ABSTRACT

Title of Document: REGULATION OF HEPATIC ER STRESS BY THE E3 UBIQUITIN LIGASE GP78 IN ZEBRAFISH

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Mammalian gp78 is an E3 ubiquitin ligase that is anchored at the membrane of the endoplasmic reticulum (ER). It regulates protein homeostasis by polyubiquitinating and targeting proteins for proteasomal degradation under both physiologic and stress conditions. To further test its role *in vivo*, we analyzed the gross embryonic morphology of zebrafish embryos in which gp78 was knocked down using morpholinos and in transgenic fish overexpressing wild-type gp78 or dominant-negative gp78. We show that gp78 is highly conserved among vertebrates. Zebrafish gp78, similar to human gp78, can colocalize with mouse MmUBC7 in HeLa cells. *In vitro* ubiquitination assays confirmed that zebrafish gp78 is indeed an E3 ubiquitin

ligase. Although *gp78* was maternally and constitutively expressed during embryonic development, with relatively high expression levels in several tissues, such as liver and brain, the knockdown of endogenous gp78 or overexpression of wild-type or dominant-negative gp78 did not result in developmental defects, suggesting compensation by other E3 ubiquitin ligases during embryonic development.

ER-associated protein degradation (ERAD) activity by the unfolded protein response (UPR) represents one of the mechanisms for restoring ER homeostasis. However, the significance of gp78 in the regulation of hepatic ER stress *in vivo* remains elusive. Here we report that zebrafish gp78 plays a key role in the regulation of hepatic ER stress under tunicamycin-induced stress, but not under physiologic conditions. Tunicamycin treatment induced ER stress and upregulated the expression of several key components of the gp78-mediated ERAD complex in the liver. Moreover, hepatic-specific overexpression of the dominant-negative form of gp78 (gp78-R2M) rendered livers more sensitive to tunicamycin-induced ER stress, suggesting a role for gp78-mediated ERAD in the regulation of hepatic protein homeostasis. Moreover, the overexpression of gp78-R2M enhanced the expression of sterol response element binding protein (Srebp) target genes in response to ER stress, while this was not observed in fish overexpressing wild-type gp78. Together, these data indicate that gp78 plays a critical role in the regulation of hepatic ER stress and lipid metabolism.

REGULATION OF HEPATIC ER STRESS BY THE E3 UBIQUITIN LIGASE GP78 IN ZEBRAFISH

By

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Abbreviations

AAA ATPase	ATPase associated with various cellular activities
AAT	α-1-antitrypsin
ACC1	Acetyl coenzyme A carboxylase a
AMF	Autocrine motility factor
AMFR	Autocrine motility factor receptor
ApoB-100	Apolipoprotein B-100
ATF6	Activating transcription factor 6
ATZ	Z variant of α-1-antitrypsin
Bp	base pair
Bip	Immunoglobulin heavy chain-binding protein
CF	Cystic fibrosis
CFTR	CF transmembrane conductance regulator
CHOP	CCAAT/-enhancer-binding protein homologous protein
CNS	Central nervous system
CT	Cholera toxin
CUE	Coupling of ubiquitin to ER degradation
CYP	Cytochrome P450
DT	Dithiothreitol
DFA	Dietary fatty acids
Dig	Digoxin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNL	*De novo* lipogenesis
Dpf	days post fertilization
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
E4	Polyubiquitin chain assembly factor
EDEMs	ER degradation-enhancing-mannosidase-like proteins
EF1α	Elongation factor 1α
eIF2α	α-subunit of eukaryotic translation initiation factor 2
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ER ManI	ER Mannosidase I
ERQC	ER quality control
Fads	Fatty acid desaturase
FASD	Fetal alcohol spectrum defects
Fasn	Fatty acid synthase
G2BR	Ube2g2-binding region
GADD34	Growth Arrest and DNA Damage-inducible 34
GFP	Green fluorescent protein
gp78C	gp78 cytosolic tail part
GSK-3	Glycogen synthase kinase 3

GST	Glutathione
HD	Huntington's disease
His	Histidine
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HMGCS1	3-hydroxy-3-methylglutaryl coenzyme A synthase 1
HRD1	HMG-CoA reductase degradation protein-1
hpf	Hours post fertilization
htt	Huntingtin
IP(3)	1,4,5-trisphosphate
Ire1α	Inositol-requiring protein 1α
kD	KiloDalton
MO	Morpholino
NEFA	Nonesterified fatty acid
OA	Oleic acid
OS	Oligomerization site
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PDI	Protein disulfide isomerase
PERK	Doublestranded RNA-activated protein kinase-like ER kinase
PMSF	Phenylmethanesulfonyl fluoride
PNGase	Peptide N-glycanase
PPARα	Peroxisome proliferator-activated receptor α
PVDF	Polyvinylidene fluoride
R2M	A mutation of two crucial residues in the RING finger domain
RACE	Rapid amplification of cDNA ends
RING	Really interesting new gene
RT-PCR	Reverse transcription polymerase chain reaction
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulfate
SERCA	Sarcoplasmic/endoplamic reticulum Ca^{2+}-dependent ATPase
SOD1	Superoxide dismutase 1
SREBP	Sterol regulatory element-binding protein
SVIP	Small VCP/p97-interacting protein
TAL	Transcription activator-like
TALENs	Transcription activator-like effector nucleases
Tg	Transgenic
TUN	Tunicamycin
U7BR	Ubc7p-binding region
Ub	Ubiquitin
UBA	Ubiquitin-associated
UBD	Ubiquitin-binding domain
UBE2G2	Ubiquitin-conjugating enzyme E2 G2
UIM	Ubiquitin-interacting motif
UPR	Unfolded protein response
UPRE	Unfolded protein response element
UTR	Untranslated region

VLDL	Very low-density lipoprotein
VIM	p97/VCP-interacting motif
WT	Wild type
Xbp1	X-box binding protein 1
ZF	Zebrafish

Chapter 1: General Introduction

1. Maintenance of protein homeostasis in the endoplasmic reticulum

Proteins destined for the secretory pathway are folded and matured in the lumen of the endoplasmic reticulum (ER) before they are transported to their final functional destinations. Proper folding is achieved by enzymes that modify proteins and by molecular chaperones that maintain polypeptide solubility and promote folding (Schubert et al 2000., Hampton et al 2002). To maintain the high fidelity of the secretory pathway, the conformations of proteins are constantly monitored by the ER quality control (ERQC) system. Proteins that eventually fail to achieve their native conformation after refolding are retained in the ER and eliminated by ER-associated degradation (ERAD) (Vembar et al 2008). Thus, ERAD is a protective mechanism to prevent the accumulation of misfolded proteins in the ER, and thereby safeguards the secretory pathway. When the load of misfolded proteins overwhelms the folding and degradation capacity of the ER, the unfolded protein response (UPR) is activated to restore ER homeostasis by limiting further loading of proteins into the ER, thus enhancing protein folding and elevating ERAD activity (Rutkowski et al 2004). Prolonged UPR, however, triggers apoptosis, which has been implicated in the pathophysiology of many diseases, such as neurodegenerative diseases, cancer, cardiovascular diseases, muscle wasting and diabetes (Malhotra et al 2007., Szegezdi et al 2006).

2. Maintenance of protein homeostasis by ERAD

ERAD is a complex process that removes misfolded proteins through degradation and thus helps maintain ER protein homeostasis. Misfolded ER proteins localize either fully (luminal proteins) or partially (membrane proteins) in the lumen of the ER, but their degradation occurs in the cytosol by the proteasomes. Thus, retrotranslocation or dislocation of these misfolded ER proteins to the cytosol is an absolute requirement for their elimination (Sommer et al 1993., Tsai et al 2002). Moreover, retrotranslocation is intimately associated with recognition of misfolded proteins on the luminal side of the ER and ubiquitination and proteasomal targeting on the cytosolic side (Vembar et al 2008., Hebert et al 1995). It is well established that ER membrane-associated ubiquitin ligase complexes coordinate substrate recognition, retrotranslocation, ubiquitination and degradation during ERAD (Kikkert et al 2005., Kostova et al 2007). gp78 is one of the ubiquitin ligases playing such a role (Fang et al 2001). Moreover, gp78 is unique among all known ERAD ubiquitin ligases in that it has multiple conserved domains that interact directly with components of both ubiquitination and retrotranslocation complexes.

3. gp78-mediated ERAD

3.1 gp78-mediated ubiquitination

Ubiquitination is a process during which proteins are modified with a single ubiquitin or a chain of ubiquitin monomers. It occurs through a cascading action of E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and then E3 ubiquitin ligase (Fang et al 2004) (**Fig. 1 A**). The complexity of ubiquitination is reflected by having two E1s, dozens of E2s and over a thousand of E3s in mammalian cells. Moreover, ubiquitination can generate nine topologically different polymeric

ubiquitin chains linked through one of the seven Lys residues (K6, K11, K27, K29, K33, K48, and K63) or the amino terminus or a mixture of Lys residues of ubiquitin (Ben-Saadon et al 2006, Behrends et al 2011). Each E3 can interact with and ubiquitinate one or several substrate proteins, and by working with different E2s, each E3 can assemble different polyubiquitin chains on its substrates. In other words, E2 determines the linkage of polyubiquitination while E3 dictates substrate specificity (Behrends et al 2011, Ye et al 2009). A number of proteins containing ubiquitin-binding domains (UBDs), such as the ubiquitin-interacting motif (UIM) and ubiquitin-associated (UBA) domains, recognize conjugated ubiquitin or polyubiquitin chains and the downstream effector proteins of signaling pathways or degradation machinery. Through these interactions UBD-containing proteins transmit ubiquitin-dependent signals to the desired biological function or proteasomal degradation (Dikic et al 2009, Winget et al 2010). The nine topologically distinct polymeric ubiquitin chains achieve a remarkably diverse range functions in ubiquitin signaling, such as targeting proteins for degradation, apoptosis, signal transduction, gene transcription, DNA repair, cell cycle progression, immune responses, virus budding, protein trafficking, and receptor and channel endocytosis (Behrends et al 2011, Ye et al 2009, Dikic et al 2009). Many of these functions control the life and death of cells. Accordingly, aberrant ubiquitination has been widely associated with development of malignancies, diabetes, cardiovascular diseases, inflammatory disorders and many neurodegenerative diseases (Schwartz et al 2009, Weissman et al 2011). gp78 exemplifies the importance of E3 ubiquitin ligases in physiology and pathology.

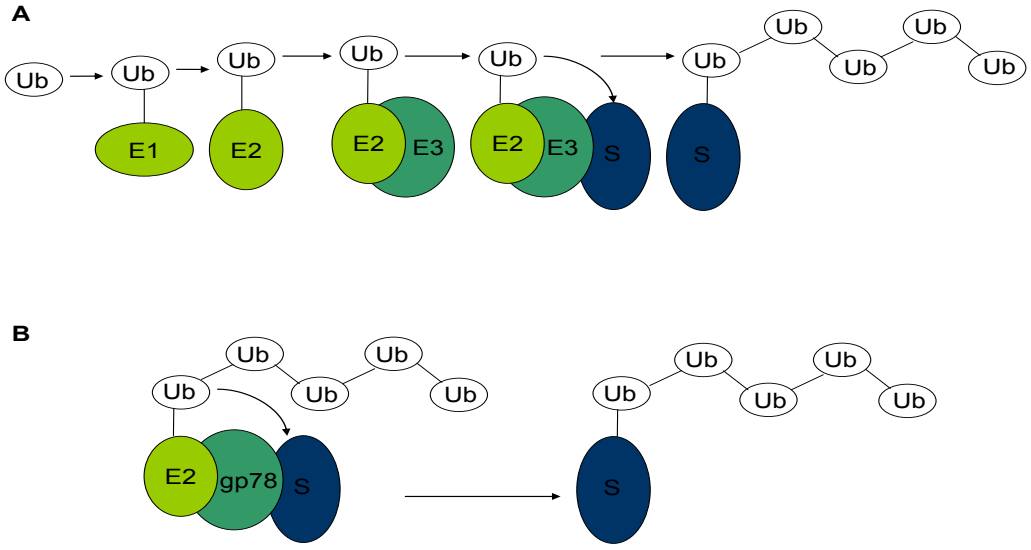


Fig. 1. Two schematic models of ubiquitination.

(A) Schematic representation of RING finger E3 ubiquitin ligase-catalyzed ubiquitination. S: substrate protein. **(B)** gp78/Ube2g2-mediated substrate (S) ubiquitination. From the left: two E2s (Ube2g2s) preassemble K48 ubiquitin chain on their active cysteines by aminolysis; the preassembled ubiquitin chain on the catalytic cysteine of the Ube2g2 transferred to a substrate.

gp78 is a polytypic RING (really interesting new gene) finger protein and is localized in the ER (Fang et al 2001). It contains five predicted transmembrane domains followed by a RING finger, an oligomerization site (OS), a coupling of ubiquitin to ER degradation (Cue) domain, a Ube2g2-binding region (G2BR) and a p97/VCP-interacting motif (VIM) (Fang et al 2001, Chen et al 2006, Li et al 2009, Ballar et al 2006) (**Fig. 2**). RING finger defines a family of E3 ubiquitin ligases (Lorick et al 1999), which led to the identification of gp78 as an E3 ubiquitin ligase acting in the ERAD pathway (Weissman et al 2001). Although the function of RING finger is to bind to ubiquitin-charged E2 to facilitate transfer of ubiquitin to a substrate, gp78 also binds ubiquitin-conjugating enzyme E2 G2 (Ube2g2) through G2BR in addition to its RING finger (Fang et al 2001, Chen et al 2006). It has been shown that its E3 activity requires the coordinated action of the RING finger, Cue domain, OS and G2BR.

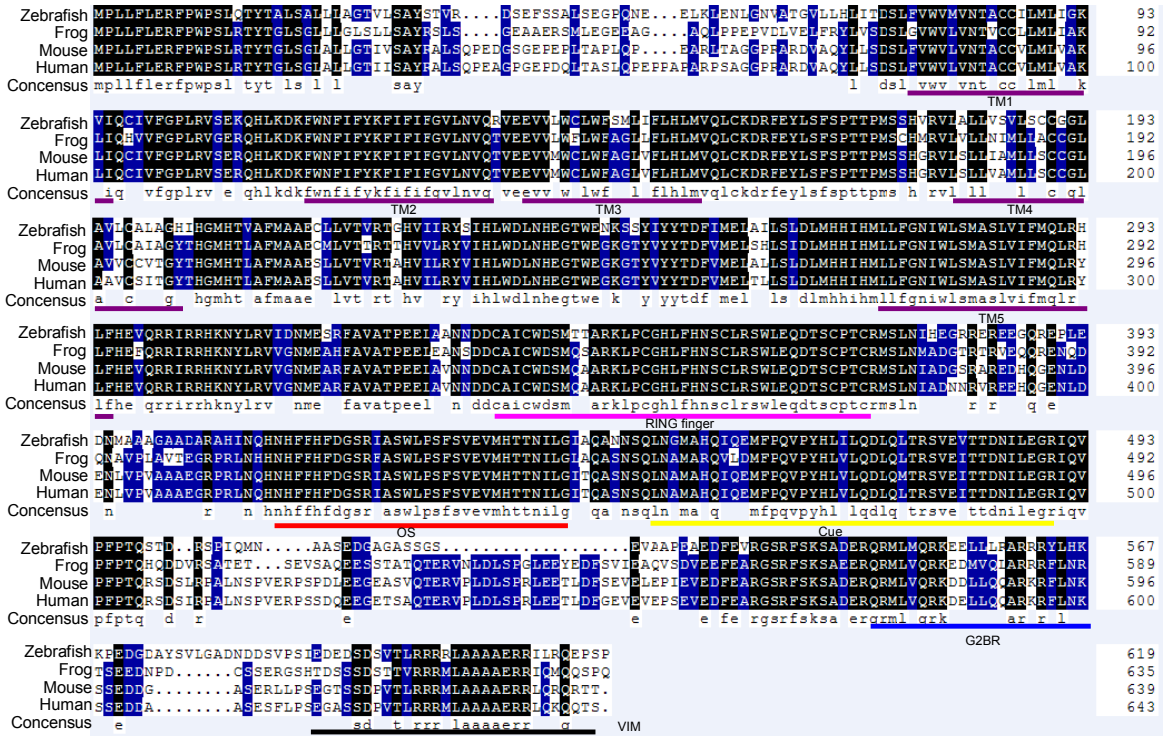


Fig. 2. Multi-species sequences alignment of gp78

Multiple sequence alignment was performed using DNAMAN. Conserved cytosolic domains are underlined. The transmembrane domains (TMs) are predicted using TMHMM-2.0.

Mechanistically, binding to G2BR leads to conformational changes in Ube2g2 that affect ubiquitin loading and significantly enhance the affinity of Ube2g2 to the RING finger (Das et al 2009). This unique dual binding mode of Ube2g2 to gp78 optimizes the efficiency of gp78-mediated ubiquitination of misfolded ER proteins. The mechanism by which gp78 cooperates with Ube2g2 to assemble polyubiquitin chains has been elegantly demonstrated (Li et al 2007) (**Fig. 1 B**). gp78/Ube2g2-mediated polyubiquitination involves preassembly of K48 polyubiquitin chains at the catalytic cysteine of Ube2g2. The extension of Ube2g2-anchored polyubiquitin chains is achieved by an aminolysis-based transfer reaction between two Ube2ge molecules that each carries a ubiquitin moiety on its active cysteine. gp78 oligomerization mediated by its OS leads to simultaneous binding of multiple Ube2g2 molecules in close proximity, which allows ubiquitin moieties to be transferred between neighboring Ube2g2s to form active site-linked polyubiquitin chains. These polyubiquitin chains are then transferred en bloc to substrate proteins (Li et al 2009).

gp78 appears to represent an example of convergent evolution with functions of both yeast ERAD E3 HMG-CoA reductase degradation protein-1 (Hrd1p) and its cofactor Cue1p found within a single molecule. Hrd1p is a polytypic RING finger E3 and Cue1p is a type III transmembrane protein (Bordallo et al 1999, Bays et al 2001, Biederer et al 1997). Cue1p contains a Cue domain and a Ubc7p-binding region (U7BR) that is functionally analogous to G2BR (Kostova et al 2009). Ubc7p is the yeast homolog of Ube2g2 and also interacts with the RING finger of Hrd1p (Deak et al 2001). Association of U7BR with Ubc7p activates the RING finger-dependent E3 activity of Hrd1p, and allows polyubiquitination of substrates (Kostova et al 2009).

