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Endoplasmic reticulum stress-induced cell death mediated by the proteasome

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Cells exposed to sustained endoplasmic reticulum (ER) stress undergo programmed cell death and display features typical of apoptosis, such as cysteine aspartyl protease (caspase) activation, cytochrome *c* release, and DNA fragmentation. Here, we show that the execution of cell death induced by ER stress is mediated via the proteasome. Inhibition of the proteasome by lactacystin prevented ER stress-induced degradation of Bcl-2, release of cytochrome *c*, processing of effector caspase-3, and exposure of phosphatidylserine. Owing to the ability of lactacystin to inhibit cytochrome *c* release, we propose that the pro-apoptotic activity of the proteasome lies upstream of mitochondrial activation. Thus, the proteasome serves as a principal mediator of ER stress-induced cell death in this system.
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The endoplasmic reticulum (ER) is the site of secretory protein synthesis, conformational maturation and quality control for correctly folded proteins. ER stress-inducing drugs disturb cellular homeostasis of protein production and lead to the accumulation of aberrant and unfolded proteins.[1] Under normal conditions, proteins destined to be secreted or targeted to any of the compartments of the secretory pathway are passaged through the membrane via the Sec61 translocon and meet molecular chaperones, which ensure proper folding and assembly of proteins and protein complexes.[2] Only proteins that achieve their native states are packaged into COPII vesicles for ER exit: proteins failing to adopt a stable conformation are dislocated through the Sec61 channel into the cytosol, where they are targeted for ubiquitylation and proteasomal degradation.[3] Protein ubiquitylation is achieved via the sequential activity of three groups of enzymes: E1, E2, and E3. Mammalian cells have one E1 enzyme, which activates ubiquitin in an ATP-dependent manner and transfers it to one of at least 15 different E2 ubiquitin carriers, from which it is then transferred to one of the hundreds of E3 ubiquitin ligases present in the cell, the latter being responsible for substrate specificity.[4] Proteasomal degradation of poorly folded ER-directed proteins is known as ER-associated degradation (ERAD), which is a cellular mechanism for achieving ER quality control.[5] Homeostasis occurs so long as the load of newly synthesized proteins is matched by stochiometric molecular chaperone activity. Conditions that lead to the accumulation of unfolded proteins result in ER stress. When challenged with ER stress, cells can re-achieve homeostasis by initiating a series of orchestrated events known as the unfolded protein response (UPR) (for review see reference[5]). The key players of the UPR are three chronologically activated ER resident signaling proteins: PERK, ATF6, and IRE1. Activation of these molecules results in the inhibition of translation (PERK), upregulation of molecular chaperones (ATF6), and upregulation of ERAD activity (IRE1).[5,6] As a consequence, the ER experiences a reduction in protein folding burden while increasing folding and quality control capacity. If a cell fails to achieve homeostasis after fully activating the UPR and its associated ERAD activity, the cell initiates programmed cell death.[7]

ER stress-induced cell death has been the focus of many studies, but a detailed molecular description of this cell death pathway has yet to emerge. From previous reports, several pro-apoptotic factors and signaling pathways have been shown to be involved in ER stress-induced cell death, including calpain, Cysteine aspartyl protease (caspase-4) (human), caspase-12 (murine), CHOP/GADD153, TRAF2, ASK-1, JNK, and pro-apoptotic Bcl-2 family members, among others.[7–10] In many instances, caspases have been implicated in this cell death pathway, on the grounds that broad-spectrum caspase inhibitors block cell death. In some cases, however, cell death is not fully blocked by caspase inhibitors, suggesting that caspase-independent mechanisms may contribute to the execution of ER stress-induced cell death. Previously, it was shown that the serine protease inhibitor Pefabloc (AEBSF) could block ER stress-induced cell death, whereas the broad-spectrum caspase inhibitor, benzyloxy-carbonyl-Val-Ala-Asp.fluoromethylketone (Z-VAD.fmk), could not, thus implicating the involvement of a serine protease (or other AEBSF-inhibitable activity) in caspase-independent ER stress-induced cell death.[11]

In this report, we show that the activity of the proteasome is necessary for ER stress-induced cell death. Proteasomal inhibition blocked ER stress-induced degradation of anti-apoptotic Bcl-2 family proteins, cytochrome *c* release,

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Abbreviations: BFA, brefeldin A; CHX, cycloheximide; caspase, cysteine aspartyl protease; GFPAn-V, His-GFP-Annexin-V; GFP^{u}, GFP appended with the short degron, CL1; PI, propidium iodide; PS, phosphatidylserine; Q-VD-OPh, Q-Val-Asp-(non-O-methylated)-OPh; Z-VAD.fmk, benzyloxy-carbonyl-Val-Ala-Asp (O-methyl) fluoromethylketone; UPR, unfolded protein response
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caspase maturation, phosphatidylserine exposure, and cell death. Furthermore, cell lines harboring temperature-sensitive E1 ubiquitin-activating enzyme were protected from ER stress-induced programmed cell death at the restrictive temperature but not at the permissive temperature. From these results, we conclude that proteasomal activity lies upstream of mitochondria and cytochrome *c* release in a pathway that links ER stress to cell death.

Results

Proteasomal inhibitors block ER stress-induced cell death induced by BFA and CHX. To elicit robust cell death in response to ER stress, BFA-treated cells were co-incubated with CHX so as to limit the activation of ATF6 and IRE1 translation-dependent survival pathways, as was done previously.[8] We reported that ER stress-induced apoptosis was not prevented by caspase inhibition with Z-VAD.fmk but could be blocked by Pefabloc, a general inhibitor of serine protease activity.[11] We therefore sought to identify the serine protease(s) responsible for cell death observed in our system. We used a mass spectrometry (MS) approach to identify proteins in cytosolic extracts bound to serine protease active site-directed inhibitors. Using two different serine protease inhibitors as bait, we repeatedly obtained hits for different proteasomal subunits in the inhibitor-enriched fractions from ER-stressed cells but did not detect any hits for ER-stress activated serine proteases (Supplementary Figure 1). Although the hits for proteasomal subunits were at the limit of significance, we tested the hypothesis that the proteasome might play a role in ER stress-induced cell death. To do so, we treated ER-stressed rat fibroblasts with several proteasomal inhibitors. Phase-contrast microscopy revealed that inhibition of the proteasome with either lactacystin, MG262, or epoxomycin, protected cells from ER stress (BFA/CHX, for 24 h) to the same extent as Pefabloc (Figure 1a). As was previously shown, we saw that caspase inhibition with either Z-VAD.fmk or Q-Val-Asp-(non-*O*-methylated)-OPh (Q-VD-Oph) (Figure 1a and data not shown) did not prevent cells from undergoing ER stress-induced cell death,[11] and CHX treatment alone did not induce cell death (Figure 1a).

