# Endoplasmic reticulum stress–induced degradation of DNAJB12 stimulates BOK accumulation and primes cancer cells for apoptosis

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**DNAJB12 (JB12) is an endoplasmic reticulum (ER)–associated Hsp40 family protein that recruits Hsp70 to the ER surface to coordinate the function of ER-associated and cytosolic chaperone systems in protein quality control. Hsp70 is stress-inducible, but paradoxically, we report here that JB12 was degraded by the proteasome during severe ER stress. Destabilized JB12 was degraded by ER-associated degradation complexes that contained HERP, Sel1L, and gp78. JB12 was the only ER-associated chaperone that was destabilized by reductive stress. JB12 knockdown by siRNA led to the induction of caspase processing but not the unfolded protein response. ER stress-induced apoptosis is regulated by the highly labile and ER-associated BCL-2 family member BOK, which is controlled at the level of protein stability by ER-associated degradation components. We found that JB12 was required in human hepatoma cell line 7 (Huh-7) liver cancer cells to maintain BOK at low levels, and BOK was detected in complexes with JB12 and gp78. Depletion of JB12 during reductive stress or by shRNA from Huh-7 cells was associated with accumulation of BOK and activation of Caspase 3, 7, and 9. The absence of JB12 sensitized Huh-7 to death caused by proteotoxic agents and the proapoptotic chemotherapeutic LCL-161. In summary, JB12 is a stress-sensitive Hsp40 whose degradation during severe ER stress provides a mechanism to promote BOK accumulation and induction of apoptosis.**

Cell viability is dependent upon maintenance of the proteome in a pristine state with defects in protein homeostasis associated with neurodegenerative diseases, cancers, and diabetes (1). Approximately 30% of total protein synthesis occurs on ER[3]-associated ribosomes, and protein biogenesis in the ER is coupled with lipid biosynthesis, the secretory pathway, and energy metabolism (2, 3). Defects in ER homeostasis are sensed in part by the unfolded protein response-signaling pathway that controls rates of protein synthesis and expression of ER-associated molecular chaperones (4). ER transmembrane Hsp40s containing a cytosolic J-domain direct cytosolic Hsp70 to function on the ER surface for action in facilitating the folding and/or degradation of polytopic membrane proteins (5–8).

Hsp40s that attract Hsp70 to the cytosolic face of the ER include HDJ-2, which is farnesylated (6), and ER transmembrane Hsp40s such as DNAJB12 (JB12) (7, 9) and DNAJB14 (JB14) (10). These Hsp40s employ a conserved J-domain to attract Hsp70 to the cytosolic face of the ER and mediate the localized regulation of Hsp70's polypeptide binding and release cycle (11–13). HDJ-2 is implicated in the folding of proteins, whereas JB12 is multifunctional. JB12 is a component of ERQC complexes and triages ERAD-sensitive and -resistant forms of misfolded membrane proteins between pathways for proteasomal degradation or ERQC autophagy (8, 14). JB12 also functions in a pathway with JB14 to facilitate the assembly of homotetrameric $K^+$ channels. Notably JB12 and JB14 appear to facilitate different steps required for conversion of $K^+$ channel monomers to tetramers (15). Likewise, requirements for JB12 in ERQC-autophagy are not shared by JB14 (8). JB12 and JB14 help specify the actions of Hsp70 in ER homeostasis and contain divergent ER luminal DUF1977 (domain of unknown function) domains that could confer the observed specificity of their actions (10).

Interestingly, JB12 and JB14 expression is not induced by the UPR or heat stress (10), and the processes that are facilitated by them in ERQC are dictated in part by the conformation of the clients to which each respective Hsp40 binds (8, 15). The study of JB12 has received more attention than JB14, and JB12 is the primary focus of this study. Depletion of JB12 from mammalian cells did not cause a strong induction of the UPR but reduced cell growth rates and also sensitized cells to death caused by challenge of the ER with misfolded membrane proteins (8). JB12, therefore, appears to participate in an ER stress–signaling circuit that senses fluctuations in loads of misfolded proteins to regulate induction of ER stress-induced apoptosis via a circuit that may lie outside of the UPR network. However, how JB12 functions to protect cells from ER stress–induced cell death is not clear.

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This article contains supplemental Materials and Methods and Tables 1–4.

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[3] The abbreviations used are: ER, endoplasmic reticulum; JB12, DNAJB12; JB14, DNAJB14; BOK, BCL-2 ovarian killer; Bort, bortezomib; ERQC, ER protein quality control; ERAD, ER-associated protein degradation; Tg, thapsigargin; Tm, tunicamycin; UPR, unfolded protein response; Huh-7, human hepatoma cell line 7; PERK, protein kinase RNA-like endoplasmic reticulum kinase.

A basic understanding of how JB12 facilitates cross-talk between the UPR and heat–shock response systems to suppress ER stress-induced apoptosis requires information about how its activity is regulated. The study of this question was initiated through examination of the impact that acute ER stress caused by proteotoxic chemical stressors on JB12 and, for comparison, JB14 expression and function. Remarkably, JB12, but not JB14, was found to be stress-sensitive, as it was rapidly destabilized during challenge of the ER with the reducing agent dithiothreitol (DTT). Reductive stress caused by DTT appeared to alter the conformation of the DUF1977 domain on JB12 and cause its selection for degradation by the ERQC factors HERP, Sel1L, and the ERAD ubiquitin ligase gp78. Regions in the DUF1977 domain of JB12 and JB14 are not well conserved, and this may account for the differential sensitivity of them to DTT. JB12 is an ER-associated Hsp40 that is selectively degraded in response to ER stress.

Huh-7 (human hepatoma cell line 7) liver carcinoma cells experience chronic ER stress as indicated by detection of high level expression of the glycoprotein chaperone calnexin, the ERQC E3 ligase RMA1, and the ER-associated BCL-2 protein BOK. BOK has an extremely short half-life and is constitutively degraded by ERAD, and its stress-dependent accumulation helps trigger the induction of ER stress-induced apoptosis (16–18). JB12 is detected in ERQC complexes that control BOK levels and stress-dependent depletion of JB12 from Huh-7 caused the accumulation of BOK, induction of caspase-9, -3, and -7 processing, and increased sensitivity to proteotoxic agents. JB12 is a multifunctional and ER stress–sensitive Hsp40 that functions to suppress BOK activity during normal growth. During severe bouts of ER stress the inactivation of JB12 appears to contribute to the elevation of BOK and the induction of ER stress–mediated apoptosis. Mechanisms by which JB12 functions to facilitate protein homeostasis and suppress ER stress-induced apoptosis will be discussed.

## Results

### *JB12 is selectively degraded in response to reductive ER stress*

To identify mechanisms for post-translational regulation of JB12 and JB14 in response to acute proteotoxic stress, COS-7 cells were challenged with chemicals that disrupt protein homeostasis in the ER membrane system, DTT, thapsigargin (Tg), or tunicamycin (Tm), or in the cytosol, bortezomib (Bort). Then, stress-dependent changes in levels of JB12, JB14, and different ERAD factors and cytosolic Hsp70 and Hsp40s were measured by Western blot (Fig. 1*A*). Under conditions where Bort inhibited the proteasome and increased the level of cytosolic Hsc70, there was no change in the level of JB12 or JB14 (Fig. 1*A*). Likewise, disruption of glycoprotein folding with Tm, which blocks *N*-linked glycosylation, had no effect on JB12 or JB14 levels. Yet, Tm clearly stressed cells because it caused an increase in the levels of the ER lumenal Hsp70 BiP, ER transmembrane calnexin, and the ERAD factors Derlin-1 and Derlin-3 (Fig. 1*A*). In stark contrast, a dramatic and selective decrease in levels of JB12, but not JB14, occurred when cells were challenged with the reducing agent DTT (Fig. 1*A*). Tg, an inhibitor of Serca2A that depletes the ER of $Ca^{2+}$, caused a reduction in JB12 levels during the 6-h challenge. Yet, the effect of Tg was modest when compared with the impact of DTT on steady-state levels of JB12.

ER stress caused by proteotoxic agents occurs in a time and dose-dependent fashion, so the impact of Tg on the apparent stability of JB12 was further explored in a time–course experiment in which the Tg concentration of 3 $\mu$M used in *panel 1A* was increased to 6 $\mu$M, and changes in JB12 levels were monitored over a longer 24-h period of challenge (Fig. 1*B*). The higher dose of Tg caused a detectable reduction in JB12 levels between 2 and 4 h of challenge, and near complete depletion occurred between 8 and 24 h. Time-dependent reductions in JB12 caused by Tg correlated with increasing levels of ER stress as indicated by increased accumulation of BiP.

JB12 levels were reduced by DTT and Tg but not Tm. JB12 is not predicted to contain a $Ca^{2+}$-binding domain, but its ER luminal DUF1977 domain contains conserved cysteine residues (7) that could be sensitive to changes in oxidative/reductive state of the ER lumen. DTT might, therefore, have a direct effect on the conformation of JB12, whereas impacts of Tg, which occur over a longer time period, could be an indirect result of the interference with function of $Ca^{2+}$-dependent chaperones such as calnexin that participate in oxidative protein folding in the ER lumen (3). Tm may not impact the stability of JB12 because it interferes with *N*-linked glycosylation, and JB12 is not a glycoprotein. For these reasons the challenge of cells with DTT was employed as a model for ER stress to study the mechanism for stress-dependent degradation of JB12.

If DTT was having a direct impact on the conformation of JB12, the destabilization of JB12 should occur before the ER becomes severely stressed (Fig. 1*C*). Indeed, in cells challenged with the low dose of 0.6 mM DTT for 2 h, the destabilization of JB12 occurs, whereas at the same time the phosphorylation of the translation initiation factor 2 $\alpha$ (eIF2$\alpha$) was not stimulated. The phosphorylation of eIF2$\alpha$ is mediated by the ER transmembrane kinase PERK in response to the ER stress-induced sequestration of BiP into complexes with misfolded proteins (3). Thus, DTT can rapidly destabilize JB12 in the absence of severe ER stress.

