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Endoplasmic Reticulum Stress Induces a Caspase-dependent N-terminal Cleavage of RBX1 Protein in B Cells*[S]

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Background: ER stress occurs physiologically in the course of B cell activation.
Results: RBX1 is N-terminally cleaved in B cells in response to physiologically and pharmacologically induced ER stress. This occurs primarily by caspase-1. Expression of cleaved RBX1 in yeast confers hypersensitivity to ER stress.
Conclusion: The N terminus of RBX1 has a functional role.
Significance: Our data indicate a novel connection between ER stress, caspase-1, and ubiquitination.

Endoplasmic reticulum (ER) stress develops when the ER is overloaded with too many proteins to fold. This elicits a signaling pathway called the unfolded protein response. The unfolded protein response is physiologically required for the terminal development of B cells into antibody-secreting plasma cells. Ring Box Protein 1 (RBX1) is a 14-kDa protein necessary for ubiquitin ligation activity of the multimeric cullin ring ubiquitin ligases (CRLs). As RBX1 is shared by a large number of CRLs, alterations in its activity may lead to global changes in protein stability. We discovered that RBX1 is cleaved in the course of LPS-induced plasma cell differentiation and in multiple myeloma cell lines upon induction of pharmacological ER stress. The cleavage is executed by several caspase proteases that cleave RBX1 eight amino acids from the N terminus. To address the possible implication of RBX1 cleavage for CRL activity, we replaced the endogenous RBX1 homolog of the yeast *Saccharomyces cerevisiae*, Roc1, with the wild type or the N-terminal Δ8 mutant human RBX1. We show that yeast expressing the cleaved RBX1 are hypersensitive to ER stress and are impaired in CRL-mediated ubiquitination and degradation. We propose a model by which N-terminal cleavage of RBX1 impairs its activity and promotes susceptibility to ER stress induction.

The endoplasmic reticulum (ER)[3] is the port of entry of proteins into the secretory pathway. Eukaryotic cells developed mechanisms to adjust the amount of secretory pathway components to meet the cellular demand. This is driven primarily by conditions of ER stress, which ensue when the amount of client proteins that emerges into the ER exceeds its overall folding capacity. ER stress in eukaryotic cells activates a cytoprotective ER-to-nucleus signaling cascade that is collectively termed the unfolded protein response (UPR), aimed at alleviating the stress conditions (1). In budding yeast, the UPR is a linear signaling pathway that controls the expression of a large number of genes in response to ER stress (2). In mammalian cells, the UPR has diversified and consists of at least three parallel signaling pathways. Activation of the UPR is required for normal embryonic development (3–5), and its dysregulation postnatal may cause a wide range of disorders (6–9). Upon ER stress, the mammalian UPR operates in two consecutive waves. The first wave causes global inhibition of protein synthesis by controlling protein translation, followed by a second wave that involves gene transcription, whose products cooperate to minimize the level of unfolded proteins by various mechanisms (1).

Most proteins in eukaryotes are degraded by the ubiquitin proteasome system (UPS). Although proteasomes reside in the cytoplasm and nucleus, they are also responsible for the degradation of ER resident proteins. The pathway of ER protein degradation, referred to as ER-associated degradation (ERAD), entails protein dislocation from the ER to the cytoplasm, where they are subjected to ubiquitination and proteasomal degradation (10). The UPR is tightly connected to the ERAD machinery. Not only that the UPR induces the expression of multiple components of the ERAD machinery (11), ERAD in yeast is dependent on intact UPR (12). However, in mammalian cells the interplay between the UPR and ERAD pathways is less straightforward. Deletion of *Xbp-1*, the main transcription factor of the mammalian UPR, did not affect the degradation of ERAD substrates in B cells (13). Nonetheless, it is clear that many ERAD components, such as EDEM1, hHRD1, HERP, and others, are under UPR control (14). Therefore, it is assumed that the UPR promotes ERAD also in mammalian cells (15, 16). Whether the UPR also regulates the UPS in a more general fashion is not well defined.

ER stress and its consequential UPR play multiple physiological roles. It is well accepted that the UPR is essential for the development of professional secretory cells (17). This feature was initially exemplified by the requirement for Xbp-1 and Ire1 in the last developmental stage of B lymphocytes into antibody-secreting cells, termed plasma cells (5, 18). The UPR promotes

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[3] The abbreviations used are: ER, endoplasmic reticulum; CRL, cullin-Ring Ligase; ERAD, endoplasmic reticulum-associated degradation; RBX-1, Ring Box Protein 1; UPR, unfolded protein response; UPS, ubiquitin proteasome system; XBP-1, X-Box Binding Protein 1; Tm, tunicamycin; Tg, thapsigargin; CHX, cycloheximide; UPRE, UPR element.

the up-regulation of immunoglobulin synthesis and is required for the vast expansion of the ER membranes (19). This in turn may promote the level of potential ERAD substrates in a manner that further enhances the UPS, thus forming a positive regulatory loop. Moreover, several studies have demonstrated that in parallel to the expansion of the ER and up-regulation of Ig synthesis, proteasome levels drop in the course of B cell activation. In fact the levels of the proteasome drop to a critical level, which confers hypersensitivity to a further proteasomal inhibition (20). This was suggested to contribute to the elimination of the vast majority of activated B cells prior to completion of development into plasma cells and may explain the exquisite sensitivity of transformed plasma cells in the form of multiple myeloma to proteasome inhibitors in the clinics (21).

We decided to investigate whether components of the UPS, upstream to the proteasome, are also affected in the course of plasma cell differentiation. As the specificity of ubiquitin conjugation to its substrates is governed by E3 ubiquitin ligases, we sought to study the level of major cellular E3 enzymes in the course of B cell activation. The largest class of ubiquitin ligases is the cullin-RING ligases (CRLs), which operate as a multimeric E3s. CRLs contain a small RING finger-containing protein (RBX1 or RBX2), a cullin scaffold protein, and one or more additional cullin-specific interacting subunits that have substrate recognition and adaptor properties (22, 23).

The human genome contains hundreds of different CRLs that are further subdivided into the specific cullin in use. Although the adaptor proteins and the cullins differ in the various CRLs, they share the small RING finger proteins. Of the two homologs, RBX1 is dominant as RBX2 is induced following stress conditions (22, 24, 25). RBX1 is a 14-kDa protein necessary for CRL activity by providing the E2-binding site and by mediating the NEDDylation of the cullin, which activates its ligase activity, and by facilitating the ubiquitin transfer from the E2 to the substrates (26). Thus, changes in RBX1 availability may affect a large spectrum of protein degradation reactions.

Here, we show that in the course of B cell activation RBX1 undergoes an N-terminal proteolytic cleavage that is mediated by several caspase enzymes. Interestingly, RBX1 cleavage can be recapitulated by enforcing ER stress in multiple myeloma cell lines but much less in other cell types. Replacement of the yeast homolog of RBX1 with the truncated human RBX1 compromises its activity and sensitizes the cells to ER stress.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Tunicamycin (Tm) and thapsigargin (Tg) were purchased from Fermentek (Jerusalem, Israel). Both compounds were dissolved in DMSO and added at the indicated concentrations. The final DMSO concentration did not exceed 0.5% (v/v). LPS was purchased from Sigma. All solvents were of analytical grade. Rabbit polyclonal antibody against RBX1 (Ab2977) was purchased from Abcam (Cambridge, MA). Anti-V5 was purchased from Serotec (Kidlington, UK). Rabbit polyclonal antibody against p97 was provided by Dr. H. Ploegh (Whitehead Institute, Cambridge, MA). Anti-cullin1 and anti-Cdc53 polyclonal antibodies were purchased from Santa Cruz Biotechnology, and anti-ubiquitin polyclonal antibodies were from Dako (Glostrup, Denmark), and anti FLAG monoclonal antibody was from Sigma.

