Sel1L-Hrd1-Mediated OS9 Degradation During Endoplasmic Reticulum Stress

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Chapter 1: INTRODUCTION AND LITERATURE REVIEW

1.1 The Endoplasmic Reticulum and Protein Quality Control

The endoplasmic reticulum (ER), a eukaryotic cellular organelle, composed of interconnected flattened sacs and tubules that is located in between the outer nucleus membrane and the Golgi. The main function of ER includes protein quality control and folding, and lipid metabolism, which are carried out by rough ER (RER) and smooth ER (SER), respectively.

The RER facilitates the folding of all secretory and membrane proteins, which include approximately one third of cell's total proteins (Brodsky and Wojcikiewicz, 2009). After being co-translationally transported into RER, nascent proteins then become fully folded, modified and transported to their destined locations. This process is tightly regulated by quality control machineries to ensure the homeostasis of the cell and the organism.

Nascent proteins enter the ER through a narrow, heterotrimeric channel called Sec61 complex (Matlack et al., 1998). For glycosylated proteins, a preassembled oligosaccharide core N-acetinglucosamine2-mannose9-glucose3 $Glc_3Man_9GlcNAC_2$ (N-linked oligosaccharides or N-glycan), derived from dolichol-PP, a lipid pyrophosphate donor on the ER membrane, is immediately added at Asn-X-Ser/Thr asparagine (ASN) motifs of nascent proteins by oligosaccharyltransferase (OST) (Parodi, 2000), (Parodi et al., 1972). The addition of N-linked oligosaccharides triggers the trimming of the two outermost glucose units by α-glucosidases I and II, leaving a N-glycan structure that can be recognized by two homologous ER lectin chaperones, calnexin (CNX) and calreticulin (CRT). CNX-CRT, along with an

oxidoreductase ERp57, interacts with glycoproteins to aid their proper folding as well as disulfide bond formation (Coe and Michalak, 2010). For well-folded glycoproteins, glucosidase II cleaves the last glucose unit, lowering the affinity and allows proteins to be released by CNX-CRT, where they can be transported to the Golgi compartment and are further modified (Malhotra and Kaufman, 2007; Molinari, 2007). Misfolded or unfolded glycoproteins, however, are recognized and re-glucosylated by ER folding sensor UDP-glucose:glycoprotein glucosyltransferase (UGT1), which allow them to stay a few more cycle of CNX-CRT and assisted for forming their protein structures (Pearse et al., 2010). If failed to achieve their destined structure, O-mannosylation is added onto the proteins by phosphoethanolamine N-methyltransferase 1 and 2 (Pmt1/Pmt2), which serve as a degradation marker to prevent overuse of chaperones and inhibits aggregation (Xu et al., 2013). These glycoproteins will eventually be cleared by ER-associated degradation (ERAD), a process that will be discuss in details in 1.2.

In contrast to glycoproteins, nascent non-glycosylated proteins are thought be assisted by a complex involving protein disufide isomerase (PDI), one of the first-identified thiol-disulfide oxidoreductases (Ferrari and Soling, 1999; Freedman et al., 2002), and binding immunoglobulin protein (BiP), a chaperone that protects immature protein from aggregation by binding to extended hydrophobic domains of nascent proteins (Hebert and Molinari, 2007). If proteins are properly folded, nonglycosylated proteins will be transported to the Golgi, similar to that of glycosylated proteins. If not, nonglycoproteins will be transported to ERAD complex by ATP-hydrolysis-powered BiP for degradation. Prior to the degradation, disulfide bonds of the misfolded non-glycosylated proteins will be cleaved by ERdj5, a protein with reductase activity at its C-terminus (Ushioda et al., 2013).

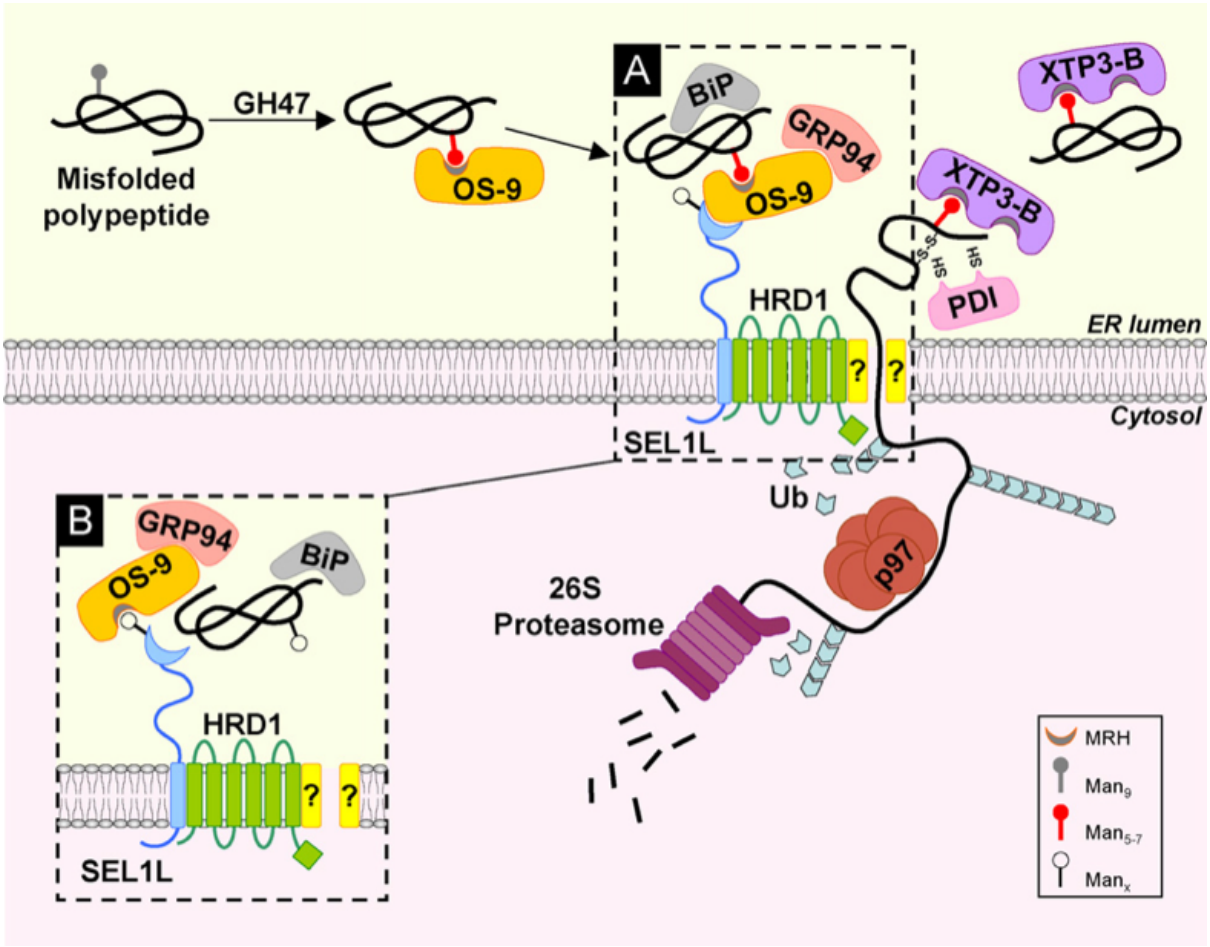
1.2 ER Associated Degradation (ERAD) – The Sel1L-Hrd1 Complex

While properly folded glycosylated proteins and nonglycosylated proteins leave the ER through COP-II coated vesicles, misfolded or unfolded proteins are retained in the ER and targeted for proteasome degradation, a process called ERAD. ERAD centers on an E3 ubiquitin ligase, which possesses variable number of transmembrane domains and a cytosolic RING finger domain and catalyze substrate ubiquitination. E3 ligase, together with multiple cofactors, forms an ERAD complex. ERAD complexes are responsible for substrates recognition, translocation and ubiquitination. In both yeast and mammalian systems, each E3 ligase predominantly degrades a specific subset of substrates, ranging from substrates with ER luminal, cytosolic and/or membrane lesions (Smith et al., 2011); however, studies have shown that overlap of substrate specificities occur in both mammals and yeast.