Like Ube2g2, Ubc7p also assembles polyubiquitin chains at its active site and ubiquitinates substrates by en bloc transferring (Ravid et al 2007).

In addition to functioning as an E3, gp78 was reported to function as a polyubiquitin chain assembly factor (E4) to catalyze polyubiquitination of CFTRΔF508 (deletion of phenylalanine 508 of the cystic fibrosis transmembrane conductance regulator) (Morito et al 2008). In this case, another RING finger protein RMA1 acts as an E3 to ubiquitinate CFTRΔF508. gp78 then recognizes the ubiquitin that is already conjugated to CFTRΔF508 via its Cue domain and catalyzes polyubiquitination of CFTRΔF508. Whether this E4 function of gp78 is specific for CFTRΔF508 or general to all its substrates remains to be explored.

3.2 gp78 directly links ubiquitination to retrotranslocation

The general scheme of ERAD has been well established. ERAD substrates are first recognized and delivered to the membrane-anchored E3 ubiquitin ligase complexes by ER luminal chaperones and lectins followed by retrotranslocation, ubiquitination and proteasomal degradation (Vembar et al 2008). In budding yeast, two complexes, one composed of the Hrd1p ubiquitin ligase degrades substrates with lesions exposed to the ER lumen or transmembrane, namely ERAD-L and ERAD-M, whereas the other composed of Doa10p ubiquitin ligase disposes of substrates with lesions on the cytosolic side of the ER, namely ERAD-C (Carvalho et al 2006, Denic et al 2006). These ERAD complexes are essentially conserved in mammalian cells, but the three ERAD pathways described in yeast are not well defined in mammalian cells, which is especially true for gp78-mediated ERAD.

gp78 has been reported to degrade all types of substrates, for example, the luminal substrate, the Z variant of α-1-antitrypsin (ATZ), the membrane substrate HMG-CoA reductase, and the cytosolic substrate mutant SOD1 (see **Table 1** for a list of substrates for gp78). How luminal substrates, such as ATZ, are targeted to gp78 is not known. As ATZ is a glycosylated substrate, mannose-trimming factors, such as ER mannosidase I (ER ManI) or one or more ER degradation-enhancing-mannosidase-like proteins **(**EDEMs) (Ruddock et al 2006, Hebert et al 2010), must be involved in ATZ degradation mediated by gp78. Binding immunoglobulin protein (BiP/grp78) and the ER lectin OS-9, whose function is to target substrates to the Hrd1 complexes, are associated with gp78 (Zhong, Y and Fang, S, unpublished data), suggesting that they may target substrates to the gp78 complex as well. In contrast, XTP3-B, another ER lectin for substrate targeting, does not associate with gp78, and thus is unlikely to function with gp78 (Hosokawa et al 2008). gp78 appears to recognize membrane substrates via different adaptor proteins, such as Derlin1, insig-1 and SPFH1/SPFH2 that recruit CFTRΔF508, HMG-CoA reductase and possibly inositol 1,4,5-trisphosphate (IP(3)) receptors, respectively (Younger et al 2006, Sun et al 2006, Wang et al 2008, Song et al 2005, Jo et al 2011, Pearce et al 2007). We do not know whether the transmembrane domains of gp78 directly recognize substrates. As expected, gp78 recognizes its cytosolic substrates, such as mutant huntingtin (htt) and SOD1 using its cytosolic tail (Yang et al 2010, Ying et al 2009). Therefore, the function of gp78 is not confined to any particular ERAD pathway defined in yeast.

After delivery to the gp78 complex (**Table 2**), luminal and probably some membrane substrates require retrotranslocation in order to be ubiquitinated. This is

determined by the topology of gp78. As in all known ERAD E3s, the E3-active domain-the RING finger of gp78 is localized on the cytosolic surface of the ER (Zhong et al 2011). Retrotranslocation enables access of luminal substrates to the E3 activity for ubiquitination. Recent studies suggest that cytosolic exposure of luminal substrates is promoted by a cooperative action of importin β and RanGDP (Zhong et al 2011), although the underlying mechanism is not known. Following ubiquitination, the cytosolic AAA ATPase (ATPase associated with various cellular activities) p97/VCP/Cdc48 and its cofactors Ufd1 and Npl4 are recruited to the cytosolic surface of the ER to extract polyubiquitinated substrates into the cytosol through hydrolysis of ATP (Tsai et al 2002, Bays et al 2002) (**Fig. 3 A**). The role of the cofactors is to enhance the binding of p97/VCP with the polyubiquitinated substrates (Ye et al 2003). The polyubiquitin chain conjugated to the substrates provides a handle for the p97/VCP complex to pull the substrate from the ER. How p97/VCP along with Ufd1 and Npl4 are recruited to the ER remains unclear. Evidence suggests that the recruitment may be a concerted effort of several proteins in the ERAD complex. For example, p97/VCP/Cdc48 interacts with Hrd1, another well-established polytypic ERAD E3, and also several other proteins that interact with Hrd1, including Derlin1-3, VIMP, Erasin, UbxD8 and Herp (Ye et al 2004, Ye et al 2005, Lilley et al 2005, Lilley et al 2004, Schulze et al 2005, Liang et al 2006, Mueller et al 2008). Although we do not know whether these multiple interactions lead to recruitment of the p97/VCP-Ufd1-Npl4 complex, functional studies in both yeast and mammalian cells have shown that the p97/VCP/Cdc48-Ufd1-Npl4 complex is required for degradation of Hrd1 substrates (Tsai et al 2002, Bays et al 2002).

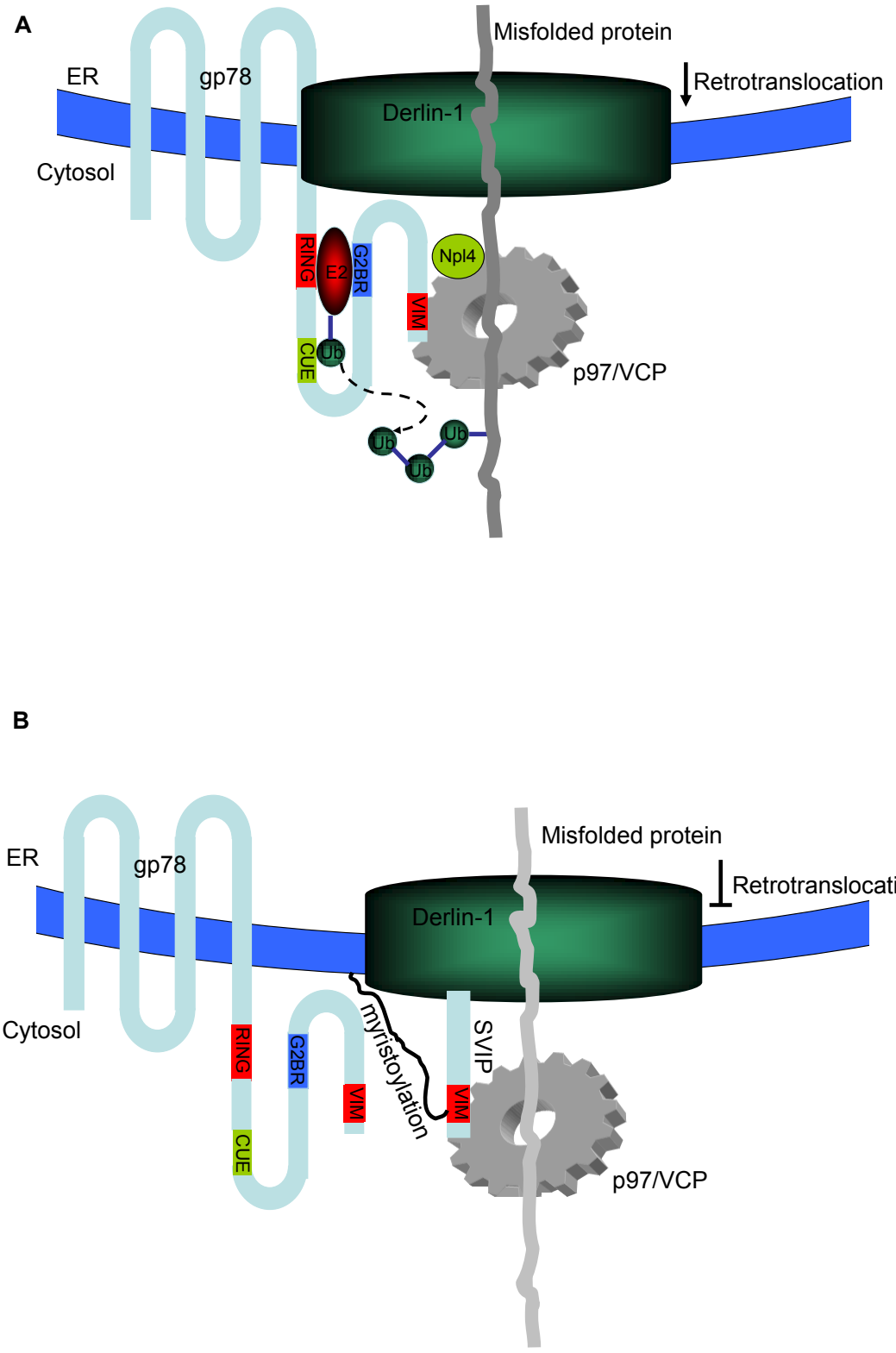
The gp78 complex contains similar membrane components to those of the Hrd1 complex. Therefore, proteins in the gp78 complex also make multiple contacts with p97/VCP. Evidence suggests that the gp78-p97/VCP interaction is most critical for coupling ubiquitination with retrotranslocation (Zhong et al 2004). gp78 contains a p97/VCP-interacting motif (VIM) near its C-terminus (Ballar et al 2006). The VIM has a high affinity towards p97/VCP and is sufficient to recruit p97/VCP to the ER surface (Ballar et al 2006, Hanzelmann et al 2011). Deletion of the VIM from gp78 stabilizes CD3δ, a well-established gp78 substrate. Moreover, the stabilized CD3δ is highly ubiquitinated, suggesting that loss of VIM in gp78 results in failure to recruit p97/VCP, which in turn results in failure to extract ubiquitinated CD3δ (Zhong et al 2004). The VIM of gp78 interacts with the ND1 domain of p97/VCP that is also the binding site for Ufd1 (Ballar et al 2006). In addition, Ufd1 bridges the interaction of the Ufd1-Npl4 dimer with p97/VCP (Meyer et al 2000). Therefore, gp78 and the Ufd1-Npl4 dimer form mutually exclusive complexes with p97/VCP (Ballar et al 2006, Stapf et al 2011). Therefore, it is unlikely that gp78 can recruit p97/VCP along with the Ufd1-Npl4 dimer. Functional studies indeed show that gp78-mediated ERAD is independent of Ufd1, but surprisingly, requires Npl4 (Ballar et al 2006, Ballar et al 2011). Nevertheless, the interaction between gp78 and p97/VCP enhances p97/VCP-polyubiquitin binding (Zhong et al 2004), suggesting that Npl4 and the Cue domain of gp78 may play an analog role to that of the Ufd1-Npl4 dimer. However, the Ufd1-Npl4 independent retrotranslocation has recently been shown for the human cytomegalovirus protein US2-mediated degradation of MHC class I heavy chain from the ER (Soetandyo et al 2010), suggesting that distinct retrotranslocation complexes

might exist. Reminiscent of ERAD, the yeast Cdc48 is recruited to stressed mitochondria, retrotranslocates ubiquitinated proteins from the outer mitochondria membrane and delivers ubiquitinated proteins to the proteasome for degradation (Heo et al 2010). Interestingly, Cdc48 is recruited to mitochondria by the VIM of Vms1. Moreover, Vms1 recruits Cdc48-Npl4 complex to retrotranslocate proteins independent of Ufd1. Moreover, Vms1 does not directly interact with Npl4. The Vms1-Npl4 interaction is bridged by Cdc48 (Heo et al 2010). The mammalian homolog of Vms1, ANKZF1, although not evaluated, is likely to play the same role (Stapf et al 2011, Heo et al 2010)**.** Since gp78-mediated ERAD requires p97/VCP and Npl4 independent of Ufd1 and gp78 contains a VIM, we predict that gp78 recruits p97/VCP-Npl4 to the ER during ERAD (**Fig. 3 A**).

Retrotranslocation is thought to occur through a proteinaceous channel. Although the identity of this channel remains elusive, it must be associated with the E3 ubiquitin ligase complexes and formed by transmembrane protein(s). Previous studies suggest that the sec61 translocon may also serve as a channel for retrotranslocation during ERAD (Wiertz et al 1996). Other transmembrane proteins, such as Derlins, gp78 and Hrd1 have been suggested to be part of the retrotranslocation channel (Schekman et al 2004, Meusser et al 2005). The yeast homolog of Hrd1, Hrd1p, has indeed been shown to be the retrotranslocation channel in yeast (Carvalho et al 2010). It is not known, however, whether Hrd1 plays the same role. Although Derlin1 was considered as the best candidate channel protein, recent studies indicate that Derlin1 is a rhomboid pseudoprotease that is unlikely to function as a retrotranslocation channel (Greenblatt et al 2011). gp78 contains five

transmembrane domains and can form large oligomers. It is tempting to speculate that its oligomerization may form the retrotranslocation channel.

It is known that at least some of the glycosylated substrates are degraded through the gp78-mediated pathway. Removal of glycans from substrates is an essential step required for degradation by the proteasomes. Indeed, gp78 is associated, via p97/VCP, with the peptide N-glycanase (PNGase), a cytosolic enzyme that deglycosylates misfolded glycoproteins, and mHR23B, a ubiquitin chaperone that delivers polyubiquitinated substrates to the proteasomes (Li et al 2005, Li et al 2008). Another important issue is how cells maintain the solubility of retrotranslocated substrates before they reach the proteasomes. Previous studies suggest that gp78 appears to play such a role (Shen et al 2006). It is now known that gp78 associates with a multiprotein complex comprising Bag6, Ubl4A and Trc35, which chaperones retrotranslocated polypeptides en route to the proteasome. Bag6 contains a chaperone-like activity capable of maintaining an aggregation-prone substrate in an unfolded yet soluble state (Wang et al 2011).


A
ER
gp78
Misfolded protein
Retrotranslocation
Derlin-1
Cytosol
RING
E2
G2BR
Npl4
VIM
CUE
Ub
p97/VCP
B
ER
gp78
Misfolded protein
Retrotranslocation
Derlin-1
Cytosol
myristoylation
SVIP
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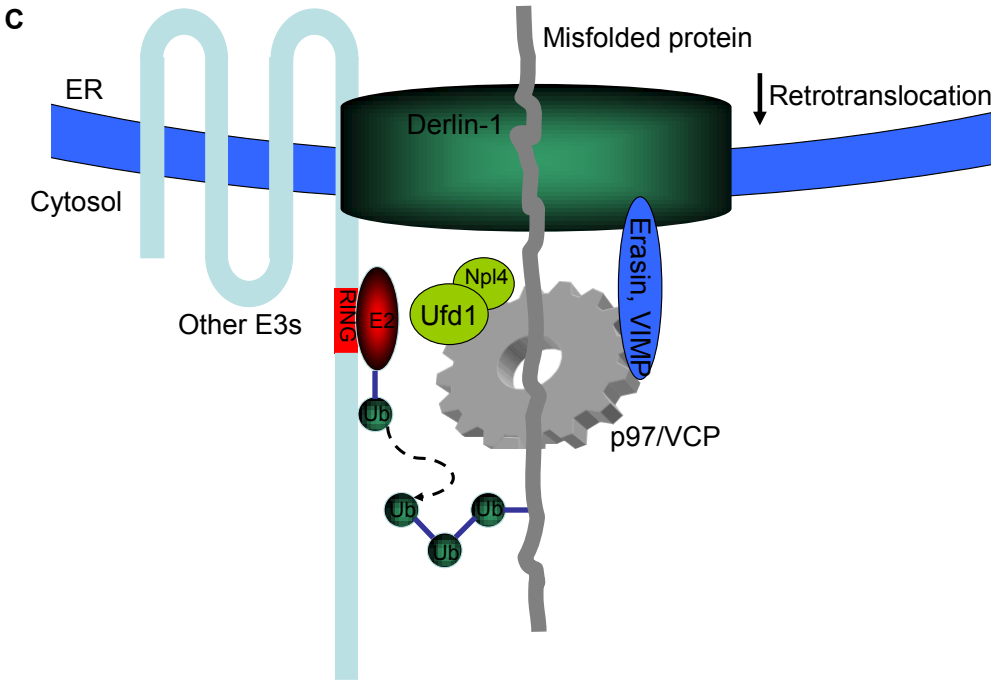

Fig. 3. A simplified view of gp78 and Hrd1-mediated ERAD pathways and their regulations by SVIP.

(A) gp78 recruits p97/VCP-Npl4(N) to the cytosolic surface of the ER for coupling ubiquitination with retrotranslocation to enhance ERAD; **(B)** SVIP(S) is anchored to membrane via myristoylation and sequesters Derlin1, p97/VCP and probably Npl4(N) away from gp78 leading to inhibition of ERAD; **(C)** p97/VCP-Ufd1(U)-Npl4(N) complex is recruited to the Hrd1 complex to couple ubiquitination with retrotranslocation. SVIP may inhibit Hrd1-mediated ERAD by sequestering p97/VCP, Npl4 and Derlin1 away from Hrd1.

