, 4 and 5), which confirmed that JNK activation under ER stress indeed requires IRE1. We next used drug-sensitized IRE1(I642G) reconstituted IRE1^{-/-} cells to dissect whether IRE1 kinase activity is required for JNK signaling. Addition of 1NM-PP1 alone was not sufficient to activate JNK (Figure 2-4B, lanes 3 and 5), but ER stress alone and combination of 1NM-PP1 with ER stress induced a modest level of JNK activation (Figure 2-4B, lanes 2, 4, and 6). 1NM-PP1-sensitized IRE1 allele does not bind ATP, hence is lack of its phospho-transfer ability. Result here suggested that IRE1 kinase phospho-transferring is required for JNK activation.

Chemically activated IRE1 overcomes IRE1 signaling attenuation

Prolonged ER stress diminishes *Xbp-1* mRNA splicing, suggesting attenuation of IRE1 signaling, despite continued accumulation of misfolded protein inside the ER (Lin, Li et al. 2007). We ask whether chemically activated IRE1 can overcome its signaling attenuation. To this end, we introduced IRE1(I642G) allele into a single locus of the genome of HEK293 cells bearing a defined *frt* site to test if we could manipulate *Xbp-1* mRNA splicing during prolonged ER stress. Addition of 1NM-PP1 alone has no effect on endogenous wildtype IRE1 but induces robust *Xbp-1* splicing in IRE1-(I642G) expressing HEK293 cells (Figure 2-5A). The UPR was not constitutively induced in these cells, as indicated by the absence of spliced *Xbp-1* mRNA at 0 hours time point (Figure 2-5A). In the absence of 1NM-PP1, robust *Xbp-1* mRNA splicing occurred in the transgenic cells after drug treatment at early time points and then diminished after prolonged ER stress (Figure 2-5B top panel). Thus the expression of IRE1(I642G) had no deleterious effect on IRE1 activation attenuation. By contrast, addition of 1NM-PP1 induced and maintained *Xbp-1* splicing. Thus artificial activation of IRE1 (I642G) allele overcame IRE1 signaling attenuation upon prolonged ER stress, sustaining *Xbp-1* mRNA splicing.

IRE1 activity enhances cell viability

Controlling IRE1 signaling by 1NM-PP1 allowed us to assess the physiological consequences of IRE1 activation and attenuation upon ER stress. In particular, we asked whether artificially extended IRE1 activation would have a beneficial

effect on cell viability upon prolonged stress. ER stress induced by tunicamycin (tm) and thapsigargin (tg) both is toxic to HEK293 cells. 48 hours post treatment, less than 2% of WT HEK293 cells survived (Fig. 2-6). The addition of 1NM-PP1 alone had no substantial effect on cell survival, though it slightly diminished the viability of WT cells). By contrast, 1NM-PP1 treatment of HEK293 cells expressing IRE1(I642G) allele significantly improved cell survival. At the 24-hours time point, IRE1(I642G) cells showed a drastic improvement of cell viability as compared to WT cells. Moreover, at 48-hours time point, where most of the WT cells were dead under tunicamycin and thapsigargin treatments, IRE1(I642G) cells showed 5 and 20 times higher in cell survival number, respectively, in the presence of 1NM-PP1 than in its absences (Figure 2-6). Even in the absence of ER stress, 1NM-PP1 addition proved beneficial in cells expressing IRE1(I642G) allele, affording a 30% enhancement of cell growth (Figure 4). Hence, IRE1 signaling directly enhanced cell viability in the face of ER stress.

Material and Methods

Cell Culture

HEK293, HeLa, MDAM468, Jurkat, and Ire1 α ^{-/-} mouse embryonic fibroblast cells were maintained at 37°C, 5% CO₂ in DMEM or RPMI media supplemented with fetal calf serum, glutamine, and antibiotics (Invitrogen, San Diego, CA).

Tunicamycin and thapsigargin were obtained from Calbiochem EMB Bioscience Inc. (Darmstadt, Germany). The generation and use of the ATP analogue, 1NM-PP1, to regulate kinase activity has been previously described (1). Transfections were performed using the FuGene reagent (Roche, Basel, Switzerland).

Molecular Biology

To construct a 1NM-PP1 sensitized IRE1 allele, QuikChange site-directed mutagenesis kit (Stratagene, San Diego, CA) was used to insert an isoleucine to glycine missense mutation in the ATP binding domain of human IRE1 α at position 642. The human IRE1[I642G] construct was integrated into HEK293 cells bearing frt sites as previously described (2). Five independent isogenic clones were analyzed with identical findings.

Protein Analysis

Cells and tissues were homogenized by sonication in 1% NP-40 or 6 M Urea, 20 mM HEPES pH=8.0, containing protease (Roche, Basel, Switzerland) and phosphatase inhibitors (Pierce, Rockford, IL). 20 to 30 µg of total cell lysates was loaded onto 10% or 4-12% SDS-PAGE minigels (NuPAGE, Invitrogen) and analyzed by Western blot. The following antibodies and dilutions were used: anti-phospho-JNK at 1:1000 (Cell Signaling, Natick, MA); anti-total JNK at 1:1000 (Cell Signaling, Natick, MA). After overnight incubation with primary antibody, membranes were washed in PBS with Tween, and incubated in horseradish peroxidase-coupled secondary antibody (Amersham, Piscataway, NJ) diluted at 1:5000 in washing buffer. Immunoreactivity was detected using the enhanced chemiluminescence assay (Pierce, Rockford, IL)

RT-PCR analysis of Xbp-1 splicing

Cells or tissues were lysed and total RNA was collected (RNAeasy, Qiagen, Germany). PolyA mRNA was reverse-transcribed using the SuperScript-RT system (Invitrogen, Carlsbad, CA). cDNA was used as template for PCR amplification across the fragment of the *Xbp-1* cDNA bearing the intron target of IRE1α ribonuclease activity. Primers used included: murine *Xbp-1*, 5'-TTACGGGAGAAAACCTCACGGC-3' and 5'-GGGTCCAAGTTGTCCAGAATGC-3'; human *Xbp-1*, 5'-TTACGAGAGAAAACCTCATGGC-3' and 5'-GGGTCCAAGTTGTCCAGAATGC-3'. PCR conditions were: 95°C for 5 min; 95°C for 1 min; 58°C for 30 sec; 72°C for 30 sec; 72°C for 5 min with 35 cycles of

amplification. A 289 bp amplicon was generated from unspliced *Xbp-1*; a 263 bp amplicon was generated from spliced *Xbp-1*. PCR products were resolved on a 2.5% agarose/1x TAE gel. As previously reported, a minor hybrid amplicon species consisting of unspliced *Xbp-1* annealed to spliced *Xbp-1* was also produced through the PCR reaction and appeared above the unspliced amplicon (4). Quantification of spliced *Xbp-1* mRNA as a percentage of total *Xbp-1* mRNA was performed using ImageQuant software (Amersham, NJ, USA).

Cell Survival

Cells (50,000 per well) were plated and, after drug treatment, well contents were trypsinized and the number of cells was counted by hemacytometer. Viable adherent cells stained with Cresyl violet. For MTT metabolism, cells were plated and after drug treatment, MTT was added to media, and colorimetric change was measured by spectrophotometer per manufacturer's protocol (Sigma, St. Louis, MO).

Discussion

ER stress inducing agents impartially activates all UPR branches at the same time, yet the three branches display surprisingly different dynamics upon prolonged ER stress (Lin, Li et al. 2007). After rapid initial onset of UPR activation through all sensors by ER stress, IRE1 signaling was selectively attenuated in human cells, despite unresolved misfolded protein accumulation. ATF6 signaling also declined with delayed timing, yet PERK branch persisted much longer in the presence of unmitigated ER stress. We proposed that the variation in signal attenuation among UPR sensors in part help cells to choose between cytoprotective and proapoptotic outputs in the face of ER stress. We used a chemical-genetic approach to artificially prolong IRE1 signaling, which enhanced cell survival, thereby demonstrating a causal link between IRE1 activity and cell survival. This result suggests a model by which individual UPR signaling pathway determines cell fate upon ER stress. The initial converged signaling events through IRE1, PERK and ATF6 produce cytoprotective outputs such as reduced translation, enhanced ER protein folding capacity and clearance of misfolded proteins. However, when ER homeostasis cannot be restored after prolonged ER stress, the proapoptotic outputs, such as induction of CHOP, outweighs the cytoprotective effort elicited by IRE1 and ATF6. This hypothesis is further supported by another work from this lab, where we applied a similar chemical-genetic strategy to selectively activate and sustain PERK signaling, and observed irreversible ER stress induced cell death (Lin, Li et al. 2009). The strategy of manipulating IRE1 signaling by a small molecule provides a powerful

tool to dissect the opposing outputs under ER stress, as well as defines their physiological scope and significance.

Urano *et al.* first reported the additional function of IRE1 through its kinase domain (Urano, Wang et al. 2000), where the authors demonstrated the requirement of IRE1 kinase domain for the recruitment of TRAF2 upon ER stress; however, it was unclear whether IRE1 kinase activity is required for JNK signaling. IRE1 kinase activation is obligatory for its RNase activity. Addition of small molecule 1NM-PP1 alone is sufficient to activate *Xbp-1* mRNA splicing despite of IRE1's lack of phospho-transfer ability. This observation is in agreement with our previous study in yeast where we proposed IRE1 kinase conformational change is sufficient to activate its downstream RNase activity (Papa, Zhang et al. 2003). By contrast, the corresponding 1NM-PP1-sensitized allele in yeast required both ER stress and the addition of 1NM-PP1 to activate *HAC1* mRNA splicing. The human drug-sensitized allele thus provided a true molecular switch that could be toggled by 1NM-PP1 regardless of ER protein folding status. Though this chemical-genetic manipulation of IRE1 kinase activity is capable of inducing IRE1 RNase activity through the conformational change, it is insufficient to activate the non-splicing branch of IRE1 signaling. Our result here suggests the requirement of IRE1's phospho-transfer activity in JNK activation. However, whether IRE1 directly phosphorylates a MAPK component that induces JNK cascade activation requires further investigation.

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Figure Legends

Figure 2-1. Summary of work from Lin et al. 2009

Various signaling dynamics of the Unfolded Protein Response (UPR) in mammals. Accumulation of misfolded proteins triggers both cell protective and cell death responses.

Figure 2-2. Chemical-genetic manipulation of human IRE1

A. The structure of the ATP analog and its antagonist 1NM-PP1. B. Alignments of the gatekeeper region of the ATP-binding domains of yeast and human IRE1. The residue (leucine or isoleucine) mutated to glycine is highlighted.

Figure 2-3. 1NM-PP1 induces IRE1 RNase activity in an ER-stress independent manner

A. *Xbp-1* mRNA splicing in IRE1^{-/-} MEFs and human IRE1 α reconstituted IRE1^{-/-} (IRE1^{-/-}-hIRE1 reconstituted) MEFs. ER stress was induced with or without 5 μ g/ml tunicamycin (Tm) for 4 hours in the presence of 5, 10 and 20 μ M of 1NM-PP1. *Xbp-1* mRNA splicing was assessed by RT-PCR. Unspliced (u) and spliced (s) *Xbp-1* mRNA products are indicated. B. *Xbp-1* mRNA splicing in IRE1^{-/-} MEFs and IRE1(I642G) reconstituted IRE1^{-/-} MEFs. 0, 2.5, 5, 10 and 20 μ M of 1NM-PP1 added to the cells for 4 hours in the presence 5 μ g/ml Tm was used to induce ER stress, *Xbp-1* mRNA splicing was determined by RT-PCR. Unspliced (u) and spliced (s) *Xbp-1* mRNA products are indicated.

Figure 2-4. IRE1 kinase phospho-transfer activity is required for JNK activation

A. IRE1^{-/-} and IRE1^{-/-}-hIRE1 reconstituted MEFs were treated with UV light for 4 hours, 500 nM thapsigargin (Tg) or 5 µg/ml tunicamycin (Tm) with 10 µM 1NM-PP1 for indicated hours. Phospho-JNK were assessed by immunoblotting. Total-JNK protein level served as a loading control. B. IRE1^{-/-}-hIRE1(I642) reconstituted cells were treated with 500 nM Tg and 10 µM 1NM-PP1 for indicated hours, treatment with 4 h of UV, as well as UV with 10 µM 1NM-PP1 as a control. Phospho-JNK were assessed by immunoblotting. Total-JNK was determined as a loading control.

Figure 2-5. Genetic control of human IRE1 in HEK293 cells

A. Parental WT and transgenic HEK293 cells expressing 1NM-PP1 sensitized IRE1 allele were treated for the indicated hours with 1NM-PP1 (5 µM), and *Xbp-1* mRNA splicing was determined by RT-PCR. B. Transgenic HEK293 cells were treated with tm (5 µg/ml) ± 1NM-PP1 (5 µM). *Xbp-1* mRNA splicing was assessed by RT-PCR.

Figure 2-6. IRE1 signaling enhances cell viability

Parental WT and transgenic HEK293 cells were treated with 300 nM thapsigargin (tg) or 5 µg/ml tunicamycin (tm). Adherent, cresyl violet-stained positive cells were counted at the indicated times and are shown relative to counts of mock-treated cells. Error bars represent SDs from three independent experiments.

The arrows indicate p value <0.01 when the corresponding samples $\pm 5 \mu\text{M}$ 1NM-PP1 were compared.

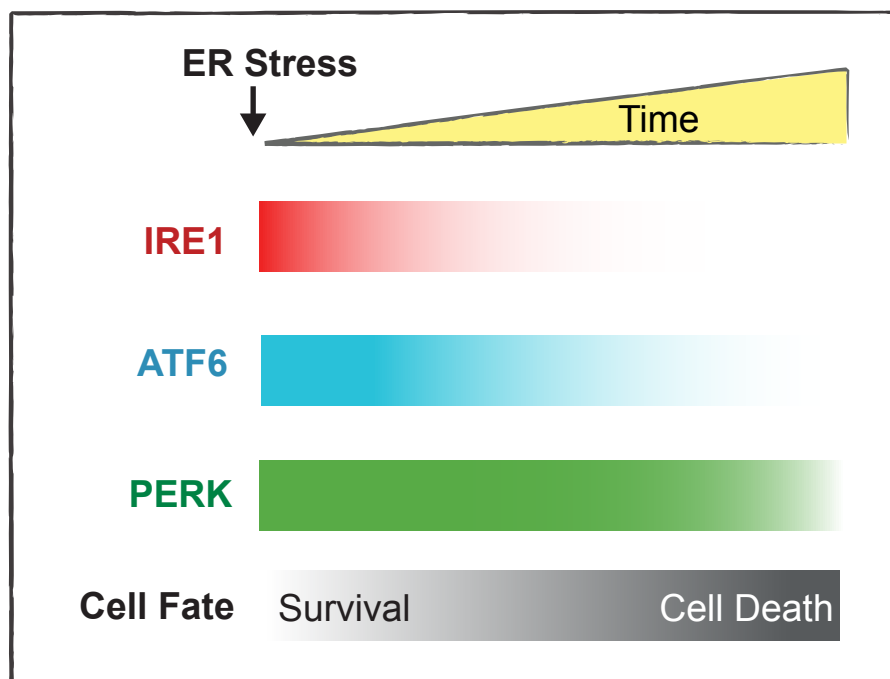
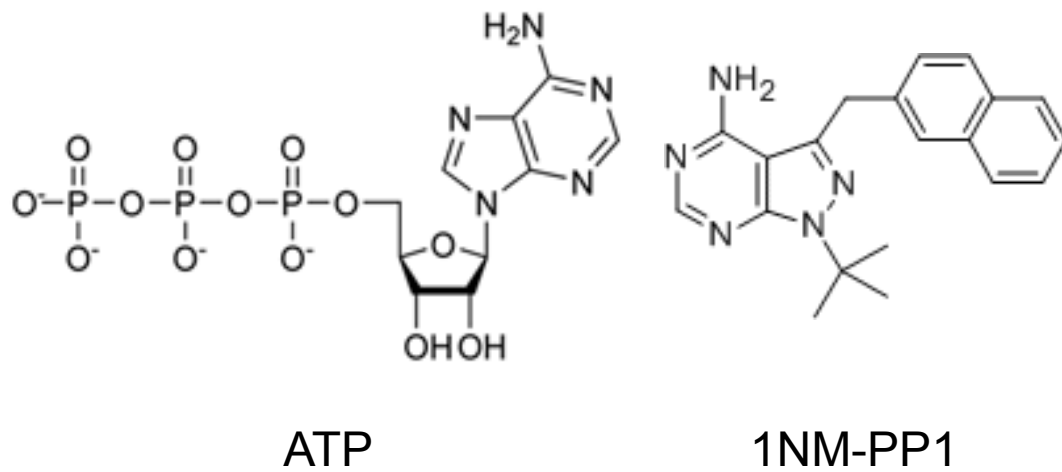


Figure 2-1

A



B

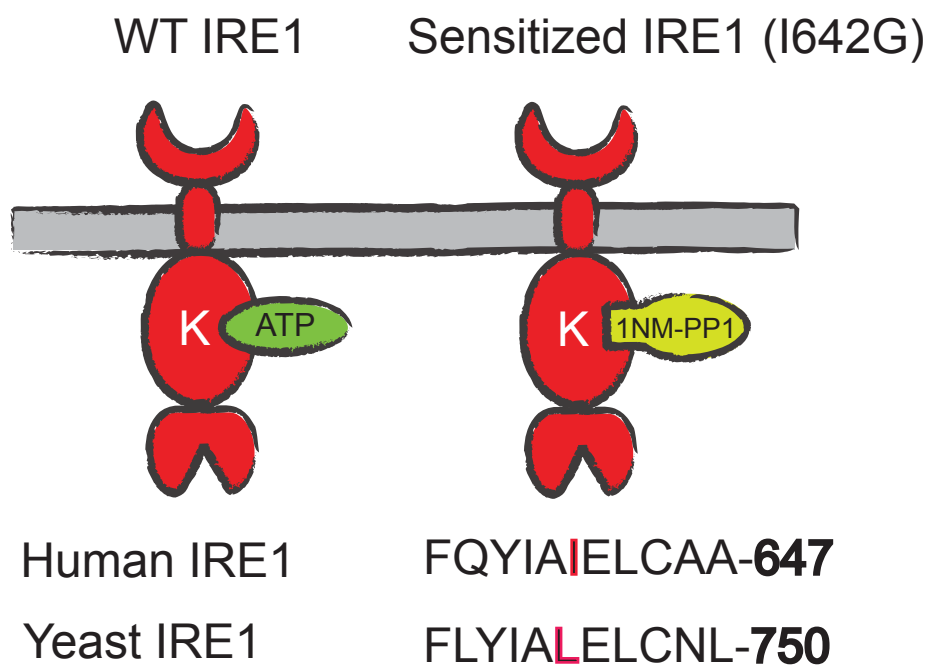
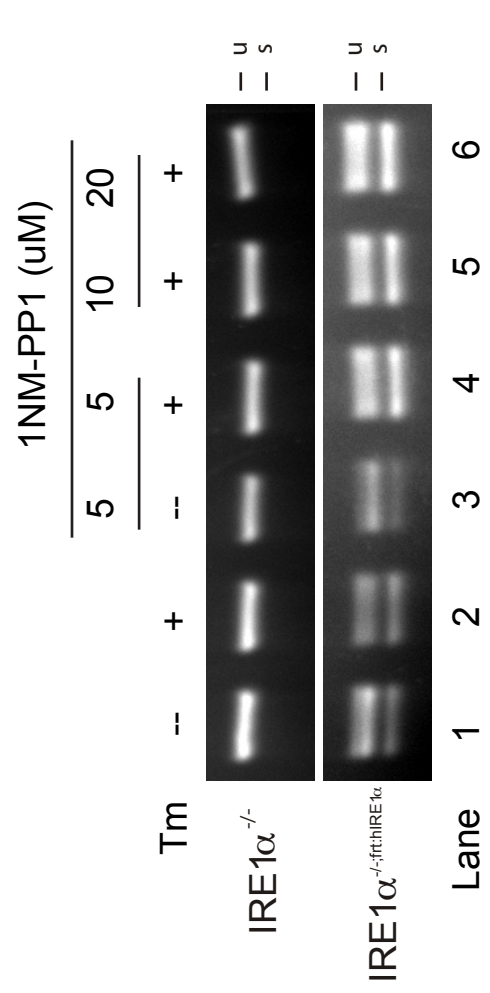