In cycloheximide chase studies, DTT reduced the half-life of JB12 from 6 h to around 1.5 h (Fig. 1*D*). In these same cells the half-life of the short-lived RMA1 (19), which is an ERQC ubiquitin ligase that interacts with JB12, was increased from 2 h to >6 h, whereas the half-life of Derlin-1, an ERQC factor present in complexes with JB12, was not detectably altered. Destabilization of JB12 occurs soon after challenge of cells with low doses of DTT and happens while its normally short-lived interaction partner RMA1 is stabilized. JB12 was identified as the first Hsp40 family member to be selectively destabilized in response to acute ER stress.

### *The ER luminal DUF1977 domain permits the stress-sensitive degradation of JB12*

JB12 contains an N-terminal J-domain, a transmembrane span, and an ER lumenal DUF1997 domain, and each could be sensitive to ER stress (Fig. 2*A*). Yet, DTT prevents oxidative folding within the ER lumen, so the logical ER stress–sensitive domain of JB12 is DUF1977. It is not clear what role the

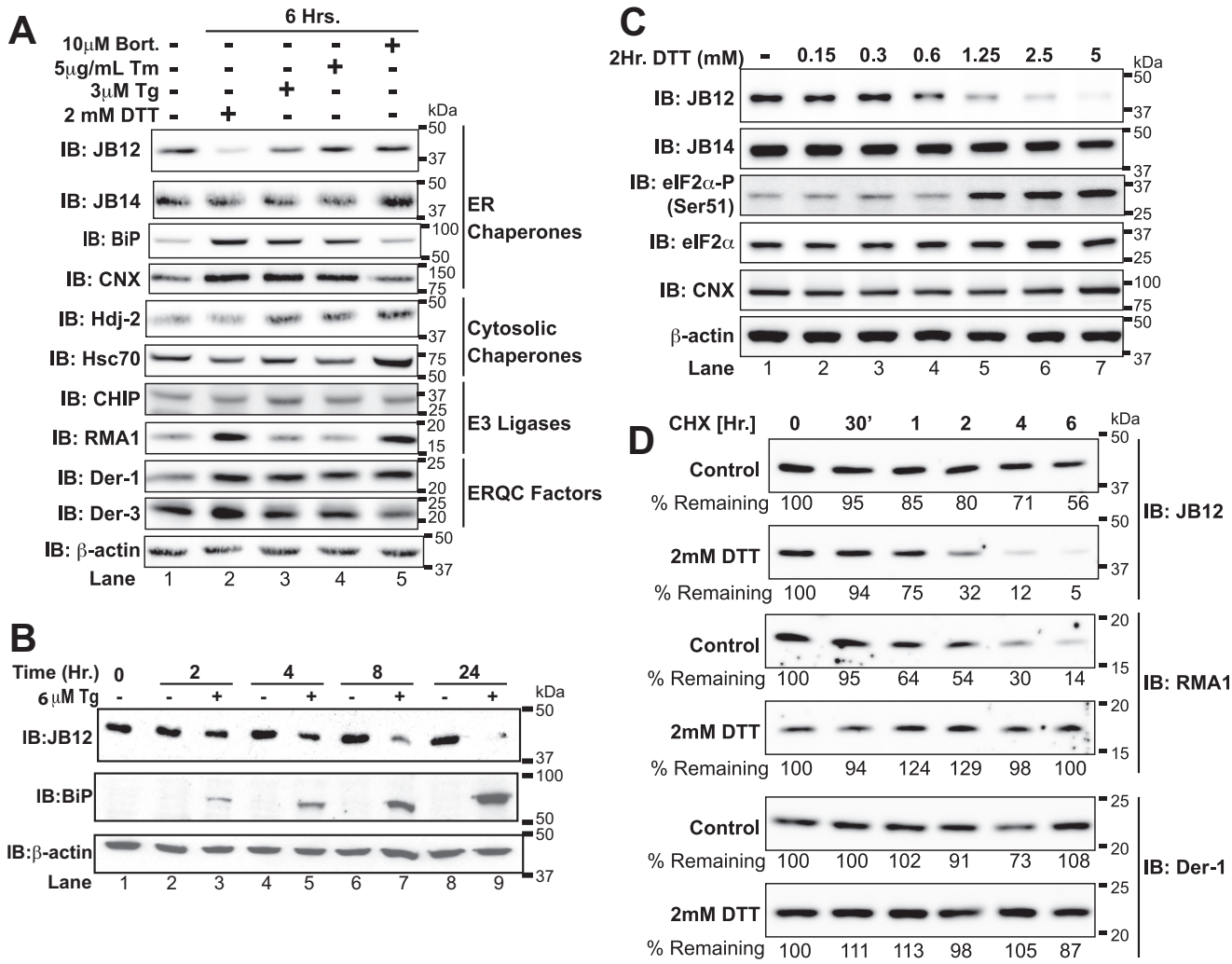



**Figure 1. JB12 is a protein quality control factor that is selectively depleted from cells in response to ER stress.** *A*, Western blot (*IB*) analysis of the impact that proteotoxic agents have on levels of cytosolic and ER-associated protein quality control factors. Extracts of COS-7 were analyzed by Western blot after a 6-h incubation with 2 mM DTT, 3 μM Tg, 5 μg/ml Tm, or 20 μM Bort. *B*, reduction of JB12 levels by 6 μM Tg during a 24 h time course. *C*, dose-dependent impact of DTT on JB12 levels during a 2-h incubation. *D*, cycloheximide (*CHX*; 150 μg/ml) chase analysis of the impact that challenge with 2 mM DTT has on levels of JB12, Derlin-1, and RMA1. *CNX*, calnexin.

DUF1997 plays in JB12 function, but it is just >110 amino acid residues long (Fig. 2*A*) and is conserved in ER transmembrane Hsp40s that are expressed in organisms from yeast to man (7, 20). JB12 and JB14 both contain a DUF1977 domain, but the stability of JB12 is sensitive to stress, whereas JB14 is resistant to challenges that destabilize JB12. The differential stress sensitivity of JB12 and JB14 is consistent with observations that these Hsp40s share some overlapping functions but also have specific non-overlapping roles in ER homeostasis (15). Sequence alignment of the JB12 and JB14 DUF1977 showed a high degree of identity in N-terminal regions, but there was a low degree of identity between their C termini (Fig. 2*A*). Such differences might explain why JB14 appears resistant to ER stress that destabilizes JB12.

To gain experimental support that the DUF1977 of JB12 is sensitive to ER stress, N- and C-terminal epitope tags were placed on JB12. Then the sensitivity to DTT of the different forms of JB12 was determined (Fig. 2*B*). Overexpressed forms of wild-type and N-terminally FLAG-tagged JB12 were destabilized by DTT in a manner similar to endogenous JB12. However, the presence of an MYC tag on the C terminus abrogated the sensitivity of JB12 to DTT. The C terminus of JB12 is highly charged, so it may be disordered. The amino acid sequence of a MYC tag (Glu-Gln-Leu-Ile-Ser-Glu-Glu-Asp-Leu) contains a number of polar and charged amino acids. Therefore, interactions between residues within DUF1977 and the MYC tag have the potential to promote conformational changes that confer resistance of JB12 to DTT.

JB12 contains two conserved cysteine residues that could be sensitive to DTT that are located at positions 329 and 363 of DUF1977 (Fig. 2*A*). The mutant C329A JB12 accumulates at a moderately lower level than JB12, but C329A JB12 remains sensitive to DTT. In contrast, accumulation of the mutant C363A JB12 was markedly reduced, and it was not sensitive to DTT. Therefore, Cys-363 appears to be important for folding of JB12 into stable conformation that is sensitive to DTT.

Why JB14, which contains the same two conserved cysteine residues as JB12, is not sensitive to DTT, is not entirely clear. Yet, Cys-363 is located near the end of JB12, and the last 40 amino acids of the DUF1977 in JB12 and JB14 are highly diver-

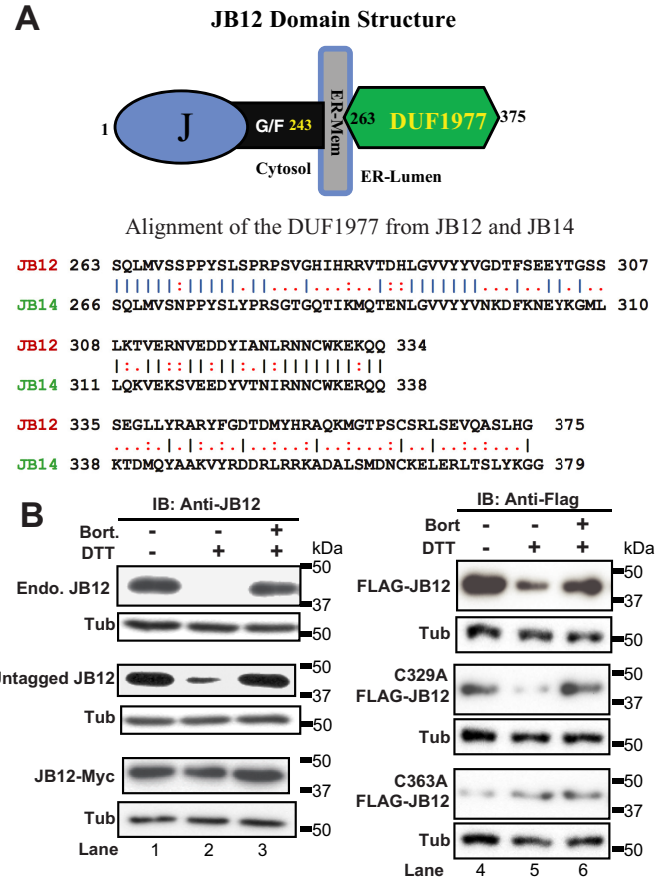



**Figure 2. Reduced sensitivity of JB12 to ER stress upon alteration of its ER luminal DUF1977 domain.** *A*, schematic of the JB12 domain structure and sequence alignment of the JB12 and JB14 DUF1977. The DUF of JB12 is located between amino acids 263 and 375 and in JB14 lies between residues 266 and 379. *B*, changes in sensitivity of JB12 to DTT (2 mM) during a 3-h incubation caused by placing an epitope tag on the JB12 N or C terminus or mutation of conserved cysteine residues in DUF1977. *IB*, immunoblot. *Tub*, tubulin.

gent (Fig. 2*A*), These differences in amino acid composition may permit JB14, but not JB12, to adopt a stress-resistant state.