4. Maintenance of protein homeostasis by gp78-mediated ERAD

4.1 Regulation of physiological processes by gp78-mediated ERAD

Increasing evidence indicates that gp78-mediated ERAD plays an important role in the regulation of physiological processes. Apolipoprotein B-100 (ApoB-100), an essential protein for the assembly and secretion of very low-density lipoproteins (VLDL) from the liver, is the first physiological substrate identified for gp78 (Liang et al 2003, Fisher et al 2011) (**Table 1**). ApoB-100 is degraded by ERAD when lipid availability limits the assembly of VLDL (Fisher et al 2011). This is part of the quality control mechanism that eliminates orphan subunits of protein complexes. gp78 and p97/VCP have been implicated in the proteasomal degradation of ApoB-100 (Liang et al 2003, Fisher et al 2011, Fisher et al 2008). Overexpression of gp78 was shown to increase ubiquitination and proteasomal degradation of ApoB-100, with reduced secretion of ApoB-100 in HepG2 cells (Liang et al 2003). By contrast, knockdown of gp78 expression decreased ApoB-100 ubiquitination and retrotranslocation. Concomitantly, VLDL assembly is enhanced and triacylglycerol secretion is increased. gp78-mediated ubiquitination commits ApoB-100 to p97/VCP-mediated retrotranslocation (Fisher et al 2011). Therefore, gp78 plays an important regulatory role in VLDL assembly through ubiquitination of ApoB-100.

gp78 also has an established role in the regulated degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), a key enzyme that catalyzes the conversion of HMG-CoA to mevalonate in the rate-limiting step of cholesterol biosynthesis (Goldstein et al 2006). The conversion is under strict feedback regulation

mediated by sterol and nonsterol metabolites of mevalonate (Goldstein et al 2006). One mechanism for the feedback regulation involves rapid degradation of HMGCR through ERAD according to studies in cultured cells (Sever et al 2003). Accumulation of sterols in the ER membrane triggers binding of the ER membrane proteins Insig-1 and Insig-2 to the sterol-sensing domain of HMGCR. Insig-1 in turn interacts the N-terminal transmembrane domains of gp78, thereby targeting HMGCR to the gp78 complex. gp78 then catalyzes polyubiquitination of HMGCR through its interaction with the E2 Ube2g2 (Song et al 2005, Goldstein et al 2006). Ufd1 acts as a cofactor for gp78 to promote HMGCR ubiquitination (Cao et al 2007). The ubiquitinated HMGCR is extracted from the ER by p97/VCP and then delivered to the proteasomes for degradation (Song et al 2005). Taken together, gp78 binds to HMGCR in an Insig1-dependent and sterol regulated manner. When cells are depleted of sterols, gp78 targets Insig-1 for degradation leading to increases in sterol synthesis by HMGCR (Lee et al 2006). It is worth noting that Hrd1p is the E3 ubiquitin ligase involved in ERAD of HMGCR in yeast (Hampton et al 1996), while Hrd1, the mammalian homolog of Hrd1p, is not involved in the regulation of HMGCR degradation in mammalian cells (Song et al 2005, Nadav et al 2003).

The liver cytochrome P450 (CYP) enzymes have recently joined the list of gp78 substrates. Specifically, CYP3A4 and CYPE21 have been shown to be substrates for gp78 (Kim et al 2010, Wang et al 2011). CYP3A4 is responsible for the metabolism of the majority of xenobiotics including anticancer agents. The levels of CYP3A4 expression have been proposed to be a factor responsible for the variability in clinical response to chemotherapy. gp78 regulates the levels of CYP3A4 by ubiquitinating

CYP3A4 leading to its degradation by the proteasomes. This finding has important clinical implications, because most anticancer agents have very narrow therapeutic windows, thus even slight changes in CYP3A4 levels could alter the exposure of the drug and result in either insufficient efficacy or toxicity (Peer et al 2011). Liver CYP2E1 is responsible for the biotransformation of clinically relevant drugs, low molecular weight xenobiotics, carcinogens and endogenous ketones. gp78 is able to ubiquitinate and target CYP2E1 for proteasomal degradation (Wang et al 2011). Phosphorylation of CYP2E1 and CYP3A4 may serve to engage the gp78/Ube2g2 complex to enhance their ubiquitination. The hepatic function of gp78 *in vivo* is further highlighted by its high level of expression in mouse liver compared with other organs (Ballar, P and Fang, S, unpublished data). Thus, gp78 may play important roles in the regulation of drug metabolism in liver.

4.2 Regulation of pathological processes by gp78-mediated ERAD

The significance of gp78-mediated ERAD is underscored by its association with not only physiological proteins but also proteins that are linked to human diseases, such as KAI1, ATZ, and CFTRΔF508, and mutant huntingtin (htt), neuroserpin, ataxin-3 and SOD1.

gp78 was originally identified as a 78-kDa glycoprotein that promotes tumor metastasis (Nabi et al 1987). Subsequently, it was shown to be the tumor autocrine motility factor (AMF) receptor (AMFR) (Silletti et al 1991, Nabi et al 1990). Consistently, gp78 has been shown to be highly expressed in various cancers, such as bladder cancer, colorectal cancer, esophageal cancer, gastric cancer and hepatocelllular carcinoma and its elevated expression is correlated with metastasis

(Fairbank et al 2009). This is in accord with the observation that patients with increased expression of gp78 have significantly worse disease-free survival rates (Endo et al 2006). The correlation of the elevated expression in tumors and increased metastasis has been solely attributed to the function of gp78 as AMFR. However, gp78 also promotes metastasis through the ERAD pathway (Tsai et al 2007). gp78 associates with and targets the transmembrane metastasis suppressor, KAI1 (also known as CD82), for degradation. Reduction of gp78 expression increases the abundance of KAI1 and reduces the metastatic potential of tumor cells, an effect that is largely abrogated by concomitant suppression of KAI1. This inverse relationship between these proteins was revealed in a human sarcoma tissue microarray (Tsai et al 2007). When overexpressed in mammary glands, gp78 promotes cell proliferation and nontumorigenic ductal outgrowth mediated by the metastasis suppressor KAI1 (Joshi et al 2010). Therefore, gp78 may promote tumor cell proliferation, invasion and metastasis by more than one mechanism.

Another pathogenic role of gp78 is in cystic fibrosis (CF). CF is a common autosomal recessive disease caused by mutations in the gene encoding CFTR, an epithelial anion channel (Ashlock et al 2011). CFTRΔF508 is the most common CF-associated mutation, which accounts for about 70% of CF alleles (101). CFTRΔF508 is retained in the ER and rapidly degraded through the ERAD pathway, which prevents its trafficking to the plasma membrane (Ostedgaard et al 2007). RMA1, an ER-anchored RING finger E3 is involved in ERAD of CFTRΔF508 (Younger et al 2006). gp78 may act as an E4 to extend the polyubiquitin chain that has been conjugated to CFTRΔF508 by RMA1 (Morito et al 2008). gp78 also enhances the

interaction of CFTRΔF508 with p97/VCP, presumably to increase CFTRΔF508 retrotranslocation. Harnessing gp78-mediated ERAD via knockdown of p97/VCP or overexpression of gp78 dominant negative mutant rescues CFTRΔF508 from ERAD and increases its trafficking to cell surface and partially restores its channel function (Vij et al 2006). By contrast, Hrd1 inhibits CFTRΔF508 degradation by acting as an E3 for gp78 (Ballar et al 2010). Knockdown of Hrd1 results in stabilization of gp78, and consequently increases in CFTRΔF508 degradation (Ballar et al 2010). Both p97/VCP and Derlin1 are critical components of the CFTRΔF508 degradation machinery. Small VCP/p97-interacting protein (SVIP) is known to sequester p97/VCP and Derlin1 away from gp78 to form an ERAD-inactive complex (Ballar et al 2007) (**Fig. 3 C**). It was shown that overexpression of SVIP leads to accumulation of CFTRΔF508 (Ballar et al 2010), supporting the idea that gp78 targets CFTRΔF508 for degradation.

gp78 is widely involved in degradation of neurodegenerative disease proteins. This function is unlikely to be specific for gp78, since Hrd1 also acts in the same spectrum of neurodegenerative disease proteins (Yang et al 2010, Ying et al 2009, Yang et al 2007, Ying et al 2011). Therefore, gp78 and Hrd1 probably recognize these mutant proteins by a quality control mechanism, although the mechanism of substrate recognition by these two E3s may be different. Polyglutamine expansion in huntingtin (htt) protein induces Huntington's disease (HD) although the mechanism remains uncertain. Some insights into the mechanism come from the discovery that mutant htt interacts with gp78 (Yang et al 2010). The HEAT repeats 2&3 of htt interact with the Cue domain of gp78. The interaction competitively reduces

polyubiquitinated protein binding to gp78 and also sterically blocks gp78 interaction with p97/VCP. These effects of htt negatively regulate the function of gp78 in ERAD and are aggravated by polyglutamine expansion. Paradoxically, gp78 is still able to ubiquitinate and facilitate degradation of htt proteins with expanded polyglutamine. When mutant htt accumulates and aggregates, it also impairs the function of p97/VCP-Ufd1-Npl4 in ERAD by sequestering them to its aggregates (Duennwald et al 2008). Therefore, it is not surprising that the impairment of ERAD by mutant htt proteins is associated with induction of ER stress (Yang et al 2010, Duennwald et al 2008). We speculate that mutant htt accumulates and gradually aggregates in neurons during HD progression, probably because the rate of mutant htt degradation is slower than the rate of its production/accumulation. The inefficiency in degradation of mutant htt proteins would preoccupy E3 proteins like gp78 and Hrd1 that might typically engage in ERAD in a futile effort toward degrading mutant htt proteins. This nonproductive interaction would lead to an accumulation of misfolded proteins in the ER leading to ER stress.

Hrd1 and gp78 are also involved in ubiquitination and degradation of mutant neuroserpin (Ying et al 2011), a secreted glycoprotein and a serine protease inhibitor of serpin family predominantly expressed in the neurons of the central nervous system (CNS) (Hastings et al 1997). The role of neuroserpin is largely unknown, but it has been suggested that neuroserpin plays a neuroprotective role and may be involved in regulation of the morphology of neuroendocrine cells and neurite outgrowth (Parmar et al 2002). Point mutations in the neuroserpin gene result in its misfolding, accumulation and formation of neuroserpin inclusion bodies in the ER,

which causes familial encephalopathy with neuroserpin inclusion bodies (Miranda et al 2004). Recent studies demonstrate that overexpression of Hrd1 and gp78 reduces the mutant neuroserpin levels, whereas knockdown of either E3 stabilizes it (Duennwald et al 2008). Impairment of p97/VCP function also stabilizes neuroserpin and increases its aggregation. These results suggest that mutant neuroserpin is a bona fide ERAD substrate for both gp78 and Hrd1 (Ying et al 2011). Therefore, gp78 and Hrd1 may play a protective role against mutant neuroserpin-induced neuronal degeneration. Similarly, gp78 has been shown to promote degradation of mutant superoxide dismutase 1 (SOD1) and ataxin-3, two neurodegenerative disease proteins, respectively associated with familial amyotrophic lateral sclerosis and Machado–Joseph disease/spinocerebellar ataxia type 3 (Ying et al 2009). The pathological common feature of these neurodegenerative disease proteins is their accumulation and aggregation in neurons during disease progression. gp78 and Hrd1 act as quality control E3s for these mutant proteins, which is another common feature. These commonalities may explain why ER stress has been increasingly recognized as a common pathogenic factor in various neurodegenerative diseases (Lindholm et al 2006). It is likely that gp78 and Hrd1 protect neurons at the early stage of the diseases when disease proteins are not in aggregates. As the diseases progress, production of mutant proteins exceeds the degradation capacity of gp78 and Hrd1, which leads to accumulation and aggregation of the mutant proteins. The disease protein aggregates interact with gp78 and Hrd1 as well as p97/VCP and impair their functions in ERAD leading to ER stress. The findings that gp78 and Hrd1 mediate degradation of

cytosolic misfolded proteins, such as mutant htt, SOD1 and ataxin-3, extend the territory of the role of gp78 and Hrd1 in quality control to cytosolic proteins.

Other substrates of gp78 include ATZ and cholera toxin (CT). Mutations of α-1-antitrypsin (AAT) lead to AAT protein retention in the ER and deficiency of circulating AAT. Accumulation of mutant AAT in the ER causes severe liver injuries, such as neonatal hepatitis, juvenile cirrhosis and hepatocellular carcinoma (Perlmutter et al 2002). gp78 was shown to ubiquitinate and facilitate degradation of ATZ, the classic deficiency variant of circulating AAT having a Z mutation (Glu 342 Lys) (Shen et al 2006). Cholera toxin (CT) is the virulence factor produced by *Vibrio cholera.* It is transported from the cell surface to the ER lumen where the catalytic CTA1 subunit is retrotranslocated to the cytosol to induce pathological water secretion. Although CTA1 is not degraded after retrotranslocation, gp78 and Hrd1 were shown to cooperate with Derlin1 and the ER luminal chaperone protein disulfide isomerase (PDI) to facilitate CTA1 retrotranslocation, suggesting that ubiquitination may be involved in CTA1 retrotranslocation (Bernardi et al 2008).

Table 1. List of identified substrates for gp78

Substrate	E3 ubiquitin ligase(s)	Comments (substrate)	References
ApoB-100	gp78	A key protein component of LDL	Stapf et al 2011, Liang et al 2003, Fisher et al 2011, Fisher et al 2008
HMG-CoA reductase	gp78	A rate-limiting enzyme in cholesterol biosynthesis	Song et al 2005, Goldstein et al 2006, Sever et al 2003, Cao et al 2007
Insig1	gp78	Regulator of cholesterol synthesis	Song et al 2005,, Lee et al 2006
CYP3A and CYPE21	gp78 and CHIP	Liver cytochrome P450 enzymes	Kim et al 2010, Wang et al 2011
KAI1	gp78	Tumor metastasis suppressor	Tsai et al 2007, Joshi et al 2010
CFTRΔF508	gp78, CHIP, Fbs1 and RMA1	The most common mutation in cystic fibrosis transmembrane conductance regulator (CFTR) causing cystic fibrosis	Morito et al 2008, Younger et al 2006, Vij et al 2006
Mutant huntingtin	gp78 and Hrd1	The Huntington's disease protein	Yang et al 2010, Yang et al 2007
Mutant neuroserpin	gp78 and Hrd1	A mutant serine protease inhibitor causing familial encephalopathy with neuroserpin inclusion bodies	Ying et al 2011
Mutant SOD1	gp78	A mutant antioxidant enzyme causing familial amyotrophic lateral sclerosis	Ying et al 2011
Ataxin-3	gp78	A mutant deubiquitinating enzyme causing Machado–Joseph disease/spinocerebellar ataxia type 3	Ying et al 2011
ATZ	gp78	Z variant of α-1-antitrypsin (ATZ) causing deficiency in circulating α-1-antitrypsin	Shen et al 2006
Cholera toxin (CT)	gp78 and Hrd1?	The virulence factor produced by *Vibrio cholera* requires retrotranslocation to exert its cytotoxicity	Bernardi et al 2008

Table 2. Proteins in the gp78 complex

Protein	Yeast Homolog	Validated	Direct vs. Indirect interaction with gp78	Function	References
gp78	Hrd1p	Yes	Direct, via OS	gp78 oligomerization required for gp78 E3 activity	Li, et al 2009
Ube2g2	Ubc7p	Yes	Direct, with G2BR	Cognate E2 for gp78	Fang et al 2001
Derlin1	Der1p	Yes	Unknown	Substrate recruitment	Ye et al 2004, Lilley et al 2004
p97/VCP	Cdc48p	Yes	Direct, with VIM	Retrotranslocation of substrates	Zhong et al 2004
Ufd1	Ufd1p	Yes	Direct	Cofactor for gp78 E3 activity towards HMG-CoA reductase	Cao et al 2007
Npl4	Npl4p	Yes	Unknown	Forms a complex with p97/VCP in gp78-mediated ERAD	Ballar et al 2011, Soetandyo et al 2010, Ballar et al 2010
PNGase mHR23B	Png1p Rad23p	Yes	Indirect, via p97/VCP	ERAD substrate-processing factors	Li et al 2005, Li et al 2008
Erasin	Ubx2p	Yes	Indirect, via p97/VCP and ubiquilin	Involved in recruiting p97/VCP and ubiquilin to ERAD complex	Liang et al 2006
Ubiquilin	Dsk2p	Yes	Indirect, via p97/VCP	Binds the proteasome and delivers the misfolded protein to proteasome	Liang et al 2006
Bag6	Unavailable	Yes	Unknown	Associates with gp78, maintains polypeptide solubility and may escort substrates to the proteasome	Wang et al 2011

Protein	Yeast Homolog	Validated	Direct vs. Indirect interaction with gp78	Function	References
UbxD8	Unavailable	Yes	Unknown	Unknown	Mueller et al 2008
Herp	Usa1p	Yes	Unknown	Unknown	Schulze et al 2005
SPFH2	Unavailable	Yes	Unknown	Unknown	Yo et al 2011
TMUB1	Unavailable	Yes	Unknown	Bridges SPFH2 to gp78 in ER membrane	Yo et al 2011
VIMP	Unavailable	Yes	Unknown	Recruits p97/VCP to ER membrane	Ye et al 2005

4.3 Regulation of gp78-mediated ERAD

4.3.1 Regulation of gp78-mediated ERAD by autoubiquitination

The function of gp78 in ERAD is subject to multilayered regulations. One of the most direct regulations is to modulate the levels of gp78 expression. ERAD prevents protein accumulation through elimination of misfolded proteins from the ER. When misfolded proteins fail to be removed efficiently by ERAD, accumulation of them will result in ER stress, which activates UPR. UPR upregulates transcription of ERAD components including E3 ubiquitin ligases (Travers et al 2000). We have demonstrated that tunicamycin-induced UPR increases the expression of *gp78* mRNA (Chen, Z, Du, S and Fang, S, unpublished data). In addition, acute ER stress enhances ERAD by stabilizing gp78 protein (Shen et al 2007). This is achieved by suppressing gp78 autoubiquitination. Autoubiquitination of gp78 targets itself for degradation by the proteasomes. Inhibition of E3 autoubiquitination may be a general mechanism by which cells rapidly respond to acute accumulation of misfolded proteins in the ER. Hrd1 exhibits the same response to that of gp78. This posttranslational response to boost ERAD activity is not limited to E3 ubiquitin ligases. For example, it has been reported that UPR boosts glycoprotein ERAD by suppressing the proteolytic downregulation of ER ManI. Stabilization of ER ManI protein enhances mannose processing, thereby facilitating ERAD (Termine et al 2009). Thus, UPR enhances gp78-mediated ERAD at both the transcriptional and posttranslational levels.

