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High ER stress in β-cells stimulates intracellular degradation of misfolded insulin

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Abstract

Endoplasmic reticulum (ER) stress, which is caused by the accumulation of misfolded proteins in the ER, elicits an adaptive response, the unfolded protein response (UPR). One component of the UPR, the endoplasmic reticulum-associated protein degradation (ERAD) system, has an important function in the survival of ER stressed cells. Here, we show that HRD1, a component of the ERAD system, is upregulated in pancreatic islets of the Akita diabetes mouse model and enhances intracellular degradation of misfolded insulin. High ER stress in β-cells stimulated mutant insulin degradation through HRD1 to protect β-cells from ER stress and ensuing death. If HRD1 serves the same function in humans, it may serve as a target for therapeutic intervention in diabetes.

Keywords: Endoplasmic reticulum stress; Unfolded protein response; Diabetes; Endoplasmic-reticulum associated protein degradation

Endoplasmic-reticulum (ER) stress is caused by the accumulation of misfolded proteins in the ER [1]. Accumulating evidence suggests that a high level of ER stress and consequently defective ER stress signaling cause chronic human diseases such as Alzheimer's disease, Parkinson's disease, and prion diseases [2]. ER stress elicits stress signaling pathways termed the unfolded protein response (UPR), which consists of three components that counteract ER stress: gene expression, translational attenuation, and ER-associated protein degradation (the ERAD system) [1,3,4]. In particular, the ERAD system has an important function in the survival of stressed cells [5,6]. It has been shown that inositol requiring 1 (IRE1), a crucial regulator of the ERAD system [5], is a sensor for unfolded and misfolded proteins in the ER. The presence of unfolded or misfolded proteins in the ER causes dimerization and trans-autophosphorylation of IRE1, leading to IRE1 activation. Furthermore, activated IRE1 splices the X-box-binding protein-1 (XBP-1) mRNA, leading to synthesis of the active transcription factor XBP-1 and upregulation of UPR genes, particularly ERAD genes [5,7].

Pancreatic β-cell death contributes to both type 1 and 2 diabetes. Recent observations suggest that chronic ER stress in β-cells plays a role in the pathogenesis of diabetes [8]. Moreover, recent reports suggest that ER stress has an important role in β-cell death in the Akita mouse model for diabetes [9–11]. The Akita mouse is a C57BL/6 mouse that is heterozygous for a mutation in the insulin-2 gene [12], which results in an amino acid substitution, cysteine 96 to tyrosine ($Ins2^{WT/C96Y}$). Cysteine 96 is involved in the formation of one of the two disulfide bonds between the A and B chains of mature insulin [13]. It is likely that this mutation causes misfolding of the insulin precursor in the ER of β-cells. Therefore, it is important to quantify ER stress levels in the β-cells

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of Akita mice to monitor their disease status. In this study, we measured the expression levels of ER stress markers and components of the ERAD system in the islets of Akita mice by quantitative real-time polymerase chain reaction (PCR).

Materials and methods

Plasmids, cell culture, and transfection. We obtained the plasmid HRD1-pCMVSPORT6 from Open Biosystems (Huntsville, AL). K. Tanaka provided ubiquitin-Flag-pcDNA3; H. Nishitoh provided insulin-2-HA-pcDNA3 and insulin-2 C96Y-HA-pcDNA3, while Dr. Seiichi Oyadomari provided insulin-2-pcDNA, and insulin-2 C96Y-pcDNA. We maintained COS7 cells and HeLa cells in DMEM with 10% fetal bovine serum and transfected them using FuGene (Roche, Basel) and HeLa Monster (Mirus, Madison, MI), respectively.

Isolating islet cells from mouse pancreas. We handpicked islet cells from collagenase P-digested whole pancreas according to the standard method [14]. Briefly, after mice are anesthesized by intraperitoneal injection of sodium pentobarbital, pancreatic islets are isolated by pancreatic duct injection of 500 U/ml collagenase solution, then digested at 37 °C for 40 min with mild shaking. Islet cells are washed several times with HBSS, separated from acinar cells on a discontinuous Ficoll 400 gradient, and then selected by under a dissecting microscope. Freshly isolated islets are cultured for 14 h in RPMI 10% FCS [15].

