In response to an accumulation of unfolded proteins in the endoplasmic reticulum (ER) lumen, three ER transmembrane signaling proteins, inositol-requiring enzyme 1 (IRE1), PRKR-like ER kinase (PERK), and activating transcription factor 6α (ATF6α), are activated. These proteins initiate a signaling and transcriptional network termed the unfolded protein response (UPR), which re-establishes cellular proteostasis. When this restoration fails, however, cells undergo apoptosis. To investigate cross-talk between these different UPR enzymes, here we developed a high-content live cell screening platform to image fluorescent UPR-reporter cell lines derived from human SH-SY5Y neuroblastoma cells in which different ER stress signaling proteins were silenced through lentivirus-delivered shRNA constructs. We observed that loss of ATF6 expression results in uncontrolled IRE1-reporter activity and increases X box-binding protein 1 (XBPI) splicing. Transient increases in both IRE1 mRNA and IRE1 protein levels were observed in response to ER stress, suggesting that IRE1 up-regulation is a general feature of ER stress signaling and was further increased in cells lacking ATF6 expression. Moreover, overexpression of the transcriptionally active N-terminal domain of ATF6 reversed the increases in IRE1 levels. Furthermore, inhibition of IRE1 kinase activity or of downstream JNK activity prevented an increase in IRE1 levels during ER stress, suggesting that IRE1 transcription is regulated through a positive feed-forward loop. Collectively, our results indicate that from the moment of activation, IRE1 signaling during ER stress has an ATF6-dependent “off-switch.”

High demands for protein synthesis, disruptions in the protein folding machinery, or defects in ER–Golgi transport may lead to an accumulation of unfolded or misfolded proteins in the ER lumen. This accumulation is sensed by three ER transmembrane signaling proteins, IRE1, PERK, and ATF6α. Activation of these proteins initiates a signaling cascade termed the unfolded protein response (UPR) that culminates in the attenuation of protein synthesis and a transcriptional response aimed at re-establishing proteostasis. However, if the stress is prolonged or severe, apoptosis ensues.

Whereas signaling of IRE1 and PERK has been shown to be critical for determining cell survival after ER stress (1, 2), functional links between ATF6α activation and cellular outcome are less well-defined. Rather than being decisive for cell fate, ATF6 signaling has been proposed to convey adaption to long-term chronic stress (3, 4). IRE1, PERK, and ATF6 are kept in an inactive state through association with ER chaperone BiP (5). As unfolded proteins accumulate in the ER, BiP dissociates allowing for the activation of the three transmembrane signaling factors. ATF6α is initially trafficked to the Golgi where it is sequentially cleaved by the proteases SP1 and SP2 (6). The N-terminal cytosolic fragment of ATF6 functions as a transcription factor and forms heterodimers with other factors such as XBP1. Transcriptional targets of ATF6 are cytoprotective ER chaperones and ERAD components, including BiP (4). Deletion of ATF6 was sufficient to attenuate ER stress–induced BiP expression (7). Furthermore, ATF6 was shown to regulate transcription of Xbp1 (8), which is spliced into an active transcription factor through IRE1.

Following BiP dissociation, IRE1 oligomerizes within the ER membrane (9). Oligomerization enables transautophosphorylation, which in turn activates the cytosolic endonuclease domain of IRE1 (10). The primary target of the IRE1 endonuclease activity is Xbp1 mRNA, splicing of which enables the translation of the active transcription factor XBP1. Thus, the IRE1 and ATF6 pathways converge on regulation of Xbp1-dependent signaling outputs, which includes ER chaperones and ERAD components (11–13).

IRE1 has also been shown to process other mRNAs, including its own, in a process termed regulated IRE1–dependent decay (14–16). Additionally the cytosolic domain of phosphorylated IRE1 can recruit the adapter protein TNF receptor 2 (TRA2), triggering the ASK1/JNK cascade (17). The timing of these different IRE1-signaling outputs is not well-understood. It has been suggested that the shift from splicing of Xbp1 to the more promiscuous degradation of mRNAs constitutes a switch from pro-survival to pro-apoptotic signaling (10). Similarly, activation of the ASK/JNK cascade has been interpreted as a pro-apoptotic signal (18). During prolonged ER stress, IRE1-mediated splicing is eventually attenuated (1, 19). This
occurred independently of the availability of the unspliced Xbp1 mRNA substrate and despite continuous ER stress.

To investigate in more detail the role of ATF6 signaling in relation to ER stress responses and cell survival, we here employed our recently established and characterized ER stress reporter cell lines (19), and monitored their responses to silencing of UPR components during ER stress using time-lapse live cell imaging. We identified cross-talk between IRE1 and ATF6 signaling and demonstrated that from the moment of activation, the ER stress signaling machinery contains an ATF6-dependent “off-switch.”

Results

Interrogating cross-talk between the UPR branches

To explore the UPR-signaling network, we silenced expression of IRE1, PERK, or ATF6 using lentivirus-delivered shRNA constructs in UPR-reporter cell lines. We previously generated and characterized these SH-SY5Y derived cell lines that stably express fluorescent reporters for XBP1 splicing, translation of ATF4, or activation of ATF6 (19, 20). The expression of the fluorescent reporters over time in response to ER stress was monitored using high content live cell imaging.

Efficient silencing of ATF6, IRE1, or PERK was confirmed by high-content imaging of ATF6-, IRE1-, or PERK-reporter activity, respectively (Fig. 1, C and D, and Fig. S1, B, C, E, and F) (19). Additionally, Western blottings and qPCR were performed to show reduced levels of ATF6 (Fig. 1, A, and B), IRE1, and PERK (Fig. S1, A and D) (19).

In the IRE1-reporter cells, IRE1 endonuclease-mediated splicing of a 26-bp intron from Xbp1–Yfp mRNA in response to ER stress leads to the expression of a spliced XBP1–YFP fusion protein (19). We found that suppressing ATF6 expression in these cells resulted in significantly increased levels of YFP-positive cells compared with control cells after treatment with thapsigargin (Tg) (Fig. 2, B and C, and Fig. S2A). Silencing of PERK expression in IRE1-reporter cells decreased the levels of XBP1 splicing. The percentage of YFP-positive cells transduced with shRNA against PERK was similar to control cells initially but decreased compared with the control cells from 20 h after ER stress (Fig. 2E and Fig. S2B).

Next, we investigated the effect of silencing ATF6 and IRE1 in the PERK-reporter cells. PERK-reporter cells express three regulatory upstream open reading frames of ATF4 in-frame with YFP from which translation is initiated under ER stress conditions through activation of PERK and subsequent phosphorylation of eIF2α. When monitoring YFP fluorescence levels in response to ER stress in the PERK-reporter cells, we found there was no difference between cells transduced with shRNA...
against ATF6 or IRE1 and scrambled control (Fig. 2, H, I, K, and L, and Fig. S2, C and D).