Lactacystin and Pefabloc block degradation of a proteasomal substrate. We reasoned that if the protective effect of Pefabloc was due to proteasomal inhibition, then we should see an accumulation of proteasomal substrates when cells are treated with Pefabloc. One well-characterized substrate of the proteasome is GFP^u, a fusion protein of GFP with a degron sequence, CL1.[12,13] We therefore transiently transfected rat fibroblasts with GFP^u and followed green fluorescence by flow cytometry. Untreated cells have low levels of green fluorescence because GFP^u is rapidly degraded by the proteasome. However, under conditions of proteasomal inhibition, as in the case of lactacystin treatment, GFP^u significantly accumulated in cells (Figure 1b). GFP^u also accumulated in cells treated with Pefabloc (Figure 1b), demonstrating that Pefabloc can inhibit proteasomal activity in cells. This is consistent with previous finding that Pefabloc inhibits the trypsin-like activity of the proteasome *in vitro*.[14,15]

Proteasomal inhibitors block ER stress-induced phosphatidylserine exposure. To validate our phase-contrast study, we followed markers of programmed cell death, namely phosphatidylserine exposure before the loss of plasma membrane integrity. Cells exposing phosphatidylserine (an early marker of apoptosis) can be labeled with a His-GFP-Annexin-V (GFPAn-V) fusion protein, which binds to phosphatidylserine in a calcium-dependent manner. Loss of plasma membrane integrity can be followed by uptake of propidium iodide (PI), a cell non-permeating nuclear stain. Analysis by fluorescence microscopy showed that lactacystin and Pefabloc, but not Z-VAD.fmk, prevented the exposure of phosphatidylserine provoked by ER stress with BFA (5 μg/ml) and CHX (10 μg/ml) for 24 h (Figure 2a). These observations were quantified by flow cytometry of GFPAn-V/PI-stained cells. After 22 h of ER stress, fluorescence-activated cell-sorting analysis revealed that 63% of the stressed cells were GFPAn-V-positive, compared to 13% in non-stressed cells (Figure 2b). Treatment with lactacystin or Pefabloc significantly protected cells from ER stress-induced cell death, reducing the number of GFPAn-V positive cells to that seen in untreated cells ($P<0.001$; BFA/CHX *versus* BFA/CHX + Pefabloc or BFA/CHX + lactacystin), whereas Z-VAD.fmk treatment only partially blocked ER stress-induced cell death (Figure 2c). The capacity of lactacystin and Pefabloc to protect cells from ER stress was not unique to BFA/CHX, as cell death induced by thapsigargin/CHX and tunicamycin/CHX was also blocked by these proteasomal inhibitors (Figure 2e and f, respectively). We could also show that in the absence of CHX, BFA killed cells in a proteasome-dependent manner (Figure 2d)

Lack of a functional E1 ubiquitin-activating enzyme delays ER stress-induced cell death. We tested the extent to which a cell line harboring a temperature-sensitive mutation in the E1 ubiquitin-activating enzyme would be protected from ER stress-induced cell death at the non-permissive temperature. Upon induction of ER stress with BFA and CHX, we quantified the fraction of GFPAn-V positive cells in ts20, a mutant cell line harboring a temperature-sensitive allele of the E1 ubiquitin-activating enzyme, as well as H38-5, a ts20 derivative in which the E1 ubiquitin-activating enzyme has been restored (Figure 3a). After 30 h of treatment with BFA (10 μg/ml) and CHX (10 μg/ml) at the permissive temperature (34°C), both H38-5 and ts20 lines were effectively killed (Figure 3a). Cell death is reported as the fold-induction because the ts20 line has a greater background level of cell death than does the H38-5 line. (For raw data, see Supplementary Figure 2a.) However, at the non-permissive temperature, only the E1-restored H38-5 cells responded to BFA/CHX, whereas the ts20 cell line did not (Figure 3a). We extended these findings by testing whether ER stress-induced cell death elicited by thapsigargin and tunicamycin would also require a functional E1 ubiquitin activating enzyme activity (Figure 3b, data are normalized as in Figure 3a. For raw data, see Supplementary Figure 2b). For each BFA, thapsigargin, and tunicamycin, cells were killed in both cell lines at 34°C and in the H38-5

