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A kinase inhibitor activates the IRE1α RNase to confer cytoprotection against ER stress

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Abstract

Unfolded proteins in the endoplasmic reticulum (ER) cause *trans*-autophosphorylation of the bifunctional transmembrane kinase IRE1α, inducing its RNase activity to splice XBP1 mRNA, in turn triggering a transcriptional program in the unfolded protein response (UPR). As we previously showed with the yeast IRE1 kinase ortholog, a single missense mutation in the ATP-binding pocket of murine IRE1α kinase sensitizes it to the ATP-competitive inhibitor 1NM-PP1, and subordinates RNase activity to the drug. This highly unusual mechanism of kinase signaling requiring kinase domain ligand occupancy—even through an inhibitor—to activate a nearby RNase has therefore been completely conserved through evolution. We also demonstrate that engagement of the drug-sensitized IRE1α kinase through this maneuver affords murine cells cytoprotection under ER stress. Thus kinase inhibitors of IRE1α are useful for altering the apoptotic outcome to ER stress, and could possibly be developed into drugs to treat ER stress-related diseases.
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Newly synthesized secretory and transmembrane proteins traversing the endoplasmic reticulum (ER) during their biogenesis fold to their native conformations in this organelle [1]. Insufficient protein folding capacity, a condition referred to as “ER stress,” leads to ER unfolded protein accumulation, and in turn triggers the unfolded protein response pathway (UPR) [2]. The UPR in mammalian cells has both transcriptional and translational outputs [3,4]. Transcriptional targets include genes encoding chaperones, oxido-reductases, phospholipid biosynthetic enzymes, ER-associated degradation (ERAD) components, and other downstream secretory pathway activities. Together, UPR outputs initially afford proteins passing through the ER an extended opportunity to fold and assemble properly, dispose of unsalvageable unfolded polypeptides, and increase the capacity for ER export by expanding organelle size and function [5]. However, if these adaptive responses do not restore homeostasis, mammalian cells switch to the alternate fate of apoptosis. While switching cellular output from adaptation to apoptosis may benefit an organism by disposing of unsalvageable cells containing misfolded ER proteins, it is also emerging that this quality control mechanism may cause disease if large numbers of functioning cells are extinguished [6].

The accumulation of unfolded proteins in the ER lumen activates the three widely expressed ER stress sensors, PERK, ATF6, and IRE1α. Activation of these sensors, when unfolded proteins accumulate, changes their oligomerization state in the ER membrane [7–9]. Upon acute activation, the downstream responses emanating from these sensors initially promote cell survival, but eventually

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these signals morph into pro-death responses if the ER stress irritants are not removed [10].

Unfolded proteins cause the most ancient ER unfolded protein sensor IRE1α to oligomerize in the ER membrane, juxtaposing its cytosolic kinase domains, which subsequently *trans* auto-phosphorylate [7,8]. Kinase auto-phosphorylation activates its C-terminal cytosolic domain, an RNase activity that initiates splicing of the XBP1 mRNA. As a consequence of removing an intron through IRE1α RNase activity, spliced XBP1 mRNA is frame-shifted and translated to produce XBP1 protein, a transcription factor whose target set includes genes encoding ER chaperones, oxido-reductases, ERAD components, and other activities that initially aid adaptation [11,12]. In addition, the IRE1α/XBP1 output is used by professional secretory cells, such as plasma cells, as they differentiate to grow an expanded ER needed for high-level protein secretion [13]. However, while IRE1α kinase activation is needed to activate the XBP1 mRNA-splicing RNase, it has also been shown that overexpression of the paralogous (gut tissue-restricted) IRE1β protein promotes apoptosis, and that this apoptotic output requires a catalytically active kinase activity [14,15]. Similarly, the widely-expressed IRE1α activity may be capable of promoting either cell survival or death.

