CELL STRUCTURE AND FUNCTION 36: 187–195 (2011)

SEL1L Is Required for Endoplasmic Reticulum-associated Degradation of Misfolded Luminal Proteins but not Transmembrane Proteins in Chicken DT40 Cell Line

Satoshi Ninagawa[1], Tetsuya Okada[1,3], Shunichi Takeda[2,3], and Kazutoshi Mori[1,3]*

[1]*Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan,*
[2]*Department of Radiation Genetics, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan,*
[3]*CREST, Japan Science and Technology Corporation, Saitama 332-0012, Japan*

ABSTRACT. **Proteins misfolded in the endoplasmic reticulum (ER) are degraded in the cytosol by a ubiquitin-dependent proteasome system, a process collectively termed ER-associated degradation (ERAD). Unraveling the molecular mechanisms of mammalian ERAD progresses more slowly than that of yeast ERAD due to the laborious procedures required for gene targeting and the redundancy of components. Here, we utilized the chicken B lymphocyte-derived DT40 cell line, which exhibits an extremely high homologous recombination frequency, to analyze ERAD mechanisms in higher eukaryotes. We disrupted the SEL1L gene, which encodes the sole homologue of yeast Hrd3p in both chickens and mammals; Hrd3p is a binding partner of yeast Hrd1p, an E3 ubiquitin ligase. SEL1L-knockout cells grew only slightly more slowly than the wild-type cells. Pulse chase experiments revealed that chicken SEL1L was required for ERAD of misfolded luminal proteins such as glycosylated NHK and unglycosylated NHK-QQQ but dispensable for that of misfolded transmembrane proteins such as NHK$_{BACE}$ and CD3-δ, as in mammals. The defect of SEL1L-knockout cells in NHK degradation was restored by introduction of not only chicken SEL1L but also mouse and human SEL1L. Deletion analysis showed the importance of Sel1-like tetratricopeptide repeats but not the fibronectin II domain in the function of SEL1L. Thus, our reverse genetic approach using the chicken DT40 cell line will provide highly useful information regarding ERAD mechanisms in higher eukaryotes which express ERAD components redundantly.**

Key words: protein degradation/ubiquitin/proteasome/gene targeting/tetratricopeptide repeat

Introduction

The endoplasmic reticulum (ER), in which secretory and transmembrane proteins are folded and assembled, possesses an efficient quality control system. The correct operation of this system is ensured by two different mechanisms, namely folding and degradation mechanisms. The ER contains a number of molecular chaperones and folding enzymes in abundance to assist the productive folding of newly synthesized proteins. However, proteins which remain improperly folded even after the assistance of the productive folding mechanism are retrotranslocated back to the cytosol, where they are ubiquitinated and degraded by the proteasome, a process collectively termed the ER-associated degradation (ERAD) mechanism (Buchberger *et al.*, 2010).

Understanding of the molecular mechanisms of ERAD has made great progress using *Saccharomyces cerevisiae* as a model organism (Xie and Ng, 2010). The power of yeast genetics has allowed many components of the ERAD machinery to be identified. The importance of molecules identified by other means is easily determined by constructing and analyzing yeast knockout strains: examples to date include Der1p, Hrd1p, Hrd3p, Usa1p, Ubx2p, Doa10p, Htm1p/Mnl1p and Yos9p. Further extensive analyses have identified three different pathways for the degradation of ERAD substrates, depending on the location of the particular substrate lesion, namely ERAD-L (lesion in the ER lumen), ERAD-M (lesion inside the ER membrane) and ERAD-C (lesion on the cytosolic side of a transmembrane protein). The ERAD-L pathway is further categorized into the ERAD-Ls (for degradation of soluble luminal proteins) and ERAD-Lm (degradation of transmembrane proteins) pathways. Interestingly, the ubiquitin ligase Hrd1p mediates ERAD-L and ERAD-M, whereas the ubiquitin ligase Doa10p mediates ERAD-C. These E3s recognize ERAD substrates

*To whom correspondence should be addressed: Kazutoshi Mori, Department of Biophysics, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-8502, Japan.
Tel: +81–75–753–4067, Fax: +81–75–753–3718
E-mail: mori@upr.biophys.kyoto-u.ac.jp
Abbreviations: ER, endoplasmic reticulum; ERAD, ER-associated degradation; NHK, null Hong Kong; SR, Sel1-like repeats.

directly or indirectly by forming a complex with other ERAD components.

In contrast to the situation in yeast, however, analysis in mammals is not straightforward (Hoseki *et al.*, 2010). Because the procedures required to obtain mouse embryonic fibroblasts deficient in even a single molecule are laborious and time-consuming, analysis of mammalian ERAD mechanisms relies mostly on knockdown experiments, in which the activity of residual proteins as well as off-target effects must also be considered. In addition, homologues of yeast ERAD components have diverged during evolution: mammalian genomes encode Derlin-1, Derlin-2 and Derlin-3 as homologues of Der1p; HRD1 and gp78 as homologues of Hrd1p; EDEM1, EDEM2, and EDEM3 as homologues of Htm1p/Mnl1p; and OS-9 and XTP3-B as homologues of Yos9p. These redundancies may obscure knockdown effects. We therefore consider that the precise identification of the roles of these paralog genes requires the use of multiple gene disruption.

We have noticed that the gene repertoire is highly conserved among vertebrates; for example, the chicken genome encodes the same set of genes as homologues of yeast Der1p, Hrd1p, Htm1p and Yos9p as mammalian genomes. We therefore considered the use of chicken DT40 cells for the analysis of ERAD mechanisms in higher eukaryotes, on the basis of their establishment as a powerful vertebrate model organism in the fields of DNA damage response and oncology (Conticello *et al.*, 2007). The exceptionally high efficiency of these cells in homologous recombination as well as their stable phenotype and availability of six selection markers provide a unique opportunity to create single, double or triple knockouts at the cellular level with relative ease. In the present study, we focused on SEL1L, the sole homologue of yeast Hrd3p in both chickens and mammals, to determine whether DT40 cells possess ERAD mechanisms similar to those of mammals.