Figure 2-2

A



B

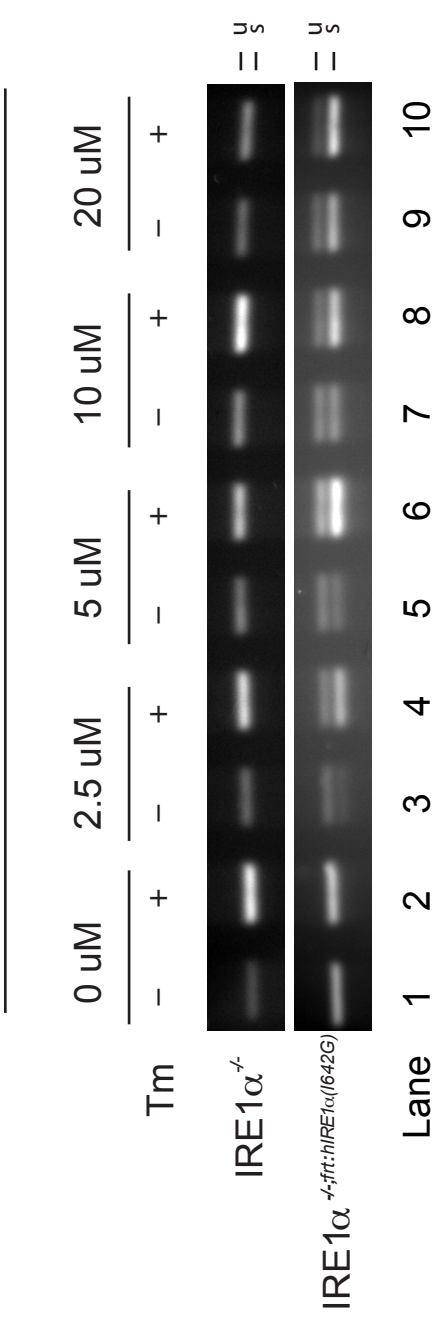
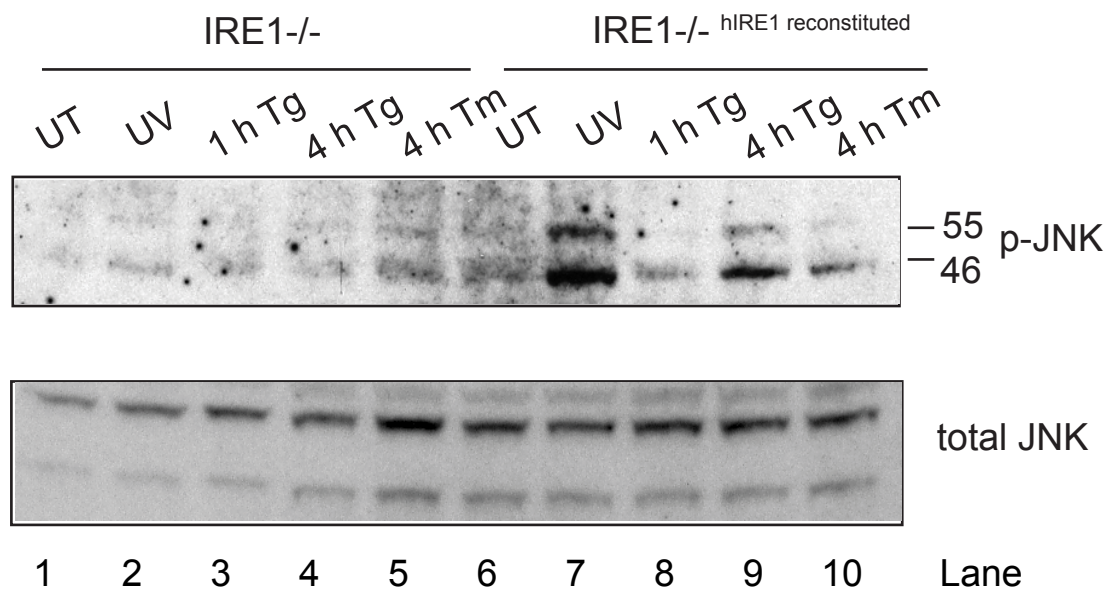


Figure 2-3

A



B

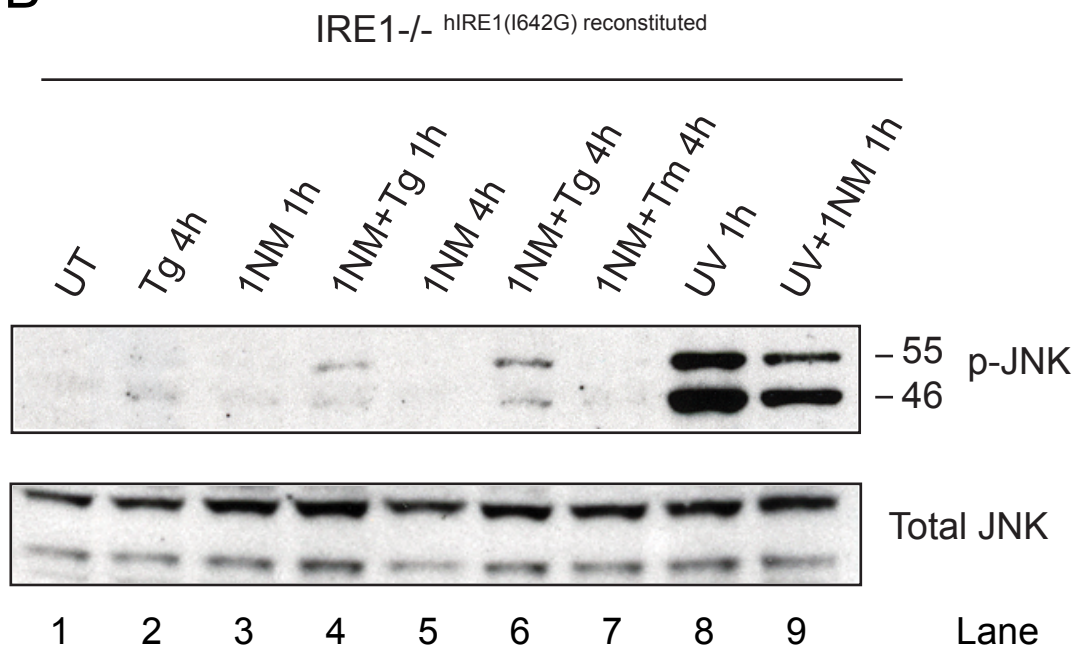
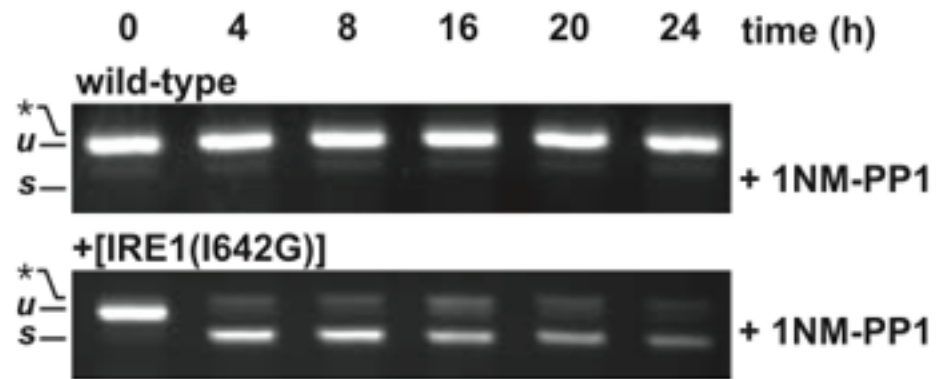


Figure 2-4

A



B

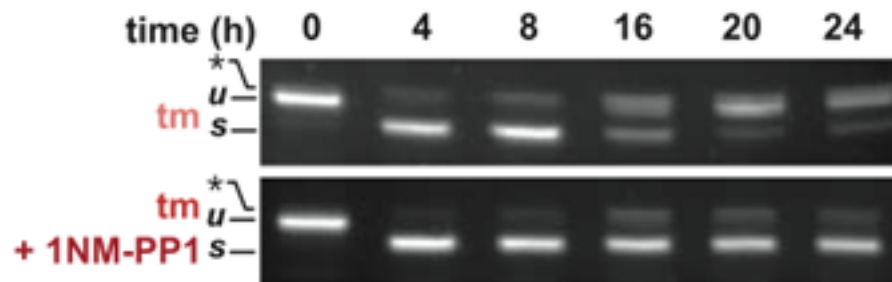


Figure 2-5

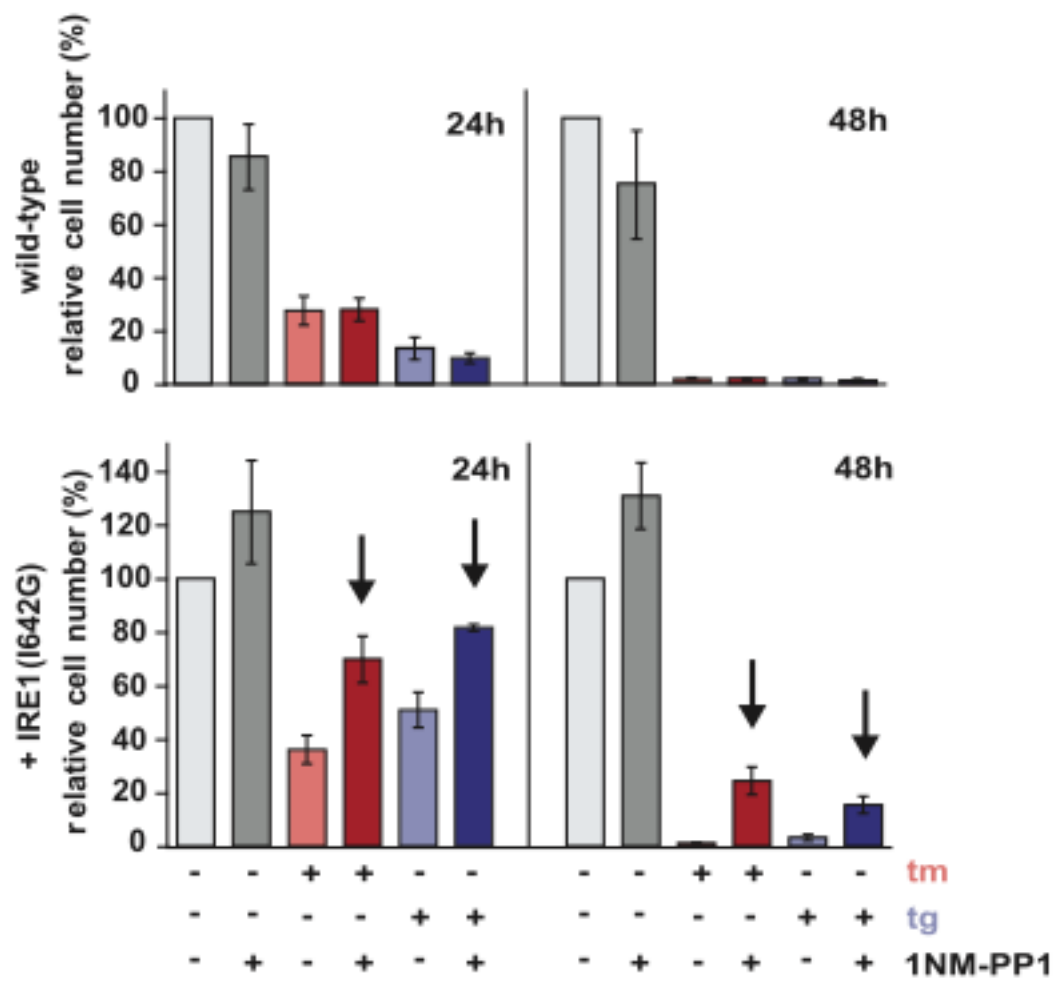


Figure 2-6

Chapter 3:

The Mammalian Endoplasmic Reticulum Stress

Sensor IRE1 Signals by Dynamic Clustering

Abstract

Accumulation of misfolded proteins in the endoplasmic reticulum (ER) triggers the unfolded protein response (UPR), an intracellular signaling pathway that adjusts the protein folding capacity of the ER according to need. If homeostasis in the ER protein folding environment cannot be reestablished, cells commit to apoptosis. The ER-resident transmembrane kinase-endoribonuclease IRE1 is the best characterized UPR signal transduction molecule. In yeast, Ire1 oligomerizes upon activation in response to an accumulation of misfolded proteins in the ER. Here we show that the salient mechanistic features of IRE1 activation are conserved: mammalian IRE1 oligomerizes in the ER membrane and oligomerization correlates with the onset of IRE1 phosphorylation and RNase activity. Moreover, the kinase/RNase module of human IRE1 activates cooperatively *in vitro*, indicating that formation of oligomers larger than four IRE1 molecules takes place upon activation. High-order IRE1 oligomerization thus emerges as a conserved mechanism of IRE1 signaling. IRE1 signaling attenuates after prolonged ER stress. IRE1 then enters a refractive state even if ER stress remains unmitigated. Attenuation includes dissolution of IRE1 clusters, IRE1 dephosphorylation, and decline in endoribonuclease activity. Thus IRE1 activity is governed by a timer that may be important in switching the UPR from the initially cytoprotective phase to the apoptotic mode.

Abbreviations

ER, endoplasmic reticulum; UPR, unfolded protein response; IRE1, inositol-requiring enzyme 1; XBP-1, x-box binding protein 1; PERK, protein kinase RNA (PKR)-like ER kinase; ATF6, activating transcription factor-6; 3F6H, 3-Flag-6-Histidine; MEFs, mouse embryonic fibroblasts

Keywords

IRE1, unfolded protein response, oligomerization, attenuation, phosphorylation

Introduction

Most secreted and transmembrane proteins are synthesized, modified and folded in the lumen of the ER. To ensure the fidelity of protein folding and maturation, cells turn on a network of signaling pathways, collectively termed the UPR, to adjust the protein-folding capacity of the ER according to need (Ron and Walter 2007). Imbalances between protein load and folding capacity is monitored by three distinct UPR sensors: IRE1 (inositol-requiring enzyme 1) (Cox, Shamu et al. 1993; Mori, Ma et al. 1993), PERK (protein kinase RNA (PKR)-like ER kinase) (Harding, Zhang et al. 1999) and ATF6 (activating transcription factor-6) (Haze, Okada et al. 2001). IRE1 is a conserved transmembrane protein with an ER-luminal domain that senses misfolded proteins in the ER, most likely by direct ligand-mediated recognition (Credle, Finer-Moore et al. 2005; Pincus, Chevalier et al. 2010). As a result, IRE1 activates its cytoplasmic kinase and endoribonuclease (RNase) domains, which initiate a nonconventional mRNA splicing reaction that results in the cleavage of the mRNA encoding the transcription factor XBP-1 (x-box binding protein 1) (Yoshida, Matsui et al. 2001; Calton, Zeng et al. 2002), a conserved effector of the UPR that drives the transcription of a plethora of ER-stress responsive genes, including ER resident chaperones and protein modifying enzymes (Lee, Iwakoshi et al. 2003).

The structural analysis of the luminal stress-sensing domain of yeast Ire1 provided insights into Ire1 activation under ER stress. Upon UPR activation, the luminal domain of Ire1 undergoes oligomerization, which brings the cytosolic

kinase domains into juxtaposition allowing their activation by trans-autophosphorylation and co-factor binding (Shamu and Walter 1996; Weiss and Schlessinger 1998). Kinase activation in turn triggers conformational changes that activate the RNase domain of the enzyme. Recent structural work suggests that the cytosolic domain of yeast Ire1 can form high-order assemblies and that this process is instrumental to activating the endoribonuclease activity of Ire1 (Korennykh, Egea et al. 2009). Furthermore, *in vivo* studies in yeast demonstrate that high-order assembly of Ire1 is driven by its luminal domain, and that Ire1 clusters into distinct foci upon activation (Kimata, Ishiwata-Kimata et al. 2007; Aragon, van Anken et al. 2009). In contrast, previous studies of human IRE1 suggested that formation of IRE1 dimers is sufficient to fully activate the enzyme (Liu, Schroder et al. 2000; Liu, Wong et al. 2002; Liu, Xu et al. 2003; Zhou, Liu et al. 2006). It has remained unclear whether oligomerization of IRE1 is necessary for its activation in metazoans.

Here we show that the salient features of IRE1 activation, including its obligate oligomerization, are conserved, and describe additional regulation that evolved in metazoan cells.

Results

IRE1 Clustering in the ER Membrane of Human Cells

To begin resolving the uncertainty and to determine whether IRE1 activation in mammalian cells involves high-order assembly, we engineered a GFP-tagged fluorescent human IRE1 α fusion construct, IRE1-3F6HGFP. The fluorescent tag was placed in the non-conserved linker between the transmembrane domain and the kinase domain (Figure 3-1A). Yeast Ire1 tagged in the corresponding position was fully functional, while C- or N-terminally GFP tagged versions were not (Aragon, van Anken et al. 2009). As expected, IRE1-3F6HGFP restored splicing of *Xbp-1* mRNA in response to ER-stress when expressed in *Ire1 α* ^{-/-} mouse embryonic fibroblasts (MEFs) (Figure 3-1B). Overexpression is known to activate wild type IRE1 constitutively, which explains the constitutive *Xbp-1* mRNA splicing observed in untreated MEFs.

We next established a stable T-REx293 cell line, in which the IRE1-3F6HGFP expression construct was integrated at a unique *frr* site in the genome, and driven by a tetracycline inducible CMV promoter (Cohen and Panning 2007; Han, Lerner et al. 2009), allowing us to control the expression level of IRE1. Increasing concentrations of doxycycline (Dox) lead to constitutive activation of *Xbp-1* mRNA splicing in the absence of ER stress (Figure 3-1C, top panel, 30 and 50 nM Dox), similar to what observed in MEFs. Based on these findings, IRE1-3F6HGFP expressed as a transgene in T-REx293 cells is therefore functional. Titration of Dox allowed us to establish conditions under which

constitutive IRE1-3F6HGFP activation in the absence of ER-stress inducer tunicamycin (Tm), an inhibitor of N-linked glycosylation, was minimized (Figure 3-1C, top panel, compare lower Dox concentrations 5 and 10 nM Dox to 30 and 50 nM Dox). No IRE1-3F6HGFP fusion protein was detected by Western blotting using anti-IRE1 antibody or by fluorescent microscopy in the absence of Dox (Figures 3-1D and 3-1E), indicating that the promoter is tightly controlled. Endogenous IRE1 protein was not detected by this antibody, likely due to low abundance of IRE1 in T-REx293 cells.

Addition of Tm robustly induced *Xbp-1* mRNA splicing at all Dox concentrations (Figure 3-1C, middle and bottom panels). The splicing observed in the absence of Dox is due to endogenous IRE1 activity, whereas the splicing at higher Dox concentrations reflects the combined activities of endogenous IRE1 and exogenous IRE1-3F6HGFP. IRE1-3F6HGFP expressed at 10 nM Dox is clearly detectable by fluorescence microscopy and found diffusely distributed in the ER membrane (Figure 3-1E, top panel), as indicated by its co-localization with the ER marker protein Cherry-KDEL (Kim, Brinkmann et al. 2008) (Figure 3-1F, “Tm, 0 h”).

In contrast, upon induction of ER stress with Tm, IRE1-3F6HGFP re-localized into discrete foci in the ER membrane (Figures. 3-1E and 3-1F, “Tm, 2 h and 4 h”). These results suggest that, like in yeast, activation of mammalian IRE1 by ER stress leads to its oligomerization in the ER membrane

Oligomerization of the Kinase/RNase Domain of Human IRE1 *in vitro*

To show that IRE1 oligomerization not only correlates with its activation but also is an intrinsic mechanistic requirement for its full activity, we characterized the enzymatic activity of human IRE1 *in vitro*. To this end, we purified the cytosolic portion of human IRE1 as previously described (Niwa, Sidrauski et al. 1999). The expressed fragment contained the kinase/RNase domains extended by 43 amino acids at its N-terminus (Figure 3-2A, “KR43”). These extra 43 amino acids are part of the linker region that serves to tether IRE1’s kinase/RNase to the transmembrane domain. The extra 43-amino acid extension was included because in yeast, a similar extension of the kinase domain is required to activate Ire1 by oligomerization (Korennykh, Egea et al. 2009).

We assayed IRE1(KR43) for its activity to site-specifically cleave a 5’-³²P-labeled stem-loop oligoribonucleotide (“HP21”) derived from the *Xbp-1* mRNA (Figure 3-S1A). The observed rate constant for RNA cleavage exhibited non-Michaelis-Menten dependence on the enzyme concentration and increased cooperatively with a Hill coefficient of $n = 3.4$ (Figure 2B), which suggests that IRE1 forms a tetramer and/or larger species to be fully activated. The theoretical curve calculated for dimerization model ($n = 2$) clearly does not conform to the data observed (Figure 3-2B, dashed line). Velocity-analytical ultracentrifugation experiments revealed the presence of high-order species of IRE1 detecting up to eight-mers (Figure 3-S1B), in agreement with the observations from the kinetic studies. The lack of odd numbered oligomers indicates that, like yeast, human

IRE1 α oligomerizes by assembly of dimeric building blocks. High-order ($n > 2$) IRE1 oligomers were also observed upon gel electrophoresis after chemical crosslinking (data not shown). We conclude that the endoribonuclease activity of human IRE1 is activated by self-association of 4 or more IRE1 monomers into an oligomeric assembly, presumably similar to that observed with yeast Ire1 (Korennykh, Egea et al. 2009).