### *Interference with BiP function in the ER lumen destabilizes JB12*

The DUF1977 of JB12 might be metastable and, therefore, undergoes a conformational change that leads to its degradation during ER stress. If so, then the action of ER luminal chaperones should be required for maintenance of JB12 during normal growth, and forms of JB12 that are destabilized by stress might become substrates of BiP. Indeed, in unstressed cells, a small pool of endogenous BiP was detected in native immunoprecipitates with FLAG-JB12 (Fig. 3*A*). Upon challenge of cells with DTT, a 4-fold increase in the association of endogenous BiP with immunoprecipitated FLAG-JB12 occurred (Fig. 3*A*). In addition, the depletion of BiP by siRNA caused a reduction in JB12 levels, and the absence of BiP also increased the sensitivity of JB12 to DTT (Fig. 3*B*). Thus, depletion of BiP partially destabilizes JB12, and forms of JB12 that are destabilized by DTT are bound by BiP.

The depletion of BiP by siRNA is nearly complete and is similar to challenge with DTT in that it caused the strong induction of ER stress (Fig. 3*B*). Levels of ER stress caused by the depletion of BiP were monitored by assaying decreases in the mobility of the kinase PERK on SDS-PAGE, which occur when PERK becomes activated through autophosphorylation (3). PERK appeared to be fully activated when BiP was depleted because BiP was no longer present to bind the ER lumenal regions of PERK and maintain it in an inactive state. Therefore, a concern about the evaluation of data from BiP siRNA knockdown studies is that the near complete loss of BiP from cells causes a stress that is severe enough to non-specifically destabilize JB12. Hence, it was asked if JB12 could also be destabilized under conditions where BiP was only partially depleted and the ER experienced low to moderate stress (Fig. 3*C*).

The partial reduction of BiP in cells was achieved through the ectopic expression the cytotoxic endoprotease subtilase A, which accumulates in the ER lumen and cleaves BiP (21). Cells were transfected with a subtilase A expression plasmid. Then at 24, 48, and 72 h post-transfection the levels of JB12, BiP, HERP, CHOP, and cleaved caspase 3 and 7 were measured by Western blot. After 24 h, JB12 levels were relatively unchanged, and levels of BiP were ~50% below normal. Yet, at the 48-h time point, JB12 levels were 60% lower, and BiP was reduced by 70%. Notably, depletion of JB12 and BiP detected 48 h after transfection of the cells with the subtilase A expression plasmid was not associated with a strong induction ER stress. This is the case because pools of active and lower mobility forms of PERK were not strongly increased. In addition, induction of CHOP, an ER stress-responsive transcription factor that controls ER chaperone levels (3), and processing of caspase 3 and 7 in subtilase A-expressing cells were well below the levels observed in DTT-treated cells. In addition, HERP expression were induced by the severe ER stress caused by DTT, but HERP levels were not increased when BiP was partially depleted by subtilase A (Fig. 3*C*).

Partial depletion of BiP, under conditions where PERK is not strongly activated, leads to destabilization of JB12. JB12 has features of a metastable protein that requires BiP to maintain its steady-state levels in the ER. In addition, destabilized JB12 appears to misfold and be bound by BiP before its delivery to ERAD for degradation.

### *Degradation of destabilized JB12 requires the ERAD factors HERP, Sel1L, and gp78*

In unstressed cells, JB12 functions in complexes with the ERQC E3 ligases RMA1 and gp78, where it acts with Hsp70 to triage non-native membrane proteins between folding and degradation pathways (7). Therefore, we asked if destabilized JB12 becomes a substrate of the ERAD machinery with which its stable forms normally interact. Depletion of RMA1, CHIP, or HRD1 by siRNA did not have a remarkable impact on the DTT-dependent degradation of JB12 (Fig. 4*A*). In contrast, the depletion of gp78, Sel1L, and HERP suppressed the degradation of JB12 caused by DTT.

HERP and Sel1L act in the ER in heteroligomeric E3 ubiquitin ligase complexes that contain either HRD1 or gp78 that degrade both soluble and membrane-inserted conformers of misfolded proteins (22, 23). HERP functions as an organizing factor (24, 25), whereas Sel1L acts in the selection of misfolded

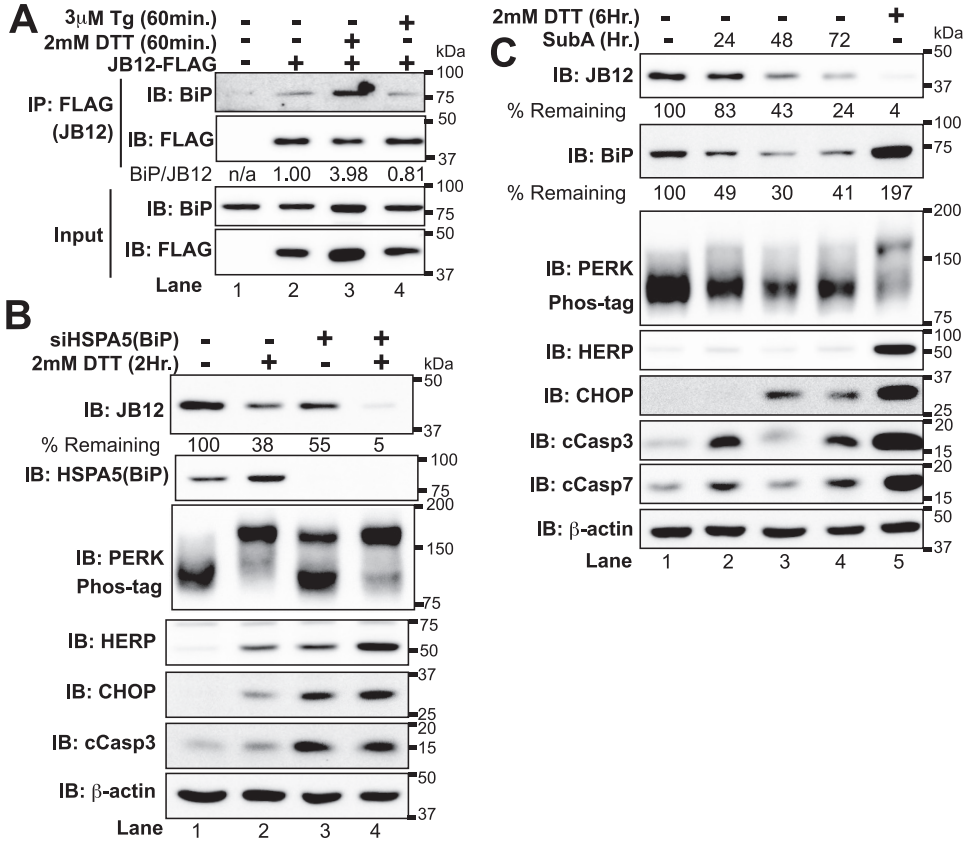



**Figure 3. Depletion of BiP from the ER lumen leads to depletion of JB12.** *A*, DTT stimulates the association of JB12 with BiP. COS-7 cells expressing JB12-FLAG were untreated or treated with DTT or Tg for 1 h. The BiP that coprecipitated (*IP*) with JB12-FLAG was then detected by Western blot. *B*, BiP depletion induces ER stress and loss of JB12. COS-7 cells were transfected with siBiP for 48 h and where indicated were treated with DTT for 6 h. *C*, subtilase A-mediated BiP depletion stimulated caspase processing and decreased JB12. Cells that were transfected with a subtilase A expression plasmid were grown for the indicated time period before preparation of extracts for use in Western blots.

proteins for ubiquitination (23). JB12 was detected in immunoprecipitable complexes with both Sel1L and HERP (Fig. 4*B*). In pulldowns where HERP-FLAG was the bait, Sel1L and JB12 were both detected. In pulldowns where MYC-Sel1L was the bait, JB12 was also detected in immunoprecipitates (Fig. 4*C*). Notably, the siRNA-mediated depletion of endogenous HERP caused an ~60% reduction in the association of JB12 with Sel1L (Fig. 4*C*). This result may explain why the knockdown of HERP hindered the degradation of JB12 caused by DTT. Treatment of cells with DTT reduced JB12 association with HERP and Sel1L, so damaged forms of JB12 appeared to be rapidly cleared from ERAD machinery.

Sel1L/gp78 has been reported to form an E3 complex that has clients that are distinct from the HRD1–Sel1L complex (26), and this helps explain why the degradation of JB12 caused by DTT is less sensitive to depletion of HRD1 than gp78. These data suggest that the stress-damaged forms of JB12 are selected for ERAD by the ERQC machinery with which native JB12 normally interacts during maintenance of ER homeostasis.