The SEL1L gene was originally isolated as a cDNA which shows sequence similarity to SEL-1, a gene identified as an extragenic suppressor of the *lin-12* hypomorphic mutant of *Caenorhabditis elegance* and shown to be most abundantly expressed in the pancreas (Biunno *et al.*, 1997). SEL1L was subsequently found to be a metazoan homologue of yeast Hrd3p, a binding partner of yeast Hrd1p. The E3 Hrd1p is localized in the ER as a transmembrane protein with its catalytic domain facing the cytoplasm (Lilley and Ploegh, 2005; Ye *et al.*, 2004). Knockdown of SEL1L blocks degradation of several ERAD-Ls substrates, such as truncated ribophorin (RI_{332}) (Mueller *et al.*, 2006); the null Hong Kong (NHK) variant of α1-proteinase inhibitor (α1-PI, also called α1-antitrypsin) (Christianson *et al.*, 2008; Hosokawa *et al.*, 2008); and unglycosylatable NHK, designated NHK-QQQ, in which the asparagine residues of all three potential glycosylation sites in NHK have been mutated to glutamine (Hosokawa *et al.*, 2008). In contrast, SEL1L knockdown shows little or only marginal effects on the degradation of ERAD-Lm substrates, such as TCRα (Mueller *et al.*, 2006), BACE476, and CD3-δ (Bernasconi *et al.*, 2010). Knockout of the SEL1L gene in mice causes embryonic lethality, and mouse embryonic fibroblasts deficient in SEL1L show blockage of both the degradation of NHK and secretion of Gaussia luciferase reporter into medium (Francisco *et al.*, 2010).

In this paper, we selected NHK and NHK-QQQ as glycosylated and unglycosylated substrates, respectively, of the ERAD-Ls pathway. NHK$_{BACE}$ and CD3-δ were selected as typical substrates of the ERAD-Lm pathway (see Fig. 1A for their schematic representation). NHK$_{BACE}$ is a transmembrane protein in the ER, in which NHK has been fused to the transmembrane and cytoplasmic regions of BACE, namely BACE501 (Bernasconi *et al.*, 2010).

Experimental Procedures

Cell culture and transfection

DT40 cells were cultured at a density of 1×10^5–1×10^6 cells per ml in RPMI1640 medium supplemented with 10% fetal bovine serum, 1% chicken serum, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 39.5°C in a humidified 5% CO_2/95% air atmosphere. DT40 cells were transfected by electroporation using a Microporator (Digital Bio) with two pulses at 1,500 V for 15 msec according to the manufacturer's instructions. Neomycin (2 mg/ml) and puromycin (0.5 μg/ml) were used for the selection and maintenance of drug-resistant clones.

pREP9-NHK and pREP9-NHK-QQQ were gifts from N. Hosokawa (Hosokawa *et al.*, 2001; Yoshida *et al.*, 2003). Plasmids for the expression of NHK$_{BACE}$-HA and CD3-δ-HA were provided by M. Molinari (Bernasconi *et al.*, 2010).

Construction of SEL1L targeting vector

The 2.7-kb fragment of the SEL1L gene used for the 5' arm was amplified by PCR from genomic DNA prepared from DT40 cells using the primers 5'-ATAAGAATGCGGCCGCGAAAATCCTTAATGAAAGCAC-3' and 5'-CGGGATCCGCATATAGAAATCCAAGGGC-3', and then subcloned between the NotI and BamHI sites of pBluescript II KS (+) vector to create the pBluescript-5' arm (SEL1L). The 3.0-kb fragment of the SEL1L gene used for the 3' arm was amplified similarly using the primers 5'-CGGGATCCGCATATAGAAATCCAAGGGC-3' and 5'-GGGGTACCGGTACAACATCACTTCCTTC-3', and then subcloned between the BamHI and KpnI sites of the pBluescript-5' arms (SEL1L) to create the pBluescript-5'-3' arms (SEL1L). The neomycin- or puromycin-resistant gene flanked by loxP sites was subcloned into the BamHI site of the pBluescript-5'-3' arms (SEL1L) to create pKO-SEL1L-neomycin or pKO-SEL1L-puromycin, respectively. These constructs were transfected into DT40 cells by electroporation after linearization.

Southern blotting

Southern blot hybridization was performed according to standard procedures (Sambrook *et al.*, 1989). Specific probes were amplified by PCR from genomic DNA prepared from DT40 cells using the primers 5'-CCTCAGAATAATGAAACAGC-3' and 5'-CTGGAACACCTCTTCCATAG-3'. Digoxigenin-labeled cDNA probes were prepared using PCR according to the manufacturer's instructions (Roche) and hybridized with DNA (3 μg) which had been digested with BamHI, treated with RNase, electrophoresed and blotted on a Hybond-N+ membrane (GE Healthcare Biosciences) using a 0.1 M NaCl/0.1 M NaOH solution. Subsequent reaction with anti-digoxigenin antibody (Roche) and treatment with the chemiluminescent detection reagent CDP-star (GE Healthcare Biosciences) were performed according to the manufacturer's specifications. Chemiluminescence was visualized using an LAS-3000mini LuminoImage analyzer (Fuji Film).

RT-PCR

Total RNA prepared from wild-type DT40 cells or various knockouts (~5×10^6 cells) by the acid guanidinium/phenol/chloroform method using ISOGEN (Nippon Gene) was converted to cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random primers. The full-length open reading frame of SEL1L was amplified using PrimeSTAR HS DNA polymerase (Takara) and a pair of primers, namely 5'-CCATCGATCAGGATGGGGTGGCGGGTGC-3' and 5'-CGGGATCCTTGCGGTGGTTGATGCTCTG-3'.

Immunological techniques

Immunoblotting analysis was carried out according to the standard procedure (Sambrook *et al.*, 1989). Approximately 2×10^6 cells were collected by centrifugation at 3,000 rpm for 2 min, washed with PBS, suspended in Laemmli's sample buffer and then boiled for 5 min. Samples were subjected to SDS-PAGE. Chemiluminescence obtained using Western Blotting Luminol Reagent (Santa Cruz Biotechnology) was detected using an LAS-3000mini LuminoImage analyzer (Fuji Film). Anti-α1 antitrypsin antibody was obtained from Dako. Anti-human SEL1L antibody was the kind gift of N. Hosokawa. Anti-Flag M2 and anti-human HRD1 antibodies were obtained from Sigma.

Metabolic labeling and immunoprecipitation

Sixteen hours after transfection, DT40 cells were starved for 10 min in methionine- and cysteine-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 2 mM glutamine and 10% dialyzed fetal bovine serum. The cells were pulse labeled for 10 min with 9.8 Mbq/dish EASY TAG™ EXPRESS Protein labeling mix [^{35}S] (PerkinElmer), centrifuged at 1,300 rpm for 2 min, and then chased in fresh complete medium. The cells were lysed in buffer A [50 mM Tris/Cl, pH 8.0, containing 1% NP-40, 150 mM NaCl, protease inhibitor cocktail (Nacalai Tesque) and 10 μM MG132]. Immunoprecipitation was performed using anti-α1 antitrypsin (Dako) or anti-HA (Santa Cruz) antibodies as well as protein A- or G-coupled Sepharose beads (GE Healthcare) according to procedures described previously (Oda *et al.*, 2006).

