



Pharmacologic IRE1/XBP1s activation confers targeted ER proteostasis reprogramming

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Activation of the IRE1/XBP1s signaling arm of the unfolded protein response (UPR) is a promising strategy to correct defects in endoplasmic reticulum (ER) proteostasis implicated in diverse diseases. However, no pharmacologic activators of this pathway identified to date are suitable for ER proteostasis remodeling through selective activation of IRE1/XBP1s signaling. Here, we use high-throughput screening to identify non-toxic compounds that induce ER proteostasis remodeling through IRE1/XBP1s activation. We employ transcriptional profiling to stringently confirm that our prioritized compounds selectively activate IRE1/XBP1s signaling without activating other cellular stress-responsive signaling pathways. Furthermore, we demonstrate that our compounds improve ER proteostasis of destabilized variants of amyloid precursor protein (APP) through an IRE1-dependent mechanism and reduce APP-associated mitochondrial toxicity in cellular models. These results establish highly selective IRE1/XBP1s activating compounds that can be widely employed to define the functional importance of IRE1/XBP1s activity for ER proteostasis regulation in the context of health and disease.

The unfolded protein response (UPR) is the primary signaling pathway activated by ER stress^{1,2}. The UPR comprises three signaling cascades activated downstream of the ER stress-sensing proteins IRE1, PERK and ATF6 (refs. ^{3,4}). In response to acute ER stress, UPR activation results in transcriptional and translational signaling to alleviate the misfolded protein load in the ER and promote adaptive remodeling of ER function and global cellular physiology^{5,6}. However, in response to chronic or severe ER insults, prolonged UPR signaling induces a pro-apoptotic response that results in cellular death^{7,8}. Through this combination of adaptive and pro-apoptotic signaling, the UPR dictates cellular function and survival in response to diverse pathologic insults.

The capacity of UPR signaling pathways to promote adaptive remodeling makes them attractive targets to ameliorate imbalances in ER proteostasis associated with etiologically diverse diseases^{9–11}. The IRE1 pathway is the most evolutionarily conserved arm of the UPR, as it is found in all eukaryotes from yeast to mammals¹². In response to ER stress, IRE1 is activated through a mechanism involving auto-phosphorylation, oligomerization and allosteric activation of a cytosolic endoribonuclease (RNase) domain^{13–15}. This RNase is involved in splicing the *XBP1* messenger RNA, resulting in a frameshift that allows translation of the active XBP1 spliced (XBP1s) transcription factor^{13,16}. XBP1s adapts ER proteostasis through the increased expression of stress-responsive genes including chaperones and degradation factors^{5,6}. The activated IRE1 RNase domain also promotes the degradation of ER-localized mRNAs through a process known as regulated IRE1-dependent decay (RIDD)^{17,18}. While the functional implications of RIDD remain to be fully established, recent results suggest that RIDD plays a protective role during ER stress through the selective degradation of mRNA encoding pro-apoptotic factors (for example, *DR5*) and increased microautophagy through the

degradation of *BLO1* (refs. ^{17,19}). Thus, IRE1 activation can promote adaptive remodeling that alleviates ER stress and enhances ER proteostasis through XBP1 splicing and/or RIDD.

Increasing IRE1–XBP1s activity offers a unique opportunity to ameliorate pathologic imbalances in ER proteostasis implicated in diverse diseases. XBP1s overexpression promotes neuroprotection in animal models of neurodegenerative disease including Parkinson's disease, Huntington's disease and peripheral nerve injury^{20–22}. Increasing XBP1s can also promote the degradation of destabilized amyloid precursor protein (APP) mutants, reducing extracellular populations of the APP cleavage product Aβ associated with Alzheimer's disease (AD)^{23,24}. Furthermore, chemical genetic activation of IRE1–XBP1s reduces the intracellular aggregation of destabilized, aggregation-prone variants of rhodopsin and α1-antitrypsin (A1AT) implicated in retinitis pigmentosa and A1AT deficiency, respectively^{5,25,26}. IRE1–XBP1s activation is also advantageous in models of other disorders including diabetes and myocardial infarction^{27,28}.

Based on this, there is substantial interest in identifying non-toxic compounds that activate IRE1–XBP1s signaling. Previous work identified compounds that allosterically activate the IRE1 RNase by binding the IRE1 kinase active site^{13–15}. While these compounds are useful for defining the mechanism of IRE1 activation, many show off-pathway activity and/or toxicity, limiting their use for defining the functional implications of IRE1–XBP1s activation in health and disease^{15,29,30}. This necessitates the development of new compounds that activate IRE1–XBP1s through an alternative mechanism.

Here, we use a high-throughput screening (HTS) approach to identify non-toxic compounds that activate IRE1-dependent *XBP1* splicing through a mechanism independent of binding the IRE1 kinase active site. We use RNA sequencing (RNA-seq)

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transcriptional profiling to demonstrate the selectivity of these compounds for IRE1–XBPs signaling over other arms of the UPR or other stress-responsive signaling pathways. These compounds, which are prioritized on the fidelity of their transcriptional response, offer an opportunity to promote adaptive ER proteostasis remodeling through selective IRE1–XBPs activation. To exemplify this, we demonstrate that our compounds promote the degradation of AD-associated APP mutants through an IRE1-dependent mechanism and mitigate APP-associated mitochondrial toxicity in cell models. Our results establish IRE1–XBPs activating compounds that provide new opportunities to explore the mechanisms by which signaling through this pathway influences cellular physiology, and to define the use of IRE1–XBPs signaling for ameliorating pathologies associated with etiologically diverse diseases.

Results

HTS to Identify IRE1–XBPs activating compounds. We used a human embryonic kidney 293^{TREX} (HEK293^{TREX}) cell line stably expressing an XBP1–Renilla luciferase (XBP1–RLuc) splicing reporter to identify compounds that activated the IRE1–XBPs signaling pathway (Fig. 1a and Extended Data Fig. 1a)^{31,32}. Activated IRE1 splices mRNA encoded by this reporter, resulting in a frameshift that allows RLuc expression³¹. We confirmed that the XBP1–RLuc reporter is activated by the ER stressor thapsigargin (Tg, a SERCA inhibitor) and is blocked by cotreatment with the selective IRE1 RNase active site inhibitor 4μ8c³³ (Extended Data Fig. 1b). We miniaturized this assay to a 1,536-well plate format and screened the 646,251 compound Scripps Drug Discovery Library at the Scripps Research Institute Molecular Screening Center (Fig. 1a and Supplementary Table 1). Our primary screen identified 10,114 compounds that activated XBP1–RLuc activity >13.83% relative to Tg (Extended Data Fig. 1c). We removed compounds previously found to activate cell-based reporters of the cytosolic heat shock response³⁴, as well as promiscuous compounds identified as positive hits in more than seven assays at the Scripps Research Institute Molecular Screening Center (Fig. 1a and Extended Data Fig. 1d,e). We then selected the top ~6,400 compounds for triplicate confirmation and toxicity screening. Compounds (5.17 μM) that reduced cell viability >26.19% were removed (Extended Data Fig. 1f). We then deprioritized compounds that also activated other UPR signaling arms by removing compounds previously reported to activate the ATF6-selective ERSE–luciferase reporter³¹ (Fig. 1a and Extended Data Fig. 1d,g). From these data, we selected 638 compounds that preferentially activate the XBP1–RLuc reporter for further characterization (Source Data Fig. 1).

We removed compounds that showed a half-maximum effective concentration (EC₅₀) for XBP1–RLuc activation >3 μM, in dose response studies and maximal activity <20% relative to Tg activation (Extended Data Fig. 1h). We then performed iterative chemical subclustering of these hits to yield a representative set of 128 compounds that reflects the diversity and relative abundance of similar scaffolds among these top hits (Fig. 1b and Supplementary Table 2). The two most represented groups in this analysis were Cluster ‘H’ containing an aryl sulfonamide moiety and Cluster ‘A’ containing an N1-phenyl substituted pyrazolopyrimidine substructure (Fig. 1b, outlined in red). These structures are commonly found in compounds that bind kinase active sites, suggesting they likely activate IRE1 by binding the IRE1 kinase domain³⁵. Since we were focused on identifying compounds that activate IRE1 independent of this mechanism, we excluded compounds in Clusters A and H in the initial characterization. Instead, we selected seven compounds that represent five different structural classes for immediate follow-up (Fig. 1c and Supplementary Table 3).

Compounds promote IRE1-dependent XBP1 splicing. We confirmed concentration-dependent activation of XBP1–RLuc in

HEK293^{TREX} cells, demonstrating reporter activation to levels 35–50% that observed with Tg and EC₅₀ values consistent with those observed during HTS (Fig. 2a, Extended Data Fig. 2a and Supplementary Table 2). We confirmed that coadministration of our compounds with the IRE1 RNase active site inhibitor 4μ8c blocked compound-dependent activation of XBP1–RLuc (Fig. 2a). Next, we showed that our compounds activate the IRE1–XBPs-selective target gene *DNAJB9* (*ERD4*, Fig. 2b and Extended Data Fig. 2b) to levels 30–50% those observed for Tg (Fig. 2b), mirroring the levels of activation observed in our reporter assay (Fig. 2a). Compound-dependent *DNAJB9* expression was inhibited in cells cotreated with the IRE1 RNase active site inhibitor 4μ8c, confirming that this effect is IRE1 dependent (Fig. 2b). Our compounds did not significantly induce the PERK-regulated target gene *CHOP* or the ATF6-regulated target gene *BiP* (*HSPA5*), indicating that our compounds are selective for the IRE1–XBPs arm of the UPR (Extended Data Fig. 2c–f). Collectively, these results suggest that our compounds selectively activate IRE1–XBPs signaling independent of other UPR signaling pathways.

We selected compounds IXA1 (1), IXA4 (4) and IXA6 (6) (Fig. 1c) as our top hits based on their selective IRE1-dependent induction of *DNAJB9*, their EC₅₀ of XBP1–RLuc activation of <3 μM and their maximal activation of IRE1–XBPs signaling to levels ~40–50% that observed with Tg. We confirmed that IXA1, IXA4 and IXA6 moderately increased IRE1-dependent *Xbp1* splicing in wild-type mouse embryonic fibroblast (MEF) cells, but not in *Ire1*-deficient MEF cells, further demonstrating that these compounds increase *Xbp1* splicing through an IRE1-dependent mechanism (Extended Data Fig. 2g).

Compound activity requires IRE1 phosphorylation. Compounds that activate the IRE1 RNase through binding the IRE1 kinase domain inhibit IRE1 autophosphorylation and can elicit off-target activity likely associated with binding to other kinases^{15,29,30}. Thus, we sought to define the effect of IXA1, IXA4 and IXA6 on IRE1 kinase activity. Treatment with APY29, an IRE1 kinase inhibitor that allosterically activates the IRE1 RNase¹⁵, blocked Tg-dependent IRE1 phosphorylation in HEK293T cells measured by a band shift by Phos-tag gel electrophoresis (Fig. 2c). In contrast, all three of our prioritized compounds increased IRE1 phosphorylation in these cells, indicating that they promote IRE1 autophosphorylation in the presence or absence of ER stress. Furthermore, cotreatment of IXA1, IXA4 and IXA6 with the IRE1 kinase inhibitor KIRA6, a compound that binds the IRE1 nucleotide-binding pocket and inhibits both IRE1 kinase and RNase activity³⁰, inhibits IRE1-dependent *XBP1* splicing in HEK293T cells (Fig. 2d). Similarly, our compounds did not increase *XBP1s* mRNA levels in *Ire1*-deficient MEF cells reconstituted with the kinase inactive P830L IRE1 (Fig. 2e). Collectively, these results indicate that our compounds do not bind the IRE1 kinase active site, but instead activate IRE1–XBPs signaling through a mechanism requiring IRE1 autophosphorylation.

IXA4 and IXA6 selectively activate IRE1–XBPs signaling. We next performed RNA-seq to define the selectivity of our compounds for IRE1–XBPs signaling (Source Data Fig. 2). Most genes induced by IXA1, IXA4 and IXA6 are known transcriptional targets of IRE1–XBPs (Fig. 3a–c). These include the ER proteostasis factors *SEC24D*, *DNAJB9* and *HERPUD1* (ref. 5). We next defined the selectivity of our compounds for IRE1–XBPs signaling relative to other arms of the UPR. We assessed selectivity by monitoring the expression of gene sets comprising of 10–20 genes that are preferentially induced by the IRE1–XBPs, ATF6 or PERK–UPR signaling pathways (Source Data Fig. 3)^{5,36}. For this analysis, we normalized the expression of individual genes to that observed in Tg-treated cells (Tg representing 100% activation of each gene). This allows us to directly compare gene expression without complications arising

