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IRE1 Signaling Affects Cell Fate During the Unfolded Protein Response

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IRE1 Signaling Affects Cell Fate During the Unfolded Protein Response

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MATERIAL AND METHODS

Cell Culture

HEK293, HeLa, MDAM468, Jurkat, and *Ire1*$\alpha^{-/-}$ 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 (*S1*). Transfections were performed using the FuGene reagent (Roche, Basel, Switzerland).

Molecular Biology

To construct a 1NM-PP1 sensitized human *Ire1*α allele, QuikChange site-directed mutagenesis (Stratagene, San Diego, CA) was used to introduce an isoleucine to glycine missense mutation in the ATP binding domain of human IRE1α at position 642. The human IRE1[I642G] construct was integrated into HEK293 cells bearing a *frt* site as previously described (*S2*). Five independent

isogenic clones were analyzed with identical findings. IRE1 mRNA was 226 ± 57-fold more abundant in the transgenic cells than in control cells. By contrast, the abundance of *BiP* mRNA and a non-UPR target gene, ribosomal protein *Rpl19* mRNA (*S3*), were not significantly altered (2.9 ± 0.2-fold and 0.9 ± 0.3-fold, respectively).

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'-TTACGGGAGAAAACTCACGGC-3' and 5'-GGGTCCAACTTGTCCAGAATGC-3'; human *Xbp-1*, 5'-TTACGAGAGAAAACTCATGGC-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 (*S4*). Quantification of spliced *Xbp-1* mRNA as a percentage of total *Xbp-1* mRNA was performed using ImageQuant software (Amersham, NJ, USA).

Quantitative Real-Time PCR Analysis

Total RNA was collected from lysed cells or frozen tissues (RNAeasy, Qiagen, Germany). PolyA mRNA was reverse-transcribed using the SuperScript RT system (Invitrogen, Carlsbad, CA) and aliquots of cDNA were used as template for quantitative PCR. Primers used included: human *BiP* mRNA, 5'-CGGGCAAAGATGTCAGGAAAG-3' and 5'-TTCTGGACGGGCTTCATAGTAGAC-3'; human *Rpl19* mRNA, 5'-ATGTATCACAGCCTGTACCTG–3' and 5'-TTCTTGGTCTCTTCCTCCTTG-3'; human *Chop* mRNA, 5'-ACCAAGGGAGAACCAGGAAACG-3' and 5'-TCACCATTCGGTCAATCAGAGC-3'; human *Ire1*α mRNA, 5'-TGGGTAAAAAGCAGGACATCTGG-3' and 5'-GCATAGTCAAAGTAGGTGGCATTCC-3'; rat *BiP* mRNA, 5'-CCTGCGTCGGTGTATTCAAG-3' and 5'-AAGGGTCATTCCAAGTGCG-3'; rat *Rpl19* mRNA, 5'-TGGACCCCAATGAAACCAAC-3' and 5'-TACCCTTCCTCTTCCCTATGCC-3'; rat *Chop* mRNA, 5'-ACGGAAACAGAGTGGTCAGTGC-3' and 5'-CAGGAGGTGATGCCAACAGTTC-3'. Quantitative PCR was performed using an MJ Opticon 2 DNA Engine (Bio-Rad, Hercules, CA). *Rpl19* mRNA levels, a ribosomal protein whose transcription is not regulated by ER stress, served as an internal normalization standard. PCR conditions were: 95°C for 5 min; 95°C for 30 sec; 55°C for 30 sec; 72°C for 1 min; 78°C for 10 sec; 72°C for 5 min, with 40 cycles of amplification.

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). Twenty 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-XBP-1^s at 1:5000 (Biolegend, San Diego, CA); anti-GAPDH at 1:10000000 (AbCAM, Cambridge, MA); anti-eIF2α at 1:2000 (Cell Signaling, Natick MA); anti-phospho-eIF2α at 1:500 (Cell Signaling, Natick,MA); anti-VCAM-1 at 1:500 (Santa Cruz Biotechnologies, Santa Cruz, CA); anti-phospho-PERK at 1:1000 (gift of Rick Locksley); anti-FLAG at 1:5000 (Sigma, St. Louis, MO); anti-ATF-4 at 1:2000 (Santa Cruz Biotechnologies, Santa Cruz, CA); 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).