Disruption of IRE1's ER-Luminal Dimerization Interface Blocks Clustering and RNase Activity of Human IRE1

IRE1 activation occurs in response to ER stress. From the paradigms established in yeast, oligomerization of Ire1's luminal domain in response to unfolded protein accumulation serves to laterally concentrate Ire1's cytoplasmic domains, such that a threshold concentration is achieved to favor their oligomerization. Oligomerization in turn leads to *trans*-autophosphorylation and Ire1 RNase activation. In yeast, there are two luminal domain interfaces that govern Ire1 dimer and oligomer formation, and mutations to either of the two interfaces disrupt Ire1 foci formation and activation of the endoribonuclease (Credle, Finer-Moore et al. 2005; Aragon, van Anken et al. 2009). Based on the crystal structure of the luminal domain of human IRE1 (Zhou, Liu et al. 2006), we identified residue lysine (K) 121, which packs tightly within the domain's dimerization interface but is predicted to be solvent-exposed in its monomeric form (Figure 3-3A). We mutated human IRE1 K121 to a bulkier tyrosine (Y) residue to disrupt oligomerization by steric hindrance. Indeed, in *Ire1 α* ^{-/-} MEFs

reconstituted with mutant IRE1-3F6HGFP(K121Y) failed to splice *Xbp-1* mRNA (Figure 3-S2A). To monitor foci formation, we next made a stable T-REx293 cell line expressing IRE1-3F6HGFP(K121Y) under the control of the Dox inducible promoter. In contrast to IRE1-3F6HGFP, mutant IRE1-3F6HGFP(K121Y) failed to cluster into foci upon Tm treatment (Figure 3-3B). The level of IRE1-3F6HGFP (K121Y) protein stayed constant over the time course of the experiment (Figure 3-3C) and the splicing of *Xbp-1* mRNA remained identical to that observed in cells that were not treated with Dox (Figure 3-S2B). In view of these results, we conclude that oligomerization of human IRE1 is required for its activation, which is controlled by its luminal domain.

Sustained ER Stress Triggers Disassembly of IRE1 Clusters and IRE1 Dephosphorylation

We previously reported that IRE1 signaling attenuates over time when HEK293 cells are exposed to persistent and unmitigated ER stress (Lin, Li et al. 2007). Here we examined whether this IRE1 signaling attenuation is governed by the same timer as IRE1 cluster formation observed in this work. To this end, we followed IRE1 localization for 8 hours post Tm treatment. IRE1-3F6HGFP clustered into many small foci after 2 hours of Tm treatment (Figure 3-4A and Supplemental Movie 1), which, as stress persisted, converted into fewer, larger foci (Figure 3-4B middle graph). After 6 hours of Tm treatment, IRE1 foci started to dissociate and vanished completely after 8 hours. A concomitant reappearance of fluorescence staining took place in the ER network (Figures. 3-4A and 3-4B),

suggesting a diffused redistribution of IRE1-3F6HGFP over the ER membrane. The total IRE1-3F6HGFP fluorescence in foci followed the same temporal trend (Figure 3-4B lower graph). Both the percentage of cells with IRE1 foci and total level of IRE1-3F6HGFP in foci correlated well with the *Xbp-1* mRNA splicing (Figure 3-4B, top panel, and Figure 3-4C), establishing a clear connection between foci dynamics and our previous observations of dynamic IRE1 activity in HEK293 cells (Lin, Li et al. 2007). During these time courses, new IRE1-3F6HGFP synthesis was prevented by removal of Dox prior to Tm addition. To further ascertain that the disappearance of foci at later time points resulted from redistribution of IRE1-3F6HGFP molecules rather than from protein degradation, we verified that IRE1-3F6HGFP protein level did not decline over the time course of the experiment (Figure 3-4D, top panel).

We next asked whether we could detect molecular changes in IRE1 that accompany its redistribution in the ER. To this end, we monitored the phosphorylation state of IRE1 by Western blotting using a phosphorylation specific antibody. IRE1 phosphorylation peaked at 4 hours of Tm treatment and then declined after 6 hours of Tm treatment (Figure 3-4D middle panel), in synchrony with foci formation and *Xbp-1* mRNA splicing.

The kinetics of the induction of BiP, a transcriptional target of XBP-1, paralleled the kinetics of IRE1-3F6HGFP foci formation (Figure 3-S3A). The level of BiP protein continued to increase even after IRE1 foci began to dissociate

presumably due to delayed attenuation of ATF6 branch (Lin, Li et al. 2007), which also targets BiP transcriptionally (Yoshida, Okada et al. 2000; Yamamoto, Yoshida et al. 2004; Acosta-Alvear, Zhou et al. 2007).

To ascertain that ER stress remained unmitigated over the time course of the experiment, we transfected T-REx293 cells with vascular cell adhesion molecule-1 (VCAM-1), a transmembrane protein that is co-translationally inserted into the ER membrane, where it becomes N-glycosylated (Garrison, Kunkel et al. 2005). The steady-state pool of VCAM-1 was fully glycosylated at the 0 hour time point and gradually became non-glycosylated during the 8 hour time course of Tm treatment with no sign of recovery (Figure 3-S3B). Moreover, all newly synthesized VCAM-1 detected after pulse-labeling was non-glycosylated at all later time points of the time course (Figure 3-4F). Therefore, the observed attenuation of IRE1 signaling after prolonged activation occurred despite the fact that Tm-induced ER stress persisted.

Reactivation of the UPR after reestablishment of ER homeostasis

To determine if IRE1 attenuation is restricted to the particular experimental conditions employed to induce ER stress (impairment of N-glycosylation by Tm), we induced the UPR with DTT, which causes ER stress by interfering with disulfide bond formation. DTT treatment resulted in IRE1-3F6HGFP foci formation (Figure 3-5A) concomitant with *Xbp-1* mRNA splicing (Figure 3-5B), which was followed by an attenuation phase in which IRE1-3F6HGFP

redistributed in the ER membrane. The results mirror those observed with Tm treatments. Therefore, taken together, our work supports that IRE1 activity attenuation is a general phenomenon (Lin, Li et al. 2007), though the timing of attenuation differs depending on the type or the strength of the stress.

If attenuation renders IRE1 insensitive to the protein folding status in the ER, we wondered whether removal of the inducing signal (as it would normally occur during homeostatic regulation when the UPR can correct the defect) would allow the signaling machinery to reset. To this end, we induced the UPR with DTT and followed IRE1-3F6HGFP foci formation and attenuation as described above. After 4 hours, we washed out the DTT and allowed the cells to rest for 12 hours. To verify that ER homeostasis was indeed restored following DTT removal, we examined the redox states of the newly synthesized pool of glycoprotein by 2-dimensional electrophoresis at 0 hours and 2 hours of DTT treatment and post 12-hour washout (Figure 3-S4A). We observed no significant change in the redox state of the glycoprotein 12 hours post DTT washout when compared to the 0 hour untreated control. By contrast, the amount of glycoproteins isolated 2 h after DTT treatment was significantly reduced (Figure 3-S4B), which can be explained by the global translational inhibition by PERK activation.

We then added fresh DTT to reintroduce ER-stress. IRE1-3F6HGFP foci formation, IRE1 phosphorylation, and *Xbp-1* mRNA splicing occurred in a manner comparable from the process observed in naïve cells that were not subjected to a

prior round of UPR induction (Figure 3-5A and 3-5B, compare panels before and after the removal of DTT). Because Dox was removed during the DTT washout to prevent new synthesis of IRE1-3F6HGFP, these results demonstrate that cells can reset IRE1 signaling and that the molecular changes that mediate IRE1 attenuation are reversible.

Discussion

Transmembrane signaling receptors often concentrate in ultrastructural signaling centers, where dimerization or oligomerization of the signaling components serves to activate the downstream pathways. Oligomers of activated signaling receptors function as molecular platforms to which effectors are recruited, leading to their activation as their local concentration surpasses the threshold for activation (Lemmon and Schlessinger 2010). Based on our current and previous work (Kimata, Ishiwata-Kimata et al. 2007; Aragon, van Anken et al. 2009), oligomerization applies for IRE1 signaling from the ER, and the salient features of IRE1 activation appear conserved from yeast to human. Specifically, we have shown that activation of human IRE1 kinase/RNase module involves at least four IRE1 molecules, and we have visualized high-order oligomeric assembly of IRE1 in living cells.

In agreement with previous work in mammalian cells (Lin, Li et al. 2007), we found that IRE1 signaling is governed by a timer: even if the inducing stress has not been mitigated, IRE1 signaling shuts off as oligomers disassemble concomitantly with IRE1 dephosphorylation. In contrast, if ER stress is resolved, IRE1 resets so that it can become reactivated. Thus similar to other signaling receptors and ion channels, IRE1 can exist in three states (Figure 3-5C): 1) an inactive state that can be activated by ER stress, 2) an active, oligomeric state, which initiates *Xbp-1* mRNA splicing, and 3) a refractive state in which IRE1 enters after prolonged activation and in which it no longer responds to

unresolved ER stress. The inactive and refractive states are both not recognized by the anti-phospho-IRE1 antibody, suggesting that dephosphorylation plays a role in entering the refractive state, yet the molecular features that distinguish the inactive and refractive states, if any, remain unknown. More subtle differences in the phosphorylation pattern or association with other yet-to-be-identified regulators may render IRE1 refractive to activation, perhaps akin to the phosphorylation and arrestin binding to G-protein coupled receptors. Mounting evidence suggests that IRE1 phosphorylation is subject to complex regulation. A recent study revealed a role for the adaptor protein, RACK1, in modulating the dynamic activation of IRE1 α signaling in pancreatic β cells in response to physiological stimulation by glucose. RACK1 may regulate IRE1 phosphorylation by forming a ternary assembly of IRE1-RACK1-PP2A phosphatase (Qiu, Mao et al. 2010), possibly resetting IRE1 foci. If modulation of IRE1's cytosolic domain drives its disassembly, it remains puzzling how the luminal domain of IRE1 would dissociate in response to prolonged ER stress, when unfolded proteins remain in the ER. A mechanism would need to exist by which this signaling event is actively shut off. One possible explanation would allow the refractive state to be established from the ER lumen, perhaps by producing or activating a putative "IRE1 oligomerization antagonist" in the ER lumen. Such a role might be played by the excessive concentrations of BiP or other chaperones that accumulate upon prolonged ER stress. The observed change in IRE1's phosphorylation status would then be a consequence rather than the cause of IRE1 becoming refractive to ER stress. The results presented here demonstrate that prolonged

ER stress instructs IRE1 to deactivate reversibly rather than causing its degradation or inflicting other irreversible damage.

We previously proposed that attenuation of IRE1 activity upon persistent ER stress serves as the tipping point in the life-death decision such that the UPR drives cells into apoptosis if ER homeostasis cannot be restored (Lin, Li et al. 2007; Han, Upton et al. 2008; Han, Lerner et al. 2009). In previous work, IRE1 signaling was experimentally prolonged by addition of activating drugs under persistent ER stress induction regimes (Lin, Li et al. 2007; Han, Upton et al. 2008), which resulted in an increased cell survival. Attenuation of IRE1 activity in the face of persistent ER stress thus may provide a timer that limits the duration of the cytoprotective phase of the UPR, which is likely to be driven by the transcriptional regulatory network overseen by XBP-1. It is interesting to note that both apoptotic and anti-apoptotic regulators, such as BAX/BAK and BAX Inhibitor-1 associate with IRE1 directly (Hetz, Bernasconi et al. 2006; Lisbona, Rojas-Rivera et al. 2009), and it is possible that their regulated association or release from IRE1 in distinct activation states may directly affects the cell's apoptotic predisposition. Analysis of the timing of recruitment of other components to IRE1 will be a crucial next step to unravel the mechanism of UPR-linked cell death.

The foci formed by active IRE1 on the ER membrane are highly dynamic, continually changing in size and intracellular localization over the time course of

UPR activation. We have recently shown in yeast by fluorescent energy transfer that IRE1 molecules come into close physical contact in these structures (Pincus, Chevalier et al. 2010). The cooperative RNase activation shown here *in vitro* and early crosslinking studies in yeast (Shamu and Walter 1996) further corroborate the notion that active IRE1 molecules are tightly packed in foci. Such packing can provide a concentrated, specialized molecular microenvironment, which could attract low affinity binders with high avidity. Thus the dynamic foci assembly may be an additional control principle in modulating the UPR and perhaps other signaling pathways. The ability to visualize IRE1 signaling centers thus signifies an important advance to study their composition and dynamics, as well as the functional consequences that arise from these features. The results reported here therefore hold great promise to inform our understanding of general design principles of signaling pathways that deploy protein oligomerization as a mechanistic principle.

Materials and Methods

Cell Culture

T-REx293, and *Ire1α*^{-/-} mouse embryonic fibroblast (MEF) cells were maintained at 37°C, 5% CO₂ in DMEM media supplemented with fetal calf serum, glutamine, and antibiotics (Invitrogen). Tunicamycin was obtained from Calbiochem EMB Bioscience Inc. (Darmstadt). Transient and stable transfections were performed using the Lipofectamine 2000 (Invitrogen) and FuGene6 reagent (Roche). Cherry-KDEL construct was derived from pShooter plasmid pCMV-Myc-GFP-KDEL (Invitrogen). IRE1-3F6HGFP T-REx293 stable cell lines were generated using 1 µg pcDNA5/FRT (pOG44) and 0.1-0.5 µg pcDNA5-IRE1-3F6HGFP-FRT. Cells were switched to hygromycin (100 µg/ml) containing media 1 day post transfection, and the media was replaced every 3 days until colonies appeared. IRE1 expression vector pEF-IRE1-3F6HGFP vector was similarly generated in MEFs.

Live Cell Imaging

T-REx293 cells were split two days before imaging onto glass bottom microwell dishes (MatTek) at 5x10⁴ cells/dish. Dox containing media was added for 24 hours, withdrawn before imaging and replaced with Imaging media that consists of HBSS (GIBCO), 2%FBS and 5 mM HEPES at pH=7. Images were acquired on a spinning-disk confocal microscope (provided by the Nikon Imaging Center, University of California, San Francisco) consisting of an inverted

microscope (TE2000U; Nikon), a spinning-disk confocal microscope (CSU22; Yokogawa), a camera (Cascade II:512; Photometrics, and a Plan Apo VC 100x/1.4 NA oil objective lens (Nikon), and was controlled by the MicroManager program.

RNA Isolation and RT-PCR

Cells were lysed and total RNA was collected (RNAeasy, Qiagen, Germany).

PolyA mRNA was reverse-transcribed using the SuperScript-RT system (Invitrogen, Carlsbad, CA). cDNA was used as template for PCR amplification

across the fragment of the *Xbp-1* cDNA bearing the intron target of IRE1 α

ribonuclease activity. Primers used included: murine *Xbp-1*, 5'-

TTACGGGAGAAAACCTCACGGC-3' and 5'-GGGTCCAACTTGTCCAGAATGC-

3'; human *Xbp-1*, 5'-TTACGAGAGAAAACCTCATGGC-3' and 5'-

GGGTCCAAGTTGTCCAGAATGC-3'. PCR conditions were: 95°C for 5 min;

95°C for 1 min; 58°C for 30 sec; 72°C for 30 sec; 72°C for 5 min with 35 cycles of

amplification. A 289-bp amplicon was generated from unspliced *Xbp-1*; a 263-bp

amplicon was generated from spliced *Xbp-1*. PCR products were resolved on a

2.5% agarose/1x TAE gel. As previously reported, a hybrid amplicon species

consisting of unspliced *Xbp-1* annealed to spliced *Xbp-1* was also produced

through the PCR reaction and is visible as a slower migrating band above the

unspliced amplicon.

Protein Expression and Purification

Human IRE1 (KR43) was expressed in SF9 insect cells similar according to established procedures (23). Briefly, expression was performed as described in BAC-TO-BAC baculovirus expression systems. Insect SF-9 cells were grown in serum-free SF-900 II SFM media at 28°C in Erlenmeyer flasks shaking at 150 rpm. The bacmid DNA was transfected into SF9 cells, and then the virus was amplified 2 more times (at low MOI) before infection of SF9 cells for expression. Cells were lysed in 20 mM Tris/HCl, pH7.5, 600 mM NaCl, 2 mM NaCl₂, 5 mM imidazole, 10% Glycerol, 1% Triton X-100 and COMPLETE protease inhibitor (Roche), sonicated and centrifuged at 100,000xg. KR43 was N-terminally tagged with 6xHis, and was purified using Ni-NTA agarose (Qiagen), washed with 30 mM imidazole and eluted with 250 mM imidazole, followed by a size exclusion chromatography on Superdex 200 HR 10/300 (GE) column in buffer contains 20 mM HEPES pH7.5, 300 mM NaCl, 2 mM MgCl₂, 5% Glycerol and 5 mM DTT. We obtained ~ 200 ug of purified KR43/ 150 ml of SF9 cells and the purified protein is stored at –80°C.

Protein Analysis and Metabolic Labeling

Cells were lysed by sonication in 6 M Urea, 20 mM HEPES pH 8.0, containing protease (Roche, Basel, Switzerland) and phosphatase inhibitors (Pierce, Rockford, IL). 20 to 30 µg of total cell lysates was loaded onto 10% SDS-PAGE minigels (NuPAGE, Invitrogen) and analyzed by Western blot. The following antibodies and dilutions were used: anti-GAPDH at 1:5000 (AbCAM, Cambridge,

MA); anti-VCAM-1 at 1:500 (Santa Cruz Biotechnologies, Santa Cruz, CA); After overnight incubation with primary antibody, membranes were washed in PBS with Tween, and incubated in horseradish peroxidase-coupled secondary antibody (Amersham, Piscataway, NJ) diluted at 1:10000 in washing buffer.

Immunoreactivity was detected using the enhanced chemiluminescence assay (Pierce, Rockford, IL). For VCAM-1 radiolabeling, cells were pulsed with 50 μ Ci of EXPRES³⁵S Protein Labeling Mix (Perkin-Elmer, Waltham, MA) for 15 minutes, lysed in 1% NP-40 with protease and phosphatase inhibitors, incubated with anti-VCAM-1 and Protein A-sepharose beads (Sigma, St. Louis, MO). After washing, immunoprecipitates were loaded onto SDS-PAGE gels, and ³⁵S-labeled VCAM-1 was detected by phospho-imaging analysis.

Preparation of RNA substrates and Kinetics

HP21 21-base polymers were purchased from Dharmacon Inc. Before use, the oligonucleotides were purified by a denaturing (8 M urea) 5–20% polyacrylamide gel electrophoresis (PAGE). Cross-linked 29:1 polyacrylamide (40%) was purchased from National Diagnostics. Gel slices containing RNA were eluted in TE buffer and ethanol-precipitated. Substrates labeled with ³²P at the 5'-terminus were prepared using T4 PNK (NEB) and gamma-³²P-ATP (Perkin Elmer). All ³²P-labelled substrates were purified by denaturing 5–20% PAGE, eluted in TE and ethanol-precipitated before using. Kinetics assays were done at 30°C and neutral pH in buffer containing 20 mM HEPES, 70 mM NaCl, 2 mM Mg(OAc)₂, 5% Glycerol and 4 mM DTT.

2-D Gel Electrophoresis

Cells were labeled with 100 μ Ci of EXPRES³⁵S Protein Labeling Mix (Perkin-Elmer, Waltham, MA) for 20 minutes, and were lysed in RIPA protein buffer with protease and phosphatase inhibitors. Total glycoprotein was isolated using a ConA Glycoprotein Isolation Kit (Pierce). Protein samples were first separated under non-reducing condition followed by reducing condition using 12% SDS-PAGE 2D Gel (Invitrogen).

MatLab Scripts

```
IRE1im = imread('1r.tif');
IRE1im = uint32(IRE1im);
BGRNDim = imread('1b.tif');
BGRNDim = uint32(BGRNDim);
IRE1_background = 1.01;
threshold_IRE1_foci = 1.16;
h = ones(5,5) / 25;
IRE1im=imfilter(IRE1im,h,'replicate');
TotIRE1 = IRE1im - IRE1_background*mean(mean(BGRNDim));
IRE1_in_foci = IRE1im - threshold_IRE1_foci*mean(mean(BGRNDim));
IRE1_foci_mask = im2bw(single(IRE1_in_foci),1);
IRE1_in_fociQ = single(TotIRE1).*IRE1_foci_mask;
Fraction_IRE1_in_Foci = mean(mean(IRE1_in_fociQ))/mean(mean(TotIRE1))
subplot(1,3,1);imagesc(IRE1im);
subplot(1,3,2);imagesc(TotIRE1);
subplot(1,3,3);imagesc(IRE1_foci_mask);
mean(mean(TotIRE1))
mean(mean(IRE1_in_fociQ))
IRE1im = imread('1r.tif');
IRE1im = uint32(IRE1im);
BGRNDim = imread('1b.tif');
BGRNDim = uint32(BGRNDim);
IRE1_background = 1.01;
threshold_IRE1_foci = 1.16;
h = ones(5,5) / 25;
IRE1im=imfilter(IRE1im,h,'replicate');
TotIRE1 = IRE1im - IRE1_background*mean(mean(BGRNDim));
IRE1_in_foci = IRE1im - threshold_IRE1_foci*mean(mean(BGRNDim));
```

```

IRE1_foci_mask = im2bw(single(IRE1_in_foci),1);
IRE1_in_fociQ = single(TotIRE1).*IRE1_foci_mask;
Fraction_IRE1_in_Foci = mean(mean(IRE1_in_fociQ))/mean(mean(TotIRE1))
subplot(1,3,1);imagesc(IRE1im);
subplot(1,3,2);imagesc(TotIRE1);
subplot(1,3,3);imagesc(IRE1_foci_mask);
mean(mean(TotIRE1))
mean(mean(IRE1_in_fociQ))

```

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Figure Legends

Figure 3-1. IRE1 Clustering in the ER Membrane of Human Cells

A. Schematic of IRE1-3F6HGFP imaging construct: The luminal domain ("LD"), transmembrane domain ("TM"), linker domain ("L"), kinase domain ("K") and endoribonuclease domain ("R") of human IRE1 and the inserted tag consisting of three FLAG epitopes followed by six histidines and a GFP ("3F6H", "GFP") are indicated. B. IRE1-3F6HGFP construct rescues *Xbp-1* mRNA splicing in *Ire1α*^{-/-} MEFs. *Xbp-1* mRNA splicing was determined by RT-PCR. The products resulting from unspliced ("u") and spliced ("s") XBP1 mRNA are indicated. The asterisk identifies a hybrid amplicon resulting from spliced and unspliced *Xbp-1* mRNA. C. Doxycycline ("Dox") titration in T-REx293 cells +/- 5 µg/ml tunicamycin (Tm). Dox was added to the cells for 24 hours and withdrawn before Tm treatment. *Xbp-1* mRNA splicing was determined by RT-PCR as in Panel B. D. IRE1-3F6HGFP protein levels were determined by Western blotting using anti-IRE1 antibodies. Blotting for the metabolic enzyme GAPDH was used as a loading control. E. Localization and clustering of IRE1-3F6HGFP in stably transfected T-REx293 cells. Fluorescent and bright field images of cells grown +/- 10 nM Dox before and after a 4 h treatment with 5 µg/ml Tm. Scale bar = 10 µm. F. Stable T-REx293 cells bearing IRE1-3F6HGFP allele were transiently transfected with Cherry-KDEL construct to co-visualize the ER.