Immunoblotting and immunoprecipitation. We lysed islet cells in ice-cold buffer (20 mM Hepes, pH 7.5, 1% Triton X-100, 150 mM NaCl, 10% glycerol, and 1 mM EDTA) containing protease inhibitors for 15 min on ice, and then clarified them by centrifugation at 14,000*g* for 10 min. Lysates were normalized for total protein, 20 μg per lane, separated using 4–20% linear gradient SDS–PAGE, and then electroblotted to nitrocellulose membranes. Flag M2 antibody and HA antibody were purchased, respectively, from Sigma (St. Louis, MO) and Roche. We immunoprecipitated lysates with the indicated antibodies and separated them using 4–20% linear gradient SDS–PAGE (Bio-Rad, Hercules, CA).

Real-time polymerase chain reaction. To isolate total RNA from the cells, we used the guanidine thiocyanate acid–phenol extraction method, in which 1 μg of total RNA from cells is reverse transcribed using Oligo-dT primer. During PCR, we used XBP-1 mRNA, mXBP1.11S: CTG AGT CCG AAT CAG GTG CAG, and mXBP1.10AS: GTC CAT GGG AAG ATG TTC TGG. To reduce the background signal, we introduced two mismatches to the native XBP-1 sequence in mXBP1.11S. To amplify the spliced form of mouse XBP-1, we used mXBP1.7S: CAG CAC TCA GAC TAT GTG CA and mXBP1.10AS. In amplification procedures, we used mBiP.3S: TTC AGC CAA TTA TCA GCA AAC TCT and mBiP.4AS: TTT TCT GAT GTA TCC TCT TCA CCA GT for mouse BiP, mHRD1.1S: CCT GCT TGT GAG TAT GGG ACC and mHRD1.2AS: TGG GTT TCC ACA GTT GGG AA for Hrd1, and mSEL1.1S: ACA GCC TTA ACC AAC TTG AGG TG and mSEL1.2AS: TCC GGG AAG CAA CGA ATC TA for Sel1L. For the thermal cycle reaction, we used the ABI prism 7000 sequencer detection system at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min.

Results and discussion

Diabetes in the Akita mouse is not associated with obesity or insulitis; rather, it develops spontaneously with dramatic reduction in β-cell mass [9,10]. Recent observations support the idea that ER stress causes β-cell death, and thus leads to diabetes in the Akita mouse ($Ins2^{WT/C96Y}$) [11,16,17]. It has been shown that the ER-resident molecular chaperone BiP is upregulated in the pancreas of the Akita mouse [11]. In this study, we found that BiP, Hrd1, and Sel1L were all upregulated in pancreatic islet cells from Akita mice (Fig. 1A), strongly suggesting that these cells are under ER stress.

Hrd1 and Sel1L are components of the ERAD system. It has been shown that the upregulation of the ERAD components is regulated by the IRE1-XBP-1 pathway. To examine the involvement of IRE1 signaling in upregulation of ERAD genes, we measured the IRE1 activation level in the islets of Akita mouse. Since it is difficult to measure the IRE1 activation level by itself, we used the XBP-1 mRNA splicing level, which reflects the IRE1 activation level, to quantify the IRE1 activation level. Spliced XBP-1 mRNA encodes the active transcription factor and activates the ERAD system [7,18,19]. We have developed a method to quantify the expression levels of the spliced and unspliced forms of XBP-1 mRNA by real-time PCR. For this purpose, we obtained the PCR efficiencies by plotting the threshold cycle (C_t), the cycle at which a significant increase in the reaction is first detected. The amplification efficiency for spliced XBP-1 was similar to that for unspliced XBP-1 (Fig. 1B).

To test this method, we measured the ratio between spliced and unspliced XBP-1 expression levels in mouse embryonic fibroblasts treated for 2 h with tunicamycin, an ER stress inducer. We successfully quantified the induction of XBP-1 splicing by ER stress in wild-type, but not in Ire1α knock-out ($Ire1\alpha^{-/-}$) mouse embryonic fibroblasts (Fig. 1C). Because there is no XBP-1 splicing in Ire1α knock-out ($Ire1\alpha^{-/-}$) mouse embryonic fibroblasts [7], this result validated our method. Using this method, we measured the ratio of spliced XBP-1 mRNA expression to unspliced XBP-1 mRNA expression, finding that the XBP-1 splicing levels were higher in Akita mice than in control animals (Fig. 1D). The data also support our prediction that the ER stress level is higher in the islets of Akita mice than in those of control mice.