Finally, we silenced PERK or IRE1 expression in the ATF6-reporter cell line, which stably expressed the ATF6-binding sequence from which transcription of YFP is induced under ER stress conditions (20, 21). Although there was no difference in YFP fluorescence levels between cells transduced with shRNA against PERK and control, we found that suppression of IRE1 expression resulted in lower YFP fluorescence in the ATF6-reporter cells (Fig. 2, N, O, R, and Q, and Fig. S2, E and F).

**ATF6 silencing results in increased XBP1 splicing in IRE1-reporter cells**

Because the above experiments indicated that ATF6 was impacting IRE1-reporter activity, we further investigated the interaction between ATF6 and IRE1. As a first step, we checked whether increased splicing of the IRE1-reporter was observed in ATF6-silenced cells treated with other ER stress–inducing agents.

We therefore transduced IRE1-reporter cells with shRNA against ATF6 and treated with tunicamycin (Tm) or brefeldin A (BFA). YFP fluorescence and cell death were monitored using high-content live cell imaging (Fig. 3). The pattern of increase in YFP-positive cells observed over time varied in response to the different ER stress–inducing drugs (Fig. 3, A–D). However, for each treatment we observed a significantly increased amount of XBP1–YFP expression in cells silenced for ATF6 compared with control cells 15 h after stimulus (Fig. 3, B and D). Furthermore, although the initial increase in YFP-positive cells in response to Tm and BFA was similar in ATF6-silenced and control cells, the amount of YFP-positive cells transduced with scrambled construct would eventually plateau and finally decrease, whereas the YFP fluorescence signal in ATF6-silenced cells continued to increase uncontrolled.

We next compared cell survival between ATF6-silenced and control cells. Cell death in the IRE1-reporter cells was monitored as percentage of propidium iodide (PI) positively-stained cells over time. Cell death increased from low initial levels after 15 h in BFA-treated cells and after 24 h in Tm- and Tg-treated cells (Fig. 3, E–J). However, there was no difference in the amount or onset of cell death between cells transduced with the ATF6-KD construct or scrambled control for either treatment.

Additionally, we investigated cell survival in SH-SY5Y parental cells transduced with shRNA against ATF6. The onset and increase in cell death were similar to the pattern seen in IRE1-reporter cells for Tm and Tg treatments, and we again found no significant difference in the amount of PI-positive cells in response to Tm or Tg between cells silenced for ATF6 or control cells (Fig. S3, A–D).

To investigate whether there were differences in early apoptosis events between ATF6-silenced cells and control cells, we were treated with Tm and harvested at different time points. Caspase-3 and cleaved caspase-3 levels were assessed using Western blotting. We could detect a decrease of caspase-3 from 24 h. The amount of cleaved caspase-3 19- and 17-kDa fragments at 40 h was slightly higher in ATF6-KD and scrambled control cells (Fig. S3E).

**ATF6 silencing results in increased XBP1 splicing and increased IRE1 levels**

To explore the underlying mechanism for the increase in XBP1 splicing in the IRE1-reporter cells, we silenced ATF6 in parental SH-SY5Y cells and analyzed XBP1 and IRE1 protein levels 4, 8, 16, and 40 h after treatment with Tm by Western blotting (Fig. 4, A and C). Levels of spliced XBP1 were increased in cells transduced with the ATF6-KD construct compared with control cells. Congruently, protein levels of IRE1 and phosphorylated IRE1 were increased in ATF6-silenced cells compared with control cells at 16 and 40 h suggesting higher amounts of activated IRE1 in these cells (Fig. 4, C and F). We did not see an increase in PERK protein levels in the ATF6-silenced cells compared with scram control when treated with Tm for the same times (Fig. S4A). Moreover, there was not a strong difference in eIF2α phosphorylation levels between ATF6-silenced and control cells (Fig. S4A). Analyzing IRE1 protein levels over time, we found that IRE1 increased in response to ER stress (Fig. 4E). ATF6-silenced cells had a higher increase in IRE1 levels compared with control cells, as well as higher amounts of phosphorylated IRE1 and spliced XBP1.

We also assessed IRE1 mRNA levels and found them to increase over time in response to ER stress. The increase was higher in ATF6-silenced cells compared with control at 16 and 24 h (Fig. 4B). Similarly, we observed an increase in spliced Xbp1 mRNA that was significantly higher in ATF6-silenced cells compared with scram control 16 and 24 h after treatment (Fig. 4D). Of note, levels of PERK mRNA only showed a small increase over time after exposure to ER stress (Fig. S4B).

We went on to assess IRE1 transcription levels in ATF6-silenced SH-SY5Y cells compared with XBP1- and ATF4-KD cells. Loss of expression in these cells was confirmed by Western blotting and quantitative real-time PCR (Fig. 4, H–J). In response to ER stress, IRE1 mRNA levels were significantly increased in ATF6-silenced cells but not in XBP1-silenced cells or control cells (Fig. 4G).

**Increased IRE1 levels are a general response to ER stress**

As we observed increased IRE1 protein levels in cells transduced with scrambled control shRNA in response to ER stress (Fig. 4, A and C), we wanted to investigate whether an increase in IRE1 levels was a general response to ER stress. We treated different cell lines with Tm or Tg and monitored IRE1 protein and mRNA levels over time. We found increased IRE1 protein

---

**Figure 2.** Employing high-content live cell imaging to interrogate cross-talk between the UPR-signaling branches, IRE1-, PERK-, or ATF6-reporter cells were transduced with shRNA against ATF6, IRE1, PERK, or scrambled control vector. 96 h after transduction, the cells were stained with Hoechst and PI and treated with 1 μM Tg. Images were taken at 1-h intervals starting immediately after treatment for 48 h using high-content time-lapse live cell imaging. **Left column:** A, D, G, J, M, and P, schematic indicating reporter cell line and silencing construct used. **Middle column:** B and E, percentage of YFP-positive cells, or H, K, N, and R, mean YFP intensity over time in response to 1 μM Tg or 0.1% DMSO in reporter cells transduced with silencing construct or scrambled control group was plotted. Error bars indicate S.E. of at least n = 2 wells of a representative experiment. **Right column:** C and F, mean percentage of YFP-positive cells, or I, L, Q, and O, mean YFP intensity 24 h after treatment with 1 μM Tg or 0.1% DMSO control. Error bars indicate S.E. of n = 3 independent experiments. Student’s t tests were performed comparing KD and scrambled groups. * indicates p < 0.05. a.u., arbitrary units.
ATF6-dependent off-switch

YFP fluorescence of IRE1-reporter cells

A) Tunicamycin

B) 15 h

C) Brefeldin A

D) 15 h

cell death

E) Tunicamycin

F) 45 h

G) Brefeldin A

H) 45 h

I) Thapsigargin

J) 45 h
levels after treatment with Tm or Tg in nontransformed SH-SY5Y cells (Fig. 5, A and B), as well as in HEK293TN and SH-EP cells (Fig. S5, A and B).