### *JB12 limits BOK accumulation and suppresses ER stress-induced apoptosis in Huh-7 cells*

Cancer cells experience chronic ER stress due in part to constant demands for oxidative protein folding in the ER lumen, and in some cancers Hsp70 molecular chaperones are essential for malignancy (27, 28). Therefore, we asked if the human hepatoma cell line 7 (Huh-7) (29) experiences chronic ER stress. The role played by JB12 in supporting the cancerous growth of Huh-7 was also examined. Huh-7 experiences a form of chronic ER stress because levels of RMA1 and calnexin were severalfold higher in Huh-7 *versus* COS-7 (Fig. 5*A*). HEK293 cells are multinucleate and express high levels of molecular chaperones, and levels of BiP, Derlin-1, and JB12 were similar in Huh-7, COS-7, and HEK293 (Fig. 5*A*).

The acute challenge of Huh-7 with DTT caused JB12 levels to plummet, BiP levels to rise, and induced cleavage of procaspase-9, -3, and -7 (Fig. 5*B*). JB12 was also found to be important for viability of unstressed Huh-7 cells because its depletion via shRNA was accompanied by an apparent induction of apoptosis that was indicated by the dramatic increase in processed caspase-9, -3, and -7 (Fig. 5*B*). Notably, depletion of JB12 by shRNA resulted in a much larger increase in caspase processing than treatment of Huh-7 with DTT.

In the absence of JB12, acute treatment of Huh-7 with DTT further stimulated accumulation of cCasp-9, -3, and -7 (Fig. 5*B*), whereas challenge of JB12 depleted Huh-7 with Tg or Bort for the same time period and only increased the accumulation of cCasp-7. JB12 plays an important role in growth of Huh-7, and loss of JB12 led to the induction of apoptosis.

ER stress-induced apoptosis is regulated in part by mechanisms that control the steady-state level of the ER-associated

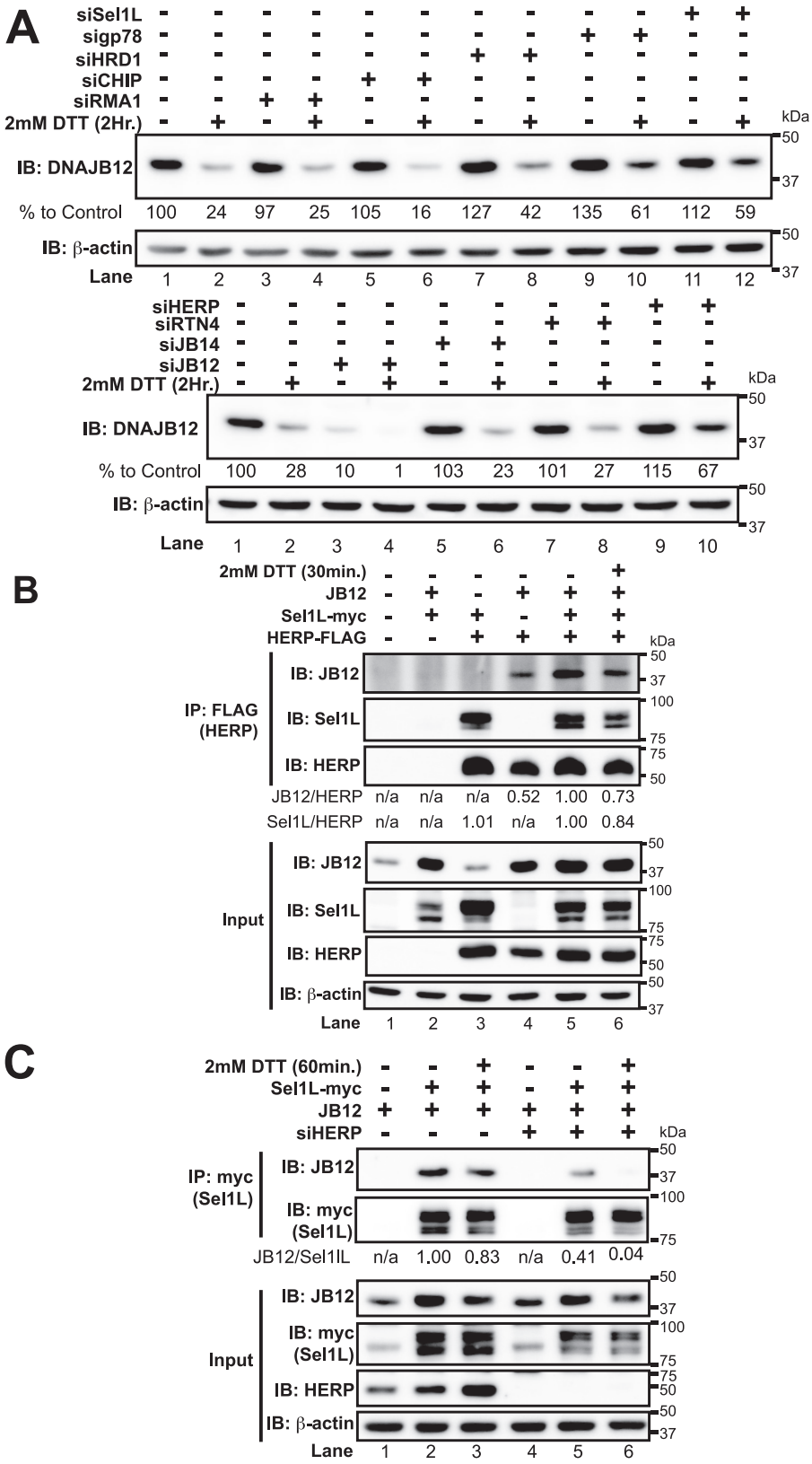



**Figure 4. Stress-stimulated turnover of JB12 required the ERQC factor gp78, Sel1, and HERP.** *A*, impact that siRNA knockdown of ERAD factors on ER stress-dependent degradation of JB12. COS-7 were treated with siRNAs for the indicated proteins as described under "Experimental Procedures" and then treated with DTT (2 mM) and cycloheximide (150 $\mu$g/ml) for 2 h. Changes in levels of the indicated proteins was then determined by Western blot (*IB*). *IP*, immunoprecipitated. *B*, JB12 is present in immunoprecipitable complexes that contain FLAG-HERP. COS-7 cells expressed exogenous and untagged JB12, HERP-FLAG, and Sel1L-myc. *C*, siRNA depletion of HERP reduced the association of JB12 with Sel1L-myc. Identification of proteins that co-immunoprecipitated with tagged bait proteins was achieved by Western blot.

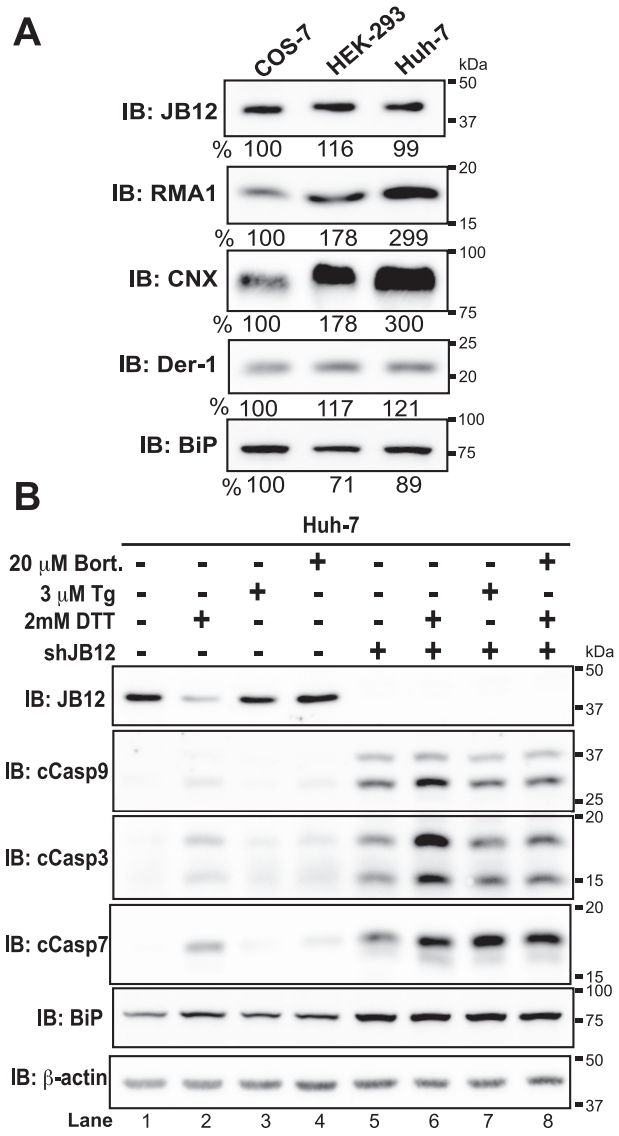



**Figure 5. Depletion of JB12 sensitized Huh-7 cancer cells to proteotoxic stress and apoptosis.** *A*, steady-state levels of ERQC factors and chaperones in Huh-7 as compared with COS-7 and HEK293. *B*, depletion of JB12 via shRNA is associated with the accumulation of cleaved caspases, cCasp-3, cCasp-7, and cCasp-9. Normal and JB12-depleted Huh-7 cells were challenged with indicated proteotoxic stressors for 6-hrs. Levels of indicated proteins were determined by Western blot (*IB*).

BCL-2 protein BOK. BOK induces caspase processing by stimulating the release of cytochrome *c* from mitochondria (16). Remarkably, BOK is expressed, but not readily detected in unstressed cells, because it is constitutively degraded and has a short half-life of 15 min (16). However, during proteotoxic stress that compromises function of the proteasome, ERQC E3 ligases such as gp78, which mediate BOK degradation, become saturated with misfolded proteins, and BOK accumulates (16).