Upregulation of the ERAD components Hrd1 and Sel1L prompted us to examine the stability of mutant insulin in Akita mice. We transfected COS7 cells with wild-type and mutant insulin-2 expression vectors, and then measured the steady-state expression level of mutant insulin, $Ins2^{C96Y}$, by immunoblot analysis. We found that $Ins2^{C96Y}$ did not accumulate to high levels in transfected cells, suggesting that it was subjected to increased intracellular degradation (Fig. 2A). The expression level of mutant insulin was increased in cells treated with the proteasome inhibitor MG132, suggesting that the ubiquitin–proteasome pathway is involved in the degradation of mutant insulin (Fig. 2A). We then co-expressed $Ins2^{C96Y}$ with a dominant negative form of ubiquitin to determine

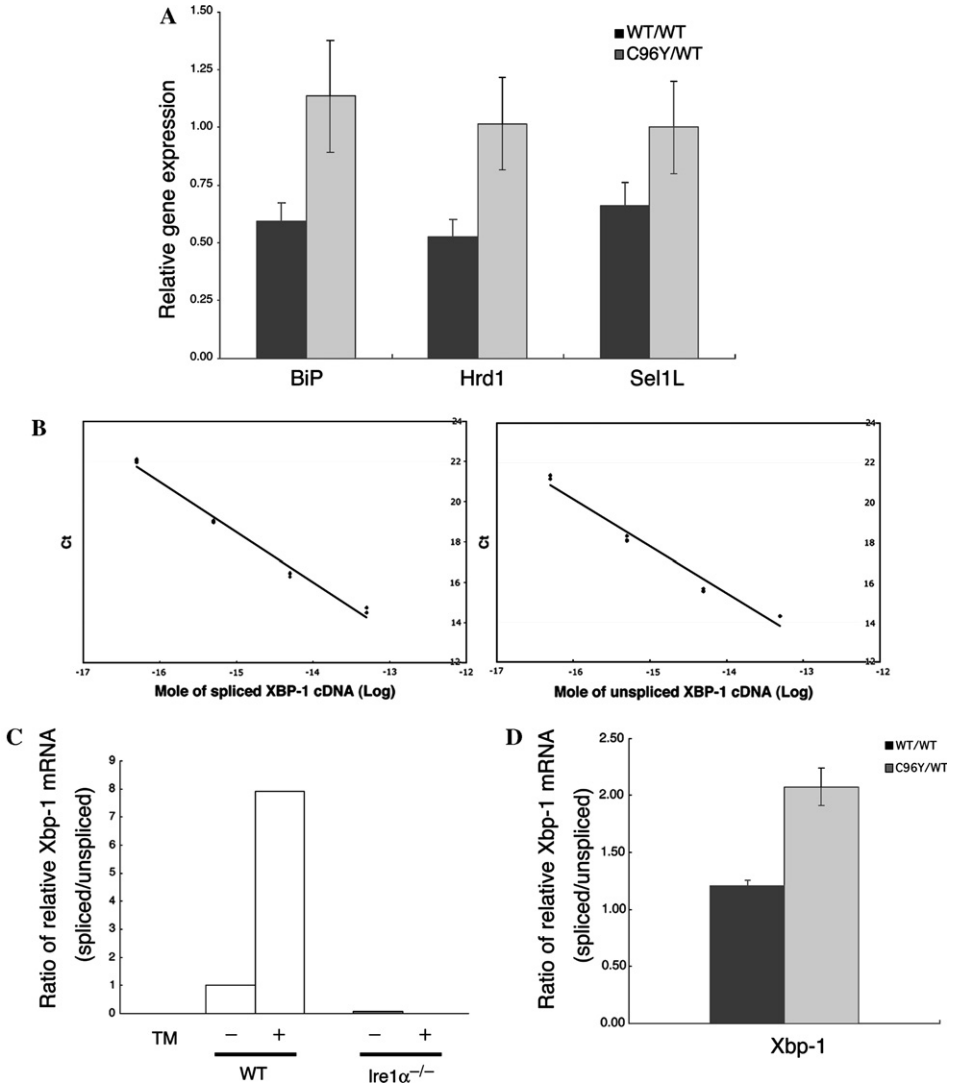

Fig. 1. (A) Expression of BiP, Hrd1, and Sel1L mRNA in the islets of Akita mice. Quantitative real-time PCR of reverse-transcribed RNA from the islets of Akita mice (Ins2 C96Y/WT) and wild-type mice (WT/WT). The amount of transcript of the gene of interest was normalized to the amount of GAPDH RNA in each sample. The means ± SEM from six animals for each genotype is shown. (B) The standard curve for amplification of the XBP-1 target detected using a cybergreen-labeled probe. C_t is the threshold cycle, defined as the cycle during which the system begins to detect an increase in the signal associated with an exponential growth of PCR product during the log-linear phase. (C) Relative expression of the active form of XBP-1 mRNA in mouse embryonic fibroblast cells. Quantitative real-time PCR of reverse-transcribed RNA from wild-type (WT) and Ire1α knockout (Ire1α $^{-/-}$) mouse embryonic fibroblast cells. Cells