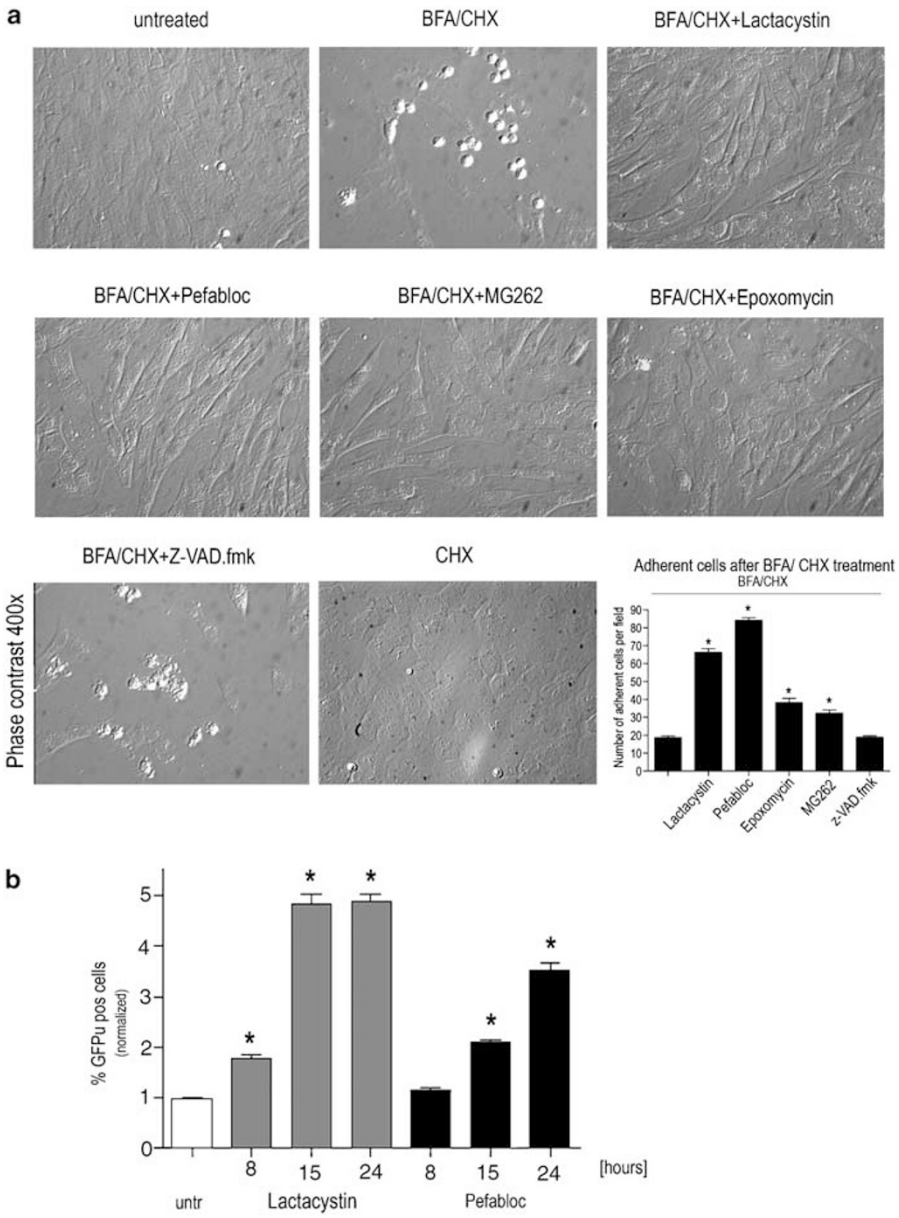

Figure 1 Cell survival of ER-stressed cells by proteasomal inhibition. (**a**) Phase-contrast microscopy ($\times$ 400 magnification) of rat fibroblasts grown to confluence and left either untreated or exposed to ER stress with BFA (5 μg/ml) and CHX (10 μg/ml) or treated with CHX alone for 24 h. Cells were pretreated either with lactacystin (5 μM), Pefabloc (300 μM), MG262 (100 nM), epoxomycin (100 nM), or Z-VAD.fmk (100 μM), 30 min before cell stress. Graph: adherent cells were counted from 10 randomly chosen fields for BFA/CHX treated samples (bar graph, error bars correspond to S.E.M. *$P<0.001$ BFA/CHX alone *versus* BFA/CHX with indicated inhibitors). (**b**) Accumulation of green fluorescence in cells transiently transfected with GFP^u. Fold increase of GFP^u fluorescence after 8, 15, or 24 h ($N=6$, 9, or 10, respectively) of treatment with lactacystin (5 μM) or Pefabloc (300 μM) compared to untreated cells ($N=11$). Error bars correspond to S.E.M. * $P<0.001$ treated *versus* untreated

line at 40°C. However, ts20 cells incubated at 40°C did not induce cell death triggered by BFA, thapsigargin, and tunicamycin. From these results, we conclude that ER stress-induced cell death is strictly dependent on a functional E1 ubiquitin-activating enzyme activity.

Proteasomal activation lies upstream of mitochondrial outer-membrane permeabilization and caspase processing. Having shown that proteasomal activity is necessary for ER stress-induced cell death, we wished to place the proteasome within the biochemical pathway that connects a stressed ER to the cell death machinery. To find out, we began by testing whether the activity of the proteasome lies upstream of effector caspase processing. Upon stimulation of cells with BFA/CHX, we detected a dramatic reduction in the 32 kDa procaspase-3 and a corresponding increase in the 17 kDa active fragment (Figure 4a). In the presence of lactacystin, we found that procaspase-3 was not processed to the same extent as that seen for BFA/CHX alone. Proteasomal inhibition with Pefabloc, on the other hand, afforded a more complete block of caspase-3 maturation. Furthermore, we found that