To define the mechanism by which JB12 suppresses the induction of apoptosis, the impact that its presence or absence in Huh-7 had on BOK levels was examined (Fig. 6*A*). Consistent with Huh-7 experiencing chronic ER stress, endogenous BOK was readily detected in Western blots of cell extracts (Fig. 6*A*). Treatment of Huh-7 with DTT was accompanied by a >2.5-fold increase in BOK and a 60% decrease in JB12 levels. The shRNA knockdown of JB12 to levels below detection by Western blot also resulted in a 3.5-fold increase in BOK (Fig. 6*A*). BOK levels were increased up to 5-fold above controls upon challenge of JB12 depleted Huh-7 with DTT. Thus, endogenous BOK is readily detected in Huh-7, and its levels increase severalfold upon depletion of JB12. The dramatic increase in BOK levels that accompanies depletion of JB12 from Huh-7 helps explain why caspase processing was activated in Huh-7 cells that lacked JB12 (Fig. 5*B*).

Next, we evaluated whether or not JB12 played a direct or indirect role in influencing the steady-state levels of BOK. To accomplish this goal, the impact on BOK of elevating the activity of WT JB12 or inhibiting JB12 function through overexpression of a dominant negative form of JB12 (QPD JB12) was measured (Fig. 6*B*). For these studies FLAG-BOK was expressed at low levels in HEK293. Because JB12 is an abundant protein, its overexpression did not reduce BOK accumulation (Fig. 6*B*). In contrast, QPD JB12, a form of JB12 that can bind ERQC factors but fails to interact with Hsp70 (7) caused a dose-dependent and severalfold increase in FLAG-BOK (Fig. 6*B*). Interference with the function of endogenous JB12 appears to block the BOK turnover.

If JB12 directly influences BOK accumulation, QPD JB12 would be expected to block BOK turnover at an intermediate stage where BOK was associated with components of the ERAD system. Indeed, QPD JB12 was detected at 4-fold higher levels than WT JB12 in immunoprecipitates with FLAG-BOK (Fig. 6*C*). Likewise, when cells were treated with Bort to inhibit proteasomal degradation of BOK, both gp78 and QPD JB12 were found to accumulate in complexes with FLAG-BOK (Fig. 6*D*).

JB12 is physically present in complexes that contain BOK and gp78. Failure of JB12 to interact with Hsp70 causes BOK levels to rise because BOK appears to become trapped in complexes with gp78. These findings suggest that JB12 and Hsp70 mediate protein assembly/disassembly events that enable gp78 to facilitate steps in BOK degradation. It is plausible that inactivation of JB12 during ER stress provides a mechanism to promote the accumulation of BOK and trigger downstream induction of caspase processing.

### *JB12 confers resistance of Huh-7 to chemotherapeutics that stimulate apoptosis*

To further test the concept that JB12 protects cells from ER stress-induced death, the requirement for JB12 in the survival of Huh-7 to challenge with proteotoxic agents was examined (Fig. 7*A*). Huh-7 tolerated treatment with 2 mM DTT for 36 h. Yet, 50% of JB12-depleted Huh-7 lost viability within 24 h of exposure to DTT (Fig. 7*A*). Huh-7 tolerated treatment with Bort for 12 h, but 50% lost viability after exposure for 24 h, whereas 50% of JB12-depleted Huh-7 lost viability within 12 h of Bort challenge. JB12 protects Huh-7 from toxic stress caused by perturbation of reductive protein folding and the global interference with proteasome function.

To further assess the function of JB12 in protecting cells from stress, we asked if reduction of its activity sensitized cells to the apoptosis inducer LCL-161. LCL-161 is a small molecule second mitochondrial activator of caspase (SMAC) mimetic (30). LCL-161 induces apoptosis by promoting the degradation of

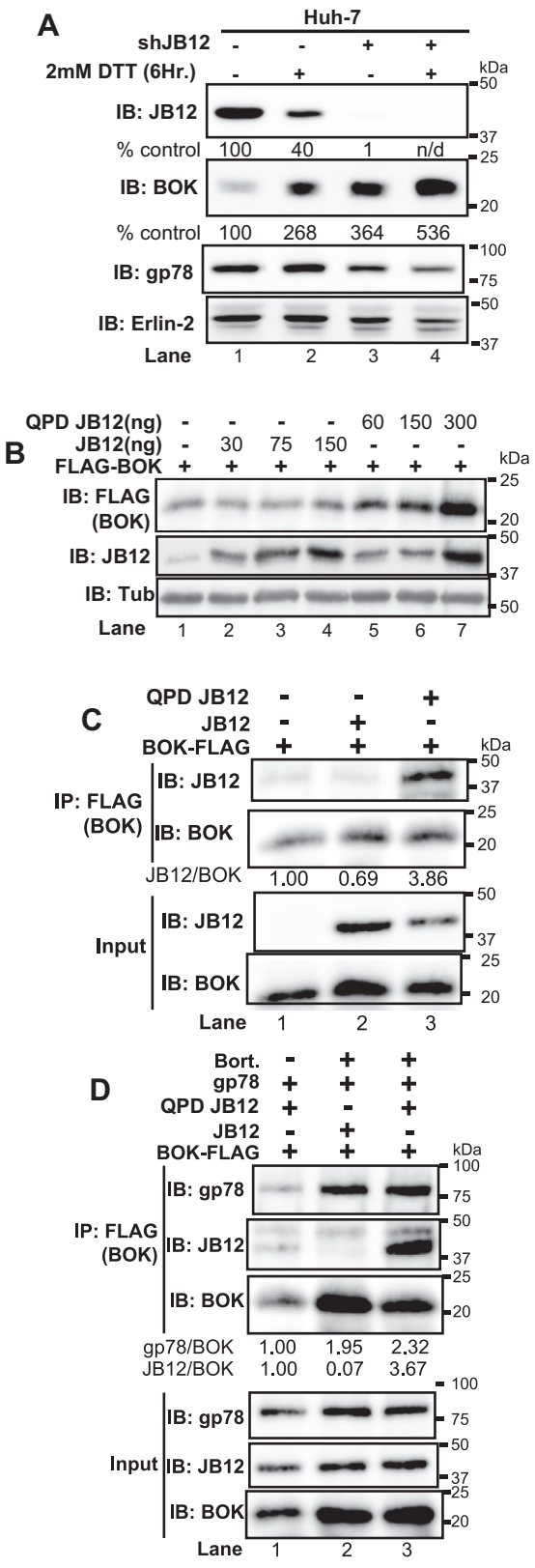



**Figure 6. Depletion of JB12 from Huh-7 were associated with an increase in BOK and induction of caspase processing.** *A*, there is an inverse relationship between BOK and JB12 levels in Huh-7 cells that are challenged with DTT. Huh-7 were treated with JB12 shRNA and/or DTT, and levels of the indicated proteins were determined by Western blot (*IB*). Blots for gp78 and Erlin-2 serve as load controls. *B*, expression of JB12-QPD, a dominant negative form of JB12, promotes BOK accumulation. HEK293 cells were transfected with the indicated quantities of wild-type JB12 or JB12-QPD, and impacts of each on FLAG-BOK accumulation was determined by Western blot. *C* and *D*, FLAG-BOK accumulated in immunoprecipitable (*IP*) complexes that contain QPD-JB12 and gp78. The indicated proteins were expressed in HEK293 cells and detected in native precipitates with FLAG-BOK by Western blot.

inhibitor of apoptosis proteins (IAPs) that bind procaspases and inhibit their processing/activation (30). However, the action of LCL-161 alone is insufficient to kill cancer cells, and a second stimulator of apoptosis is required for it to induce cancer cell death (31). Indeed, we found that treatment of Huh-7 with LCL-161 caused the depletion of cIAP-1 (Fig. 2*B*) but did not reduce the viability of Huh-7 (Fig. 7*A*). Yet, the depletion of JB12 from Huh-7 sensitized them to LCL-161-induced death (Fig. 7*A*).