4.3.2 Regulation of gp78-mediated ERAD by HRD1

The crosstalk between E3 ubiquitin ligases has been shown to be involved in regulation of the ubiquitination activity of gp78 during ERAD (Ballar et al 2010, Shmueli et al 2009). gp78 is a substrate for the Hrd1 ubiquitin ligase (Ballar et al 2010, Shmueli et al 2009). Autoubiquitination of gp78 requires its functional RNIG finger while the ubiquitination of gp78 by Hrd1 is solely dependent on the RING finger activity of Hrd1 but not on that of gp78 (Shmueli et al 2009). The regulation of gp78 by Hrd1 is underscored by the observation that gp78 is stabilized in Hrd1 knockdown cells and embryonic fibroblasts of Hrd1 homozygous knockout mice ($Syvn^{-/-}$) (Shmueli et al 2009). An interesting question is how the cells determine when and to what extent gp78 is regulated by autoubiquitination or Hrd1.

4.3.3 Regulation of gp78-mediated ERAD by SVIP

The function of gp78 is also regulated at the step of retrotranslocation. This regulation is mediated by the SVIP (Ballar et al 2007). SVIP does not have transmembrane domain and is localized to the ER membrane through myristoylation. SVIP contains a well-conserved VIM that competes with gp78 for binding to p97/VCP leading to interruption of gp78-p97/VCP interaction. Moreover, SVIP in fact sequesters p97/VCP and Derlin1 away from gp78. Derlin1 is a substrate-recruiting protein for gp78. As expected, SVIP also inhibits the ubiquitination of the gp78 substrate, CD3δ. Thus, dependent on the relative levels of gp78 and SVIP, p97/VCP and Derlin1 can either form an ERAD-inhibitory complex with SVIP or an ERAD-active complex with gp78. Therefore, SVIP regulates gp78-mediated ERAD by regulating the assembly of the gp78-Derlin1-p97/VCP complex. This regulation

might not be limited to gp78-mediated ERAD, since p97/VCP is a converging point for probably all ERAD pathways (**Fig. 3**).

In addition to its role in ERAD, SVIP is also a regulator of the autophagy pathway. As an ERAD inhibitor, SVIP facilitates autophagy by promoting LC3 lipidation, enhancing p62 expression, sequestration of polyubiquitinated proteins to autophagosomes and increasing starvation-induced degradation of LC3II and p62 proteins (Wang et al 2011). The opposite roles of SVIP in ERAD and autophagy may be important mechanisms by which cells handle ER stress. It was shown that ER stress causes an early downregulation of the SVIP protein and prolonged ER stress markedly increases SVIP protein levels. We speculate that when SVIP is downregulated, gp78 is upregulated, which leads to increases in ERAD activity. Prolonged ER stress causes a significant accumulation and aggregation of misfolded proteins in the ER, and ERAD is expected not to be effective under these conditions. Thus, prolonged ER stress upregulates SVIP to enhance autophagic removal of aggregated proteins from the ER. Therefore, SVIP may be a switch from ERAD to autophagy during the course of ER stress.

5 ER stress and its association with lipid metabolism in the liver

The unfolded protein response (UPR) activated by ER stress has been associated with the pathogenesis of many diseases, including diabetes mellitus due to insufficient insulin function, viral infections that require a large number of membrane proteins, neurodegenerative diseases associated with the accumulation of misfolded

proteins, and cancer, which is characterized by hypoxia, a disturbed oxidative environment within the ER (Lin et al 2008).

Recently, the roles of ER stress and the UPR in hepatic steatosis have been under extensive study. First, the liver, as well as several other tissues, such as the pancreas, plasma, salivary gland, and mammary gland, are characterized by the expression of a large number of secretory proteins. Interestingly, in these tissues, it is common to observe high expression levels of chaperones or ERAD components under physiologic conditions. This indicates high levels of ER stress and a protective mechanism for the UPR. The reason for this could be explained by the fact that the liver is rich in rough ER, an important organelle for the proper folding of secretory proteins, calcium storage, and lipid and cholesterol synthesis, making the ER in the liver susceptible to an overload of misfolded proteins.

5.1 ER stress in the liver

In general, factors that trigger ER stress include, but are not limited to, free fatty acids, alcohol abuse, alcohol-induced toxic acetaldehyde production, alcohol-induced cytokine production, alcohol-induced toxic homocysteine, oxidative stress, perturbations of calcium or iron homeostasis, alterations of S-adenosylmethionine to Sadenosylhomocysteine ratio, and abnormal epigenetic modifications, glucosamine, lipogenic diet, glucose starvation, hypoxia, cell differentiation, protein overproduction, diabetes, obesity, cancer, inflammation, virus infection, and chemicals such as glycosylation inhibitors, reductive agents, antibiotics, anticancer agent, and so on. (Ji 2012).

5.2 Lipid metabolism in the liver

5.2.1 Sources of lipids in non-alcoholic and alcoholic hepatic steatosis

In general, several factors can trigger hepatic steatosis, such as insulin resistance, alcohol abuse, drug abuse, smoking, virus infection and genetic diseases. However, the sources of triglycerides are important factors in both non-alcoholic and alcoholic fatty livers. *De novo* lipogenesis (DNL, about 25%), dietary fatty acids (DFA, about 15%), plasma nonesterified fatty acid (NEFA, about 60%) from lipolysis of adipose tissue are three main sources of accumulation of triglycerides (Donnelly et al 2005).

In terms of the mechanism, increased *de novo* synthesis, increased uptakes and decreased fatty acids oxidation and decreased export all contribute to accumulation of triglyceride. (Donnelly et al 2005). First, increase of triglyceride synthesis is one of the major causes of hepatic steatosis. This could occur by increased availability of glycerol and fatty acids. Second, increased uptakes, for example, prolonged exposure of free fatty acid results in accumulation of triglycerides, hepatic steatosis, and induction of ER stress in McA-RH7777 liver cells (Ota et al 2008). In addition to this, free fatty acids are toxic to cells. Indeed, free fatty acid has been shown to induce ER stress and apoptosis in pancreatic β cells and liver cells (Kharroubi et al 2004., Wei et al 2006., Wang et al 2006.). Third, reduced fatty acid oxidation contributes to development of fatty liver diseases. For example, accelerated fatty acid oxidation in other tissues, such as muscle, can reduce hepatic steatosis after 24 h fast in SJL/J mice (Guan et al 2009.). Fourth, decrease of export worsen the lipid accumulation. For example, excess accumulation of hepatic lipid may inhibit hepatic apolipoprotein B100 secretion, which worsen the hepatic steatosis (Ota et al 2008). It was also showed that intravenous infusion of oleic acid (OA) in mice resulted in increased ER

stress and inhibition of ER stress that restored OA-stimulated ApoB secretion after prolonged OA infusion (Ota et al 2008).

Upregulation of several genes involved in lipid metabolism also contributes to alcoholic hepatic steatosis in response to alcohol exposure. Mice exposed to chronic alcohol feeding showed hepatic steatosis in which many genes are upregulated (Yin et al 2007). These genes are involved in (i) transport of glucose into hepatocytes and glycolysis to produce acetyl-CoA for fatty acid *de novo* synthesis, (ii) fatty acid transporters such as CD36, Slc27a1 and Slc27a4, and *de novo* synthesis through activation of Srebp1c pathway, (iii) fatty acid esterification to triglycerides, (iv) cholesterol transport, rate-limiting enzyme of *de novo* cholesterol synthesis, HMGCR through activation of Srebp2 pathway and bile acid synthesis (Yin et al 2007).

5.2.2 Role of insulin resistance in development of non-alcoholic hepatic steatosis

Regulation of insulin levels also plays an important role in the development of non-alcoholic fatty livers. First, insulin resistance might stimulate delivery of glucose and fatty acids to liver and adipose tissues, where excess of glucose is converted into fatty acids and triglyceride during glycolysis and lipogenesis and they are stored in the form of glycogen and lipids respectively. Second, insulin resistance might inhibit glucogeneogenesis and glycogenolysis. Third, insulin inhibits lipolysis in the liver, but insulin resistance in adipocytes might reversely overstimulate lipolysis (Yang et al 2009), leading to an increase in the plasma non-esterified fatty acid flux (Lewis et al 2002., Zhou et al 2009., Sanyal et al 2001). Fourth, insulin stimulates fatty acid synthesis by upregulation of its transcripts, enhancing processing of mature SREBPs (Kim et al 1998., Horton et al 1998., Shimomura et al 1999., Foufelle et al 2002.,

Hegarty et al 2005). Last but not the least, clinical studies showed that treatment of diabetic patients with drugs to improve insulin sensitivity successfully, in part, reduced the fat accumulation in the liver (Polyzos et al 2009).

5.2.3 Acetaldehyde affects lipid metabolism

Acetaldehyde is toxic to hepatocytes and may play a critical role in development of fatty liver diseases. Acetaldehyde, an ethanol intermediate metabolite, is toxic to cells if not further metabolized. Moreover, acetaldehyde was proposed to activate (peroxisome proliferator-activated receptor α) PPARα, a key nuclear hormone receptor that when activated, forms heterodimer with retinoid X receptor (RXR), binds to peroxisome proliferator response element of genes involved in the fatty acid oxidation pathways, and activates their transcription (Crabb et al 2006). Here it is worth pointing out that posttranslational modification of PPARα or RXR or its ligand by acetaldehyde, a highly reactive chemical to amino group of proteins, might underlie its molecular mechanism. Not surprisingly, PPARα knockout mice displayed impaired fatty acid oxidation, and accumulation of lipid with fasting, accompanied with hypoglycemia and increased serum free fatty acids (Le May et al 2000). Consistent with this, evidence showed that treatment of PPARα agonists reduced alcoholic fat accumulation in mice fed with ethanol (Marche et al 2011).

5.2.4 Cytokine production triggers development of hepatic steatosis

Ethanol-induced cytokine production by Kupffer cells, e.g. TNFα, seems to play a critical role in development of alcoholic liver diseases. A detailed review on the roles of Kupffer cells, TNFα, and other factors, such as adiponectin, osteopontin, interleukin 6, and plasminogen activator inhibitor 1 on alcoholic fatty liver diseases

has been reviewed (Crabb et al 2006). These evidences of cytokine production in the development of alcoholic fatty livers include but are not limited to the following aspects. First, mice depleted of Kupffer cells showed resistance to ethanol-induced liver injury (Adachi et al 1994). Second, cytokines result in imbalance of calcium homeostasis through inhibition of SERCA (sarcoplasmic/endoplamic reticulum Ca^{2+}-dependent ATPase) pump expression in the ER and thus induce ER stress in pancreatic β-cells (Cardozo et al 2005). Third, anti-TNFα antibody treatment attenuated hepatic inflammation and necrosis observed in ethanol-fed rats without improvement of hepatic steatosis (Limuro et al 1997). Fourth, clinically, plasma inflammation has been shown to be associated with hepatic steatosis and serum adipokine levels are proposed to be a marker of nonalcoholic fatty liver disease. (Lemoine et al 2009., Tarantino et al 2009).

5.2.5 Other pathways involved in regulation of lipid metabolism

The AMP-activated protein kinase (AMPK) pathway is involved in regulation of lipid metabolism in liver. AMPK signaling inhibits ACC and HMG CoA reductase and thus reduces fatty acid and cholesterol synthesis respectively. Upon alcohol feeding, AMPK activity is reduced, leading to reduced level of phosphorylated acetyl coenzyme A carboxylase a (ACC1) and increased ACC1 activity, and consequently increased fatty acid synthesis (Davies et al 1992).

Estrogens also play a role in regulation of hepatic cholesterol. It is believed that females are more protected from hypercholemia-related diseases because of the hypolipidemic estrogens they produce (Farhat et al 1996., Bär et al 1997).

5.3 Association of ER stress with hepatic steatosis

Recently, more and more studies are focusing on the question of whether ER stress plays a direct and sufficient role in the development of fatty livers. Indeed, tunicamycin treatment in zebrafish larvae induces the upregulation of ER stress markers in the liver and mimics the hepatic steatosis seen in the *hi559* mutant (Thakur et al 2011). In humans, the attenuated expression of ER stress markers, such as BiP, spliced X-box binding protein 1 (XBP1), phosphorylated α-subunit of eukaryotic translation initiation factor 2 (eIF2α) , and JNK1, was observed in the liver, adipose tissue, and skeletal muscle of obese subjects after weight loss (Gregor et al 2009). In a mouse model of type 2 diabetes, the treatment of obese and diabetic mice with the chemical chaperones 4-phenyl butyric acid and taurine-conjugated ursodeoxycholic acid reduces ER stress, restores insulin sensitivity, resolves fatty liver disease, and enhances insulin action in the liver, muscle, and adipose tissue (Ozcan et al 2006). Adipose triglyceride lipase knockout mice show protection from ER stress in response to tunicamycin treatment, along with increased lipid accumulation in the liver, probably due to the inability to produce free fatty acids (Fuchs et al 2012). All together, these studies suggest a causal role for ER stress in hepatic steatosis.

The molecular mechanism of alcohol-induced hepatic ER stress is complicated and remains largely unknown. Some of the factors that trigger alcohol-induced hepatic ER stress include toxic acetaldehyde and homocysteine, oxidative stress, perturbations of calcium or iron homeostasis, the alteration of the S-adenosylmethionine to S-adenosylhomocysteine ratio, and abnormal epigenetic modifications (Ji et al 2006).

An open question that remains to be further investigated is why ER stressed cells display lipid accumulation. It is believed that ER stressed cells undergo not only an increased protein load, but also an expansion of the lipid components of their membranes. The expansion of the ER might play a beneficial role in terms of reducing the protein load to the ER lumen.

How ER stress results in hepatic steatosis also remains poorly understood. Several studies have shown that ER stress induces the activation of SREBPs, master regulators of *de novo* lipid biosynthesis (Colgan et al 2007., Lee et al 2004). However, this does not provide a detailed mechanism and does not clarify whether and how the UPR pathway is involved in the development of hepatic steatosis. There are 3 branches of the UPR and each arm seems to play a role in hepatic lipid metabolism in a particular way (**Fig. 4**).

5.4 Activation of unfolded protein response by ER stress plays a role in hepatic steatosis

5.4.1 Role of the IRE1-XBP1 pathway in hepatic steatosis

Overexpression of the splicing-form of *Xbp1* is sufficient to induce phosphatidylcholine biosynthesis, suggesting a dual role for XBP1 and a link between the UPR and lipid metabolism (Sriburi et al 2004). On the other hand, conditional knockout of *Xbp1* in the liver caused hypotriglyceridemia and hypocholesterolemia due to defects in *de novo* lipogenesis without affecting protein secretory functions (Lee et al 2008). This also indicates that the dual functions of XBP1 in both lipogenesis and the UPR are probably independent of each other (Basseri et al 2008).

The exposure of mice with a liver-specific deletion of *inositol-requiring protein 1α* (*Ire1*α) to tunicamycin causes the upregulation and nuclear localization of CCAAT/-enhancer-binding protein homologous protein (CHOP), which was proposed to interfere with the function of C/EBPα (Rutkowski et al 2008). CHOP is a novel developmentally regulated nuclear protein that dimerizes with the transcription factors CCAAT enhancer-binding protein (C/EBP) and the full-length isoform of the C/EBPβ protein (LAP) and functions as a dominant-negative inhibitor of gene transcription (Rutkowski et al 2008).

5.4.2 Role of the PERK-eIF2α pathway in hepatic steatosis

The inhibition of the doublestranded RNA-activated protein kinase-like ER kinase (PERK)-eIF2α arm of the UPR pathway through Growth Arrest and DNA Damage-inducible 34 (GADD34), a eIF2α-specific phosphatase, causes low glycogen and susceptibility to fasting hypoglycemia in lean mice, and resistance to hepatic steatosis in animals fed a high-fat diet. This phenomenon is correlated with the reduced expression of the adipogenic nuclear receptor PPARgamma and C/EBPα and C/EBPβ (Oyadomari et al 2008). Challenging mice that express dominant-negative eIF2α (S51A) in the liver specifically with tunicamycin caused attenuated lipid accumulation in the liver (Oyadomari et al 2008). This attenuated lipid accumulation could be explained by the observed reduction in the expression of C/EBPα (Oyadomari et al 2008). On the other hand, the deletion of PERK resulted in the decreased expression of lipogenic enzymes and reduced lipid content in the mouse mammary gland (Bobrovnikova-Marjon et al 2008). The suppression of apolipoprotein secretion might explain this phenomenon. Indeed, another group

observed the attenuated translation of ApoB-100 via the PERK pathway in glucosamine treated HepG2 cells, leading to the suppressed secretion of ApoB-100 (Qiu et al 2009).

5.4.3 Role of the ATF6 pathway in hepatic steatosis

Activating transcription factor 6 (ATF6) seems to play pathological and protective roles in chronic and acute ER stress-induced hepatic steatosis respectively. Acute treatment of *Atf6* knockout mice with tunicamycin treatment causes hepatic steatosis, most likely due to the increase of SREBP1 target genes, reduced oxidation of fatty acids, and decreased secretion of ApoB-100 (Yamamoto et al 2010). Consistent with this, morphorlino knockdown of *Atf6* showed increased expression levels of ER stress markers in response to acute tunicamycin treatment and thus protected against hepatic steatosis in zebrafish embryos (Cinaroglu et al 2011). On the other hand, morphorlino depletion of *Atf6* displayed reduced expression levels of ER stress markers in response to chronic tunicamycin treatment and thus protect against hepatic steatosis in zebrafish embryos (Cinaroglu et al 2011). The pathological role of ATF6 is evidenced by the observation that overexpression of an active form of ATF6α stimulates fatty acid synthesis (Bommiasamy et al 2009).

5.4.4 Roles of some other players of the UPR pathway in hepatic steatosis

Despite each arm of the UPR functioning in hepatic lipid metabolism under physiologic and high-fat diet conditions, whether ER stress plays a direct role in hepatic lipid metabolism remains unknown (Rutkowski et al 2008). Studies on some critical players of the UPR pathway might provide useful insights into this question.