For VCAM-1 radiolabeling, cells were pulsed with 100 μCi of EXPRE^{35}S^{35}S 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, immunoprecipitate was loaded onto SDS-PAGE gels, and ^{35}S-labeled VCAM-1 was detected by phospho-imaging analysis.

Metabolic Labeling

Cells were pulsed with 50 μCi of EXPRE^{35}S^{35}S Protein Labeling Mix (Perkin-Elmer, Waltham, MA) for 15 minutes, lysed in 1% NP-40, and aliquots were loaded on SDS-PAGE gels. Radioisotope incorporation was analyzed via ImageQuant (Amersham, Piscataway, NJ).

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).

Animals and Histology Analysis

Transgenic rats with P23H mutations in rhodopsin were produced by Chrysalis DNX Transgenic Sciences (now Xenogen Biosciences, Cranbury, NJ) on an albino Sprague-Dawley background and maintained at UCSF in a 12h: 12h light-dark environment at an in-cage illuminance level of approximately 120-160 lux. The P23H rats express a mouse opsin gene with a proline to histidine substitution at codon 23. Experimental rats carry a single copy of the transgene and two copies of the wild-type rhodopsin gene. Different levels of mutant opsin compared to native opsin are expressed in different lines of transgenic rats, with those having a higher level of expression (P23H-1) showing a faster rate of photoreceptor degeneration than those with a lower level of expression of the mutant opsin (P23H-3). The rats are more fully described at: http://www.ucsfeye.net/mlavailRDratmodels.html.

At the indicated ages, rats were euthanized by overdose of carbon dioxide. Immediately after death, they were either perfused with mixed aldehydes and their eyes embedded in epoxy resin for histological sectioning (*S5*), or the retinas were removed from the eyes for molecular analysis by the "winkling" method (*S6*) and immediately frozen in liquid nitrogen. All procedures

were approved by and followed the guidelines of the University of California San Francisco Institutional Animal Care and Use Committee.

One-μm thick plastic sections were cut along the vertical meridian of the embedded eyes. The mean outer nuclear layer (ONL) thickness, a measure of photoreceptor number (*S7*), was obtained with a computer-assisted method from 54 measurements around each eye as described elsewhere (*S8*). Statistical comparisons between measurements of transgenic and wild-type retinas were made using a two-tailed, unpaired Student's *t*-test.

Supplemental Figure 1 | Attenuation of *Xbp-1* mRNA splicing in multiple human cell lines. HeLa, MDAM468, and Jurkat cells were treated with tunicamycin (5 μg/ml) for the indicated times (hours), and *Xbp-1* mRNA splicing was determined by RT-PCR. For comparison, splicing of *Xbp-1* mRNA in HEK293 cells is also shown (Fig 1A).

Supplemental Figure 2 | 1NM-PP1 does not activate JNK in transgenic cells. Transgenic HEK293 cells expressing IRE1(I642G) were treated with 1NM-PP1 (5 μM) for the indicated duration. *Xbp-1* mRNA splicing was assessed by RT-PCR, and phospho-JNK protein levels were assessed by immunoblotting. Total JNK protein levels are shown as a loading control.

Supplemental Figure 3 | IRE1 signaling enhances cell viability (metabolic activity). Parental wild-type and transgenic cells were treated with agents, and mitochondrial dehygrogenase activity was measured via the MTT assay and is shown relative to mock-treated cells. Errors represent standard deviation from

three experiments. The arrows indicate P value < 0.01 compared to the sample in which no 1NM-PP1 was added, using the Student's t-test.