Using a chemical-genetic strategy, we previously deciphered the functional relationship between the kinase and the RNase in the yeast IRE1 ortholog. We showed that the yeast IRE1 kinase domain acts as a conformational switch when bound by a ligand to activate the attached RNase. The ligand can even be an ATP-competitive kinase inhibitor, demonstrating that kinase phosphotransfer activity itself is unnecessary for IRE1 kinase function [16]. Given that there is great interest in potentially manipulating the UPR pathway to protect against human protein folding diseases, we applied a similar chemical-genetic approach to study mammalian IRE1α kinase. Our results convincingly show that the unprecedented mechanism of kinase activation of the attached RNase using a small ligand, first discovered in yeast IRE1, has not only been conserved in mammalian IRE1 orthologs, but that similar provision of a kinase ligand to the mouse ortholog additionally confers significant survival advantage in mouse embryonic fibroblasts (MEFs) under ER stress.

Materials and methods

Cell culture and retroviral transduction. Mouse embryonic fibroblasts (MEFs) were grown in DMEM 10% FCS, 50 U/mL penicillin and 50 μg/mL streptomycin. For retroviral expression, a cDNA encoding wild type or mutant mouse Ire1α was cloned into pBABEpuro, and site-directed mutagenesis carried out using Quickchange (Invitrogen). Virus production was carried out in the 293-GPG cell line. Briefly, retroviral expression constructs were transiently transfected into 293-GPG cells using Lipofectamine 2000 (Invitrogen). Virus was collected on a daily basis and filtered through a 0.45 μm syringe filter. Virus collected on day 6 or 7 was used to infect either wild-type MEF or IRE1α−/− MEF. Stable retroviral-transduced colonies were selected in 2 μg/mL puromycin.

XBP-1 splicing assay. XBP-1 splicing assay was performed as described previously Calfon et al. [12]. After treatment, total RNA was extracted using Trizol reagent (Invitrogen) per the manufacturer's instruction. After DNaseI treatment, a 600 bp cDNA product enocompassing the IRE1 cleavage site was amplified by one-step RT-PCR kit (Invitrogen) using the sense primer mXBP1.3S (5′-AAACAGAGTAGCAGCGCAGACTGC-3′) and the antisense primer mXBP1.2AS (5′-GGATCTCTAAAACTA GAGGCTTGGTG-3′). This fragment was further digested by PstI to reveal a restriction site that is lost upon splicing of XBP-1 by Ire1α. The products were resolved on a 2% agarose gel.

Apoptosis assays. Cells were seeded into 24-well plates at a density of 2×10^4 cells per well and either left untreated or pre-treated for 24 h with 2 μM or 5 μM 1NM-PP1. After this time the cells were incubated with 0.0 μg/ml, 0.075 μg/ml or 0.1 μg/ml tunicamycin (Tm) for a further 24 h. After this second incubation, any floating cells were collected and saved. The remaining adherent cells were harvested through treatment with 100 μl 0.25% trypsin for 5 min. Floating and adherent cells were combined and pelleted at 1500 rpm for 5 min. The cell pellet was washed twice in 1 ml cold phosphate buffered saline. The cells were resuspended in 500 μl Annexin-V binding buffer (0.1 M Hepes/NaOH, pH 7.4, 1.4 M NaCl, and 25 mM $CaCl_2$) supplemented with 0.5 μl of Annexin V-FITC reagent (BioVision, USA). Cells were incubated at room temperature with the binding buffer/Annexin-V reagent for 5 min in the dark, then passed through a 70 μm nylon cell strainer (BD Falcon, USA). Apoptosis was quantified by flow cytometric detection of Annexin V-FITC staining on a BD Bioscience FACSCalibur machine, using BD Bioscience CellQuest Pro acquisition software. Each data point represents three independent replicates. Statistical significance was determined using the Student's *t*-test at the following website http://www.physics.csbsju.edu/stats/t-test.html.

Results and discussion

To investigate the effects of inhibiting the IRE1α kinase, we reconstituted *Ire1α−/−* mouse embryonic fibroblasts (MEFs) using a retrovirus bearing a cDNA encoding mouse IRE1α mutated at Ile642 to Gly. This substitution is predicted to sensitize IRE1α to the ATP-competitive drug 1-*tert*-butyl-3-naphthalen-1-ylmethyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine (1NM-PP1) by creating an enlarged active site pocket not found in wild-type kinases [17]. While this rational protein engineering method is tolerated by many kinases without decrement in catalytic activity, other kinases are partially or severely impaired by the space-creating substitution [18].