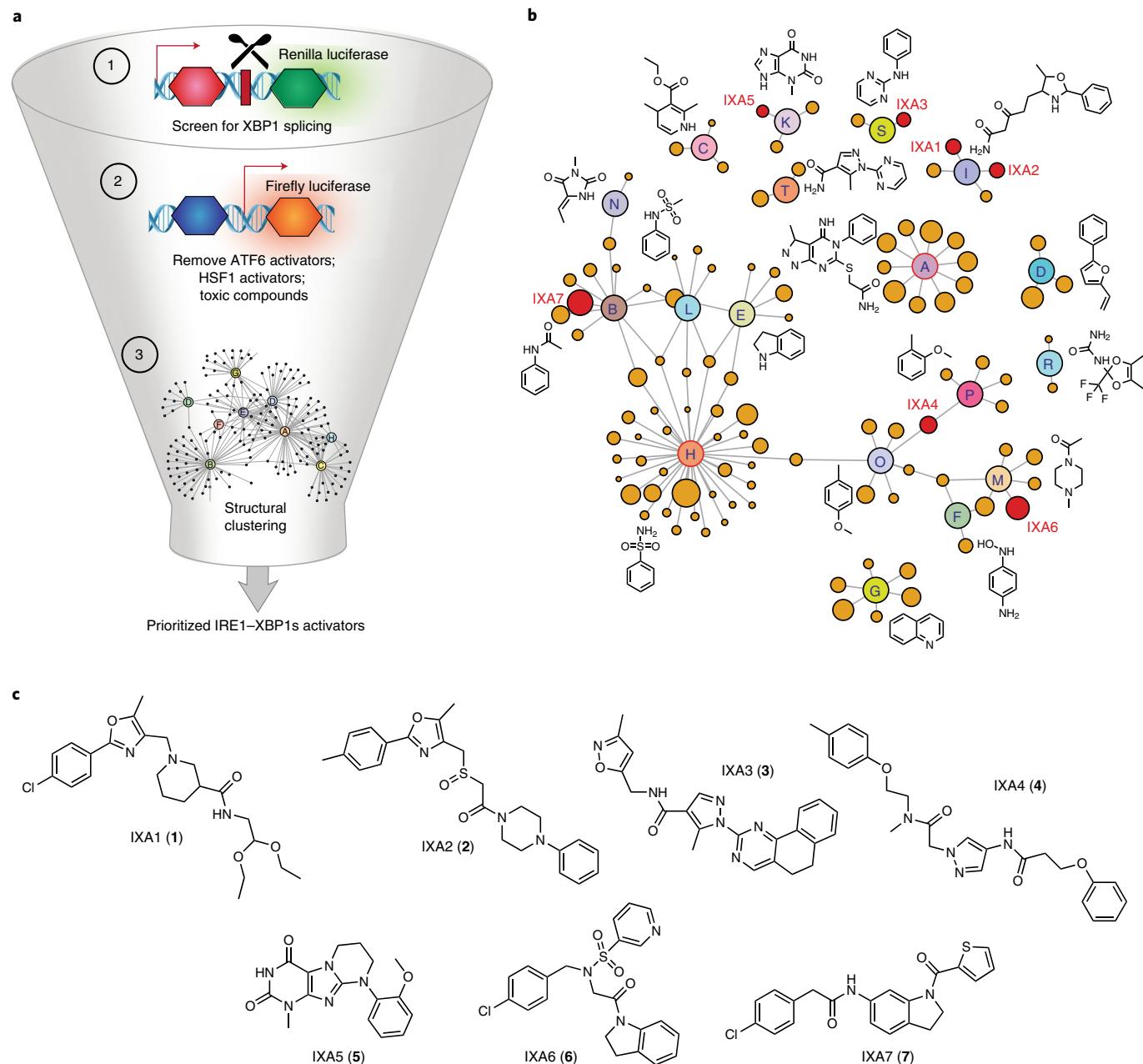


Fig. 1 | HTS to identify preferential IRE1-XBP1s activating compounds. **a**, Illustration of the screening pipeline employed to identify prioritized compounds that selectively activate the IRE1-dependent XBP1-RLuc reporter. This pipeline includes a primary screen to identify compounds that activate the XBP1-RLuc reporter, removal of compounds that activate reporters of other stress-responsive signaling pathways (for example, the ATF6 arm of the UPR and the HSR) and structural clustering of selective activators into defined structural classes. **b**, Network plot illustrating shared structural motifs among a subset of the 128 compounds identified to preferentially activate the XBP1-RLuc reporter >20%, display a maximal EC₅₀ for reporter activation of <3 μM and show a half-maximum inhibitory concentration (IC₅₀) for toxicity of >3 μM. Prioritized compounds identified for subsequent studies are shown in red. **c**, Chemical structures of our top seven prioritized IRE1-XBP1s activators identified via HTS.

from differential expression³⁶. We found that IXA1, IXA4 and IXA6 activate the IRE1-XBP1s geneset to levels ~30–40% that observed for Tg (Fig. 3d–f); levels nearly identical to those observed in other experiments (Fig. 2a,b). Our compounds showed only a modest increase in the activation of the ATF6 target geneset (<20% that observed with Tg), which is consistent with previous reports showing overlap between genes primarily regulated by ATF6 and their mild induction by IRE1-XBP1s (for example, *BiP*; Extended Data Fig. 2f)⁵. Thus, these results indicate that our compounds do not substantially activate ATF6 transcriptional signaling. However, IXA1 did increase expression of the PERK geneset, indicating mild

activation of the PERK pathway (Fig. 3d). Notably, IXA4 and IXA6 did not activate the PERK geneset (Fig. 3e,f). Collectively, these results indicate that compounds IXA4 and IXA6 preferentially activate IRE1-XBP1s signaling, while compound IXA1 shows some promiscuous activity in the context of UPR signaling.

We additionally evaluated IRE1 RIDD activity using our RNA-seq dataset. Despite observing robust IRE1-XBP1s transcriptional activity following 4 h of treatment, we did not observe significant reductions in the RIDD targets *SCARA3*, *BLOC1S1* or *COL6A1* (ref. ¹⁷), although the levels of these mRNA are reduced in Tg-treated cells (Extended Data Fig. 3a–c). This may reflect a

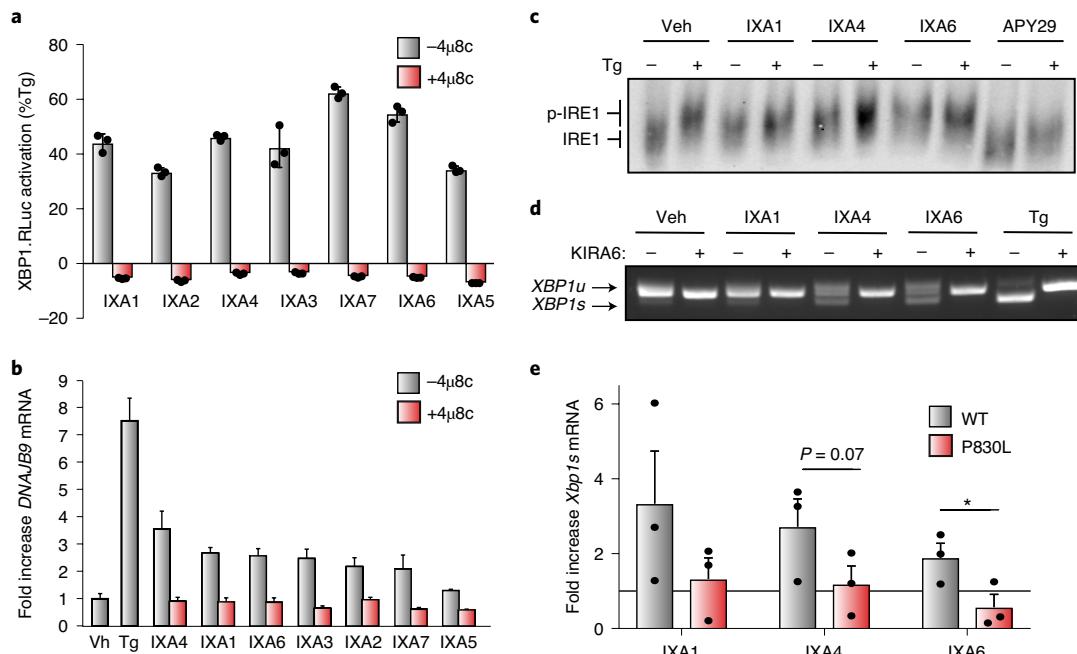


Fig. 2 | Compound-dependent IRE1-XBP1s activation requires IRE1 autophosphorylation. **a**, Luminescence in HEK293^{TREX} cells stably expressing the XBP1-RLuc splicing reporter treated with the indicated IRE1-XBP1s activators (10 μM) in the presence or absence of the IRE1 active site inhibitor 4μ8c (32 μM) for 18 h. Luminescence is shown as the percentage signal relative to Tg (500 nM, 18 h). Error bars represent s.d. for $n=3$ replicates. **b**, Graph showing qPCR of the XBP1s target gene *DNAJB9* in HEK293T cells treated for 4 h with the indicated compound (10 μM) or Tg (500 nM) in the presence or absence of 4μ8c (32 μM). Error bars show 95% confidence interval for $n=3$ replicates. **c**, Immunoblot of IRE1 following Phos-tag SDS-PAGE to separate phosphorylated and unphosphorylated IRE1 in lysates prepared from HEK293T cells treated for 4 h with 10 μM IXA1, IXA4 or IXA6 or 1 μM APY29 in the presence or absence of 500 nM Tg. Phosphorylated (p-IRE1) and unphosphorylated (IRE1) are indicated on the gel. **d**, cDNA gel showing splicing of *XBP1* mRNA in HEK293T cells treated for 4 h with 10 μM IXA1, IXA4, IXA6 or 500 nM Tg in the presence or absence of 10 μM IRE1 kinase inhibitor KIRA6 for 4 h. **e**, Graph showing qPCR of *Xbp1s* mRNA in *Ire1*^{-/-} MEFs reconstituted with WT or kinase inactive P830L IRE1, treated for 4 h with 10 μM IXA1, IXA4 and IXA6. Fold increase for each condition is presented relative to vehicle-treated control. Error bars represent s.e.m. for $n=3$ replicates. P values were calculated using one-tailed Student's *t*-test. * $P < 0.05$.

dependence of RIDD on PERK-dependent eIF2α phosphorylation.³⁷ Taken together, these results indicate that our prioritized compounds promote adaptive IRE1-XBP1s signaling, but not RIDD, following 4 h of treatment in HEK293T cells.

Compounds activate the XBP1s transcriptional response. Gene Ontology analysis shows that our IRE1-XBP1s activating compounds primarily induce expression of genes annotated with Gene Ontology terms related to ER stress and the UPR (Fig. 4a,b, Extended Data Fig. 4a and Supplementary Table 4). This suggests that our compounds do not activate other stress-signaling pathways. Consistent with this, applying a geneset approach similar to that described in Fig. 3d–f³⁶, we show that our compounds do not significantly activate stress-responsive proteostasis pathways such as the cytosolic heat shock response, the oxidative stress response, the mitochondrial UPR or the NFκB inflammatory response (Extended Data Fig. 4b–d and Source Data Fig. 3). These results indicate that IXA4 and IXA6 (and to a lesser extent IXA1) do not significantly activate other stress-responsive signaling pathways apart from IRE1-XBP1s.

Next, we compared the expression of the top 100 genes significantly induced by our compounds to the expression of these genes following stress-independent XBP1s or ATF6 transcription factor activation in HEK293^{TREX} cells, where chemical genetic activation of XBP1s or ATF6 transcriptional signaling can be achieved by addition of different activating ligands⁵. Most of the top 100 genes induced by IXA4 and IXA6 overlapped with genes induced by genetic XBP1s activation (Extended Data Fig. 5a,b), although, as expected, genetic XBP1s activation induces these genes to higher extents (Extended

Data Fig. 4e). However, substantial overlap with ATF6 target genes was not observed, further indicating that our compounds do not significantly activate this UPR pathway (Extended Data Fig. 5a,b). The high level of overlap observed for genes induced by XBP1s and our compounds is further evident when comparing the groups of genes induced >1.2-fold by either IXA4 or IXA6 and genetic XBP1s activation. Compound IXA4 shows 88% overlap of genes induced >1.2-fold, while compound IXA6 shows 64% overlap (Fig. 4c,d). In contrast, compound IXA1 shows less overlap with genetic XBP1s activation (Extended Data Figs. 4f and 5c), reflecting the more promiscuous nature of this compound (Fig. 3d). Of the ten nonoverlapping genes identified for IXA4 (Fig. 4c), nine are also induced by IXA6, likely reflecting a similar mechanism of induction (Extended Data Fig. 4g). Two of these genes, *IDII* and *SLC35A3* are reported to be regulated by IRE1-XBP1s^{38,39}. Furthermore, the mild IXA4-dependent increase in *LRRCC1* appears to be reduced by cotreatment with 4μ8c, suggesting that this gene is also regulated by IRE1 (Extended Data Fig. 4h). In contrast, *HSPA1A* and *HSPA1B* are induced by many different stress pathway activators through an undefined mechanism³⁶ and the mild IXA4-dependent increase in *CETN3* does not appear sensitive to 4μ8c (Extended Data Fig. 4i). This indicates that IXA4 may induce a small subset of genes through an IRE1-independent mechanism. Taken together, the results in Fig. 4 confirm that IXA4 is the most selective for the IRE1-XBP1s signaling pathway. Thus, we prioritized IXA4 for further mechanistic scrutiny and phenotypic studies based on the fidelity of its transcriptional response.

We confirmed the identity and chemical purity of IXA4 using nuclear magnetic resonance and high-performance liquid

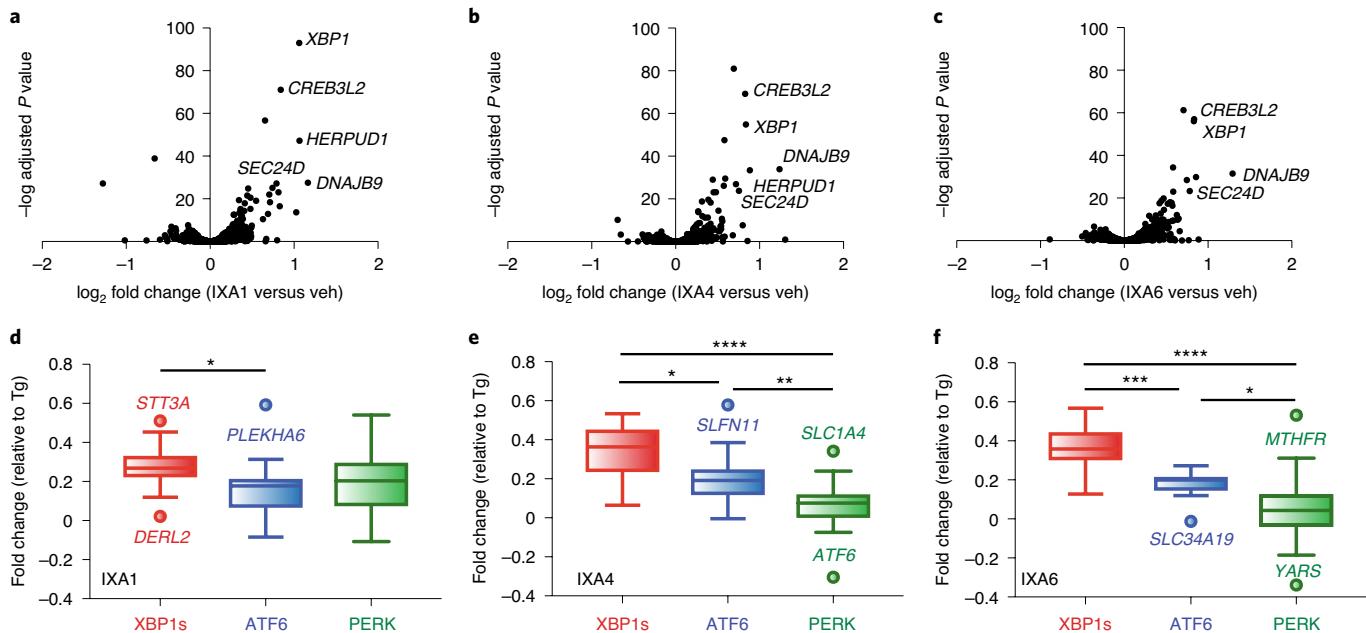


Fig. 3 | Transcriptional profiling of compounds IXA1, IXA4 and IXA6 shows preferential induction of IRE1-XBP1s target genes. **a**, Volcano plots from whole-transcriptome RNA-seq showing negative log transformed adjusted P values for gene expression (y axis) versus \log_2 transformed fold change (x axis) in HEK293T cells treated for 4 h with IXA1 (10 μ M). **b**, Volcano plots from whole-transcriptome RNA-seq showing negative log transformed adjusted P values for gene expression (y axis) versus \log_2 transformed fold change (x axis) in HEK293T cells treated for 4 h with IXA4 (10 μ M). **c**, Volcano plots from whole-transcriptome RNA-seq showing negative log transformed adjusted P values for gene expression (y axis) versus \log_2 transformed fold change (x axis) in HEK293T cells treated for 4 h with IXA6 (10 μ M). **d**, Plots showing fold-change values from whole-transcriptome RNA-seq of target genes regulated downstream of the IRE1-XBP1s (red), ATF6 (blue) or PERK (green) signaling arms of the UPR expressed as fold change relative to Tg treatment (1 μ M, 4 h) in HEK293T cells treated with IXA1 (10 μ M, 4 h). Center line reflects median, box limits reflect upper and lower quartiles, whiskers reflect 1.5x interquartile range (IQR) and points reflect outliers as calculated by Tukey method. The composition of these gene sets is shown in Source Data Fig. 3. **e**, Plots showing fold-change values from whole-transcriptome RNA-seq of target genes regulated downstream of the IRE1-XBP1s (red), ATF6 (blue) or PERK (green) signaling arms of the UPR expressed as fold change relative to Tg treatment (1 μ M, 4 h) in HEK293T cells treated with IXA4 (10 μ M, 4 h). Center line reflects median, box limits reflect upper and lower quartiles, whiskers reflect 1.5x IQR and points reflect outliers as calculated by Tukey method. The composition of these gene sets is shown in Source Data Fig. 3. **f**, Plots showing fold-change values from whole-transcriptome RNA-seq of target genes regulated downstream of the IRE1-XBP1s (red), ATF6 (blue) or PERK (green) signaling arms of the UPR expressed as fold change relative to Tg treatment (1 μ M, 4 h) in HEK293T cells treated with IXA6 (10 μ M, 4 h). Center line reflects median, box limits reflect upper and lower quartiles, whiskers reflect 1.5x IQR and points reflect outliers as calculated by Tukey method. The composition of these gene sets is shown in Source Data Fig. 3.