Importantly, the overexpression of BiP, the ER molecular chaperone upregulated during activation of the UPR, protects against the hepatic steatosis induced by insulin and ER stress in *ob/ob* mice (Kammoun et al 2009). This prevention, at least in part, works through the inhibition of SREBP-1c activation, reducing the expression of SREBP-1c target genes, and thus reducing the levels of triglyceride and cholesterol (Kammoun et al 2009). On the other hand, it was shown that the liver specific deletion of BiP resulted in ER stress, apoptosis, fat accumulation, sensitivity to alcohol, high-fat diet, and toxin-induced hepatic disorders (Ji et al 2011). In this study, it was shown that the molecular chaperone 4-phenylbutyrate can attenuate the fat accumulation that ensues after the above treatments (Ji et al 2011). CHOP, another important transcription factor activated by ER stress, was also consistently shown to function in the regulation of hepatic steatosis. CHOP knockout mice showed reduced apoptosis despite the appearance of hepatic steatosis upon ethanol feeding (Ji et al 2005).

Despite these studies, whether the activation of the UPR induced by ER stress plays a direct role in hepatic lipid accumulation is not completely understood and requires further investigation. Studies on the role of downstream effectors of the UPR, such as E3 ubiquitin ligases, on the regulation of hepatic protein homeostasis and lipid metabolism might provide useful insight.

5.4.5 Role of the ubiquitin-proteasome system in the regulation of key regulators of lipid metabolism

Several proteins involved in lipid metabolism, such as HMGCR, ApoB-100, INSIG1, and SREBPs, are highly regulated at different levels. The translation and

secretion of ApoB is highly regulated. Apolipoproteins are synthesized in both the rough and smooth ER (Glaumann et al 1975). Their secretion is largely regulated at the translational and posttranslational level rather than at the transcriptional level (Ginsberg et al 1995).

The mature form of SREBP can be regulated by the ubiquitin-proteasome system (Hirano et al 2001). Glycogen synthase kinase 3 (GSK-3) phosphorylates SREBP, leading to a conformational change in SREBP, and thus promotes the binding of SREBP to the SCFFbw7 (SKP1-cullin-1-F-box complex that contains FBW7 as the F-box protein) E3 ubiquitin ligase, which targets it for subsequent 26S proteasomal degradation (Sundqvist et al 2005., Punga et al 2006). This suggests a critical role for signal transduction by SREBP in cholesterol metabolism (Bengoechea-Alonso et al 2007).

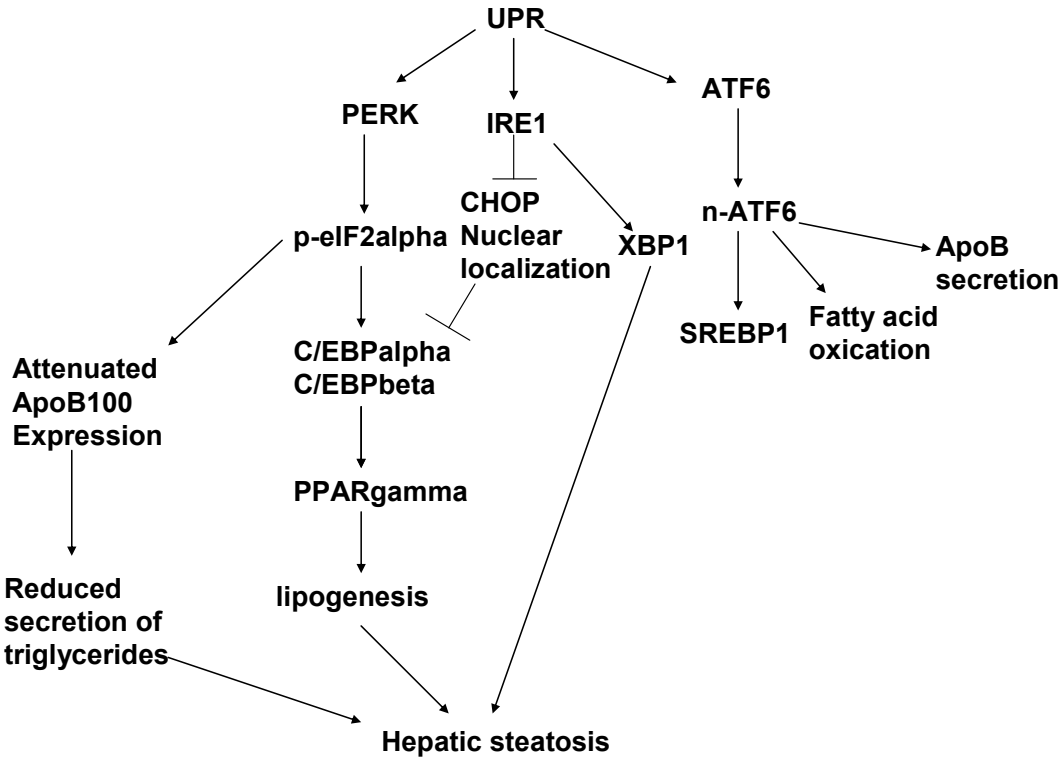

Fig. 4. Role of the ER stress-activated unfolded protein response (UPR) in hepatic steatosis.

PERK phosphorylates eIF2α, inhibits protein synthesis, including that of APOB100, and thus causes reduced secretion of triglycerides in the liver. The phosphorylation of eIF2α increases the expression of adipogenic nuclear PPARγ, C/EBPα and C/EBPβ. IRE-1 inhibits the nuclear localization of CHOP, which is believed to interfere with the transcription factors C/EBPα and C/EBPβ in the nucleus. C/EBPα and C/EBPβ induce lipogenesis through another transcription factor PPARγ. XBP1 might have a role in inducing lipogenesis that is independent of the UPR pathway. ATF6 induces hepatic steatosis probably though the increased expression of SREBP1 target genes, reduced oxidation of fatty acids, and decreased secretion of ApoB-100. ATF6 might also function to protect against hepatic steatosis induced by acute ER stress.

6. Zebrafish as a model for the study of hepatic ER stress and lipid metabolism

The zebrafish has become a popular model in biomedical research for studying lipid metabolism, fatty liver diseases, and tumorigenesis. Originally, the zebrafish was used as a model for liver development and recently it has been gaining popularity in the fields of cancer research, lipid metabolism in the liver, and lipid transport in the circulation system. For example, the zebrafish was proven to be an important and popular model in cancer research (Feitsma H & Cuppen E, 2008). Histological studies showed that zebrafish tumors resemble many types of human tumors. Moreover, several well-established tools, such as the high throughput screening of oncogenes, forward genetic screening of tumor suppressors, transplantation of human tumor cells, and the generation of transgenic fish that express human oncogenes, have rendered the zebrafish especially useful in oncology.

There are several advantages of using the zebrafish as a model system in lipid metabolism. First, the metabolic pathways of zebrafish, mice, and humans are relatively conserved (Hölttä-Vuori et al 2010).

Second, many relevant mutant zebrafish lines are available. These mutants might either display resistance to alcohol-induced hepatic steatosis or more severe fatty liver diseases, and will be useful tools for studying the mechanism of the development of hepatic steatosis. Moreover, chemical screening based on a defined genetic background was shown to be a promising approach for identifying chemical suppressors of defined genetic mutations (Kaufman et al 2009). Various readouts,

such as immunohistology, *in situ* hybridization, and other morphological analyses can be used to score phenotypes.

Third, the zebrafish is a good model for the high-throughput screening of drugs for obesity treatment. It has been shown that simple staining of fat in live transparent zebrafish embryos using Nile red containing media might be a promising tool for screening anti-obesity drugs (Jones et al 2008).

Fourth, the transparency of the zebrafish during early embryonic development makes it an excellent model for adipocyte research. One can use *in vivo* imaging analyses to study adipocyte development and the formation of adipose tissue and can directly visualize neutral lipid droplets with Nile red in the live animal in real time (Flynn et al 2009). In addition to Nile red, another useful dye is BCθ, a theta-toxin produced by *Clostridium perfringens*, which when conjugated with biotin and avidin-conjugated fluorescent dyes is more stable for fluorescent microscopy as compared with conventional filipin staining because it shows less photobleaching (Reid et al 2004). This could be especially useful for monitoring lipid metabolism in real time in the live animal. Another sensitive fluorescent cholesterol, BODIPY-cholesterol, was used to visualize sterol movement in living cells and organisms (Hölttä-Vuori et al 2008). Using this technique and others, another group showed that the zebrafish is a good model to detect plaques in live animals. Confocal microscopy can detect vascular lipid accumulation in adult zebrafish fed with a high-cholesterol diet supplemented with a fluorescent cholesteryl ester. Lipid accumulation was found to be accompanied by lipoprotein oxidation and macrophage lipid uptake, suggesting

that the zebrafish is a good model for studying the development of atherogenesis *in vivo* (Stoletov et al 2009).

Fifth, easy alcohol exposure makes the zebrafish a good model for studying alcoholic fatty livers. The exposure of zebrafish embryos to alcohol at early stages causes phenotypes that mimic fetal alcohol spectrum defects (FASD). Supplementation with cholesterol rescues the phenotypes caused by impaired hedgehog signal transduction (Li et al 2007). Another study showed that 45 h ethanol exposure causes developmental defects, such as delayed development, axial malformation, cyclopia, otolith defects, pericardial edema, yolk sac edema, and axial blistering in zebrafish and the internal ethanol concentration reached about 340 mM. Exposure to acetaldehyde, a toxic intermediate product of ethanol metabolism if not catalyzed, caused similar phenotypes (Reimers et al 2004).

Sixth, direct knockout models in zebrafish have become more and more feasible. Heritable targeted gene disruption using designed zinc-finger nucleases has been successfully accomplished (Doyon et al 2008). More recently, transcription activator-like (TAL) effector nucleases (TALENs), which contain a TAL effector DNA binding domain and a *Fok*I nuclease cleavage domain, were shown to be a more specific way of causing targeted gene modification compared to zinc finger nucleases (Sander et al 2011).

Chapter 2: Functional Characterization of gp78 during Early Embryonic Development in Zebrafish

1. Abstract

Mammalian gp78 is an E3 ubiquitin ligase that is anchored at the membrane of the ER. It regulates protein homeostasis by polyubiquitinating and targeting proteins for proteasomal degradation under both physiologic and stressed conditions. To test its role *in vivo*, we analyzed the gross embryonic morphology of zebrafish embryos in which gp78 was knocked down using morpholinos and of transgenic zebrafish overexpressing wild-type gp78 or dominant-negative gp78. We show that gp78 is highly conserved among vertebrates. Zebrafish gp78, like human gp78, can colocalize with mouse MmUBC7 in HeLa cells. *In vitro* ubiquitination assays confirmed that zebrafish gp78 indeed is an E3 ubiquitin ligase. Although gp78 was maternally and constitutively expressed during embryonic development, with relatively high expression levels in several tissues, such as the liver and brain, knockdown of gp78 or overexpression of wild-type or dominant-negative gp78 did not result in developmental defects, suggesting a compensation by other E3s during embryonic development.

2. Introduction

ER-associated protein degradation (ERAD) is critical for the maintenance of ER protein homeostasis. Proteins destined for the secretory pathway must be properly folded before transportation to their final, functional destinations. The quality of proteins is tightly monitored by the ER quality control (ERQC) system, which

achieves the proper folding of the majority of proteins using unique enzymes and molecular chaperones, and retains misfolded and unassembled proteins in the ER (Ellgaard et al 2003). Misfolded proteins are recognized, retrotranslocated, polyubiquitinated, and then targeted for degradation by the 26S proteasome in the cytosol; a complicated process termed ER-associated protein degradation (ERAD) (Vembar et al 2008).

ERAD plays a critical role in cell survival. It has been shown that the simultaneous loss of ERAD and UPR results in dramatic cell death (Travers et al 2000). Furthermore, the overexpression of parkin, an E3 ubiquitin ligase, protects against cell death induced by ER stress (Imai et al 2000). All this evidence indicates an important role for ERAD in cell survival.

Recently, we and several other groups showed that gp78, an E3 ubiquitin ligase, plays an important role in the regulation of physiological and pathological processes by targeting its substrates for proteasomal degradation (Chen, Du, and Fang 2011). gp78 was the first mammalian ER membrane-bound E3 ubiquitin ligase to be discovered that mediates the ubiquitination of unwanted proteins during ERAD (Fang et al 2001). It was originally isolated as a 78 kiloDalton (kDa) membrane glycoprotein from murine melanoma cells and as a tumor autocrine motility factor receptor, AMFR, mediating tumor invasion and metastasis (Nabi et al 1987, Silletti et al 1991, Nabi et al 1990). Recently, gp78 was reported to promote sarcoma metastasis and regulate cell proliferation by targeting a metastasis suppressor KAI1 for degradation (Tsai et al 2007, Joshi et al 2010). More importantly, the targeted disruption of *Hrd1*, a homolog of gp78 in mammalian cells, caused embryonic

lethality with aberrant hematopoiesis and increased apoptosis in the liver (Yagishita et al 2005). Consistent with this, the deletion of gp78 homolog, *Hrd1* in *Caenorhabditis elegans* affected growth rate (Sasagawa et al 2007). However, in yeast, unlike in mice, the deletion of *Hrd1p* did not result in lethality (Bordallo et al 1998). Importantly, deletion of gp78 ortholog, *Hrdl-1*, in *C. elegans*, did not affect growth rate (Sasagawa et al 2007). These led us to the question of whether gp78 is required for embryonic development. So far, there is no literature on gp78 knockout animal models. Thus, the *in vivo* function of gp78 remains largely unknown. Here we designed experiments to characterize gp78 in zebrafish and to determine its *in vivo* function during embryonic development. Surprisingly, the dysfunction of gp78 did not result in developmental defects. Our results indicate that other E3 ligases may compensate for the loss of gp78 in zebrafish.

3. Materials and Methods

1) Maintenance of zebrafish

Adult zebrafish were raised and maintained at the zebrafish facility of the Aquaculture Research Center, Institute of Marine and Environmental Technology as previously described (Li et al 2011). Briefly, the fish were maintained at 28 °C with 14 h of light and 10 h of dark, in 8 gallon aquaria supplied with freshwater and aeration.

2) Isolation of gp78 cDNA from zebrafish

Total RNA was extracted from 5 days post fertilization (dpf) zebrafish larvae using Trizol reagent (Invitrogen, CA, USA). A rapid amplification of cDNA ends (RACE) cDNA library was made from purified total RNA using a cDNA kit

(Fermentas, MD, USA). Zebrafish *gp78* cDNA was cloned from the 5' RACE library using a 5' GP78-P1 primer and a 3' GP78-P2 primer that has a C-terminal *myc* tag sequence followed by the stop codon. The PCR products were purified and cloned into the pGEM-T easy vector to generate the *pGEM-gp78* plasmid.

GP78-P1, 5'- ATGCCTCTGCTGTTTCTGGAGCG-3';

GP78-P2, 5'-CTACAGATCCTCTTCAGAGATGAGTTTCTGCTCGAATGG-GGAAGGCTCCTGCCTCA-3'.

3) Whole-mount in situ hybridization

The *gp78* antisense probe was synthesized using the *pGEM-gp78* plasmid as a template. *pGEM-gp78* was linearized with *Nco*I and transcribed with SP6 RNA polymerase. Whole-mount *in situ* hybridizations were performed as described (Du et al 2001).

4) Synthesis of morpholino-modified antisense oligos for translation blockers

The *gp78* translation blocker morpholino (gp78-5'-UTR-MO) was made on the basis of the antisense sequence of the 5'-untranslated region (UTR). The gp78-5'-UTR-MO (CAGTCCACACGTACAGCAGTCTTCT) was purchased from Gene Tools (Philomath, OR, USA) and used as described previously (Nasevicius & Ekker 2000).

The sequence of gp78-5'-UTR-MO, CAGTCCACACGTACAGCAGTCTTCT.

5) Construction of Tol2-ef1α:gp78-wt^{myc}, Tol2-ef1α:gp78-R2M^{myc}

To generate a DNA construct expressing myc-tagged gp78, the *gp78* coding sequence with the myc tag sequence was re-amplified using *Bam*HI-gp78-p1 and *Not*I-gp78-myc-p2. The amplified PCR product was directly cloned into pBSSK(+)

through blunt end ligation using the *Eco*RV cut sites in the vector. The insert was then released from the pBSSK-*gp78* plasmid by *Not*I and *B*amHI digestion. The DNA insert of *gp78* was then subcloned into the *Not*I and *Bam*HI sites of the T2AL200R150G vector (Urasaki et al 2006), which contains an *ef1α* promoter, to produce the plasmid *ef1α:gp78*^{myc}. gp78-R2M mutations were generated by using the QuickChange site-directed mutagenesis kit (Stratagene, CA, USA). All constructs were confirmed by sequencing.

BamHI-gp78-p1, GGATCCATGCCTCTGCTGTTTCTGGAGCGT

NotI-gp78-myc-p2, GCGGCCGCCTACAGATCCTCTTCAGAGATGAGT

gp78-Mu-P1, CCTGCGGAaACCTTTTCaACAATTCCTG

gp78-Mu-P2, CAGGAATTGTTGAAAAGGTTTCCGCAGG.

6) Generation of ef1α:gp78^{myc} and ef1α:gp78-R2M^{myc} transgenic fish

To generate transgenic fish, the constructs were co-injected with the *Tol2* transposase mRNA into zebrafish embryos at the 1-cell stage. Germ-line transgenic founders were screened by PCR by using DNA from 100 pooled F1 embryos at 24 hours post ferilization (hpf). Adult F1 transgenic fish were identified by PCR by using DNA from caudal fin as previously described (Tan et al 2006). The expression of exogenous *gp78* transcripts were detected by RT-PCR using primers IVS-E1E2-P1 and gp78-I-P3.

IVS-E1E2-P1 GATCCTGAGAACTTCAGGCTCCT
Gp78-I-P3 TGTGCATGACCTCCACTGAGAAACT

7) Morpholino and DNA microinjection in zebrafish embryos

Morpholino antisense oligos were dissolved in Danieau buffer (Nasevicius & Ekker 2000) to a final concentration of 0.5 mM. Zebrafish embryos were injected at

the 1- or 2- cell stage with 1–2 nL (5 or 10 ng) of morpholino (MO). 1–2 nL of DNA (100 ng/μL) was injected into zebrafish embryos at the 1- or 2- cell stage.