Yeast contains two ubiquitin ligases, Doa10 and Hrd1p, and both contain large multispanning membrane domains (Mehnert et al., 2010). Hrd1p interacts with both substrates with membrane or luminal lesions and ERAD components on the luminal side, while promotes polyubiquitination and the delivery of substrates on the cytoplasmic side (Mehnert et al., 2010). Hrd1p associates with substrate recruitment factor Hrd3p, the ubiquitin-conjugating enzyme Ubc7, as well as Cdc48p ATPase (Gauss et al., 2006b). Kar2p and Yos9p are two chaperones binding to substrates and are in association with Hrd1p. Yos9p was shown to bind to glycosylated substrates at its MRH domain and form a complex with BiP, thus defining substrates of Hrd1p (Carvalho et al., 2006; Denic et al., 2006; Hosokawa et al., 2001). In fact,

both Yos9 and Hrd3p serve as “gatekeepers” in recognizing substrates (Ismail and Ng, 2006). In addition, the degradation of all soluble, luminal proteins also depends on the binding of a small integral protein Der1 to Hrd1p via Usa1p (Carvalho et al., 2006; Horn et al., 2009). The Doa10 ligase removes ER-membrane proteins with cytoplasmic lesions, while whether it also removes soluble proteins remain controversial (Kaufman et al., 2002; Mehnert et al., 2010). In order to degrade proteins, the Doa10 complex requires ubiquitin-conjugating enzyme Ubc6, Ubc7, along with Ubc cofactor Cue1 and Cdc48p ATPase complex (Ismail and Ng, 2006; Kaufman et al., 2002).

To date, many distinct ERAD pathways have been found in the mammalian system, such as HMG-CoA reductase degradation protein 1 (Hrd1), glycoprotein 78 (Gp78), Kf-1 and TRC8 (Maruyama et al., 2008; Mehnert et al., 2010; Stagg et al., 2009). Yet, only Hrd1 and Gp78 were studied in details, and several exogenous and endogenous substrates were identified in each pathway ((Hirsch et al., 2009). Hrd1 is the mammalian homolog of Hrd1p in yeast, which form a stoichiometric complex with sel-1 suppressor of lin-12-like (Se1lL), the mammalian homolog of Hrd3p, for its own stability and for substrate recognition. Chaperones and lectins also recognize specific subset of substrates and deliver them to either Hrd1 or Sel1L for degradation.



Hebert. D.N., Bernasconi. R., & Molinari. M. (2009). *Seminars in Cell & Developmental Biology*

In details, substrates are proteins that have lesions or fail to achieve their native states. When glycosylated proteins/substrates fail to form their proper structures, they are removed from CNX-CRT cycle by mannosidases and/or ER degradation enhancer, mannosidase alpha like (EDEM), enzymes that can remove sugar units. EDEM interacts with CNX and possibly receives glycosylated substrates during their interaction (Hirao et al., 2006; Hosokawa et al., 2001; Mast et al., 2005; Molinari et al., 2003; Oda et al., 2003; Olivari et al., 2005). Sequentially, substrates are transferred to osteosarcoma amplified 9 (OS9)/transactivated gene B protein (XTP3B), which are mammalian homologs of Yos9. Interestingly, OS9 and XTP3B seem to have different functions from one another. While OS9 interacts with 94 kDa

glucose-regulated protein (GRP94) and Sel1L (Christianson et al., 2008), XTP3B forms a complex with BiP and interacts with Se1L-Hrd1 complex (Hosokawa et al., 2008). Nevertheless, both lectins deliver glycosylated substrates to the Sel1L-Hrd1 complex. In contrast to glycosylated substrates, non-glycosylated substrates are recognized by BiP, followed by the cleavage of disulfide bound by ERdj5. After ATP hydrolysis, BiP delivers substrates to Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein (Herp) complex that composed of p97 ATPase, Hrd1, Sel1L and Derlin-1 (Ushioda et al., 2013).

Substrates are then translocated through a channel from ER to cytosol. Previously, Sec61 was thought to be the channel for the translocation of substrates. However, it was shown under real-time fluorescence that ERAD substrates were retained in the ER when Derlin-1, the homolog of Der1 in yeast was blocked, but not when Sec61 was blocked (Wahlman et al., 2007). Hence, Derlin-1 now is considered as the most possible channel for the retrotranslocation. Derlin-1 associates with different substrates and interacts with VIMP that recruits the p97 ATPase and its cofactors (Ye et al., 2004). Ubiquitination are thought to be occurred on the cytosolic domain of substrates once they enter the channel, which is carried out by an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and the E3 ligase (Richly et al., 2005). The ring domain on Hrd1 and other E3 ligases add ubiquitin (Ub) to substrates powering by ATPase P97. After translocation and polyubiquitination, substrates are finally degraded by cytosolic 26S proteasome. Deubiquitination is also required in the process of dislocation (Ernst et al., 2009).

1.3 The IRE1α-XBP1 pathway of the Unfolded Protein Response (UPR)

Dysfunction of ERAD leads to the accumulation of misfolded proteins, leading to ER stress and unfolded protein response (UPR), which include three major ER-to-nucleus signaling pathways (Shen et al., 2004). Unlike ERAD that operates in both normal and stress conditions, UPR is activated only in stressed state. Three UPR pathways, the inositol-requiring protein-1 (IRE1α), activating transcription factor-6 (ATF6) and protein kinase RNA-like ER kinase (PERK) pathways work synergically to first reduce protein load in the ER, then increase capacity of the ER to handle unfolded proteins, and finally trigger cell death if ER stress becomes irreversible (Ron and Walter, 2007). IRE1α is a bifunctional transmembrane kinase and endoribonuclease that is activated through autophosphorylation and oligomerization under ER stress (Ron and Walter, 2007). Activated IRE1α cleaves a unique mRNA called X-box binding protein 1 (XBP1/XBP1u) in metazoans, which cause the excision of an intron and lead to a spliced, lower molecular weight mRNA XBP1s. XBP1s is a transcription factor that targets a diverse ranges of genes, including ER chaperones and ERAD components, tissue specific metabolic genes, disease-associated genes and so on (Acosta-Alvear et al., 2007). Thus, the activation of IRE1α enhances protein-folding capacity of the ER and alleviates ER stress; ATF6 is an ER-membrane embedded transcription factor that pinched off and transported through vesicle upon ER stress. In there, ATF6 is cleaved sequentially by site-1 and site-2 proteases (S1P and S2P) (Haze et al., 1999; Ye et al., 2000), leaving a N-terminal domain (ATF6N) that is translocated to the nucleus and up-regulates UPR genes such as BiP, Grp94 and PDI (Walter and Ron, 2011). Similar to the activation of IRE1α, the last UPR sensor PERK is activated by autophosphorylation and oligomerization (Kebache et al., 2004). Unlike IRE1α, PERK inhibits the potent translation initiation factor eukaryotic translation

initiation factor-2 (eIF2α), thus inhibiting translation and protein load in the ER (Ron and Walter, 2007).

Although both ERAD and UPR are powerful tools cells utilize to relief ER stress, they can be insufficient in the presence of severe ER stress. In this condition, cell death will be triggered. Under prolonged and severe ER stress, calcium is transferred from ER to mitochondria mainly through ligand-gated calcium channel called inositol 1,4,5-triphosphate receptors (IP3Rs), initiating a calcium-dependent caspase apoptotic pathway that eventually leads to cell death (Mendes et al., 2005).