Construction of plasmids

Human, mouse and chicken SEL1L cDNA were obtained by RT-PCR amplification using total RNA prepared from HEK293 cells, mouse embryonic fibroblasts and DT40 cells, respectively, using the following paired primers: 5'-CCATCGATAGGATGCGGGTCCGGATAGGGC-3' and 5'-CGGGATCCCTGTGGTGGCTGCTGCTCTG-3' for human SEL1L, 5'-CCATCGATAGGATGCAGGTCCGCGTCAGGCT-3' and 5'-CGGGATCCCTGTGGTGGCTGCTGCTCTG-3' for mouse SEL1L, and 5'-CCATCGATCAGGATGGGGTGGCGGGTGC-3' and 5'-CGGGATCCTTGCGGTGGTTGATGCTCTG-3' for chicken SEL1L. These cloned genes were inserted between the ClaI and BamHI sites of p3*flag-CMV™-14 Expression vector (Sigma) to create pCMV-hSEL1L-Flag, pCMV-mSEL1L-Flag and pCMV-gSEL1L-Flag, in which the Flag tag is attached to the C-terminus of each SEL1L. pCMV-hSEL1L and pCMV-gSEL1L to express untagged versions of human and chicken SEL1L, respectively, were made by creating a stop codon at the original SEL1L stop codon site in pCMV-hSEL1L-Flag and pCMV-gSEL1L-Flag.

Deletion mutants of SEL1L were constructed by the site-directed mutagenesis (DpnI) method and amplified accordingly using Prime Star (TAKARA) and paired primers 5'-GAAGAGCAGTCCAATAAGAGG-3' and 5'-CTGACCTGCTACCACTTGAG-3' for gSEL1L-ΔFNII, 5'-CTGACTGGTGGAACAGTGG-3' and 5'-AGTTTCACAGAAGCCCCAC-3' for gSEL1L-ΔSR1-4, 5'-ATTCTGGCTTTTTATAATCTGG-3' and 5'-TGAAATATCACTAGCAACATG-3' for gSEL1L-ΔSR5–8, and 5'-GATGCCCAGGTTCCAGTCTTCC-3' and 5'-ATGGCCACCCTGGGAGGCC-3' for gSEL1L-ΔSR9–11.

Results

Disruption of chicken SEL1L locus

The chicken SEL1L gene product is highly homologous to human and mouse SEL1L gene products (Fig. 1B). Each contains the N-terminal signal sequence, a domain similar to type II module present in fibronectin, eleven copies of a short tetratricopeptide called Sel1-like repeats (SR1–11) (Fig. 1C), and a C-terminal transmembrane domain. The transmembrane domain is required for the retention of SEL1L in the ER, whereas the SR5-11 region is required for association with HRD1, OS-9 and XTP3-B in HEK293 cells (Christianson *et al.*, 2008). This conservation prompted us to knockout the SEL1L gene in the DT40 cell line to determine whether chicken cells are suitable for analysis of ERAD mechanisms in higher eukaryotes.

The chicken SEL1L gene consists of 21 exons (Fig. 2A).