Figure 3-2. Oligomerization of the Kinase/RNase Domain of Human IRE1 *in vitro*

A. Schematic of the IRE1(KR43) construct expressed in insect SF9 cells. IRE1 (KR43) consists a 43 amino acid portion of linker, kinase (“K”) and endoribonuclease (“R”) domains of human IRE1. B. Cooperative activation profile for IRE1(KR43) obtained using 5’-³²P-HP21. The solid curve indicates the profile of IRE1(KR43) with $n = 3.4$, and the dashed curve indicates the predicted activation profile with $n = 2$ for comparison purpose. Schematic representation of RNA substrate (HP21) used in the *in vitro* experiments. Arrowhead marks the site of specific cleavage by human IRE1 (KR43).

Figure 3-3. Disruption of IRE1’s ER-lumenal Dimerization Interface Blocks Clustering and RNase Activity of Human IRE1

A. Crystal structure of human IRE1 luminal domain dimer and monomer (PDB ID code 2HZ6). The structure identifies K121 as a tightly packed residue at the dimerizing interface. B. IRE1-3F6HGFP(K121Y) mutant construct localization with Dox (10 nM) and Tm (5 ug/ml) treatment for indicated hours. C. The level of IRE1-3F6HGFP(K121Y) protein expressed is examined via immunoblotting against total 3F6HGFP-IRE1 under 10 nM Dox induction.

Figure 3-4. Sustained ER Stress Triggers Disassembly of IRE1 Clusters and IRE1 Dephosphorylation

Stably transformed T-REx293 cells were grown in media containing 10 nM Dox. At time 0, the medium was replaced to media containing no Dox but 5 µg/ml Tm to induce ER stress. A. IRE1-3F6HGFP localization over 8 h time course. B. Quantification of the time course experiment: percentage of cells with IRE1 foci, average number of IRE1 foci per cell and percentage of IRE1 in foci were determined as described in Methods. Error bars represent the standard error of the mean (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$). The statistical significance of the difference between later time points and 0 h is indicated. C. *Xbp-1* mRNA splicing was determined by RT-PCR and quantified. D. Total and phospho-IRE1-3F6HGFP protein levels were determined by immunoblotting. Histograms represent the ratio of phosphorylated-IRE1-3F6HGFP and total IRE1-3F6HGFP. E. T-REx293 cells were pulse-labeled for 1 hour with ^{35}S -methionine at the indicated times after beginning of the Tm treatment. Radiolabeled VCAM-1 (“g”: glycosylated, “ng”: nonglycosylated) was detected after immunoprecipitation and SDS gel electrophoresis.

Figure 3-5. Reactivation of the UPR After Restoration of ER Homeostasis

A. T-REx293 cells were treated with 10 nM Dox and DTT (1 mM) for the indicated time. After 4 hours of DTT treatment, DTT-containing media was exchanged for regular media for 12 hours. Cells were then re-exposed to ER stress by treatment with either DTT (1 mM) or Tm (5 µg/ml). B. Splicing was determined by

RT-PCR. Immunoreactive bands for total IRE1 and phosphorylated IRE1 were detected by Western blotting as in Figure 4. The normalized ratio of the signals from the two blots is displayed. C. Three state model for activation of mammalian IRE1.

Figure 3-S1. Oligomerization of kinase/RNase domain of human IRE1 in vitro

A. *in vitro* cleavage of 5' labeled HP21 derived from *Xbp-1* mRNA by IRE1 (KR43). B. Analytical ultracentrifugation. IRE1(KR43) was sedimented for 6 h in Beckman Optima XL-A ultracentrifuge. Sedimentation profiles were analyzed with UI-trascan (by B. Demeler) to determine the distribution of oligomeric IRE1 (KR43) species in solution.

Figure 3-S2. IRE1-3F6HGFP(K121Y) fails to splice *Xbp-1* mRNA

A. *Ire1α*^{-/-} MEFS were reconstituted with the IRE1-3F6HGFP(K121Y) allele. *Xbp-1* mRNA splicing was determined by RT-PCR. IRE1-3F6HGFP(K121Y) protein level is examined using total IRE1 antibody. B. *Xbp-1* mRNA splicing in T-REx293 cells stably transfected with IRE1-3F6HGFP(K121Y) construct under 0 or 10 uM Dox induction. *Xbp-1* mRNA splicing was determined by RT-PCR. Asterisks demote hybrid PCR product resulting from the spliced (s) and unspliced (u) *Xbp-1* mRNA

Figure 3-S3. ER stress remained unmitigated after IRE1 activity attenuation

A. BiP protein expression level is also examined under the same conditions as panel 4D. B. VCAM-1 was transiently transfected into T-REx293 cells that harbor a stably integrated allele of IRE1-3F6HGFP. Cells were treated with 10 nM Dox and 5 ug/ml Tm for indicated time. Mature and non-glycosylated VCAM-1 species were determined by immunoblotting.

Figure 3-S4. ER homeostasis was restored after 12-hour washout

A. Total glycoproteins were isolated from T-REx293 cells before and after DTT (1 mM) treatment and removal at the indicated times. 2-D electrophoresis was performed in first non-reducing (1D) and then reducing (2D) conditions. B. Quantification of integrated total signal from the pulse experiment at indicated time.

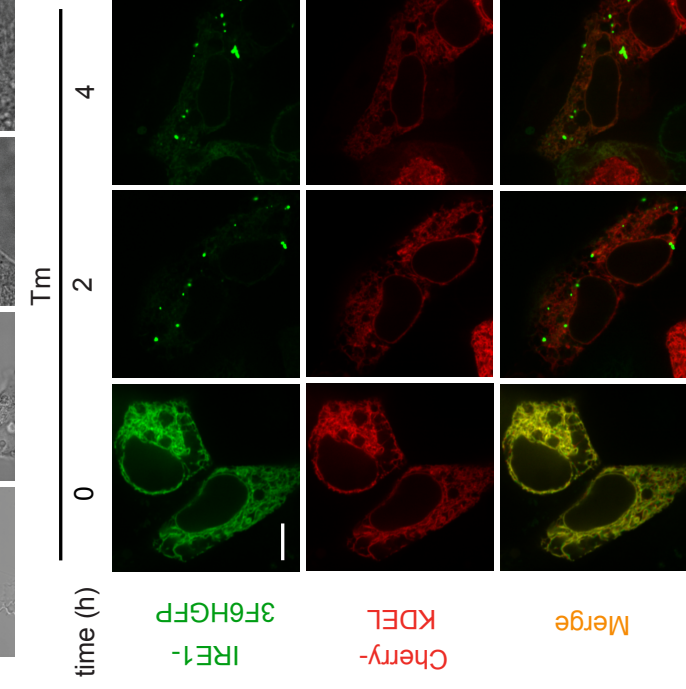
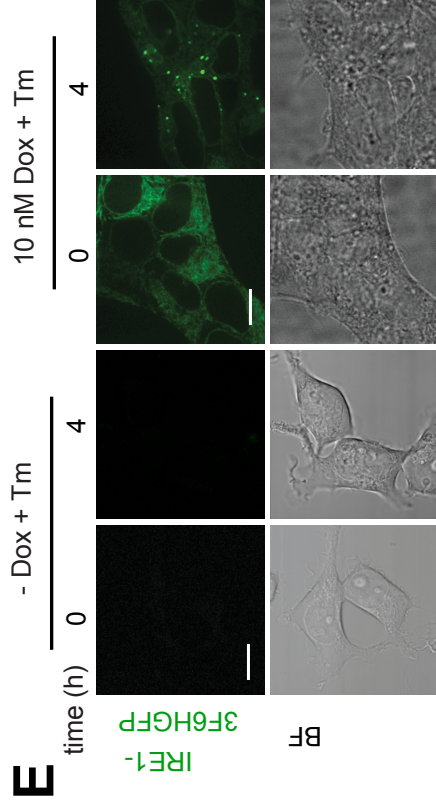
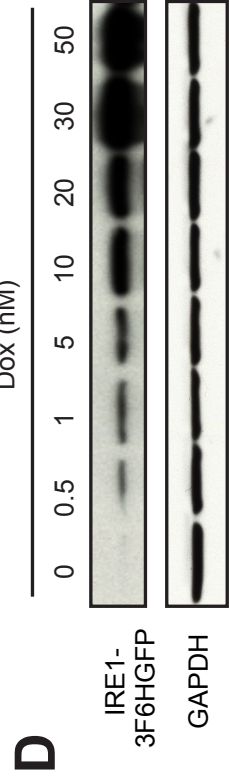
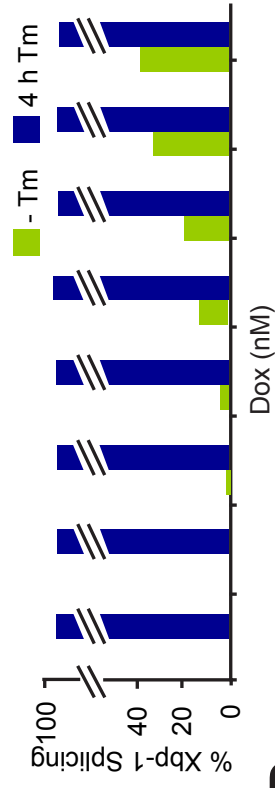
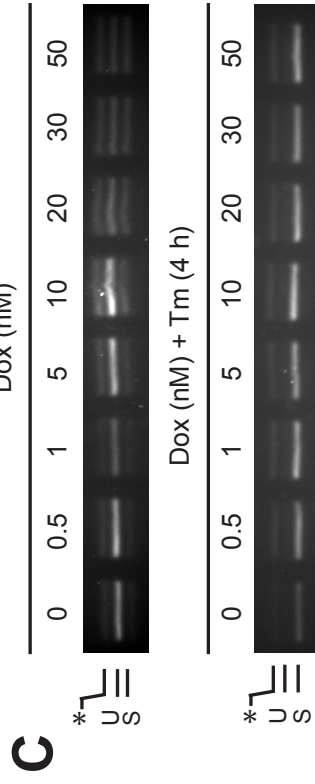
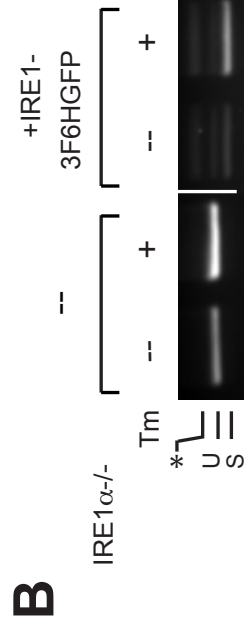