The ER protein glycosylation inhibitor tunicamycin (Tm) causes ER stress to activate the wild-type IRE1α kinase activity, in turn triggering the attached RNase activity to produce spliced XBP1 mRNA (detected through an RT-PCR assay, Fig. 1 compare lanes 5 and 6). A functioning IRE1α kinase activity is needed to activate the XBP1 mRNA-splicing RNase [12,19]. Substitution of Ile642 to Gly in the retrovirally-delivered IRE1α completely abrogated XBP1 mRNA-splicing activity when ER stress was induced using Tm, making the *Ire1α−/−* MEFs reconstituted with IRE1α (I642G) indistinguishable from the parent *Ire1α−/−* MEFs in this property (Fig. 1, compare lane 10 with lanes 2 and 6). This result suggested that the I642G mutation severely abrogated kinase function. Ile642 is predicted to lie deep in the ATP-binding pocket of kinase domain, and as adenosine nucleotide binding in this pocket is central to activation of the attached RNase

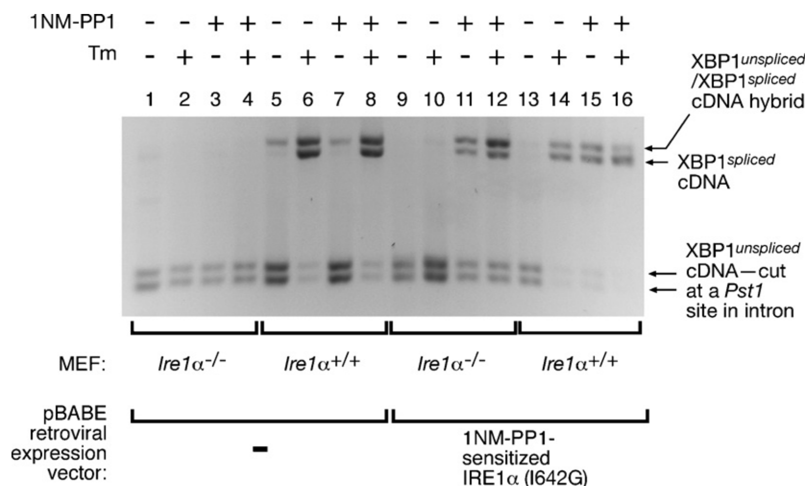

Fig. 1. XBP1 splicing in 1NM-PP1-sensitized IRE1α-reconstituted MEFs. MEFs were treated with 5 μg/mL tunicamycin (Tm) and/or 10 μM 1NM-PP1 for 8 h. The XBP1 cDNA products of PstI digestion were revealed on a 2% agarose gel. Unspliced XBP1 mRNA produces the two lower bands (291 bp and 310 bp), whereas spliced XBP-1 mRNA gives one 575 bp band. The highest band is a previously described hybrid of spliced and unspliced XBP1 cDNA fragments.

in the yeast ortholog, mutation of this conserved residue to Gly in the mammalian ortholog may have created a destabilized ATP-binding cavity. Thus, severely compromising the kinase activity of IRE1α abrogates its RNase activity.

Surprisingly, addition of 1NM-PP1, an ATP-competitive inhibitor, to the *Ire1α*−/− MEFs reconstituted with IRE1α (I642G) substantially restored XBP1 mRNA splicing (Fig. 1, compare lanes 10 and 11). In these reconstituted cells we could be assured that XBP1 mRNA splicing occurred due to the transgenic IRE1α (I642G) version, and not through an indirect mechanism of activating endogenous IRE1α, since these cells lacked both chromosomal IRE1α copies. Furthermore, 1NM-PP1 by itself had no effect on XBP1 splicing in *Ire1α*+/+ cells, indicating that it does not induce the UPR in MEFs by indirectly causing ER stress (Fig. 1, lane 7). Additionally we noted that expressing IRE1α (I642G) in *Ire1α*+/+ MEFs confers on these cells two independent properties: the ability to splice XBP1 mRNA in response to 1NM-PP1 through the drug-sensitized version (Fig. 1, lane 15), while still retaining the ability to respond to ER stress (Fig. 1, lane 14). This indicated that the presence of the transgenic drug-sensitized IRE1α does not interfere with signaling by endogenous IRE1α, and furthermore that the drug-sensitized IRE1α version remains quiescent until it encounters 1NM-PP1.