1.4 OS9

OS9 is a mammalian lectin in the ER lumen that contains Mannose-6-phosphate receptor homology (MRH) domain and is an important component of the Sel1L-Hrd1 ERAD complex (Hosokawa et al., 2010a). OS9 mRNA is expressed ubiquitously and is amplified in sarcomas (Hosokawa et al., 2010a). Human OS9 has four variants as a result of alternative splicing: full-length version of the protein OS9.1 comprises 667 amino acid; OS9.2 lacks exon 13 and is 55 amino acid shorter with a glutamate to glycine conversion in the splice region; OS9.3, which is 70 amino acid shorter than the full length, lacks exon 13 and last part of exon 11; OS9.4 lacks final part of exon 11 only and is 15 amino acid shorter (Kimura et al., 1998). However, only OS9.1 and OS9.2 have been observed in human/mice cell lines and tissues (Bernasconi et al., 2008). Interestingly, reverse transcription (RT) and quantitative polymerase chain reaction (qPCR) analysis revealed that the transcripts of OS9.1 at normal cellular state are significantly lower than those of OS9.2 in both mouse embryonic fibroblasts (MEF) and human embryonic kidney 293 T cells (HEK

293T) (Bernasconi et al., 2008). It also has been reported that the transcription of OS9 gene is induced upon ER stress (Bernasconi et al., 2008).

OS9, as well as its yeast homolog Yos9, has been identified to deliver substrates, especially luminal glycosylated substrates to the Sel1L-Hrd1/Hrd3p-Hrd1p complex for degradation (Hosokawa et al., 2010a). The substrate recognizing ability of OS9 lies in its MRH domain, which is homologous to mannose-6-phosphate receptors (MPRs) (Munro, 2001). Both cation-dependent (CD-MPR) and cation-independent MPRs (CI-MPR) recognizes terminal mannose-6-phosphate (M6P) on the N-linked glycans of lysosomal enzymes (Satoh et al., 2010), which lead to the recognition that proteins containing an MRH domain are lectins that have sugar (glycan) binding ability (Hosokawa et al., 2010a). Alignment of Yos9, OS9 and XTP3B, another homolog of Yos9 found in mammals, revealed that several amino acid and six cysteine residues are well conserved; yet, only two of the six interact with mannose in CD-MPR (Roberts et al., 1998). Despite the N-terminal of MRH domains of Yos9 and OS9 share 20% identity, they only share 12% overall identity, suggesting only the functional part of MRH has been well conserved (Friedmann et al., 2002). Crystallized human OS9 MRH domain showed that OS9 has a flattened B-barrel structure with a P-type lectin fold and contains a double tryptophan residues at its 117 and 118 position, which resides in its sugar binding site (Satoh et al., 2010). The same study showed that this double trypophan motif (WW) recognizes specific trimmed-glycan structure presented on misfolded glycoproteins, allowing OS9 to bind and sequentially deliver substrates to the ERAD complex (Satoh et al., 2010).

Yos9 in yeast is a luminal membrane-associated ER protein that is required for the degradation of glycosylated proteins, but not non-glycosylated proteins in

yeast (Munro, 2001). The lectin domain of Yos9 is required for its interaction with substrates (Gauss et al., 2006a; Kim et al., 2005), but not for its interaction with the ERAD complex. Yos9 interacts with a well-defined ERAD glycosylated substrate carboxypeptidase Y (CPY) only when CPY is misfolded (Munro, 2001); however, this interaction was abolished when MRH domain of Yos9 was deleted. It is also found that without MRH domain, misfolded CPY was retained in the ER instead of being degraded, suggesting the MRH domain of Yos9 is needed for the delivery of misfolded glycoproteins to the Hrd1p-Hrd3p ERAD complex. Despite the deletion of MRH abolish the interaction of Yos9 and substrates, it does not affect the interaction of Yos9 and Hrd3p (Gauss et al., 2006a) The question then lies at how Yos9 interact with the complex. Following study confirmed that Yos9 physically interact with the last TPR triplet of Hrd3p, but does not directly interact with Hrd1p (Gauss et al., 2006b). It has been proposed that Yop9 confirms the misfolding state of substrates before handing them to the ERAD complex by proofreading the exposed hydrophobic residues on the surface of substrates (Munro, 2001), thus acting as a “gatekeeper” to ensure quality of ERAD.

Although Yop9 is well characterized in yeast, mammalian OS9 in ERAD is less clear and more controversial. First of all, the two variants of OS9, OS9.1 and OS9.2, seem to have its own special function and effect, apart from their common function. From the observation in our laboratory, the protein level of the two variants changes by unknown variable in both MEF and HEK 293T cells, despite the level of OS9.2 transcripts is higher in both cell lines (Bernasconi et al., 2008). Functionally, it has been shown that when Sel1L is silenced by RNAi, substrate BACE476 delta binds specifically to OS9.1, but not OS9.2. RNAi silencing of both OS9.1 and OS9.2 delays the clear of substrate NHK, but not when only OS9.1 is silenced. Sucrose

gradient analysis also suggested OS9.2 preferentially binds to Sel1L (Hosokawa et al., 2010b). Secondly, confusing results have been found when OS9.1 is knocked-down or overexpressed. While knock down of OS9 delays degradation of human α1-antitrypsin variant null Hong Kong (NHK) (Bernasconi et al., 2008; Christianson et al., 2008), it does not affect the degradation of several other ERAD substrates such as RI332 (Christianson et al., 2008). Overexpression of OS9 instead of facilitating the degradation of substrates, were found to either delay the degradation of substrates RI332 or NHK (Bernasconi et al., 2008; Mueller et al., 2008), (Mueller et al., 2008) or remain HNK in the ER (Christianson et al., 2008). Recent studies have shown that both OS9 and XTP3B are required for the degradation of soluble ERAD luminal substrates BACE and CD3 without transmembrane domains, since the knockdown of either OS9 or XTP3B does not affect degradation while the knock down of both blocks it (Bernasconi et al., 2010).

Moreover, how OS9 interact with the ERAD complex and deliver substrates to the complex remind unclear. Thus far, two main models have been suggested. One model suggests that OS9 and XTP3B stably incorporated in the complex through stably interacting with Sel1L. In this model, MRH of OS9 recognizes trimmed N-glycan on misfolded glycoproteins that are in proximity with OS9 (Munro, 2001). In the other model, OS9-GRP94 forms a complex in the ER lumen and actively recognizing misfolded glycoproteins, followed by delivering substrates to ERAD complex. The interaction of OS9 with the complex in this model is dynamic rather than stable (Munro, 2001). Further studies are needed to confirm how OS9 deliver substrates. Just like in yeast, OS9 has been shown to interact with Sel1L. Latest study found that OS9 has reduced interaction with Sel1L when the MRH domain activity is abolished in OS9, suggesting that besides interacting with Sel1L on its

MRH domain, OS9 also interact with Se1lL on other residues (Hosokawa et al., 2009). In concordance, our data also showed that nonglycosylated Sel1L interests with OS9.

Analysis of Yos9 and OS9 revealed the role of both lectins in ERAD. The MRH domain of OS9 is needed for the recognition and the degradation of certain ERAD substrates, but is not required for its interaction with Sel1L. However, how OS9 delivers substrates, the classes of substrates as well as the function of its variants remain controversial. Hence, more future studies are needed to understand this intriguing protein.