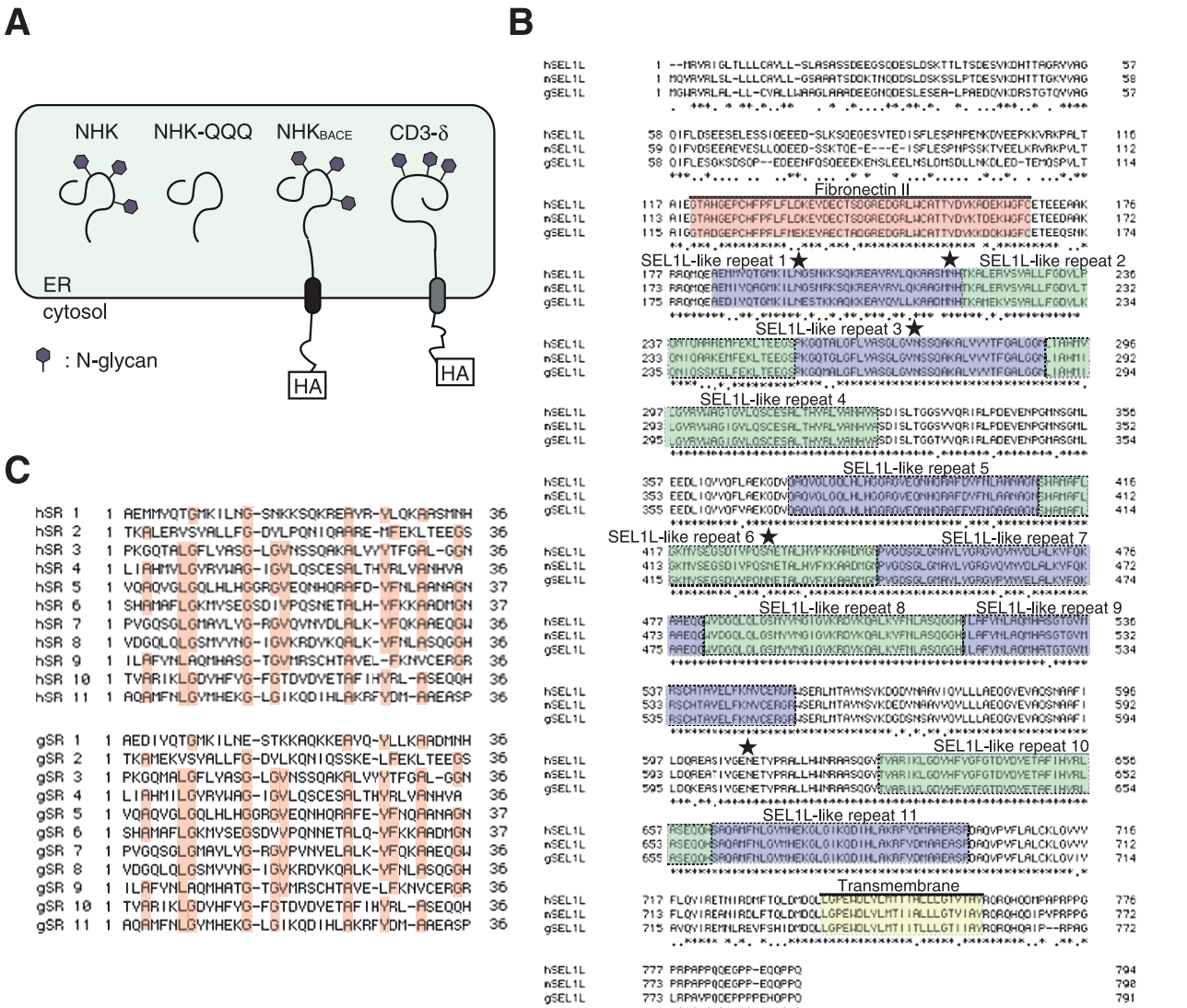

Fig. 1. Schematic presentation of the four ERAD substrates analyzed and conservation of SEL1L among vertebrates. (A) NHK and NHK-QQQ are substrates of the ERAD-Ls pathway, whereas NHKBACE and CD3-δ are substrates of the ERAD-Lm pathway. NHK, NHKBACE and CD3-δ are N-glycosylated, whereas NHK-QQQ is not. (B) Amino acid sequences of human, mouse and chicken SEL1L (designated hSEL1L, mSEL1L and gSEL1L, respectively) are aligned. Asterisks and periods underneath the sequences indicate amino acids identical in the three and two species, respectively. Stars denote potential N-glycosylation sites. The fibronectin II domain, Sel1-like repeats 1-11 and transmembrane domain are highlighted. (C) Amino acid sequences of human and chicken Sel1-like repeats (hSR and gSR, respectively) 1–11 are aligned and highly conserved amino acids are highlighted.

Exons 7–10, which encode a majority of SR3 and the entire SR4, were replaced with the neomycin-resistant gene (Fig. 2B); because SR1 and SR2 are less conserved in both human and chicken SEL1L compared with other repeats (Fig. 1C), we thought that the N-terminal fragment from the signal peptide to SR2 would not be functional even if it is expressed. We verified gene disruption by Southern blotting, and obtained 4 clones heterozygous for the SEL1L locus from 18 clones resistant to neomycin (Fig. 2C, lane 2). Exons 7–10 in the other allele in this heterozygous clone were replaced with the puromycin-resistant gene (Fig. 2B). Homologous recombination was again checked by Southern blotting, and we obtained 4 clones in which the SEL1L locus was correctly recombined from 20 clones resistant to puromycin (Fig. 2C, lanes 3 and 4). RT-PCR and immunoblotting analyses clearly showed the absence of SEL1L mRNA and protein, respectively, in the two SEL1L-knockout clones (Fig. 1D and E). SEL1L-knockout cells grew only slightly more slowly than the wild-type cells (Fig. 1F).

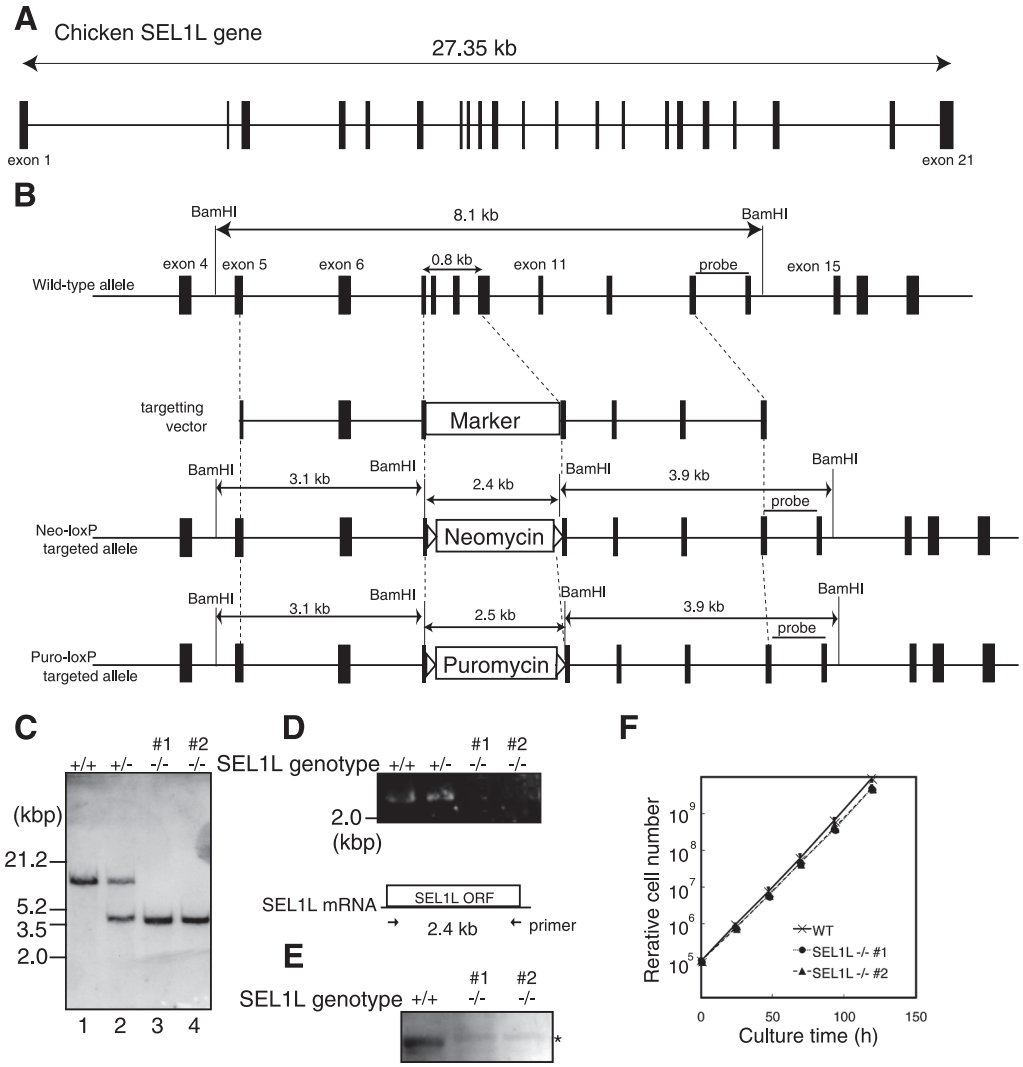

Fig. 2. Generation and characterization of DT40 cells deficient in SEL1L. (A) Structure of the chicken SEL1L gene is shown schematically with its length indicated at the top. It consists of 21 exons indicated by vertical lines. (B) Structures of wild-type and targeted alleles of the SEL1L gene as well as the SEL1L-targeting vector are shown schematically. Restriction enzyme sites and the positions of the 3'-probe for Southern blot hybridization are indicated. Locations and sizes of the fragments expected to be detected are shown at the top of each allele. The open arrowheads flanking the neomycin- or puromycin-resistant gene indicate *loxP* sites. (C) Genomic DNA isolated from parental DT40 cells (+/+), targeted cells by the neomycin-resistant gene (+/–), and targeted cells by the neomycin- and puromycin-resistant genes (–/–, #1 and #2) were digested with BamHI and analyzed by Southern blot hybridization using the 3'-probe to confirm the recombination of the SEL1L gene. (D) RT-PCR analysis was performed to amplify cDNA corresponding to SEL1L mRNA, as schematically shown at bottom, from total RNA which had been prepared from indicated genotypes. (E) Immunoblotting analysis of cell lysates prepared from indicated genotypes was performed using anti-human SEL1L antibody. The asterisk indicates a non-specific band. (F) Wild-type clone and two lines of SEL1L knockout (–/–) were cultured and their cell numbers were counted every 24 hr. Each value represents the mean of triplicate determinations.