Plasmid Construction and Transient Transfection—Human RBX1 and its N-terminal truncated variants were cloned into pcDNA3.1(+) between BamHI/XhoI sites. 293T cells were transfected with 5 μg of plasmid using standard calcium-Phosphate methods. V5 tag, in which aspartic acid was replaced with glutamic acid (termed V5(D2E)) was added to the N terminus of Rbx1 by PCR and cloned in the same manner to pcDNA3.1. Expression vector for GST-Cul1 was provided by Dr. Zhenqiang Pan (Mount Sinai School of Medicine, New York). For the expression in yeast, hRBX1 or ΔRBX1 were cloned into pRS415 (CEN/LEU) and pRS416 (CEN/URA) yeast expression vectors, harboring a GPD promoter, between BamHI/XhoI sites.

In Vitro Recombinant Caspase Assays—Recombinant caspases kit was purchased from Calbiochem, and the assay was done as described previously (27). Briefly, 293T cells were lysed in protease buffer (100 mM Hepes, 10 mM DTT, pH 7.5). 35 μg of total protein (25 μl) were incubated for 1 h at 37 °C with 1 unit of each caspase. The reaction was stopped; sample buffer was added, and samples were processed by immunoblotting.

GST Fusion Protein Purification and in Vitro Caspase Assay—Human RBX1 and its site-directed mutants (harboring replacement of Asp to Ala) were cloned into pGEX6p-1 vectors in-frame to GST. Competent BL21 bacteria were transformed with the vectors, and protein expression was induced by IPTG for 4 h at 37 °C. Bacteria were lysed in STE lysis buffer (150 mM NaCl, 10 mM Tris (pH 8), 1 mM EDTA), which contained 1.5% of sodium lauryl sarcosine. Following sonication and clearing by centrifugation (10,000 × *g*, 30 min, 4 °C), lysates were loaded onto glutathione beads (GST·Bind resin, Novagen) and incubated at 4 °C for 90 min. The beads were then washed three times with TGEM1 buffer (NaCl 1 M, 20 mM Tris-HCl (pH 7.9), 20% glycerol, 1 mM EDTA, 5 mM magnesium chloride, 0.1% Nonidet P-40) followed by washes with TGEM0.1 buffer (as TGEM1, except 0.1 M NaCl). Beads were kept at 4 °C as 50% v/v suspension in TGEM0.1 until the experiment. Equal amounts of proteins were incubated with 20 units of rCaspase3 according to the manufacturer's protocol for 2 h at 37 °C.

Cell Culture and Cell Lines—Mature B cells were purified from mouse splenocytes by magnetic depletion with anti-CD43 (Miltenyi Biotec). Cells were plated at 1.5×10^6 cells/ml in complete medium containing RPMI 1640 (Invitrogen) supplemented with 10% FBS (Biological Industries, Israel), 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 50 μM β-mercaptoethanol, 25 mM 1× nonessential amino acids, and 1 mM sodium pyruvate (Biological Industries). RPMI8226 cells were propagated in the B cell medium. HEK293T cells were maintained in DMEM (Invitrogen) supplemented with 10% FCS, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml of streptomycin (Biological Industries). Cells were maintained at 37 °C in a 5% CO_2 incubator.

Western Blot Analysis—Cells were washed twice with cold PBS, and whole cell lysates were prepared in 1% SDS. The lysate was sonicated briefly and cleared by centrifugation. Total protein concentration was determined using the BCA protein assay reagent kit (Pierce). Following SDS-PAGE analysis under reducing conditions, gels were electrotransferred to PVDF

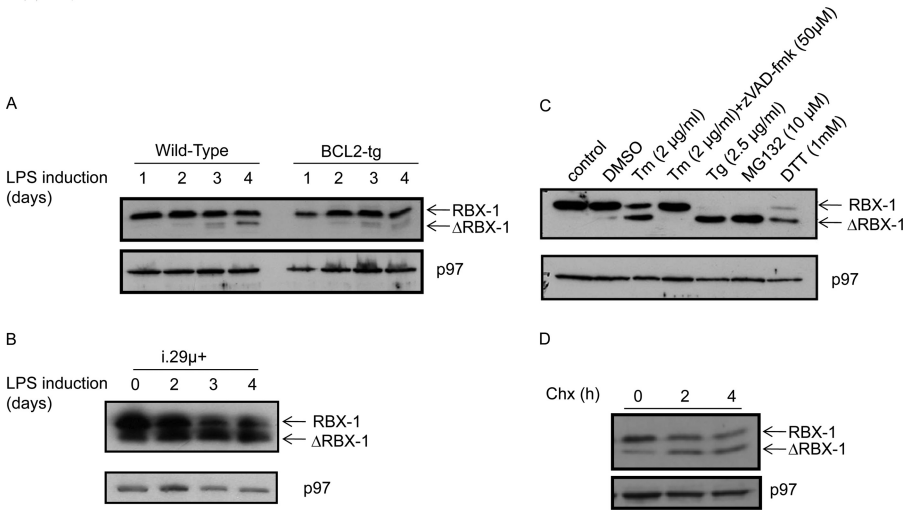

FIGURE 1. **Rbx1 is cleaved during B cell differentiation and ER stress induction.** *A,* B cells were purified from spleens of WT or *Bcl2* Tg mice and incubated for up to 4 days with LPS. Total cell lysates were prepared, analyzed on 15% SDS-PAGE, and blotted with anti-RBX1 antibody. *B,* i.29μ+ cells were incubated with LPS, and samples were analyzed as in *A*. *C,* RPMI8226 cells were treated overnight with the indicated compound. Total cell extract was prepared and analyzed as in *A*. *D,* RPMI8226 cells were treated overnight with 2.5 μg/ml and subjected to CHX treatment for up to 4 h. Samples of equal numbers of cells were withdrawn and analyzed for RBX1 as in *A*. Shown are typical experiments of at least three repetitions.

membranes. Membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 (TBST), 5% milk powder and probed with the specific antibodies, followed by secondary horseradish peroxidase-conjugated antibodies. ECL reagents (Biological Industries, Beit Haemek, Israel) were used to develop the blots by chemiluminescence. p97 was used for controlling protein loading.

Roc1 Shuffle System—Replacement of wild type Roc1 with mutant variants was done by the gene shuffling technique. First, a single copy of Roc1 open reading frame was knocked out in diploid yeast cells. This was done by PCR amplification of a fragment of the *KanMX4* gene with flanking ends compatible to Roc1 open reading frame, followed by transformation. Because *Roc1* is an essential gene, only one of the two *Roc1* copies in the diploids was deleted. Next, a pRS416 (CEN/URA) plasmid containing hRBX1 and a GPD promoter was then transformed into the Roc1-deleted diploid strain, followed by sporulation induction. Spores lacking the *Roc1* gene but expressing hRBX1 from a plasmid were separated by tetrad dissection and selected for G418 resistance and growth on SD-URA media. The resulting haploid strain served as a founder strain for expressing the various RBX1 derivatives. hRBX1 or ΔRBX1 on a pRS415 (CEN/LEU) plasmid replaced hRBX1 on pRS416 by transformation, followed by selection on SD-Leu and on 5-fluoroorotic acid to remove the Ura-expressing plasmid.

β-Gal Activity Assay—Yeast cells were transformed with a plasmid containing β-gal, with a UPRE promoter, as described previously (11). Cells were grown to mid-log phase; Tm was added (2 μg/ml), and samples were collected at the indicated time points. The samples were spun down for 30 s at 14,000 × *g*, and the pellet was resuspended in 1 ml of buffer Z (16.1 g/liter $Na_2HPO_4{\cdot}7H_2O$, 5.5 g/liter $NaH_2PO_4{\cdot}H_2O$, 0.75 g/liter KCl, 0.246 g/liter $MgSO_4{\cdot}7H_2O$, 2.7 ml/liter β-mercaptoethanol). Three drops of chloroform and two of 0.1% SDS (w/v) were added. Samples were vortexed and incubated at 30 °C for 5 min. Then 200 μl of *o*-nitrophenyl β-D-galactopyranoside (4 mg/ml in water) was added, and samples were incubated at 30 °C until color change. For reaction termination, 0.5 ml of 1 M Na_2CO_3 was added; cell debris was removed by centrifugation, and absorbance was determined at 405 nm.