We previously showed that the kinase phosphotransfer activity in yeast IRE1 is not required for its RNase activity as long as a small ligand (even an inhibitor) is bound to the kinase domain [16]. Since then, it has remained unclear if this unprecedented mode of kinase signaling is simply a curiosity of the IRE1 kinase in this lower eukaryote. The aforementioned results convincingly demonstrate that this unusual mechanistic relationship between the kinase and RNase domains of IRE1 is a general feature of this bifunctional enzyme in many eukaryotes. However, in striking contrast to our previous experience with yeast IRE1 [16], the mammalian drug-sensitized IRE1α could be activated simply through provision of 1NM-PP1 (Fig. 1, compare lane 11 with 9, and lanes 15 with 13), irrespective of whether or not ER stress was additionally induced. It is possible that because the mammalian IRE1α protein in this study was expressed under the control of a strong viral promoter, its supraphysiological levels may have led to it becoming pre-oligomerized through its ER lumenal domain, thereby making 1NM-PP1 an instructive trigger for kinase activation. It is also conceivable that the mammalian drug-sensitized versions become tightly bound with 1NM-PP1, and therefore come with a pre-activated kinase domain that can be triggered when unfolded proteins accumulate—even transiently—in the ER. Our chemical-genetic systems will be invaluable tools to decipher the mechanistic connections between IRE1α kinase and its RNase domains in the future.

In this report, we chose to use these tools to address a critical physiological question: namely whether pharmacologically manipulating IRE1α could affect cell fate. Signaling through IRE1α in the mammalian UPR has been proposed to have opposing physiological outputs. As mentioned, IRE1α kinase activation is normally needed to trigger the RNase [19]. Yet overexpression of IRE1β containing a functionally active kinase has also been shown to promote cell death [14,15]. Indeed we noted that repeated attempts to retrovirally reconstitute *Ire1α*−/− MEFs with wild-type IRE1α caused the resulting cell lines to be unstable, such that the clones that grew out under antibiotic selection had lost the transgene. This was presumably because the strong viral promoter constitutively driving the transgene led to intolerably high levels of the active IRE1α kinase. On the hand, retroviral reconstitution of either *Ire1α*−/− or *Ire1α*+/+ MEFs with the kinase-dead IRE1α (I642G) was straightforward, and the resulting

clones stably expressed the transgene at consistent levels indefinitely. Furthermore, the RNA and protein levels of the mutant IRE1α (I642G) in these cells was many fold enriched over corresponding levels of endogenous IRE1α (not shown). Previous work established that the IRE1α RNase activity negatively regulates its own mRNA [20]. Since the IRE1α (I642G) RNase does not respond to ER stress (Fig. 1, lane 10), it is likely that it does not down regulate its own message.

The ability to forcibly activate the RNase of IRE1α (I642G), independent of ER stress, allowed us to study the physiological consequence of triggering the XBP1 mRNA splicing activity on cell survival. We first noted that triggering of XBP1 mRNA splicing in either the *Ire1α*+/+ or the *Ire1α*−/− MEFs reconstituted with IRE1α (I642G) for up to 24 h using 5 μM 1NM-PP1 led to no significant increases in apoptosis as measured by percentage of Annexin-V positive cells (Fig. 2A and B). This indicated that forcible IRE1α-mediated XBP1 mRNA splicing is not pro-apoptotic *per se*. In contrast, provision of the ER stress inducer Tm at 0.075 μg/ml to wild-type cells induced significant apoptosis by 24 h post-treatment with about 40% of the cells consistently displaying Annexin-V staining, and this was preceded by many hours of vigorous and sustained splicing of XBP1 mRNA in these cells (Fig. 2C).