Figure 3-1

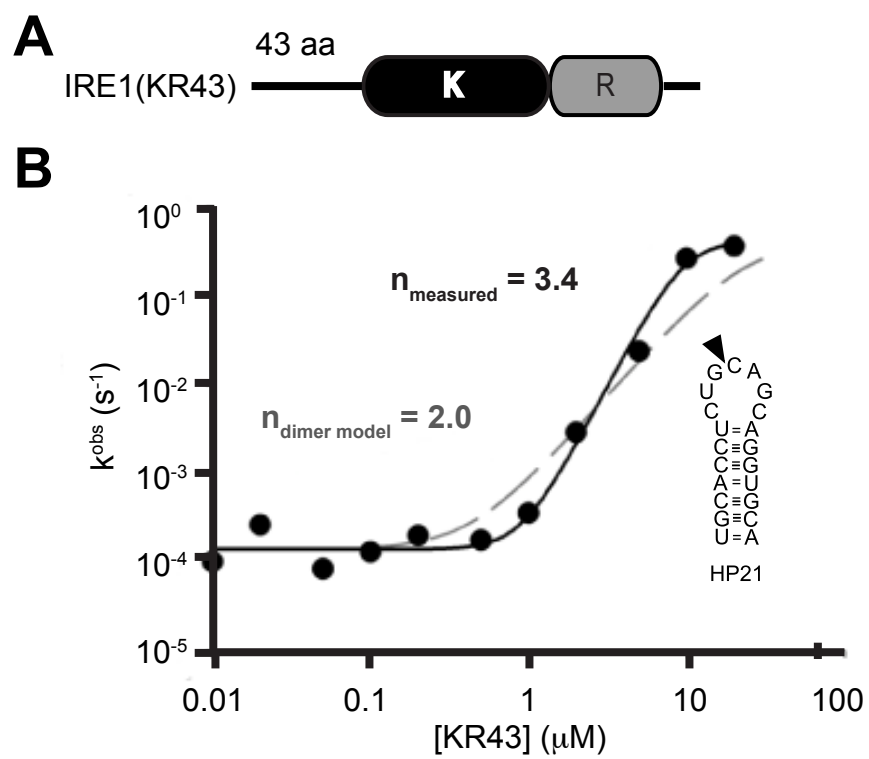


Figure 3-2

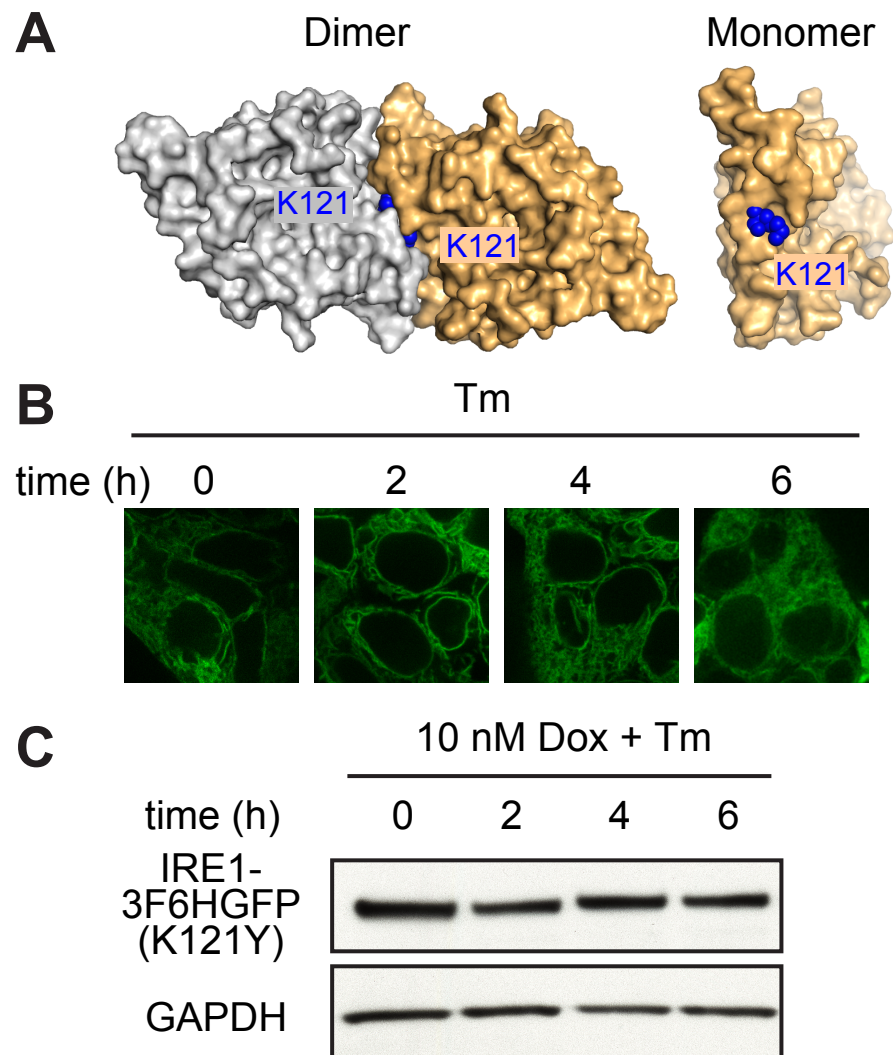


Figure 3-3

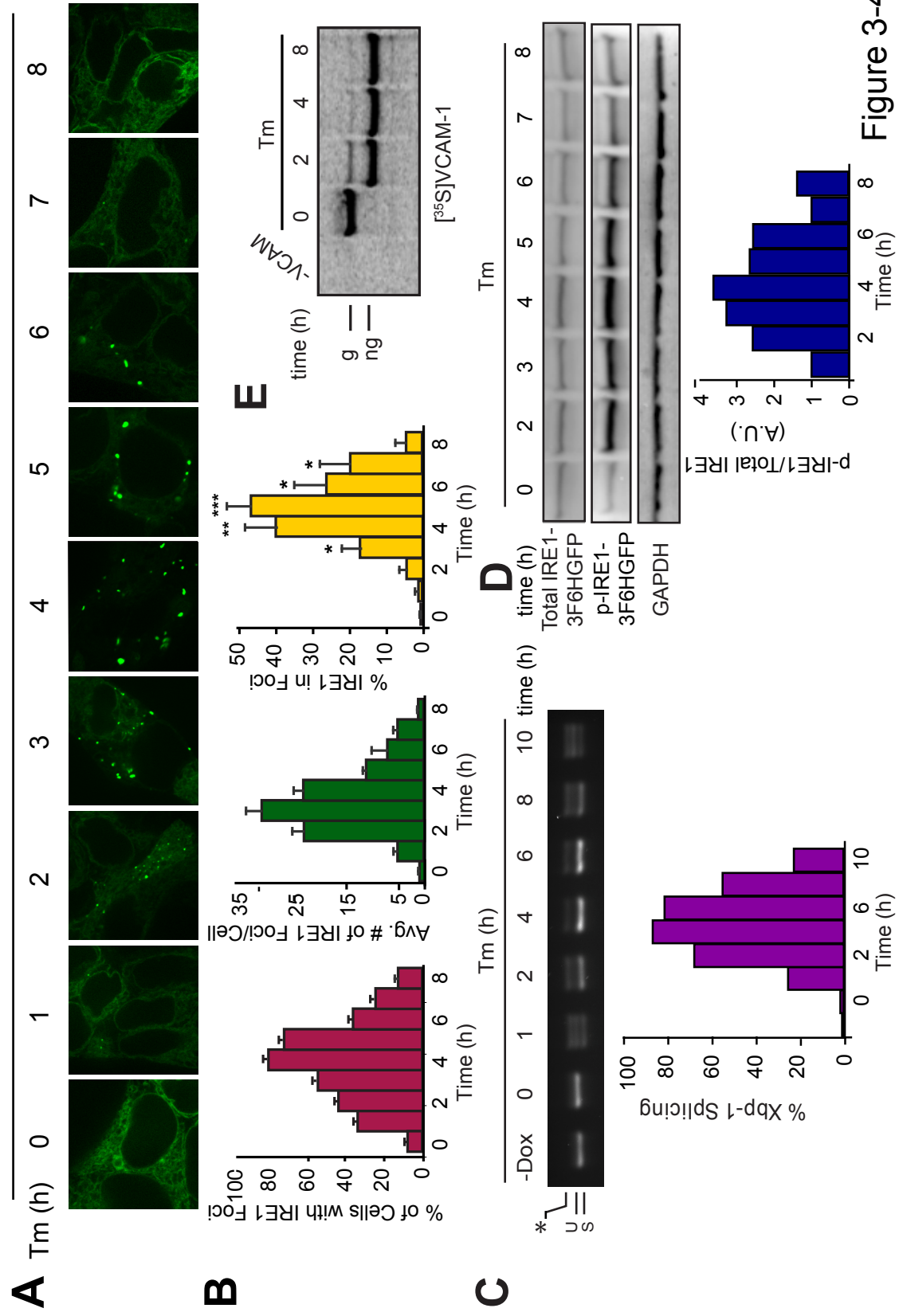


Figure 3-4

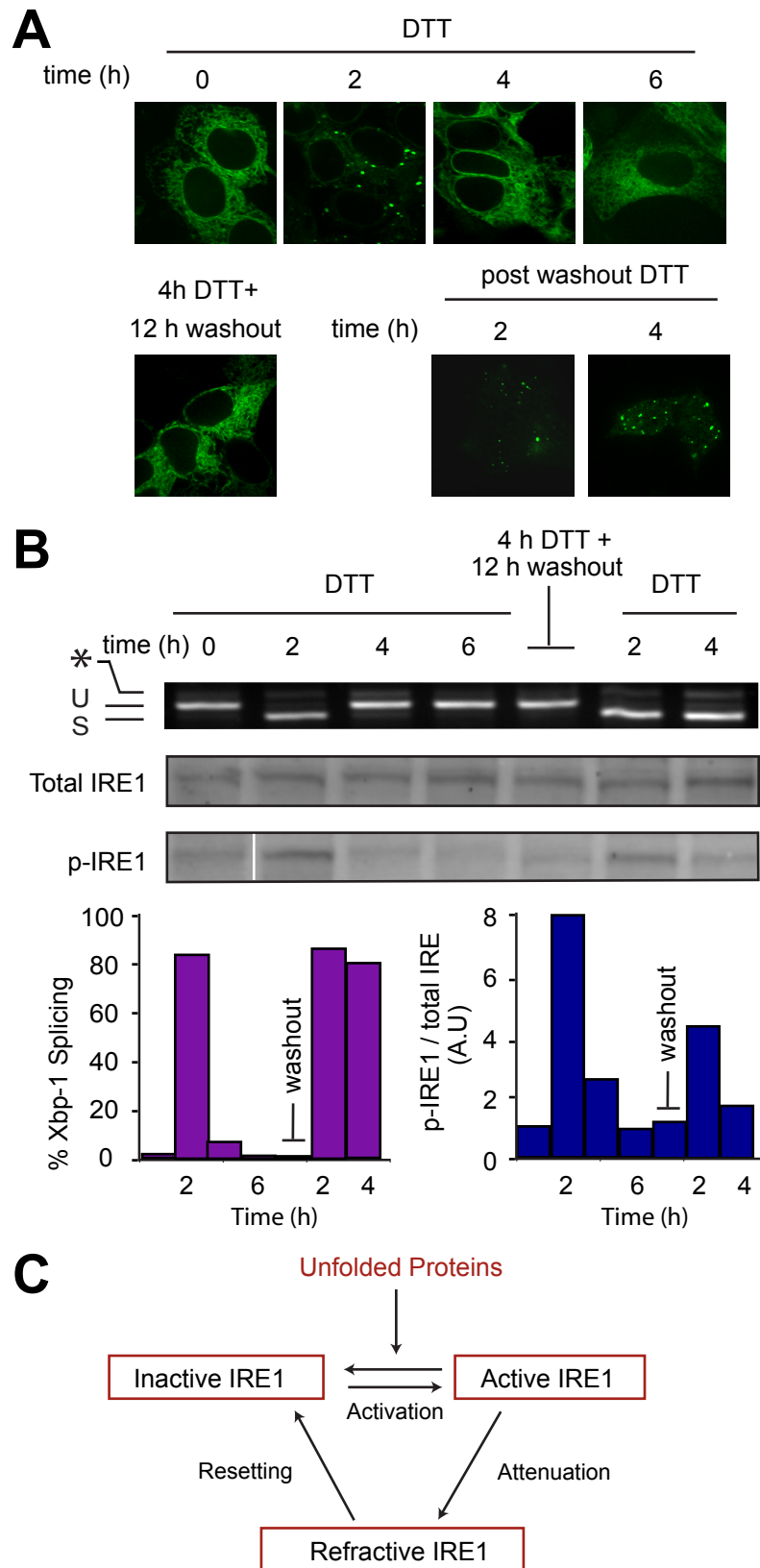


Figure 3-5

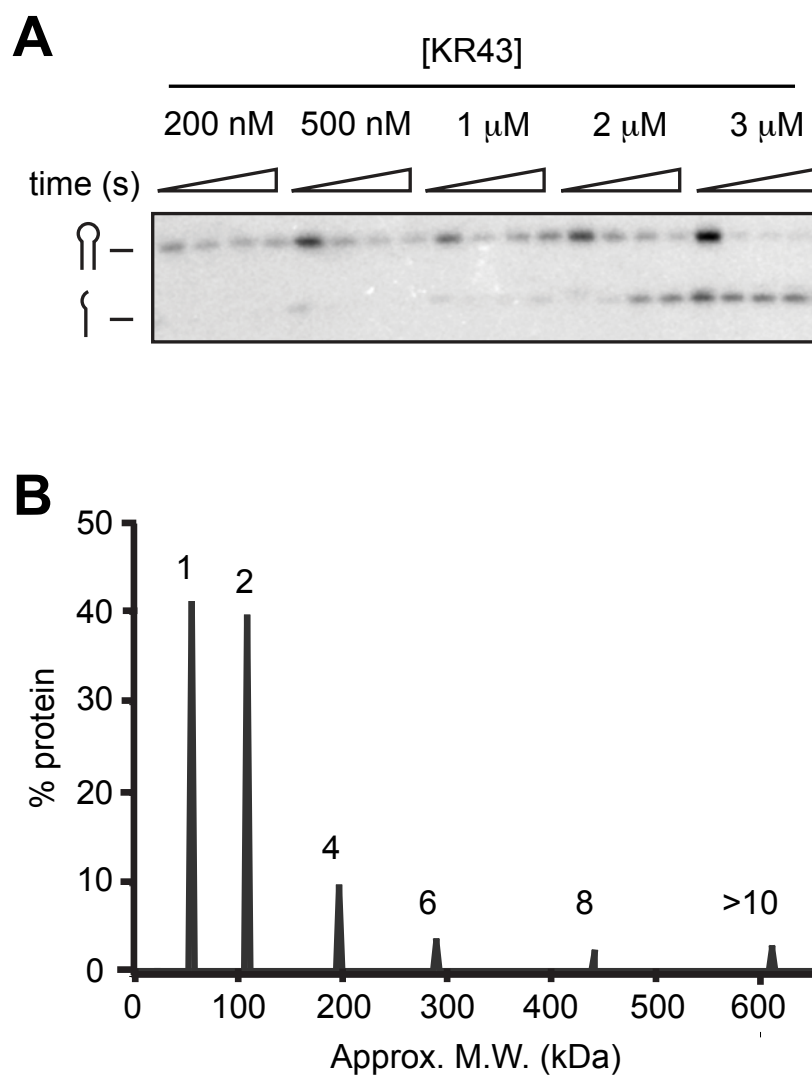


Figure 3-S1

A

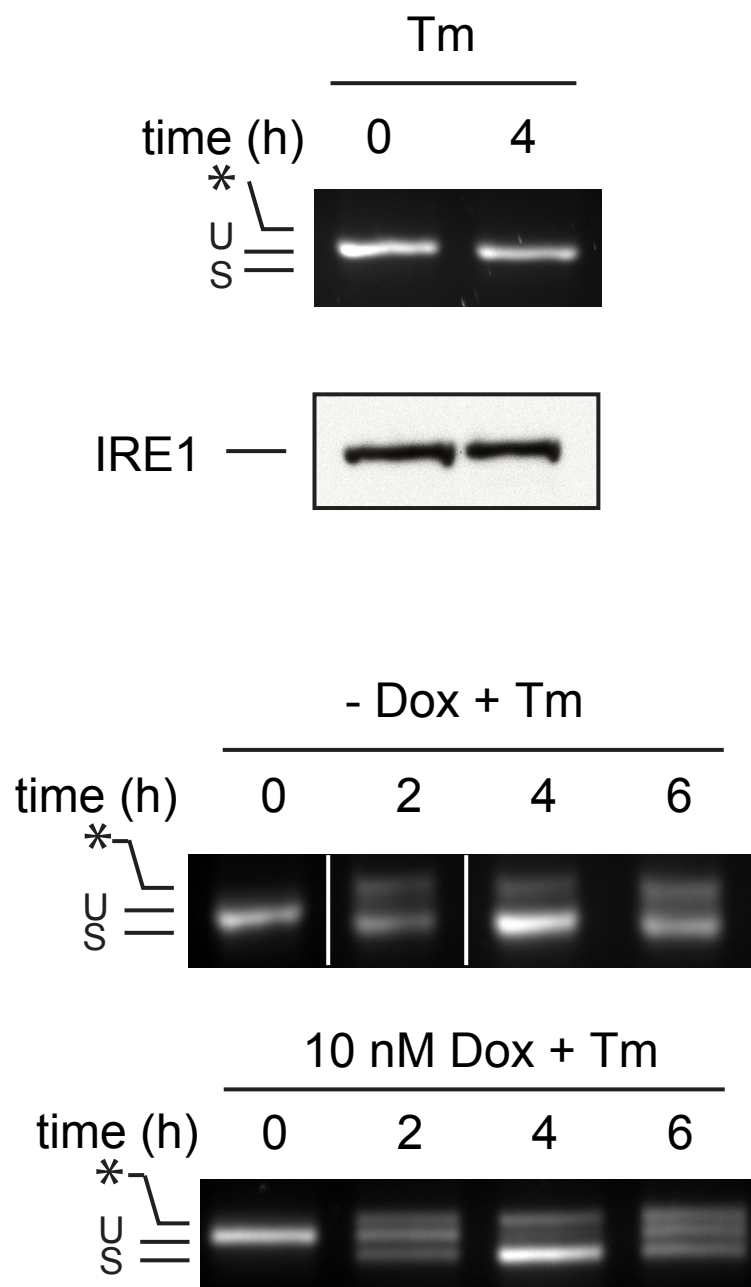
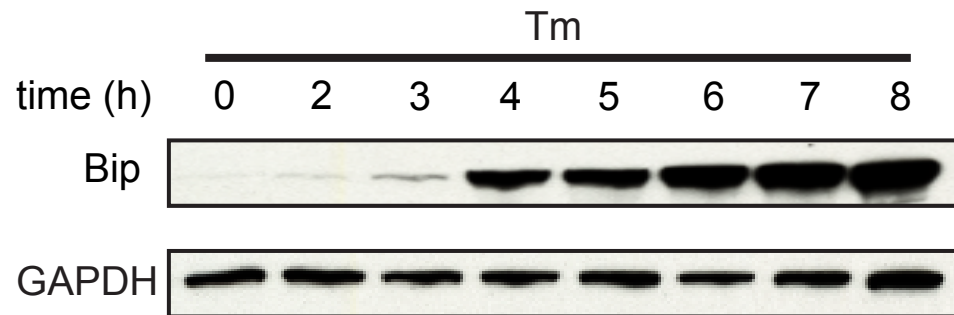


Figure 3-S2

A



B

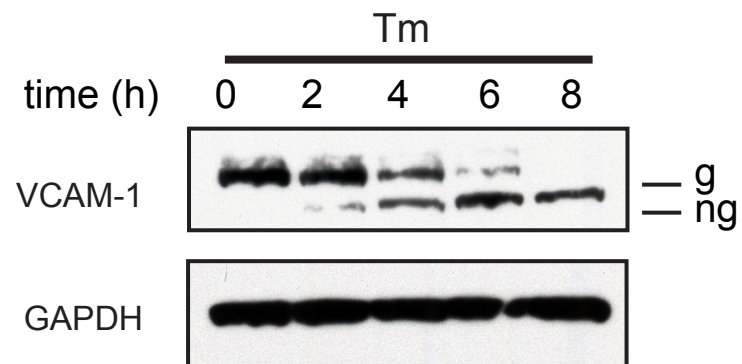


Figure 3-S3

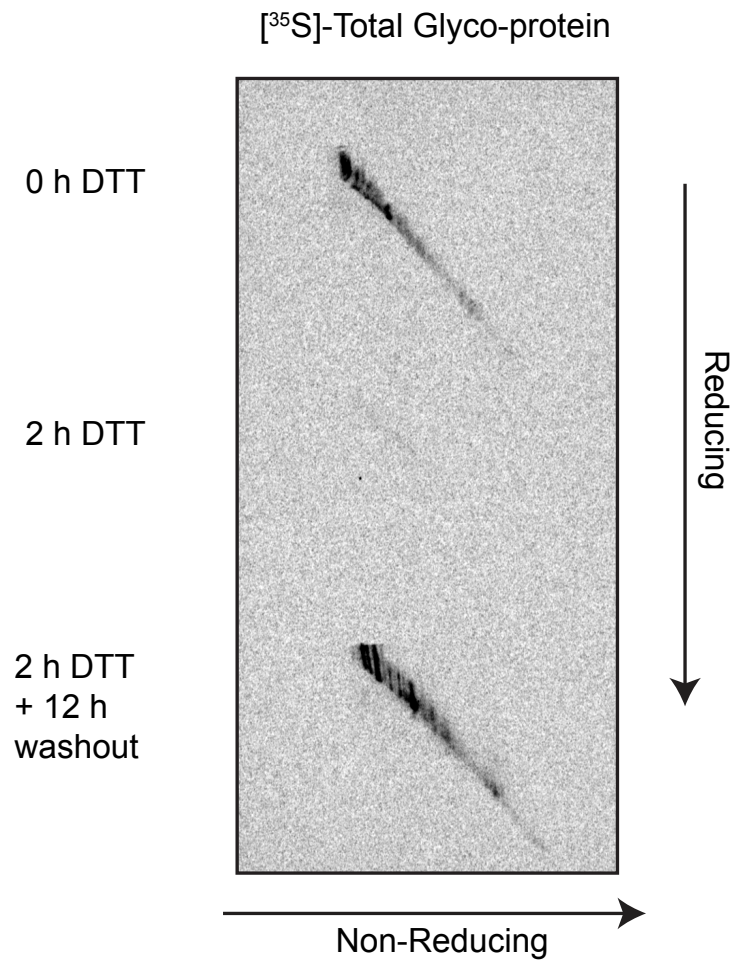
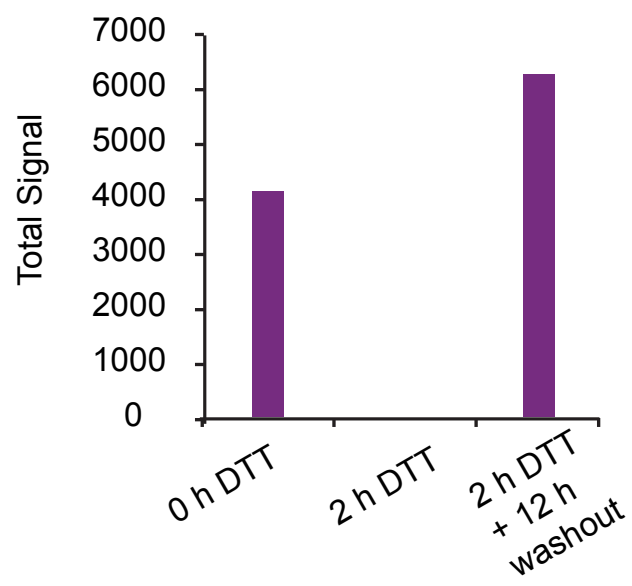
A**B**

Figure 3-S4

Appendix A:

**Human IRE1 *in vitro* enzymatic activity
characterization**

In yeast, the length of the linker region controls the oligomerization and activation of Ire1 (Korennykh, Egea et al. 2009). Korennykh *et al.* prepared variants of the cytosolic portion of yeast Ire1 that contains the kinase and RNase domains as well as 24, 32 and 120 amino acids linker region. *In vitro* RNase cleavage result revealed that only Ire1 construct extended by 32 a.a linker exhibited high-order oligomer assembly, 24 and 120 a.a. linker construct both formed predominantly dimers.

We purified human IRE1 cytosolic domain to verify that oligomerization is an intrinsic mechanistic requirement for its full activation. We included 43 amino acids of the linker region of human IRE1 (KR43), which serves to tether IRE1's kinase/RNase to the transmembrane domain. The extra 43-a.a. extension was included because it is homologous to the 32-a.a. construct in yeast. In addition to KR43, we purified other variants of human IRE1, including IRE1 KR105, and KR105-pro. KR105 contains human IRE1 full length linker region. KR105-pro contains IRE1 full length linker and a deletion of C-terminus proline rich region. In mammals, the Src homology 2/3 (SH2/SH3) domain-containing protein Nck-1 was found to interact with IRE1 and regulates IRE1 signaling and IRE1-dependent activation of ERK (Nguyen, Kebache et al. 2004). The classical SH3 domain is usually found in proteins that interact with other proteins and mediate assembly of specific protein complexes, typically via binding to proline-rich peptides in their respective binding partner. Hence, we set out to examine whether deletion of proline-rich region *in vitro* affects human IRE1 self-assembly.

Unlike in yeast, the length of linker has no effect on IRE1 self-association. KR105 and KR43 exhibited non-Michaelis-Menten dependence on enzyme concentration and increase cooperatively with a Hill coefficient of $n=2.4$ and 2.3 respectively, suggesting that that IRE1 activation requires at least dimers (Figure A1 red and black curves). Deletion of proline-rich region also had no effect on IRE1 activity (Figure A1 green curve).

In yeast, addition of cofactor ADP increased Ire1 enzymatic activity but reduced it cooperativity (Korennykh, Egea et al. 2009). We next explored whether cofactors affect human IRE1 *in vitro* activity in a similar fashion. To this end, we used KR43 construct to perform the *in vitro* experiment in the presence of various cofactors: ADP-Mg, ATP-Mg or ASC24, a proven activator of yeast Ire1. Addition of ASC24 showed a slight increase in human KR43 RNase activity, but neither ADP-Mg or ATP-Mg had any effect on the rate of the cleavage reaction (Figure A2). Furthermore, none of the cofactors affected the cooperativity of IRE1 activation, all reactions exhibited the same Hill coefficient, $n=2.5$.

We later improved human IRE1 purification protocol. Newly purified KR43 exhibited an improved Hill coefficient, $n=3.75$, indicating that IRE1 forms more than tetramers for its activation (Figure A3). This purification method was later used in our manuscript Li *et al.* 2010 and IRE1 production was scaled up to produce milligram quantity of functional human IRE1 for an *in vitro* drug screen.

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Figure legends

Figure A1. Human IRE1 *in vitro* construct variants and their activation profiles

Cooperative activation profile for KR43 obtained using 5'-³²P-HP21 substrates. Black curve indicates profile of KR105 with $n=2.4$, red curve indicates profile of KR43 of $n=2.3$, and green curve indicates profile of KR105-pro with $n=2.4$.

Figure A2. Cofactors has no effect on KR43 *in vitro* cleavage activity

Cooperative activation profile for KR43 in the presence of cofactors using 5'-³²P-HP21 substrates. Black curve indicates the activation profile for KR43 only. Green curve denotes the profile of KR43 with 2 mM ATP-Mg with $n=2.5$. Red curve indicates the profile of KR43 with 2 mM ADP-Mg with $n=2.5$. Magenta curve indicates the profile of KR43 with 2 mM ASC24 with $n=2.5$. Chemical structure of ASC24 is shown as well.

Figure A3. Improved KR43 *in vitro* activity

Cooperative activation profile new method purified KR43 vs old method purified KR43 using 5'-³²P-HP21 substrates. Black curve indicates the activation profile for old method purified KR43 with $n=2.2$. Magenta curve indicates the profile for the new method purified KR43 with $n=3.75$.