Chapter 2: ER STRESS INDUCES OS9 DEGRADATION VIA THE SEL1L-HRD1 ERAD COMPLEX

2.1 Abstract

Misfolded or unfolded proteins generated by inefficient protein folding or ER stress is translocated from the ER to cytosol for proteasome degradation, a process termed endoplasmic reticulum associated degradation (ERAD). OS9, a lectin protein, has been shown to deliver misfolded or unfolded glycoproteins to the most conserved ERAD complexes in the mammalian system, the Sel1L-Hrd1 ERAD complex (Mehnert et al., 2010). Our study on OS9 shows that despite being an effector of the complex, OS9 itself is degraded by the same complex in response to ER stress. OS9 protein level decreases drastically upon the treatment of Tunicamycin (TM), Thapsigargin (Tg) or Dithiothreitol (DTT) . The addition of MG132 completely blocked the degradation, showing OS9 is degraded by proteasome. We then show the degradation of OS9 is abolished in Hrd1 knock-out ($Hrd1^{-/-}$) MEF cells, and significantly attenuated in $Sel1L^{-/-}$ cells when cells were treated with 6hr of cycloheximide (CHX), suggesting both Sel1L and Hrd1 are required for the degradation of OS9, but Hrd1 plays a more important role in the process. We also find that the protein level of OS9 is significantly higher in Sel1L-inducible knock out (IKO) mice than in WT mice, confirming that OS9 is degraded by Sel1L-Hrd1 complex *in vivo*. Using co-immunoprecipitation (CO-IP), we find that Sel1L associates with OS9 in a glycosylation independent manner and that it can be ubiquitinated by overexpressed Hrd1. In addition, our data suggest the protein stability of OS9 is mediated through the XBP1-Hrd1 axis. These data suggest that

OS9 is degraded through the Sel1L-Hrd1 complex under ER stress. Not only does this study identifies a potential endogenous substrate of the Sel1L-Hrd1 complex, given the fact that OS9 is an effector of the complex, this study may also provide insights into a possible self-regulatory mechanism of the complex through regulating OS9.

2.2 Introduction

The ER is the major site for the synthesis of membrane proteins as well as proteins destined to the secretory pathway. To monitor protein quality, folding of nascent polypeptides in the ER is tightly controlled by several cellular mechanisms. Terminally misfolded and unfolded proteins (substrates) are degraded by ERAD, a conserved process characterized by the recognition and translocation of substrates, followed by the degradation by cytosolic proteasome (McCracken and Brodsky, 1996).

ERAD is centered on E3 ligases, which are proteins that facilitate the polyubiquitination of substrates. Since only polyubiquitinated substrates can be recognized and degraded by cytosolic proteasome, E3 ligases serve an essential role in this process (Smith et al., 2011). To date, the best characterized and the only conserved E3 ligase in mammalian system is Hrd1 (Iida et al., 2011). Hrd1 physically associates with Sel1L (Lilley and Ploegh, 2005), a critical adaptor protein of Hrd1, and Hrd1-Sel1L forms a stable transmembrane ERAD complex by interacting with Derlin 1&2, chaperones such as OS9, p97/VCP and Herp (Lilley and Ploegh, 2005).

In order for degradation to occur, stringent and accurate substrate recognition and delivery system must present. OS9, a lectin protein that processes MRH

domain, plays an important role in recognizing misfolded glycoproteins and bring them to the Sel1L-Hrd1 complex. It is proposed that the MRH domain of OS9 recognizes and binds to specific sugar units on substrates that are partially processed by other chaperones such as EDEMs (Christianson et al., 2008). Since OS9 is shown to physically associate with Sel1L (Christianson et al., 2008), it is believed that OS9 delivers substrates to the Sel1L-Hrd1 complex through their interaction (Christianson et al., 2008). However, whether OS9 stably interacts with Sel1L remains unclear (Hosokawa et al., 2009). Understanding recognition pathways throw lights on the identification of substrate classes and the actual endogenous substrates, especially when only a few endogenous substrates of the Sel1L-Hrd1 complex have been identified thus far.

In this study, we used Hrd1$^{-/-}$/Sel1L$^{-/-}$ MEF cells and ER stress inducing drugs to study the dynamic of OS9 under those conditions. Our data suggest that OS9, despite being part of the Sel1L-Hrd1 complex, is an endogenous substrate of the exact same complex.

2.3 Experimental Procedures

Cell lines and reagents. Hrd1$^{-/-}$ MEF cells were generous gift from Dr. Hosokawa of Kyoto University. Sel1L$^{-/-}$ MEF cells were generous gift from Dr. Qiaoming Long of Cornell University. HEK 293T an MEF cells were maintained in DMEM supplemented with 10% FBS (Hyclone) and 1% penicillin/streptomycin. TM, MG132 and stock CHX (Sigma) were dissolved in DMSO and ethanol, respectively. Cells were treated with TM at 2.5μg/ml for 4.5 hrs and immediately snap-frozen in liquid nitrogen.

Site-directed mutagenesis. Site-directed mutagenesis to generate Sel1L Arginine (N) to Glutamine (Q) single mutants at residues 191, 213, 269, 427, 604, respectively and Sel1L non-glycosylated mutant (N5Q) at all five residues were performed using pcDNA3/GFP-Sel1L as template (see supplement for primer sequences). The PCR included mutagenic primers, as well as Pfu polymerase (Stratagene) and the program consisted of 16 cycles of 95°C for 30sec, 55°C for 30s and 72°C for 5 min. After DpnI digestion for 2-3 hr, the PCR product was transformed into XL-Blue competent cells by incubating PCR products with the cells for 10 min, followed by a heat-shock at 42°Cfor 30sec. All mutations were confirmed by DNA sequencing.

Transfection and immunoprecipitation. HEK 293T were plated on to 10-cm-diameter dishes the day before transfection. Transfection was performed by preparing polyethylenimine (PEI, Sigma) (1mg/ml in 0.9% NaCl) with plasmids at 5:1 (µl/ug) ratio and adding to the cell culture. Transfected cells were using Triton X-100 reagent as previous described (Chen and Qi, 2010). Anti- Flag agarose beads (Sigma-Aldrich) were added to the supernatants and rocked gently at 4◦C overnight. Following 4-6 times wash with washing buffer [20mM Tris/HCl, pH7.5, 137mM NaCl, 1% (v/v) Triton X-100, 2mM EDTA and 10% (v/v) glycerol], the beads were boiled and analyzed by Western Blotting.

Western Blotting. Western blotting was performed using 10-20ug of total cell lysates. Antibodies used in this study: HSP90 (rabbit, 1:6000), FLAG-HRP from Santa Cruz; Sel1L (rabbit, 1:2000), OS9 (rabbit, 1:5000) from Abcam; Hrd1 (rabbit, 1:8000) from Novus Biologicals; Calnexin (rabbit, 1:10000) from Assay Design. Band density was quantified using the Image Lab software on the ChemiDOC XRS+ system (Bio-Rad).

RNA extraction, RT and q-PCR. Total RNA of mice tissues and cells were extracted using TRIzol reagent as previously described (Sun et al., 2014) and reverse transcribed using the Superscript III kit (Invitrogen). cDNAs were analyzed using the SYBR Green PCR system on the Roche LightCycler 480 machine. All quantitative PCR (qPCR) data were normalized to ribosomal l32 gene in the corresponding sample.

Statistical Analysis. Results are expressed as mean ± SEM. Comparisons between groups were made by unpaired two-tailed Student t test, where $P<0.05$ was considered as statistically significant.

2.4 Results

ER Stress Induces OS9 Degradation.