More intriguing was the possibility that pre-emptive XBP1 mRNA splicing using 1NM-PP1 in the designer lines could afford cytoprotection against ER stress. The XBP1 transcription factor has downstream targets that are proposed to expand ER function, which should make the organelle more robust and better equipped to tolerate high levels of ER stress [21]. To test this hypothesis, we pre-treated all the aforementioned cell lines for 24 h to two different concentrations of 1NM-PP1 (2 μM or 5 μM, or as a negative control left them untreated), before exposing the cells to three different concentrations of Tm (0.00 μg/ml, 0.075 μg/ml or 0.10 μg/ml), for another 24 h.

We then measured apoptosis in the treated cells by Annexin-V labeling—in triplicate for each treatment in each cell line. The results are expressed as the ratio of viable cells in the presence versus absence of 1NM-PP1 pretreatment when exposed to Tm (Fig. 3.). Pre-treatment with 1NM-PP1 at 2 μM clearly afforded cytoprotection only to *Ire1α*−/− MEFs reconstituted with IRE1α (I642G), with 1.7-fold and 1.6-fold enhancement of viability at Tm concentrations of 0.075 and 0.1 μg/ml, respectively (Fig. 3D). These concentrations of 1NM-PP1 did not improve viability in any of the other cell lines. Dose escalation of 1NM-PP1 to 5 μM led, in fact, to small but statistically significant cytotoxicity in both the *Ire1α*−/− and *Ire1α*+/+ parent MEFs (Fig. 3A and B). Strikingly, this enhanced dose of 1NM-PP1 caused much larger rates of cytoprotection, but again only in the *Ire1α*−/− MEFs reconstituted with IRE1α (I642G), with 3.5-fold enhancement of viability at both Tm concentrations of 0.075 and 0.1 μg/ml, respectively (Fig. 3D). In contrast, the *Ire1α*+/+

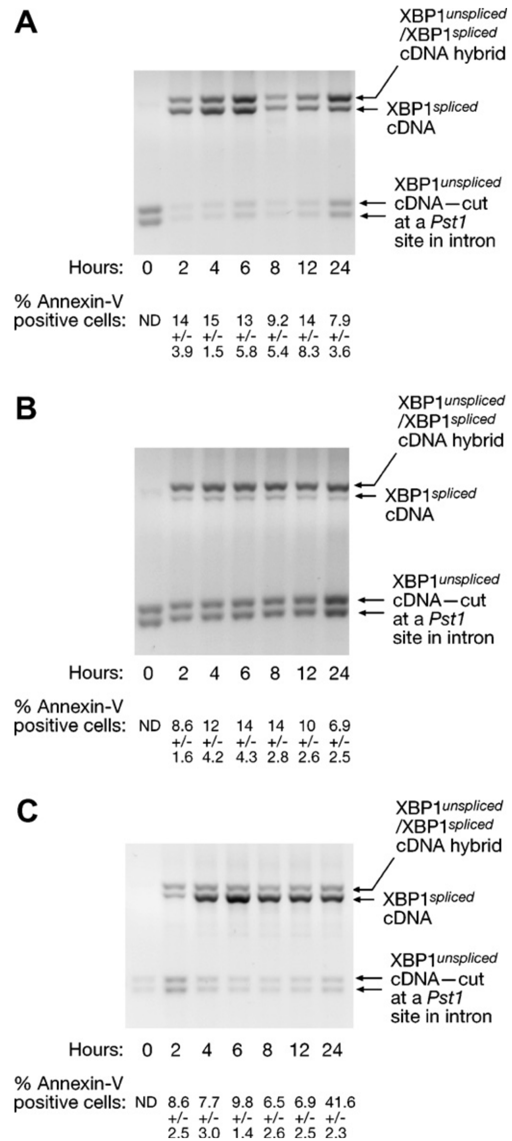

Fig. 2. Temporal splicing of XBP1 mRNA induced by 1NM-PP1 versus ER stress. *Ire1α*+/+ (A) or *Ire1α*−/− MEFs (B) reconstituted with IRE1α (I642G) were treated with 5 μM 1NM-PP1. Wild-type MEFs were treated with 0.075 μg/ml tunicamycin (C). The cleavage of XBP1 mRNA and the percentage of apoptotic cells (Annexin-V positive) in triplicate experiments was determined at the indicated times.