Measurement of Protein Turnover—Yeast cells were transformed with an HA-Hac1p-expressing vector (kindly provided by Dr. Peter Walter, University of California, San Francisco). Cells were grown to mid-log phase, when cycloheximide (CHX) was added at 0.5 mg/ml. 1 *A* of yeast culture (600 nm) was collected at the indicated time points. Samples were lysed in protein sample buffer, loaded on SDS-PAGE as described above, and immunoblotted with anti-HA antibody (Roche Applied Science).

Caspase-1 Activity Assay—Cells were treated as described. Caspase activity was determined using the commercial SensoLyte™ AFC Caspase profiling kit (AnaSpec, CA) according to manufacturer's orders.

RESULTS

Rbx1 Is Cleaved during LPS-driven B Cell Differentiation and in Multiple Myeloma Cell Lines upon ER Stress—Activation of naive splenic B cells with LPS *in vitro* recapitulates many of the features seen for plasma cell differentiation *in vivo*. Within 3–4 days of stimulation, B cells expand their ER and acquire secretory capacity for antibodies (13). To test the possible effect of LPS treatment on RBX1 expression, primary splenic B cells were incubated in the presence of LPS for 3 days. When the expression of Rbx1 was analyzed by immunoblotting, besides the intact Rbx1, an additional protein with lower molecular weight reacted to the antibody (Fig. 1*A*). Because this protein appeared at the late stages of LPS stimulation, we hypothesized that the differentiation state of the cells dictates its appearance rather than LPS signaling *per se*. In the course of LPS stimulation, a portion of the B cells succumb to apoptosis, which may promote the expression of the smaller RBX1 in a manner not related to the differentiation of plasma cells. These apoptotic

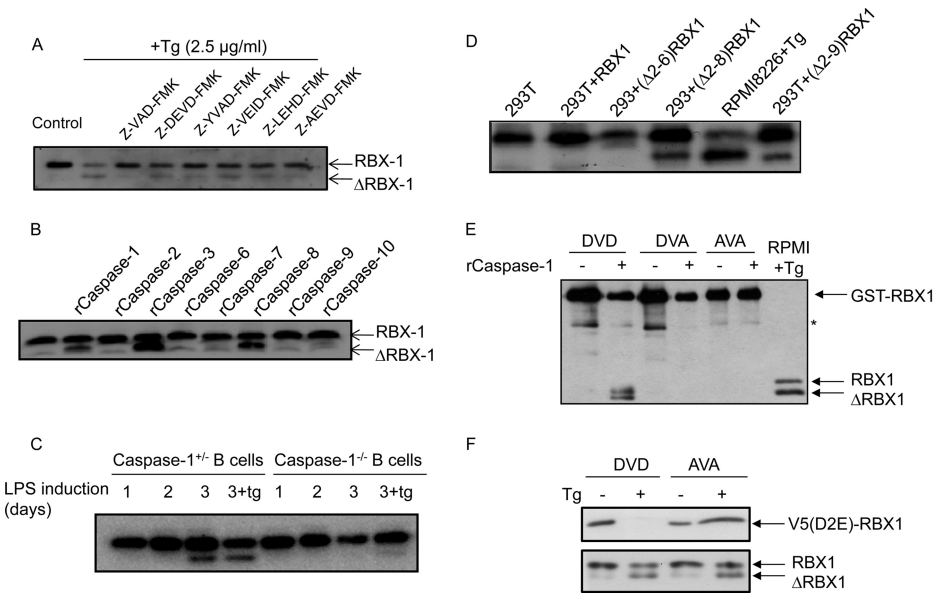

FIGURE 2. **RBX1 is cleaved by caspases after Asp-8.** *A,* RPMI8226 cells were treated with 2.5 μg/ml Tg, and in the presence of the indicated caspase inhibitors (50 μM), RBX1 cleavage was assessed by immunoblotting of cell lysates with anti-RBX1. *B,* 293T cells were lysed in protease buffer. 35 μg of total proteins incubated each of the caspases individually, and reaction was stopped by boiling in sample buffer. The reaction was then analyzed by Western blotting with anti-RBX1 antibody. *C,* splenic B cells were purified from heterozygous or caspase-1 KO mice and incubated for 3 days with LPS. 2.5 μg/ml Tg was added at the last 24 h where indicated. Cell lysates were separated on 15% SDS-PAGE and blotted with anti-RBX1 antibody. *D,* pcDNA3.1 vector expressing the indicated RBX1 mutant was transfected into 293T cells, and following 48 h cell lysates were analyzed by Western blotting with anti-RBX1. *E,* recombinant GST-RBX1 with Asp residue substitutions at position 6 and/or 8 were expressed in BL21 *E. coli* and incubated with rCaspase-1. Cleavage was detected using Western blotting with anti-RBX1. *F,* pcDNA3.1 that encodes V5(D2E)-tagged Rbx1 or its AVA mutant was electroporated into RPMI8226 cells and treated or not overnight with Tg. Total cell extracts were immunoblotted for V5 and Rbx1.

cells are eliminated in B cells derived from mice transgenic for *Bcl2* under the Ig promoter (28). Interestingly, the smaller Rbx1 protein was also observed in *Bcl2* Tg B cells (Fig. 1*A*). This indicates that the change in Rbx1 expression is most likely not related to the apoptotic cells, rather to the physiological process of plasma cell differentiation. The reduction in proteasomal activity was demonstrated in the lymphoma i.29μ+ cells upon LPS stimulation (20). We therefore examined whether these cells also display the lower band of RBX1 in an LPS-dependent manner. Indeed, a smaller RBX1 polypeptide was already observed at nonstimulated conditions. As the stimulation proceeded, the relative levels of the smaller RBX1 increased, similar to their occurrence in the primary cells (Fig. 1*B*).

One of the hallmarks of plasma cell differentiation is the involvement of ER stress, which develops as of day 2 (29), exactly when the additional form of RBX1 appeared. We therefore hypothesized that ER stress may be the underlying cause for this observation. We decided to test this hypothesis directly by applying various modes of drug-induced ER stress to the multiple myeloma cell line RPMI8226. We detected the shorter version of RBX1 following treatments with Tm, Tg, the proteasome inhibitor MG132, and DTT, indicating the direct involvement of ER stress in its generation (Fig. 1*C*).

Two main options may underlie the production of the smaller RBX1 protein. It is either a newly synthesized protein, whose synthesis is instigated by ER stress, or rather a proteolytic cleavage product of the full-length RBX1. To distinguish between these possibilities, we performed a CHX chase assay. Accumulation of the new band in the course of the chase would indicate a proteolytic cleavage as the synthesis of new proteins is arrested. RPMI8226 cells were treated with Tg in a manner that robustly induced the smaller RBX1. Cells were then treated with CHX for 4 h. The shorter form of RBX1 accumulated over time, indicating that the smaller RBX1 is not a newly synthesized protein but rather is a cleavage product of pre-formed RBX1 (Fig. 1*D*). Following this observation, we examined the capability of several protease inhibitors for their potential to block the cleavage of RBX1. We found that following Tm treatment, the co-application of the pan-caspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone substantially abrogated the generation of the cleaved RBX1 protein (Fig. 1*C*, *4th lane*). Thus, caspases are likely to be the proteases that cleave RBX1 under ER stress.