To examine the protein stability of OS9 under ER stress, we treated MEF cells with ER stress inducer TM, a drug that blocks glycosylation. Drug treatment revealed that while Sel1L and Hrd1 remain stable upon 4 hr of TM treatment, OS9 levels were largely reduced (Fig. 2.1A and B). The reduction was more pronounced in OS9.2, in which the protein level decreased by 70% (Fig.2.1A and B), indicating OS9 is less stable than Sel1L and Hrd1, and is subject to degradation under ER stress. Interestingly, this effect was completely blocked by the addition of MG132, a proteasome inhibitor (Fig, 2.1A), suggesting OS9 is degraded by proteasome. In order to verify the effect of OS9 degradation was not drug-specific, we treated MEF cells with other classes of ER stress inducers, namely Tg that blocks Ca2+ ATPase, and DTT that blocks disulfide bond formation. As expected, both Tg and DTT induced the degradation of OS9, and the effects were reversed by the co-addition of

MG132 (Fig. 2.1 C and D). However, Our data suggested OS9 respond differently with different classes of drugs (Fig. 2.1 C and D). Also, OS9.2 was more affected than OS9.1 in all three cases, hinting that OS9.2 is either less stable than OS9.1, or it may more likely to be associated with the Sel1L-Hrd1 complex, as previously reported (Hosokawa et al., 2010a).

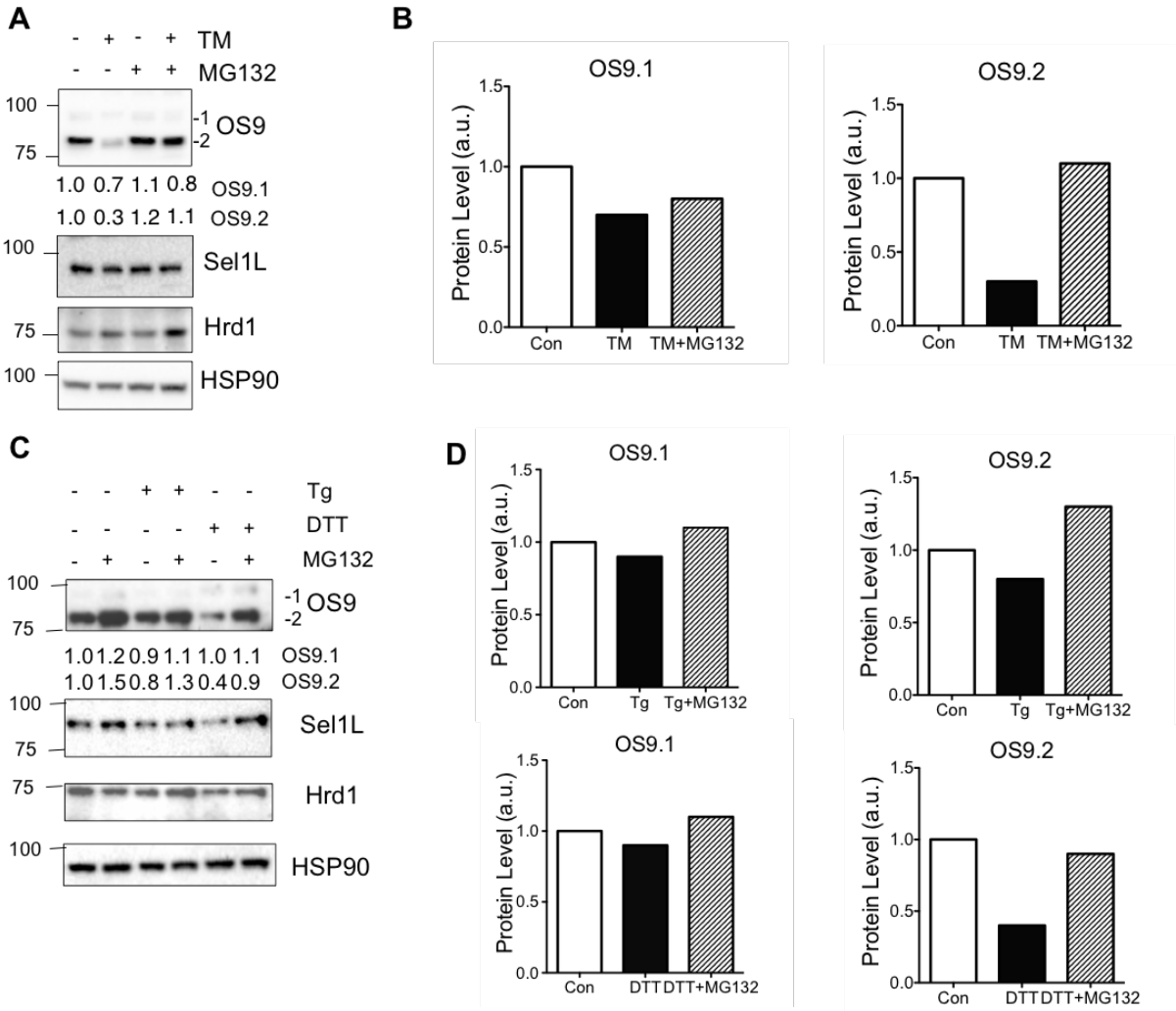


Figure 2.1. ER stress induces OS9 degradation. (A) Western blot analysis of OS9, Sel1L and Hrd1 in MEF cells that were either untreated (Con), treated with MG132, treated with 2.5ug/ml TM or treated with TM and MG132 for 4 hr. Quantitation of OS9 upon being normalized to the loading control HSP90 were shown under western blot of OS9 as indicated. OS9 protein levels in untreated samples, TM treated samples, and TM+MG132 treated samples were presented in bar graph in (B) as 'Con', 'TM' and 'TM+MG132' respectively. (C) Western blot analysis of OS9,

Sel1L and Hrd1 in MEF cells that were either untreated (Con), treated with MG132, treated with 300ng/ul Tg, treated with 1mM DTT, treated with Tg and MG132 or treated with DTT and MG132 for 4hr. OS9 protein levels in untreated samples, Tg treated samples and Tg + MG132 treated samples were quantified in bar graph shown in (D); OS9 protein levels in untreated samples, DTT treated samples, and DTT + MG132 treated samples were also quantified in separate bar graph shown in (D).

OS9 Degradation Is Mediated by the Sel1L-Hrd1 ERAD Complex *in Vitro*.

We next asked whether the degradation of OS9 is mediated by the Sel1L-Hrd1 complex. In $Hrd1^{-/-}$ MEF cells, the protein levels of both Sel1L and OS9 were increased at basal level comparing to those of wide type (WT) MEFs (Fig. 2.2A). The basal level of both OS9.1 and OS9.2 increased by almost 2 fold in $Hrd1^{-/-}$ cells (Fig. 2.2B), while the mRNA only increased for 50% (Fig.2.2C), pointing that the increase in OS9 protein level was partly due to post-transcriptional regulation. This supports our hypothesis that OS9 is degraded by the Hrd1 complex. Interestingly, the protein level of Sel1L also had 2 fold increase in $Hrd1^{-/-}$ cells (Fig, 2.2B). Upon the induction of cell death by adding CHX to WT, $Se1lL^{-/-}$ and $Hrd1^{-/-}$ cells, we found that the degradation of OS9 was attenuated in Sel1L KO cells and was completely abolished in Hrd1 KO cells (Fig. 2.2D and E). These data suggested that OS9 is indeed degraded by the Sel1L-Hrd1 ERAD complex.

A recent study from our laboratory demonstrated that Sel1L regulates the stability of Hrd1 (Sun et al., 2014), just as the situation in yeast (Kaneko et al., 2012). Consistently, our data showed that Hrd1 was much less stabled and drastically reduced in $Sel1L^{-/-}$ cells (Fig.2.2D). The remained Hrd1 in $Sel1L^{-/-}$ cells might explain

the observation that OS9 was still subject to degradation under CHX chase experiment in the absence of Sel1L.