Essential role of SEL1L in the ERAD-Ls but not ERAD-Lm pathway

We expressed NHK by transfection, and determined its fate by pulse chase experiments in DT40 cells. NHK disappeared from wild-type DT40 cells with a half-life of less than 1.5 h, but only approximately 10% of pulse-labeled NHK was secreted into medium (Fig. 3A). Importantly, treatment of wild-type DT40 cells with lactacystin, an inhibitor of the proteasome, significantly slowed the disappearance of NHK without affecting its secretion, indicating that NHK was subjected to ERAD, as in mammalian cells.

We then determined the fate of NHK in two clones of SEL1L knockout. Results showed that the degradation rate of NHK in SEL1L knockout cells was significantly slowed to the same extent as that observed in wild-type cells treated

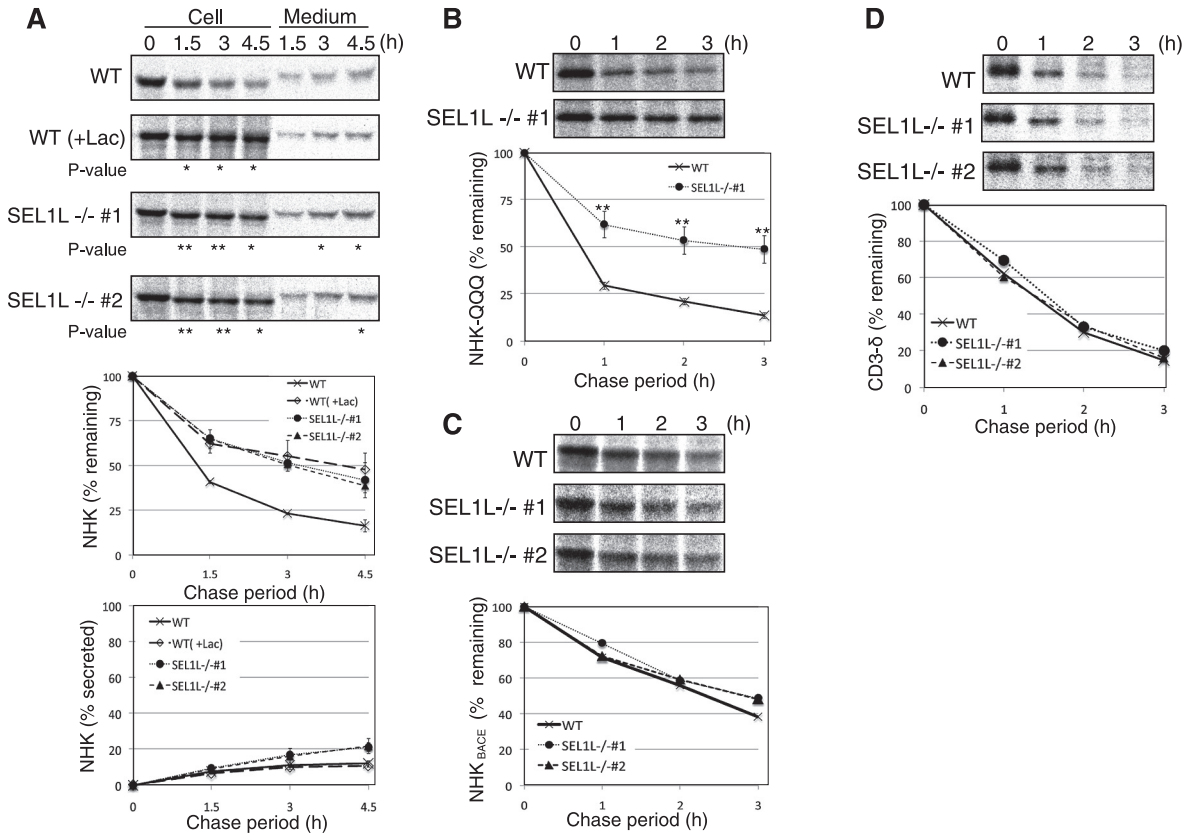

Fig. 3. Effect of SEL1L knockout on degradation of NHK, NHK-QQQ, NHKBACE and CD3-δ. (A) Wild-type cells and two lines of SEL1L knockout (–/–) were transfected with plasmid to express NHK. Sixteen hours later, cells were pulse labeled with ^{35}S-methionine and cysteine for 10 min and then chased for the indicated periods. An aliquot of wild-type cells was treated with 20 μM lactacystin (+Lac) 1 h prior to pulse labeling. Cells lysed with buffer containing 1% NP-40 as well as medium were subjected to immunoprecipitation using anti-α1-PI antibody. The radioactivity of each band was determined and normalized with the value at chase period 0 h. The means from three independent experiments with standard deviations (error bars) are plotted against the chase period. P value: * <0.05, ** <0.01. (B) Wild-type and SEL1L knockout cells were transfected with plasmid to express NHK-QQQ. Pulse-chase experiments followed by immunoprecipitation were carried out as in (A). The means from three independent experiments are plotted against the chase period. P value: ** <0.01. (C) Wild-type cells and two lines of SEL1L knockout were transfected with plasmid to express NHKBACE. Pulse-chase experiments followed by immunoprecipitation were carried out as in (A). (D) Wild-type cells and two lines of SEL1L knockout were transfected with plasmid to express CD3-δ. Pulse-chase experiments followed by immunoprecipitation using anti-HA antibody were carried out as in (A).

with lactacystin (Fig. 3A), indicating that degradation of NHK depends on SEL1L. Similarly, the degradation rate of unglycosylatable NHK-QQQ was markedly and significantly slowed in SEL1L knockout cells compared with wild-type cells (Fig. 3B). In marked contrast, degradation of NHKBACE and CD3-δ was not affected by the presence or absence of SEL1L (Fig. 3C and D). We therefore concluded that SEL1L is essential for the efficient degradation of misfolded soluble luminal proteins but dispensable for that of transmembrane proteins, even if the misfolded part is located in the ER lumen, as in mammals.