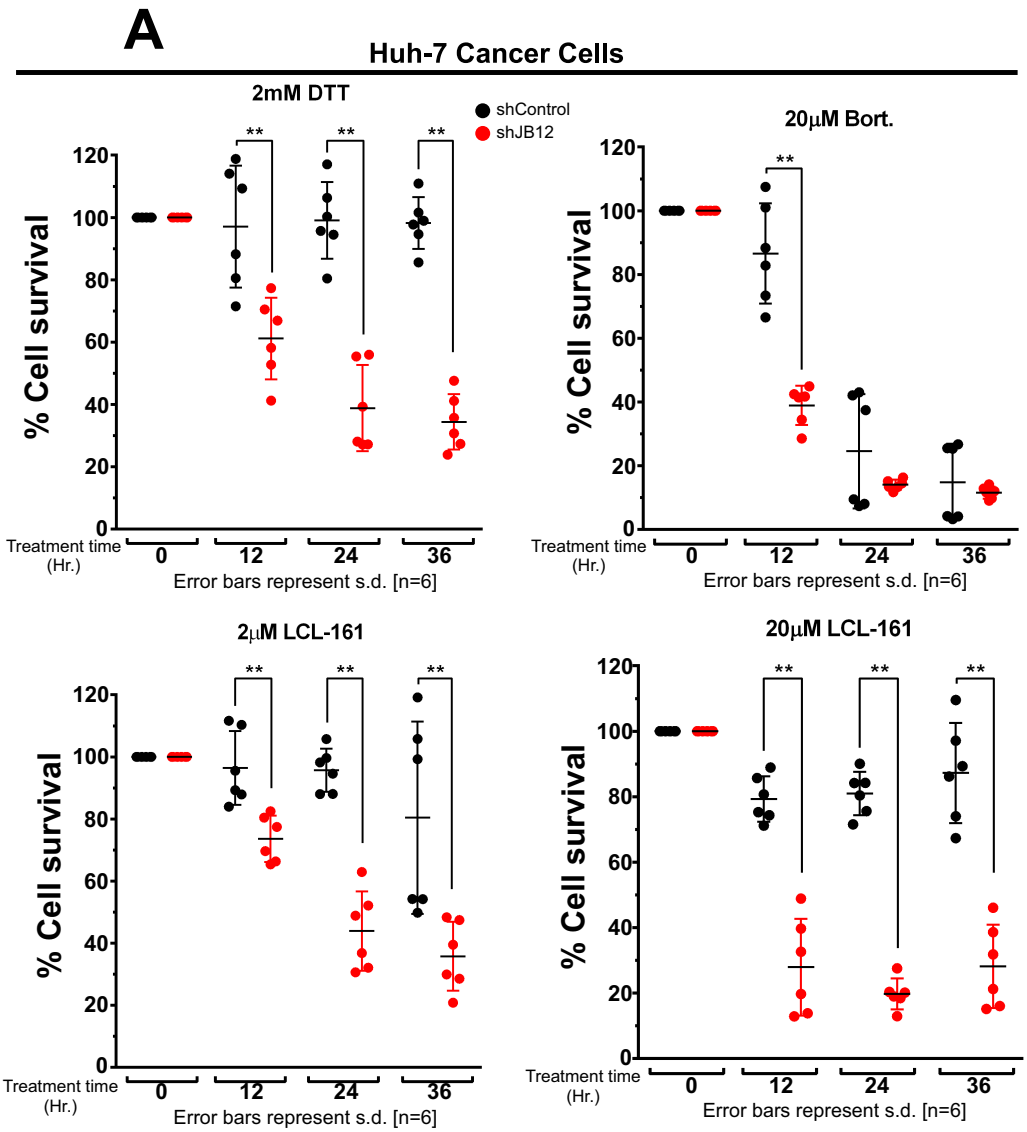
Consistent with the shRNA depletion of JB12 causing the accumulation of proapoptotic BOK (Fig. 6*A*), reduction of JB12 permitted LCL-161 to drive a dose-dependent fashion increase in caspase activation (Fig. 7*B*). These findings indicate that JB12 is required to protect Huh-7 from proteotoxic stress via a mechanism that involves the suppression of BOK accumulation. The loss of JB12 function in Huh-7 contributes to the accumulation of BOK, activation of procaspase processing, and induction of ER stress-induced apoptosis.

## Discussion

Hsp70 and Hsp40s are constitutively expressed, and a subset of them are induced to protect cells from proteotoxicity (1, 32). JB12 recruits Hsp70 to the ER surface to facilitate protein folding and protein triage and has unique features among other members of the Hsp40 family because it is destabilized by ER stress. Destabilized JB12 is degraded via an ERAD pathway that utilizes HERP, the E3 ligase gp78, and the ERAD substrate selector Sel1L. Alteration of Cys-363 in the ER lumenal DUF1977 domain prevents JB12 from adopting a stress-sensitive conformation. Proper folding/assembly of JB12 appears to be required for it to adopt a stress-sensitive conformation.

JB12 was found to function in association with gp78 to mediate the constitutive degradation of BOK, a stress-sensitive and short-lived BCL-2 family member that triggers ER stress-induced apoptosis (Fig. 8). Stress-dependent degradation of JB12 is associated with increased BOK, induction of caspase processing, and sensitization of Huh-7 liver cancer cells to proteotoxic agents and a chemotherapeutic agent. JB12 has features of an ER stress sensor whose inactivation during acute stress permits the accumulation of BOK and thereby triggers the initiation of ER stress-induced apoptosis.

JB12 and JB14 perform overlapping functions in ER homeostasis but are not functionally redundant, as each respective Hsp40 can facilitate a unique aspect of ER protein homeostasis that is not facilitated by the other (8, 10, 15). The observation that JB12, but not JB14, stability is sensitive to acute stress is, therefore, consistent with JB12 and JB14 having unique roles in maintenance of protein homeostasis. The mechanism for the differential sensitivity of JB12 and JB14 to stress is not clear, but the DUF1977 of both JB12 and JB14 is highly charged, although not identical, and monomeric forms are predicted to be dynamically unfolded (7). Therefore, differences in the dynamic conformational states of JB12 and JB14 may permit JB12 to popu-


A
Huh-7 Cancer Cells
2mM DTT
shControl
shJB12
20μM Bort.
2μM LCL-161
20μM LCL-161
% Cell survival
Treatment time (Hr.)
0
12
24
36
Error bars represent s.d. [n=6]
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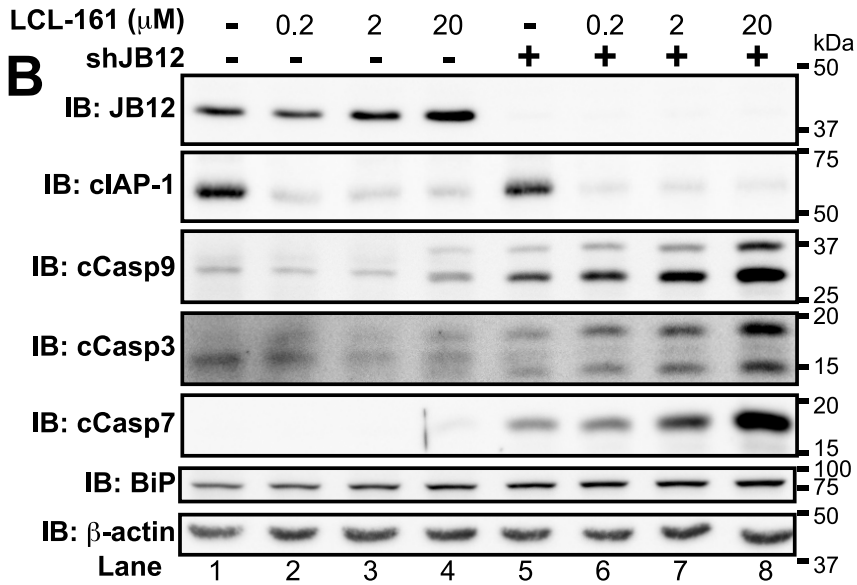
B
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IB: JB12
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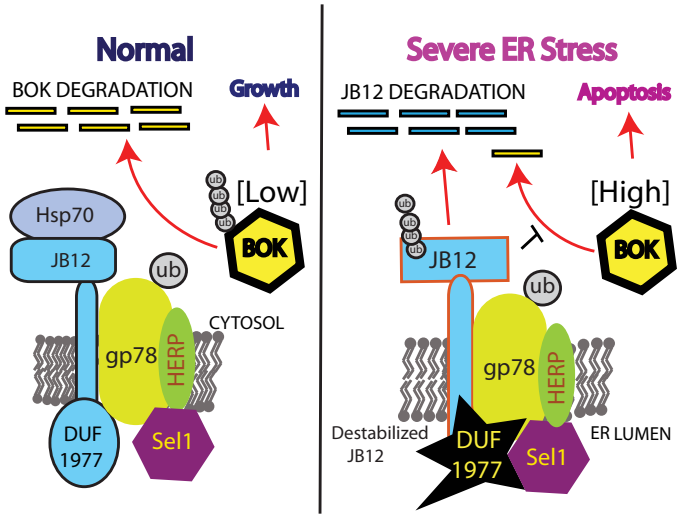


**Figure 8. Model depicting how ER stress impacts JB12 and BOK in normal and stressed cells.** During normal growth JB12 cooperates with Hsp70 and gp78 to mediate the constitutive degradation of BOK. Upon inactivation of JB12 during acute and severe ER stress, the efficiency of BOK degradation is reduced. The resultant degradation of JB12 and accumulation of BOK then contributes to the induction of ER stress-induced apoptosis.

late a stress-sensitive state that is not populated by JB14. The biogenesis of JB12 is sensitive to mutation of Cys-363 in the DUF1977 of JB12 because C363A JB12 accumulated at low levels and was insensitive to DTT. JB12 also contains Cys-329, but C329A JB12 remains sensitive to DTT, so destabilization of JB12 by DTT does not appear to result from disruption of intrachain disulfide bonds. However, Hsp40s function as homo- and heterodimers (33, 34), so assembly of JB12 into a stable oligomeric complex may require disulfide bond formation that involves Cys-363. The disruption of JB12's conformation or assembly with other proteins during ER stress could provide a mechanism for its destabilization.

JB12 facilitates aspects of ERAD that involve the selection of misfolded membrane proteins for ubiquitination by E3 ligase complexes that contain RMA1/RNF5, gp78, Hrd1, and possibly others (7, 35–37). When JB12 associates with misfolded ER proteins that are inefficiently retrotranslocated out of the ER, it can also mediate the transfer of its ERAD-resistant clients to the ERQC autophagy pathway (8). JB12, therefore, functions as a protein triage factor, and loss of JB12 should cause serious negative side effects on cell biology. Indeed, COS-7 and HEK293 cells that lack JB12 die from apoptosis when challenged with large pools of misfolded membrane proteins (8). Consistently, Huh-7 liver cancer cells that lack JB12 were reported herein to be hypersensitive to challenge with a proteasome inhibitor, DTT, and the apoptosis inducer LCL-161.

Strikingly, reduction of JB12 in Huh-7 is associated with a modest elevation of BiP, a strong increase in the accumulation of BOK, and robust activation of caspase processing. These data suggest that the inability of JB12 to function in protein triage triggers early steps in ER stress-induced apoptosis. Such a scenario may occur during proteotoxic stress encountered by cells and may also occur in disease states where mutant forms of membrane proteins misfold and aggregate in the ER bilayer (5–8). Aggregated membrane proteins are resistant to degradation by ERAD or ERQC autophagy (8), so they have the potential to sequester JB12 and inhibit its function in ER homeostasis. Sequestration of JB12 would decrease its availability to facilitate degradation of BOK and set the stage for induction of ER stress-induced apoptosis.