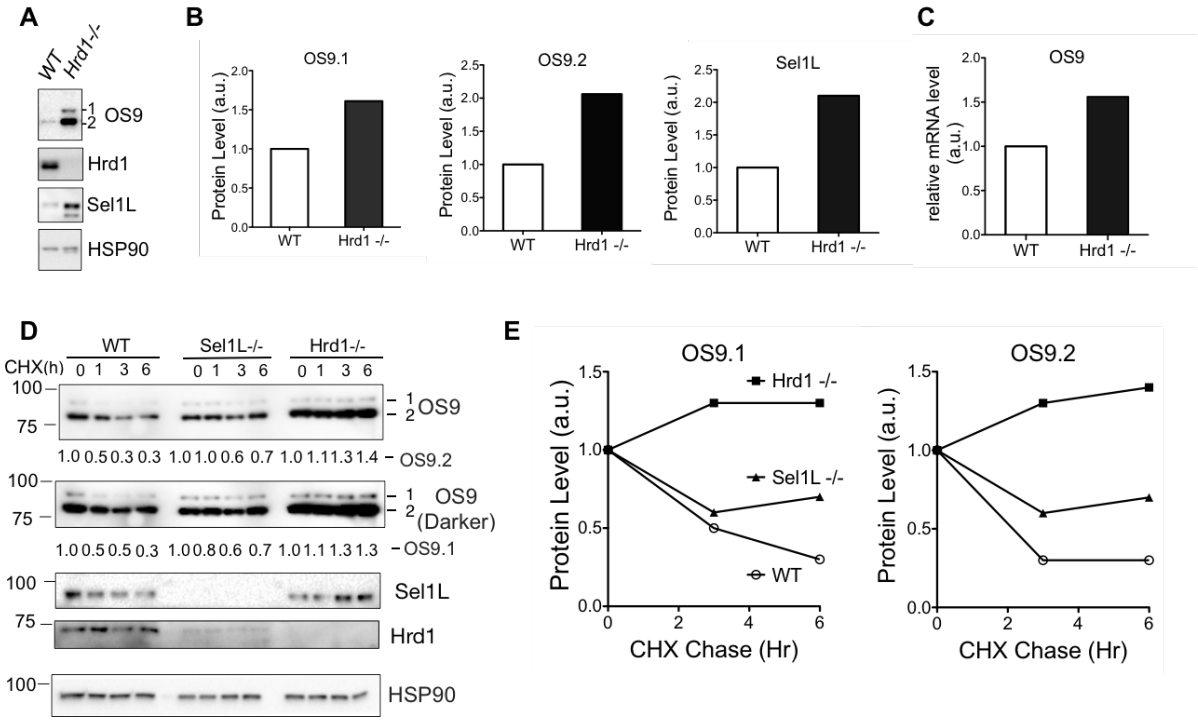


Figure 2.2. OS9 degradation is mediated by the Sel1L-Hrd1 complex *in Vitro*. (A) Western blot analysis of OS9, Hrd1 and Sel1L in untreated WT and Hrd1$^{-/-}$ MEF cells with quantitation shown in B. (C) qPCR analysis of OS9 in WT and Hrd1$^{-/-}$ MEF cells. (D) OS9 half-life analysis in MEF cells treated with CHX at time 0, 1, 3 and 6hr. Darker exposure of OS9 blot is included to show the levels of OS9.1. Quantitation shown in (E) upon normalization to the loading control HSP90.

OS9 Degradation Is Mediated by the Sel1L-Hrd1 ERAD *in Vivo*.

To determine the effect of ERAD deficiency on the degradation of OS9 *in vivo*, we next analyzed OS9 protein levels in recently generated tamoxifen-inducible knockout mice (IKO) (Sun et al., 2014). Indeed, in line with those in vitro findings, OS9 protein levels were significantly elevated in tissues lacking Sel1L such as the pancreas, ileum and kidney (Fig 2.3 A-C). The mRNA level of OS9 was decreased in

IKO mice (Fig 2.3 D), providing further support to the notion that Sel1L controls OS9 degradation. Therefore, OS9 is degraded by Sel1L-containing ERAD complex in vivo.

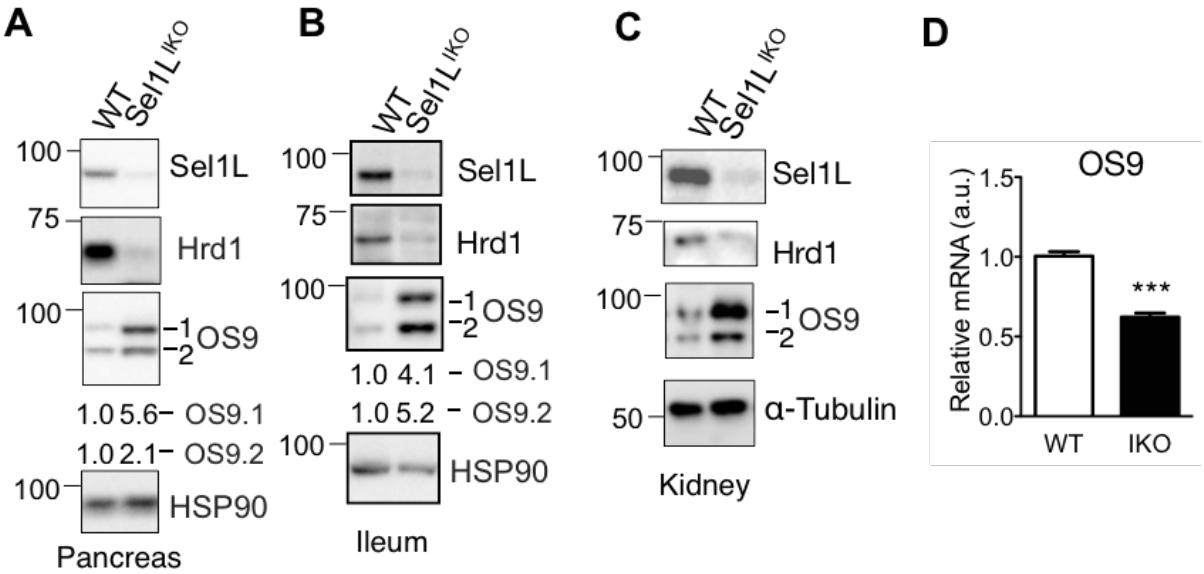


Figure 2.3. OS9 Degradation Is Mediated by the Sel1L-Hrd1 ERAD *in Vivo*. (A-C) Western blot analysis of Sel1L, Hrd1 and OS9 in pancreas (A), ileum (B) and kidney (C) from WT and IKO mice. Quantitation of OS9 upon being normalized to the loading control HSP90 were shown under western blot of OS9 as indicated. (D) qPCR analysis of OS9 in the kidney of WT and IKO mice. The data is a courtesy of Iris Sun and Dr. Guojun Shi in the Qi laboratory.

OS9 Interacts With Sel1L In a Glycosylation Independent Manner.

Previous studies have shown that OS9 physically interacts with Sel1L (Christianson et al., 2008; Hosokawa et al., 2010a; Satoh et al., 2010), however it remains unclear whether the interaction is glycosylation dependent. To this end, we

first performed transfection in HEK293T cells to demonstrate that both isoforms of OS9 (OS9.1 and OS9.2) interacted with Sel1L (Fig. 2.4A and B).

To assess whether their interactions are glycosylation dependent, we generated a Sel1L mutant with no potential glycosylation sites (Sel1L N5Q). IP data showed that both Sel1L and Sel1L N5Q readily interacted with OS9 (Fig.2.4C), suggesting that Sel1L interacts with OS9 independently of glycans on Sel1L protein.