chromatography (Supplementary Fig. 1). We then used immunoblotting to show that IXA4 increases XBP1s protein in HEK293T cells to levels ~40% that with Tg, but did not increase phosphorylation of PERK or eIF2 α , further highlighting the selectivity of this compound (Extended Data Fig. 6a). Similarly, we showed IXA4 selectively upregulates XBP1s mRNA, relative to genes regulated by ATF6 (for example, *BiP*) or PERK (for example, *CHOP*), in other cell lines including Huh7 and SHSY5Y cells (Extended Data Fig. 6b,c). Similar results were observed for IXA6 (Extended Data Fig. 6a–c). Lastly, phosphokinase profiling shows that IXA4 did not increase phosphorylation of c-Jun N-terminal kinase (JNK) or c-Jun (Extended Data Fig. 6d)—two kinase targets phosphorylated on chronic IRE1 activation through an XBP1s-independent mechanism⁴⁰. Other kinase targets measured in this assay were also not significantly affected by IXA4 treatment. These data further support the selective activation of the IRE1-XBP1s UPR signaling pathway afforded by IXA4.

IXA4 reduces secretion of APP through IRE1 activation. IRE1-XBP1s signaling promotes targeted remodeling of ER proteostasis pathways comprising chaperones, folding enzymes, trafficking proteins and degradation factors^{5,6}. Consistent with its selectivity for IRE1-XBP1s, IXA4 also promotes selective transcriptional remodeling of ER proteostasis pathways, relative to cytosolic or mitochondrial pathways (Fig. 5a and Source Data Figs. 2 and 3). Quantitative

immunoblotting of the IRE1-XBP1s trafficking factor SEC24D confirmed that IXA4-dependent increases in ER proteostasis factor gene expression correspond to increased protein levels (Extended Data Fig. 7a). Similar results were observed with IXA6 (Extended Data Fig. 7a,b).

The potential of our compounds to remodel the ER proteostasis network through IRE1-XBP1s activation suggests that these compounds could correct pathologic imbalances in ER proteostasis for disease-relevant proteins such as APP. APP is a secretory protein that undergoes proteolytic processing to produce aggregation-prone cleavage products including the amyloidogenic peptide A β (ref. ⁴¹). Enhancing ER proteostasis through XBP1s overexpression reduces secretion of toxic A β (refs. ^{23,24}). Thus, we predicted that the enhanced ER proteostasis environment afforded by IRE1-XBP1s activation by our compounds would similarly reduce A β secretion.

We show that IXA4 reduced A β levels 50% in conditioned media prepared on CHO^{PA2} cells expressing the V717F APP (APP^{V717F}) mutant (Fig. 5b)⁴². We confirmed compound-dependent IRE1-XBP1s activation in these cells by quantitative PCR (qPCR) (Extended Data Fig. 7c). IXA4 did not significantly influence CHO^{PA2} viability measured by CellTiterGlo (Extended Data Fig. 7d). Similarly, we did not observe reductions in cell proliferation or increases in poly(ADP-ribose) polymerase (PARP) cleavage in IXA4-treated CHO^{PA2} cells (Extended Data Fig. 7e,f). These results indicate that the compound-dependent reduction in A β secretion

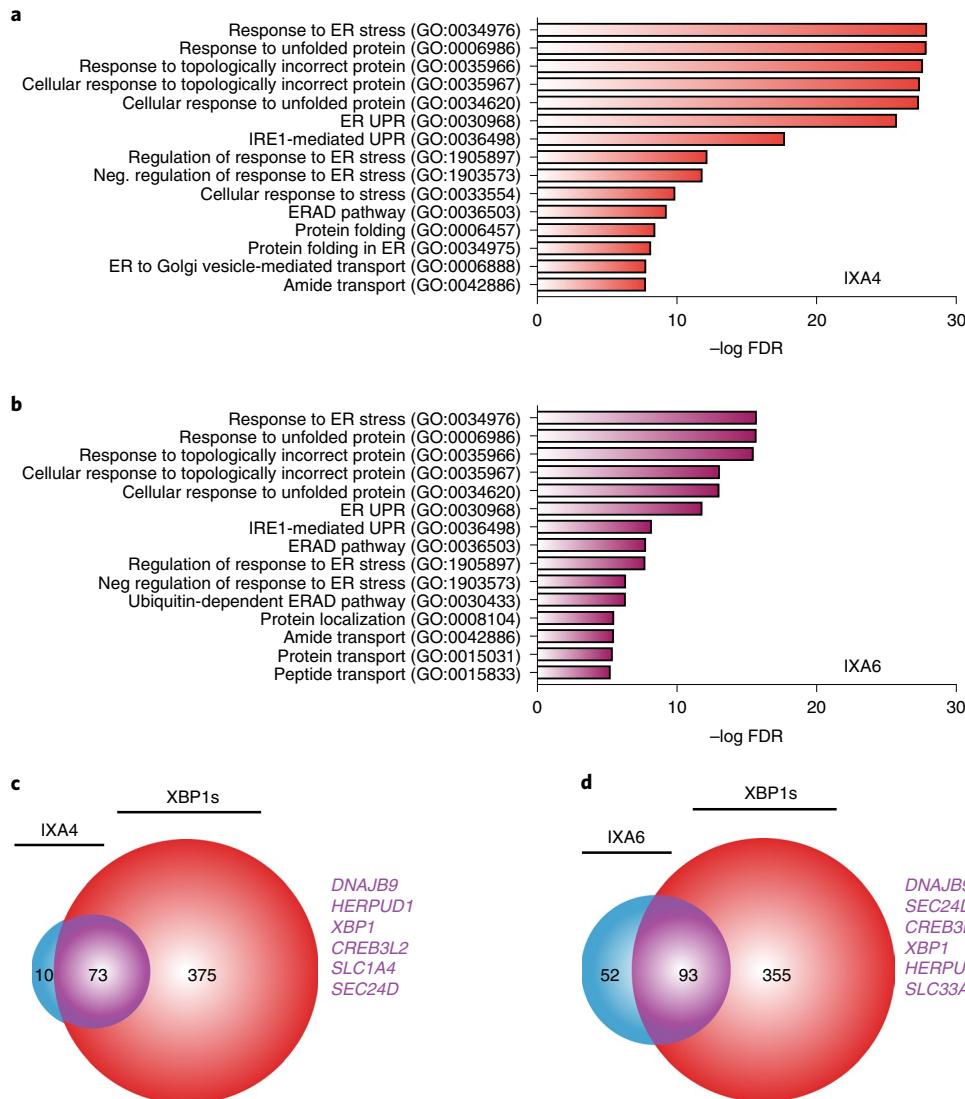


Fig. 4 | Compounds IXA4 and IXA6 show selectivity for IRE1-XBP1s-dependent ER proteostasis remodeling. **a**, Gene Ontology (GO) analysis of differentially expressed genes from RNA-seq for HEK293T cells treated with IXA4 (10 μ M, 4 h). Top 15 entries with the lowest false discovery rates are shown. See Supplementary Table 4 for full GO analysis. **b**, Gene Ontology (GO) analysis of differentially expressed genes from RNA-seq for HEK293T cells treated with IXA6 (10 μ M, 4 h). Top 15 entries with the lowest false discovery rates are shown. See Supplementary Table 4 for full GO analysis. **c**, Venn diagram of genes upregulated >1.2-fold (adjusted $P < 0.05$) in HEK293T cells treated with compound IXA4 (10 μ M) for 4 h in comparison to genes induced >1.2-fold (adjusted $P < 0.05$) in HEK293^{DAx} cells treated with doxycycline (1 μ g ml⁻¹) for 4 h. Genes listed in purple are top overlapping targets between conditions. **d**, Venn diagram of genes upregulated >1.2-fold (adjusted $P < 0.05$) in HEK293T cells treated with compound IXA6 (10 μ M) for 4 h in comparison to genes induced >1.2-fold (adjusted $P < 0.05$) in HEK293^{DAx} cells treated with doxycycline (1 μ g ml⁻¹) for 4 h. Genes listed in purple are top overlapping targets between conditions.

cannot be attributed to cell death. The IXA4-dependent reduction in A β secretion is blocked by cotreatment with 4 μ 8c, confirming this reduction is dependent on IRE1 RNase activity (Fig. 5b). Similar results were observed in CHO^{7WD10} cells stably expressing wild-type APP (APP^{WT}) and in cells treated with the alternative IRE1-XBP1s activator IXA6 (Extended Data Fig. 7d–h).

XBP1s overexpression has been shown to reduce A β secretion through the increased targeting of APP to ER-associated degradation (ERAD)^{23,24}. IXA4 reduced APP^{V717F} protein levels in both lysates and conditioned media prepared on CHO^{7PA2} cells, indicating that this compound increases APP degradation (Fig. 5c and Extended Data Fig. 8a). This reduction was reversed by cotreatment with either 4 μ 8c or the proteasome inhibitor MG132 (Fig. 5c and Extended Data Fig. 8a–c), indicating that APP^{V717F} levels were reduced through a mechanism involving both IRE1 RNase activity

and ERAD via the proteasome. This is consistent with reports demonstrating that XBP1s overexpression increases APP ERAD²⁴. We further demonstrated that IXA4 reduces APP^{V717F} secretion and increases APP^{V717F} degradation using [³⁵S] metabolic labeling (Fig. 5d–f). Similar results were observed with IXA6 (Extended Data Fig. 8d). Collectively, these results demonstrate that our compounds reduce A β secretion and increase APP degradation through an IRE1-dependent mechanism.

IXA4 prevents APP-associated mitochondrial dysfunction. Previous results show that overexpression of APP^{WT} or the Swedish APP (APP^{SW}) double mutant (K595N/M596L) induces mitochondrial dysfunction in SHSY5Y cells through the production of toxic APP cleavage products that localize to mitochondria and mitochondria-associated ER membranes (MAMs)^{13,44}. Consistent

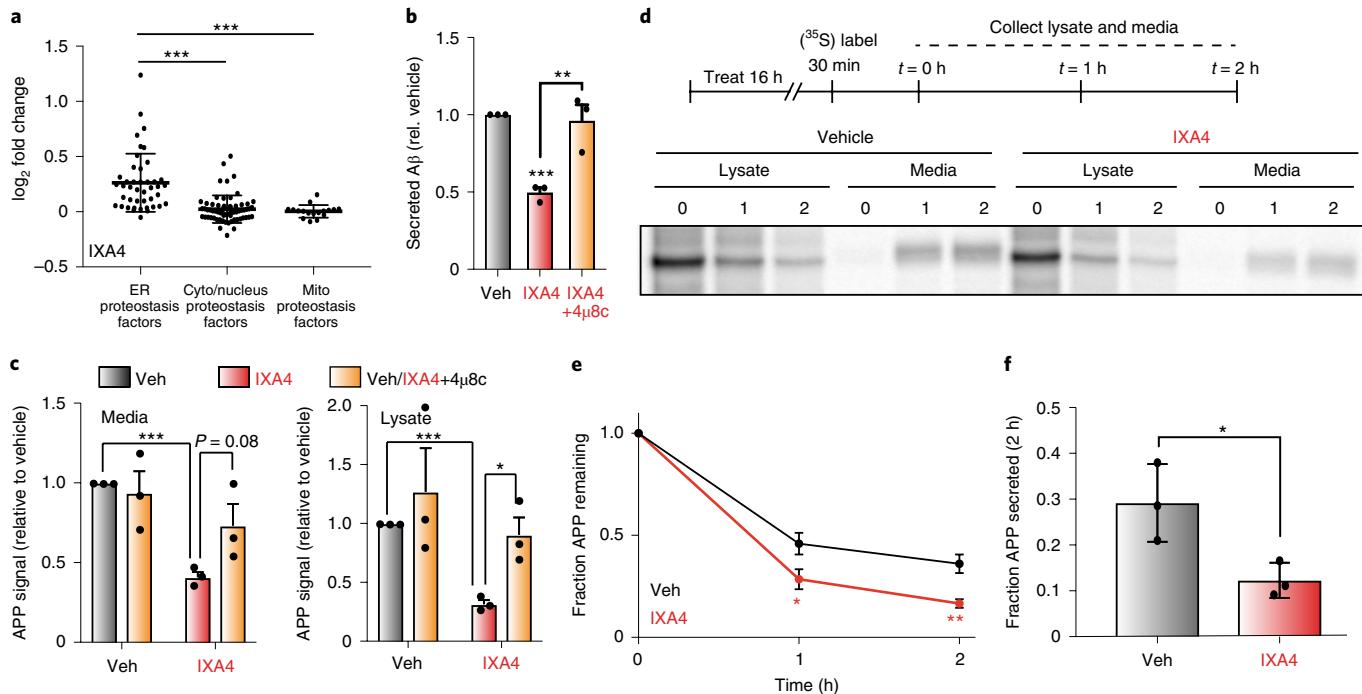


Fig. 5 | Compound IXA4 increases degradation of APP mutants. **a**, Plot of \log_2 fold-change mRNA levels from RNA-seq in cells treated with IXA4 (10 μ M, 4 h) of proteostasis factors found in the ER, cytosol/nucleus or mitochondria. The composition of these proteostasis gene sets is shown in Source Data Fig. 3. **b**, Graph showing relative signal from ELISA of secreted A β peptide in conditioned media from CHO 7PA2 cells treated with IXA4 (10 μ M) in the presence or absence of 4 μ 8c (32 μ M). Cells were pretreated for 18 h with compounds. Media was then replaced and conditioned in the presence of compounds for 24 h before harvesting conditioned media for ELISA. Secreted A β was normalized to that observed in untreated controls. Error bars represent s.e.m. for $n=3$ replicates. P values calculated from one-tailed Student's t -test. ** $P < 0.01$, *** $P < 0.001$. **c**, Quantification of mutant APP relative to vehicle-treated controls in media and lysate isolated from CHO 7PA2 cells treated with IXA4 (10 μ M) and/or 4 μ 8c (32 μ M) as in **b**, measured by immunoblotting. A representative immunoblot is shown in Extended Data Fig. 8a. Error bars represent s.d. for $n=3$ replicates. P values calculated from one-tailed Student's t -test. * $P < 0.05$, *** $P < 0.001$. **d**, Representative autoradiogram showing the [35 S] metabolic labeling of mutant APP in CHO 7PA2 cells treated with IXA4 (10 μ M) for 16 h before 30 min labeling. Media and lysates were collected at 0, 1 or 2 h and [35 S]-labeled mutant APP was isolated by immunopurification. The experimental protocol is shown above. **e**, Plot showing fraction mutant APP remaining at each time point of the metabolic labeling experiment shown in **d**. Fraction remaining was calculated using the following equation: (APP in lysate at time = t + APP in media at time = t) / (APP in lysates at $t=0$ + APP in media at $t=0$). Error bars represent s.d. for $n=3$ replicates. P values were calculated from one-tailed Student's t -test. * $P < 0.05$, ** $P < 0.01$. **f**, Plot showing fraction of fraction APP secreted at 2 h of the metabolic labeling experiment shown in **d**. Fraction secretion was calculated using the following equation: (APP in media at time = t) / (APP in lysates at $t=0$ + APP in media at $t=0$). Error bars represent s.d. for $n=3$ replicates. P values were calculated from one-tailed Student's t -test. * $P < 0.05$.