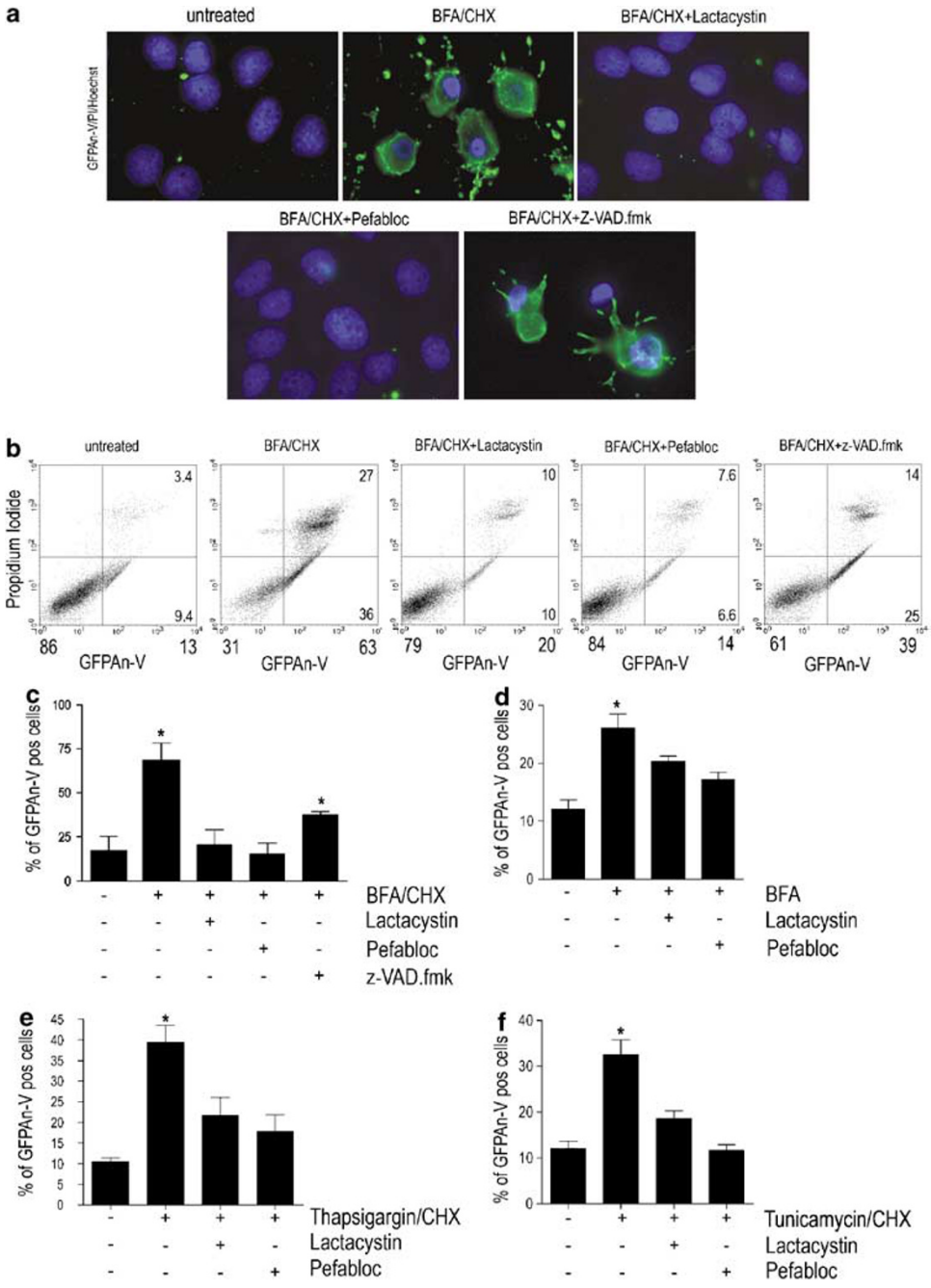

Figure 2 Inhibition of ER stress-induced exposure of PS by proteasomal inhibition. Rat fibroblasts were grown to confluence and left either untreated or exposed to ER stress. Cells were pretreated either with lactacystin (5 μM), Pefabloc (300 μM), or Z-VAD.fmk (100 μM), 30 min before cell stress. (**a**) GFPAn-V-, PI-, and Hoechst 33342-stained cells were visualized by fluorescence microscopy after 24 h treatment with BFA (5 μg/ml) CHX (10 μg/ml). (**b**) Representative flow cytometry dot plots from rat fibroblasts stained with GFPAn-V/PI after 22 h of ER stress. The average cell counts are listed in each dot-plot quadrant and are expressed as a percentage. (**c**) Statistical evaluation of apoptotic cells (GFPAn-V positive): Untreated ($N=34$), treated with BFA/CHX ($N=17$), or stressed and pretreated with either lactacystin ($N=16$), Pefabloc ($N=17$), or Z-VAD.fmk ($N=9$) 30 min before cell stress. Error bars correspond to S.E.M. *$P<0.001$ treated *versus* untreated group. (**d–f**) Statistical evaluation of apoptotic cells (GFPAn-V positive) after ER stress with BFA (10 μg/ml) alone (72 h, **d**), thapsigargin/CHX (24 h, **e**), or tunicamycin/CHX (48 h, **f**) and pretreated with either lactacystin or Pefabloc, 30 min before cell stress. Data were collected from three independent experiments. Error bars correspond to S.E.M. *$P<0.001$ treated *versus* untreated group

Z-VAD.fmk and Q-VD-OPh did not impose a complete block in ER stress-induced cell death (Figures 1 and 2, Ref.[11] and data not shown). This suggests that some fraction of the caspase-3 processing associated with BFA/CHX treatment might only be an epiphenomenon, and is not a key player in this pathway. Nevertheless, the fact that both lactacystin and Pefabloc decreased ER stress-induced caspase-3 maturation indicated that the proteasome lies upstream of caspase-3 processing. Moreover, we saw a similar processing pattern for caspase-2 (Supplementary

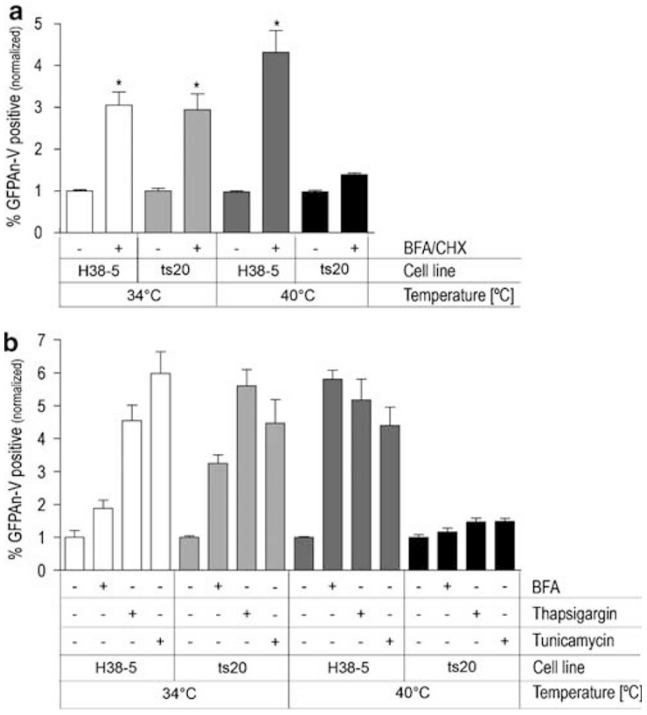

Figure 3 ER stress-induced cell death is delayed in E1-deficient cells. (**a**) Two cell lines, ts20 and H38-5, were treated for 30 h with BFA (10 μg/ml) and CHX (10 μg/ml). The ts20 cell line carries a temperature-sensitive E1 enzyme, whereas the H38-5 cell line is a derivative of the ts20 line in which E1 activity has been restored. Plots show the fold increase of GFPAn-V cells (determined by flow cytometry) normalized to the untreated condition in each case. H38-5, 34°C: ($N=25$), ts20, 34°C: ($N=24$), H38-5, 40°C: ($N=15$), ts20, 40°C: ($N=21$), error bars correspond to S.E.M., $^*P<0.001$ for treated *versus* the corresponding untreated control. (**b**) Cells were treated for 36 h with BFA (1 μg/ml), thapsigargin (100 nM), or tunicamycin (0.5 μg/ml), and the fraction of GFPAn-V-positive cells were determined by flow cytometry as in (**a**). For raw data see Supplementary Figure 2. Data were collected from three independent experiments, error bars correspond to S.E.M.