Importantly, introduction of flag-tagged or untagged wild-type chicken SEL1L gene into SEL1L knockout cells fully restored the degradation rate of NHK, as assessed by cycloheximide chase experiments (Fig. 4A). Furthermore, introduction of flag-tagged wild-type mouse SEL1L gene and untagged wild-type human SEL1L gene also restored the degradation rate of NHK (Fig. 4B), although the flag-tagged wild-type human SEL1L gene somehow did not do so (data not shown). These results clearly showed the conservation of ERAD mechanisms among vertebrates.

We then constructed deletion mutants of chicken SEL1L and analyzed whether they could restore the ability of SEL1L knockout cells to degrade NHK (Fig. 5A). Immunoblotting analysis showed that full-length as well as various deletion mutants were expressed at comparative levels, and that all were sensitive to endoglycosidase H, indicating that they were correctly localized in the ER (Fig. 5B and C). Cycloheximide chase experiments revealed that full-length SEL1L and a mutant lacking the fibronectin II domain (gSEL1L-ΔFNII) restored NHK degradation, whereas three mutants lacking Sel1-like repeats (Δ1–4, Δ5–8, and Δ9–11)

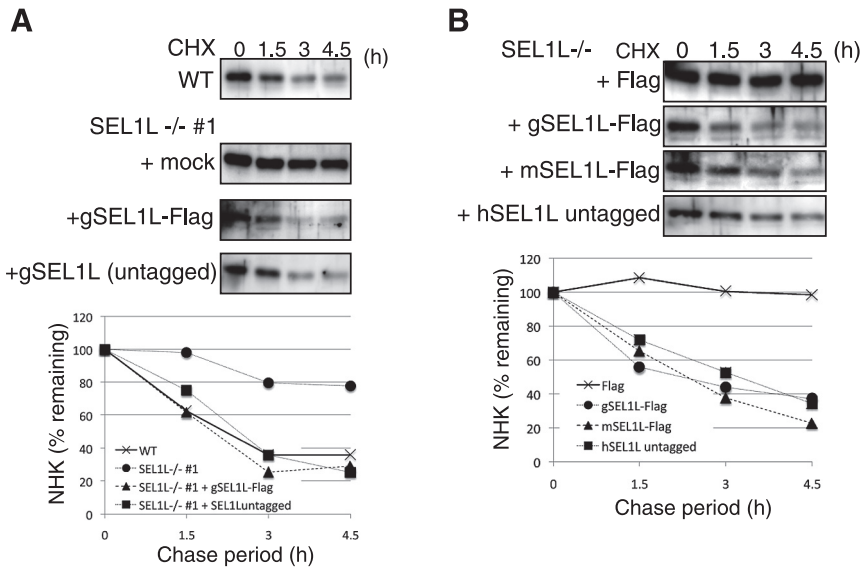

Fig. 4. Effect of introduction of chicken, mouse and human SEL1L on NHK degradation in SEL1L knockout cells. (A) Wild-type and SEL1L knockout cells were transfected with plasmid to express NHK. SEL1L knockout cells were also transfected with plasmid to express Flag-tagged chicken SEL1L (gSEL1L-Flag) or untagged chicken SEL1L. Fifteen hours later, transfected cells were treated with cycloheximide (CHX, 50 μg/ml) for the indicated periods. Cell lysates were prepared and analyzed by immunoblotting using anti-α1-PI antibody. The intensity of each band was determined and normalized with the value at chase period 0 h. (B) SEL1L knockout cells were transfected with vector alone (Flag) or plasmid to express Flag-tagged chicken SEL1L (gSEL1L-Flag), Flag-tagged mouse SEL1L (mSEL1L-Flag), or untagged human SEL1L (hSEL1L untagged) together with plasmid to express NHK. Cycloheximide chase experiments followed by immunoblotting was carried out as in (A).

were defective (Fig. 5D), implicating the importance of Sel1-like repeats in the function of SEL1L.

Discussion

SEL1L is highly conserved among vertebrates, with the amino acid sequence of human SEL1L 83% identical to that of chicken SEL1L (Fig. 1B). In contrast, human SEL1L is only 6% identical to Hrd3p, the yeast homologue. Both human and chicken SEL1L contain 11 tetratricopeptide repeats called Sel1-like repeats (Fig. 1C), whereas yeast Hrd3p contains 6 tetratricopeptide repeats. We therefore considered that this high conservation, as well as the relative ease of gene targeting, would make chicken DT40 cells a highly useful model for investigating the molecular mechanisms governing mammalian ERAD. Indeed, 4 of 18 and 4 of 20 clones isolated based on their resistance to neomycin and puromycin, respectively, were correctly targeted (Fig. 2C). The availability of further four selection markers as well as the ability to remove an incorporated selection marker from the DT40 genome using the Cre-loxP system will allow us to assess the function of redundant proteins by constructing complete knockouts and analyzing their phenotypes.

In this paper we show that, similarly to mammalian SEL1L, chicken SEL1L is essential to the degradation of ERAD-Ls substrates but not to that of ERAD-Lm substrates (Fig. 3). This finding further supports the appropriateness of DT40 cells in the analysis of ERAD mechanisms. It was previously reported that the yeast ortholog protein Hrd3p is required for the degradation of both ERAD-L and ERAD-M substrates (Carroll and Hampton, 2010; Carvalho *et al.*, 2006). This discrepancy is very likely due to the secondary effect of Hrd3p knockout on Hrd1p, an E3 which is essential for both ERAD-L and ERAD-M pathways (Bordallo *et al.*, 1998) and which is destabilized through its self-ubiquitination in the absence of Hrd3p (Carroll and Hampton, 2010; Gardner *et al.*, 2000). This stability control of Hrd1p by Hrd3p probably plays an important role in yeast cells in avoiding undesired degradation of proteins which are still functional in the ER. Under normal conditions, for example, the temperature-sensitive *sec61-2* mutant is rapidly degraded by the ERAD-M pathway at temperatures above 38°C only (Plemper and Wolf, 1999), but becomes highly unstable even at the low temperature of 25°C, when Hrd1p is overexpressed (Bordallo *et al.*, 1998). In contrast, this stability control of Hrd1p by Hrd3p does not seem to occur in vertebrates, because the SEL1L knockdown in mammals in previous publications or SEL1L knockout in chickens shown here had little effect on the ERAD-Lm pathway, which requires HRD1; although SEL1L knockdown in mammals exhibited a marginal effect on the degradation of ERAD-Lm substrates, such as BACE476