8) Reverse Transcription (RT)-PCR

Total RNA was isolated from zebrafish embryos at 0, 3, 6, 12, 14, 19, and 24 hpf and 2, 3, 4, 5, and 6 dpf with the Trizol reagent (Invitrogen). 1 μg of total RNA was used for the synthesis of cDNA using the first-strand cDNA synthesis kit (Fermentas). 1 μL of synthesized cDNA was used for PCR reactions. 12.5 μL of PCR reaction solutions were used in DNA agarose gel electrophoresis. Primers and PCR cycles were listed in **Table 3**.

9) Immunostaining of whole-mount fish embryos

Immunostaining was carried out using whole-mount zebrafish embryos as described previously (Tan et al 2006) with an anti-myc antibody (90E10α, A7811; Sigma, MO, USA). The secondary antibody was a biotinylated anti-mouse IgG (Vector Laboratories, CA, USA). Signal was detected using the avidin-biotin-peroxidase/diaminobezidine (ABC-DAB) assay (ABC, Vector Laboratories, Burlingame, CA). The embryos were photographed under an upright microscope (Leica MZ12, Heerbrugg, Switzerland) equipped with an Olympus DP70 Digital Microscope Camera (Olympus America Inc., NY, USA).

10) Cell culture and immunostaining

To produce a flag-tagged zebrafish gp78, the *gp78* coding sequence with flag tag sequence was re-amplified using Hind III-gp78-p1 and BamH I-gp78-p2 primers. The amplified PCR product was subcloned to pFLAG-CMV6a vector through *Hind*III *and* *Bam*HI cutting sites of the vector. Flag-tagged human gp78 and myc-tag mouse

MmUBC7 constructs were described previously (Chen et al 2006, Fang et al 2001). HeLa cells grown on slide glass were transiently co-transfected plasmids encoding flag-h-gp78 or flag-zf-gp78 with myc-MmUBC7. 24 h after transfection, cells were fixed in 4% paraformaldehyde for 30 min at 4 °C and blocked in 0.1 % saponin, 0.1 % human serum albumin. The cells were labeled with mouse monoclonal anti-FLAG antibody and rabbit monoclonal anti-myc antibody for 1 h following labeled with Alexa ® Fluor 488 conjugated goat anti-mouse IgG (H+L) and Alexa ® Fluor 594 conjugated with goat anti rabbit IgG (H+L) for 1 h. Fluorescence microscopy was performed using a Zeiss 510 Laser Scanning Confocal Microscope.

HindIII-gp78-P1	CCCGAAGCTTATGCCTCTGCTGTTTCTGGA
BamHI-gp78-p2	AGGCCGGATCTCAGAATGGGGAAGGCTCCT

11) Western blot analysis

Wild-type or MO-injected zebrafish embryos at 24 hpf (100 embryos each) and 48 hpf (50 embryos each) were dechorionated manually and crushed gently to remove the yolk by triturating with a glass pipette. Embryos at 96 hpf (30 embryos each), and 120 hpf (20 embryos each) were directly used for protein extraction. All embryos were solubilized in 200 µL sodium dodecyl sulfate (SDS) loading buffer (0.125 M Tris-Cl, pH 6.8; 4 % SDS; 20 % glycerol; 0.2 M dithiothreitol (DTT); 0.02 % bromophenol blue) containing phenylmethanesulfonyl fluoride ((PMSF), 1 mM) as a protease inhibitor. Samples (20 µL each) were vortexed, and the proteins were separated on a 10 % SDS-polyacrylamide gel electropheresis (PAGE) gel. Adult fish liver, brain, heart, pancreas, ovaries, and muscle were dissected from 3-month-old female wild-type fish and snap-frozen in liquid nitrogen and stored at -80°C.

Dissected organs were homogenized in buffer consisting of 20 mM HEPES-KOH (pH 7.4), 100 mM KCl, 2 mM EDTA, 1 mM PMSF, 1 mM dithiothreitol, 10 μg/mL leupeptin, 1 μg/mL pepstatin A, and 1 % Triton X-100. 20–40 μg of liver samples were used in each lane. Proteins were electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilion-P; Millipore, MA, USA). Immunodetections were carried out by incubating the membrane with primary antibodies recognizing gp78 (1F1; Yang et al 2010) and γ-tubulin (1:2000; Sigma) followed by incubation with peroxidase-conjugated secondary antibodies.

12) In vitro ubiquitination

pET28a-gp78C was described previously (Yang et al 2010). To generate a *pGEX-4T-gp78C* construct that encodes GST-tagged cytosolic part of zebrafish gp78, primers BamHI-gp78C-P1 and XhoI-gp78C-P2 were designed. Expression and purification of 6×histidine (his)-taggged gp78C and GST-tagged-zf-gp78C were performed as previously described (Yang et al 2010).

BamHI-gp78C-P1	AGGCGGATCCAGACATAAAAACTACCTGCGTGTC
XhoI-gp78C-P2	TCAGCTCGAGTCAGAATGGGGAAGGCTCCT

In vitro ubiquitination was performed as described (Li et al 2009). In brief, E1 (60 nM), Ube2g2 (200 nM), ubiquitin (10 μM) were incubated with gp78C (2 μg) in a 20 μl reaction system at 37 °C in buffers containing 25 mM Tris/HCl (pH 7.4), 2 mM ATP, and 2 mM $MgCl_2$. 2 μl of the reaction mix were sampled at indicated time and subjected to treatment with the same volume of 2×SDS sample buffern at 95 °C for 5 min. Ubiquitin (Ub) chains were detected by immunostaining with anti-ub monoclonal antibodies.

4. Results

1) Isolation and characterization of zebrafish gp78

Full-length *gp78* cDNA was isolated from zebrafish 5 dpf larvae by RT-PCR. The PCR product encodes a protein of 620 amino acids that shares high sequence identity with gp78 from frog, mouse, and humans, suggesting that it is a gp78 ortholog (**Fig. 2**). Zebrafish gp78 is predicted to have 5 N-terminal transmembrane domains and 5 other known domains in the cytosolic-tail (**Fig. 5)**. Sequence comparison shows that all these domains are highly conserved among zebrafish, frog, mouse, and human (**Fig. 2**). Amino acid sequence comparison reveals that zebrafish and human gp78 have 94 %, 97 %, 81 %, 50 %, and 48 % identity for the RING, oligomerization site (OS), Cue, G2BR, and p97 binding domains (also named as VIM), respectively (**Fig. 5**).

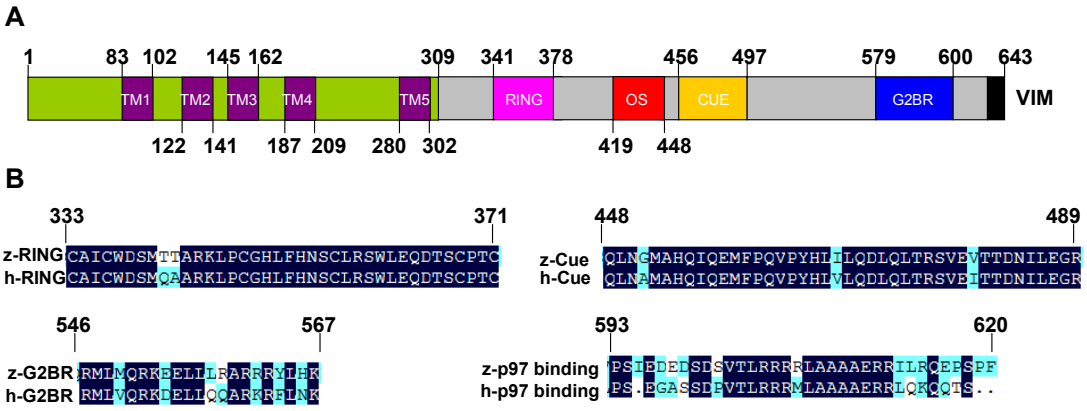

Fig. 5. Schematic of the domains of zebrafish gp78, and sequence alignment of gp78 between zebrafish and humans.

The zebrafish gp78 contains 5 transmembrane domains (TMs) and 5 conserved domains in the cytosolic tail. The conserved cytosolic domains, really interesting new gene (RING) finger, oligomerization site (OS), coupling of ubiquitin to ER degradation (Cue) domain, Ube2g2-binding region (G2BR) and a p97/VCP-interacting motif (VIM), are compared between zebrafish and human gp78. The TMs were predicted using TMHMM-2.0. Multiple sequence alignment was performed using DNAMAN.

2) E3 ubiqutin ligase activity of zebrafish gp78

Since zebrafish contains all the conserved domains of mammalian gp78, we determined whether zebrafish gp78 is an E3 ubiquitin ligase. We assayed for ER colocalization by immunostaining and by *in vitro* polyubiquitination assays using recombinant proteins. The results showed that zebrafish gp78, like human gp78, colocalized with mouse MmUBC7 in HeLa cells (**Fig. 6 A-B**). To test whether zebrafish gp78 has an E3 ubiquitin ligase activity, we expressed and purified glutathione (GST)-tagged zebrafish gp78 cytosolic part (zf-gp78C-GST) and 6xhis-tagged human gp78 cytosolic part (h-gp78C-his) (**Fig. 6 C**). *In vitro* ubiquitination assays using purified recombinant zf-gp78C-GST and h-gp78C-his confirmed that zebrafish gp78 is indeed an E3 ubiquitin ligase that can promote the formation of polyubiquitin chains (**Fig. 6 D**).

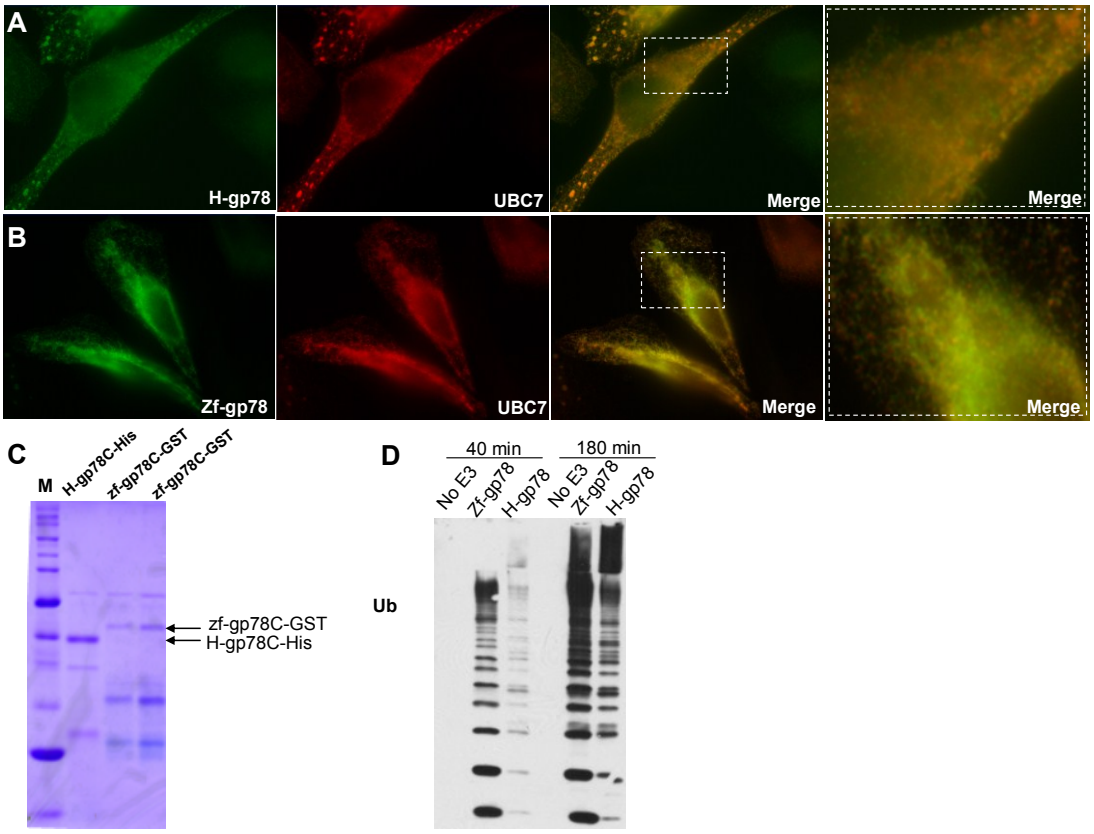

Fig. 6. ER localization and E3 ubiquitin ligase activity of zebrafish gp78.

(A, B) Flag-tagged zebrafish gp78 (flag-zf-gp78) and flag-tagged human gp78 (flag-h-gp78) colocalize with myc-tagged mouse MmUBC7 by immunostaining using anti-flag or anti-myc antibodies in HeLa cells. **(C)** Recombinant His-tagged human gp78 (H-gp78C-his) and GST-tagged zebrafish gp78 (zf-gp78C-GST) were expressed and purified from *E. coli*. **(D)** In vitro ubiquitination showed that recombinant His-tagged human gp78 (H-gp78C-his) and GST-tagged zebrafish gp78 (zf-gp78C-GST) can promote the formation of polyubiquitin chains.

3) Expression patterns of zebrafish gp78

The temporal expression of *gp78* was determined in zebrafish embryos by RT-PCR (**Fig. 7 A**). *gp78* transcripts were detected in fertilized eggs, suggesting that it was expressed maternally. The expression of *gp78* remained constitutive at all embryonic and larval stages analyzed, from fertilization up to 6 dpf.

To determine the spatial patterns of expression, we analyzed *gp78* expression in zebrafish embryos by whole-mount *in situ* hybridization (**Fig. 7 B–E**). A 1125 base pair (bp) antisense probe complementary to the 3' end of the *gp78* RNA transcript was generated for *in situ* hybridization. *In situ* hybridization using the probe revealed expression in several tissues including the brain, eyes, liver, gut, and pancreas of zebrafish embryos at 4 dpf and 5 dpf (**Fig. 7 B–E**). Western blot analysis showed expression of gp78 in several of the tissues tested and a relatively high expression in the brain, liver, and ovary (**Fig. 7 F**). Similar expression patterns were found in tissues of adult mice by Western blotting (**Fig. 7 G**).

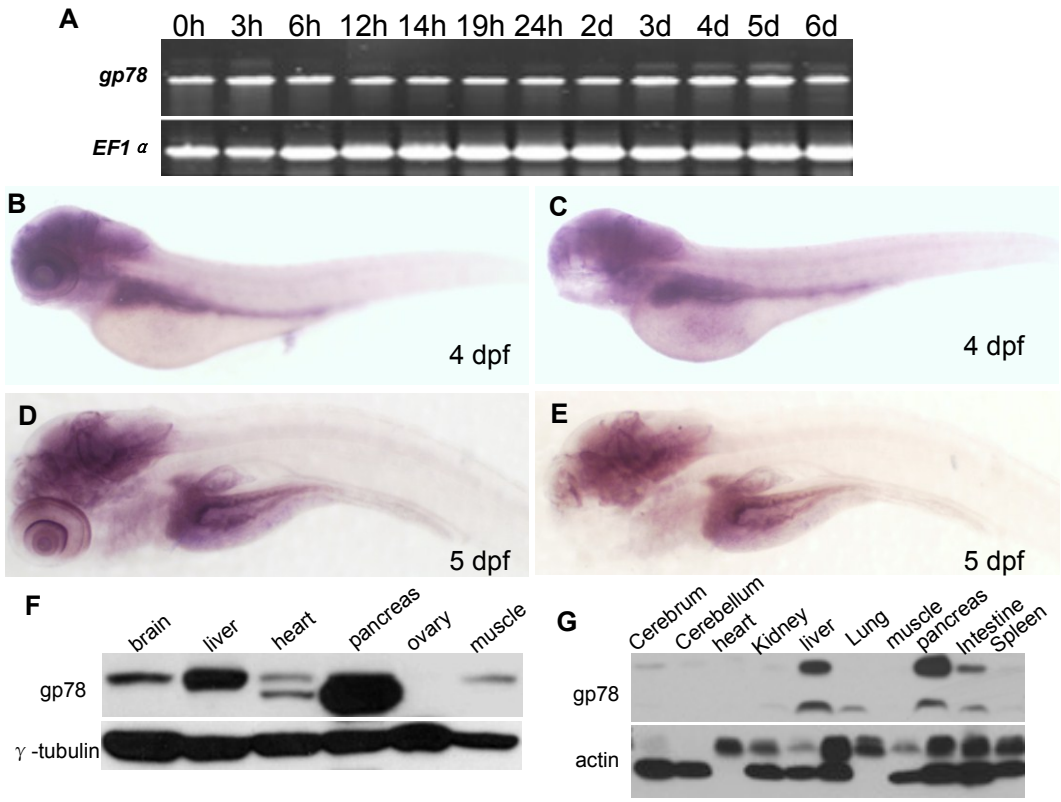

Fig. 7. Temporal and spatial expression patterns of gp78 in larval and adult zebrafish.

(A) The *gp78* transcript in zebrafish embryos was detected by RT-PCR to be expressed maternally and zygotically during the 6 day development period. **(B-E)** The spatial expression pattern of *gp78* was analyzed by *in situ* hybridization using a dig-labeled *gp78* antisense probe. 4 dpf (*B* with eyes, *C* with eyes removed) and 5 dpf (*D* with eyes, *E* with eyes removed) embryos were analyzed. **(F)** The tissue expression pattern of gp78 in adult zebrafish was analyzed by Western blot using the anti-gp78 monoclonal antibody 1F1. **(G)** A similar tissue expression pattern of gp78 in adult mice was also confirmed by Western blot.