Cancer cells suffer from constitutive ER stress due to dysregulation of normal growth control and heavy flux through pathways for oxidative protein folding in the ER lumen (3, 38). Recent studies have demonstrated the importance of the Hsp70 and Hsp90 systems in promoting the cancerous growth of cells in tumors (39). Data presented suggest that one function of Hsp70 in fostering cancerous cell growth is limiting the accumulation of the cell death inducer BOK. Hence, the inhibitors of Hsp70 are under development as drugs with chemotherapeutic potential (40). However, the Hsp70 system is essential for cell viability, so Hsp70 inhibitors are likely to have broad side effects that limit their utility as drugs. Yet, Hsp40s such as JB12 are expressed at substoichiometric levels to Hsp70 and direct specific Hsp70 family members to facilitate distinct subsets of reactions required for protein homeostasis. Therefore, targeting a specific Hsp40, such as JB12, for inactivation to promote BOK accumulation has the potential to reduce the side effects of current drugs designed to modulate Hsp70 that globally antagonize Hsp70 function (41). Because loss of JB12 primes Huh-7 cancer cells for death by causing BOK accumulation for killing by LCL-161, it is possible that a compound that

**Figure 7. JB12 protects Huh-7 from apoptosis induced by chemical stressors and the chemotherapeutic LCL-161.** *A*, impact that depletion of JB12 from Huh-7 has on survival from challenge with proteotoxic agents and the apoptosis inducer LCL-161. Changes in cell viability caused by stressors were estimated after 12, 24, and 36 h of exposure through assay for the reduction of ATP levels in treated cells (see "Experimental Procedures" for details). Values are normalized as the % of the total ATP in control cells. Statistical significance was evaluated by Student's *t* test. A *p* value of <0.01 was considered statistically significant (**). 2 mM DTT: 12 h, $p = 0.0039$; 24 h, $p = <0.0001$; 36 h, $p = <0.0001$. 20 μM Bort: 12 h, $p = < 0.0001$. 2 μM LCL-161, 12 h, $p = 0.0026$; 24 h, $p = <0.0001$; 36 h, $p = 0.0078$. 20 μM LCL-161: 12 h, $p = <0.0001$; 24 h, $p = <0.0001$, 36 h, $p = <0.0001$. *B*, the dose-dependent fashion impact of LCL-161 on caspase processing in control and JB12-depleted Huh-7 cells. Shown are Western blots (*IB*) of cell extracts where levels of the indicated proteins were measured.

selectively drives the degradation of JB12 could be used in the clinic to induce apoptotic death of cancer cells.

## Experimental procedures

### *Cell culture and transfection*

African green monkey kidney (COS-7), human hepatocellular carcinoma (Huh-7), and human embryonic kidney (HEK293) cells were cultured in Dulbecco's Eagle's minimum-enriched medium (DMEM) at 37 °C and 5% $CO_2$. Cells were seeded in 6-well plates at a density of $3 \times 10^5$ cells/well. Plasmid transfections into COS-7 cells were performed by using Effectene (Qiagen, Hilden, Germany) at 20-h post-seeding. Expression plasmids included pcDNA3.1-DNAJB12, pcDNA3.1-DNAJB12-FLAG, pcDNA3.1-Sel1L-myc, pCMV-HERP-myc-DDK (Cat.RC217811, Origene), and pcDNA3.1-SubtilaseA (Plasmid 35948, Addgene) (see supplemental Materials and Methods for additional plasmid information). Cells were cultured for 48 h before being challenged with stress-inducing chemicals (see supplemental Tables 1–3 for a list of chemicals and tools used in this study). Supplemental Table 4 contains the molecular masses of the proteins that were detected by Western blot.

For knockdown experiments, all siRNAs were purchased from Thermo Fisher Scientific (Waltham, MA). For some target genes, two siRNAs to the same target gene were used to increase knockdown efficiency. COS-7 cells that were seeded into 6-well plate at $3 \times 10^5$ cells/well for 20 h were transfected at 5 pmol/well siRNA with Lipofectamine2000® (Thermo Fisher Scientific). Twenty hours post-transfection, cells were split at 1:2 ratios and allowed to recover for 48 h. JB12 knockdown in Huh-7 cells was achieved by using lentiviral shJB12 expressed from pLKO.1 (TRCN0000022297, Open Biosystems, Huntsville, AL). Huh-7 cells were grown for 48 h and then incubated with 10 $\mu$l of raw lentiviral stock/ml of serum-free DMEM for 24 h. The infected Huh-7 cells were then grown an additional 24 h in complete media and then split 1:2 into a new plate and grown for 48 h before analysis.

### *Lysate preparation for Western blotting*

COS-7 cells or Huh-7 cells were collected and then solubilized in 100 $\mu$l of Nonidet P-40 lysis solution (1% (v/v) Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% (w/v) SDS) supplemented with 1 mM PMSF and 1× complete protease inhibitor mixture (Roche Applied Science) and 1× PHOSstop phosphatase inhibitor mixture (Roche Applied Science). The lysates were then homogenized by digital sonicator at 26% intensity for 7 s. The homogenized lysates were diluted to 1 $\mu$g/$\mu$l total protein in 1× SDS-PAGE sample buffer containing 20 mM DTT. The mixed protein samples were incubated at 37 °C for 30 min before resolving by SDS-PAGE. The material in resolved SDS-PAGE gels was transferred onto 0.22 $\mu$M nitrocellulose membranes (Bio-Rad). Membranes were analyzed by Western detection with the indicated antibodies (see supplemental Table 3 for antibodies used in this study and supplemental Table 4 for the molecular mass of the respective proteins).

Protein signal detection was performed by using Clarity™ Western ECL substrate (Bio-Rad) and visualized by LAS4000 imager (GE Healthcare). Image data were analyzed by ImageJ (National Institutes of Health) and quantitated with ImageQuant (GE Healthcare).

Analysis of PERK phosphorylation was enhanced by the use of the Phos-Tag™ reagent (Wako, Osaka, Japan). This required cell lysate preparation in Nonidet P-40 lysis buffer that was supplemented with EDTA-free 1× complete protease inhibitor mixture (Roche Applied Science). Electrophoresis was performed with use of EDTA-free 6% SDS-PAGE that contained 50 $\mu$M $MnCl_2$ and 3.5 $\mu$M Phos-Tag™ (Wako), and proteins were resolved at a constant current of 10 mA.

### *Immunoprecipitation of proteins from cell extracts prepared with non-ionic detergents*

Transfected HEK293 or COS-7 cells were harvested from plates with ice-cold citric saline (135 mM KCl, 15 mM sodium citrate) and then lysed in Nonidet P-40 buffer that was supplemented with 1 mM PMSF. Insoluble material was removed by centrifugation at 14,000 rpm at 4 °C for 5 min. Antibodies against bait proteins were added to the cleared lysates and incubated for 30 min at 4 °C. The target protein–antibody complex was isolated with protein G-agarose (Roche Applied Science) during an incubation at 4 °C for 30 min. Material bound to agarose beads was washed 3 times with Nonidet P-40 lysis buffer was then recovered from beads via incubation with 2× SDS-PAGE sample buffer at 37 °C for 30 min. To pull down FLAG-containing proteins, anti-FLAG M2 beads (Sigma) were used instead of protein G-agarose. Material that was bound to FLAG beads was then eluted with FLAG peptide, and the liberated materials were probed by Western blot.

In studies of the effect of HERP on interaction between DNAJB12 and Sel1L, COS-7 cells grown for 24 h in a 6-well plate were transfected with siHERP. After 24 h, cells were split 1:2, allowed to grow for another 20 h, then transfected with pcDNA3.1-Sel1L-myc and pcDNA3.1-DNAJB12. After a period of 24 h cells were harvested, and immunoprecipitations and Western blot analysis was conducted.

### *Assay for changes in cell viability*

COS-7 or Huh-7 cells were seeded at $1 \times 10^5$ cells/well in a 96-well plate and grown for 48 h. Media were then exchanged with new media containing ER stressors or anti-cancer drug LCL-16. Cell viability was indirectly measured by changes in cellular ATP levels using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) following the manufacturer's protocol.

*Author contributions*—P. S., D. E. G., and H. Y. R. designed and carried out experiments and also prepared the figures and text of the manuscript. D. M. C. designed the study and assisted with manuscript preparation.