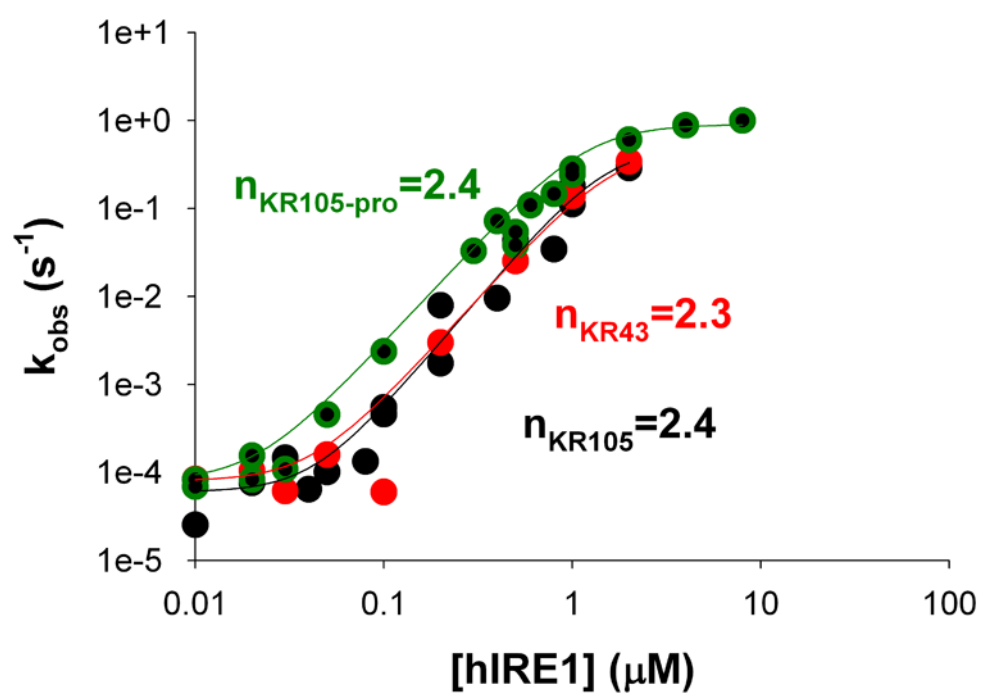


Figure A1

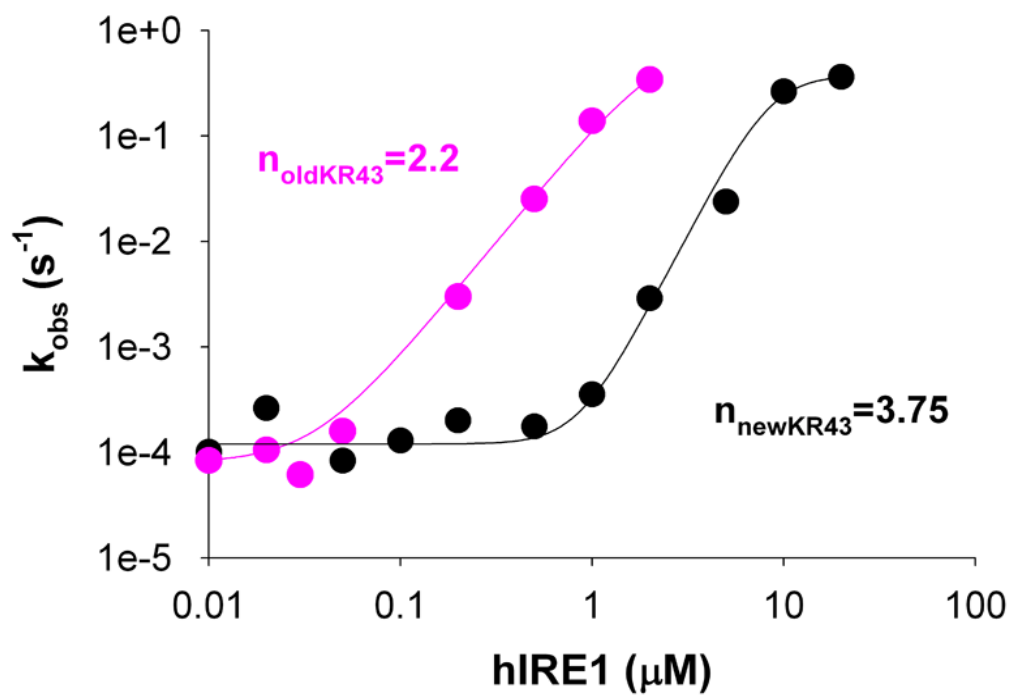


Figure A3

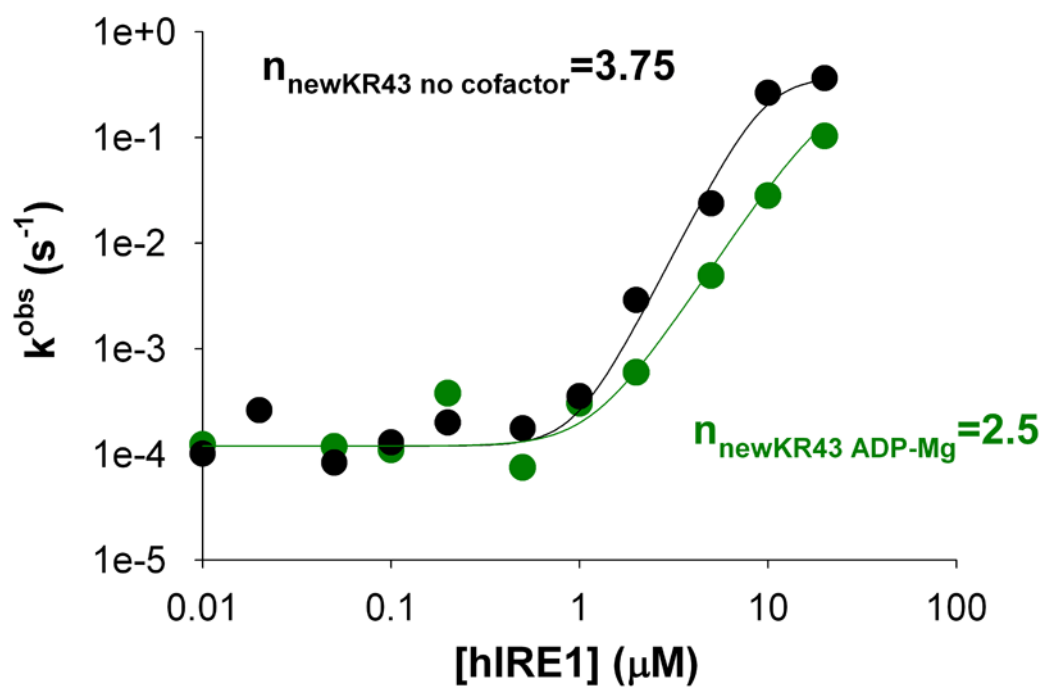


Figure A4

Appendix B:
Dissecting IRE1 signaling regulation mechanism

Many studies have suggested that IRE1 activity is regulated by different factors, including the phosphatase PTP-1B (Gu, Nguyen et al. 2004), ASK1-interacting protein 1 (AIP1) (Luo, He et al. 2008), members of pro- and anti-apoptotic families (Baillly-Maitre, Fondevila et al. 2006; Hetz, Bernasconi et al. 2006; Lisbona, Rojas-Rivera et al. 2009), as well as scaffold protein receptor for activated C-kinase 1 (RACK1) complex that includes protein phosphatase 2A (PP2A) (Qiu, Mao et al. 2010). All of these factors were found to associate with IRE1 upon ER stress; however, all of these interactions were determined by immunoprecipitation IRE1 and immunoblotting proteins of interest that are always over-expressed. It is interesting to note that both apoptotic and anti-apoptotic factors, such as BAX/BAK and BAX inhibitor-1 (BI-1), associate with IRE1 directly. We've shown that IRE1 signal attenuates over prolonged ER stress; hence, the timing of the recruitment of these components to IRE1 is crucial to unravel the mechanism of UPR-linked cell death.

To dissect IRE1 signaling regulation mechanism, we took an unbiased proteomic approach to identify IRE1 interacting partners under prolonged ER stress via immunoprecipitation followed by mass-spectrometry (MS). We initially carried out our strategy in yeast because the ease of generating stable cell lines and validating the method. To this end, we placed a 3-FLAG-6-His (3F6H) epitope at the C-terminus of endogenous yeast Ire1. The tagged Ire1 showed no growth defect, as compared to endogenous untagged Ire1 (Figure B1). We also assessed Ire1-3F6H's ability to cleave *HAC1* mRNA under ER stress. Even

though the northern blot showed a decreased total mRNA level, the percentage of spliced *HAC1* mRNA is comparable to that of endogenous wildtype Ire1 (Figure B2, compare lanes 2 and 6). C-terminally tagged GFP construct IRE1-GFP3F6H, on the other hand, showed a significant decline in *HAC1* mRNA cleavage, suggesting the large epitope might hinder Ire1 activity possibly by blocking *HAC1* mRNA's accessibility to Ire1 RNase domain (Figure B2). Both growth phenotype and splicing results suggested that 3F6H tagged Ire1 is functional.

We next immunoprecipitated Ire1-3F6H from yeast cells, which was followed by the mass-spectrometry analysis of precipitated protein complexes. We first tested two different detergents (TritonX-100 and Empigen) at different concentrations on solubilizing Ire1 since Ire1 is an ER membrane protein. TritonX-100 worked more effectively at extracting Ire1 and its interacting proteins from the membrane, as more Ire1 is precipitated together with a much larger protein complex (Figure B3, compare lanes 3, 4, 5 with 6,7,8). We used the optimized IP protocol, immunoprecipitated Ire1 in the presence and absence of DTT (Figure B4), and the samples were analyzed by tandem mass-spectrometry (MS/MS). Even though Kar2, the molecular chaperone that's known to interact with Ire1, was detected in both -DTT and +DTT conditions, the number of peptides detected in the presence of ER stress was significantly less, suggesting the dissociation of Kar2 upon misfolded protein accumulation, which is in agreement to previous studies (Bertolotti, Zhang et al. 2000; Kimata, Kimata et

al. 2003). Other MS identified proteins were mostly house-keeping proteins that are in high abundance and have little connection to ER stress or Ire1, hence they were considered insignificant hits. Learning a great lesson from yeast about immunoprecipitating Ire1 interacting complex from whole cell extract, we continued our pursuit of IRE1 regulators in mammalian cells.

To this end, we generated a stable T-REx293 cell line, in which IRE1-3F6H expression construct was integrated at a unique *frt* site in the genome, and was driven by a tetracycline-inducible CMV promoter (Cohen and Panning 2007; Han, Lerner et al. 2009). We immunoprecipitated human IRE1-3F6H from T-REx293 cells at 0, 4 and 8 hours of tunicamycin (Tm) treatment (Figure B5), and we used non-induced cells as a control. We were looking for proteins recruited to or dissociated from IRE1 in a stress and stress duration dependent manner. Samples from immunoprecipitation were analyzed by tandem mass spectrometry (MS/MS), the list of hits were shown in the supplemental table 1. Among the hits, potentially interesting proteins were re-organized in supplemental table 2.

One of the proteins that we decided to follow up on is phosphoglycerate mutase 5 (PGAM5), which was only present when IRE1 expression was induced and ER stress was absent (Table BS2). A recent study showed that PGAM5 functioned as a Ser/Thr phosphatase and served as a specialized activator of ASK1 (Takeda, Komuro et al. 2009). ASK1 has previously been linked to ER stress induced JNK activation previously (Nishitoh, Matsuzawa et al. 2002). We asked whether PGAM5 plays a role in regulating IRE1's phosphorylation state. We

monitored the level of total PGAM5 protein over prolonged ER stress (Figure B6A). In agreement with the MS data, the level of PGAM5 decreased over 8 hours of ER stress. Since PGAM5 dephosphorylates the inhibitory site of ASK1, thus induces ASK1 activation, we next examined whether ASK1 activation correlates with PGAM5 level upon ER stress. We followed phospho- and total-ASK1 protein level over the same time course. Phospho-ASK1 protein level peaked at 4 hours and declined after 6 hours of tunicamycin treatment (Figure B6B), and the total ASK1 level stayed constant over the time course. This result is consistent with our previous observation on JNK activation (Lin, Li et al. 2007) and IRE1 signaling (Li, Korennykh et al. 2010) upon ER stress. Hence, PGAM5 level does not correlate with ASK1 activation upon ER stress. It is possible that PGAM5 is only responsible for initial releasing ASK1 from the inhibitory state, and is dispensable at later time point under prolonged ER stress.

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Figure Legends

Figure B1. 3F6H- and 3F6HGFP- tagged yeast Ire1 growth phenotype

Yeast cell viability assay by 1:5 serial dilution of wildtype, *ylre1*-3F6H and *ylre1*-3F6HGFP strains in normal YPD media, under inositol deprivation or in the presence of 0.2 µg/ml Tunicamycin (Tm).

Figure B2. 3F6H- and 3F6HGFP- tagged yeast Ire1 functionality

Northern blotting of *HAC1* mRNA splicing before and after induction with DTT (10 mM) for 45 minutes. Unspliced *HAC1* mRNA is denoted as “u”, and spliced *HAC1* mRNA is denoted as “i”. Scr1 is a loading control. The percentage of *HAC1* cleavage is quantified in the bottom panel.

Figure B3. Immunoprecipitation of yeast Ire1

Immunoprecipitation of Ire1-3F6H from yeast cells. Cells were lysed in 2%, 0.5% and 0.1% of TritonX-100 and Empigen respectively, and subjected to immunoprecipitation with Dynabeads conjugated with Flag antibody. Associated protein were eluted with Flag peptides. Untagged Ire1 strain was used as a control. Gel was stained with coomassie blue G250 dye. The amount of total Ire1 in the soluble (s), pellet (p), flowthrough (ft) fractions of cell extract was determined using anti-Flag antibody under indicated conditions.

Figure B4. Yeast Ire1 mass spectrometry analysis

Immunoprecipitated samples in the presence and absence of 10 mM DTT were analyzed by tandem MS/MS. Cells were lysed with 2% TritonX-100.

Components of protein complex associated with Ire1 are shown on the coomassie blue stained gel.

Figure B5. Immunoprecipitation of human IRE1

Immunoprecipitation of IRE1-3F6H from T-REx293 cells. IRE1-3F6H expression was induced with 10 nM of Doxycycline (Dox). Upon harvesting, cells were crosslinked with 1% para-formaldehyde (PFA) and the crosslinking reaction was quenched by addition of glycine. Cells were lysed with 1% TritonX-100 and subjected to immunoprecipitation with Dynabeads conjugated with Flag antibody. Associated proteins were eluted with Flag peptides and analyzed by tandem mass spectrometry (MS/MS). Cells without Dox induction were used as a control. Cells with Dox induction were subjected to 5 µg/ml tunicamycin (Tm) treatment for indicated hours.

Figure B6. PGAM5 and ASK1 protein level upon ER stress

A. Total PGAM5 protein level was assessed by western blotting against total PGAM5 antibody at indicated times, ER stress was induced with 5 µg/ml tunicamycin (Tm). B. Phospho- and total- ASK1 protein levels were determined via western blotting using phospho-specific and total- ASK1 antibodies at indicated times. ER stress was induced with 5 µg/ml tunicamycin (Tm).

Tables B1-1 to B1-7. Lists of protein identified via MS/MS

Proteins are ranked by their confidence scores. The ranking reflects the relative abundance of proteins, and homologous proteins are grouped together.

Numbers indicate the relative numbers of peptides detected.

Table B2. List of potential IRE1 interacting proteins

The list of protein was selected from Table B1 based on their function and cellular location. The ranking reflects the relative abundance of proteins, and homologous proteins are grouped together. Numbers of peptides detected are indicated.

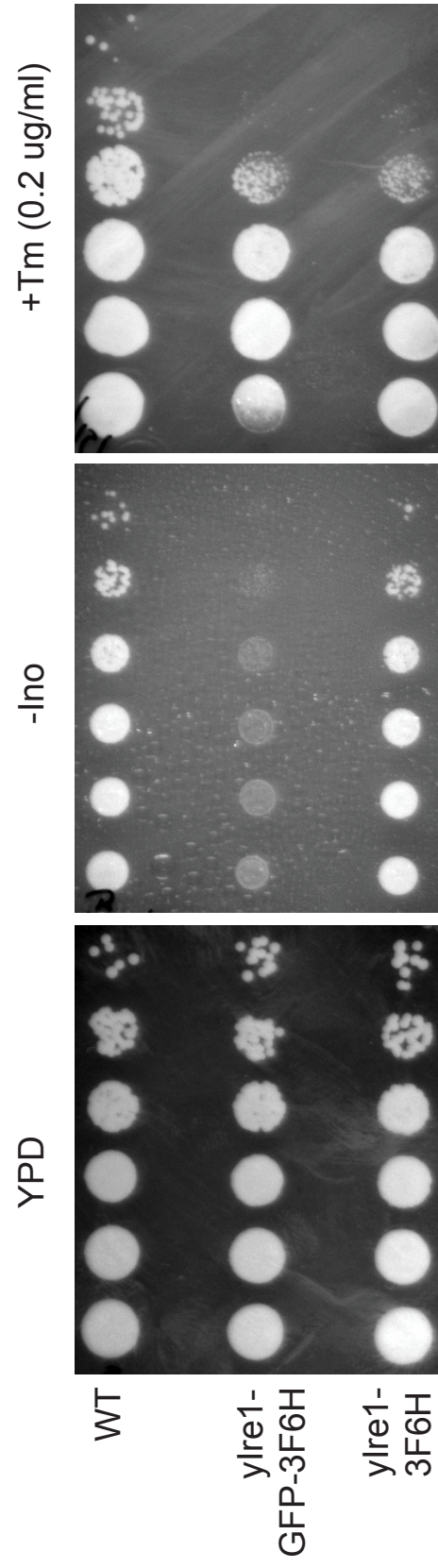


Figure B1

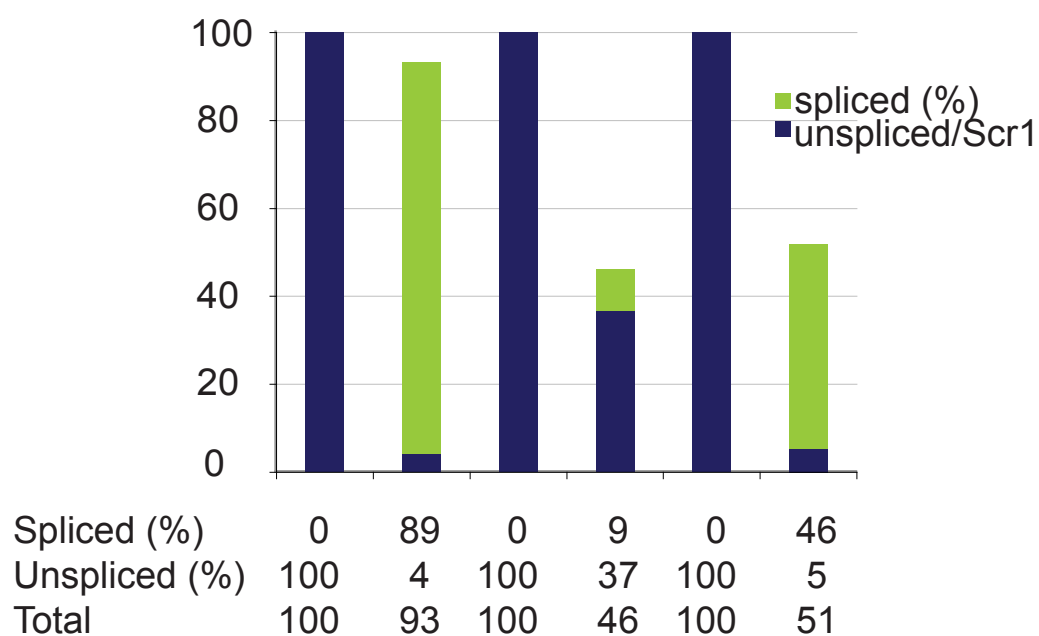
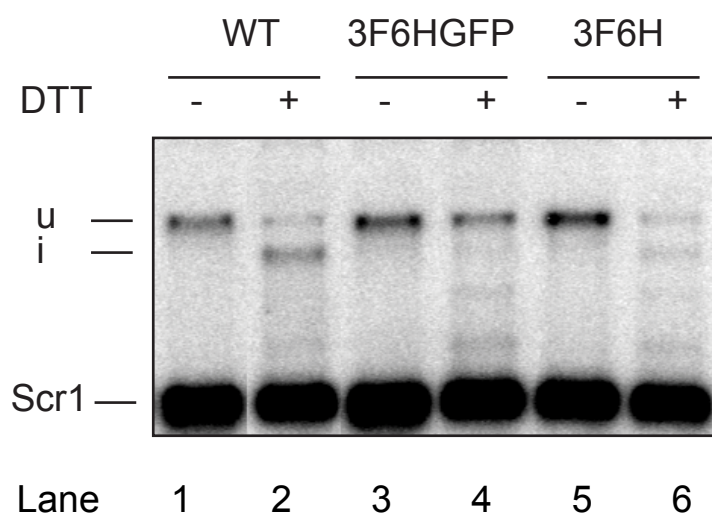


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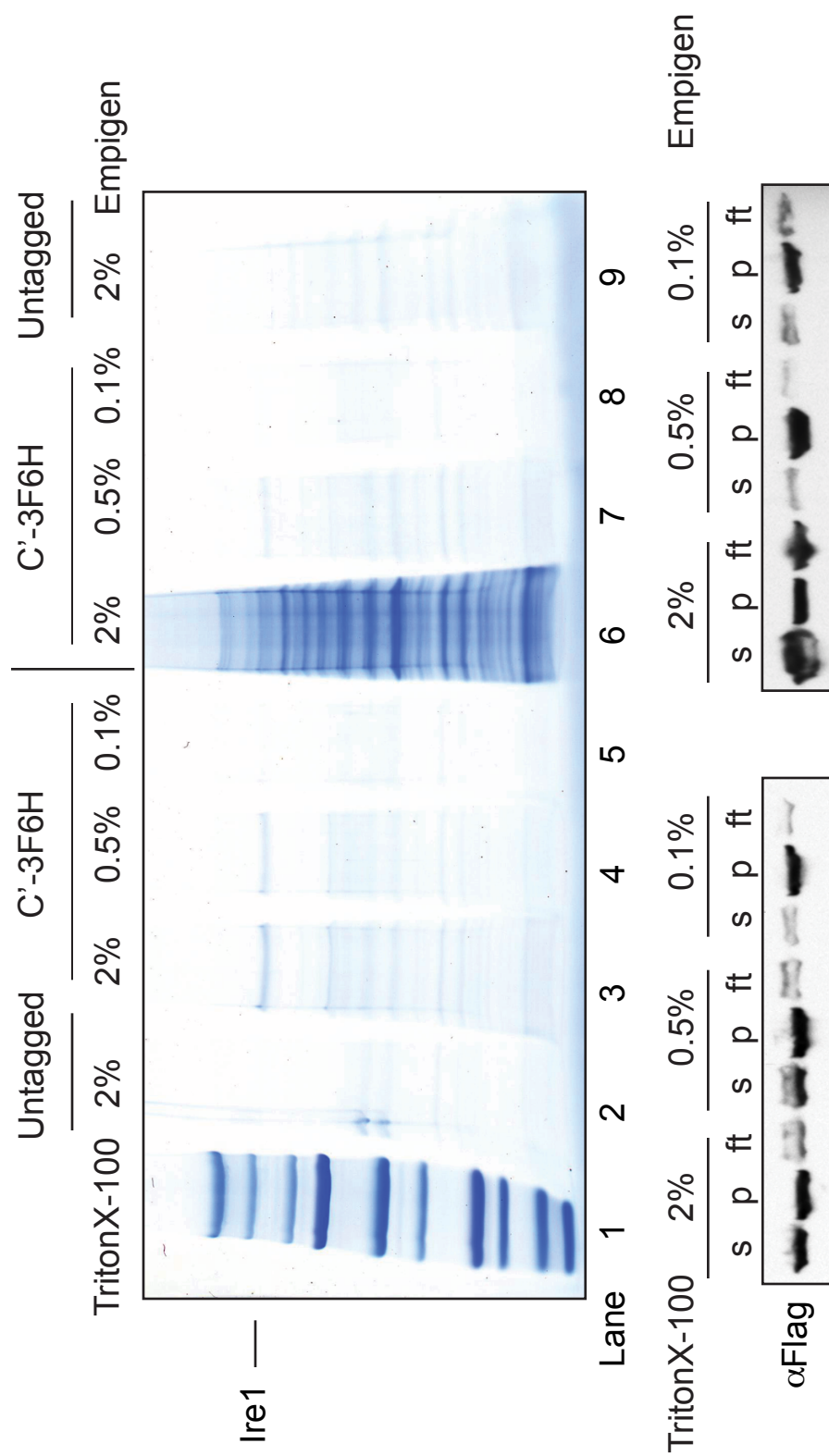