Supplemental References and Notes

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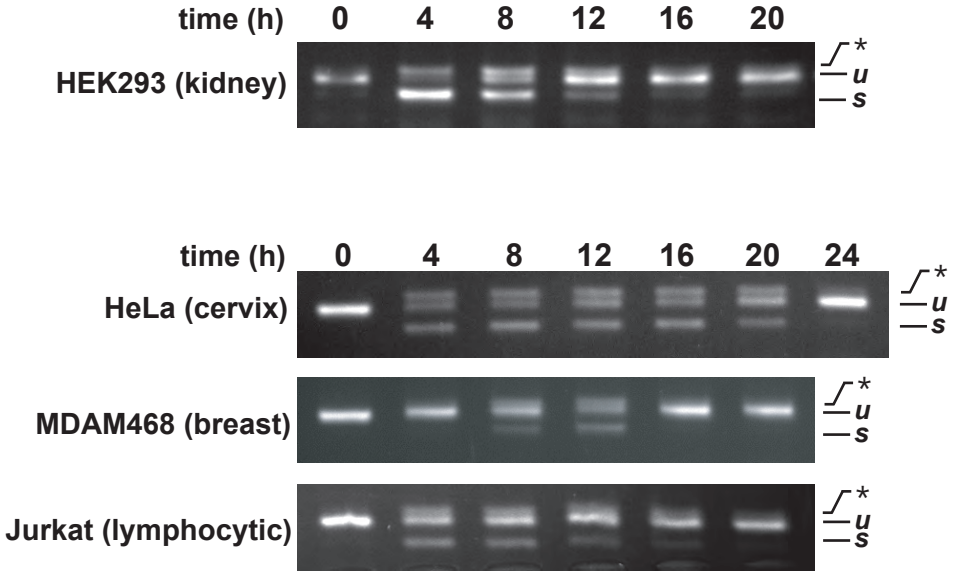
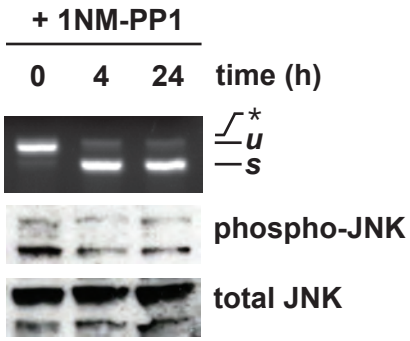
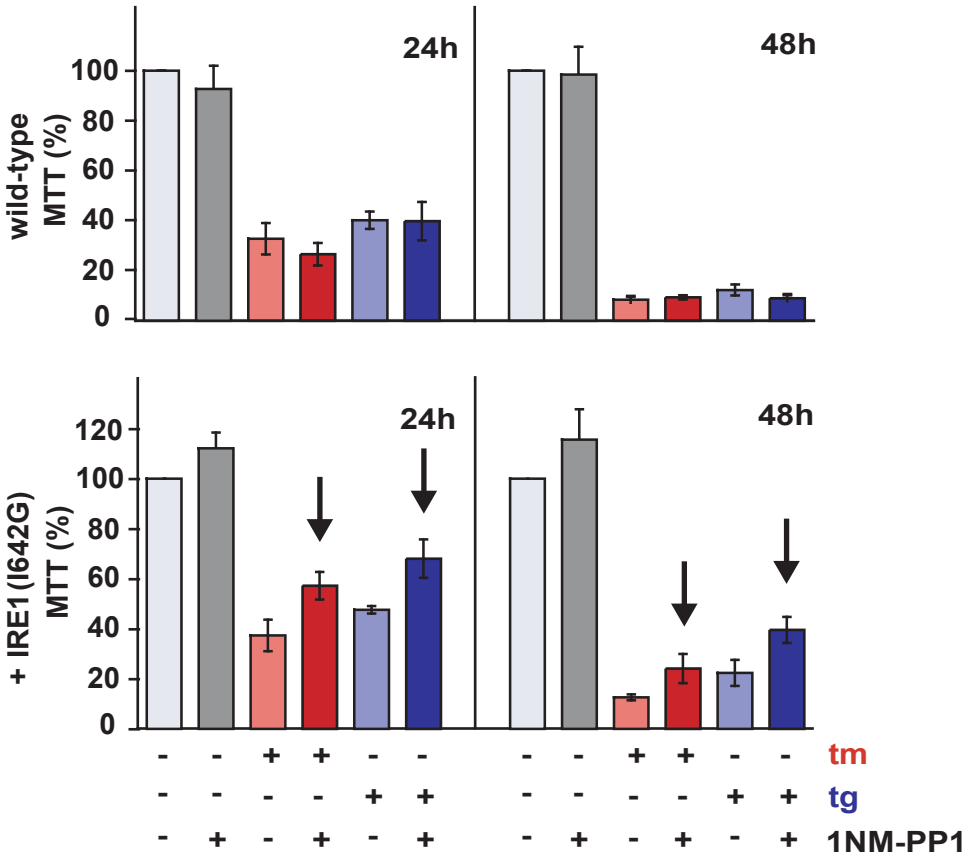


Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3