Accumulation of IRE1 protein during ER stress was recently reported to be caused by attenuation of BiP-dependent degradation of IRE1 (22). However, we observed that mRNA levels of IRE1 were elevated in response to Tm or Tg in SH-SY5Y as well as in HEK293TN cells (Fig. 5, C and D, and Fig. S5, C and D), suggesting that transcription was contributing to the accumulation of IRE1 under ER stress. We further tested this by inhibiting translation using cycloheximide (CHX) in Tg-treated SH-SY5Y cells. Although there was some increase in IRE1 protein levels in response to Tg in the CHX-treated cells, it was markedly lower compared with the increase in cells just treated with Tg (Fig. 5E). GRP78 protein levels remained stable over time in the Tg- and CHX-treated cells, confirming effective inhibition of translation.

**Increase in IRE1 levels depends on IRE1 phosphorylation and JNK-dependent signaling**

As our data indicated that increases in IRE1 levels under ER stress were due to transcriptional up-regulation, we next wanted to analyze closer the regulation of IRE1 transcription. ER stress is known to be coupled to JNK activation through JNK-dependent signaling (17). Recently, the transcription factor JunB was shown to interact with AP1-binding sites in the IRE1 promoter and to be involved in regulation of IRE1 expression during osteoblast differentiation (23). We thus hypothesized that IRE1 self-regulates its expression under ER stress through the activity of its kinase domain and subsequent activation of JNK and JNK-dependent transcription.

To test this hypothesis, we pre-treated SH-SY5Y cells with the JNK inhibitor SP600125 (24) or the IRE1 kinase inhibitor APY29 (25) before inducing ER stress, and we analyzed whether IRE1 levels were affected (Fig. 6, A and B). In cells treated with JNK or IRE1 kinase inhibitor APY29 and Tg, IRE1 protein levels were lower compared with cells treated with Tg, demonstrating that ER stress-dependent induction of IRE1 expression was impaired in these cells. There was no IRE1 phosphorylation detectable in APY29-treated cells, indicating effective inhibition of IRE1 kinase. We also observed lower levels of phosphorylated IRE1 in SP600125-treated cells, which could be due to lower overall IRE1 levels, reduced IRE1 clustering and autophosphorylation, and reduced downstream IRE1 activation. Deficient IRE1 activation was further illustrated by low levels of spliced XBP1 in the SP600125- and APY29-treated cells. c-JUN phosphorylation was reduced in SP600125- and APY29-treated cells compared with Tg only–treated cells, indicating inhibition of JNK and suggesting that IRE1 kinase-dependent phosphorylation was an upstream event. Additionally, we used a second IRE1 kinase inhibitor, KIRA6 (26). We found little increase in IRE1 levels in cells treated with Tg and KIRA6 (Fig. S6A).

We also monitored IRE1 levels in response to ER stress in cells with or without the JNK inhibitor or IRE1 kinase inhibitor KIRA6 over time. In the cells treated with Tm and SP600125, IRE1 protein levels only increased slightly compared with Tm only–treated cells (Fig. 6C). Similarly, we did not see an increase in IRE1 levels in Tm- and KIRA6-treated cells (Fig. S6B). To exclude that the increase in IRE1 expression depended on XBP1 splicing, we treated cells with the IRE1–endonuclease inhibitor 4μC (27) before inducing ER stress with Tm (Fig. 6C). There was no difference in IRE1 protein levels, which increased over time in both groups. Spliced XBP1, however, was only detectable in Tm-treated but not in Tm- and 4μC-treated cells.

Additionally, we measured IRE1 activity in IRE1-reporter cells continuously for 48 h employing high-content time-lapse imaging. Cells were pre-treated with APY29 and 4μC, and subsequently challenged with Tm or Tg (Fig. 6, D–G). The increase in fluorescent cells in response to Tm or Tg was slower, and levels remained lower in response to Tm or Tg in cells pre-treated with APY29, suggesting lower XBP1 splicing. There was no splicing of fluorescent XBP1–YFP reporter detectable in cells pre-treated with 4μC. Taken together, our results suggest that the observed increase in IRE1 protein levels depends on IRE1 phosphorylation and activation of JNK and JNK-dependent signaling.

**Expression of transcriptionally active ATF6 rescues IRE1 levels**

As the increase in IRE1 protein levels was a common feature during ER stress and appeared exacerbated in cells silenced for ATF6, we wanted to further characterize the role of ATF6 in ER stress-dependent expression of IRE1.

To investigate whether overexpression of the transcriptionally active, cytoplasmic domain of ATF6 had the inverse effect of decreasing IRE1-dependent splicing in IRE1-reporter cells, we cloned the N-terminal 373 amino acids encoding the 5′
ATF6-dependent off-switch

A) XBP1s and IRE1 protein levels

B) IRE1 mRNA levels in over time

C) IRE1 mRNA levels in over time

D) Spliced XBP1 mRNA levels in over time

E) IRE1 protein levels

G) IRE1 mRNA levels in different kds

H) XBP1-kd

I) ATF4-kd

J) IRE1- and XBP1-kd

---

18276 J. Biol. Chem. (2018) 293(47) 18270–18284
Figure 5. IRE1 levels increase in response to ER stress. SH-SYSY cells were treated with 3 μM Tm or Tg for times indicated. Increase in IRE1 protein levels in response to Tm (A) or Tg (B) were analyzed by Western blotting using antibodies against IRE1 and spliced XBP1. β-Actin served as loading control. Experiment was repeated with similar results. Real-time qPCR analysis of IRE1-mRNA in SH-SYSY cells treated with 3 μM Tm (C) or 3 μM Tg (D) for times indicated. Results were normalized to β-actin levels and expressed relative to 0 h control cells (mean of n = 3, and error bars indicate S.E). Experiments were repeated with similar results. E, IRE1 expression levels were analyzed in SH-SYSY cells treated with 3 μM Tg and with or without 1 μg/ml cycloheximide. Protein levels were analyzed by Western blotting using antibodies against IRE1 and KDEL antibody against GRP78 and GRP94. β-Actin served as loading control. Experiment was repeated with similar results.