were treated or untreated with tunicamycin (TM), an ER stress inducer, for 2 h. The ratio of relative XBP-1 mRNA levels (spliced versus unspliced) is shown. (D) Expression of the active form of XBP-1 mRNA in the islets of Akita mice. Quantitative real-time PCR of reverse-transcribed RNA from the islets of Akita mice (Ins2 C96Y/WT) and wild-type mice (WT/WT). The ratio of relative XBP-1 mRNA levels (spliced versus unspliced) is shown. The means ± SEM from six animals for each genotype is shown.

whether or not polyubiquitination is required for $Ins2^{C96Y}$ degradation. The Lys-48 residue of ubiquitin, which is the site of isopeptide linkage of other ubiquitin molecules, is essential for the formation of multi-ubiquitin chains. Mutant ubiquitin in which this invariant lysine is replaced by arginine (K48R) is a polyubiquitin chain terminator that reduces the efficiency of proteasome-mediated degradation and stabilizes polyubiquitinated substrates [20]. Co-expression of $Ins2^{C96Y}$ and $ubiquitin^{K48R}$ increased the $Ins2^{C96Y}$ expression level (Fig. 2B), indicating that $Ins2^{C96Y}$ is degraded by the ubiquitin–proteasome system.

Because HRD1 is upregulated in the islets of Akita mice and encodes an E3 ubiquitin ligase required for the ERAD system [6,21–23], we explored the question of whether or not mutant insulin is ubiquitinated by HRD1. For this purpose, we co-transfected COS7 cells with HRD1 expression vector and either wild-type or C96Y insulin-2 with Flag-tagged ubiquitin. HRD1 expression did not increase the ubiquitination of wild-type insulin-2, but did increase that of C96Y insulin-2 (Fig. 3), demonstrating that mutant insulin-2 is susceptible to HRD1-mediated ubiquitination, and degradation.