Specific Caspases Remove 8 Amino Acids from the N Terminus of RBX1—As caspase enzymes are diverse in their biological roles and substrate specificity, we wanted to know which of the caspases are involved in the cleavage of RBX1 and where exactly the cleavage occurs. To this end, we used a set of tetrapeptide caspase-specific inhibitors. RPMI8226 cells were treated with Tg overnight in the presence or absence of the different caspase inhibitors (50 μM). All tested inhibitors exhibited inhibitory effect on the cleavage to various degrees (Fig. 2*A*). Thus, this experiment did not produce valuable information on the identity of the specific caspases involved. This is probably due to the limited specificity of the tetrapeptide inhibitors. We therefore decided to examine the ability of recombinant caspases to cleave RBX1 in cell lysates. Lysates from 293T cells were incubated eight different recombinant caspases, and RBX1 cleavage was examined by immunoblotting. Of the eight different caspases tested, caspases-1, -3, and -8 cleaved RBX1 (Fig. 2*B*).

Caspases-3 and -8 are known to play a pivotal role in programmed cell death. However, the cleavage of RBX1 by

caspase-1 was surprising, because this enzyme belongs to the inflammatory caspases that primarily engage in the cleavage of the proform of IL-1β (30) and IL-18 (31, 32). To elucidate the possible role of caspase-1 in the cleavage of Rbx1 in primary B cells, we analyzed splenic B cells from caspase-1 knock-out mice. B cells were extracted and incubated with LPS for 3 days. On the last day, we enhanced the cleavage of Rbx1 by Tg treatment. We found that the cleavage of Rbx1 was substantially reduced in caspase-$1^{-/-}$ B cells, compared with the heterozygous mice. However, caspase-1 is not the sole RBX1 protease, because the cleaved form was still detected in the KO cells after Tg treatment, albeit to a lesser extent (Fig. 2*C*). We conclude that in primary B cells RBX1 is cleaved, at least in part, by caspase-1. To further substantiate the role of caspase-1 in the cleavage of RBX1, we tested Rbx1 cleavage in Raw246.7 cells that secrete IL-1β upon LPS stimulation (33) and hence activate caspase-1. Indeed, upon their stimulation with LPS, Rbx1 cleavage was discerned (supplemental Fig. S1). To corroborate the involvement of caspase-1 in the ER stress-induced cleavage of Rbx1, we performed activity assays for caspase-1 using fluorogenic peptides. Caspase-1 activity was increased by 20-fold upon induction of ER stress conditions, which is ~6-fold higher than treatment with etoposide, which is a well established activator of apoptosis (supplemental Fig. S2).

Because the anti-RBX1 antibodies used in our study were raised against the C terminus of the protein, the cleavage must occur at the N terminus. Caspases cleave exclusively after an aspartic acid residue, and RBX1 contains five aspartic acids. The first two are at positions 6 and 8. The third one in the sequence is far downstream at position 40. Based on the size difference between the cleaved RBX1 and the full-length protein, we estimated the cleaved fragment to be about 1 kDa. Accordingly, the cleavage is most likely to occur at positions 6 or 8. To map the specific RBX1 cleavage site, we have constructed several vectors encoding full-length RBX1 and N-terminal truncations. 293T cells, in which RBX1 is not cleaved endogenously (data not shown), were transfected with the different RBX1-encoding vectors, and samples were analyzed by SDS-PAGE and immunoblotting for RBX1. Deletion of amino acids 2–8 yielded a fragment with indistinguishable mobility as endogenous cleaved RBX1 of RPMI8226 cells (Fig. 2*D*). A deletion of an additional amino acid (Δ2–9) resulted in a protein slightly smaller than the cleaved RBX1. Based on these data, we mapped the cleavage site to the aspartic acid at position 8.

To further support this conclusion, we expressed a GST fusion of full-length RBX1 (denoted as DVD) and aspartic acid mutants in bacteria. In the mutants, the second aspartic acid was replaced with alanine (DVA) or both (AVA). RBX proteins were isolated from the bacterial lysates using glutathione-conjugated Sepharose beads and then subjected to on-bead cleavage reaction with recombinant caspase-1. Only WT RBX1 was cleaved, resulting in the release of two polypeptides as follows: one at the expected size and a second one slightly smaller that migrated at the front of the gel (Fig. 2*E*). The nature of the smaller fragment is unknown; however, both mutant proteins lacking the second aspartic acid were left intact, thus reinforcing the identification of the cleavage site as Asp-8. To verify that the AVA mutant is indeed resistant to cleavage in live cells, it

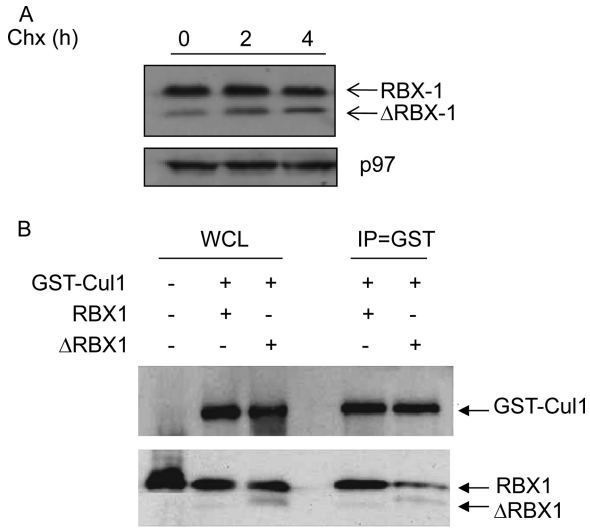

FIGURE 3. **ΔhRBX1 binds to Cul1.** ΔRBX1 is stable upon transfection with pcDNA3.1 that encodes ΔRBX1, followed by immunoblotting in the presence of CHX for 4 h (*A*). Plasmids expressing GST-cullin1 and RBX1 or ΔhRBX1 were co-transfected into 293T cells. Cells were harvested after 48 h. After mild lysis, whole cell extracts (*WCL*) were pulled down with GST beads. Samples were separated on 10 and 16% SDS-PAGE and blotted with anti-Cul1 and anti-RBX1 antibodies, respectively (*B*).

was required to express a tagged version of the WT and AVA in cells to distinguish them from endogenous RBX1. We searched for a tag that does not contain an aspartic acid residue to avoid confounding issues of cleavage of the tag itself by caspases. After screening several epitope tags, V5 was selected. V5 contains a single aspartic acid residue *versus* FLAG or HA tags that contain multiple residues. Replacement of the aspartic acid with glutamic acid, termed V5(D2E), does not affect recognition by the V5 monoclonal antibody. The V5(D2E)-tagged WT (DVD) and AVA mutants of Rbx1 were cloned into expression vectors and transfected by electroporation into RPMI8226 cells. Cells were then treated or not with Tg to promote the cleavage of RBX1. Transfection yield was no greater than 10%. Although the effect on endogenous RBX1 was similar for both transfectants, the tagged WT Rbx1 disappeared upon Tg treatment, because of the cleavage, whereas the AVA mutant was resistant to the treatment (Fig. 2*F*). This indicates directly that cleavage of RBX1 occurs at the aspartic acid residues of the N terminus. Combined, our data implicate the second aspartic acid as the site of cleavage. We termed the cleaved RBX1 at position 8 as ΔRBX1.