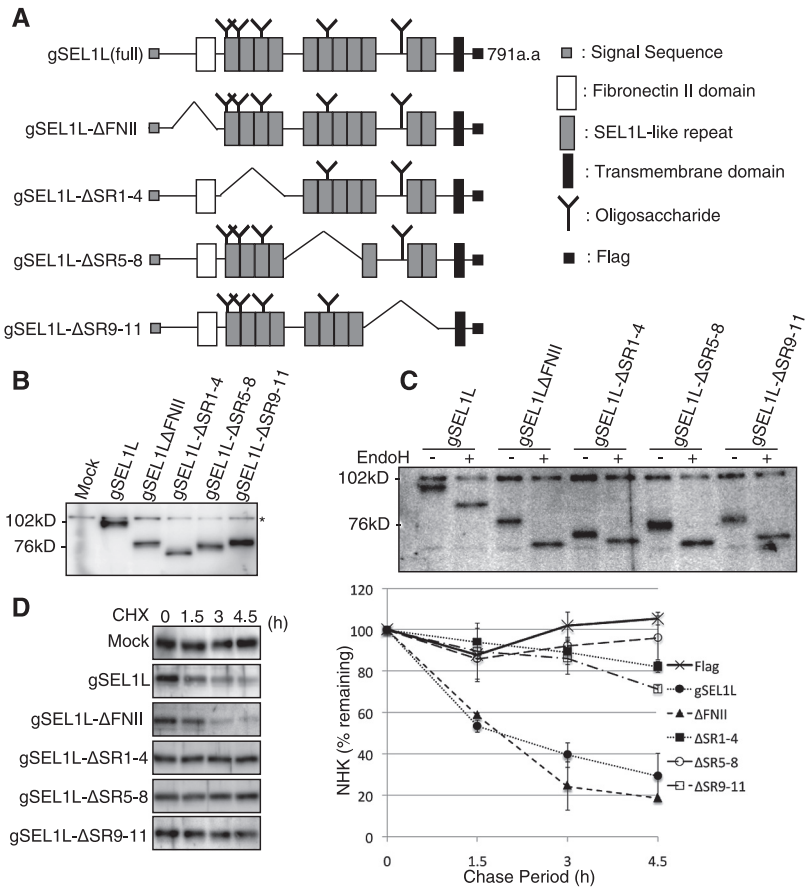

Fig. 5. Effect of deleting fibronectin II domain or Sel1-like repeats on the function of SEL1L. (A) Schematic structures of full-length SEL1L and its various deletion mutants analyzed. (B) SEL1L knockout cells were transfected with plasmid to express full-length or deletion mutant of SEL1L. Cell lysates were prepared and analyzed by immunoblotting using anti-Flag antibody. The asterisk denotes a non-specific band. (C) Cell lysates prepared as in (B) were untreated (–) or treated (+) with endoglycosidase H (Endo H), and then analyzed by immunoblotting as in (B). (D) SEL1L knockout cells were transfected with plasmid to express full-length or deletion mutant of SEL1L together with plasmid to express NHK. Cycloheximide chase experiments were performed as in Fig. 4A. The means from two independent experiments with standard deviations (error bars) are plotted against the chase period.

(Bernasconi *et al.*, 2010), tyrosinase (C89R) (Ballar *et al.*, 2011) and TCRα (Cattaneo *et al.*, 2008), overexpression of a dominant negative form of HRD1 blocked degradation of TCRα (Kikkert *et al.*, 2004). As overexpression of human HRD1 rather slows degradation of the ERAD-Lm substrate TCRα (Kikkert *et al.*, 2004), the activity of HRD1 is probably well self-controlled, which has allowed the development of vertebrate HRD1 free from any stability control by SEL1L. Unfortunately, we were unable to demonstrate this notion directly because the anti-human HRD1 antibody we obtained detected human HRD1 protein in HEK293 cells but multiple bands in both wild-type and SEL1L knockout DT40 cells at similar levels (data not shown), and we were unable to assign which band represented chicken HRD1 protein because the still-incomplete chicken genome database contains no information on the HRD1 gene, mRNA and protein. It was also previously shown that yeast Hrd1p was able to recognize specifically misfolded membrane proteins through its transmembrane domain, independently of Hrd3p or any other ERAD factor (Sato *et al.*, 2009). Such a characteristic feature might perhaps be conserved in vertebrate HRD1, which would allow the degradation of misfolded membrane proteins in the absence of SEL1L.

Molinari and colleagues proposed that mammalian OS-9, a homologue of yeast Yos9p, plays a dual role in the quality control of proteins in the ER, namely inhibition of the secretion of misfolded protein conformers and enhancement of their disposal, based on the observation that knockdown of OS-9 resulted in a delay in NHK degradation (half life of ~90 min in control cells versus ~140 min in knockdown cells) and an increase in NHK secretion (25% in control cells versus 38% in knockdown cells after 3 h chase) (Bernasconi *et al.*, 2008). However, an increase in NHK secretion may simply result from an increase in the level of intracellular NHK due to the delay in NHK degradation. Interestingly, SEL1L knockout in DT40 cells showed the

same phenotype as OS-9 knockdown: NHK degradation was delayed (half life of ~1 h in wild-type cells versus ~3 h in knockout cells) and NHK secretion was increased (12% in wild-type cells versus 21% in knockout cells after 4.5 h chase) (Fig. 3A). Importantly, treatment of wild-type cells with lactacystin delayed NHK degradation to the same extent as that observed in SEL1L knockout cells but had no effect on NHK secretion, which was 11% after 4.5 h chase (Fig. 3A), indicating that the level of intracellular NHK is not the main determinant of NHK secretion. Our results indicate that SEL1L is a component of not only ERAD but also of the ER protein quality control machinery, which is aimed at blocking the secretion of misfolded proteins.

Defect of SEL1L knockout cells in NHK degradation was restored by introduction of not only chicken SEL1L but also mouse and human SEL1L (Fig. 4B). We further showed the importance of the Sel1-like repeats but not the fibronectin II domain in the function of SEL1L (Fig. 5). In conclusion, our reverse genetic approach using the chicken DT40 cell line provides highly useful information regarding ERAD mechanisms in higher eukaryotes which express ERAD components redundantly.