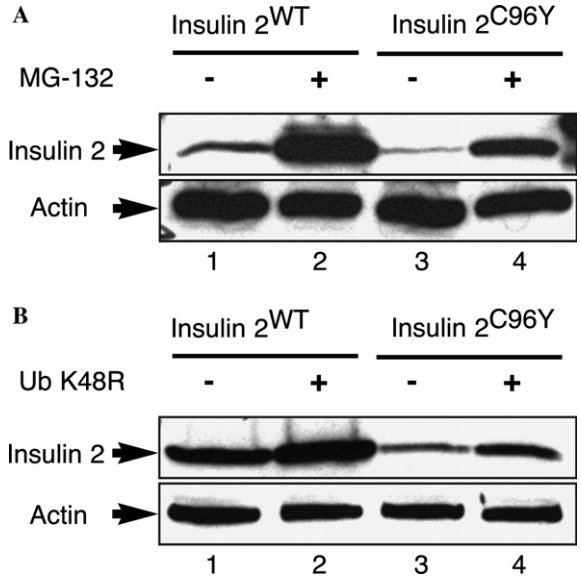

Fig. 2. (A) The effect of proteasome inhibitor on the steady-state expression level of wild-type or C96Y insulin-2. Lanes 1 and 3: COS7 cells were transfected with wild-type or C96Y insulin-2 expression vector alone. Lanes 2 and 4: COS7 cells were transfected with Flag-tagged ubiquitin^{K48R} (Ub K48R) expression vector and treated with MG132 (20 μM). (B) The effect of expression of ubiquitin^{K48R} on the expression level of wild-type or C96Y insulin-2. Lanes 1 and 3: COS7 cells transfected with wild-type or C96Y insulin-2 expression vector alone. Lanes 2 and 4: COS7 cells cotransfected with Flag-tagged ubiquitin^{K48R} (Ub K48R) expression vector.

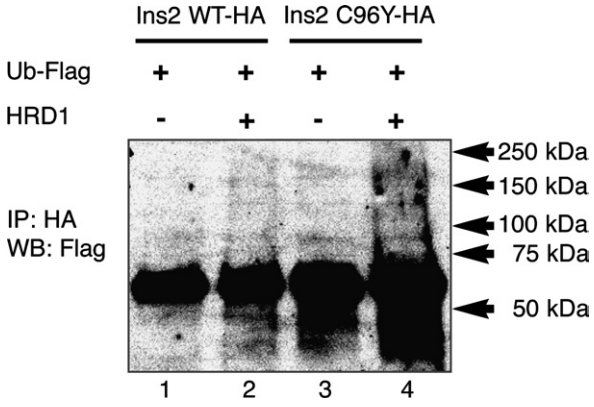

Fig. 3. Ubiquitination of insulin by HRD1. COS7 cells were transfected with expression vectors for HA-tagged wild-type or C96Y mutant insulin-2, HRD1, and Flag-tagged ubiquitin. Cell were lysed in detergent, immunoprecipitated with anti-HA antibody, and immunoblotted with anti-Flag antibody.

Taken together, these findings suggest that misfolded insulin produced in Akita mouse is selectively ubiquitinated and degraded by a HRD1-mediated ERAD pathway and that HRD1 protects cells against the toxic effects of misfolded insulin. In addition, our results provide useful methods to quantify ER stress level in the islets of Akita mice. There is a high baseline level of ER stress in pancreatic β-cells because of the heavy load of client protein, insulin (K. Lipson and F. Urano, manuscript in preparation). This means that only a slight increase in ER stress could lead to β-cell death. Thus, our new methods to quantify ER stress level could be useful to measure the vulnerability of β-cells to ER stress-mediated cell death and could ultimately lead to the development of methods for the early diagnosis of diabetes. Our results indicate not only that HRD1 is upregulated in the diabetes mouse model, but that HRD1 may be central to the protection of β-cells from ER stress-mediated death. This raises the possibility that small molecules that activate or enhance the HRD1-mediated ERAD pathway may be therapeutically beneficial to patients with diabetes.

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References

[1] H.P. Harding, M. Calfon, F. Urano, I. Novoa, D. Ron, Transcriptional and translational control in the mammalian unfolded protein response, Annu. Rev. Cell Dev. Biol. 18 (2002) 575–599.
[2] R.J. Kaufman, Orchestrating the unfolded protein response in health and disease, J. Clin. Invest. 110 (2002) 1389–1398.
[3] R.J. Kaufman, D. Scheuner, M. Schroder, X. Shen, K. Lee, C.Y. Liu, S.M. Arnold, The unfolded protein response in nutrient sensing and differentiation, Nat. Rev. Mol. Cell Biol. 3 (2002) 411–421.
[4] K. Mori, Tripartite management of unfolded proteins in the endoplasmic reticulum, Cell 101 (2000) 451–454.
[5] H. Yoshida, T. Matsui, N. Hosokawa, R.J. Kaufman, K. Nagata, K. Mori, A time-dependent phase shift in the mammalian unfolded protein response, Dev. Cell 4 (2003) 265–271.
[6] M. Kaneko, M. Ishiguro, Y. Niinuma, M. Uesugi, Y. Nomura, Human HRD1 protects against ER stress-induced apoptosis through ER-associated degradation, FEBS Lett. 532 (2002) 147–152.
[7] M. Calfon, H. Zeng, F. Urano, J.H. Till, S.R. Hubbard, H.P. Harding, S.G. Clark, D. Ron, IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA, Nature 415 (2002) 92–96.
[8] H.P. Harding, D. Ron, Endoplasmic reticulum stress and the development of diabetes: a review, Diabetes 51 (Suppl. 3) (2002) S455–S461.
[9] T. Kayo, A. Koizumi, Mapping of murine diabetogenic gene mody on chromosome 7 at D7Mit258 and its involvement in pancreatic islet and beta cell development during the perinatal period, J. Clin. Invest. 101 (1998) 2112–2118.