Figure 6. ATF6 silencing results in increased XBP1 splicing and IRE1 levels. SH-SYSY cells were transduced with vectors encoding shRNA against ATF6 or scrambled control (scram) followed by treatment with 3 μM Tm or DMSO for 4 and 8 h (A) or 16 and 40 h (C). XBP1s and IRE1 protein levels were analyzed by Western blotting using antibodies against spliced XBP1, IRE1, and IRE1-p (Ser-714). β-Actin served as loading control. (Please note the membrane was further incubated with antibodies against PERK, eIF2α, and eIF2α-P as shown in Fig. S4A.) Real-time qPCR analysis of Ire1-spliced (B) or Xbp1-spliced (D) mRNA levels in cells silenced for ATF6 or scram control treated with 3 μM Tm for the times indicated. Results were normalized to β-actin levels and expressed relative to DMSO-treated scram control cells (mean of n = 3, error bars indicate S.E). Student’s t tests were performed to compare Tm-treated and DMSO control group (* indicates p < 0.05). F, quantification of IRE1 protein levels at 40 h using densitometry. ATF6 levels were normalized to actin (mean of n = 3 independent experiments, error bars indicate S.E.). Real-time qPCR analysis of Ire1 mRNA (G), Xbp1 mRNA (H), and Atf4 mRNA (I) in cells transduced with shRNA against ATF6, XBP1, ATF4, or scram control, treated with 3 μM Tm or DMSO for 24 h. Results were normalized to β-actin levels and expressed relative to respective DMSO-treated cells (mean of n = 3 from independent cultures, error bars indicate S.E.). Student’s t tests were performed to compare Tm-treated and DMSO control group (* indicates p < 0.05) or XBP1-KD or ATF4-KD and scram control cells. (# indicates p < 0.05). J, XBP1s and IRE1 protein levels were analyzed by Western blotting using antibodies against spliced XBP1 and IRE1. β-Actin served as loading control.
ATF6-dependent off-switch

A IRE1-and JNK-kinase inhibitors

0 h - - APV29 Tg SP600 Tg - - - - APV29 Tg SP600 Tg - - 8 h - - APV29 Tg SP600 Tg - - - - APV29 Tg SP600 Tg - -

IRE1-P 130 kDa
IRE1 130 kDa
XBP1s 72 kDa
cJUN-P 55 kDa
JNK 36 kDa
ACTIN 36 kDa

B JNK-kinase inhibitor

0 h 4 h 8 h 16 h 24 h 0 h 4 h 8 h 16 h 24 h

IRE1-P 130 kDa
IRE1 130 kDa
XBP1s 72 kDa
JNK 36 kDa
ACTIN 36 kDa

C IRE1-endonuclease inhibitor

0 h 4 h 8 h 16 h 24 h 0 h 4 h 8 h 16 h 24 h

IRE1-P 130 kDa
IRE1 130 kDa
XBP1s 72 kDa
JNK 36 kDa
ACTIN 36 kDa

D IRE1-kinase and -endonuclease Inhibitors in IRE1 reporter cells

Tunicamycin

E

F Thapsigargin

G
tor, indicating lower levels of XBP1 splicing in the ATF6(1–373)-expressing cells (Fig. 7, A and B). A similar effect was observed in response to Tg (Fig. 7, C and D).

Additionally, we tested the effect of overexpression of ATF6(1–373) in PERK-reporter cells where an increase in fluorescence intensity indicates PERK activation and translation of ATF4. We found that the fluorescence intensity in response to Tm was higher in cells transduced with the ATF6(1–373)-expression construct compared with control vector. However, the difference was not significant (Fig. S7, A and B). When treated with Tg, there was little or no difference in fluorescence intensity between cells overexpressing the N-terminal ATF6(1–373) and control (Fig. S7, C and D). Next, we investigated whether expression of transcriptionally active ATF6(1–373) was sufficient to reinstate normal IRE1 levels in cells following loss of ATF6 expression. Employing the shRNA construct against ATF6, we generated a stable cell line silenced for ATF6 and a respective scrambled control cell line. These cells were transduced with ATF6(1–373)-expression construct or control, and mRNA levels of ATF6 were analyzed through quantitative real-time PCR. The primer pairs used in the reaction were designed to anneal in the 5’ region of ATF6, which encodes the N-terminal cytosolic domain or in the 3’ region coding for the C-terminal endoplasmic domain of ATF6, respectively.

The effective silencing of ATF6 in the stable cell line was confirmed with the 3’ primer pair. mRNA levels of ATF6 were significantly lower than in the scrambled control cell line, independent of transduction with the ATF6(1–373) construct (Fig. 7F). Real-time PCR using the 5’ primer pair yielded significantly lower levels of ATF6 mRNA in the ATF6-silenced cells transduced with the control construct, but not in the ATF6(1–373)-transduced ATF6-silenced cells. Additionally, ATF6 levels were higher in the scrambled cells transduced with the ATF6(1–373) construct compared with control vector-transduced cells, indicating effective expression of cytosolic ATF6 in these cells (Fig. 7G).

We finally analyzed IRE1 levels in these cells following treatment with Tm. IRE1 mRNA levels and protein levels in the ATF6-silenced cells transduced with empty control vector were increased compared with scrambled cells. In contrast, IRE1 levels in ATF6-silenced cells transduced with the ATF6(1–373) construct were similar to levels in the scrambled cells (Fig. 7H and I). These experiments provided evidence that the transcriptionally active cytosolic domain of ATF6 was sufficient to suppress the increase of IRE1 levels in response to ER stress, suggesting a role for ATF6 in the down-regulation of IRE1 transcription under ER stress.

**Figure 6. Increase in IRE1 levels in response to ER stress depends on IRE1 and c-JUN phosphorylation.** A, SH-SY5Y cells were pre-treated with 1 µM IRE1 kinase inhibitor APY29, 30 µM JNK inhibitor SP600125 for 1 h, followed by treatment with 3 µM Tg for 3 or 8 h. Protein levels were analyzed by Western blotting using antibodies against IRE1, and IRE1-p (Ser-714), spliced XBP1, c-JUN-p (Ser-63) and JNK, β-Actin served as loading control. SH-SY5Y cells were treated with 20 µM SP600125 (B) or 50 µM IRE1-endonuclease inhibitor 4µB (C) and/or 3 µM Tm for times indicated. Protein levels were analyzed by Western blotting using antibodies against IRE1, and IRE1-p (Ser-714), and spliced XBP1. β-Actin served as loading control. Experiments were repeated with similar results. D–G, IRE1-reporter cells were pre-treated with 1 µM APY29 or 50 µM 4µB for 1 h, followed by treatment with Tm or Tg and staining with Hoechst and PI. Cells were incubated on stage, and images were taken at 1-h intervals for 48 h using high-content time-lapse live cell imaging. The percentage of YFP-positive cells plotted over time in response to 1 µM APY29 or 50 µM 4µB pre-treatment and 0.3 µM Tm (D) or 1 µM Tg (F) and 0.1% DMSO. Error bars indicate S.E. of n = 4 wells. The mean percentage of YFP-positive cells 8 h after treatment with Tm (E) or Tg (G) is shown. Error bars indicate S.E. of n = 3 independent experiments. Student’s t tests were performed comparing Tm and DMSO treated for each pre-treatment (* indicates p < 0.05) and comparing APY29 or 4µB pretreatment to control group (# indicates p < 0.05).

**Discussion**

Here, we set up a high-content screening platform using fluorescent-reporter cell lines in conjunction with shRNA-mediated silencing of UPR pathway components to interrogate cross-talk between the UPR-signaling pathways during ER stress. We found that ATF6 silencing resulted in increased XBP1 splicing, which was due to an increase in IRE1 mRNA and protein levels. Further investigation showed that the increase in IRE1 levels appeared to be a general response to ER stress, which depended on IRE1 kinase activity and subsequent JNK-dependent signaling. Our experiments also showed that overexpression of transcriptionally active ATF6 restored IRE1 levels in ATF6-silenced cells suggesting that ATF6 plays a role in the regulation of IRE1 activity.