with this, overexpression of APP^{WT} or APP^{SW} in SHSY5Y resulted in a 25 or 40% reduction of mitochondrial membrane potential, respectively, as measured by tetramethylrhodamine (TMRE) staining (Fig. 6a,b). Overexpression of APP^{SW} in SHSY5Y cells modestly increased expression of the IRE1–XBP1s target gene *DNAJB9*, but not other UPR genes such as the PERK-regulated target gene *CHOP* (Extended Data Fig. 9a,b). Treatment with IXA4 further increased *DNAJB9* expression, indicating that our compounds are active in this model. Treatment with IXA4 prevented the APP^{WT}- or APP^{SW}-dependent reductions in mitochondrial membrane potential in these cells (Fig. 6c,d and Extended Data Fig. 9c). This indicates that pharmacologically enhancing IRE1–XBP1s activity can block APP-associated mitochondrial depolarization.

Mitochondrial depolarization decreases the capacity for cells to produce ATP through oxidative phosphorylation at the inner mitochondrial membrane. Thus, APP^{SW}-dependent mitochondrial depolarization should reduce mitochondrial ATP production. To confirm this, we demonstrated that APP^{SW} overexpression reduced ATP levels in SHSY5Y cells cultured in galactose-containing media, where ATP is primarily produced by mitochondria, but not in SHSY5Y cells cultured in glucose media, where ATP is primarily produced by glycolysis (Fig. 6e)⁴⁵. Treatment with IXA4 rescued ATP levels

in APP^{SW}-overexpressing cells cultured in galactose media, further demonstrating that this compound mitigates mitochondrial dysfunction induced by toxic APP^{SW} overexpression (Fig. 6f).

Discussion

IRE1 activating compounds have traditionally been developed by targeting the IRE1 nucleotide-binding pocket to induce allosteric activation of the IRE1 RNase^{13–15}. While these types of compounds have provided important insights into the molecular mechanism of IRE1 activation, their use for defining the functional implications of IRE1–XBP1s signaling is limited due to off-pathway activity, likely due to their binding to other kinases^{15,29,30}. This indicates that new strategies are required to establish highly selective IRE1–XBP1s activating compounds that can be used to probe the biological and potentially therapeutic benefit of IRE1–XBP1s signaling in the context of diverse diseases. The implications of finding these highly selective IRE1–XBP1s activators is evident from recent work establishing non-toxic, selective activators of the ATF6–UPR signaling pathway, which have revealed new insights into the functional importance of ATF6 activity in diverse biological functions including ER proteostasis remodeling, mammalian development, and cardiac protection against myocardial infarction^{31,46,47}.

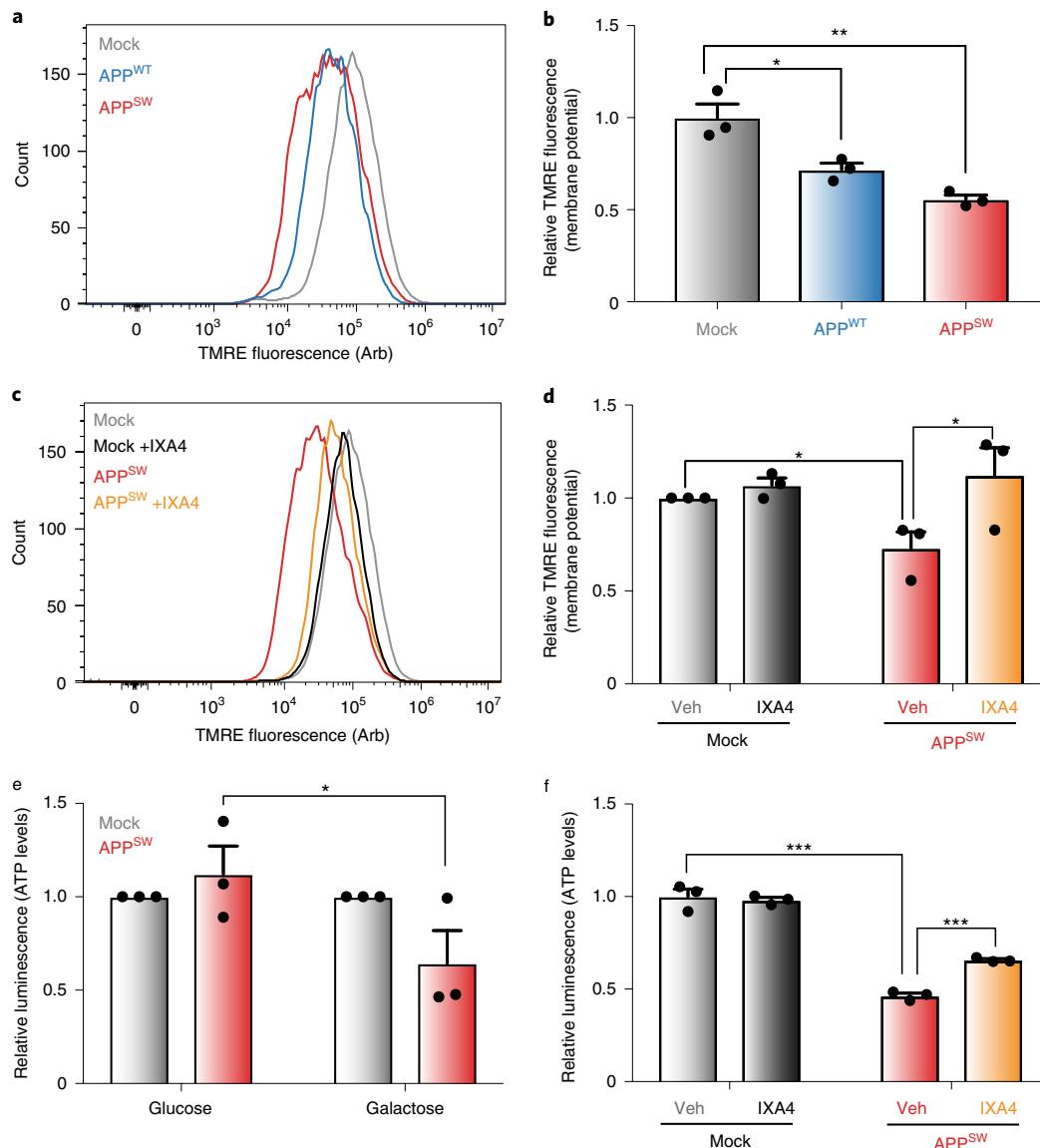


Fig. 6 | The IRE1-XBP1s activator IXA4 rescues mitochondrial defects in SHSY5Y cells expressing disease-relevant APP mutants. **a**, Representative histograms showing TMRE staining of SHSY5Y cells transiently expressing empty vector (Mock), APP^{WT} or Swedish mutant APP (APP^{SW}). **b**, Quantification of TMRE staining from **a**. TMRE normalized to geometric mean from cells transiently expressing empty vector (Mock). Error bars represent s.d. for $n=3$ replicates. P values were calculated from one-tailed Student's t -test. * $P<0.05$, ** $P<0.01$. **c**, Representative histograms showing TMRE staining of SHSY5Y cells transiently expressing empty vector (Mock) or APP^{SW} in the presence or absence of IXA4 (10 μ M) for 72 h. **d**, Quantification of TMRE staining from **c**. TMRE normalized to geometric mean from cells transiently expressing empty vector (Mock). Error bars represent s.e.m. for $n=3$ replicates. P values were calculated from one-tailed Student's t -test. * $P<0.05$. **e**, Graph showing relative ATP levels measured by CellTiterGlo luminescence in SHSY5Y cells transiently expressing empty vector (mock) or APP^{SW} cultured in either normal high-glucose media or glucose-free media supplemented with galactose for 72 h. Luminescence signal was normalized to that observed in cells transiently expressing empty vector (Mock) cultured in glucose- or galactose-containing media. Error bars represent s.e.m. for $n=3$ replicates. P values were calculated from one-tailed Student's t -test. * $P<0.05$. **f**, Graph showing relative ATP levels measured by CellTiterGlo luminescence in SHSY5Y cells transiently expressing empty vector (Mock) or APP^{SW} cultured in galactose media for 72 h in the presence or absence of IXA4 (10 μ M). Luminescence signal was normalized to that observed in cells transiently expressing empty vector (Mock) incubated in the absence of IXA4. Error bars represent s.d. for $n=3$ replicates. P values were calculated from one-tailed Student's t -test. *** $P<0.001$.

Here, we employ a screening strategy that prioritizes transcriptional profiling over mechanism-based activation to identify non-toxic compounds that selectively activate the IRE1-XBP1s arm of the UPR. Our compounds (for example, IXA4) activate IRE1-XBP1s signaling without globally activating the UPR or other stress-responsive signaling pathways (for example, the heat shock response or oxidative stress response). Our compounds increase IRE1 RNase activity through a mechanism independent of binding

the IRE1 nucleotide-binding pocket. This demonstrates that our compounds are distinct from currently available IRE1 activators, which bind the kinase active site. We showed that treatment of HEK293T cells with our prioritized compound IXA4 does not induce XBP1s-independent IRE1 signaling such as RIDD or JNK phosphorylation—two aspects of IRE1 activity often associated with chronic activation of this signaling pathway^{37,40}. The lack of RIDD and JNK phosphorylation could reflect the moderate activation of

IRE1–XBPs signaling afforded by our compounds or requirements of other signaling inputs (for example, PERK activation³⁷) to promote their activation. However, it is possible that our compounds could activate these aspects of IRE1 signaling in other cell types or experimental conditions, although this could likely be controlled through varying the compound dosage and administration.

While the mechanism by which our compounds activate IRE1–XBPs signaling requires further exploration, we show that they induce adaptive ER proteostasis remodeling that mimics that observed with chemical genetic XBPs activation. This provides new opportunities to define the functional implications of IRE1–XBPs signaling in the context of health and disease. We demonstrate this potential by showing that our prioritized compound IXA4 improves ER proteostasis of AD-relevant APP mutants, reducing the secretion of A β through the increased targeting of APP to degradation. Further, we show that IXA4 reduces mitochondrial dysfunction associated with mutant APP overexpression, likely reflecting the reduced intracellular APP afforded by compound treatment. These results highlight the potential for targeting ER proteostasis to mitigate pathologic disruption in other organelles, including the mitochondria, which can be induced by destabilized, disease-associated proteins.

Genetic activation of IRE1–XBPs signaling promotes protection against different types of pathologic insult associated with multiple diseases^{20–22,25,28,48}. This indicates that IRE1 represents a potential therapeutic target for these disorders. However, in the context of ER stress, chronic IRE1 activity can promote detrimental phenotypes such as increased apoptosis and inflammation in models of diseases such as atherosclerosis and sepsis^{49,50}, potentially limiting the therapeutic applications for IRE1–XBPs activating compounds. Pharmacologic IRE1–XBPs activation afforded by our compounds (for example, IXA4) offers advantages over genetic strategies to probe the therapeutic potential for IRE1–XBPs activation to intervene in these diseases. Our compounds allow selective activation of IRE1–XBPs in disease-relevant models, independent of genetic manipulation. Furthermore, they allow dosable and temporal control over IRE1–XBPs activity using different dosing regimens, potentially limiting the adverse outcomes associated with chronic IRE1 activation. Thus, the highly selective, non-toxic IRE1–XBPs activating compounds established herein (for example, IXA4) provide new opportunities to probe the functional implications of this signaling pathway in diverse contexts and define the therapeutic potential for activating IRE1 to mitigate pathologic imbalances in cellular physiology implicated in diverse diseases.

Online content

Any Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41589-020-0584-z>.