MEFs reconstituted with IRE1α (I642G) did not benefit from 1NM-PP1 treatment at either dose, despite the fact that XBP1 mRNA splicing was even more vigorous in this background than in the *Ire1α*−/− MEFs (Fig. 2A and B). This suggests that a dominant pro-death signal in response to ER stress may continue to proceed unchecked through the endogenous wild-type IRE1α, which harbors an active kinase. Through expressing 1NM-PP1-sensitized IRE1α in the *Ire1α*−/− MEFs, we could cleanly ascribe cytoprotective effects to the sole drug-sensitized variant. Although these results are consistent with previous observations that the kinase phosphotransfer activity of wild-type IRE1α may itself have pro-death outputs, perhaps through kinase activation of pro-apoptotic ASK1 and JNK [22], more

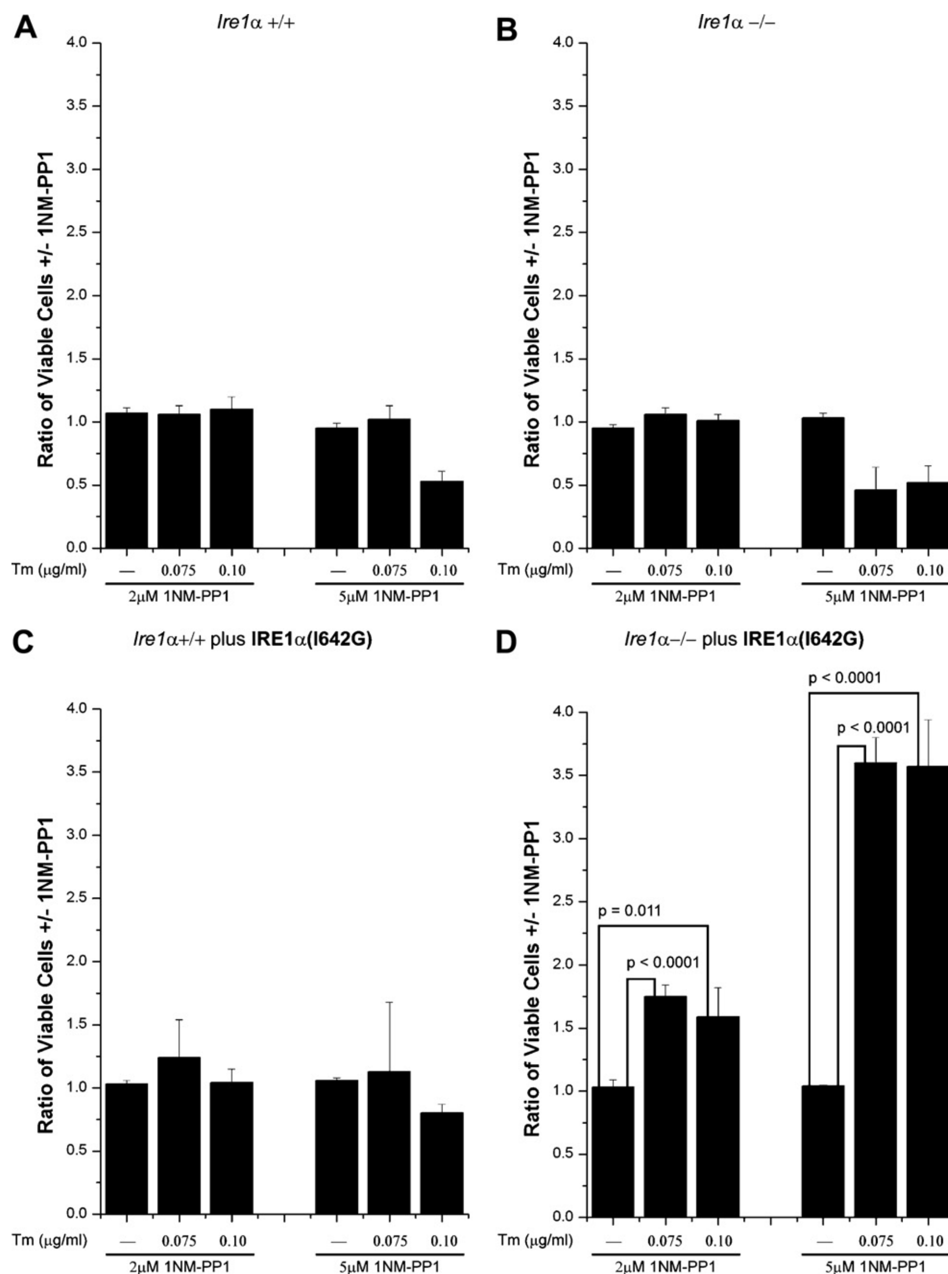

Fig. 3. Cell viability under ER stress affected by 1NM-PP1. The indicated cells were left untreated or pre-treated with 2 μM or 5 μM 1NM-PP1 for 24 h. After this time, cells were treated with 0.00 μg/ml, 0.075 μg/ml, or 0.10 μg/ml tunicamycin (Tm) for a further 24 h. Data are represented as the ratio of viable cells (Annexin-V negative) pretreated with 1NM-PP1 compared to those without 1NM-PP1 treatment. Each experiment was conducted in triplicate.

detailed mechanistic studies will be needed to investigate this further.

Using the strong ER stress-inducing agent, Tm, at doses that cause significant apoptosis, these are among the first proof-of-concept studies showing that engagement of the IRE1α kinase with an inhibitor has pro-life consequences in the cellular response to ER stress. While this work was in preparation, another report was published demonstrating some similar findings, although the authors of that study relied on ectopic production of the drug-sensitized IRE1α kinase in wild-type HEK cells containing endogenous IRE1α, and did not address apoptotic endpoints *per se* [23].