Δ*RBX1 Maintains Its Association with cullin 1 (Cul1)*—Next we examined whether the Δ8 truncation affects RBX1 association with the CRL E3 ligase complex. To operate as an activator for CRL E3 ligases, RBX1 must associate with the cullin scaffold protein. In fact, unbound RBX1 is rapidly removed by proteasomal degradation, most likely because of self-ubiquitination (34). To assess whether ΔRBX1 remains associated with the cullin scaffold proteins, we initially examined its stability by CHX chase. 293T were transfected with pcDNA3.1 that encodes ΔRBX1, and its turnover was estimated by immunoblotting. We observed that ΔRBX1 was stable, inferring association with the cullins (Fig. 3*A*). To test RBX1 direct interaction with the cullin, we transfected 293T cells with expression vector that encodes GST-Cul1 and pcDNA3.1-RBX1 or

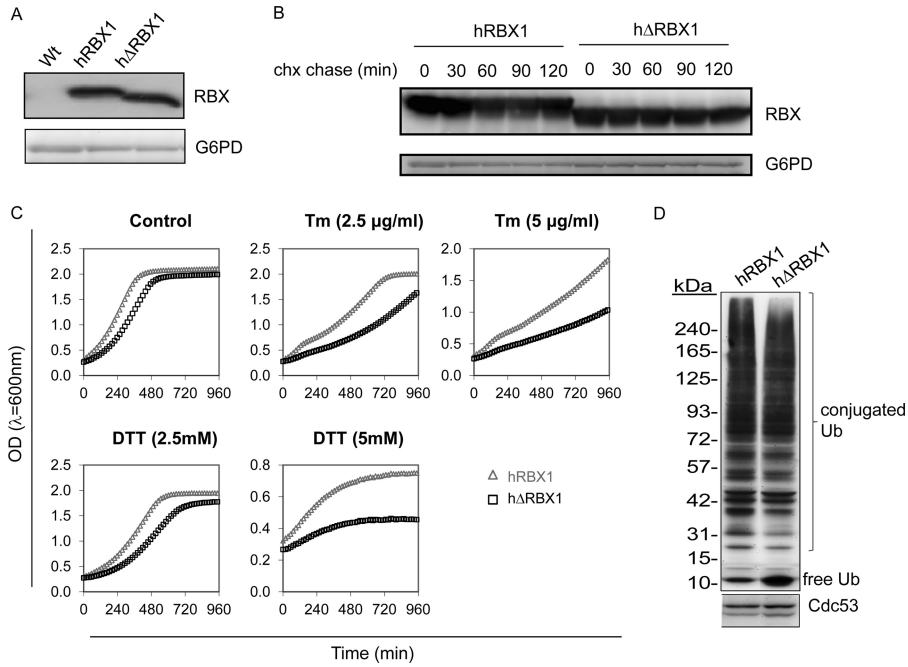

FIGURE 4. **Yeast expressing hΔRBX1 are hypersensitive to ER stress.** Yeasts deleted of their endogenous homolog of RBX1 and transgenic for either RBX1 or ΔhRBX1 were lysed and immunoblotted with anti-hRBX1 antibody, where wild type yeast served as a negative control (*A*). Both RBX1 forms were stable for 2 h during CHX chase (*B*). Growth tests were performed using a Tecan plate reader in a flat-bottomed 96-well plate. Absorbance measurements were taken in 15-min intervals. Chemicals were added to the wells at $t = 0$ (*C*). Shown is immunoblot analysis for ubiquitin and Cdc53 from hRBX1 or ΔhRBX1 expressing strains, harvested at the logarithmic growth phase at equal absorbance. Densitometry analysis of four repeats shows ~15% reduction in ubiquitination in the ΔhRBX1 strain and a similar increase at the level of free ubiquitin (*D*).

pcDNA3.1ΔhRBX1. Cells were lysed using mild detergent, and the lysates were subjected to a GST pulldown. Western blot analysis revealed that both the full-length and the ΔhRBX1 proteins co-precipitate with GST-Cul1 (Fig. 3*B*), suggesting that N-terminal cleavage does not interfere with binding to the cullin.

ΔhRBX1 Confers Reduced Polyubiquitination and Higher Sensitivity to ER Stress Conditions in Saccharomyces cerevisiae—RBX1 protein is highly abundant, and attempts to overexpress the mutated ΔhRBX1 by standard transfection methods did not yield a sufficient expression level to confer a clear phenotype on top of the wild type. Because human RBX1 is homologous to the budding yeast Roc1 protein and can fully reconstitute Roc1-deficient strains (35), we decided to explore the functionality of ΔhRBX1 in *S. cerevisiae*. This approach was appealing because the budding yeasts do not have caspase activity; thus RBX1 is not expected to undergo proteolytic cleavage in this cellular system. Applying gene shuffling techniques, endogenous Roc1p was replaced with hRBX1 or ΔhRBX1. Under these conditions both strains were viable, indicating that ΔhRBX1 complements Roc1 deficiency in yeast.

Next, hRBX1 and ΔhRBX1 expression was verified using immunoblotting with anti-hRBX1 antibody. Each strain exclusively expressed its transduced form (Fig. 4*A*), and both were expressed to similar levels. Proteolytic stability was examined in both strains using CHX chase. We found that both proteins were stable in the course of the chase (Fig. 4*B*). Next, the growth of hRBX1 and ΔhRBX1 strains under various stress conditions was compared. Under normal incubation conditions, both strains showed similar kinetics of replication (Fig. 4*C*, *left panel*). However, induction of ER stress with either Tm or DTT caused a delayed and impaired replication in the ΔhRBX1 strain (Fig. 4*B*). Interestingly, other stress inducers, such as 3% EtOH or heat shock, did not attenuate ΔhRBX1 cell growth compared with the wild type (supplemental Fig. S3). These data suggest that RBX1 cleavage specifically causes hypersensitivity to ER stress

Because in yeast CRLs are the largest E3 family members, we wanted to test if the expression of ΔhRBX1 in yeast affects the overall ubiquitination pattern in a way that contributes to the growth sensitivity phenotype. We therefore analyzed the level of ubiquitination at the logarithmic growth phase by probing total cell extracts with anti-ubiquitin antibodies (Fig. 4*D*). Ubiquitin conjugates in the ΔhRBX1 strain were lower by 14.5% compare with the full-length RBX1-expressing cells (S.D. = 3.38). This was accompanied by a corresponding increase in the cellular pool of the free ubiquitin (Fig. 4*D*). These data links the N-terminal region of RBX1 with the ubiquitination process.

The basis for hypersensitivity to ER stress of the ΔhRBX1 strain might be related to changes in the intracellular distribution of the CRLs. To test this possibility, yeast microsomes were purified, and RBX1 association with the microsomal fraction was examined. We found that a large portion of RBX1 was tightly associated with the microsome fraction (~50%). To assess the strength of RBX1 association to the ER membrane, we mixed the microsomes in a 1:1 ratio from both strains and subjected them to a wash with increasing concentrations of NaCl. We found that 1 M of NaCl was needed to fully dissociate RBX1 from the membrane, indicating that CRLs are strongly attached to the ER. However, the relative ratio between RBX1 and ΔRBX1 remained similar in the microsomal fractions under the salt wash whether ER stress was inflicted by Tm or

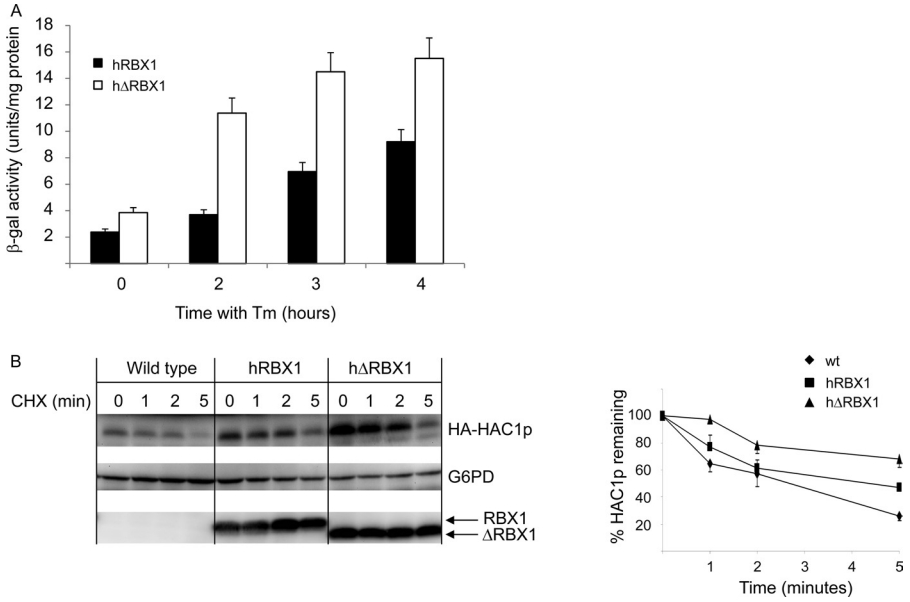

FIGURE 5. **ER stress causes higher UPR induction in ΔhRBX1-expressing cells.** Yeast cells were transformed with a plasmid encoding β-gal, under the control of a UPRE promoter. Samples were collected at the indicated times after ER stress induction by Tm (2 μg/ml). β-Gal activity was assayed and normalized to the sample total protein concentration (*A*). Yeast were transformed with HA-tagged HAC1p, and CHX (0.5 mg/ml) was added to mid-log phase culture. Samples were collected for immunoblotting at indicated time points. Hac1p levels were quantified and normalized against glucose-6-phosphate dehydrogenase (*G6PD*) levels (*B*). Immunoblotting to hRBX1 was performed to confirm strain identity.

not (supplemental Fig. S4*A*). We conducted a similar experiment with RPMI8226 under Tg treatment and saw a similar trend (supplemental Fig. S4*B*). This indicates that the hypersensitivity to ER stress in ΔRBX1 cells is not due to impaired association with the ER membrane.