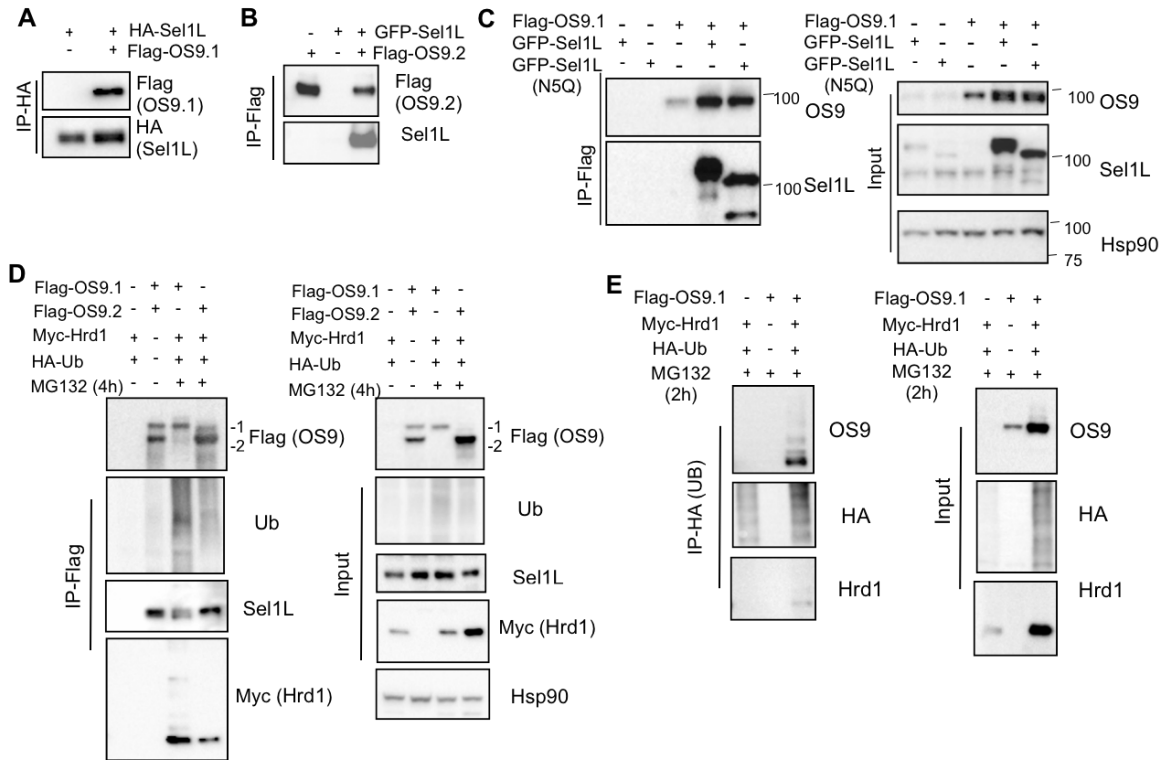


Figure 2.4. Physical interactio between OS9 and Sel1L, and ubiquitination of OS9. (A) Inmunoprecipitation and Western blot analyses of the interactions between (A) Sel1L and OS9.1, (B) Sel1L and OS9.2, and (C) Sel1L N5Q and OS9.1, in HEK293T cells transfected with different combinations of plasmids as indicated. (D-E) Inmunoprecipitation and Western blot analyses of ubiquitination of OS9 in HEK293T cells transfected with different combinations of plasmids as indicated. MG132 were added to the cells 2 or 4 hr before harvesting.

OS9 Is Ubiquitinated by Hrd1

Hrd1 is an E3 ligase that ubiquitinates substrates and target them for degradation (Tsai and Weissman, 2011). To study whether OS9 can be ubiquitinated by Hrd1, we overexpressed OS9.1 or OS9.2 with Hrd1 and Ub. MG132 was added to inhibit proteasome activity, allowing the detection of ubiquitinated substrates. Our data showed that both OS9.1 and OS9.2 were ubiquitinated. However, we noticed that the extent of ubiquitination may be different (Fig 2.4 D). A more intense smeared band indicated that OS9.1 may be ubiquitinated more efficiently than OS9.2 (Fig.2.4 D). To confirm the ubiquitination of OS9, we then overexpressed OS9.1, Ub, Hrd1 and immumopreticipated with HA (UB). Indeed, mono-ubiquitinated OS9.1 was presented in the IP sample (Fig.2.4 E). Hence, we concluded that OS9 can be ubiquitinated by Hrd1.

Regulation of OS9 stability via the XBP1-Hrd1 signaling axis.

It is known that the IRE1α-XBP1 pathway of the UPR controls the expression of some ERAD genes (Alcock and Swanton, 2009; Bernasconi et al., 2008). We next addressed whether the IRE1α-XBP1 pathway regulates the stability of OS9. To this end, we treated WT, IRE1$\alpha^{-/-}$ and XBP1$^{-/-}$ cells with CHX to study the degradation rate of OS9. Here we showed that the degradation of both OS9.1 and OS9.2 were attenuated in IRE1$\alpha^{-/-}$ and XBP1$^{-/-}$ cells under CHX treatment (Fig 2.5A and B). In consistent with previous report that the IRE1α-XBP1 pathway regulate Hrd1, Hrd1 expression level was extremely low without the presence of XBP1, which may explain the attenuated degradation of OS9 in XBP1$^{-/-}$ cells compare to those of the WT cells. Since XBP can also transcriptionally induce OS9, the counter-intuitive finding of the increased OS9 basal protein level further suggests that OS9 is

degraded by the Sel1L-Hrd1 complex. Thus, our data suggest a dynamic regulation of OS9 stability through the XBP1-Hrd1 axis.

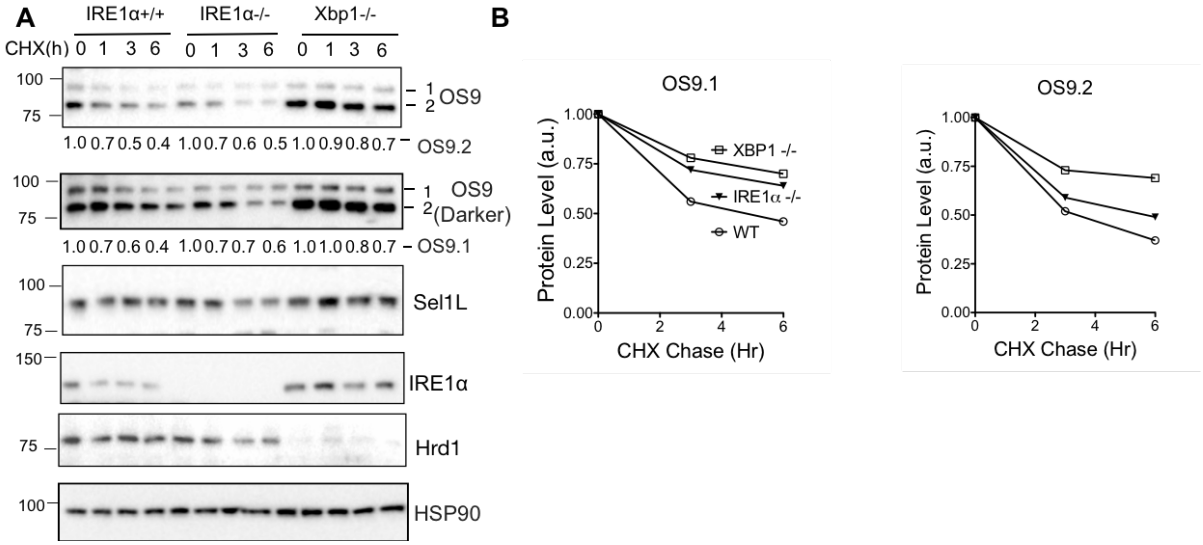


Figure 2.5. Regulation of OS9 stability via the XBP1-Hrd1 axis. (A) OS9 half-life analysis in MEF cells treated with CHX at time 0, 1, 3 and 6hr. Darker exposure of OS9 blot is included to show the levels of OS9.1. Quantitation shown in (B) upon normalization to the loading control HSP90.