[10] M. Yoshioka, T. Kayo, T. Ikeda, A. Koizumi, A novel locus, Mody4, distal to D7Mit189 on chromosome seven determines early onset NIDDM in nonobese C57BL/6 (Akita) mutant mice, Diabetes 46 (1997) 887–894.
[11] S. Oyadomari, A. Koizumi, K. Takeda, T. Gotoh, S. Akira, E. Araki, M. Mori, Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes, J. Clin. Invest. 109 (2002) 525–532.
[12] J. Wang, T. Takeuchi, S. Tanaka, S.K. Kubo, T. Kayo, D. Lu, K. Takata, A. Koizumi, T. Izumi, A mutation in the insulin-2 gene induces diabetes with severe pancreatic beta-cell dysfunction in the mody mouse, J. Clin. Invest. 103 (1999) 27–37.
[13] U. Masharani, J.H. Karam, Pancreatic hormones and diabetes mellitus, in: F.S. Greenspan, D.G. Gardner (Eds.), Basic and Clinical Endocrinology, McGraw-Hill, 2001, pp. 623–698.
[14] P.E. Lacy, M. Kostianovsky, Method for the isolation of intact islets of Langerhans from the rat pancreas, Diabetes 16 (1967) 35–39.
[15] A. Andersson, Isolated mouse pancreatic islets in culture: effects of serum and different culture media on the insulin production of the islets, Diabetologia 14 (1978) 397–404.
[16] F. Urano, X. Wang, A. Bertolotti, Y. Zhang, P. Chung, H.P. Harding, D. Ron, Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1, Science 287 (2000) 664–666.
[17] H. Nishitoh, A. Matsuzawa, K. Tobiume, K. Saegusa, K. Takeda, K. Inoue, S. Hori, A. Kakizuka, H. Ichijo, ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats, Genes Dev. 16 (2002) 1345–1355.
[18] X. Shen, R.E. Ellis, K. Lee, C.Y. Liu, K. Yang, A. Solomon, H. Yoshida, R. Morimoto, D.M. Kurnit, K. Mori, R.J. Kaufman, Complementary signaling pathways regulate the unfolded protein response and are required for *C. elegans* development, Cell 107 (2001) 893–903.
[19] H. Yoshida, T. Matsui, A. Yamamoto, T. Okada, K. Mori, XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor, Cell 107 (2001) 881–891.
[20] D. Finley, S. Sadis, B.P. Monia, P. Boucher, D.J. Ecker, S.T. Crooke, V. Chau, Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant, Mol. Cell. Biol. 14 (1994) 5501–5509.
[21] M. Kikkert, R. Doolman, M. Dai, R. Avner, G. Hassink, S. van Voorden, S. Thanedar, J. Roitelman, V. Chau, E. Wiertz, Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum, J. Biol. Chem. 279 (2004) 3525–3534.
[22] E. Nadav, A. Shmueli, H. Barr, H. Gonen, A. Ciechanover, Y. Reiss, A novel mammalian endoplasmic reticulum ubiquitin ligase homologous to the yeast Hrd1, Biochem. Biophys. Res. Commun. 303 (2003) 91–97.
[23] N.W. Bays, R.G. Gardner, L.P. Seelig, C.A. Joazeiro, R.Y. Hampton, Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation, Nat. Cell Biol. 3 (2001) 24–29.