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Methods

Materials and reagents. Antibodies used were APP (6E10, Fisher Scientific cat no. 501029533), APP (horseradish peroxidase- (HRP)-4G8, Fisher Scientific catalog no. 501029498), SEC24D (mouse) antibody was provided as a generous gift from the Balch Laboratory at TSRI, XBP1s (Cell Signaling catalog no. 12782S), KDEL (Enzo catalog no. ADI-SPA-827-F), P-eIF2α (Cell Signaling catalog no. 9721S), Tot-eIF2α (Abcam catalog no. ab5369), PERK (Cell Signaling catalog no. 3192S), PARP (Cell Signaling catalog no. 9542S) and Tubulin (Sigma catalog no. T6074-200UL)

Pharmacologics used were thapsigargin (Fisher Scientific catalog no. 50-464-295), 4μ8c (EMD Millipore catalog no. 412512), IXA1 (ChemDiv catalog no. C522-3739), IXA2 (2) (ChemDiv catalog no. C527-0672), IXA3 (3) (Hit2Lead catalog no. SC-46317020), IXA4 (Hit2Lead catalog no. SC-91093541), IXA5 (5) (Life Chemicals catalog no. F3164-0105), IXA6 (Life Chemicals catalog no. F5120-0005), IXA7 (7) (Life Chemicals catalog no. F5098-0023), KIRA6 (Selleck Chemicals catalog no. S8658), Staurosporine (LKT catalog no. S7600-1 mg) and MG132 (Selleck Chem catalog no. S3619)

HTS (including all the filtering steps and the chemical clustering). HEK293^{TREX} cells incorporating either the XBP1-Rluc or ERSE-FLuc reporters were collected by trypsinization and resuspended at a density of 500,000 cells per ml. The assay was started by dispensing 5 μl of cell suspension into each well of white, solid-bottom 1,536-well plates using a flying reagent dispenser and placed into an online incubator for 3 h. Cells were then treated with 34 nL per well of test compounds to give final concentrations of 5.17 μM, DMSO (low control, final concentration 0.68%, 0% activation) or 37 μM of Delta-7 thapsigargin (high control, final concentration 500 nM, 100% activation). Plates were incubated for 18 h at 37°C, removed from the incubator and equilibrated to room temperature for 10 min. Luciferase activity was detected by addition of 5 μl of Renilla-Glo reagent (Promega) to each well. After a 10 min incubation time, light emission was measured with the ViewLux reader (PerkinElmer). Tg exhibited a robust signal to noise ratio (4.06 ± 0.23) and was used to confirm consistent assay performance across experimental plates ($Z' = 0.69 \pm 0.01$). Compound (5.17 μM) dependent XBP1-Rluc activation was additionally normalized to Tg (assigned to be 100% activation) to allow comparisons between compounds across screening plates. The percentage activation of each test compound was calculated as follows: Percentage activation = $100 \times (\text{test compound} - \text{median low control}) / (\text{median high control} - \text{median low control})$. Primary screening of the 646,275 compound library at Scripps Florida yielded 10,114 hits for XBP1-Rluc activity at $\geq 13.83\%$ activation by thapsigargin (three times the standard deviation of the negative DMSO control in the assay), an approximate 1.5% hit rate. Compounds that hit in more than seven screens (promiscuity score) were eliminated, as well as those that elicited HSP70 activation. The top 6,391 remaining compounds were moved forward to triplicate confirmation screening and HEK293^{TREX} CTG cytotoxicity counterscreening. Toxic compounds found to reduce cell viability $> 26.19\%$ (5.17 μM) relative to doxorubicin were removed, leaving 6,185 nontoxic compounds showing reproducible XBP1-Rluc activation. Duplicates were removed from the resulting list, and the top 638 activating compounds were moved forward for triplicate titration screening and HEK293^{TREX} CTG titration counterscreening. These 638 compounds were subjected to hierarchical clustering using the Library MCS application from the ChemAxon JChem Suite, grouping 551 of these by 20 conserved structural motifs, with 87 singletons. All 638 compounds were also subject to quality control measurements by liquid chromatography–mass spectrometry, ultraviolet-visible light spectroscopy, mass spectrometry and evaporative light scattering detection to confirm purity and mass. Those that did not pass both of these quality checks were eliminated. From titration data of the remaining compounds, those with $< 20\%$ reporter activation and EC₅₀ values $> 3 \mu\text{M}$ were eliminated. Additionally, compounds from the HEK293^{TREX} CTG counterscreen with EC₅₀ values $< 3 \mu\text{M}$ were also eliminated. Remaining clustered compounds were iteratively subclustered so that the diversity of the cluster would be captured by a smaller representative group, comprising only compounds that activated the reporter to a practical degree for *in vitro* measurements ($> 30\%$). Remaining singleton compounds that passed quality control and showed reporter activation $> 30\%$ were also included for *in vitro* assays.

RNA-seq analysis (including Geneset analysis and GO-term analysis). Cells were lysed and total RNA collected using the RNeasy mini kit, according to the manufacturer's instructions (Qiagen). Conventional RNA-seq was conducted via BGI Americas on the BGI Proprietary platform, providing single-end 50 bp reads at 20 million reads per sample. Alignment of sequencing data was done using DNAsStar Lasergene SeqManPro to the GRCh37.p13 human genome reference assembly, and assembly data were imported into ArrayStar 12.2 with QSeq (DNAStar Inc.) to quantify the gene expression levels and normalization to reads per kilobase per million. Differential expression analysis and statistical significance calculations between different conditions was assessed using 'DESeq' in R, compared to vehicle-treated cells, using a standard negative binomial fit of the reads per kilobase per million data to generate fold-change quantifications.

Cell culture and transfections. Briefly, all cell lines were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Corning Cellgro) supplemented

with 10% fetal bovine serum (FBS) (Omega Scientific), 2 mM L-glutamine (Gibco), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Gibco). SHSY5Y cells in galactose conditions were cultured in glucose-free DMEM (Corning Cellgro) supplemented with 10% FBS (Omega Scientific), 2 mM L-glutamine (Gibco), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Gibco) and 5 mM galactose. All cells were cultured under typical tissue culture conditions (37°C, 5% CO₂). Cells were routinely tested for mycoplasma every 6 months. No further authentication of cell lines was performed by the authors. Cells were transfected with calcium phosphate precipitation. All plasmids for transfection were prepared using the Qiagen Midiprep kit according to the manufacturers protocol. 7PA2 cells were kindly provided by E. Koo (University of California, San Diego).

qPCR, transcriptional profiling. Primers:

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hDNAJB9 (F: GGAAGGAGGAGCCTAGGTC, R:
ATCCTGCACCCCTCCGACTAC)
hBiP (F: GCCTGTATTCTAGACCTGCC, R:
TTCATCTGCCAGCCAGTTG)
hCHOP (F: ACCAAGGGAGAACAGGAAACG, R:
TCACCATTCCGGTAATCAGAGC)
hLRRCCI (F: TCATCGAGAAAGAGAACAGCGC, R:
GATTATGACAGAGAACAGCCACAGG)
hCETN3 (F: GATTATGACAGAGAACGCCACAGG, R:
ATGCCTTGAGTATTCTCATGG)
hRiboPro (F: CGTCGCCTCCTACCTGCT, R:
CCATTCACTCACTGATAACCTTG)
mXBPs (F: ACGAGGTTCCAGAGGTGGAG, R:
TGTCAGGAATCCCCAAAAGG)
mRiboPro (F: TGTCATCGCTCAGGGTGTG, R:
AAGCCAATCCCAGTCGTC)
hamXBPU (F: CTCCAGAGACGGAGTCCAAG, R:
CAGAGGTGACGTAGTCTAGTGTG)
hamXBPs (F: CTCCAGAGACGGAGTCCAAG, R:
CACCTGCTCGGGACTC)
hamActin (F: AGCTGAGAGGGAAATTGTGCG, R:
GCAACCGAACCGCTCATT)

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The relative mRNA expression levels of target genes were measured using qPCR with reverse transcription (RT-qPCR). Cells were treated as described at 37°C, washed with Dulbecco's phosphate-buffered saline (Gibco) and then RNA was extracted using the RNeasy Minikit (Qiagen). qPCR reactions were performed on complementary DNA prepared from 500 ng of total cellular RNA using the QuantiTect Reverse Transcription Kit (Qiagen). The FastStart Universal SYBR Green Master Mix (Roche), cDNA and primers purchased from Integrated DNA Technologies were used for amplifications (6 min at 95°C then 45 cycles of 10 s at 95°C, 30 s at 60°C) in an ABI 7900HT Fast Real Time PCR machine. Primer integrity was assessed by a thermal melt to confirm homogeneity and the absence of primer dimers. Transcripts were normalized to the housekeeping genes *RiboPro* and all measurements were performed in triplicate. Data were analyzed using the RQ Manager and DataAssist 2.0 softwares (ABI). qPCR data are reported as mean $\pm 95\%$ confidence interval as calculated in DataAssist 2.0.

Immunoblotting, SDS-PAGE and Phos-tag SDS-PAGE. Cell lysates were prepared as previously described in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X100, 0.5% deoxycholate and protease inhibitor cocktail (Roche)). Total protein concentration in cellular lysates was normalized using the Bio-Rad protein assay. Lysates were then denatured with 1× Laemmli buffer + 100 mM DTT and boiled before being separated by SDS-PAGE. Samples were transferred onto nitrocellulose membranes (Bio-Rad) for immunoblotting and blocked with 5% milk in tris-buffered saline, 0.5% Tween-20 (TBST) following incubation overnight at 4°C with primary antibodies. Membranes were washed in TBST, incubated with IR-Dye conjugated secondary antibodies and analyzed using Odyssey Infrared Imaging System (LI-COR Biosciences). Quantification was carried out with LI-COR Image Studio software.

Phosphokinase array. The Proteome Profile Human Phospho-kinase Array Kit from R&D Systems (catalog no. ARY003C) was used to assess phosphorylation status of a panel of human kinases in HEK293T cells treated with IXA4 compared to vehicle. The array was performed per the manufacturer's instructions. Briefly, cell lysates were prepared after 4 h of treatment and incubated on membranes containing antibodies for kinases of interest. Each antibody had been dotted in duplicate. After washing, HRP-conjugated secondary antibody cocktail was incubated on membranes. HRP substrate was then incubated on membranes and dot blots were visualized via chemiluminescence with a Bio-Rad ChemiDoc XRS+.

PCR and agarose gel electrophoresis. To amplify the spliced and unspliced XBP1 mRNA, XBP1 primers were used as described previously¹⁶. PCR products were electrophoresed on 2.5% agarose gel. Glyceraldehyde 3-phosphate dehydrogenase (forward 5'GGATGATGTTCTGGAGAGCC3', reverse 5'CATCACCCTTCCAGGAGC3') was used as a loading control. The size difference between the spliced and the unspliced XBP1 is 26 nucleotides.

A β ELISA. 7PA2 or 7WD10 cells were cultured on 96-well plates (Corning) and treated with IRE1 activating compounds $\pm 4\mu\text{M}$ overnight. The medium was then replaced with fresh medium containing treatment at a reduced volume (50%), culture medium was collected after 24 h. The medium was analyzed by an A β enzyme-linked immunosorbent assay (ELISA) as follows. Monoclonal 6E10 anti-A β (residues 1–17) mouse IgG1 (Biolegend) was coated in 50 mM carbonate buffer, pH 9.6, at 4°C overnight on high binding assay black plates (Costar), washed with TBST (tris-buffered saline with 0.05% Tween-20) and blocked with 5% nonfat milk in TBST. Samples and standards (condition 7PA2 media) were incubated for 1.5 h, followed by addition of 4G8 antibody (anti-A β residues 17–24, mouse IgG2b (Biolegend)) conjugated to HRP and incubated for 1.5 h at 25°C. After washing, ABTS substrate was added, followed by detection with an absorbance plate reader.

[³⁵S] Metabolic labeling. [³⁵S] metabolic labeling experiments were performed as previously described⁶. Briefly, transfected CHO^{7PA2} cells were plated and treated on poly-D-lysine coated six-well plates and metabolically labeled in DMEM-Cys-/Met (Corning CellGro, Mediatech Inc.) supplemented with glutamine, penicillin/streptomycin, dialyzed FBS and EasyTag EXPRESS [³⁵S] Protein Labeling Mix (Perkin Elmer) for 30 min. Cells were washed twice with complete media and incubated in prewarmed DMEM for the indicated times. Media or lysates were harvested at the indicated times. Lysates were prepared in RIPA buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X100, 0.5% sodium deoxycholate, 0.1% SDS) containing proteases inhibitors cocktail (Roche). APP species were immunopurified using protein G sepharose beads bound with 6E10 antibody and washed four times with RIPA buffer. The immunisolates were then eluted by boiling in 6× Laemmli buffer and separated on 12% SDS-PAGE. Gels were stained with Coomassie Blue, dried, exposed to phosphorimager plates (GE Healthcare) and imaged by autoradiography using a Typhoon Trio Imager (GE Healthcare). Band intensities were quantified by densitometry in ImageQuant. Fraction secreted was calculated using the equation: fraction secreted = (extracellular [³⁵S]-APP signal at t)/(extracellular [³⁵S]-APP signal at $t=0$ + intracellular [³⁵S]-APP signal at $t=0$). Fraction remaining was calculated using the equation: ((extracellular [³⁵S]-APP signal at t + intracellular [³⁵S]-APP signal at t)/(extracellular [³⁵S]-APP signal at $t=0$ + intracellular [³⁵S]-APP signal at $t=0$)).

CellTiterGlo viability assays. For determination of relative cellular ATP levels, SHSY5Y cells were seeded into flat black, poly-D-lysine coated 96-well plates (Corning). Cells were treated as indicated then lysed by the addition of CellTiter-Glo reagent (Promega). Samples were dark adapted for 10 min to stabilize signals. Luminescence was then measured in an Infinite F200 PRO plate reader (Tecan) and corrected for background signal. All measurements were performed in biologic triplicate.

TMRE staining and flow cytometry. Cells were treated as indicated then incubated with TMRE dye (200 nM) for 30 mins at 37°C. Samples were collected by trypsinization. Trypsin was neutralized by washing into cell culture media and then samples were washed twice in DPBS. Cell pellets were suspended into DPBS supplemented with 0.1% BSA. Fluorescence intensity of TMRE was recorded on the PE channel of a Novocyt Flow Cytometer (ACEA Biosciences, Inc.). Data are

presented as mean of the fluorescence intensity from three experiments. For each experiment, 10,000 cells per condition in triplicate were recorded.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The raw data that support the findings of this study are available within the paper and its Extended Data files (pertaining to Figs. 1, 3 and 4 and Extended Data Figs. 1, 3, 4 and 5), and/or are available from the corresponding author upon reasonable request. The RNA-seq data have been deposited to the public National Center for Biotechnology Information Gene Expression Omnibus repository under the data identifier [GSE148802](#). Source data are provided with this paper.

Code availability

Code for standard open-source DESeq differential gene expression RNA-seq analysis used in R statistical software is available from the corresponding author upon reasonable request. Source data are provided with this paper.

Acknowledgements

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Author contributions

J.M.D.G., A.M., C.B.C., L.P., L.S., T.P.S., J.W.K. and R.L.W. designed the research. J.M.D.G., L.C., B.O.S., A.M., R.J.P., C.B.C. and L.P. performed the chemical, cell biological and biochemical experiments described in this manuscript. E.S. and L.S. performed the HTS to identify IRE1-XBP1s activating compounds. L.S., T.P.S., J.W.K. and R.L.W. provided oversight on the H.T.S. and compound selection. J.M.D.G., E.T.P. and R.L.W. analyzed the RNA-seq data. R.L.W. provided oversight on all aspects of this work. J.M.D.G. and R.L.W. wrote the manuscript with input from other coauthors.

Competing interests

J.W. Kelly is a cofounder of Proteostasis Therapeutics Inc. A patent has been submitted for compounds identified through work described in this manuscript. This patent includes R.L. Wiseman, J.W. Kelly, J.M.D. Grandjean, L. Plate and C.B. Cooley as coinventors. No other author has competing interests.

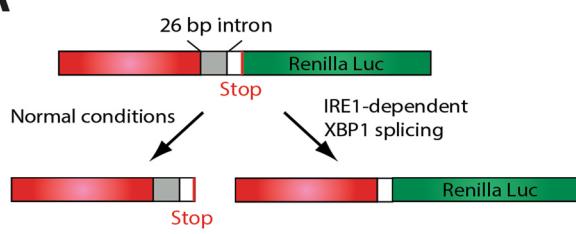
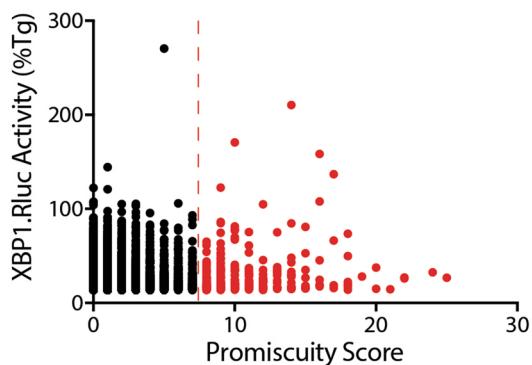
Additional information

Extended data is available for this paper at <https://doi.org/10.1038/s41589-020-0584-z>.