UPR Is Hyperactivated in the ΔhRBX1 Strain upon ER Stress—In yeast, upon ER stress, *HAC1* mRNA is spliced to yield the transcription factor Hac1p, which activates transcription of genes containing UPRE in their promoter. It is therefore possible to assess the level of UPR activation by using a UPRE containing promoter linked to a reporter gene (11). RBX1 or ΔhRBX1 strains were transformed with a UPRE-controlled β-galactosidase (gal) construct, and β-gal activity was measured at several time points after the addition of Tm. As anticipated, β-gal activity was induced faster and stronger in the ΔhRBX1-expressing strain, compared with cells expressing the intact protein (Fig. 5*A*). This raises the question whether the increase in the levels of UPR induction is due to stabilization of Hac1p, which is known to be mediated by the Cul1 CRL, SCF-cdc34, in a Roc1-dependent manner (36). To examine it, HA-tagged Hac1p was transformed into the cells, and by using CHX chase experiments, we showed that the rate of Hac1p degradation was slightly slower in the ΔhRBX1 strain (Fig. 5*B*), compared with wild type and hRBX1 strains. Hac1p half-life is ~2 min for wild type and hRBX1 strains and is more than 5 min for ΔhRBX1. This was also reflected in higher Hac1p levels at steady state. These data suggest that ΔhRBX1-containing CRLs are less efficient in protein ubiquitination, and thus degradation, and may explain their hypersensitivity to ER stress.

DISCUSSION

The UPR was originally described as a cellular measure against ER stress conditions. However, accumulating evidence demonstrates that in mammals the ER stress and its consequential UPR control biological pathways not strictly related to protein folding in the ER. In this respect the UPR was shown to affect signaling pathways, cell cycle, apoptosis, and autophagy (reviewed in Ref. 37). Numerous studies connected the UPR to protein degradation primarily via the ERAD pathway (14). However, it is reasonable to assume that ER stress and the UPR may also manipulate the UPS in a more general manner.

In humans, CRLs are a family of over 300 ubiquitin ligases and require either RBX1 or RBX2 for their function. Of the two RING proteins, RBX1 is the major one as it is essential for the function of Cul1, Cul2, Cul3, Cul4A, and Cul4B. It performs two activities as follows: NEDDylation of the cullin and recruitment of the ubiquitin-conjugating enzyme. For this purpose, RBX1 juggles between two E2 enzymes, the NEDD8-specific E2s (Ubc12 or UBE2F) and the ubiquitin E2s. Accordingly, RBX1 adopts several configurations (38, 39), and in all of them RBX1 maintains association with the cullin via its RING domain. Whether the N terminus of RBX1 is required for its functions has not been rigorously explored.

We found that 8 amino acids are clipped from the N terminus of RBX1 in an ER stress-driven pathway in the course of B cell activation by LPS (Fig. 1). *In vitro*, the cleavage is mediated by caspase-8, an initiator of apoptosis, caspase-3, the executor of apoptosis, and caspase-1, an inflammation-related caspase. Unexpectedly, in B cells caspase-1 is most likely the predominant one (Fig. 2). In myeloid cells, caspase-1 is activated by the inflammasomes in response to stimulation of pattern recognition receptors (40). In contrast, evidence that naive B cells contain inflammasomes and caspase-1 is missing. However, various pharmacological treatments and specifically interferons promote caspase-1 activity in B cells (41). This may explain the delay in Rbx1 cleavage seen in the LPS-treated B cells. ER stress has been shown to activate various caspases, such as caspases-

12, -7, and -2 (42, 43). The fact that caspase-1 activity was upregulated upon ER stress to a higher degree than upon apoptosis induction (supplemental Fig. S2) might indicate that in B cells apoptotic mechanisms are engaged to connect ER stress to caspase-1. Indeed, a recent study demonstrates that in myeloid cells, in addition to the activation of caspase-1 by innate signaling pathways, ER stress promotes the activity of NLRP3 inflammasomes in a mechanism that does not require any of the UPR canonical arms (44). The exact mechanisms by which caspase-1 activity is connected to ER stress await clarification.

ΔhRBX1 associates with Cul1 (Fig. 3). The binding of RBX1 to cullin1 C-terminal domain is mediated by its RING domain that spans amino acids 20–108. Moreover, the association of RBX1 with Cul1 is stable enough to endure co-purification from *Escherichia coli* extracts (39). Thus, we did not expect that the small deletion at the N terminus would compromise the binding to Cul1. However, the N terminus of RBX1 may still play a functional and physiological role. We encountered technical problems to assess its functionality in mammalian cells. As can be seen in Fig. 2*D*, overexpression in 293T did not yield expression levels comparable with that of the endogenous RBX1, thus limiting the utility of this approach in gaining a clear phenotype. Attempts to combine overexpression with knocking down the endogenous RBX1 using shRNA always resulted in cells in which the full-length RBX1 still trumped the expression of ΔhRBX1.

To obtain functional information on the role of the N terminus of RBX1, we resorted to yeast, in which previous studies have already demonstrate that human RBX1 rescues the lethality of *Roc1*Δ cells (45). Furthermore, yeast do not express caspase proteins, and thus it was expected that the full-length RBX1 will not undergo cleavage under stress. Because Roc1 is an essential gene, we replaced it by gene shuffling with either hRBX1 or ΔhRBX1. This system enabled us to dissect the unique effect of each protein on cellular physiology. As Roc1p is stable and highly abundant, we used a high level expression promoter. ΔhRBX1-expressing yeast propagated normally, indicating that ΔhRBX1-bound CRLs are proficient in ubiquitination associated with cell cycle control. Nonetheless, when tested under various stress conditions, only ER stress resulted in severe retardation in the growth of ΔhRBX1 cells relative to cells expressing the full-length RBX1 (Fig. 4). The particular sensitivity of ΔhRBX1 toward ER stress and not toward other stress modes, such as heat shock or inclusion of ethanol, suggests that ER stress, at least for DTT or tunicamycin treatments, inflicts higher pressure on the UPS than other stress modalities. In support of this assumption, ΔhRBX1 displayed handicapped ubiquitination patterns as indicated by reduced levels of ubiquitin conjugates and enhanced levels of free ubiquitin (Fig. 4). These data provide the first indication that the N terminus of RBX1 has a biological significance.