Acknowledgment. We thank Ms. Kaoru Miyagawa and Ms. Yayoi Yamamoto for their technical and secretarial assistance. We are grateful to Dr. Hosokawa (Kyoto University, Japan) for providing anti-human SEL1L antibody and various plasmids, and to Dr. Molinari (Institute for Research in Biomedicine, Switzerland) for providing various plasmids. This work was financially supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (20247026 to K.M.) and also in part by the Global Center of Excellence Program "Formation of a Strategic Base for Biodiversity and Evolutionary Research: from Genome to Ecosystem" of the Ministry of Education, Culture, Sports and Technology of Japan. Satoshi Ninagawa was a recipient of Research Fellowships from the Japan Society for the Promotion of Science for Young Scientists (2008–2009).

References

Ballar, P., Pabuccuoglu, A., and Kose, F.A. 2011. Different p97/VCP complexes function in retrotranslocation step of mammalian ER-associated degradation (ERAD). *Int. J. Biochem. Cell Biol.*, **43**: 613–621.

Bernasconi, R., Pertel, T., Luban, J., and Molinari, M. 2008. A dual task for the Xbp1-responsive OS-9 variants in the mammalian endoplasmic reticulum: inhibiting secretion of misfolded protein conformers and enhancing their disposal. *J. Biol. Chem.*, **283**: 16446–16454.

Bernasconi, R., Galli, C., Calanca, V., Nakajima, T., and Molinari, M. 2010. Stringent requirement for HRD1, SEL1L, and OS-9/XTP3-B for disposal of ERAD-LS substrates. *J. Cell Biol.*, **188**: 223–235.

Biunno, I., Appierto, V., Cattaneo, M., Leone, B.E., Balzano, G., Socci, C., Saccone, S., Letizia, A., Della Valle, G., and Sgaramella, V. 1997. Isolation of a pancreas-specific gene located on human chromosome 14q31: expression analysis in human pancreatic ductal carcinomas. *Genomics*, **46**: 284–286.

Bordallo, J., Plemper, R.K., Finger, A., and Wolf, D.H. 1998. Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. *Mol. Biol. Cell*, **9**: 209–222.

Buchberger, A., Bukau, B., and Sommer, T. 2010. Protein quality control in the cytosol and the endoplasmic reticulum: brothers in arms. *Mol. Cell*, **40**: 238–252.

Carroll, S.M. and Hampton, R.Y. 2010. Usa1p is required for optimal function and regulation of the Hrd1p endoplasmic reticulum-associated degradation ubiquitin ligase. *J. Biol. Chem.*, **285**: 5146–5156.

Carvalho, P., Goder, V., and Rapoport, T.A. 2006. Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell*, **126**: 361–373.

Cattaneo, M., Otsu, M., Fagioli, C., Martino, S., Lotti, L.V., Sitia, R., and Biunno, I. 2008. SEL1L and HRD1 are involved in the degradation of unassembled secretory Ig-μ chains. *J. Cell. Physiol.*, **215**: 794–802.

Christianson, J.C., Shaler, T.A., Tyler, R.E., and Kopito, R.R. 2008. OS-9 and GRP94 deliver mutant alpha1-antitrypsin to the Hrd1-SEL1L ubiquitin ligase complex for ERAD. *Nat. Cell Biol.*, **10**: 272–282.

Conticello, S.G., Langlois, M.A., Yang, Z., and Neuberger, M.S. 2007. DNA deamination in immunity: AID in the context of its APOBEC relatives. *Adv. Immunol.*, **94**: 37–73.

Francisco, A.B., Singh, R., Li, S., Vani, A.K., Yang, L., Munroe, R.J., Diaferia, G., Cardano, M., Biunno, I., Qi, L., Schimenti, J.C., and Long, Q. 2010. Deficiency of suppressor enhancer Lin12 1 like (SEL1L) in mice leads to systemic endoplasmic reticulum stress and embryonic lethality. *J. Biol. Chem.*, **285**: 13694–13703.

Gardner, R.G., Swarbrick, G.M., Bays, N.W., Cronin, S.R., Wilhovsky, S., Seelig, L., Kim, C., and Hampton, R.Y. 2000. Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. *J. Cell Biol.*, **151**: 69–82.

Hoseki, J., Ushioda, R., and Nagata, K. 2010. Mechanism and components of endoplasmic reticulum-associated degradation. *J. Biochem. (Tokyo)*, **147**: 19–25.

Hosokawa, N., Wada, I., Hasegawa, K., Yorihuzi, T., Tremblay, L.O., Herscovics, A., and Nagata, K. 2001. A novel ER α-mannosidase-like protein accelerates ER-associated degradation. *EMBO Rep.*, **2**: 415–422.

Hosokawa, N., Wada, I., Nagasawa, K., Moriyama, T., Okawa, K., and Nagata, K. 2008. Human XTP3-B forms an endoplasmic reticulum quality control scaffold with the HRD1-SEL1L ubiquitin ligase complex and BiP. *J. Biol. Chem.*, **283**: 20914–20924.

Kikkert, M., Doolman, R., Dai, M., Avner, R., Hassink, G., van Voorden, S., Thanedar, S., Roitelman, J., Chau, V., and Wiertz, E. 2004. Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. *J. Biol. Chem.*, **279**: 3525–3534.

Lilley, B.N. and Ploegh, H.L. 2005. Multiprotein complexes that link dislocation, ubiquitination, and extraction of misfolded proteins from the endoplasmic reticulum membrane. *Proc. Natl. Acad. Sci. USA*, **102**: 14296–14301.

Mueller, B., Lilley, B.N., and Ploegh, H.L. 2006. SEL1L, the homologue of yeast Hrd3p, is involved in protein dislocation from the mammalian ER. *J. Cell Biol.*, **175**: 261–270.

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.

Plemper, R.K. and Wolf, D.H. 1999. Retrograde protein translocation: ERADication of secretory proteins in health and disease. *Trends Biochem. Sci.*, **24**: 266–270.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sato, B.K., Schulz, D., Do, P.H., and Hampton, R.Y. 2009. Misfolded membrane proteins are specifically recognized by the transmembrane domain of the Hrd1p ubiquitin ligase. *Mol. Cell*, **34**: 212–222.

Xie, W. and Ng, D.T. 2010. ERAD substrate recognition in budding yeast. *Semin. Cell Dev. Biol.*, **21**: 533–539.

Ye, Y., Shibata, Y., Yun, C., Ron, D., and Rapoport, T.A. 2004. A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature*, **429**: 841–847.

Yoshida, H., Matsui, T., Hosokawa, N., Kaufman, R.J., Nagata, K., and Mori, K. 2003. A time-dependent phase shift in the mammalian unfolded protein response. *Dev. Cell*, **4**: 265–271.

(Received for publication, June 22, 2011, accepted, August 8, 2011 and published online, August 20, 2011)