To gain a better understanding of the role of ATF6 during the unfolded protein response, we started with a systematic analysis of individual UPR components using live cell imaging, which had the distinct advantage of allowing us to monitor ER stress responses over long periods of time, imaging early and late events. This approach uncovered a new interaction between the ATF6- and the IRE1-signaling pathway. We found that silencing of IRE1 expression in ATF6-reporter cells resulted in lower YFP fluorescence. The ATF6-reporter cell lines stably express the 5xATF6–YFP reporter plasmid, also termed UPRE reporter in the literature, which contains ATF6-binding sites first identified by Wang et al. (21). However, we and others have found that spliced XBP1s can also induce transcription from the ATF6-binding sequence (7, 8, 12). Therefore, loss of IRE1 expression and downstream loss of spliced XBP1 resulted in lower induction of YFP fluorescence in the ATF6-reporter cells. Congruently, the remaining activation of the ATF6-reporter construct observed in the ATF6-silenced cells is likely due to a redundancy in binding and transcription initiation by spliced XBP1 (Fig. 1, C and D).

More interestingly, we also observed that silencing of ATF6 in IRE1-reporter cells robustly resulted in increased splicing of the Xbp1–Yfp mRNA. Similarly, we found that parental SH-SY5Y cells silenced for ATF6 had higher amounts of endogenous spliced XBP1 in response to ER stress as well. This was surprising as ATF6 is known to induce transcription of XBP1 (8, 28). In cells with limited ATF6 expression, we therefore expected less XBP1 splicing due to less Xbp1 transcription.

We found that in control cells the amount of spliced XBP1 plateaued or decreased after 20–24 h, whereas in ATF6-silenced cells, XBP1 splicing continued unchecked. IRE1-splicing activity has been previously shown to be down-regulated despite continuous ER stress and independent of Xbp1 tran-
ATF6-dependent off-switch

ATF6-overexpression in IRE1-reporter cells

A Tunicamycin

B Thapsigargin

ATF6-overexpression in ATF6 silenced cells

E

F

G

H

I

ATF6 mRNA levels

IRE1 mRNA levels

IRE1 protein levels
Activation of IRE1 is linked to formation of oligomers and trans-autophosphorylation of its cytosolic domains, which in turn activates the endonuclease domain (9, 10). Bax-inhibitor-1 (BI-1) was reported to have a role in the inhibition of IRE1 signaling. IRE1 inactivation was found to be delayed in BI-1 knockout mouse embryo fibroblasts (29). Our data suggest that ATF6 is necessary for this shutdown process by controlling IRE1 transcript levels as we found IRE1 transcription to be increased in ATF6-silenced cells. We observed that cells with limited ATF6 expression had higher Ire1–mRNA and protein levels and also higher levels of phosphorylated IRE1 in response to ER stress, but only at later time points (16 and 40 h).

An increase in IRE1 protein levels under ER stress conditions was identified as a common feature during ER stress occurring across a range of different cell types in our experiments. This is in line with a previous study that showed IRE1 expression to be induced in mouse livers following intraperitoneal injection of tunicamycin (30). Furthermore, accumulation of IRE1 protein was suggested to be caused by decreased degradation under ER stress conditions when BiP dissociated from the luminal domain of IRE1 (22).

Our experiments provided evidence that IRE1 transcription was also directly up-regulated in response to ER stress. We found IRE1 mRNA levels to be greatly exacerbated in cells silenced for ATF6 compared with control cells. But again, this de-regulation did not occur initially but only at the later time points measured (16 and 24 h). Furthermore, we showed that ATF6 is involved in controlling IRE1 transcription as we found that the expression of the transcriptionally active N-terminal fragment of ATF6 was sufficient to restore normal IRE1 levels in ATF6-silenced cells. We therefore identified a new level of regulation of IRE1 levels during ER stress (Fig. 8).

Very little is known about regulation of IRE1 transcription. Our experiments provide evidence that IRE1 up-regulates its own transcription through activation of the JNK signaling cascade. Of note, the IRE1 promoter contains AP1-binding sites that could provide a site for ATF6 interaction to curb IRE1 transcription (23). Alternatively, ATF6 might control Ire1 levels indirectly through its transcriptional target BiP. BiP was shown to target IRE1 for ERAD degradation under basal conditions. As BiP dissociates from IRE1 in response to increased levels of unfolded proteins, IRE1 can accumulate (22).

Furthermore, ATF6 is known to induce BiP transcription (7). Lower levels of BiP expression could thus result in accumulation of IRE1 protein as less protein can be targeted for degradation.

Oligomerization is a necessary step for the activation of the IRE1 kinase domain, autophosphorylation, and subsequent activation of the endonuclease domain (9). Prolonged ER stress was proposed to lead to increased phosphorylation of the IRE1 kinase, which in turn hyperactivates the endonuclease, causing its substrate specificity to relax and its outputs to become pro-apoptotic (10, 26). We observed higher amounts of phosphorylated IRE1 in ATF6-silenced cells compared with control cells, which could be associated with higher overall amounts of

Figure 7. Expression of transcriptionally active ATF6 rescues IRE1 levels. IRE1-reporter cells were transduced with vector expressing HA-ATF6(1–373) or empty control vector. 96 h after transduction, the cells were stained with Hoechst and PI and treated with Tm or Tg. Images were taken at 1-h intervals for 48 h using high-content time-lapse live cell imaging. The percentage of YFP-positive cells over time in response to 0.3 μM Tm (A) or 0.3 μM Tg (C) and 0.1% DMSO in ATF6(1–373) and control group was plotted. Error bars indicate S.E. of n = 6 wells. The mean percentage of YFP-positive cells 15 h after treatment with 0.1, 0.3, or 1 μM Tm (B) or Tg and 0.1% DMSO control (D). Error bars indicate S.E. of n = 3 independent experiments. Student’s t tests were performed comparing ATF6-oe and control groups for each treatment. * indicates p < 0.05. E, IRE1-reporter cells were transduced with vector expressing HA-ATF6(1–373) or empty control vector. 72 h after transduction, cells were harvested, and HA-ATF6 levels were analyzed by Western blotting using antibody against the HA-tag. β-Actin served as loading control. F, SH-SYSY cells stably transfected with shRNA against ATF6 or scram control were transduced with vector expressing HA-ATF6(1–373) or empty control vector. 72 h after transduction, cells were harvested, and HA-ATF6 levels were analyzed by Western blotting using antibody against the HA-tag. β-Actin served as loading control. G, SH-SYSY cells stably transfected with shRNA against ATF6 or scram control were transduced with vector expressing HA-ATF6(1–373) or empty control vector. IRE1 mRNA levels were analyzed by real-time qPCR using primer annealing in the 3’ region of endogenous Atf6 and HA-Atf6(1–373). Results were normalized to β-actin levels and expressed relative to scrm control cells transfected with empty vector; mean of n = 3, error bars indicate S.E. Student’s t tests were performed comparing all groups to scram control cells transfected with empty vector control; * indicates p < 0.05. Additonally ATF6-KD cells transfected with HA-ATF6(1–373) were compared with ATF6-KD cells expressing empty vector; # indicates p < 0.05. I, Western blot analysis of IRE1 protein levels in SH-SYSY cells stably transfected with shRNA against ATF6 or scrm control and transfused with vector expressing HA-ATF6(1–373) or empty control vector followed by treatment with 3 μM Tm or DMSO for 24 h. Results were normalized to β-actin levels and expressed relative to scrm control cells transfected with empty vector (mean of n = 3, error bars indicate S.E.). J, Western blot analysis of IRE1 protein levels in SH-SYSY cells stably transfected with shRNA against ATF6 or scrm control and transfused with vector expressing HA-ATF6(1–373) or empty control vector followed by treatment with 3 μM Tm or DMSO for 40 h. Antibodies against IRE1 and HA were used. β-Actin served as loading control.
ATF6-dependent off-switch