What is the mechanism by which ΔhRBX1 exhibits a higher level of ER stress (Fig. 5*A*)? The defect in ubiquitination, although subtle, may be more pronounced for proteins with an extremely short half-life. To examine this, Hac1p degradation was followed as readout for two reasons. First is that Hac1p is degraded at an exceedingly high rate, which allows the detection of relatively small changes in degradation efficiencies. Second, we wanted to examine whether Hac1p stability coincides with the higher UPRE-dependent transcription activity observed in the ΔhRBX1 strain. Indeed, we saw that Hac1p degradation was moderately retarded in the ΔhRBX1 strain compare with the wild type (Fig. 5*B*). It should be emphasized that the higher but subtle changes in Hac1p levels measured in the ΔhRBX1 strain cannot rectify the ER stress conditions conferred by blocking *N*-linked glycosylation with Tm. Thus, the hypersensitivity to ER stress is more likely to result from the role CRLs play in clearing ERAD substrates (46). Moreover, because CRLs are involved in a plethora of degradation reactions, even a slight delay in their activity may contribute to this phenotype. It was recently proposed that RBX1 might adapt two conformations through RING domain rotation to enable its dual roles in ubiquitination and NEDDylation (47). It is possible that the RBX1 gymnastics utilize the unstructured, loose N terminus of RBX1, which is removed in some conditions as we described.

The differentiation of naive B lymphocytes into plasma cells is a dynamic process that involves multiple elements of cell biology. In brief, at the initial phase B cells following activation through their toll-like receptors undergo rapid divisions. This is accompanied by changes in cell surface markers and acquisition of migratory capabilities. The ER undergoes expansion; antibodies are secreted, and finally cell cycle is arrested (48). It has been shown that plasma cells at sites of inflammation actively secrete IL-1β (49). In addition, IL-1β is a poor prognostic marker for multiple myeloma patients (50). Therefore, caspase-1, which is responsible for IL-1β secretion, is most likely expressed in plasma cells under certain conditions, which may also result in the cleavage of RBX1. It remains to be examined whether the cleavage of RBX1 is important to the physiology or pathology of plasma cell differentiation. This should be done by replacing the full-length RBX1 with its truncated form similarly to what we performed in yeast. At this stage we cannot provide a definite answer to this question. We can postulate that if the cleavage of RBX1 in mammalian cells also confers sensitivity to ER stress, as seen in yeast, it may contribute to either slowing down proliferation or promoting ER stress-mediated apoptosis of the B cells. This biochemical reaction may provide an additional pressure of selection to cells that eventually may endure better ER stress. Alternatively, RBX1 cleavage might only be a side product of caspase-1 activation without affecting the differentiation pathway to a significant level.

REFERENCES

1. Ron, D., and Walter, P. (2007) Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol.* **8,** 519–529
2. Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S., and Walter, P. (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* **101,** 249–258
3. 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
4. Zhang, K., Wong, H. N., Song, B., Miller, C. N., Scheuner, D., and Kaufman, R. J. (2005) The unfolded protein response sensor IRE1α is required at two distinct steps in B cell lymphopoiesis. *J. Clin. Invest.* **115,** 268–281
5. Reimold, A. M., Iwakoshi, N. N., Manis, J., Vallabhajosyula, P., Szomo-

lanyi-Tsuda, E., Gravallese, E. M., Friend, D., Grusby, M. J., Alt, F., and Glimcher, L. H. (2001) Plasma cell differentiation requires the transcription factor XBP-1. *Nature* **412,** 300–307
6. Kaser, A., Lee, A. H., Franke, A., Glickman, J. N., Zeissig, S., Tilg, H., Nieuwenhuis, E. E., Higgins, D. E., Schreiber, S., Glimcher, L. H., and Blumberg, R. S. (2008) XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. *Cell* **134,** 743–756
7. Lee, A. H., Scapa, E. F., Cohen, D. E., and Glimcher, L. H. (2008) Regulation of hepatic lipogenesis by the transcription factor XBP1. *Science* **320,** 1492–1496
8. Rutkowski, D. T., Wu, J., Back, S. H., Callaghan, M. U., Ferris, S. P., Iqbal, J., Clark, R., Miao, H., Hassler, J. R., Fornek, J., Katze, M. G., Hussain, M. M., Song, B., Swathirajan, J., Wang, J., Yau, G. D., and Kaufman, R. J. (2008) UPR pathways combine to prevent hepatic steatosis caused by ER stress-mediated suppression of transcriptional master regulators. *Dev. Cell* **15,** 829–840
9. Harding, H. P., Zeng, H., Zhang, Y., Jungries, R., Chung, P., Plesken, H., Sabatini, D. D., and Ron, D. (2001) Diabetes mellitus and exocrine pancreatic dysfunction in perk$^{-/-}$ mice reveals a role for translational control in secretory cell survival. *Mol. Cell* **7,** 1153–1163
10. Meusser, B., Hirsch, C., Jarosch, E., and Sommer, T. (2005) ERAD. The long road to destruction. *Nat. Cell Biol.* **7,** 766–772
11. Friedlander, R., Jarosch, E., Urban, J., Volkwein, C., and Sommer, T. (2000) A regulatory link between ER-associated protein degradation and the unfolded-protein response. *Nat. Cell Biol.* **2,** 379–384
12. Casagrande, R., Stern, P., Diehn, M., Shamu, C., Osario, M., Zúñiga, M., Brown, P. O., and Ploegh, H. (2000) Degradation of proteins from the ER of *S. cerevisiae* requires an intact unfolded protein response pathway. *Mol. Cell* **5,** 729–735
13. Tirosh, B., Iwakoshi, N. N., Glimcher, L. H., and Ploegh, H. L. (2005) XBP-1 specifically promotes IgM synthesis and secretion but is dispensable for degradation of glycoproteins in primary B cells. *J. Exp. Med.* **202,** 505–516
14. Tsai, Y. C., and Weissman, A. M. (2010) The unfolded protein response, degradation from endoplasmic reticulum, and cancer. *Genes Cancer* **1,** 764–778
15. Termine, D. J., Moremen, K. W., and Sifers, R. N. (2009) The mammalian UPR boosts glycoprotein ERAD by suppressing the proteolytic down-regulation of ER mannosidase I. *J. Cell Sci.* **122,** 976–984
16. Oda, Y., Okada, T., Yoshida, H., Kaufman, R. J., Nagata, K., and Mori, K. (2006) Derlin-2 and Derlin-3 are regulated by the mammalian unfolded protein response and are required for ER-associated degradation. *J. Cell Biol.* **172,** 383–393
17. Lee, A. H., Chu, G. C., Iwakoshi, N. N., and Glimcher, L. H. (2005) XBP-1 is required for biogenesis of cellular secretory machinery of exocrine glands. *EMBO J.* **24,** 4368–4380
18. Iwawaki, T., Akai, R., and Kohno, K. (2010) IRE1α disruption causes histological abnormality of exocrine tissues, increase of blood glucose level, and decrease of serum immunoglobulin level. *PLoS One* **5,** e13052
19. Shaffer, A. L., Shapiro-Shelef, M., Iwakoshi, N. N., Lee, A. H., Qian, S. B., Zhao, H., Yu, X., Yang, L., Tan, B. K., Rosenwald, A., Hurt, E. M., Petroulakis, E., Sonenberg, N., Yewdell, J. W., Calame, K., Glimcher, L. H., and Staudt, L. M. (2004) XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles and increases protein synthesis in plasma cell differentiation. *Immunity* **21,** 81–93
20. Cenci, S., Mezghrani, A., Cascio, P., Bianchi, G., Cerruti, F., Fra, A., Lelouard, H., Masciarelli, S., Mattioli, L., Oliva, L., Orsi, A., Pasqualetto, E., Pierre, P., Ruffato, E., Tagliavacca, L., and Sitia, R. (2006) Progressively impaired proteasomal capacity during terminal plasma cell differentiation. *EMBO J.* **25,** 1104–1113
21. Bianchi, G., Oliva, L., Cascio, P., Pengo, N., Fontana, F., Cerruti, F., Orsi, A., Pasqualetto, E., Mezghrani, A., Calbi, V., Palladini, G., Giuliani, N., Anderson, K. C., Sitia, R., and Cenci, S. (2009) The proteasome load *versus* capacity balance determines apoptotic sensitivity of multiple myeloma cells to proteasome inhibition. *Blood* **113,** 3040–3049
22. Sun, Y., Tan, M., Duan, H., and Swaroop, M. (2001) SAG/ROC/Rbx/Hrt, a zinc RING finger gene family. Molecular cloning, biochemical properties, and biological functions. *Antioxid. Redox. Signal.* **3,** 635–650
23. Gu, Q., Tan, M., and Sun, Y. (2007) SAG/ROC2/Rbx2 is a novel activator protein-1 target that promotes c-Jun degradation and inhibits 12-*O*-tetradecanoylphorbol-13-acetate-induced neoplastic transformation. *Cancer Res.* **67,** 3616–3625
24. Tan, M., Gu, Q., He, H., Pamarthy, D., Semenza, G. L., and Sun, Y. (2008) SAG/ROC2/RBX2 is a HIF-1 target gene that promotes HIF-1α ubiquitination and degradation. *Oncogene* **27,** 1404–1411
25. Duan, H., Wang, Y., Aviram, M., Swaroop, M., Loo, J. A., Bian, J., Tian, Y., Mueller, T., Bisgaier, C. L., and Sun, Y. (1999) SAG, a novel zinc RING finger protein that protects cells from apoptosis induced by redox agents. *Mol. Cell. Biol.* **19,** 3145–3155
26. Kamura, T., Conrad, M. N., Yan, Q., Conaway, R. C., and Conaway, J. W. (1999) The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates Rub1 modification of cullins Cdc53 and Cul2. *Genes Dev.* **13,** 2928–2933
27. Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholson, D. W. (1997) A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J. Biol. Chem.* **272,** 17907–17911
28. Strasser, A., Whittingham, S., Vaux, D. L., Bath, M. L., Adams, J. M., Cory, S., and Harris, A. W. (1991) Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 8661–8665
29. Iwakoshi, N. N., Lee, A. H., Vallabhajosyula, P., Otipoby, K. L., Rajewsky, K., and Glimcher, L. H. (2003) Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. *Nat. Immunol.* **4,** 321–329
30. Kronheim, S. R., Mumma, A., Greenstreet, T., Glackin, P. J., Van Ness, K., March, C. J., and Black, R. A. (1992) Purification of interleukin-1β-converting enzyme, the protease that cleaves the interleukin-1β precursor. *Arch. Biochem. Biophys.* **296,** 698–703
31. Ghayur, T., Banerjee, S., Hugunin, M., Butler, D., Herzog, L., Carter, A., Quintal, L., Sekut, L., Talanian, R., Paskind, M., Wong, W., Kamen, R., Tracey, D., and Allen, H. (1997) Caspase-1 processes IFN-γ-inducing factor and regulates LPS-induced IFN-γ production. *Nature* **386,** 619–623
32. Gu, Y., Kuida, K., Tsutsui, H., Ku, G., Hsiao, K., Fleming, M. A., Hayashi, N., Higashino, K., Okamura, H., Nakanishi, K., Kurimoto, M., Tanimoto, T., Flavell, R. A., Sato, V., Harding, M. W., Livingston, D. J., and Su, M. S. (1997) Activation of interferon-γ-inducing factor mediated by interleukin-1β-converting enzyme. *Science* **275,** 206–209
33. Obermeier, F., Gross, V., Schölmerich, J., and Falk, W. (1999) Interleukin-1 production by mouse macrophages is regulated in a feedback fashion by nitric oxide. *J. Leukocyte Biol.* **66,** 829–836
34. Ohta, T., Michel, J. J., and Xiong, Y. (1999) Association with cullin partners protects ROC proteins from proteasome-dependent degradation. *Oncogene* **18,** 6758–6766
35. Ohta, T., Michel, J. J., Schottelius, A. J., and Xiong, Y. (1999) ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. *Mol. Cell* **3,** 535–541
36. Pal, B., Chan, N. C., Helfenbaum, L., Tan, K., Tansey, W. P., and Gething, M. J. (2007) SCFCdc4-mediated degradation of the Hac1p transcription factor regulates the unfolded protein response in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **18,** 426–440
37. Wei, D., and Sun, Y. (2010) Small RING finger proteins RBX1 and RBX2 of SCF E3 ubiquitin ligases. The role in cancer and as cancer targets. *Genes Cancer* **1,** 700–707
38. Duda, D. M., Borg, L. A., Scott, D. C., Hunt, H. W., Hammel, M., and Schulman, B. A. (2008) Structural insights into NEDD8 activation of cullin-RING ligases. Conformational control of conjugation. *Cell* **134,** 995–1006
39. Zheng, N., Schulman, B. A., Song, L., Miller, J. J., Jeffrey, P. D., Wang, P., Chu, C., Koepp, D. M., Elledge, S. J., Pagano, M., Conaway, R. C., Conaway, J. W., Harper, J. W., and Pavletich, N. P. (2002) Structure of the Cul1-Rbx1-Skp1-F box Skp2 SCF ubiquitin ligase complex. *Nature* **416,** 703–709
40. Franchi, L., and Núñez, G. (2008) The Nlrp3 inflammasome is critical for