4) Knockdown of gp78 expression did not result in developmental defects

To test whether or not gp78 functions in early development, we performed a knockdown analysis of gp78 in zebrafish embryos. A 5'-UTR translational blocker was designed to target the 5'-UTR region of the transcript. 5–10 ng morpholino was injected into zebrafish embryos at 1- to 2-cell stage and the effect on gp78 was analyzed at different stages by Western blotting (**Fig. 8 A-B**). As shown in Fig. 8A, injection of the 5'-UTR morpholino dramatically knocked down gp78 at 24 and 48 hpf (**Fig. 8 A**). To determine the efficacy of this morpholino, the expression levels were analyzed in wild-type and morphant embryos (embryos injected with 5'-UTR morpholino at the 1- or 2-cell stage) at 96 hpf and 120 hpf (**Fig. 8 B**). Compared with wild-type embryos, the gp78 expression levels were significantly reduced in the morphant embryos at 96 hpf and 120 hpf (**Fig. 8 B**), indicating that the morpholino effectively knocks down the expression of gp78 in the cells of zebrafish embryos. To determine whether or not the knockdown of gp78 caused developmental defects, the morphant embryos were examined morphologically every day for 7 days after the injection. The morphant embryos were morphologically normal at 48 and 72 hpf (**Fig. 8 C-D**) and at the early developmental stages up to 7 dpf (data not shown). Moreover, we did not observe any developmental defects in the liver in wild-type fish embryos at 72 hpf (**Fig. 8 D**). To further investigate whether the knockdown of gp78 affects liver development, a transgenic fish line (*hfe2:gfp* also named as RGM-GFP) expressing green fluorescent protein (GFP) specifically in the liver was used for the knockdown experiments to assay the effect of the knockdown on liver development. The morphant embryos showed normal liver morphology at 96 and 125 hpf (**Fig. 8 E-**

F) and at the early developmental stages up to 7 dpf (data not shown). The morphant embryos could be raised without any defects. These data suggest that gp78 might not be essential for the normal development of zebrafish embryos.

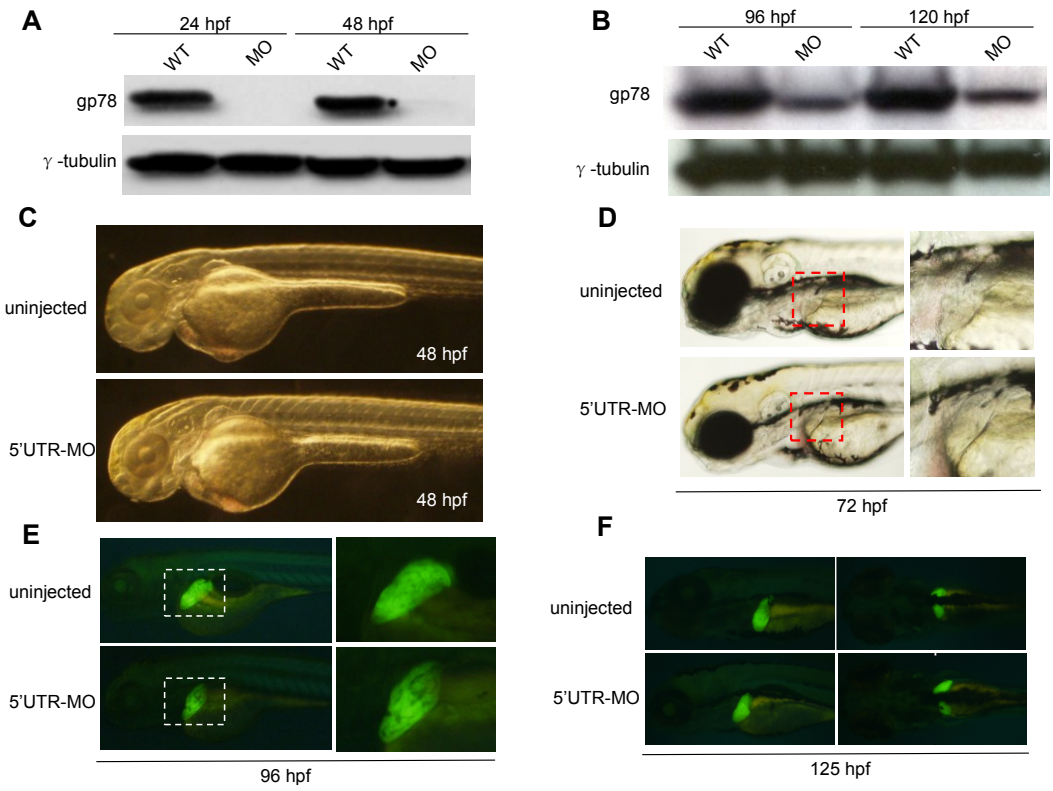

Fig. 8. Normal development of gp78-knockdown embryos.

(A) Western blotting showed a dramatic reduction in the protein levels of gp78 in morpholino (MO)-knockdown embryos at 24 hpf and 48 hpf. **(B)** The gp78 protein levels in 96 hpf and 120 hpf larvae were also analyzed and were significantly reduced by 5'-UTR-MO. **(C, D)** The morphant embryos were morphologically normal at 48 and 72 hpf and normal liver morphology was seen in the morphant embryos at 72 hpf. **(E, F)** A transgenic fish line (*hfe2:gfp*, also called RGM-GFP) expressing GFP specifically in the liver was injected with the 5'-UTR-MO and the morphant embryos showed normal liver morphology at 96 and 125 hpf.

5) Overexpression of gp78-wt or gp78-R2M did not result in developmental defects

To further test whether gp78 plays a role in early embryonic development, we generated transgenic fish lines that ubiquitously expressed a myc-tagged wild-type *gp78 (gp78-wt^{myc})* or dominant-negative *gp78* (*gp78-R2M^{myc}*) minigene driven by the *ef1α* promoter. As shown, 2 histidine residues, H354 and H357 in the RING finger domain, critical for the E3 ubiquitin ligase activity of gp78, were mutated to asparagine residues (N354 and N357) in the *Tol2-ef1α:gp78-R2M^{myc}* construct (**Fig. 9 A**). Transient expression of the *gp78-wt^{myc}* minigene was detected in 24 hpf wild-type embryos injected with the *Tol2-ef1α:gp78-wt ^{myc}* construct (**Fig. 9 B**). Expression of the *gp78-wt^{myc}* minigene was detected in the 24 hpf embryos of 2 *ef1α:gp78-wt ^{myc}* transgenic lines by RT-PCR (**Fig. 9 C**). Overexpression of total gp78 (the expression of endogenous gp78 and that of the ectopic *gp78-wt^{myc}* minigene) in these 2 lines was confirmed by Western blot using the monoclonal antibody 1F1 as previously described (Yang et al 2010) (**Fig. 9 D**). Overexpression of total gp78 (the expression of endogenous gp78 and that of the ectopic *gp78-R2M^{myc}* minigene) was also observed in 2 *ef1α:gp78-R2M^{myc}* transgenic lines (lines #11 and #12) (**Fig. 9 E**). Together we successfully showed genetic manipulation of gp78 expression in our study. Consistent with morpholino knockdown analysis, overexpression of *gp78-wt^{myc}* or *gp78-R2M^{myc}* did not cause morphological defects during development (data not shown).

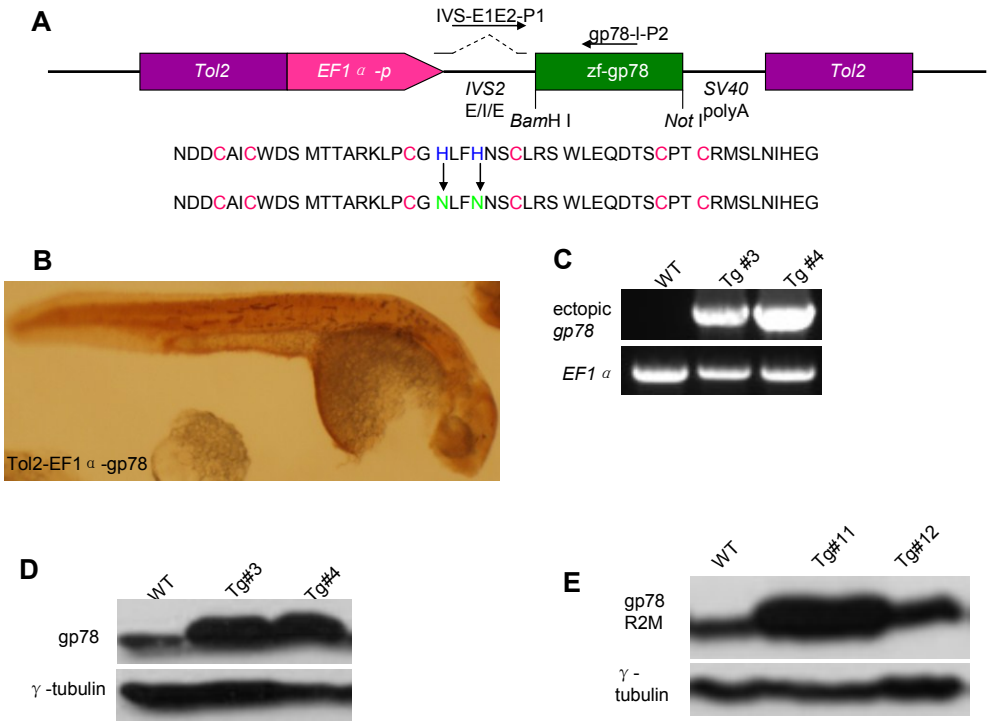

Fig. 9. Overexpression of gp78-wt and gp78-R2M in transgenic lines.

(A) Schematic showing the *Tol2-ef1α:gp78-wt*^{myc} and *Tol2-ef1α:gp78-R2M*^{myc} constructs. 2 critical histidine residues H354 and H357 in the RING finger domain were mutated to asparagine residues (N354 and N357) to generate the R2M mutant. **(B)** The *Tol2-ef1α:gp78-wt*^{myc} construct was injected into embryos at the 1- or 2-cell stage and the expression of gp78-myc was detected by whole mount anti-myc tag antibody staining. **(C)** Expression of the *gp78-wt-myc* minigene was detected in 24-hpf embryos of 2 *ef1α:gp78-wt*^{myc} transgenic lines by RT-PCR. **(D)** Overexpression of gp78 in these 2 lines was confirmed in the embryos at 24 hpf by Western blot using the monoclonal antibody 1F1. **(E)** Overexpression of gp78-R2M in the 2 lines was also confirmed in the embryos at 24 hpf by Western blot using the monoclonal antibody 1F1.

5. Discussion

In this study, we demonstrated that gp78 does not play a major role in embryonic development. Neither the knockdown of gp78 at early stages of development nor the overexpression of wild-type or dominant-negative gp78 resulted in developmental defects. To our knowledge, this is the first demonstration of a lack of a role for gp78 in embryonic development in animals. Despite the fact that *gp78* is maternally and continuously expressed during early embryonic development and *Hrd1* (SYVN1) homozygous knockout mice are embryonic lethal due to severe anemia probably caused by enhanced apoptosis of embryonic erythrocytes in the liver (Yagishita et al 2005), why the genetic manipulation of *gp78* does not disrupt the normal development of zebrafish remains unclear.

Interestingly, XBP1 homozygous knockout mice are also embryonic lethal due to defects in liver development and plasma cell differentiation (Reimold et al 2000). It is possible that the XBP1 transcription factor plays a critical role in liver development and plasma cell differentiation while one of its UPR target genes, Hrd1, controls apoptosis of embryonic erythrocytes in the liver, and gp78 has no role in these processes. Given that Hrd1 and gp78 are homologs, it is still possible that Hrd1 might regulate some exclusive substrates that are critical for controlling apoptosis in embryonic erythrocytes in the liver, which might explain the lack of a phenotype in gp78-knockdown embryos. Substrates both common and distinct to gp78 and Hrd1 have been identified (Ballar et al 2010). Alternatively, abnormal phenotypes in gp78 knockdown embryos might become evident under certain environmental conditions,

e.g. induction of ER stress by alcohol treatment. In terms of embryonic ER stress, we showed high expression of spliced *xbp-1* in eggs dissected from female adult zebrafish, indicating strong ER stress in the unfertilized egg (Chen, Z, Fang, S, and Du, S, unpublished data). It is possible that the maternal expression of gp78 may provide an advantage in facilitating the degradation of misfolded proteins in unfertilized eggs. However, although we do not observe developmental defects, we cannot rule out the possibility that other abnormal phenotypes are present but yet to be discovered.

Table 3. List of primers, their sequences and PCR cycles.
The primer names refer to the gene which they were designed against and restriction site if added.

Primer Name	Sequence (5' to 3')	Semi-quantitative RT-PCR Cycles
GP78-P1	ATGCCTCTGCTGTTTCTGGAGCG	
GP78-P2	CTACAGATCCTCTTCAGAGATGAGTTT CTGCTCGAATGGGGAAGGCTCCTGCCTCA	
BamHI-GP78-p1	GGATCCATGCCTCTGCTGTTTCTGGAGCGT	
NotI-GP78-myc-p2	GCGGCCGCCTACAGATCCTCTTCAGAGATGAGT	
GP78-Mu-P1	CCTGCGGAAACCTTTTCAACAATTCCTG	
GP78-Mu-P2	CAGGAATTGTTGAAAAGGTTTCCGCAGG	
IVS-E1E2-P1	GATCCTGAGAACTTCAGGCTCCT	
Gp78-I-P3	TGTGCATGACCTCCACTGAGAAACT	
HindIII-gp78-P1	CCCGAAGCTTATGCCTCTGCTGTTTCTGGA	
BamHI-gp78-p2	AGGCCGGATCTCAGAATGGGGAAGGCTCCT	
BamHI-gp78C-P1	AGGCGGATCCAGACATAAAAACTACCTGCGTGTC	
XhoI-gp78C-P2	TCAGCTCGAGTCAGAATGGGGAAGGCTCCT	
RGM-p-XhoI-P1	CTCGAGTCCTGGAGGCCAAATATAGACAAGCA	
RGM-p-XhoI-P2	CTCGAGCCATACAGACACAGGCAGGACGGCCT	
AMFR-P3	GGAGGTGGTGCTGTGGTGTC	30
AMFR-P4	ATGACCTCCACTGAGAAACT	30
Bip-p1	ATGCGGTTGCTTTGCCTGTTTTTGCTG	27
Bip-p2	CTACAGCTCGTCCTTCTCTTCGGCCTCTTCA	27
Chop-P1	AGTTGGAGGCGTGGTATGA	30
Chop-P2	AGATCTCCGGATGAGGTGTT	30
Derlin1-P1	GGTTTGCTGGCTCCATTGCT	30
Derlin1-P2	GGCATGGGTCTCCTGCTTGG	30
VCP-P1	TCGTCAGGCTGCTCCTTGTG	30
VCP-P2	TCCTTGGTTACTGGATGGGAAT	30
Xbp1-P1	GCAGGAGATCAGACTCAGAGTCTG	30
Xbp1-P2	CGAGACAAGACGAGTGATCTGCT	30
EF1α-P1	GCATACATCAAGAAGATCGGC	18
EF1α-P2	GCAGCCTTCTGTGCAGACTTTG	18
Acc1-P1	CAACAACTACGCTAATGTGGAACT	30

Acc1-P2	GCTGCTGCCATCATACGAGA	30
Fasn-P1	GAGAAAGCTTGCCAAACAGG	23
Primer Name	Sequence (5’ to 3’)	Semi-quantitative RT-PCR Cycles
Fasn-P2	GAGGGTCTTGCAGGAGACAG	23
Fads2-P1	GCAGACAGACCGAATCACCG	22
Fads2-P2	CGCAAATGCTCCGTACAAGG	22
Hmgcs1-P1	GGTCGTTACGCTCTGGTTGT	30
Hmgcs1-P2	GATACGGGGCATCTTCTTGA	30
Hmgcra-P1	CTGAGGCTCTGGTGGACGTG	25
Hmgcra-P2	ATCGGTTGCGGTCTGAAAAT	25
Hmgcrb-p P1	GCCTGTTAGCCGTCAGTGGA	25
Hmgcrb- P2	TCGTGTCGTCGCTGCCTTGT	25
Srebp1-P1	GTAGCATCGCCCTGCATTACAACA	27
Srebp1-P2	CCAGCGGGTTAAAGGACAGAAACA	27
Srebp2-P1	AACGCTACCGCTCCTCCATCAA	27
Srebp2-P2	CTCGTGCCTCCCTCCAACCA	27
Cyp2e1-P1	GGTGGACCAGGCTGACGACT	27
Cyp2e1-P2	CGATCTATCTCCCTCTGCATTT	27

Chapter 3: Functional Characterization of the Role of gp78 in Hepatic ER Stress and Lipid Metabolism in Zebrafish

1. Abstract

The enhancement of ERAD activity by the UPR represents one of the mechanisms used to restore ER homeostasis. However, the significance of gp78 in the regulation of hepatic ER stress *in vivo* remains elusive. Here we report that zebrafish gp78 plays a key role in the regulation of hepatic ER stress under tunicamycin-induced stress conditions but not under physiologic conditions. Tunicamycin treatment induces ER stress and upregulates the expression of several key components of the gp78-mediated ERAD complex in the liver. Moreover, hepatic specific overexpression of the dominant-negative form of gp78 (gp78-R2M) renders the liver more sensitive to ER stress induced by tunicamycin, suggesting a role for gp78-mediated ERAD in the regulation of hepatic protein homeostasis. Furthermore, the overexpression of gp78-R2M enhanced the expression of Srebp target genes in response to ER stress, while this was not observed in fish overexpressing wild-type gp78 (gp78-wt). Together, these data indicate that gp78 plays a critical role in the regulation of hepatic ER stress and lipid metabolism.

2. Introduction

The transcriptional upregulation of ERAD components represents one of the mechanisms used to maintain ER protein homeostasis. Under several conditions, the inefficient clearance of misfolded proteins leads to their accumulation in the ER lumen, a condition termed ER stress, which results in the activation of the UPR, a

signaling transduction pathway that restores ER protein homeostasis (Schröder, et al 2005). The UPR has been shown to exist in organisms from yeast to humans. Under stress conditions, the dissociation of BiP from 3 ER transmembrane proteins called IRE1α, PRKR-like endoplasmic reticulum kinase (PERK), and ATF6, causes a change in their conformation and activates the UPR pathway (Schröder M & Kaufman RJ 2005).

These 3 transmembrane proteins serve as sensors that transmit information about the protein folding status in the ER lumen to the cytosol, where active forms of transcription factors are generated via distinct mechanisms. The active forms of transcription factors then go into the nucleus to activate the expression of their target genes, including chaperones and components of the ERAD pathway, leading to the maintenance of ER protein homeostasis (Ye 2005, Lee 2005).