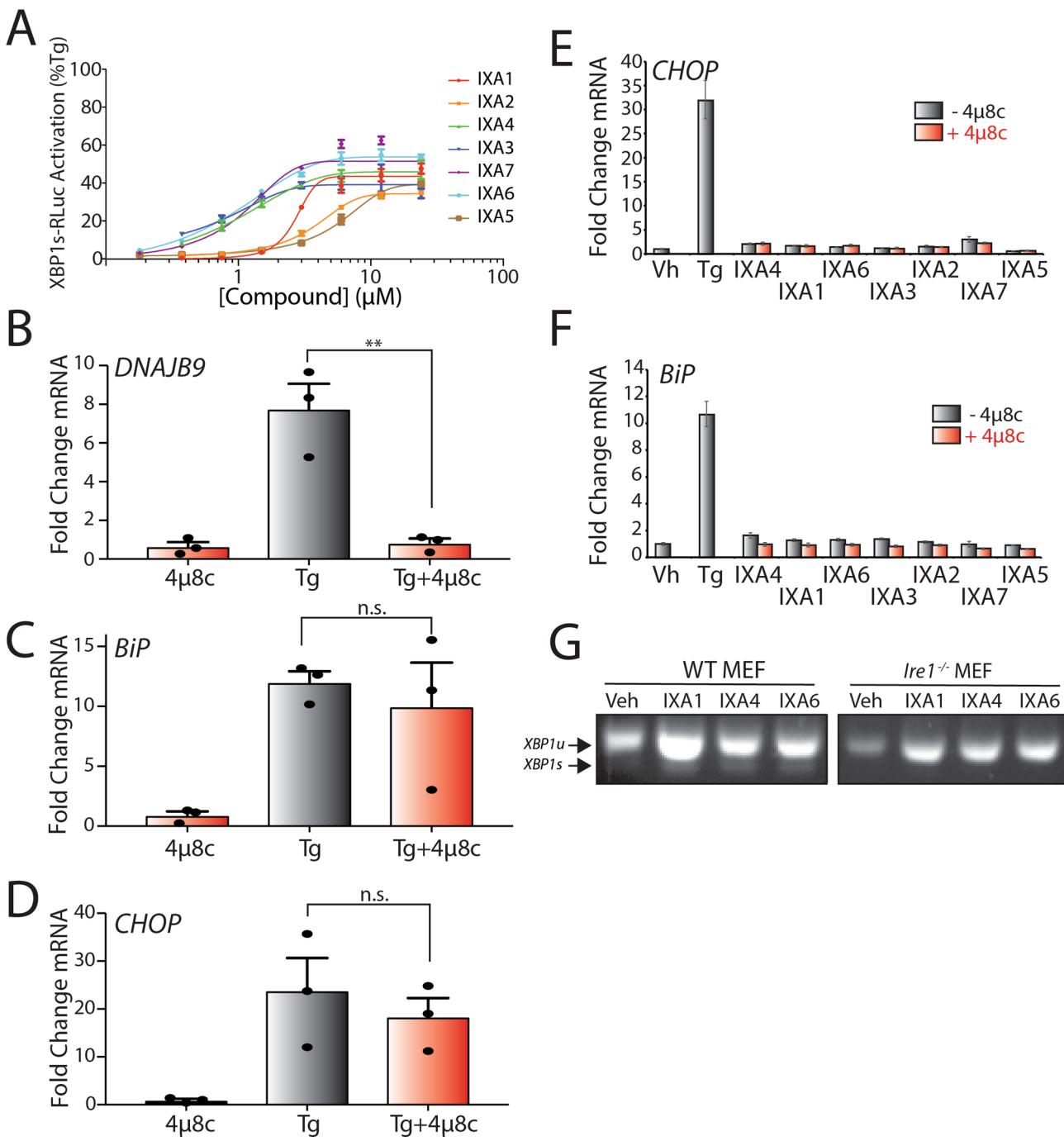
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Correspondence and requests for materials should be addressed to R.L.W.

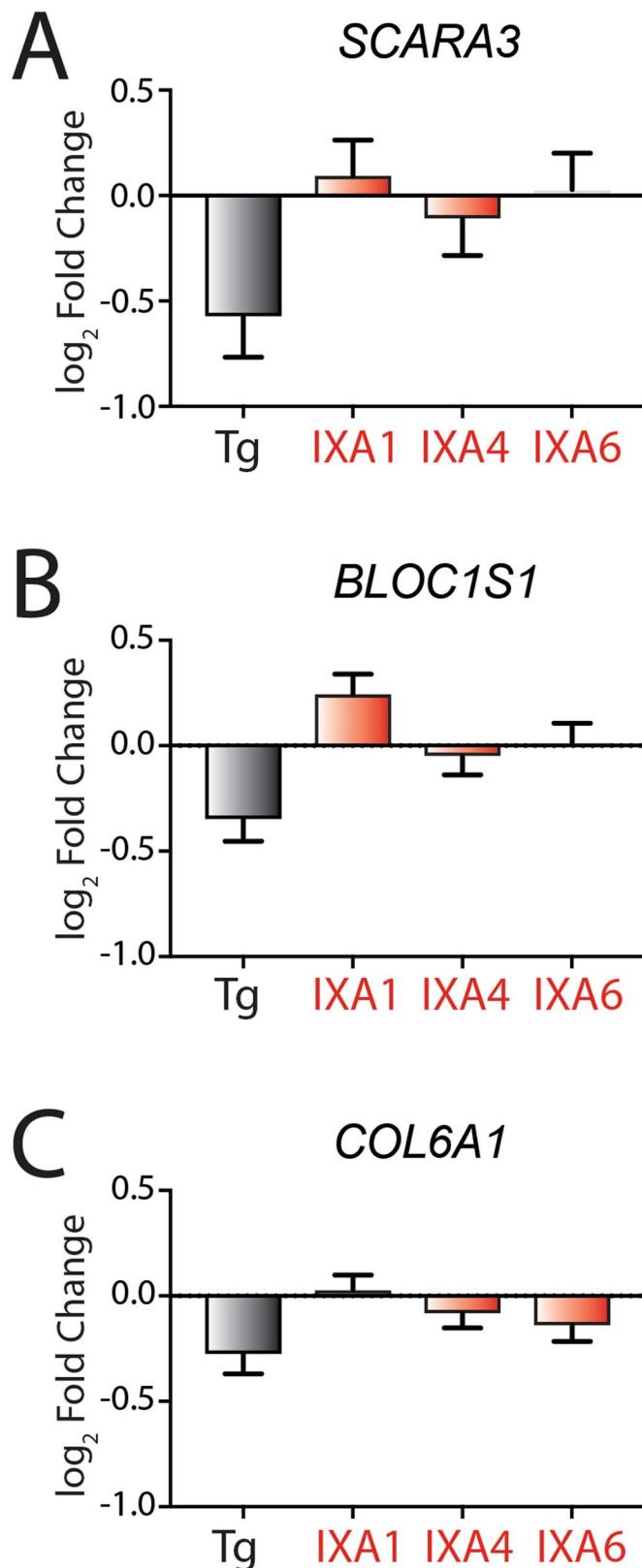
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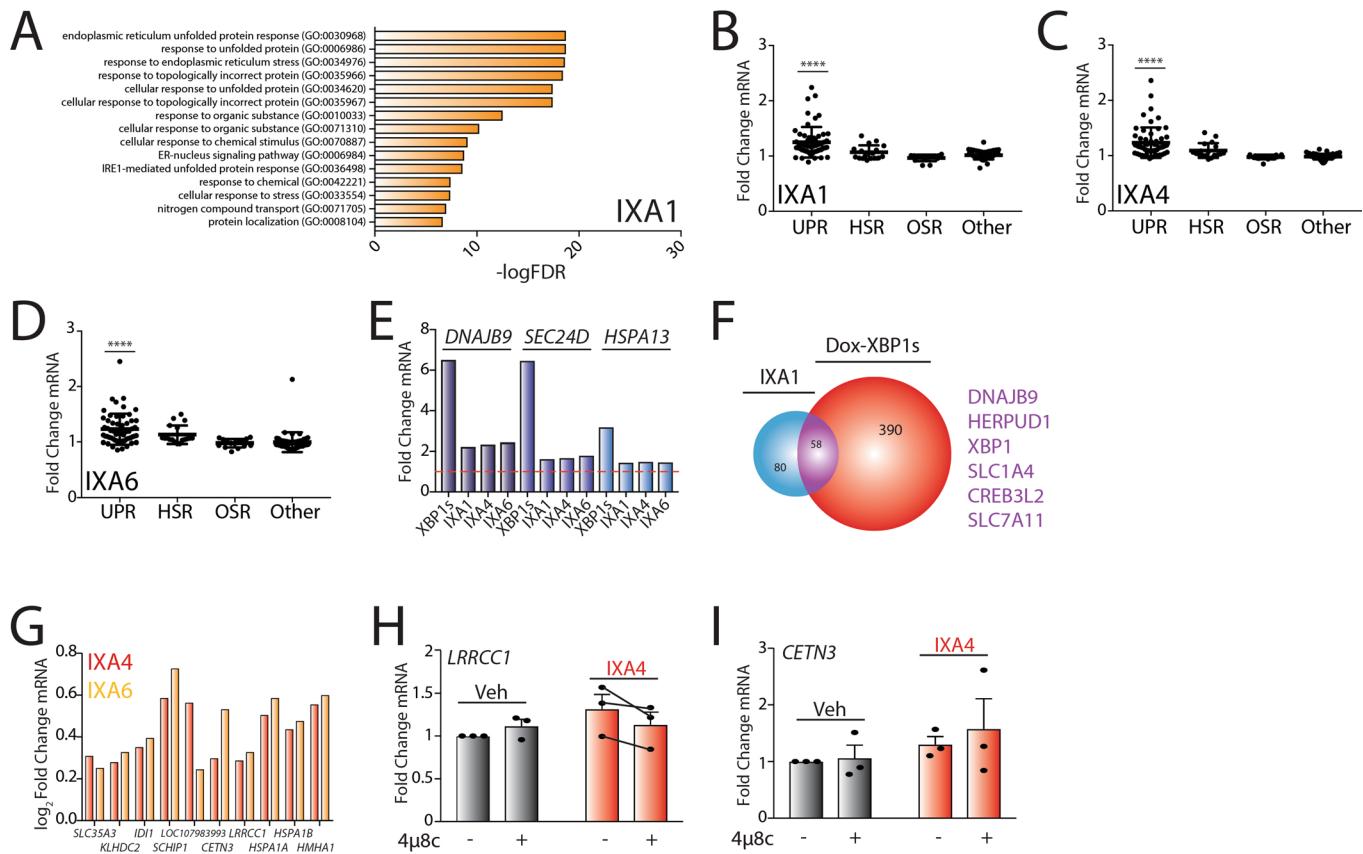
Extended Data Fig. 1 | HTS to identify preferential IRE1-XBP1s activating compounds. **a**, Schematic of the XBP1-Renilla luciferase (XBP1-RLuc) splicing reporter used in our high-throughput screen to identify small molecule activators of IRE1/XBP1s signaling. **b**, Graph showing XBP1-RLuc splicing reporter activation in HEK293^{TREX} cells incubated with Tg (500 nM) in the presence or absence of 4μ8c (64 μM) for 16 hrs. Error bars show SD for n=3 replicates. **c**, Plot showing XBP1-RLuc activation in HEK293^{TREX} cells stably expressing the XBP1-RLuc reporter treated with the 10,114 small molecules (6 μM; 18 hrs) identified as hits in the primary screen. Luminescence is shown as % signal relative to treatment with Tg (500 nM; 18 hrs). **d**, Venn diagram of overlap of compounds identified to activate the IRE1-dependent XBP1-RLuc splicing reporter, the ATF6-selective ERSE-FLuc reporter, or the HSF1-dependent HSP70-FLuc reporter via high-throughput screening. **e**, Plot showing XBP1-RLuc activation in HEK293^{TREX} cells (% signal compared to that observed with 500 nM Tg, 18 hrs) versus promiscuity score for our top 638 compounds identified by HTS. The promiscuity score reports on the number of assays performed at the TSRI-FL Screening Center where each compound was identified as a positive hit. **f**, Plot showing IC₅₀ for toxicity as measured by CellTiterGlo luminescence calculated from titration screening of 638 hit compounds. The dashed red lines indicates IC₅₀ = 3 μM. **g**, Plot showing IRE1-dependent XBP1-RLuc activation versus ATF6-selective ERSE-Fluc activation in HEK293^{TREX} cells (% signal compared to that observed with 500 nM Tg, 18 hrs) for the subset of our top 638 compounds that were also identified to activate the ATF6-selective ERSE-FLuc reporter. The dashed red line indicates equal XBP1s-RLuc and ERSE-Fluc activation. **h**, Plot showing XBP1-RLuc activation in HEK293^{TREX} cells (% signal compared to that observed with 500 nM Tg, 18 hrs) versus EC₅₀ of XBP1-RLuc activation in HEK293^{TREX} cells calculated from titration screening of hit 638 compounds. The dashed red lines indicates 20% Rluc activation and Rluc EC₅₀ = 3 μM.