Figure B3

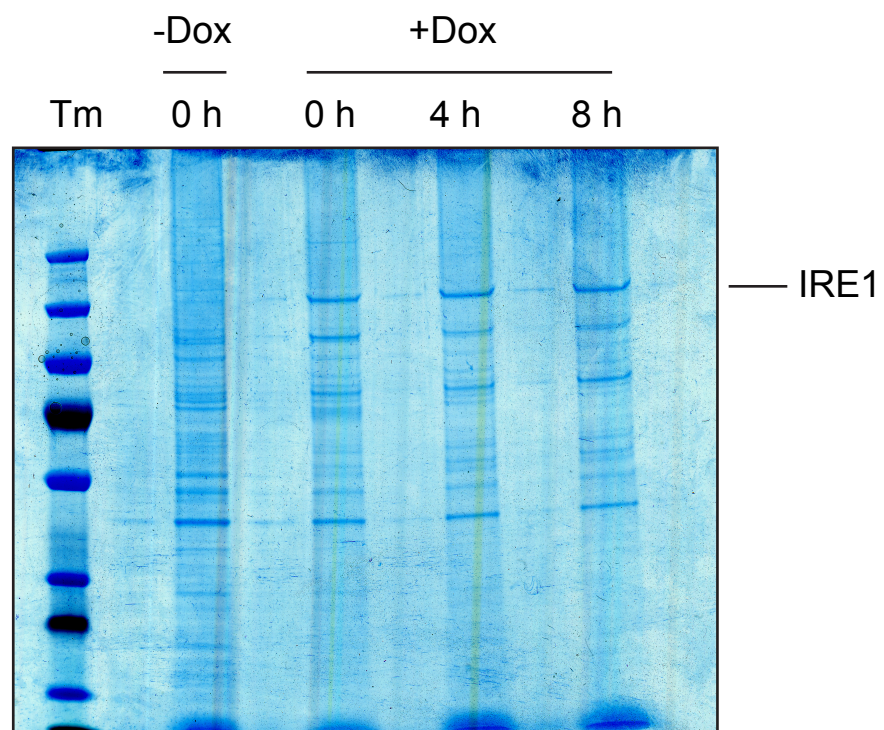


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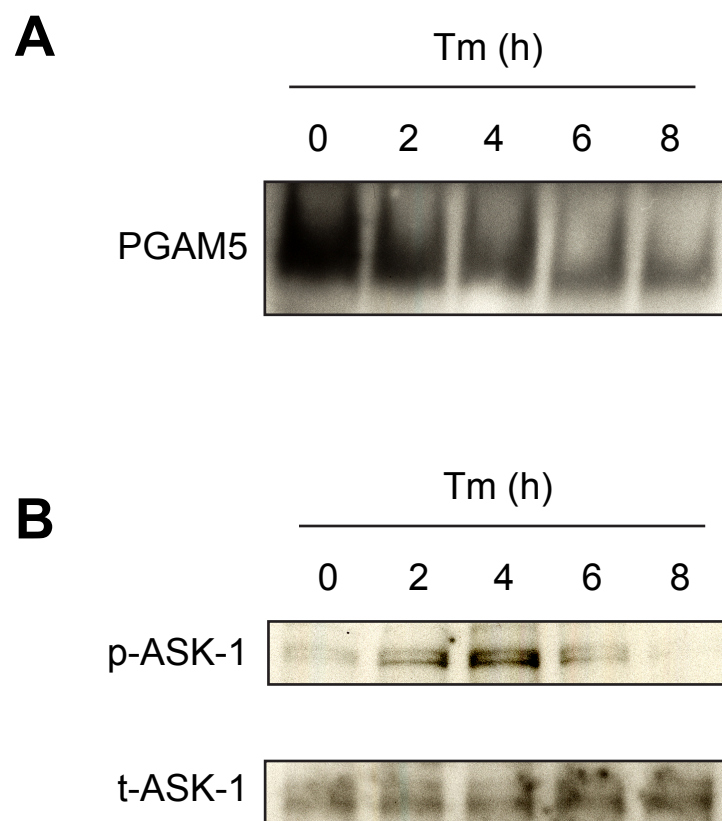


Figure B6

# of Peptides							Protein Name
Rank	Uniq Pep	Acc #	non-induced	0 h Tm	4 h Tm	8 h Tm	
[1]		O75460	21	47	54	53	Serine/threonine-protein kinase/endoribonuclease IRE
[2]		P60709	37	35	31	31	Actin, cytoplasmic 1
[2-1]	3	P63261	37	34	31	32	Actin, cytoplasmic 2
[2-2]	5	P62736	23	22	20	22	Actin, aortic smooth muscle
[2-3]	4	P63267	23	21	20	22	Actin, gamma-enteric smooth muscle
[2-4]	5	P68032	23	23	20	22	Actin, alpha cardiac muscle 1
[2-5]	5	P68133	23	23	20	22	Actin, alpha skeletal muscle
[2-6]	1	Q6S8J3	9	9	8	8	POTE ankyrin domain family member E
[2-7]	1	Q9BYX7	6	6	5	5	Beta-actin-like protein 3
[3]		P11021	26	32	36	37	78 kDa glucose-regulated protein
[3-1]	31	P11142	26	26	24	17	Heat shock cognate 71 kDa protein
[3-2]	25	P08107	22	16	17	15	Heat shock 70 kDa protein 1
[3-3]	10	P34931	10	8	8	8	Heat shock 70 kDa protein 1L
[3-4]	10	P54652	10	10	7	7	Heat shock-related 70 kDa protein 2
[3-5]	8	P17066	7	6	7	5	Heat shock 70 kDa protein 6
[3-6]	4	P48741	4	4	4	4	Putative heat shock 70 kDa protein 7
[4]		P14625	28	32	35	35	Endoplasmic
[4-1]	24	P08238	23	14	16	13	Heat shock protein HSP 90-beta
[5]		P04264	18	33	22	24	Keratin, type II cytoskeletal 1
[6]		P13667	14	25	24	23	Protein disulfide-isomerase A4
[7]		P07900	25	15	19	20	Heat shock protein HSP 90-alpha
[7-1]	9	Q58FF7	11	6	6	4	Putative heat shock protein HSP 90-beta-3
[7-2]	2	Q58FF8	6	4	3	3	Putative heat shock protein HSP 90-beta 2
[7-3]	4	Q12931	2	3	3	3	Heat shock protein 75 kDa, mitochondrial
[8]		P14618	21	22	8	9	Pyruvate kinase isozymes M1/M2
[9]		Q99832	22	15	8	8	T-complex protein 1 subunit eta
[10]		P35527	9	19	11	13	Keratin, type I cytoskeletal 9
[11]		P68371	25	21	21	15	Tubulin beta-2C chain
[11-1]	4	P07437	24	21	20	16	Tubulin beta chain
[11-2]	6	Q13885	24	20	20	15	Tubulin beta-2A chain
[11-3]	5	Q9BVA1	23	20	20	15	Tubulin beta-2B chain
[11-4]	2	Q13509	16	15	15	11	Tubulin beta-3 chain
[11-5]	3	Q9BUF5	11	9	7	3	Tubulin beta-6 chain
[12]		P13639	24	15	18	14	Elongation factor 2
[12-1]	1	Q15029	2				116 kDa U5 small nuclear ribonucleoprotein componen
[13]		P30101	13	20	20	21	Protein disulfide-isomerase A3
[14]		P50990	22	16	13	11	T-complex protein 1 subunit theta
[15]		P78371	20	20	13	11	T-complex protein 1 subunit beta
[16]		O43707	19	18	16	15	Alpha-actinin-4
[16-1]	4	P12814	11	4	3	4	Alpha-actinin-1
[16-2]	1	P35609	6	4	4	4	Alpha-actinin-2
[17]		P50991	16	17	13	10	T-complex protein 1 subunit delta
[18]		P17987	11	14	10	6	T-complex protein 1 subunit alpha
[19]		P06733	15	14	10	11	Alpha-enolase
[20]		Q71U36	14	13	15	15	Tubulin alpha-1A chain
[20-1]	1	Q9BQE3	13	12	14	14	Tubulin alpha-1C chain
[20-2]	1	P68366	10	9	11	12	Tubulin alpha-4A chain
[21]		Q15084	6	10	9	9	Protein disulfide-isomerase A6
[22]		P49327	13	5	2	5	Fatty acid synthase
[23]		P07237	9	11	12	13	Protein disulfide-isomerase
[24]		P49368	19	12	8	7	T-complex protein 1 subunit gamma
[25]		P16615	1	11	13	11	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
[26]		Q9Y230	15	7	5	6	RuvB-like 2
[27]		P54886	13	8	11	14	Delta-1-pyrroline-5-carboxylate synthetase
[28]		P40227	12	9	10	9	T-complex protein 1 subunit zeta
[29]		Q9UQ80	14	8	11	9	Proliferation-associated protein 2G4
[30]		P11586	12	6	6	8	C-1-tetrahydrofolate synthase, cytoplasmic
[31]		P60842	8	5	8	7	Eukaryotic initiation factor 4A-I
[31-1]	1	Q14240	5	3	6	4	Eukaryotic initiation factor 4A-II
[32]		Q01813	7		2	1	6-phosphofructokinase type C
[32-1]	1	P17858	3		1	1	6-phosphofructokinase, liver type
[33]		P18206	9	7	5	4	Vinculin
[34]		P38646	8	7	5	8	Stress-70 protein, mitochondrial
[35]		Q02790	12	8	5	5	FK506-binding protein 4
[36]		P08670	8	10	13	18	Vimentin
[37]		Q15366	5	7	3	4	Poly(rC)-binding protein 2
[37-1]	1	P57721	3	5	2	4	Poly(rC)-binding protein 3
[37-2]	5	Q15365	5	5	3	6	Poly(rC)-binding protein 1
[38]		P11940	10	3	4	5	Polyadenylate-binding protein 1
[38-1]	1	Q13310	6	2	3	2	Polyadenylate-binding protein 4
[39]		P51659	8	5	3	3	Peroxisomal multifunctional enzyme type 2
[40]		P68104	12	8	9	9	Elongation factor 1-alpha 1
[41]		P12277	6	6	5	4	Creatine kinase B-type

Table B1-1

# of Peptides							
Rank	Uniq Pep	Acc #	non-induced	0 h Tm	4 h Tm	8 h Tm	Protein Name
[47-2]	2	P35241	6	1	4	2	Radixin
[48]		P08865	4	7	5	4	40S ribosomal protein SA
[49]		Q9Y4L1		7	6	6	Hypoxia up-regulated protein 1
[50]		Q9UBS4	3	9	4	3	DnaJ homolog subfamily B member 11
[51]		P23526	7	5	3	3	Adenosylhomocysteinase
[52]		Q9Y262	5	6		1	Eukaryotic translation initiation factor 3 subunit L
[53]		P54136	8	1	5	5	Arginyl-tRNA synthetase, cytoplasmic
[54]		P04075	8	9	5	3	Fructose-bisphosphate aldolase A
[54-1]	1	P09972	2				Fructose-bisphosphate aldolase C
[55]		O00116	8	2			Alkylidihydroxyacetonephosphate synthase, peroxisomal
[56]		P36578	9	4	4	4	60S ribosomal protein L4
[57]		P48643	16	9	3	1	T-complex protein 1 subunit epsilon
[59]		Q9P2J5	7	2	2		Leucyl-tRNA synthetase, cytoplasmic
[60]		P12268	8	3	2	1	Inosine-5'-monophosphate dehydrogenase 2
[60-1]	2	P20839	3				Inosine-5'-monophosphate dehydrogenase 1
[61]		P27797		8	5	8	Calreticulin
[62]		P04843	2	4	8	5	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 4
[63]		P55884	6	1	2	3	Eukaryotic translation initiation factor 3 subunit B
[64]		P54577	9	9	6	4	Tyrosyl-tRNA synthetase, cytoplasmic
[65]		P27824		5	8	8	Calnexin
[66]		Q8NC51	4	5	3	4	Plasminogen activator inhibitor 1 RNA-binding protein
[67]		P06744	7	6	4	4	Glucose-6-phosphate isomerase
[68]		P62258	6	7	5	3	14-3-3 protein epsilon
[68-1]	4	P63104	2	4			14-3-3 protein zeta/delta
[68-2]	4	P27348	2	3	3	2	14-3-3 protein theta
[68-3]	2	P61981			3		14-3-3 protein gamma
[68-4]	1	P31946		2			14-3-3 protein beta/alpha
[69]		Q14974	7	3	4	1	Importin subunit beta-1
[70]		P04406	8	6	6	7	Glyceraldehyde-3-phosphate dehydrogenase
[71]		P06576	4	3	4	6	ATP synthase subunit beta, mitochondrial
[72]		O00303	6	4	5	4	Eukaryotic translation initiation factor 3 subunit F
[73]		P47897	8	1	3	2	Glutamyl-tRNA synthetase
[74]		O75083	4	2			WD repeat-containing protein 1
[75]		O43175	5	4	4	4	D-3-phosphoglycerate dehydrogenase
[76]		P26640	5	1			Valyl-tRNA synthetase
[77]		P23396	7	4	7	4	40S ribosomal protein S3
[78]		P39656		5	5	4	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 4
[79]		P02768	4	6	4	5	Serum albumin
[80]		Q9Y6U3	7	3	2	3	Adseverin
[81]		Q6P3W7	3	4	3	6	SCY1-like protein 2
[82]		P26641	8	4	3	2	Elongation factor 1-gamma
[83]		P21333	3		6	3	Filamin-A
[84]		Q07065	4	3	6	2	Cytoskeleton-associated protein 4
[85]		P14868	6	4	3	2	Aspartyl-tRNA synthetase, cytoplasmic
[86]		P53396	7	1			ATP-citrate synthase
[87]		O95573	1	5	6	4	Long-chain-fatty-acid--CoA ligase 3
[88]		P61163	4	2	3	2	Alpha-centractin
[89]		O43242	5	5	5	4	26S proteasome non-ATPase regulatory subunit 3
[90]		Q9Y3F4	3	4		3	Serine-threonine kinase receptor-associated protein
[91]		P16989	4	2	3		DNA-binding protein A
[91-1]	4	P67809	5	3	3	3	Nuclease-sensitive element-binding protein 1
[92]		P49588	7	5	2	3	Alanyl-tRNA synthetase, cytoplasmic
[93]		P22314	5	2			Ubiquitin-like modifier-activating enzyme 1
[94]		P61221	4				ATP-binding cassette sub-family E member 1
[95]		P60228	4	4	4	6	Eukaryotic translation initiation factor 3 subunit E
[96]		Q9Y265	5	5	4	2	RuvB-like 1
[97]		P37268		3	5	2	Squalene synthetase
[98]		Q14444	4	2	2	3	Caprin-1
[99]		Q92499	5		1		ATP-dependent RNA helicase DDX1
[100]		Q01518	5	1			Adenylyl cyclase-associated protein 1
[101]		P10809	7	3	3	6	60 kDa heat shock protein, mitochondrial
[102]		P25705	3	4	3	3	ATP synthase subunit alpha, mitochondrial
[103]		P63244	4	3	4	4	Guanine nucleotide-binding protein subunit beta-2-like 1
[104]		P08237	3				6-phosphofructokinase, muscle type
[105]		P05388	3	2	3	5	60S acidic ribosomal protein P0
[106]		Q8WUM4	4		1	1	Programmed cell death 6-interacting protein
[107]		P55060	5	5	5	3	Exportin-2
[108]		P23921	4				Ribonucleoside-diphosphate reductase large subunit
[109]		P30050	3	1	3	3	60S ribosomal protein L12
[110]		Q14204	5				Cytoplasmic dynein 1 heavy chain 1
[111]		Q15019	3	1	1	2	Septin-2
[112]		Q8NBS9	2	4	4	3	Thioredoxin domain-containing protein 5
[113]		P61978	2	4	2	3	Heterogeneous nuclear ribonucleoprotein K

Table B1-2

# of Peptides							
Rank	Uniq Pep	Acc #	non-induced	0 h Tm	4 h Tm	8 h Tm	Protein Name
[114]		P09960	4				Leukotriene A-4 hydrolase
[115]		P13796	3	4	2	2	Plastin-2
[115-1]	2	P13797	2		3	1	Plastin-3
[116]		Q15046	4				Lysyl-tRNA synthetase
[117]		P30153	6	4	5	2	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit
[118]		P62913	1	2	1	3	60S ribosomal protein L11
[119]		Q99613	4	3	2	4	Eukaryotic translation initiation factor 3 subunit C
[120]		Q13283	4	3			Ras GTPase-activating protein-binding protein 1
[121]		P04844	2	6	3	3	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase
[122]		P28482	3	2	3	4	Mitogen-activated protein kinase 1
[123]		Q9Y285	6	2		3	Phenylalanyl-tRNA synthetase alpha chain
[124]		P35268	1	3	1		60S ribosomal protein L22
[125]		P23381	2	2			Tryptophanyl-tRNA synthetase, cytoplasmic
[126]		P61160	7	2	4		Actin-related protein 2
[127]		Q16643	3	1	3	3	Drebrin
[128]		P50395	5	3	2	4	Rab GDP dissociation inhibitor beta
[129]		P30566	4	2	3		Adenylosuccinate lyase
[130]		P50454	2	4	3	3	Serpin H1
[131]		Q9BSJ8	2	2	2	1	Extended synaptotagmin-1
[132]		Q13765	2	2	2	2	Nascent polypeptide-associated complex subunit alpha
[133]		Q92747	2	1		1	Actin-related protein 2/3 complex subunit 1A
[134]		Q14CX7	4				N-terminal acetyltransferase B complex subunit MDM20
[135]		P35606	2	2	4	3	Coatomer subunit beta'
[136]		O00571		2	4		ATP-dependent RNA helicase DDX3X
[137]		P46060	2				Ran GTPase-activating protein 1
[138]		P62937	3	3		2	Peptidyl-prolyl cis-trans isomerase A
[139]		Q99615	4				DnaJ homolog subfamily C member 7
[140]		P16403			4	3	Histone H1.2
[140-1]	1	P10412			3	4	Histone H1.4
[141]		Q10567	4				AP-1 complex subunit beta-1
[141-1]	1	P63010	2				AP-2 complex subunit beta
[142]		Q13200	3				26S proteasome non-ATPase regulatory subunit 2
[143]		P31948	4	3	1		Stress-induced-phosphoprotein 1
[144]		Q9UJU6	4				Drebrin-like protein
[145]		Q13561	2	1	1	3	Dynactin subunit 2
[146]		Q96QK1	4		1		Vacuolar protein sorting-associated protein 35
[147]		Q9NZI8	3		2	1	Insulin-like growth factor 2 mRNA-binding protein 1
[147-1]	3	O00425	4				Insulin-like growth factor 2 mRNA-binding protein 3
[148]		P49411	4	2	2	2	Elongation factor Tu, mitochondrial
[149]		P07355	3	1		2	Annexin A2
[150]		P16298	3				Serine/threonine-protein phosphatase 2B catalytic subunit beta
[151]		Q14318		3	3	2	FK506-binding protein 8
[152]		P62241	2	2	2	2	40S ribosomal protein S8
[153]		P55072	3	3	2	4	Transitional endoplasmic reticulum ATPase
[154]		P35221	3				Catenin alpha-1
[155]		P22234	6	4			Multifunctional protein ADE2
[156]		Q15393	2				Splicing factor 3B subunit 3
[157]		Q13838	1	2	3	2	Spliceosome RNA helicase BAT1
[158]		P22307	3				Non-specific lipid-transfer protein
[159]		Q9BY44	3				Eukaryotic translation initiation factor 2A
[160]		Q00610	2		1	3	Clathrin heavy chain 1
[162]		P50402	1		2		Emerin
[163]		P52907	2	1	2	2	F-actin-capping protein subunit alpha-1
[164]		Q06830	5	5	3	3	Peroxisomal protein PEX1
[164-1]	2	P32119	1	3	2	2	Peroxisomal protein PEX2
[164-2]	2	Q13162		3			Peroxisomal protein PEX4
[165]		Q9BS26		1	2	2	Endoplasmic reticulum resident protein ERp44
[166]		P11413	1				Glucose-6-phosphate 1-dehydrogenase
[167]		O75131	4				Copine-3
[168]		P34932	1	3		1	Heat shock 70 kDa protein 4
[169]		P41091	3	3	4	3	Eukaryotic translation initiation factor 2 subunit 3
[170]		P07737	3				Profilin-1
[171]		P12532	1	2			Creatine kinase U-type, mitochondrial
[172]		P62269	1	3	2		40S ribosomal protein S18
[173]		P61619		2	4	2	Protein transport protein Sec61 subunit alpha isoform 1
[174]		P61158	6	1			Actin-related protein 3
[175]		O00154	2		1		Cytosolic acyl coenzyme A thioester hydrolase
[176]		Q9NTJ5		4	4	4	Phosphatidylinositol 3-kinase SAC1
[177]		P62988	2	2	1	1	Ubiquitin
[178]		Q9NUU7	2				ATP-dependent RNA helicase DDX19A
[179]		O60506	3		1		Heterogeneous nuclear ribonucleoprotein Q
[180]		P62888		2	3	2	60S ribosomal protein L30
[181]		Q9UBB4	3	2			Ataxin-10