IRE1 enabling more clustering of the proteins within the ER membrane.

It is important to note that we did not identify ATF6-silenced SH-SY5Y cells to be more sensitive to ER stress–induced cell death. This is in line with our previously published experiments in single cells where we found that a reduction in IRE1 endonuclease activity occurs in both dying and resistant cells and thus is not decisive for cell outcome (19). Similarly, our current high-content imaging experiments indicate that the increase in IRE1 activity caused by silencing of ATF6 is not a predictor for cell fate. This also aligns with previous observations in ATF6–/– cells that demonstrated a role for ATF6 signaling in the long-term adaptation to chronic stress rather than being decisive for cell death or survival (3, 4).

Taken together, our experiments show that ATF6 interacts with the Ire1-signaling arm of the UPR not only as previously known by regulating Xbp1 transcription but also by regulating Ire1 levels. ATF6 appears to be necessary to accomplish Ire1 deactivation during prolonged ER stress. As ATF6 and Ire1 are both activated upon the accumulation of unfolded protein in the ER, our experiments demonstrate that the Ire1-signaling arm of the UPR contains an integrated off-switch.

Experimental procedures

Cell culture and treatments

SH-SY5Y neuroblastoma cell lines were grown in DMEM/F-12 (Sigma, Wicklow, Ireland) supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin/streptomycin. The SH-SY5Y–derived Ire1-reporter and Perk-reporter cell lines were generated as described previously (19). SH-SY5Y cell lines stably expressing pLVX-HA-ATF6(1–373) or pLVX-control vector were selected in medium containing 5 μg/ml puromycin (Sigma) for up to 2 weeks. HEK293TN cells were grown in DMEM (5 g/liter glucose) (Lonza) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin. SH-EP cells were cultivated in RPMI 1640 medium (Sigma) supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin/streptomycin. Cells were treated with tunicamycin (Sigma), thapsigargin (Sigma), brefeldin A, SP600125 (Sigma), APY29 (Tocris Bioscience, Bristol, UK), 4μ8C (Sigma), KIRA6 (Merck Millipore, Cork, Ireland), and cycloheximide (Sigma) at indicated concentrations.

Plasmid construction

The pCGN-ATF6(1–373) was a gift from Ron Prywes (Addgene plasmid no. 27173) (Addgene, Cambridge, MA). For generation of viral particles, HA-ATF6(1–373) was amplified using forward primer 5′-ATCGCCCTAGGATGCTCTAGCTA-3′ and reverse primer 5′-TACGGATCTACTGGGACTTTGAAGC-3′ and cloned into vector pLVX using the XhoI and BamHI restriction sites.

shRNA and lentivirus production/transduction

MISSION® shRNA vectors for gene silencing of ATF6 (clone number TRCN0000017855), Ire1 (TRCN0000000530), Perk (TRCN0000001399), Xbp1 (TRCN00000019804), and ATF4 (TRCN0000013573) in mammalian cells and scrambled control containing plasmids were obtained from Sigma. Lentiviral particles were produced by co-transfecting HEK293TN cells with viral envelope protein encoding vector pMD2.G (Addgene) and packaging protein-coding vector psPAX2 (Addgene) together with the MISSION® shRNA containing pLKO.1-puro or pLVX-HA-ATF6(1–373) vector. For transduction with viral particles, SH-SY5Y cells were seeded at a density of 3 × 10^4 cells/well in a 12-well plate and incubated with 250 μl of virus suspension in 1 ml of medium and 5 μg/ml Polybrene per well. The cells were centrifuged at 1000 × g at 20 °C for 90 min. Medium was changed after 24 h. Transduced cells were cultivated for 72 h before being moved to imaging plates or 96 h before being subjected to treatment or selection.

Western blotting

Cells were lysed, and Western blotting was performed as described previously (31). Blots were probed with the following primary antibodies: rabbit polyclonal anti-IRE1α (1:1000) (catalog no. 3294; Cell Signaling Technology, Inc., Danvers, MA); rabbit monoclonal anti-IRE1-p (Ser-724) (1:500) (catalog no. 124945; Abcam, Cambridge, UK); mouse monoclonal anti-XBP1 (1:500) (catalog no. 647501; BioLegend, San Diego, CA); mouse monoclonal anti-KDEL (10C3) (1:500) (catalog no. ADI-SPA-827; Enzo Life Sciences, Exeter, UK); rabbit polyclonal anti-ATF-6α (H-280) (1:100) (catalog no. sc-22799; Santa Cruz Biotechnology, Inc., Dallas, TX); rabbit polyclonal anti-c-Jun-p (Ser-63) II (1:1000) (catalog no. 9261S; Cell Signaling); rabbit polyclonal anti-JNK2 (56G8) (1:1000) (catalog no. 9258; Cell Signaling); mouse monoclonal anti-HA probe (F-7) (1:200) (catalog no. sc-7392; Santa Cruz Biotechnology); rabbit polyclonal anti-caspase-3 (catalog no. 9662S; Cell Signaling); rabbit polyclonal anti-cleaved caspase-3 (catalog no. 9661S; Cell Signaling); rabbit polyclonal anti-Perk (catalog no. 3192S; Cell Signaling); rabbit polyclonal anti-Elf2α (catalog no. 9722S; Cell Signaling); rabbit polyclonal anti-P-Elf2α (catalog no. 9721S; Cell Signaling); and mouse monoclonal anti-β-actin (1:10,000) (catalog no. A5441; Sigma). Secondary antibodies conjugated to horseradish peroxidase (ThermoFisher Scientific, Dublin, Ireland) were detected using Immobilon chemiluminescent horseradish peroxidase substrate (Millipore, Cork, Ireland). Blots were imaged using LAS-4000 imaging system (Fujifilm, Sheffield, UK).