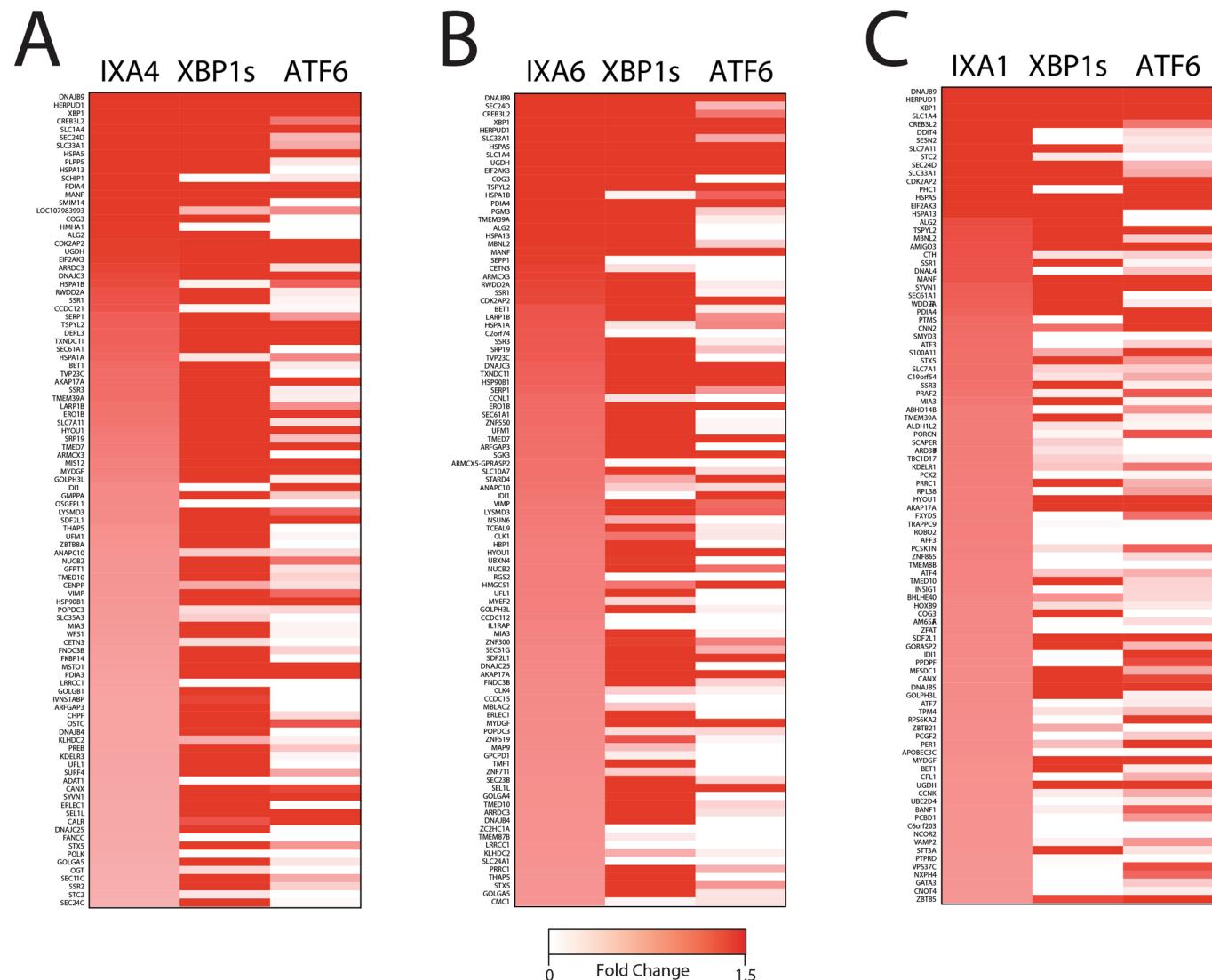
Extended Data Fig. 2 | Compound-dependent IRE1-XBP1s activation requires IRE1 autophosphorylation. **a**, Plot showing XBP1-RLuc activation in HEK293^{TREX} cells (% signal compared to that observed with 500 nM Tg, 18 hrs) treated for 18 hrs with the indicated concentrations of prioritized IRE1/XBP1s activator. Error bars represent SD for $n=3$ replicates. **b**, Graph showing qPCR of the XBP1s target gene *DNAJB9* in HEK293T cells treated for 4 hrs with Tg (500 nM) in the presence or absence of 4 μ 8c (64 μ M). Error bars show SE for $n=3$. P-values were calculated using one-tailed Student's t-test. ** $p < 0.01$. **c**, Graph showing qPCR of the ATF6 target gene *BiP* in HEK293T cells treated for 4 hrs with Tg (500 nM) in the presence or absence of 4 μ 8c (64 μ M). Error bars show SE for $n=3$. P-values were calculated using one-tailed Student's t-test. **d**, Graph showing qPCR of the PERK target gene *CHOP* in HEK293T cells treated for 4 hrs with Tg (500 nM) in the presence or absence of 4 μ 8c (64 μ M). Error bars show SE for $n=3$. P-values were calculated using one-tailed Student's t-test. **e**, Graph showing qPCR of the PERK target gene *CHOP* in HEK293T cells treated for 4 hrs with prioritized IRE1/XBP1s activators (10 μ M) or Tg (500 nM), in the presence or absence of 4 μ 8c (32 μ M). Error bars show 95% CI for $n=3$ replicates. **f**, Graph showing qPCR analysis of the ATF6 target gene *BiP* in HEK293T cells treated for 4 hrs with indicated compound (10 μ M), or Tg (500 nM), in the presence or absence of 4 μ 8c (32 μ M). Error bars show 95% CI for $n=3$ replicates. **g**, cDNA gel showing splicing of *XBP1* mRNA in WT MEF or *Ire1*^{-/-} MEF cells treated with **IXA1**, **IXA4**, or **IXA6** (10 μ M) for 4 hrs.



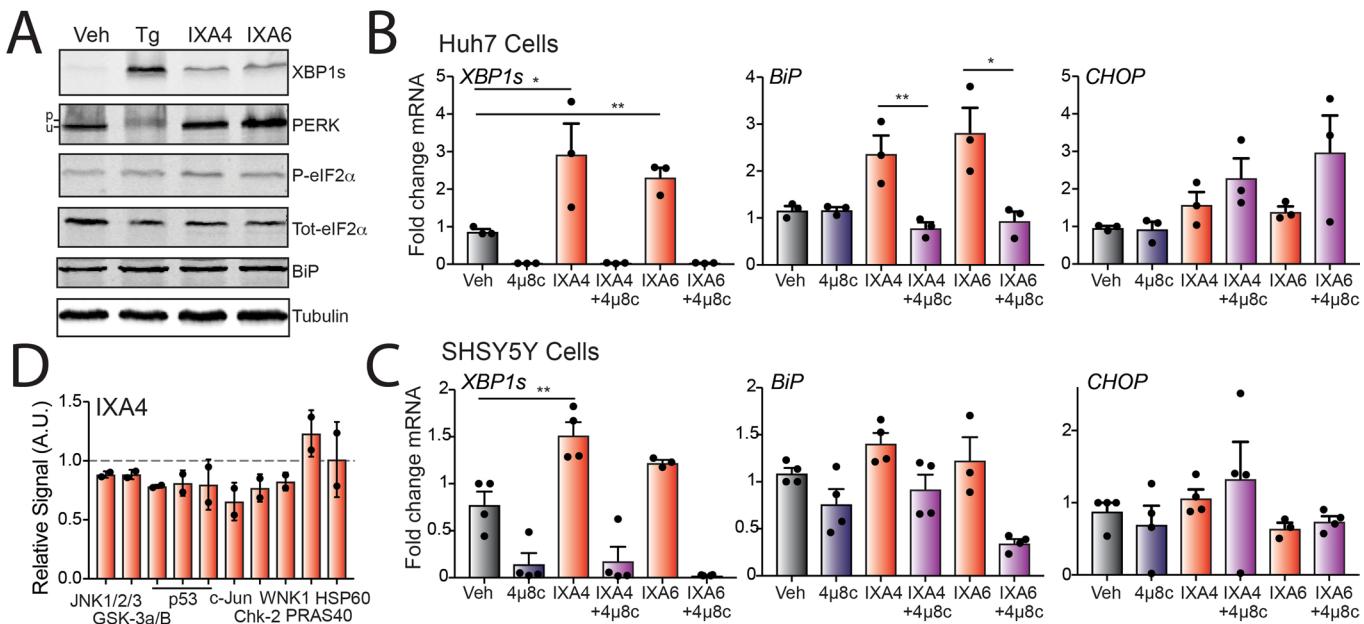
Extended Data Fig. 3 | Transcriptional profiling of compounds IXA1, IXA4 and IXA6 shows preferential induction of IRE1-XBP1s target genes. **a**, Graph showing log₂ Fold change mRNA levels from RNA-seq for the RIDD target SCARA3 in HEK293T cells treated with 10 μ M IXA1, IXA4, IXA6, or 500 nM Tg for 4 hrs. Error bars represent SD for n=3 replicates. **b**, Graph showing log₂ Fold change mRNA levels from RNA-seq for the RIDD target BLOC1S1 in HEK293T cells treated with 10 μ M IXA1, IXA4, IXA6, or 500 nM Tg for 4 hrs. Error bars represent SD for n=3 replicates. **c**, Graph showing log₂ Fold change mRNA levels from RNA-seq for the RIDD target COL6A1 in HEK293T cells treated with 10 μ M IXA1, IXA4, IXA6, or 500 nM Tg for 4 hrs. Error bars represent SD for n=3 replicates.



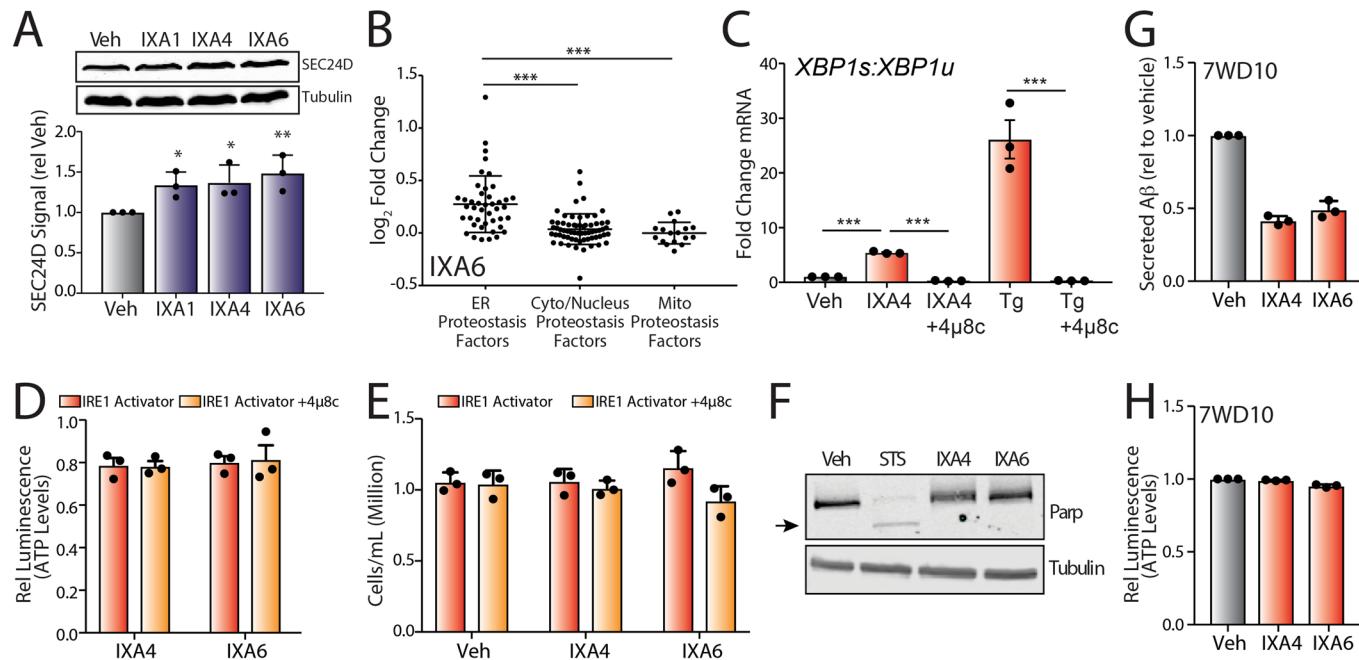
Extended Data Fig. 4 | Compounds IXA4 and IXA6 show selectivity for IRE1-XBP1s-dependent ER proteostasis remodeling (part 1). **a**, Gene Ontology (GO) analysis of differentially expressed genes from RNA-seq for HEK293T cells treated with **IXA1** (10 μ M, 4 hrs). Top 15 entries with lowest FDR are shown. See Supplementary Table 4 for full GO analysis. **b**, Graph showing fold change mRNA levels from RNA-seq of target genes activated downstream of the UPR, HSR, OSR, and other stress signaling pathways in HEK293T cells treated with **IXA1** (10 μ M) for 4 hrs. The composition of these gene sets is shown in Source Data Fig. 3. P-values were calculated using one-way ANOVA compared to 'Other'. ***p < 0.0001. **c**, Graph showing fold change mRNA levels from RNA-seq of target genes activated downstream of the UPR, HSR, OSR, and other stress signaling pathways in HEK293T cells treated with **IXA4** (10 μ M) for 4 hrs. The composition of these genesets is shown in Source Data Fig. 3. P-values were calculated using one-way ANOVA compared to 'Other'. ***p < 0.0001. **d**, Graph showing fold change mRNA levels from RNA-seq of target genes activated downstream of the UPR, HSR, OSR, and other stress signaling pathways in HEK293T cells treated with **IXA6** (10 μ M) for 4 hrs. The composition of these genesets is shown in Source Data Fig. 3. P-values were calculated using one-way ANOVA compared to 'Other'. ***p < 0.0001. **e**, Bar graph showing fold change mRNA levels of the IRE1/XBP1s targets *DNAJB9*, *SEC24D*, and *HSPA13* from RNA-seq of HEK293^{DAX} cells expressing dox-inducible XBP1s treated with dox (1 μ g/mL) for 4 hr or HEK293T cells treated with compounds **IXA1**, **IXA4**, or **IXA6** (10 μ M) for 4 hrs. **f**, Venn diagram of genes upregulated >1.2 fold (adjusted p-value < 0.05) in HEK293T cells treated with compound **IXA1** (10 μ M) for 4 hrs in comparison to genes induced >1.2 fold (adjusted p-value < 0.05) in HEK293^{DAX} cells treated with doxycycline (1 μ g/mL) for 4 hrs. Genes listed in purple are top overlapping targets between conditions. **g**, Graph showing \log_2 Fold Change mRNA levels from RNA-seq of the 10 non-overlapping genes activated in cells treated with **IXA4** (10 μ M) compared to dox-inducible XBP1s. \log_2 Fold change mRNA levels of these genes in cells treated with **IXA6** (10 μ M) are also included. **h**, Graph showing qPCR of the *LRRCC1* gene in HEK293T cells treated with **IXA4** (10 μ M) in the presence or absence of 4 μ 8c (64 μ M) for 4 hrs. Error bars show SE for n=3. **i**, Graph showing qPCR of the *CETN3* gene in HEK293T cells treated with **IXA4** (10 μ M) in the presence or absence of 4 μ 8c (64 μ M) for 4 hrs. Error bars show SE for n=3.