Figure 3), placing this caspase downstream of the proteasome as well.

We previously showed in our model of ER stress-induced cell death using the BFA/CHX cocktail that over expression of Bcl-2 was protective, indicating mitochondrial involvement in this pathway. Furthermore, BFA/CHX treatment resulted in cytochrome *c* release from mitochondria (Figure 4b, and Ref[11]). Thus, we asked the question whether the death-inducing activity of the proteasome could affect the outer-membrane permeability of the mitochondria. With the proteasome inhibitors lactacystin and Pefabloc, we followed the BFA/CHX-induced change in localization of cytochrome *c* from mitochondrial-associated punctate structures (Figure 4b) to a diffuse pattern. At 5 μM, lactacystin blocked the release of cytochrome *c* from mitochondria to the same extent as Pefabloc (300 μM), whereas Z-VAD.fmk (100 μM) had no effect. When co-stained with Hoechst 33342, cells with cytosolic cytochrome *c* also had condensed and fragmented nuclei typical of apoptotic cells. In contrast, the nuclei of untreated cells or stressed cells treated with either Pefabloc or lactacystin were normal in appearance (Figure 4b).

The fact that proteasome inhibitors prevented the release of cytochrome *c* from mitochondria in response to ER stress indicated that the death-promoting activity of the proteasome lies upstream of mitochondrial outer-membrane permeabilization. The Bcl-2 family members are the key regulators of mitochondrial outer-membrane permeabilization. We therefore expanded our search of proteasome-regulated apoptotic events to include Bcl-2 family members because it seemed plausible that in reponse to prolonged ER stress, the proteasome could function to degrade anti-apoptotic Bcl-2 family members. This would tip the balance of pro-apoptotic versus anti-apoptotic Bcl-2 family members towards programmed cell death. To test the possibility that proteasomal activity could cause degradation of anti-apoptotic Bcl-2 family members in response to ER stress, we followed the expression levels of Bcl-2, Bcl-w, and Bcl-xL by Western blot analysis. After treating cells for 24 h with BFA/CHX, we observed a dramatic reduction in Bcl-2, Bcl-w, and Bcl-xL protein levels. What is more, the reduced abundance of these proteins was reversed by proteasomal inhibition with lactacystin and pefabloc (Figure 4c). This effect did not require CHX in the experiment because extracts prepared from cells treated with BFA alone showed that Bcl-2 was degraded after 72 h of ER stress in a proteasome-dependent manner (Figure 4c). Whereas we did observe a loss of the full-length Mcl-1 during conditions of ER stress, we did not see an accumulation of this species when proteasomal inhibitors were included (data not shown).

Discussion

In the present study, we demonstrate a requirement for the proteasome in ER stress-induced cell death. We determined that the pro-apoptotic activity of the proteasome lies in a pathway upstream of Bcl-2, Bcl-w, and Bcl-xL degradation, mitochondrial release of cytochrome *c*, caspase-3 maturation, and phosphatidylserine exposure. In each case, the proteasome inhibitors lactacystin and Pefabloc are effective at blocking these markers of a pathway that connects protracted ER stress to cellular destruction. As further indication that the proteasome is a player in this pathway, we utilized a mutant cell line that harbors a temperature-sensitive allele of the E1 ubiquitin-activating enzyme. ER stress-induced cell death proceeded normally at the permissive temperature, but was blocked at the restrictive temperature, indicating that one of the functions of the ubiquitin proteasome system is to initiate a pro-apoptotic signaling cascade in response to prolonged ER stress.

Previously, ER stress-induced apoptosis was shown to be mediated by a serine protease-like activity that was blocked by Pefabloc, a general inhibitor of serine proteases.[11] We therefore sought to elucidate the identity of the Pefabloc-inhibitable activity required for ER stress-induced cell death. Toward this end, we developed two affinity labeling methods, one with biotinylated and the other with fluorogenic active site-directed serine protease inhibitors and subsequently analyzed the samples by mass spectrometry (Supplementary Figure 1). Although we were unable to identify any serine proteases in either approach, we repeatedly obtained hits for proteasomal subunits among the proteins found both in the