Real-time qPCR

RNA was extracted using RNeasy mini kit (Qiagen). cDNA was generated from 500 ng of total RNA using random hexamer primers (ThermoFisher Scientific) and Superscript III reverse transcriptase (Invitrogen). Real-time quantitative PCR was carried out using SYBR Green PCR Master Mix. The following primer pairs were used: ATF6 (5′-end primer), 5′-ACCTCAGCCACTTCTCATCACTAGATCTAGCTA-3′ and reverse primer 5′-TACGGATCTACTGGGACTTTGAAGC-3′ and cloned into vector pLVX using the XhoI and BamHI restriction sites.

shRNA and lentivirus production/transduction

MISSION® shRNA vectors for gene silencing of ATF6 (clone number TRCN00000017855), Ire1 (TRCN0000000530), Perk (TRCN0000001399), Xbp1 (TRCN00000019804), and ATF4 (TRCN0000013573) in mammalian cells and scrambled control containing plasmids were obtained from Sigma. Lentiviral particles were produced by co-transfecting HEK293TN cells with viral envelope protein encoding vector pMD2.G (Addgene) and packaging protein-coding vector psPAX2 (Addgene) together with the MISSION® shRNA containing pLKO.1-puro or pLVX-HA-ATF6(1–373) vector. For transduction with viral particles, SH-SY5Y cells were seeded at a density of 3 × 10^4 cells/well in a 12-well plate and incubated with 250 μl of virus suspension in 1 ml of medium and 5 μg/ml Polybrene per well. The cells were centrifuged at 1000 × g at 20 °C for 90 min. Medium was changed after 24 h. Transduced cells were cultivated for 72 h before being moved to imaging plates or 96 h before being subjected to treatment or selection.
CACTCTGTATGT-3’ and 5’-CAATGGTCAGCCCATCA-GTCT-3’; Grp78, 5’-CTGGCAAGTGAAGCTCC-3’ and 5’-GGGTTCAAGGGCATAGGA-3’; ATF4, 5’-ACAGCA-AGGAGGATCCTTCT-3’ and 5’-GGCTGTTAATGTC-TCTGAGC-3’; PERK, 5’-GGAAGGAGACCGGATT-ATT-3’ and 5’-ACTATGTCATTATGGCAGCTT-3’; and actin, 5’-TCAACCACTGTGCCCCATCTAGA-3’ and 5’-CAGCGGAAAGCTTCATGAGGTG-3’. Reactions were performed on the LightCycler (Roche Diagnostics). The data were analyzed using the LightCycler Software 4.0. All samples were normalized to β-actin and expressed relative to untreated control.

**High-content time-lapse imaging and data analysis**

SH-SY5Y- or UPR-reporter cells were cultivated for 24 h in Nunc® microwell 96-well optical bottom plates. Prior to imaging, cells were stained with 100 ng/ml Hoechst 33582 and 2 μg/ml propidium iodide and treated with tunicamycin, thapsigargin, or the same volume of DMSO. The plate was incubated on stage at 37 °C, 5% CO2. Nine fields of view per well were imaged at 1-h increments for 48 h using a Cellomics ArrayScan VTi instrument (ThermoFisher Scientific) equipped with a ×10 PlanApo objective lens (NA 0.45), a 120-watt Hg arc illumination source (EXFO), and a monochrome CCD camera (Orca AG, Hamamatsu, Japan). The following filters sets were used: Hoechst excitation 387 ± 11 nm, quad band emission filter 440/521/607/700; PI excitation 504 ± 12 nm and emission 645 ± 75 nm; YFP excitation 504 ± 12 nm and emission 542 ± 27 nm (Chroma and Semrock, AHF, Germany). A 25% ND filter was used in the excitation light path of all channels. Images were taken of nine fields of view per well in 1-h intervals for 48 h, with 1024 × 1024 pixel resolution (6.45-μm pixel size). CellProfiler 2.0 cell image analysis software (32) was used to identify individual objects and measure their fluorescence intensity. The mean intensity per object of all objects identified in the Hoechst channel was measured in the input PI images and the cross-talk corrected YFP images. A purpose-built Matlab script (Matlab; MathWorks, Inc., Cambridge, UK) was used to calculate and plot the fraction of dying cells and the mean of the mean YFP fluorescence intensity values of all cells per treatment group and time point as well as standard errors of these values.

**Author contributions**—F. W., C. G. C., and J. H. P. conceptualization; F. W. and H. D. data curation; F. W. and C. G. C. formal analysis; F. W. and J. H. P. supervision; F. W. validation; F. W. investigation; F. W. visualization; F. W. and A. O. methodology; F. W. and J. H. P. writing-original draft; H. D. software; J. H. P. funding acquisition; A. O. experiment preparation for high-content imaging.

**References**

1. Lin, J. H., Li, H., Yasumura, D., Cohen, H. R., Zhang, C., Panning, B., Shokat, K. M., Lavail, M. M., and Walter, P. (2007) IRE1 signaling affects cell fate during the unfolded protein response. *Science* **318**, 944–949

2. Han, J., Back, S. H., Hur, J., Lin, Y. H., Gildersleeve, R., Shan, J., Yuan, C. L., Krokowski, D., Wang, S., Hatzoglou, M., Kilberg, M. S., Sartor, M. A., and Kaufman, R. J. (2013) ER stress-induced transcriptional regulation increases protein synthesis leading to cell death. *Nat. Cell Biol.* **15**, 481–490

3. Wu, J., Rutkowski, D. T., Dubois, M., Swathirajan, J., Saunders, T., Wang, J., Song, B., Yau, G. D., and Kaufman, R. J. (2007) ATF6α optimizes long-lasting endoplasmic reticulum function to protect cells from chronic stress. *Dev. Cell* **13**, 351–364

4. Shoulders, M. D., Ryno, L. M., Genereux, J. C., Moresco, J. J., Tu, P. G., Wu, C., Yates, J. R., 3rd., Su, A. L., Kelly, J. W., and Wiseman, R. L. (2013) Stress-independent activation of XBP1s and/or ATF6 reveals three functionally diverse ER proteostasis environments. *Cell Rep.* **3**, 1279–1329

5. Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P., and Ron, D. (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat. Cell Biol.* **2**, 326–332

6. Ye, J., Rawson, R. B., Komuro, R., Chen, X., Davé, U. P., Prywes, R., Brown, M. S., and Goldstein, J. L. (2000) ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Cell* **66**, 1355–1364

7. Yamamoto, K., Sato, T., Matsu, T., Sato, M., Okada, T., Yoshida, H., Harada, A., and Morii, K. (2007) Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6α and XBP1. *Dev. Cell* **13**, 365–376