aluminum hydroxide-mediated IL-1β secretion but dispensable for adjuvant activity. *Eur. J. Immunol.* **38,** 2085–2089
41. Lee, C. K., Smith, E., Gimeno, R., Gertner, R., and Levy, D. E. (2000) STAT1 affects lymphocyte survival and proliferation partially independent of its role downstream of IFN-gamma. *J. Immunol.* **164,** 1286–1292
42. Szegezdi, E., Fitzgerald, U., and Samali, A. (2003) Caspase-12 and ER stress-mediated apoptosis. The story so far. *Ann. N.Y. Acad. Sci.* **1010,** 186–194
43. Gu, H., Chen, X., Gao, G., and Dong, H. (2008) Caspase-2 functions upstream of mitochondria in endoplasmic reticulum stress-induced apoptosis by bortezomib in human myeloma cells. *Mol. Cancer Ther.* **7,** 2298–2307
44. Menu, P., Mayor, A., Zhou, R., Tardivel, A., Ichijo, H., Mori, K., and Tschopp, J. (2012) ER stress activates the NLRP3 inflammasome via an UPR-independent pathway. *Cell Death Dis.* **3,** e261
45. Swaroop, M., Wang, Y., Miller, P., Duan, H., Jatkoe, T., Madore, S. J., and Sun, Y. (2000) Yeast homolog of human SAG/ROC2/Rbx2/Hrt2 is essential for cell growth, but not for germination. Chip profiling implicates its role in cell cycle regulation. *Oncogene* **19,** 2855–2866
46. Yoshida, Y., Tokunaga, F., Chiba, T., Iwai, K., Tanaka, K., and Tai, T. (2003) Fbs2 is a new member of the E3 ubiquitin ligase family that recognizes sugar chains. *J. Biol. Chem.* **278,** 43877–43884
47. Calabrese, M. F., Scott, D. C., Duda, D. M., Grace, C. R., Kurinov, I., Kriwacki, R. W., and Schulman, B. A. (2011) A RING E3-substrate complex poised for ubiquitin-like protein transfer. Structural insights into cullin-RING ligases. *Nat. Struct. Mol. Biol.* **18,** 947–949
48. van Anken, E., Romijn, E. P., Maggioni, C., Mezghrani, A., Sitia, R., Braakman, I., and Heck, A. J. (2003) Sequential waves of functionally related proteins are expressed when B cells prepare for antibody secretion. *Immunity* **18,** 243–253
49. Hassan, G., Moreno, S., Massimi, M., Di Biagio, P., and Stefanini, S. (1997) Interleukin-1-producing plasma cells in close contact with hepatocytes in patients with chronic active hepatitis. *J. Hepatol.* **27,** 6–17
50. Cozzolino, F., Torcia, M., Aldinucci, D., Rubartelli, A., Miliani, A., Shaw, A. R., Lansdorp, P. M., and Di Guglielmo, R. (1989) Production of interleukin-1 by bone marrow myeloma cells. *Blood* **74,** 380–387