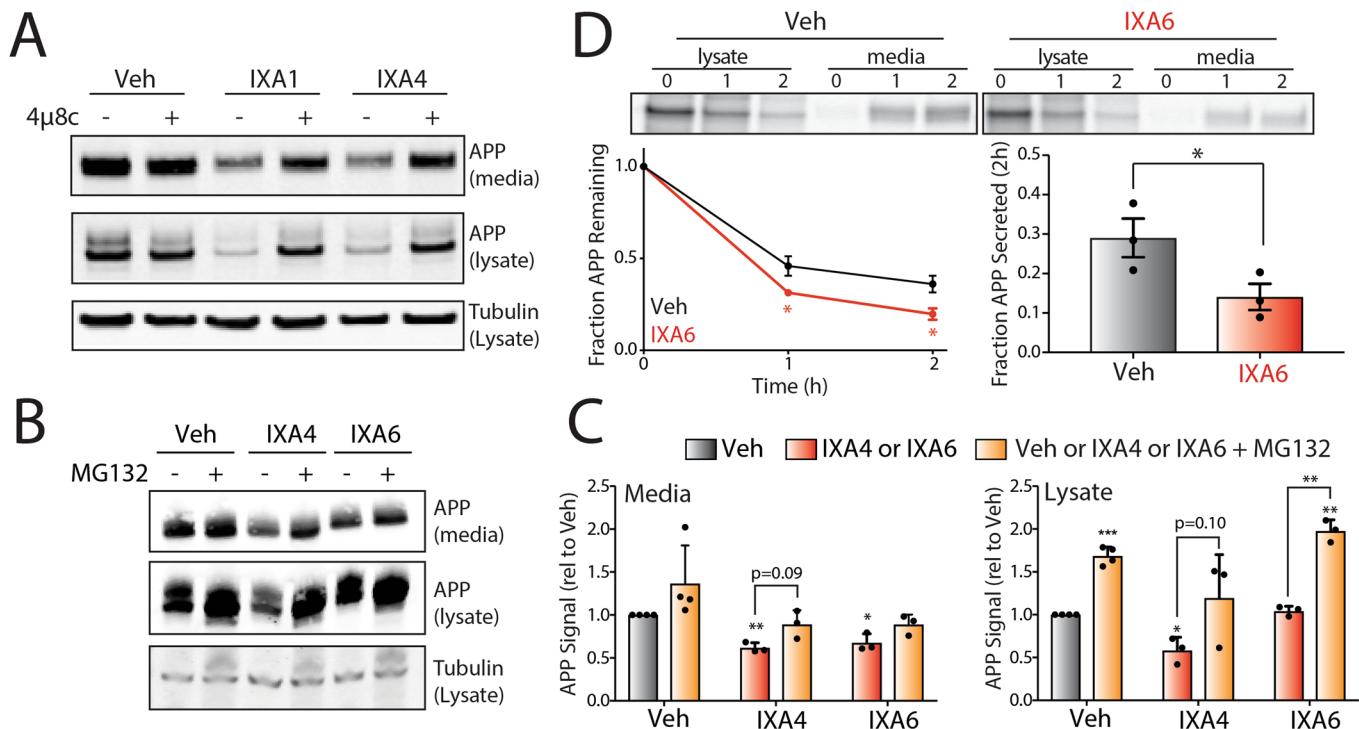
Extended Data Fig. 5 | Compounds IXA4 and IXA6 show selectivity for IRE1-XBP1s-dependent ER proteostasis remodeling (part 2). **a**, Heat map of top 100 genes upregulated in HEK293T cells treated for 4 hrs with **IXA4** (10 μM) compared to dox-inducible XBP1s or TMP-stabilized DHFR.ATF6 in HEK293^{DAX} cells treated with doxycycline (1 μg/mL) or TMP (10 μM) for 4 hr. **b**, Heat map of top 100 genes upregulated in HEK293T cells treated for 4 hrs with **IXA6** (10 μM) compared to dox-inducible XBP1s or TMP-stabilized DHFR.ATF6 in HEK293^{DAX} cells treated with doxycycline (1 μg/mL) or TMP (10 μM) for 4 hrs. **c**, Heat map of top 100 genes upregulated in HEK293T cells treated for 4 hr with **IXA1** (10 μM) compared to dox-inducible XBP1s or TMP-stabilized DHFR.ATF6 in HEK293^{DAX} cells treated with doxycycline (1 μg/mL) or TMP (10 μM) for 4 hrs.



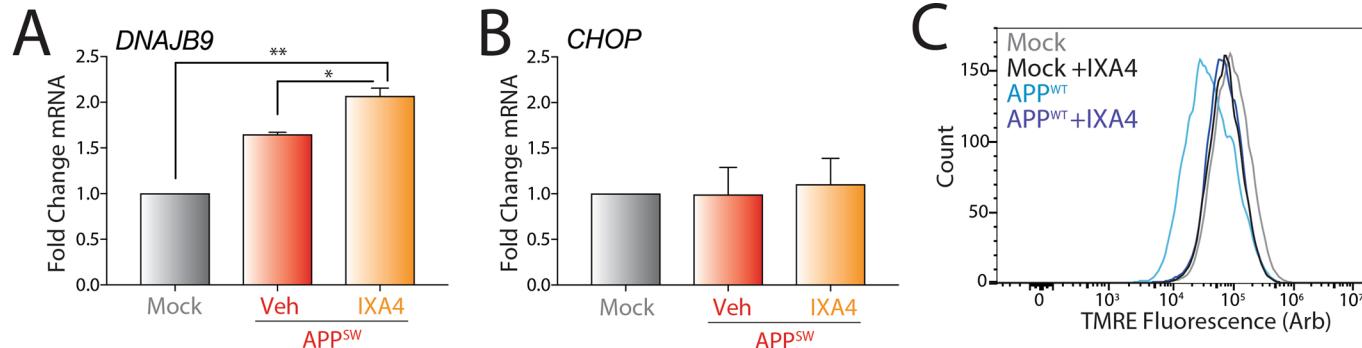
Extended Data Fig. 6 | Compounds IXA4 and IXA6 show selectivity for IRE1-XBP1s-dependent ER proteostasis remodeling (part 3). **a**, Representative immunoblots of XBP1s, PERK, eIF2 α , and BiP in lysates prepared from HEK293T cells treated with vehicle, thapsigargin (1 μ M), **IXA4** (10 μ M), or **IXA6** (10 μ M) for 4 hrs. **b**, Graph showing qPCR of the XBP1 target gene *DNAJB9*, ATF6 target gene *BiP*, and PERK target gene *CHOP* in Huh-7 cells treated with **IXA4** (10 μ M) or **IXA6** (10 μ M) in the presence or absence of 4 μ 8c (64 μ M) for 4 hrs. Error bars show SE for n=3 replicates. Statistics calculated from one-tailed Student's t-test. *p < 0.05, **p < 0.01. **c**, Graph showing qPCR of the XBP1s target gene *DNAJB9*, ATF6 target gene *BiP*, and PERK target gene *CHOP* in SHSY5Y cells treated with **IXA4** (10 μ M) or **IXA6** (10 μ M) in the presence or absence of 4 μ 8c (64 μ M) for 4 hrs. Error bars show SE for n=4 replicates. Statistics calculated from one-tailed Student's t-test. *p < 0.05, **p < 0.01. **d**, Graph showing relative signal of phosphorylated target proteins by phosphokinase array dot immunoblotting in HEK293T cells treated with **IXA4** (10 μ M) for 4 hrs. Error bars show SD for n=2.



Extended Data Fig. 7 | Compound IXA4 increases degradation of APP mutants (part 1). **a**, Representative immunoblots and quantification of SEC24D in lysates prepared from HEK293T cells treated with vehicle, **IXA1**, **IXA4**, or **IXA6** (10 μM, 18 hrs). Error bars show SD for n=3 replicates. P-values were calculated using one-tailed Student's t-test. *p < 0.05, **p < 0.01. **b**, Plot of log₂ Fold Change mRNA levels from RNA-seq in cells treated with **IXA6** (10 μM, 4 hrs) of proteostasis factors found in the ER, cytosol/nucleus, or mitochondria. The composition of these proteostasis genesets is shown in Source Data Fig. 3. **c**, Graph showing the Fold change mRNA ratio of *Xbp1s:Xbp1u* by qPCR in CHO^{7PA2} cells treated with **IXA4** (10 μM) or thapsigargin (1 μM) in the presence or absence of 4μ8c (64 μM) for 4 hrs. Error bars show SE for n=3 replicates. Statistics calculated from one-tailed Student's t-test. *p < 0.05, **p < 0.01. **d**, Graph showing relative CellTiterGlo luminescence from CHO^{7PA2} cells treated with IRE1/XBPIs activators **IXA4**, or **IXA6** (10 μM) in the presence or absence of 4μ8c (32 μM). Cells were treated for 18 hrs, media was then replaced and conditioned in the presence of treatments for 24 hrs before measuring ATP levels. Luminescence signal was normalized to that observed in untreated controls. Error bars represent SE for n=3 replicates. **e**, Graph showing cell counts (10⁶cells/mL) of CHO^{7PA2} cells treated with **IXA4** or **IXA6** (10 μM) in the presence or absence of 4μ8c (64 μM) for 24 hrs. **f**, Immunoblot of PARP in lysates collected from CHO^{7PA2} cells treated with **IXA4** or **IXA6** (10 μM) or staurosporine (STS) (1 μM) for 24 hrs. **g**, Graph showing relative signal from ELISA of secreted Aβ peptide from conditioned media prepared on CHO^{7WD10} cells treated with **IXA4** or **IXA6** (10 μM). Cells were pretreated for 18 hrs with compounds. Media was then replaced and conditioned in the presence of compounds for 24 hrs before harvesting the media for ELISA. Secreted Aβ was normalized to that observed in untreated controls. Error bars represent SE for n=3 replicates. **h**, Graph showing relative CellTiterGlo luminescence from CHO^{7WD10} cells treated with IRE1/XBPIs activators **IXA4** or **IXA6** (10 μM). Cells were treated for 18 hrs, media was then replaced and conditioned in the presence of treatments for 24 hrs before measuring ATP levels. Luminescence signal was normalized to that observed in untreated controls. Error bars represent SE for n=3 replicates.



Extended Data Fig. 8 | Compound IXA4 increases degradation of APP mutants (part 2). **a**, Representative immunoblot of mutant APP in media and lysates collected from CHO^{7PA2} cells treated with **IXA1** or **IXA4** (10 μ M) in the presence or absence of 4 μ 8c (64 μ M). Cells were treated for 18 hrs, media was then replaced and conditioned in the presence of treatments for 24 hrs before harvesting. **b**, Representative immunoblot of mutant APP in media and lysates collected from CHO^{7PA2} cells treated with **IXA4** or **IXA6** (10 μ M) in the presence or absence of MG132 (10 μ M) for 18hrs. **c**, Quantification of immunoblots represented in panel **b** of relative APP signal in lysates or conditioned media from CHO^{7PA2} cells treated with IRE1/XBP1s activators **IXA4** or **IXA6** (10 μ M) in the presence or absence of MG132 (10 μ M) for 18hrs. Error bars represent SE for n = 4 replicates. Statistics calculated from one-tailed Student's t-test. *p < 0.05, **p < 0.01, ***p < 0.001. **d**, Representative autoradiogram showing the [³⁵S]-metabolic labeling of mutant APP in CHO^{7PA2} cells treated with **IXA6** (10 μ M) for 16 hrs prior to 30 min labeling. Media and lysates were collected at 0, 1, or 2 hrs and [³⁵S]-labeled mutant APP was isolated by immunopurification. The experimental protocol is shown above. Fraction remaining was calculated as described in Fig. 5d and fraction secretion was calculated as in Fig. 5e. Error bars represent SD for n = 3 replicates. P-values were calculated from one-tailed Student's t-test. *p < 0.05.



Extended Data Fig. 9 | The IRE1-XBP1s activator IXA4 rescues mitochondrial defects in SHSY5Y cells expressing disease-relevant APP mutants. **a**, Graph showing qPCR of the XBP1s target gene *DNAJB9* in SHSY5Y cells transiently expressing empty vector (Mock) or Swedish mutant APP (APP^{SW}) in the presence or absence of **IXA4** (10 μ M) for 72 hrs. Error bars show SE for n=2. Statistics calculated from one-tailed Student's t-test. *p < 0.05, **p < 0.01. **b**, Graph showing qPCR of the PERK target gene *CHOP* in SHSY5Y cells transiently expressing empty vector (Mock) or APP^{SW} in the presence or absence of **IXA4** (10 μ M) for 72 hrs. Error bars show SE for n=2. **c**, Representative histograms showing TMRE staining of SHSY5Y cells transiently expressing empty vector (Mock) or wild-type APP (APP^{WT}) in the presence or absence of **IXA4** (10 μ M) for 72 hrs.

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Code for standard open-source DESeq differential gene expression RNAseq analysis used in R statistical software is available from the corresponding author upon reasonable request.

Data analysis

All data analysis was performed as described in the Materials and Methods.

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DATA AVAILABILITY

The raw data that support the findings of this study are available within the paper and its Extended Data files (pertaining to Figures 1, 3, 4, and Extended Data Figures 1, 3, 4, and 5), and/or are available from the corresponding author upon reasonable request. The RNA-seq data have been deposited to the public National Center for Biotechnology Information GEO repository under the data identifier GSE148802.

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<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology	<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging
<input checked="" type="checkbox"/>	<input type="checkbox"/> Animals and other organisms		
<input checked="" type="checkbox"/>	<input type="checkbox"/> Human research participants		
<input checked="" type="checkbox"/>	<input type="checkbox"/> Clinical data		

Antibodies

Antibodies used	Antibodies: APP (6E10, Fisher Scientific Cat #501029533), APP (HRP-4G8, Fisher Scientific Cat #501029498), SEC24D (mouse) antibody was provided as a generous gift from the Balch Lab at TSRI, XBP1s (Cell Signaling Cat #12782S), KDEL (Enzo Cat # ADI-SPA-827-F), eif2a-P (Cell Signaling Cat #9721S), eif2a-total (Abcam Cat # ab5369), PERK (Cell Signaling Cat #3192S), PARP (Cell Signaling Cat #9542S), tubulin (Sigma Cat # T6074-200UL)
Validation	All antibodies have been previously validated in the Wiseman lab using knockout lines and/or overexpression.

Eukaryotic cell lines

Policy information about cell lines	
Cell line source(s)	SHSY5Y, HEK293T, and Huh7 cells were purchased from ATCC. HEK293DAX cells, XBP1.Rluc stable HeK293T cells, and ERSE.Fluc HEK293T cells were established in the Wiseman lab (see Shoulders et al (2013) Cell Reports; Plate et al (2016) ELIFE). CHO7PA2 and CHO7WD10 cells were obtained from Edward Koo's lab at UCSD.
Authentication	Cells were routinely tested for mycoplasma every 6 months. No further authentication of cell lines was performed by the authors.
Mycoplasma contamination	Cells were routinely tested for mycoplasma every 6 months. No further authentication of cell lines was performed by the authors.
Commonly misidentified lines (See ICLAC register)	Name any commonly misidentified cell lines used in the study and provide a rationale for their use.

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

TMRE Staining and Flow Cytometry

Cells were treated as indicated then incubated with TMRE dye (200 nM) for 30 mins at 37°C. Samples were collected by trypsinization. Trypsin was neutralized by washing into cell culture media and then samples were washed twice in DPBS. Cell pellets were suspended into DPBS supplemented with 0.1% BSA. Fluorescence intensity of TMRE was recorded on the PE channel of a Novocyte Flow Cytometer (ACEA Biosciences, Inc). Data are presented as mean of the fluorescence intensity from 3 experiments. For each experiment, 10,000 cells per condition in triplicates were recorded.

Instrument

Novocyte Flow Cytometer (ACEA Biosciences, Inc).

Software

Flowjo

Cell population abundance

Experiments were performed by scanning >10,000 cells per sample.

Gating strategy

No gating strategies were employed apart from FSC/SSC scattering for viable cells. The presented data show only the histogram of fluorescence or the normalized geometric mean.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.