8. Lee, K., Tiraposphon, W., Shen, X., Michalak, M., Prywes, R., Okada, T., Yoshida, H., Morii, K., and Kaufman, R. J. (2002) IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 signaling in the unfolded protein response. *Genes Dev.* **16**, 452–466

9. Li, H., Korennykh, A. V., Behrman, S. L., and Walter, P. (2010) Mammalian endoplasmic reticulum stress sensor IRE1 signals by dynamic clustering. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 16113–16118

10. Han, D., Lerner, A. G., Vande Walle, L., Upton, J. P., Xu, W., Hagen, A., Backes, B. J., Oakes, S. A., and Papa, F. R. (2009) IRE1α kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates. *Cell* **138**, 562–575

11. Calfon, M., Zeng, H., Urano, F., Till, J. H., Hubbard, S. R., Harding, H. P., Clark, S. G., and Ron, D. (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* **415**, 92–96

12. Lee, A. H., Iwakoshi, N. N., and Glimcher, L. H. (2003) XBP1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol. Cell. Biol.* **23**, 7448–7459

13. Jonikas, M. C., Collins, S. R., Denic, V., Oh, E., Quan, E. M., Schmid, V., Weibezahn, J., Schwappach, B., Walter, P., Weissman, J. S., and Schuldiner, M. (2009) Comprehensive characterization of genes required for protein folding in the endoplasmic reticulum. *Science* **323**, 1693–1697

14. Hollen, J., and Weissman, J. S. (2006) Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science* **313**, 104–107

15. Hollen, J., Lin, J. H., Li, H., Stevens, N., Walter, P., and Weissman, J. S. (2009) Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. *J. Cell Biol.* **186**, 325–331

16. Tiraposphon, W., Lee, K., Callaghan, B., Welihinda, A., and Kaufman, R. J. (2000) The endoribonuclease activity of mammalian IRE1α autoregulates its mRNA and is required for the unfolded protein response. *Genes Dev.* **14**, 2725–2736

17. Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., and Ron, D. (2000) Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 9280–9285

18. Hetz, C., and Glimcher, L. H. (2009) Fine-tuning of the unfolded protein response: assembling the IRE1 interactome. *Mol. Cell* **35**, 551–561

19. Walter, F., Schmid, J., Düssmann, H., Concannon, C. G., and Prehn, J. H. (2015) Imaging of single cell responses to ER stress indicates that the relative dynamics of IRE1/XBP1 and PERK/ATF4 signalling rather than a switch between signalling branches determine cell survival. *Cell Death Differ.* **22**, 1502–1516
ATF6-dependent off-switch

20. Nolan, K., Walter, F., Tuffy, L. P., Poeschel, S., Gallagher, R., Haunsberger, S., Bray, I., Stallings, R. L., Concannon, C. G., and Prehn, J. H. (2016) Endoplasmic reticulum stress-mediated upregulation of miR-29a enhances sensitivity to neuronal apoptosis. *Eur. J. Neurosci.* 43, 640–652 CrossRef Medline

21. Wang, Y., Shen, J., Arenzana, N., Tirasophon, W., Kaufman, R. J., and Prywes, R. (2000) Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. *J. Biol. Chem.* 275, 27013–27020 Medline

22. Sun, S., Shi, G., Sha, H., Ji, Y., Han, X., Shu, X., Ma, H., Inoue, T., Gao, B., Kim, H., Bu, P., Guber, R. D., Shen, X., Lee, A. H., Iwawaki, T., et al. (2015) IRE1α is an endogenous substrate of endoplasmic-reticulum-associated degradation. *Nat. Cell Biol.* 17, 1546–1555 CrossRef Medline

23. Guo, F. J., Jiang, R., Xiong, Z., Xia, F., Li, M., Chen, L., and Liu, C. J. (2014) IRE1α constitutes a negative feedback loop with BMP2 and acts as a novel mediator in modulating osteogenic differentiation. *Cell Death Dis.* 5, e1239 CrossRef Medline

24. Bennett, B. L., Sasaki, D. T., Murray, B. W., O’Leary, E. C., Sakata, S. T., Xu, W., Leisten, J. C., Motiwala, A., Piero, S., Satoh, Y., Bhagwat, S. S., Manning, A. M., and Anderson, D. W. (2001) SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci. U.S.A.* 98, 13681–13686 CrossRef Medline

25. Wang, L., Perera, B. G., Hari, S. B., Bhattarai, B., Backes, B. J., Seeliger, M. A., Schürer, S. C., Oakes, S. A., Papa, F. R., and Maly, D. J. (2012) Divergent allosteric control of the IRE1α endoribonuclease using kinase inhibitors. *Nat. Chem. Biol.* 8, 982–989 CrossRef Medline

26. Ghosh, R., Wang, L., Wang, E. S., Perera, B. G., Igbaria, A., Morita, S., Prado, K., Thamsen, M., Caswell, D., Macias, H., Weibert, K. F., Gliedt, M. J., Alavi, M. V., Hari, S. B., Mitra, A. K., et al. (2014) Allosteric inhibition of the IRE1α RNase preserves cell viability and function during endoplasmic reticulum stress. *Cell* 158, 534–548 CrossRef Medline

27. Cross, B. C., Bond, P. J., Sadowski, P. G., Jha, B. K., Zak, J., Goodman, J. M., Silverman, R. H., Neubert, T. A., Baxendale, I. R., Ron, D., and Harding, H. P. (2012) The molecular basis for selective inhibition of unconventional mRNA splicing by an IRE1-binding small molecule. *Proc. Natl. Acad. Sci. U.S.A.* 109, E869–E875 CrossRef Medline

28. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 to produce a highly active transcription factor. *Cell* 107, 881–891 CrossRef Medline

29. Lisbona, F., Rojas-Rivera, D., Thielen, P., Zamorano, S., Todd, D., Martinon, F., Glavic, A., Kress, C., Lin, J. H., Walter, P., Reed, J. C., Glimcher, L. H., and Hetz, C. (2009) BAX inhibitor-1 is a negative regulator of the ER stress sensor IRE1α. *Mol. Cell* 33, 679–691 CrossRef Medline

30. Tsuru, A., Imai, Y., Saito, M., and Kohno, K. (2016) Novel mechanism of enhancing IRE1α-XBP1 signalling via the PERK-ATF4 pathway. *Sci. Rep.* 6, 24217 CrossRef Medline

31. Ramapathiran, L., Bernas, T., Walter, F., Williams, L., Düssmann, H., Concannon, C. G., and Prehn, J. H. (2014) Single-cell imaging of the heat-shock response in colon cancer cells suggests that magnitude and length rather than time of onset determines resistance to apoptosis. *J. Cell Sci.* 127, 609–619 CrossRef Medline

32. Carpenter, A. E., Jones, T. R., Lampecht, M. R., Clarke, C., Kang, I. H., Friman, O., Guertin, D. A., Chang, J. H., Lindquist, R. A., Moffat, J., Golland, P., and Sabatini, D. M. (2006) CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* 7, R100 CrossRef Medline