Kinase activation in signaling pathways is often a transitory and reversible event, and persistent signaling by kinases has been shown in numerous instances to have strongly deleterious effects in cells. IRE1α kinase activation is obligatory for RNase activation, which initially should have adaptive outputs. IRE1α's RNase could reduce ER stress through multiple mechanisms including cleavage of mRNAs encoding secretory and transmembrane proteins as was recently demonstrated [24], or through the execution of the transcriptional program controlled by the XBP1 transcription factor, the pro-life outputs of which have been previously demonstrated [25]. Yet persistent kinase signaling by IRE1α could signal that homeostasis has not

been reached, instead promoting the switch to apoptosis under continuous ER stress. Drug-based inhibition of the IRE1α kinase causing simultaneous and persistent activation of its RNase therefore may afford cytoprotection by uncoupling the obligatory mechanistic link between the kinase and the RNase, while biasing towards adaptive outputs emanating from the RNase. Our observations and the novel tools we describe in this work should permit both the mechanistic dissection of these opposing outputs, as well as the definition of their physiological scope.

Among known multi-domain proteins containing kinase activities, this unusual mechanistic relationship between the kinase domain and another nearby catalytic activity thus far remains a unique and conserved feature of IRE1 proteins. We suggest that this unique feature in mammalian IRE1α's kinase is fortuitous from the standpoint of drug development, and should be exploited. To date, pharmacological means to produce specific transcription factors in cells are lagging. However, numerous highly specific kinase inhibitors have been developed, and are already in clinical use. If small molecule inhibitors of the wild-type IRE1α kinase activity could be developed, we predict that such compounds will be tremendously useful for fundamental research in UPR signaling, and may eventually find clinical application in the treatment of myriad cellular diseases now being found to proceed from ER stress.

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