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### References

1. Balchin, D., Hayer-Hartl, M., and Hartl, F. U. (2016) In vivo aspects of protein folding and quality control. *Science* **353,** aac4354

2. Yamamoto, K., Sato, T., Matsui, T., Sato, M., Okada, T., Yoshida, H., Harada, A., and Mori, 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
3. Walter, P., and Ron, D. (2011) The unfolded protein response: from stress pathway to homeostatic regulation. *Science* **334,** 1081–1086
4. Hetz, C., Chevet, E., and Oakes, S. A. (2015) Proteostasis control by the unfolded protein response. *Nat. Cell Biol.* **17,** 829–838
5. Meacham, G. C., Patterson, C., Zhang, W., Younger, J. M., and Cyr, D. M. (2001) The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat. Cell Biol.* **3,** 100–105
6. Meacham, G. C., Lu, Z., King, S., Sorscher, E., Tousson, A., and Cyr, D. M. (1999) The Hdj-2/Hsc70 chaperone pair facilitates early steps in CFTR biogenesis. *EMBO J.* **18,** 1492–1505
7. Grove, D. E., Fan, C. Y., Ren, H. Y., and Cyr, D. M. (2011) The endoplasmic reticulum-associated Hsp40 DNAJB12 and Hsc70 cooperate to facilitate RMA1 E3–dependent degradation of nascent CFTRΔF508. *Mol. Biol. Cell* **22,** 301–314
8. Houck, S. A., Ren, H. Y., Madden, V. J., Bonner, J. N., Conlin, M. P., Janovick, J. A., Conn, P. M., and Cyr, D. M. (2014) Quality control autophagy degrades soluble ERAD–resistant conformers of the misfolded membrane protein GnRHR. *Mol. Cell* **54,** 166–179
9. Yamamoto, Y. H., Kimura, T., Momohara, S., Takeuchi, M., Tani, T., Kimata, Y., Kadokura, H., and Kohno, K. (2010) A novel ER J-protein DNAJB12 accelerates ER-associated degradation of membrane proteins including CFTR. *Cell Struct. Funct.* **35,** 107–116
10. Sopha, P., Kadokura, H., Yamamoto, Y. H., Takeuchi, M., Saito, M., Tsuru, A., and Kohno, K. (2012) A novel mammalian ER-located J-protein, DNAJB14, can accelerate ERAD of misfolded membrane proteins. *Cell Struct. Funct.* **37,** 177–187
11. Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C., and Zylicz, M. (1991) *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 2874–2878
12. Cyr, D. M., Lu, X., and Douglas, M. G. (1992) Regulation of Hsp70 function by a eukaryotic DnaJ homolog. *J. Biol. Chem.* **267,** 20927–20931
13. Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M. K., and Hartl, F. U. (1992) Successive action of DnaK, DnaJ, and GroEL along the pathway of chaperone-mediated protein folding. *Nature* **356,** 683–689
14. Houck, S. A., and Cyr, D. M. (2012) Mechanisms for quality control of misfolded transmembrane proteins. *Biochim. Biophys. Acta* **1818,** 1108–1114
15. Li, K., Jiang, Q., Bai, X., Yang, Y. F., Ruan, M. Y., and Cai, S. Q. (2017) Tetrameric assembly of $K^+$ channels requires ER-located chaperone proteins. *Mol. Cell* **65,** 52–65
16. Llambi, F., Wang, Y. M., Victor, B., Yang, M., Schneider, D. M., Gingras, S., Parsons, M. J., Zheng, J. H., Brown, S. A., Pelletier, S., Moldoveanu, T., Chen, T., and Green, D. R. (2016) BOK Is a Non-canonical BCL-2 Family effector of apoptosis regulated by ER-associated degradation. *Cell* **165,** 421–433
17. Chipuk, J. E., and Luna-Vargas, M. P. (2016) Cell biol.: ERADicating survival with BOK. *Curr. Biol.* **26,** R473–R476
18. Fernandez-Marrero, Y., Ke, F., Echeverry, N., Bouillet, P., Bachmann, D., Strasser, A., and Kaufmann, T. (2016) Is BOK required for apoptosis induced by endoplasmic reticulum stress? *Proc. Natl. Acad. Sci. U.S.A.* **113,** E492–E493
19. Younger, J. M., Chen, L., Ren, H. Y., Rosser, M. F., Turnbull, E. L., Fan, C. Y., Patterson, C., and Cyr, D. M. (2006) Sequential quality-control checkpoints triage misfolded cystic fibrosis transmembrane conductance regulator. *Cell* **126,** 571–582
20. Kampinga, H. H., and Craig, E. A. (2010) The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nat. Rev. Mol. Cell Biol.* **11,** 579–592
21. Nakajima, S., Hiramatsu, N., Hayakawa, K., Saito, Y., Kato, H., Huang, T., Yao, J., Paton, A. W., Paton, J. C., and Kitamura, M. (2011) Selective abrogation of BiP/GRP78 blunts activation of NF-κB through the ATF6 branch of the UPR: involvement of C/EBPβ and mTOR-dependent dephosphorylation of Akt. *Mol. Cell Biol.* **31,** 1710–1718
22. Christianson, J. C., Shaler, T. A., Tyler, R. E., and Kopito, R. R. (2008) OS-9 and GRP94 deliver mutant α1-antitrypsin to the Hrd1-SEL1L ubiquitin ligase complex for ERAD. *Nat. Cell Biol.* **10,** 272–282
23. Kanehara, K., Xie, W., and Ng, D. T. (2010) Modularity of the Hrd1 ERAD complex underlies its diverse client range. *J. Cell Biol.* **188,** 707–716
24. Horn, S. C., Hanna, J., Hirsch, C., Volkwein, C., Schütz, A., Heinemann, U., Sommer, T., and Jarosch, E. (2009) Usa1 functions as a scaffold of the HRD-ubiquitin ligase. *Mol. Cell* **36,** 782–793
25. Schulze, A., Standera, S., Buerger, E., Kikkert, M., van Voorden, S., Wiertz, E., Koning, F., Kloetzel, P. M., and Seeger, M. (2005) The ubiquitin-domain protein HERP forms a complex with components of the endoplasmic reticulum associated degradation pathway. *J. Mol. Biol.* **354,** 1021–1027
26. Zhang, T., Xu, Y., Liu, Y., and Ye, Y. (2015) gp78 functions downstream of Hrd1 to promote degradation of misfolded proteins of the endoplasmic reticulum. *Mol. Biol. Cell* **26,** 4438–4450
27. Bhat, T. A., Chaudhary, A. K., Kumar, S., O'Malley, J., Inigo, J. R., Kumar, R., Yadav, N., and Chandra, D. (2017) Endoplasmic reticulum–mediated unfolded protein response and mitochondrial apoptosis in cancer. *Biochim. Biophys. Acta* **1867,** 58–66
28. Hazari, Y. M., Bashir, A., Haq, E. U., and Fazili, K. M. (2016) Emerging tale of UPR and cancer: an essentiality for malignancy. *Tumour Biol.* **37,** 14381–14390
29. Kausar, H., Gull, S., Ijaz, B., Ahmad, W., Sarwar, M. T., Iqbal, Z., Nawaz, Z., Riazuddin, S., and Hassan, S. (2011) Huh-7 cell line as an alternative cultural model for the production of human like erythropoietin (EPO). *J. Transl. Med.* **9,** 186
30. Qin, Q., Zuo, Y., Yang, X., Lu, J., Zhan, L., Xu, L., Zhang, C., Zhu, H., Liu, J., Liu, Z., Tao, G., Dai, S., Zhang, X., Ma, J., Cai, J., and Sun, X. (2014) Smac mimetic compound LCL161 sensitizes esophageal carcinoma cells to radiotherapy by inhibiting the expression of inhibitor of apoptosis protein. *Tumour Biol.* **35,** 2565–2574
31. West, A. C., Martin, B. P., Andrews, D. A., Hogg, S. J., Banerjee, A., Grigoriadis, G., Johnstone, R. W., and Shortt, J. (2016) The SMAC mimetic, LCL-161, reduces survival in aggressive MYC-driven lymphoma while promoting susceptibility to endotoxic shock. *Oncogenesis* **5,** e216
32. Cyr, D. M., Langer, T., and Douglas, M. G. (1994) DnaJ-like proteins: molecular chaperones and specific regulators of Hsp70. *Trends Biochem. Sci.* **19,** 176–181
33. Sha, B., Lee, S., and Cyr, D. M. (2000) The crystal structure of the peptide–binding fragment from the yeast Hsp40 protein Sis1. *Structure Fold Des.* **8,** 799–807
34. Nillegoda, N. B., and Bukau, B. (2015) Metazoan Hsp70-based protein disaggregases: emergence and mechanisms. *Front. Mol. Biosci.* **2,** 57
35. Han, S., Liu, Y., and Chang, A. (2007) Cytoplasmic Hsp70 promotes ubiquitination for endoplasmic reticulum–associated degradation of a misfolded mutant of the yeast plasma membrane ATPase, PMA1. *J. Biol. Chem.* **282,** 26140–26149
36. Nakatsukasa, K., Huyer, G., Michaelis, S., and Brodsky, J. L. (2008) Dissecting the ER-associated degradation of a misfolded polytopic membrane protein. *Cell* **132,** 101–112
37. Morito, D., Hirao, K., Oda, Y., Hosokawa, N., Tokunaga, F., Cyr, D. M., Tanaka, K., Iwai, K., and Nagata, K. (2008) Gp78 cooperates with RMA1 in endoplasmic reticulum-associated degradation of CFTRΔF508. *Mol. Biol. Cell* **19,** 1328–1336
38. Lee, A. S., and Hendershot, L. M. (2006) ER stress and cancer. *Cancer Biol. Ther.* **5,** 721–722
39. Sabnis, A. J., Guerriero, C. J., Olivas, V., Sayana, A., Shue, J., Flanagan, J., Asthana, S., Paton, A. W., Paton, J. C., Gestwicki, J. E., Walter, P., Weissman, J. S., Wipf, P., Brodsky, J. L., and Bivona, T. G. (2016) Combined chemical–genetic approach identifies cytosolic HSP70 dependence in rhabdomyosarcoma. *Proc. Natl. Acad. Sci. U.S.A.* **113,** 9015–9020
40. Assimon, V. A., Gillies, A. T., Rauch, J. N., and Gestwicki, J. E. (2013) Hsp70 protein complexes as drug targets. *Curr. Pharm. Des.* **19,** 404–417
41. Miyata, Y., Li, X., Lee, H. F., Jinwal, U. K., Srinivasan, S. R., Seguin, S. P., Young, Z. T., Brodsky, J. L., Dickey, C. A., Sun, D., and Gestwicki, J. E. (2013) Synthesis and initial evaluation of YM-08, a blood-brain barrier permeable derivative of the heat shock protein 70 (Hsp70) inhibitor MKT-077, which reduces tau levels. *ACS Chem. Neurosci.* **4,** 930–939