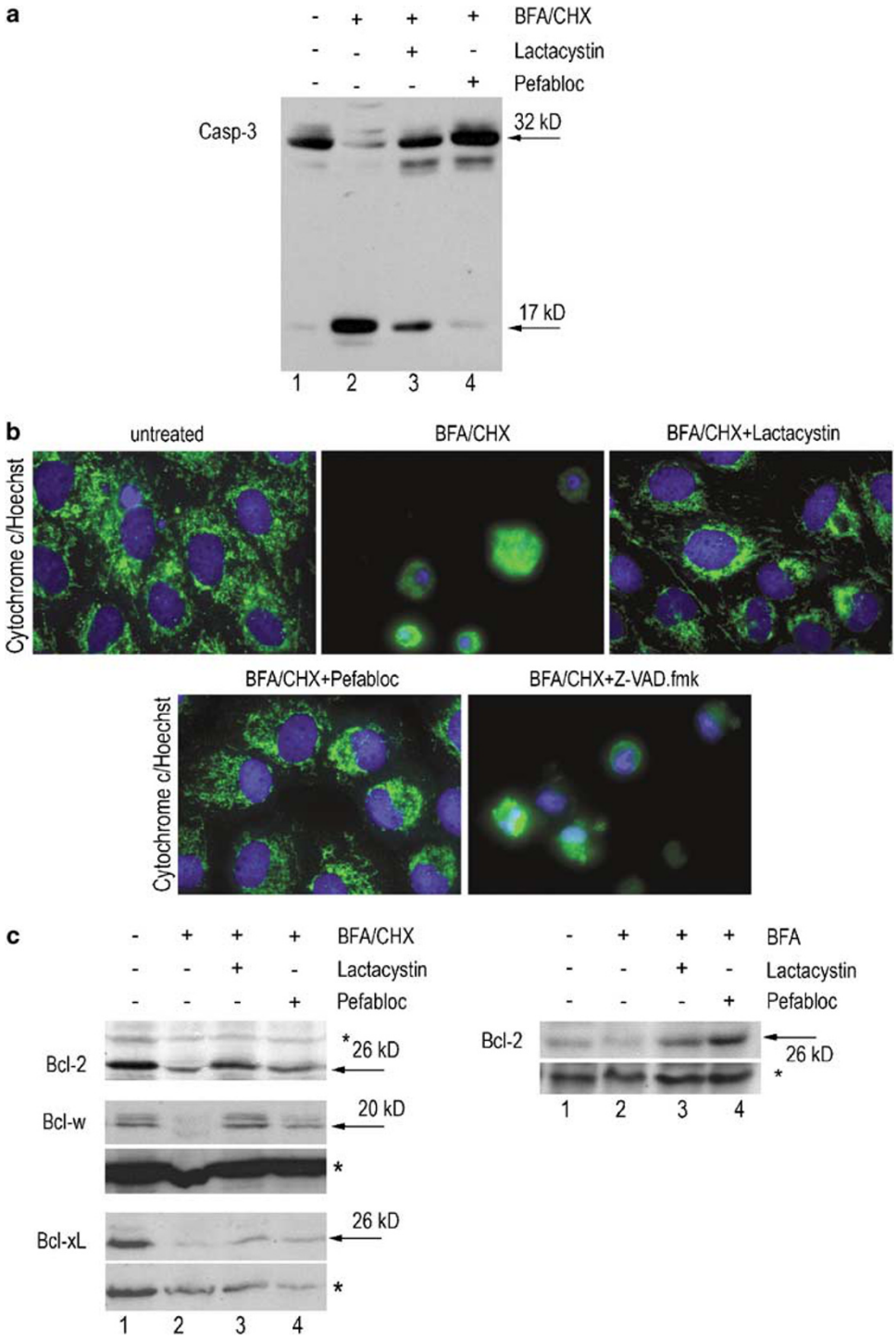

Figure 4 Proteasomal inhibitors block ER stress-induced caspase-3 processing, cytochrome *c* release, and degradation of antiapoptotic Bcl-2 family proteins. (**a**) Western blot showing pro- (p32) and processed- (p17) forms of caspase-3, in either untreated, treated with BFA (5 μg/ml) and CHX (10 μg/ml) for 24 h, or co-treated with either lactacystin (5 μM), or Pefabloc (300 μM). (**b**) Anti-cytochrome *c* immunocytochemistry and Hoechst 33342 stain of ER-stressed rat fibroblasts treated with BFA (5 μg/ml) and CHX (10 μg/ml) for 22 h. Cells were preincubated either with lactacystin (5 μM), Pefabloc (300 μM), or Z-VAD.fmk (100 μM) 30 min before cell stress. (**c**) Western blot showing the levels of Bcl-2, Bcl-w, and Bcl-xL, in either untreated, treated with BFA (5 μg/ml) and CHX (10 μg/ml) for 24 h, or treated with either lactacystin (5 μM), or Pefabloc (300 μM). Western blot showing Bcl-2 levels after BFA (10 μg/ml) treatment alone for 72 h with the same inhibitors. Unspecific bands from the same gel are shown as loading controls. Asterisks(*) mark the position of non-specific bands recognized by the antibody, which serve as loading controls. Arrows point to the position of molecular weight markers at the size indicated

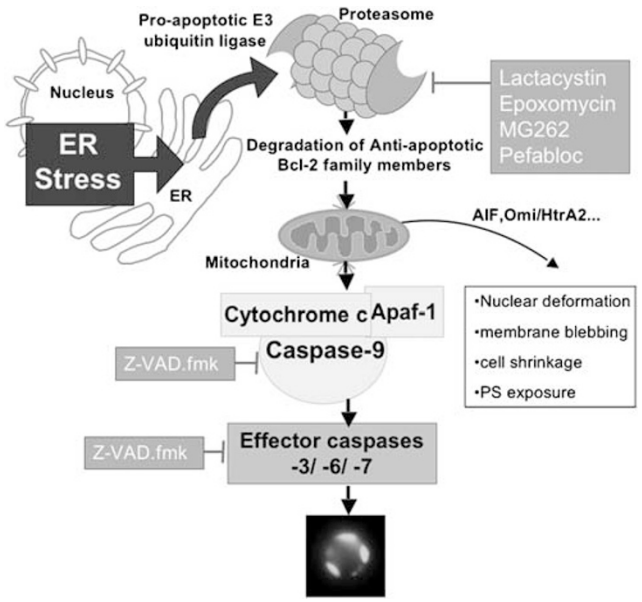

Figure 5 Putative model of proteasome-mediated ER stress-induced cell death. ER stress activates a pro-apoptotic ER ubiquitin ligase which tags anti-apoptotic Bcl-2 family members with ubiquitin. Subsequently, the proteasome degrades these anti-apoptotic molecules, thereby tipping the balance between pro-and anti-apoptotic factors towards apoptosis. Subsequently, mitochondria are activated, and pro-apoptotic factors such as cytochrome *c*, AIF, Omi, and others are released. As a result, caspase-dependent and independent signaling cascades ensue, eventually leading to cell death. Inhibition of proteasomal activity with lactacystin, epoxomycin, MG262, or Pefabloc prevents the degradation of anti-apoptotic factors, and therefore protect the cells from ER stress-induced cell death

biotinylated inhibitor-enriched fraction as well as those that were labeled by the fluorogenic active site-directed serine protease inhibitor, albeit near the limit of detection in both cases. That the proteaseome, a threonine protease, could be identified in our mass spectrometry approaches with serine protease inhibitors serving as bait is not surprising because several serine protease inhibitors, among them Pefabloc, have been shown to inhibit the proteasome non-selectively.[14,15] Whereas it was previously proposed that the inhibitory effect of Pefabloc on ER stress-induced cell death was due to inhibition of a serine protease,[11] we now believe that at the concentrations used (300 μM), Pefabloc inhibits the activity of the proteasome, and that proteasomal inhibition prevents cells from undergoing apoptosis in response to ER stress. This hypothesis is supported by the fact that Pefabloc treatment results in an accumulation of an engineered proteasomal substrate, GFP^u. However, we cannot exclude the possibility that additional Pefabloc-inhibitable serine protease activities might also play a role in the novel ER stress-induced apoptotic cascade described herein.