Table B1-3

# of Peptides							
Rank	Uniq Pep	Acc #	non-induced	0 h Tm	4 h Tm	8 h Tm	Protein Name
[182]		Q9NYL9			3		Tropomodulin-3
[183]		P84098	2				60S ribosomal protein L19
[184]		O15372	3			1	Eukaryotic translation initiation factor 3 subunit H
[185]		Q09028		2		1	Histone-binding protein RBBP4
[186]		P12956	4	1			ATP-dependent DNA helicase 2 subunit 1
[187]		P23284	1	3	2	2	Peptidyl-prolyl cis-trans isomerase B
[188]		Q92598	2		2	2	Heat shock protein 105 kDa
[189]		Q9ULV4	5	3	3	1	Coronin-1C
[190]		Q32P28	1	2	1	2	Prolyl 3-hydroxylase 1
[191]		P29966	2				Myristoylated alanine-rich C-kinase substrate
[192]		Q9UGP8			2		Translocation protein SEC63 homolog
[193]		P26196	3	2			Probable ATP-dependent RNA helicase DDX6
[194]		P33176	2	1	1	1	Kinesin-1 heavy chain
[195]		Q7L2H7	1			2	Eukaryotic translation initiation factor 3 subunit M
[196]		P12081	3	3			Histidyl-tRNA synthetase, cytoplasmic
[197]		O00231	3	2			26S proteasome non-ATPase regulatory subunit 11
[198]		P07195	2	4	3	3	L-lactate dehydrogenase B chain
[198-1]	3	P00338	2	2		2	L-lactate dehydrogenase A chain
[199]		Q15645	3				Thyroid receptor-interacting protein 13
[200]		P47755	1	2	2	2	F-actin-capping protein subunit alpha-2
[201]		P39023	3	2			60S ribosomal protein L3
[202]		Q04837		2	2		Single-stranded DNA-binding protein, mitochondrial
[203]		O75396		2	3	2	Vesicle-trafficking protein SEC22b
[204]		O75534	3	2	3	2	Cold shock domain-containing protein E1
[205]		P31939	4	2			Bifunctional purine biosynthesis protein PURH
[206]		Q8WU90	2		1		Zinc finger CCCH domain-containing protein 15
[207]		P60174		2	1		Triosephosphate isomerase
[208]		P06396	2				Gelsolin
[209]		P22102	3				Trifunctional purine biosynthetic protein adenosine-3
[210]		P34897	3	1		1	Serine hydroxymethyltransferase, mitochondrial
[211]		P49419	2				Alpha-aminoacidic semialdehyde dehydrogenase
[212]		Q8WVM8	1	1			Sec1 family domain-containing protein 1
[213]		O15371	2				Eukaryotic translation initiation factor 3 subunit D
[214]		Q9NVI7			5		ATPase family AAA domain-containing protein 3A
[215]		P61011	1	2	1	1	Signal recognition particle 54 kDa protein
[216]		P29401	1	2	1	2	Transketolase
[217]		O94874	1		3	4	UPF0555 protein KIAA0776
[218]		P29692		1	1	2	Elongation factor 1-delta
[219]		Q9NVA2	2				Septin-11
[219-1]	2	Q16181	3				Septin-7
[220]		Q04637	4				Eukaryotic translation initiation factor 4 gamma 1
[221]		P30040		2	3	3	Endoplasmic reticulum protein ERp29
[222]		O95831	3	2	2		Apoptosis-inducing factor 1, mitochondrial
[223]		P31943	1	2	2	2	Heterogeneous nuclear ribonucleoprotein H
[224]		Q9BXJ9	3				NMDA receptor-regulated protein 1
[225]		Q92615	2				La-related protein 4B
[226]		P35611	3		1		Alpha-adducin
[227]		P62195	3				26S protease regulatory subunit 8
[228]		O95793	2				Double-stranded RNA-binding protein Staufen homolog 1
[229]		Q16555	3				Dihydropyrimidinase-related protein 2
[229-1]	1	Q14195	2				Dihydropyrimidinase-related protein 3
[230]		Q9Y490	1				Talin-1
[231]		P35579	3				Myosin-9
[232]		P62263	2	2	2		40S ribosomal protein S14
[233]		Q9Y266	3	2	2	2	Nuclear migration protein nudC
[234]		P56192	2	2	2	1	Methionyl-tRNA synthetase, cytoplasmic
[235]		Q13347	1	2	2	4	Eukaryotic translation initiation factor 3 subunit I
[236]		O95292		2	2		Vesicle-associated membrane protein-associated protein B/C
[237]		Q8N1G4	3				Leucine-rich repeat-containing protein 47
[238]		P19367	1		3		Hexokinase-1
[239]		O43324	1	1	1	2	Eukaryotic translation elongation factor 1 epsilon-1
[240]		P18031		2	2		Tyrosine-protein phosphatase non-receptor type 1
[241]		P78344	2				Eukaryotic translation initiation factor 4 gamma 2
[242]		Q13618	1				Cullin-3
[243]		Q96SB4	2				Serine/threonine-protein kinase SRPK1
[243-1]	1	P78362	2				Serine/threonine-protein kinase SRPK2
[244]		Q8N766		2		1	Uncharacterized protein KIAA0090
[245]		Q02978		1	1	1	Mitochondrial 2-oxoglutarate/malate carrier protein
[246]		O75436	1	1	1	2	Vacuolar protein sorting-associated protein 26A
[247]		O75521	3				Peroxisomal 3,2-trans-enoyl-CoA isomerase
[248]		P23528	2		2		Cofilin-1
[249]		O00232	2				26S proteasome non-ATPase regulatory subunit 12
[250]		Q8NE71	1			1	ATP-binding cassette sub-family F member 1

Table B1-4

# of Peptides							
Rank	Uniq Pep	Acc #	non-induced	0 h Tm	4 h Tm	8 h Tm	Protein Name
[251]		P83731			1	1	60S ribosomal protein L24
[252]		P60660	1	2			Myosin light polypeptide 6
[253]		Q01844			1	1	RNA-binding protein EWS
[254]		P55145		1	1	1	Mesencephalic astrocyte-derived neurotrophic factor
[255]		P17812		1			CTP synthase 1
[256]		P19338		2	2	1	Nucleolin
[257]		P09874	1		1		Poly [ADP-ribose] polymerase 1
[258]		P63220	3		2		40S ribosomal protein S21
[259]		P24752			1		Acetyl-CoA acetyltransferase, mitochondrial
[260]		P55209		1	1	1	Nucleosome assembly protein 1-like 1
[261]		Q9Y5M8		1		1	Signal recognition particle receptor subunit beta
[262]		O75821	1	1	1	1	Eukaryotic translation initiation factor 3 subunit G
[263]		Q00341	1				Vigilin
[264]		O75688	1	2			Protein phosphatase 1B
[265]		P48444	4	2	2	2	Coatome subunit delta
[266]		Q709F0	3				Acyl-CoA dehydrogenase family member 11
[267]		Q15691				1	Microtubule-associated protein RP/EB family member 1
[268]		P43378	1				Tyrosine-protein phosphatase non-receptor type 9
[269]		P13489	2			1	Ribonuclease inhibitor
[270]		P51648		2	2	2	Fatty aldehyde dehydrogenase
[271]		P62829		2			60S ribosomal protein L23
[272]		P00558	5	4	2	3	Phosphoglycerate kinase 1
[273]		Q92890		1			Ubiquitin fusion degradation protein 1 homolog
[274]		Q00796	2				Sorbitol dehydrogenase
[275]		Q15417	1			1	Calponin-3
[276]		Q9HCN8		1			Stromal cell-derived factor 2-like protein 1
[277]		Q9HCN4			1		GPN-loop GTPase 1
[278]		P62917	1	1			60S ribosomal protein L8
[279]		Q9Y2Z0		1		1	Suppressor of G2 allele of SKP1 homolog
[280]		P51114	1				Fragile X mental retardation syndrome-related protein 1
[281]		P59998	2			2	Actin-related protein 2/3 complex subunit 4
[282]		Q9UBS3		1	1	1	DnaJ homolog subfamily B member 9
[283]		P18085	2		3	2	ADP-ribosylation factor 4
[284]		P82979		2			SAP domain-containing ribonucleoprotein
[285]		P54619		2	2	1	5'-AMP-activated protein kinase subunit gamma-1
[286]		Q86VP6	2				Cullin-associated NEDD8-dissociated protein 1
[287]		Q8N1F7	1			1	Nuclear pore complex protein Nup93
[288]		A6NL28	1			1	Putative tropomyosin alpha-3 chain-like protein
[289]		Q14157	2				Ubiquitin-associated protein 2-like
[290]		Q9Y295	2		1	1	Developmentally-regulated GTP-binding protein 1
[291]		Q07020				2	60S ribosomal protein L18
[292]		Q9NQP4	1				Prefoldin subunit 4
[293]		O76094			1		Signal recognition particle 72 kDa protein
[294]		P31689	1	1			DnaJ homolog subfamily A member 1
[295]		Q9HD20		2			Probable cation-transporting ATPase 13A1
[296]		P53618			2		Coatome subunit beta
[297]		Q9BWD1		1			Acetyl-CoA acetyltransferase, cytosolic
[298]		Q9Y5A9	3				YTH domain family protein 2
[298-1]	1	Q7Z739	2				YTH domain family protein 3
[299]		Q92973	2				Transportin-1
[300]		Q9UHB6	1		1		LIM domain and actin-binding protein 1
[301]		Q9BVC6		1	1	1	Transmembrane protein 109
[302]		Q8NBJ5	1	1			Procollagen galactosyltransferase 1
[303]		Q9H3P7			1		Golgi resident protein GCP60
[304]		P13010	2		2		ATP-dependent DNA helicase 2 subunit 2
[305]		Q86U42	1		1		Polyadenylate-binding protein 2
[306]		P23246	1				Splicing factor, proline- and glutamine-rich
[307]		P99999	1	1	1	1	Cytochrome c
[308]		Q9BR76	2	1			Coronin-1B
[309]		P35998	1				26S protease regulatory subunit 7
[310]		P62495	3	3			Eukaryotic peptide chain release factor subunit 1
[311]		P62701	3		2	3	40S ribosomal protein S4, X isoform
[312]		P49189		1			4-trimethylaminobutyraldehyde dehydrogenase
[313]		Q96JB5		1			CDK5 regulatory subunit-associated protein 3
[314]		P54578	1				Ubiquitin carboxyl-terminal hydrolase 14
[315]		P25205	1		1	2	DNA replication licensing factor MCM3
[316]		P31040	1				Succinate dehydrogenase [ubiquinone] flavoprotein subunit
[317]		P00505				2	Aspartate aminotransferase, mitochondrial
[318]		Q9Y678	2				Coatome subunit gamma
[319]		P62333	1				26S protease regulatory subunit S10B
[320]		Q9BT78	3		1		COP9 signalosome complex subunit 4
[321]		P58546	1	1	1		Myotrophin
[322]		P33993				2	DNA replication licensing factor MCM7

Table B1-5

# of Peptides							Protein Name
Rank	Uniq Pep	Acc #	non-induced	0 h Tm	4 h Tm	8 h Tm	
[323]		Q9UL25		1			Ras-related protein Rab-21
[324]		O00151		1			PDZ and LIM domain protein 1
[325]		Q9H3N1		1		1	Thioredoxin-related transmembrane protein 1
[326]		P62857	1				40S ribosomal protein S28
[327]		Q14739			1		Lamin-B receptor
[328]		Q86X55	1				Histone-arginine methyltransferase CARM1
[329]		P43034	1				Platelet-activating factor acetylhydrolase IB subunit alpha
[330]		O14737		1	2	1	Programmed cell death protein 5
[331]		Q8IVM0	1				Coiled-coil domain-containing protein 50
[332]		Q9UEY8	1				Gamma-adducin
[333]		P63151	1				Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B
[334]		Q96AG4				1	Leucine-rich repeat-containing protein 59
[335]		P50914	1			1	60S ribosomal protein L14
[336]		Q8NF37		1	2		Lysophosphatidylcholine acyltransferase 1
[337]		Q9H223	1				EH domain-containing protein 4
[338]		P09622	2				Dihydrolipoyl dehydrogenase, mitochondrial
[339]		P18124	2		1	1	60S ribosomal protein L7
[340]		P46776	1			1	60S ribosomal protein L27a
[341]		P46783	1				40S ribosomal protein S10
[342]		Q15436				1	Protein transport protein Sec23A
[343]		Q13724				1	Mannosyl-oligosaccharide glucosidase
[344]		O14744	2				Protein arginine N-methyltransferase 5
[345]		Q92616	1				Translational activator GCN1
[346]		P61803			2		Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit
[347]		P35637	1		2		RNA-binding protein FUS
[348]		P21964		1			Catechol O-methyltransferase
[349]		P42677	3		1		40S ribosomal protein S27
[350]		P50570	1	1	1		Dynamin-2
[351]		P26599	3		1		Polypyrimidine tract-binding protein 1
[352]		Q92900	3				Regulator of nonsense transcripts 1
[353]		P60866	1				40S ribosomal protein S20
[354]		Q9HB71	1	2			Calcyclin-binding protein
[355]		P00390	1				Glutathione reductase, mitochondrial
[356]		P62899				1	60S ribosomal protein L31
[357]		Q02878	1		2		60S ribosomal protein L6
[358]		Q96HS1		1			Serine/threonine-protein phosphatase PGAM5, mitochondrial
[359]		P26373	1				60S ribosomal protein L13
[360]		P62424	1				60S ribosomal protein L7a
[361]		P51149			1		Ras-related protein Rab-7a
[362]		Q14671	1				Pumilio homolog 1
[363]		O14979		1		1	Heterogeneous nuclear ribonucleoprotein D-like
[364]		P05023	1				Sodium/potassium-transporting ATPase subunit alpha-1
[365]		O60749	1				Sorting nexin-2
[366]		P39019				1	40S ribosomal protein S19
[367]		Q15293		2			Reticulocalbin-1
[368]		P08240			2		Signal recognition particle receptor subunit alpha
[369]		P49736		1			DNA replication licensing factor MCM2
[370]		P62826				1	GTP-binding nuclear protein Ran
[371]		O00410	2	1			Importin-5
[372]		Q00325		1	1		Phosphate carrier protein, mitochondrial
[373]		Q9Y4P3			1		Transducin beta-like protein 2
[374]		P30419			1	1	Glycylpeptide N-tetradecanoyltransferase 1
[375]		O14745	2				Na(+)/H(+) exchange regulatory cofactor NHE-RF1
[376]		P61513	1				60S ribosomal protein L37a
[377]		Q86UE4	1	1		1	Protein LYRIC
[378]		A8MTJ3	1				Guanine nucleotide-binding protein G(t) subunit alpha-3
[379]		O60361	1		1	1	Putative nucleoside diphosphate kinase
[380]		P42766	1				60S ribosomal protein L35
[381]		P0C7T9	1				Putative basic leucine zipper and W2 domain-containing protein 1-like
[382]		P37802		2	1		Transgelin-2
[383]		Q8WWH5		1			Probable tRNA pseudouridine synthase 1
[384]		O43776	2	2			Asparaginyl-tRNA synthetase, cytoplasmic
[385]		P46782			1		40S ribosomal protein S5
[386]		Q9UNM6			1		26S proteasome non-ATPase regulatory subunit 13
[387]		P53004	1				Biliverdin reductase A
[388]		Q15819		1			Ubiquitin-conjugating enzyme E2 variant 2
[389]		P62854			2		40S ribosomal protein S26
[390]		P62942		1			Peptidyl-prolyl cis-trans isomerase FKBP1A
[391]		Q9Y2A7	1				Nck-associated protein 1
[392]		P26639	1				Threonyl-tRNA synthetase, cytoplasmic
[393]		P14314		1	2		Glucosidase 2 subunit beta
[394]		P16949		1			Stathmin
[395]		Q13813	1				Spectrin alpha chain, brain

Table B1-6

# of Peptides							
Rank	Uniq Pep	Acc #	non-induced	0 h Tm	4 h Tm	8 h Tm	Protein Name
[396]		Q12904				1	Aminoacyl tRNA synthetase complex-interacting multifunctional
[397]		Q92783	1				Signal transducing adapter molecule 1
[398]		P50995	2				Annexin A11
[399]		Q13217		1			DnaJ homolog subfamily C member 3
[400]		Q53EL6	1				Programmed cell death protein 4
[401]		P40429	1				60S ribosomal protein L13a
[402]		Q13564	1				NEDD8-activating enzyme E1 regulatory subunit
[403]		P06493				1	Cell division control protein 2 homolog
[404]		P52306	1				Rap1 GTPase-GDP dissociation stimulator 1
[405]		Q02543				2	60S ribosomal protein L18a
[406]		O60925		1			Prefoldin subunit 1
[407]		Q86Y56	1				HEAT repeat-containing protein 2
[408]		P38606	2				V-type proton ATPase catalytic subunit A
[409]		Q9NQX3	1				Gephyrin
[410]		O00178	1				GTP-binding protein 1
[411]		P47756		2			F-actin-capping protein subunit beta
[412]		P07814	1				Bifunctional aminoacyl-tRNA synthetase
[413]		P81605		1			Dermcidin
[414]		Q9Y5K6	1				CD2-associated protein
[415]		P63241			1		Eukaryotic translation initiation factor 5A-1
[416]		P05387		2		1	60S acidic ribosomal protein P2
[417]		O43684		1			Mitotic checkpoint protein BUB3
[418]		Q96CS3			2		FAS-associated factor 2
[419]		P09110	1				3-ketoacyl-CoA thiolase, peroxisomal
[420]		P31350	1				Ribonucleoside-diphosphate reductase subunit M2
[421]		Q13617	1				Cullin-2
[422]		Q9UBF2	2				Coatomer subunit gamma-2
[423]		P06753		2		2	Tropomyosin alpha-3 chain
[424]		Q5TDH0	1				Protein DDI1 homolog 2
[425]		Q07021				2	Complement component 1 Q subcomponent-binding protein, m
[426]		P36871	1				Phosphoglucomutase-1
[427]		Q8IZ83	1				Aldehyde dehydrogenase family 16 member A1
[428]		P0CB38	1				Polyadenylate-binding protein 4-like
[429]		Q15750	1				Mitogen-activated protein kinase kinase kinase 7-interacting pr
[430]		P43487	1				Ran-specific GTPase-activating protein
[431]		P49257				1	Protein ERGIC-53
[432]		Q9P2W9			2		Syntaxin-18
[433]		P14174			2		Macrophage migration inhibitory factor

Table B1-7

	# of	Peptides		
non-induced	0 h Tm	4 h Tm	8 h Tm	Hits
23	14	16	13	Heat shock protein HSP 90-beta
25	15	19	20	Heat shock protein HSP 90-alpha
11	6	6	4	Putative heat shock protein HSP 90-beta-3
6	4	3	3	Putative heat shock protein HSP 90-beta 2
1	11	13	11	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
8	5	8	7	Eukaryotic initiation factor 4A-I
5	3	6	4	Eukaryotic initiation factor 4A-II
5	6		1	Eukaryotic translation initiation factor 3 subunit L
6	1	2	3	Eukaryotic translation initiation factor 3 subunit B
4	4	4	6	Eukaryotic translation initiation factor 3 subunit E
4	3	2	4	Eukaryotic translation initiation factor 3 subunit C
3				Eukaryotic translation initiation factor 2A
3	3	4	3	Eukaryotic translation initiation factor 2 subunit 3
3			1	Eukaryotic translation initiation factor 3 subunit H
1			2	Eukaryotic translation initiation factor 3 subunit M
2				Eukaryotic translation initiation factor 3 subunit D
4				Eukaryotic translation initiation factor 4 gamma 1
1	2	2	4	Eukaryotic translation initiation factor 3 subunit I
1	1	1	2	Eukaryotic translation elongation factor 1 epsilon-1
2				Eukaryotic translation initiation factor 4 gamma 2
1	1	1	1	Eukaryotic translation initiation factor 3 subunit G
		1		Eukaryotic translation initiation factor 5A-1
3	4		3	Serine-threonine kinase receptor-associated protein
4		1	1	Programmed cell death 6-interacting protein
4	3	1		Stress-induced-phosphoprotein 1
3	2	2		Apoptosis-inducing factor 1, mitochondrial
	1	2	1	Programmed cell death protein 5
1				Programmed cell death protein 4
	4	4	4	Phosphatidylinositol phosphatase SAC1
	2	2		Tyrosine-protein phosphatase non-receptor type 1
1	2			Protein phosphatase 1B
1				Tyrosine-protein phosphatase non-receptor type 9
6	4	5	2	Ser/thr-protein phosphatase 2A 65 kDa regulatory subunit A alpha
1				Ser/thr-protein phosphatase 2A 55 kDa regulatory subunit B alpha
3				Ser/thr-protein phosphatase 2B catalytic subunit beta
	1			Ser/thr-protein phosphatase PGAM5, mitochondrial
3		2	1	Insulin-like growth factor 2 mRNA-binding protein 1
4				Insulin-like growth factor 2 mRNA-binding protein 3
		1	1	RNA-binding protein EWS
1		1		Polyadenylate-binding protein 2
1		2		RNA-binding protein FUS
5	5	3	3	Peroxiredoxin-1
1	3	2	2	Peroxiredoxin-2
	3			Peroxiredoxin-4

Table B2

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