2.5 Discussion

Although previous study has speculated that the degradation of OS9 may depend on the Sel1L-Hrd1 ERAD complex (Tyler et al., 2012), this is the first study providing direct evidence of this hypothesis. Here we identified that OS9 is an endogenous ERAD substrate by manipulating cellular stress conditions and by depleting critical ERAD components Sel1L and Hrd1. Our data showed that OS9 is degraded under ER stress, but the degradation was blocked when proteasome was inhibited. In the absence of Sel1L and Hrd1, OS9 degradation was attenuated or abolished under CHX treatment, respectively. Our data further showed that OS9 is physically associated with Sel1L, and that it can also be ubiquitinated by Hrd1.

These data suggested that the degradation of OS9 is mediated by the Sel1L-Hrd1 ERAD complex. Moreover, we found that the stability of OS9 is regulated through the the IRE1α-XBP1 pathway, suggesting a possible self-regulatory scheme of the Sel1L-Hrd1 complex.

OS9 was first found as a gene that is amplified in osteosarcoma with unknown function (Hosokawa et al., 2010a); however, it is now widely recognized that it is part of a large macromolecular ERAD complex that centered on Sel1l-Hrd1 (Christianson et al., 2008; Hosokawa et al., 2010a). The function of OS9 lies on its MRH domain, which recognizes specific trimmed glycan structures on ERAD substrates and bring them to the Sel1L-Hrd1 complex (Hosokawa et al., 2010a). Our data showed that either Sel1L or Hrd1 deficiency interfere with the degradation of OS9 both in cell lines and in mice, suggesting the dependence of OS9 degradation on Sel1L and Hrd1. Given that the transcription of OS9 was uncoupled with the increased protein level in IKO mice, it is highly possible that OS9 is degraded through the Sel1L-Hrd1 complex. Previous study shows when Sel1L was depleted, OS9 strongly associated with identified endogenous substrate CD147 through its MRH domain (Tyler et al., 2012). Together with our data, we suggest that OS9 may form a “suicidal” complex with substrates of the Sel1L-Hrd1 complex, followed by co-degradation.

This hypothesis explains that fact that OS9 is degraded under ER stress. Given the importance of OS9 in recognizing and delivering substrates to the Sel1L-Hrd1 complex, it is intuitively unreasonable that it is degraded under ER stress, a situation where chaperones like OS9 are needed to degraded accumulated misfolded proteins and relieve ER stress. However, if OS9 is co-degraded with substrates, as we suggested, OS9 protein level should decrease. Given that OS9 is transcriptionally upregulated under ER stress (Alcock and Swanton, 2009; Sun et al.,

2014), the turnover rate of OS9 seemed to be higher than that of the transcriptional regulation.

It is well established that Hrd1 is an E3 ligase that plays the most important role in the ubiquitination and degradation of substrates (Vembar and Brodsky, 2008). Our data herein showed that OS9 is ubiquitinated by the overexpression of Hrd1 and Ub, further supporting our hypothesis. Also, our data showed that the degradation of OS9 was completely abolished in Hrd1$^{-/-}$ MEFs, while that was only retarded in Sel1$^{-/-}$ MEFs, suggesting Hrd1 is responsible for degrading OS9. In concordance with previous finding of our lab, we showed that Hrd1 protein level is drastically reduced in Sel1L$^{-/-}$ MEFs, suggesting Sel1L regulates the stability of Hrd1. Since Hrd1 was still presented in Sel1L$^{-/-}$ cells, OS9 could still be degraded at a slower rate. Taking together, we believe that the degradation of OS9 is mediated by Hrd1, while Sel1L plays a critical role in stabilizing Hrd1.

Besides stabling Hrd1, Sel1L is critical in physically bring OS9 to Hrd1, as previous studies and our data showed that OS9 interacts with Sel1L, but not with Hrd1(Hosokawa et al., 2010b; Satoh et al., 2010) Moreover, here we showed that OS9 interact with Sel1L in a glycosylation independent manner, suggesting the MRH domain of OS9 is not required for their interaction. This also suggests the possibility that OS9 may physically interact substrates at its MRH domain, and bring substrates to Hrd1 by physically interact with Sel1L at some other residues. Hence, our study also provides information on the mechanism of substrate degradation mediated by the OS9-Sel1L-Hrd1 pathway.

It is well known that UPR can induce the transcription of ERAD genes, such as Hrd1 and OS9, through the IRE1α-XBP signaling pathway (Alcock and Swanton, 2009; Bernasconi et al., 2010). However, other regulatory pathways of ERAD

proteins may also exist. Here our data suggests that the absence of XBP1 drastically decrease the expression of Hrd1, which in turn inhibits the degradation of OS9 and leads to the accumulation of OS9 in XBP1$^{-/-}$ cells. Hence, it is possible that OS9 is under the dual regulation of the IRE1α-XBP signaling pathway at the transcriptional level and the Sel1L-Hrd1 degradation pathway at protein level. If this finding is true, the regulation between UPR and ERAD, and within ERAD maybe more dynamic and complex than we once thought.

2.6 Summary and Future Directions

In summary, our study identified a potential endogenous substrate of the Sel1L-Hrd1 complex. OS9, an effector involved in substrate recognition and recruitment of the Sel1L-Hrd1 complex, is itself a substrate of the exact same complex. Given the substrate-recruitment role of OS9, our data suggests that OS9 may be co-degraded with other Se1lL-Hrd1 ERAD substrates by forming a complex with them. Hence, this study not only identifies an endogenous substrate for the Sel1L-Hrd1 complex, but also sheds light on the mechanism underlying substrate degradation, an area that is still need to be further investigated. Moreover, our data suggests that the regulation of OS9 is very dynamic and beyond the simple IRE1α-XBP1 regulatory pathway, which may serves as a mechanism for the self-regulation of the Sel1L-Hrd1 complex. Hence, future studies should focus on 1) identifying substrates of the Sel1L-Hrd1 complex; and 2) studying the dynamics among substrates, OS9 and the Sel1L-Hrd1 complex, and 3) elucidating the intricate crosstalk between ERAD and UPR. We believe these studies will enrich our understanding on the maintenance of ER homeostasis.

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Supplement

Table S1: Primer Sequences used in this study

Primers	Forward	Reverse
Genotyping primer		
flox/flox	CTGACTGAGGAAGGGTCTC	GCTAAAAACATTACAAAGGGGCA
Cre	AGCGATGGATTTCCGTCTCT	CACCAGCTTGCATGATCTCC
qPCR primers		
sel1l	TGGGTTTTCTCTCTCTCCTCTG	CCTTTGTTCCGGTTACTTCTTG
os9	GCTGGCTGACTGATGAGGAT	CGGTAGTTGCTCTCCAGCTC
l32	GAGCAACAAGAAAACCAAGCA	TGCACACAAGCCATCTACTCA
Mutagenesis primers		
Sel1L (N191Q)	CCGGGATGAAGATACTGCAGGGAAGCAATAGGAAGAG	CTCTTCCTATTGCTTCCCTGCAGTATCTTCATCCCGG
Sel1L (N213Q)	GAAGGCAGCAGGCATGCAGCACACCAAAGCCCTG	CAGGGCTTTGGTGTGCTGCATGCCTGCTGCCTTC
Sel1L (N268Q)	CTTCTGGGCTTGGTGTTCAGTCAAGTCAGGCAAAGG	CCTTTGCCTGACTTGACTGAACACCAAGCCCAGAAG
Sel1L (N427Q)	CATCGTACCTCAGAGTCAGGAGACGGCACTTCAC	GTGAAGTGCCGTCTCCTGACTCTGAGGTACGATG
Sel1L (N604Q)	CAACCATTGTAGGTGAGcAGGAAACTTACCCCAGAGC	GCTCTGGGGTAAGTTTCCTGCTCACCTACAATGGTTG