To date, the mediators that link ER stress to the apoptotic machinery have not been fully elucidated. Caspase-12 was proposed to function as the apical caspase responsible for initiating an apoptotic cascade in response to ER stress. However, *caspase-12*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were shown to be only partially protected from ER stress-inducing agents.[16] Furthermore, caspase-12 processing was shown to be downstream of the Bax/Bak gateway, as indicated by the fact that maturation of caspase-12 was blocked when *Bax*/*Bak* double knockout MEFs were treated with BFA.[9] These findings make it unlikely that caspase-12 could be a principal mediator of ER stress-induced cell death. CHOP/GADD153, the pro-apoptotic translational-dependent mediator of ER stress-induced cell death,[17] is not a player in our model system because we co-treated cells with CHX so as to bypass the expression of protective UPR genes, as was done previously.[8,11] Therefore, we have defined an ER stress-induced cell death pathway that is independent of CHOP/GADD153. We also excluded calpains from playing a role in this paradigm because none of the calpain inhibitors tested had any protective effect on ER stress-induced apoptosis (data not shown).

Our finding that the proteasome is a mediator of ER stress-induced cell death is surprising because many reports document that proteasome inhibitors alone are toxic,[18,19] and toxicity is exacerbated when cells also experience ER stress.[20] What is more, proteasomal inhibitors can actually cause ER stress, as shown by the upregulation of Grp78/Bip and CHOP/GADD153, thus providing further evidence that proteasomal activity is intimately coupled to ERAD as the last leg of the UPR.[21,22] Recently, a potent proteasome inhibitor, PS-341 (Bortezomib), was shown to be effective in the treatment of multiple myeloma, and is now approved by the US Food and Drug Administration. The toxicity of PS-341 in multiple myeloma cells is very likely to be the result of ER stress-induced apoptosis.[22]

Pro-apoptotic functions of the proteasome have been described in several instances including the constitutive death of neutrophils as well as NGF withdrawal, DNA damage, glucocorticoid treatment and reduced extracellular potassium.[23–27] Additionally, the proteasome has been implicated in the early stages of wallerian degeneration after axotomy.[28] In response to DNA damage, the large Bcl-2 homology domain-only protein Mule/ARF-BP1 was shown to ubiquitylate Mcl-1 – an anti-apoptotic Bcl-2 family member – thereby causing its degradation via the proteasome.[29] Also, the Bcl-2 family members Bcl-2, Mcl-1, and Bfl-1 have been shown to undergo proteasomal degradation when critical serine or threonine residues become dephosphorylated in response to treatment with paclitaxel or TNF.[30–35] In these examples, proteasomal activity tips the balance of pro- and anti-apoptotic Bcl-2 family members toward apoptosis, and thereby serves as a mediator of apoptosis.

The induction of UPR and ERAD in response to ER stress is protective. But at some point, after failing to achieve cellular homeostasis, cells induce programmed cell death. The mechanism of this switch is not fully understood, but likely involves the expression of CHOP/GADD153, which can cause downregulation of Bcl-2.[36] However, even under translation-inhibitory conditions, where CHOP/GADD153 is not expressed, we find that proteasomal activity is required for ER stress-induced cell death. Despite the fact that the prosurvival function of ERAD requires proteasomal activity, it seems that the later pro-apoptotic arm of ER stress also requires the activity of the proteasome. Therefore, the proteasome is involved both in protecting cells from ER stress (as in the case of ERAD) and in the activation of programmed cell death. We have speculated that these two opposing

activities of the proteasome could be the result of the alternative regulation of multiple E3 ubiquitin-ligase activities. In the protective phase of ER stress (UPR and ERAD), the E3 ubiquitin ligases SCF^{FBS1} and SCF^{FBS2} recognize misfolded proteins that have been dislocated from the ER, thereby directing the proteasome to degrade these malfolded - and potentially toxic-proteins.[5] Later, the cell could commit to cell death by upregulating an E3 ubiquitin-ligase activity that may target an anti-apoptotic molecule, such as the anti-apoptotic molecules Bcl-2, Bcl-w, and Bcl-xL. One candidate for such an activity is Mule/ARF-BP1, which has been shown to ubiquitylate Mcl-1, thus tagging Mcl-1 for proteasomal degradation and tipping the balance toward apoptosis.[29] However, we did not find that full-length Mcl-1 accumulates in the presence of proteasomal inhibitors (data not shown). Therefore, the putative ER stress-activated E3 ubiquitin-ligase activity is not likely to be Mule/ARF-BP1 (Figure 5).

Manipulation of the cell's decision to induce cell death in response to protracted ER stress may be possible with the development of specific E3 ubiquitin ligase inhibitors: Those that would inhibit SCF^{FBS1} and SCF^{FBS2} would be toxic, whereas those that would inhibit pro-apoptotic E3 ubiquitin ligases – like Mule/ARF-BP1 – would be protective. As evidence has emerged that ER stress plays a role in neurodegenerative diseases like Alzheimer's, Huntington's, Parkinson's and ALS,[37] reagents that block the proteasomal degradation of anti-apoptotic molecules may prove to be neuroprotective.

Materials and Methods

Cells and drug treatments. Rat 6 embryo fibroblasts (R6) were cultured in DMEM with 5% FCS, 1000 mg/l glucose, 60 μg/ml penicillin, and 100 μg/ml streptomycin (Mediatech, Inc. Herndon, US). The temperature-sensitive ts20 Balb/C 3T3 clone A31 fibroblast cell line and its E1 corrected H38-5 derivative were cultured in DMEM with 10% FCS, 4500 mg/l glucose, 60 μg/ml penicillin, and 100 μg/ml streptomycin.[38] Cells were maintained at 34°C. Where indicated, they were shifted to 40°C for 30 h before treatment with brefeldin A (BFA), or 36 h for treatment with tunicamycin or thapsigargin, with or without cycloheximide (CHX). Where indicated, cells were pre-incubated with 100 μM Z-VAD.fmk (MP Biomedicals, OH, US), 150–300 μM Pefabloc (AEBSF; Roche Diagnostics; Mannheim; Germany), 50 nM–5 μM lactacystin (Calbiochem, Darmstadt, Germany), 100 nM epoxomycin (EMD Bioscience, San Diego, USA), or 100 nM Z-Leu-Leu-Leu-B$(OH)_2$ (MG262; Biomol, USA) for 30 min before adding 5–10 μg/ml BFA and/or 10 μg/ml CHX alone (both drugs: Sigma, St Louis, MO, USA).

Counting of viable cells. Adherent cells from 10 randomly chosen fields ($\times$ 400 of magnification) were counted after 24 h of treatment with BFA/CHX in the absence or presence of inhibitors.

Preparation of cytosol. Stressed or non-stressed monolayer cells were scraped from 100 mm plates, washed in PBS and lysed by 3–4 cycles of freeze–thawing in buffer A (25 mM Hepes, pH 7.4, 2 mM EGTA, 2 mM $MgCl_2$, 2 mM DTT, 100 μM PMSF, 10 μg/ml leupeptin, 400 ng/ml pepstatin, 10 μg/ml aprotinin, 5 μg/ml cytochalasin B). The homogenate was centrifuged at 100 000 $\times$ g and the supernatant was used for Western blotting.

Preparation of total protein extracts. Media from stressed or non-stressed monolayer cells were collected and combined with the cells after trypsinization. After pelleting, cells were re-suspended in RIPA buffer (50 mM Tris, pH 7.5, 0.5% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl) containing protease inhibitors (complete, Mini; Roche; Penzberg; Germany). Extracts were used for Western blotting.

SDS-PAGE and Western blotting. Cytosol (40–50 μg) or total extracts were run on 10–20% SDS-PAGE, transferred to Nitrocellulose (Whatman; NJ; US) and immunodetected by anti-caspase-3 (Cell Signaling; Danvers; US), anti-Bcl-2, or anti-Bcl-xL (BD Biosciences, San Jose, CA, USA), anti-Bcl-w, or anti-caspase-2 (a kind gift from Andreas Strasser; WEHI; Melbourne, Australia), with primary antibodies followed by peroxidase-coupled secondary antibodies (Sigma, St Louis, MO, USA). Proteins were visualized by enhanced chemiluminescence (PIERCE, Rockford, USA).

Generation of His-GFP-Annexin-V and purification. His-GFP-Annexin-V (GFPAn-V) was generated by subcloning the GFP-Annexin-V cDNA[39] into the *Xho*I/*Bam*H1 sites of pET15b (Novagen , Darmstadt, Germany). The construct was transfected into the *Escherichia coli* strain BL21(DE3) and protein expression was induced with 1 mM IPTG at 25°C overnight. Bacteria were lysed in TBS-T (0.1% Tween), lysosyme (0.8 mg/ml), protease inhibitor cocktail (Roche; Penzberg; Germany), and Triton X-100 (1%). The protein was purified by a His-Bind® purification kit (Novagen) as provided by the manufacturer.

GFPAn-V- and PI-stainings. After growing cells to confluency on 12 mm glass coverslips, stressed, and unstressed cells were directly stained without prior washing (to avoid losing detached cells) with 3 μg/ml GFPAn-V and 2.5 μg/ml propidium iodide (PI, Sigma) in Annexin-V binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and subsequently fixed in 4% paraformaldehyde (PFA) containing 2 μg/ml of the Hoechst 33342 stain (Molecular Probes, Carlsbad, CA, USA). The coverslips were viewed under an Eclipse TE300 microscope (Nikon) and pictures were taken with a Photometrics, Roper Scientific Inc., Germany, Cool Snap EZ camera at a magnification of $\times$ 1000. For flow cytometry, media, and cells from untreated and treated samples were collected by trypsinization, stained with 3 μg/ml GFPAn-V and 2.5 μg/ml PI in Annexin-V binding buffer and incubated at room temperature for 15 min, diluted in 200 ml of AnnexinV binding buffer, and read on a BD LSR flow cytometer (BD Biosciences, San Jose, CA, USA). Data were processed with CellQuest Pro (BD Biosciences).

Mitochondrial cytochrome *c* release. Rat 6 cells were fixed on coverslips in 4% PFA and permeabilized for 5 min with 0.05% saponin and 15 min in ice-cold acetone. Cells were washed in PBS and incubated with primary anti-cytochrome *c* antibodies (BD) at a dilution of 1:200 in PBA (PBS with 1% bovine albumin). After incubation with secondary FITC-conjugated antibodies (Molecular Probes) 1:200 in PBA, cells were fixed in 4% PFA containing 2 μg/ml of Hoechst 33342 stain.

GFP^u transfection. By appending a short degron sequence, CL1[12] to the C terminus of GFP, the protein is constitutively degraded by the proteasome; this fusion protein is referred to as GFP^u.[13] Rat 6 fibroblasts (5×10^6) were mixed gently with 3 μg of GFP^u DNA,[13] electroporated with a Nucleofector Kit Number V for cell lines according to the manual (Amaxa; Köln; Germany). Electroporated cells were re-suspended in culture medium, seeded onto 12-well culture dishes at a density of 2.7×10^5 cells per well, and recovered overnight at 37°C before drug treatment.

Statistical analysis. Data passed the d'Agostino and Pearson omnibus normality test (K2) test for normal distribution and are represented as mean and S.E.M. Groups were compared by one-way ANOVA, followed by Tukey test ($P<0.0001$), using the Prism software package (version 4, GraphPad Prism).

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Supplementary Information accompanies the paper on Cell Death and Differentiations website (http://www.nature.com/cdd)