The activation of the UPR by ER stress has been shown to be one of the causes of hepatic steatosis and fatty liver disease (Basseri & Austin 2008). All 3 branches of the UPR play roles in hepatic steatosis (Rutkowski et al 2008). Interestingly, the overexpression of BiP, a heat-shock protein 70 homolog that represents a type of target gene of the UPR pathway, has been shown to protect against insulin and ER stress-induced hepatic steatosis by reducing the activation of SREBP-1c in mice (Kammoun et al 2009). gp78, an E3 ubiquitin ligase that represents the other type of target gene of the UPR pathway, may play a role in the protection against hepatic steatosis and fatty liver disease.

In addition, recent studies have demonstrated that gp78 might play a role in ER stress. We have previously reported that acute ER stress enhances the activity of

gp78-mediated ERAD by suppressing autoubiquitination of gp78 and leading to its stabilization (Shen et al 2007). In addition, gp78 counteracts ER stress induced by mutant superoxide dismutase 1 (SOD1) and ataxin-3 by repressing their aggregation and enhancing their degradation (Ying et al 2009). In contrast, compromising the function of gp78 in ERAD with mutant huntingtin via interaction with its CUE domain results in the inhibition of the interaction between gp78 and polyubiquitinated proteins and p97/VCP, thus triggering ER stress in cultured cells (Yang et al 2010). Similarly, cells with reduced levels of gp78 show increased sensitivity to cell death induced by ER stress (Tsai et al 2007). Besides, it has been reported that autocrine motility factor (AMF), the ligand of gp78, protects against tunicamycin-induced ER stress and this protection is gp78-dependent in HEK293 cells (Fu et al 2011).

Moreover, it has become clear that gp78 is involved in targeting several hepatic proteins for degradation through the ERAD pathway according to studies using cultured cells. These substrates include HMG CoA reductase, a rate-limiting enzyme in cholesterol biosynthesis in the liver, and Apo-B100, a protein mainly synthesized in the liver according to *in vitro* studies (Song et al 2005, Liang et al 2003). gp78 also increases the solubility of and facilitates the degradation of the liver disease asscociated Z variant α(1)-antitrypsin protein, ATZ, in cultured cells (Shen et al 2006). gp78 has been reported to be involved in the proteasomal degradation of CYP3A, a dominant liver cytochrome P450 enzyme responsible for drug metabolism, and liver CYP2E1, which is responsible for the biotransformation of clinically relevant drugs, low molecular weight xenobiotics, carcinogens and endogenous ketones, in cultured rat hepatocytes (Kim et al 2010, Wang et al 2011). The hepatic

function of gp78 *in vivo* is further demonstrated by its high level of expression in the liver as compared with other organs (Ballar, P and Fang, S, unpublished data). So far, no literature on *gp78*-knockout mice has been published. Thus, the significance of gp78 in the regulation of hepatic ER stress under physiologic or stress conditions *in vivo* remains unknown.

The zebrafish has been shown to be an ideal system for studying hepatic ER stress, lipid metabolism, hepatic steatosis, and alcoholic or non-alcoholic fatty liver disease for the following reasons: facility of exposing to chemicals, the sensitivity of its liver to environmental or genetic perturbation, the lack of effect of external nutrients on its liver functions during early developmental stages, easy genetic manipulation, such as the generation of transgenic models or transient morpholino knockdown of gene expression, relatively low cost, a large number of offspring, external development of embryos, functional conservation of metabolic pathways with mammals, transparency, and quick generation time (Lieschke & Currie 2007, Nasevicius & Ekker 2000, Passeri et al 2009, Hölttä-Vuori et al 2010). Using transgenic fish that overexpress the dominant-negative form of gp78 in the liver and using morpholino knockdown of gp78, we report here that the gp78-mediated ERAD pathway plays a key role in the regulation of hepatic ER stress and lipid metabolism. These findings not only provide evidence of links between the UPR and lipid metabolism but also hold therapeutic potential for liver steatosis and alcoholic or non-alcoholic fatty liver diseases.

3. Materials and Methods

1) Maintenance of zebrafish and exposure of adult fish to tunicamycin

Adult zebrafish were raised and maintained as described in Chapter 2 until treatment with either tunicamycin. 2 to 3 month-old fish were exposed to 1 ug/mL tunicamycin dissolved in dimethyl sulfoxide (DMSO), in 50 mL fresh fish water for the indicated time described in the result section at 28 °C during the day.

2) Whole-mount in situ hybridization

Probes were generated by PCR amplification from a cDNA library generated from 5 dpf larvae. All primers are listed in the **Table 3**. Each PCR product was cloned into pGEMT-easy (Promega) and was sequenced. Followed by linearization with restriction enzymes, the constructs were transcribed with either SP6 or T7 RNA polymerase using a digoxigenin RNA labeling mix (Roche). In detail, the *gp78* antisense probe was synthesized using the *pGEM-gp78* plasmid as a template. *pGEM-gp78* was linearized with *Nco*I and transcribed with SP6 RNA polymerase. The *bip* antisense probe was synthesized using the *pGEM-bip* plasmid as a template. The *pGEM-bip* plasmid was linearized with *Eco*RV and transcribed with SP6 RNA polymerase. The *chop* antisense probe was synthesized using the *pGEM-chop* plasmid as a template. The *pGEM-chop* plasmid was linearized with *Sac* II and transcribed with SP6 RNA polymerase. The *p97* antisense probe was synthesized using the *pGEM-p97* plasmid as a template. The *pGEM-p97* was linearized with *Sal* I and transcribed with T7 RNA polymerase. The *derlin1* antisense probe was synthesized using the *pGEM-derlin1* plasmid as a template. The *pGEM-derlin1* plasmid was linearized with *Sac* II and transcribed with SP6 RNA polymerase. The antisense probe for *fabp10a* was generated as described previously (Bian et al 2011). Whole-mount *in situ* hybridizations were performed as described (Du et al 2001).

Bip-p1	ATGCGGTTGCTTTGCCTGTTTTTGCTG
Bip-p2	CTACAGCTCGTCCTTCTCTTCGGCCTCTTCA
Chop-P1	AGTTGGAGGCGTGGTATGA
Chop-P2	AGATCTCCGGATGAGGTGTT
Derlin1-P1	GGTTTGCTGGCTCCATTGCT
Derlin1-P2	GGCATGGGTCTCCTGCTTGG
P97/VCP-P1	TCGTCAGGCTGCTCCTTGTG
VCP/VCP-P2	TCCTTGGTTACTGGATGGGAAT

3) Semi-quantitative RT-PCR

Total RNA was isolated from dissected livers with the Trizol reagent (Invitrogen, CA, USA). 1 ug of total RNA was used for the synthesis of cDNA using the first-strand cDNA synthesis kit (Fermentas). The synthesized cDNA was diluted 10-fold and 1 ul of diluted cDNA was used for PCR reactions. All 12.5 ul of PCR reaction solutions were used in DNA agarose gel electrophoresis. Primers and PCR cycles are listed in **Table 3**. The band intensities were quantified with free NIH Image J software.

4) Generation of RGM:gp78-wt^{myc}, RGM:gp78-R2M^{myc} constructs and transgenic fish

To generate DNA constructs expressing a myc-tagged gp78 under a liver specific promoter, the *ef1α* promoter in *Tol2-eflα:gp78-wt ^{myc}* and *Tol2-eflα:gp78-R2M ^{myc}* constructs were replaced with a liver specific promoter, RGM promoter (Bian et al 2011) to produce the plasmids *Tol2-RGM:gp78-wt^{myc}* and *Tol2-RGM:gp78-R2M^{myc}*. In brief, a 6-kbp RGM promoter was amplified with primers RGM-p-XhoI-p1 and RGM-p-XhoI-p2 and then subcloned to pGEMT-easy vector to generate pGEMT-RGMp construct. *Xho*I-digested RGM-promoter was released from pGEMT-RGMp and subcloned to *Xho*I digested *Tol2-eflα:gp78-wt^{myc}* to produce a plasmid *Tol2-RGM:gp78-wt^{myc}*. *Tol2-RGM:gp78-R2M^{myc}* construct was generated by

site-directed mutagenesis using primers gp78-Mu-P1 and gp78-Mu-P2. The generation of transgenic fish and detection of exogenous transcript were performed as described in Chapter 2.

RGM-p-XhoI-p1	CTCGAGTCCTGGAGGCCAAATATAGACAAGCA
RGM-p-XhoI-p2	CTCGAGCCATACAGACACAGGCAGGACGGCCT

5) Immunostaining of whole-mount fish embryos

Immunostaining was carried out using whole-mount zebrafish embryos as described in Chapter 2.

6) Western blot analysis

Adult fish livers were dissected from female wild type (WT) or transgenic fish at 2 to 3 months of age and snap-frozen in liquid nitrogen and stored at -80 °C. Dissected livers were homogenized in buffer consisting of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES-KOH (pH 7.4), 100 mM KCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μg/mL leupeptin, 1 μg /mL pepstatin A and 1 % Triton X-100. 20-40 μg of liver samples were used each lane. Proteins were electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilion-P; Millipore). Immunodetections were carried out by incubating with primary antibodies recognizing gp78 (1F1; Hui Yang et al 2010), Bip (1:3000; sigma), and γ-tubulin (1:2000; Sigma) followed by incubation with peroxidase-conjugated secondary antibodies. The band intensities were quantified with NIH Image J software.

4. Results

1) Tunicamycin treatment induces hepatic ER stress in larval and adult zebrafish

Molecular chaperones and components of ERAD are 2 major types of UPR target genes. However, whether or not components of gp78-mediated ERAD are upregulated by the UPR remains undetermined. To establish a model for studying ER stress, we exposed fish embryos to 1 μg/mL of tunicamycin, a protein N-glycosylation inhibitor and a well-known ER stress inducing agent, starting at 3–3.5 dpf as previously described (Passeri et al 2009). After 48 h treatment, the fish larvae were sampled and subjected to whole mount *in situ* hybridization for the ER stress markers *bip* and *chop*. Interestingly, the results showed elevated expression of *bip* and *chop* mainly in the liver of fish larvae treated with tunicamycin (**Fig. 10 A-B**). No change in expression was observed using a liver specific probe *Fabp10a* (**Fig. 10 F**). Next, we examined whether tunicamycin could induce ER stress in the livers of adult zebrafish. RT-PCR results showed that adult fish exposed to 2.5 μg/mL of tunicamycin for the indicated periods of time displayed elevated levels of *bip*, *chop*, and *xbp1*-s at the transcript level (**Fig. 11 A**). Consistently, a marked increase of *bip* was observed at the protein level (**Fig. 11 B**). All together, these data suggest that tunicamycin induces ER stress in the liver of larval and adult zebrafish.

2) Tunicamycin treatment upregulates components of the gp78 complex in the liver

To determine whether or not the activation of UPR by ER stress upregulates components of the gp78 complex in our model system, fish embryos similarly treated tunicamycin were subjected to whole mount *in situ* hybridization for the genes encoding the known components of the gp78 complex, *gp78*, *p97/VCP* and *derlin1*. Elevated mRNA expression of them these transcripts was observed in response to tunicamycin treatment in the livers of fish larvae (**Fig. 10 C-E**). In the livers of adult

fish treated with 2.5 μg/mL tunicamycin for the indicated periods of time, elevated levels of these transcripts were also seen (**Fig. 11 A**). Consistently, an increase of gp78 was observed at the protein level (**Fig. 11 B**). All together, these data suggest that activation of the UPR by tunicamycin results in the upregulation of gp78 and its parterns, which might coordinately enhance degradation during gp78-mediated ERAD.

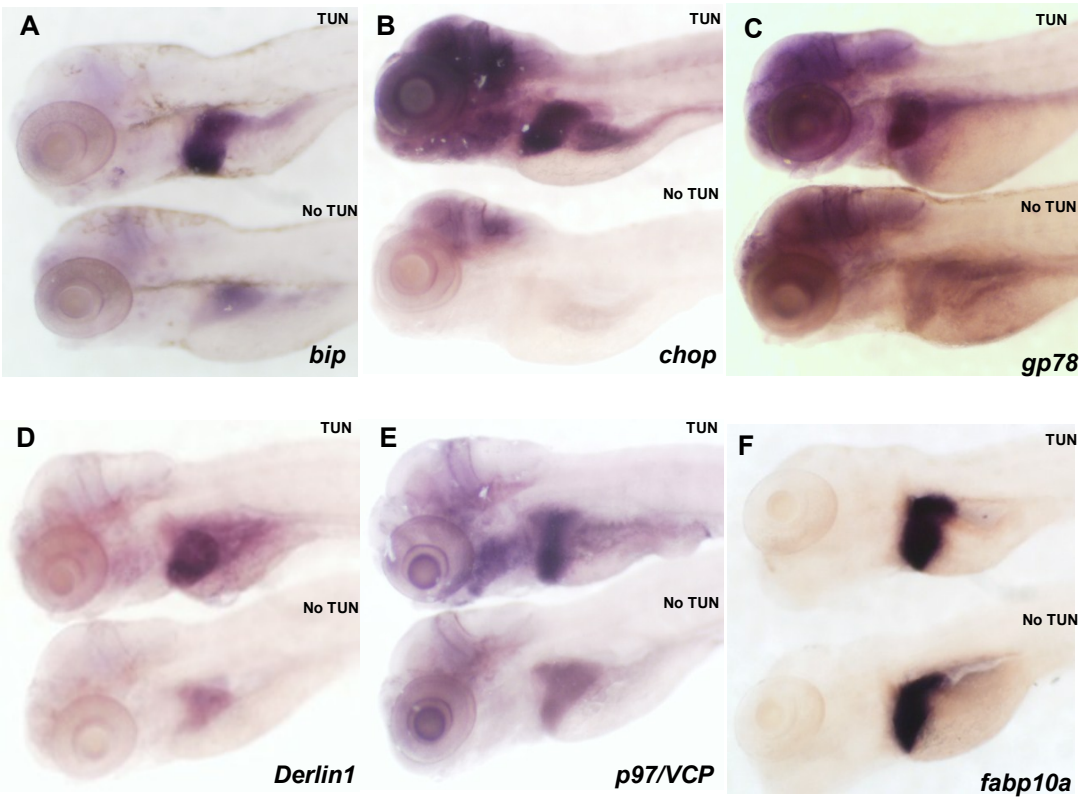

Fig. 10. Induction of hepatic ER stress and upregulation of the components of the gp78 complex by tunicamycin treatment in zebrafish larvae.

(A, B) *In situ* hybridization results showing the upregulation of the ER stress marker *bip* in the livers of 5.5 dpf larvae treated with 1 μg/mL tunicamycin for 48 h. **(C-E)** *In situ hybridization* results showing the upregulation of the components of the gp78 complex, namely *gp78*, *derlin1*, and *p97/VCP*, in the livers of 5.5 dpf larvae treated with 1μg/mL tunicamycin for 48 h. **(F)** *In situ hybridization* results showing the same expression level of the *fabp10a* transcript, a gene specifically expressed in the liver as a control.

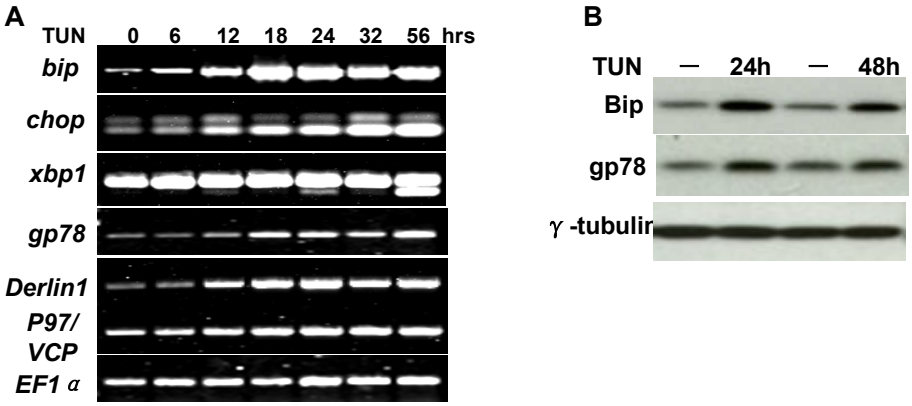

Fig. 11. Induction of hepatic ER stress and increased expression of the components of the gp78 complex upon tunicamycin treatment in adult zebrafish.

(A) RT-PCR result showing the upregulation of the ER stress markers *bip*, *chop*, and the spliced form of *xbp1* (*s-xbp1*) in the livers of adult fish treated with 2.5 μg/mL tunicamycin for the indicated periods of time. The results also showed the upregulation of *gp78*, *derlin1*, and *p97/VCP* in the livers of adult fish treated with 2.5 μg/mL tunicamycin for the indicated periods of time. **(B)** Consistently, an increase of Bip and gp78 was observed at the protein level in the livers of adult fish treated with 2.5 μg/mL tunicamycin for 24 h and 48 h.

3) Overexpression of the dominant-negative form of gp78 (gp78-R2M) in the liver renders adult zebrafish more sensitive to tunicamycin-induced hepatic ER stress

To test the liver specific function of gp78 in hepatic ER stress and to study the long-term effects in adult fish, transgenic fish lines expressing dominant-negative gp78 (gp78-R2M) specifically in the liver were generated (**Fig. 12**). It has been shown that a mutation in the RING finger domain of gp78, giving rise to the mutant gp78-R2M, exhibits dominant-negative effects on the degradation of several substrates, including CD3δ, CFTRΔF508, and HMG CoA reductase (Fang et al 2001, Zhong et al 2004, Pallar et al 2010). We asked whether the overexpression of this dominant-negative gp78 (gp78-R2M) specifically in the liver renders the liver more sensitive to tunicamycin-induced ER stress. We detected the transient expression of gp78-R2M^{myc} in by whole mount immunostaining in wild-type fish embryos injected with the *Tol2-RGM:gp78-R2M^{myc}* construct and the ectopic expression of the transgene by RT-PCR (**Fig. 12 B-C**). The overexpression of the transgene was confirmed by Western blot using dissected livers from adult fish and whole mount *in situ* hybridization (**Fig. 12 D-E**).

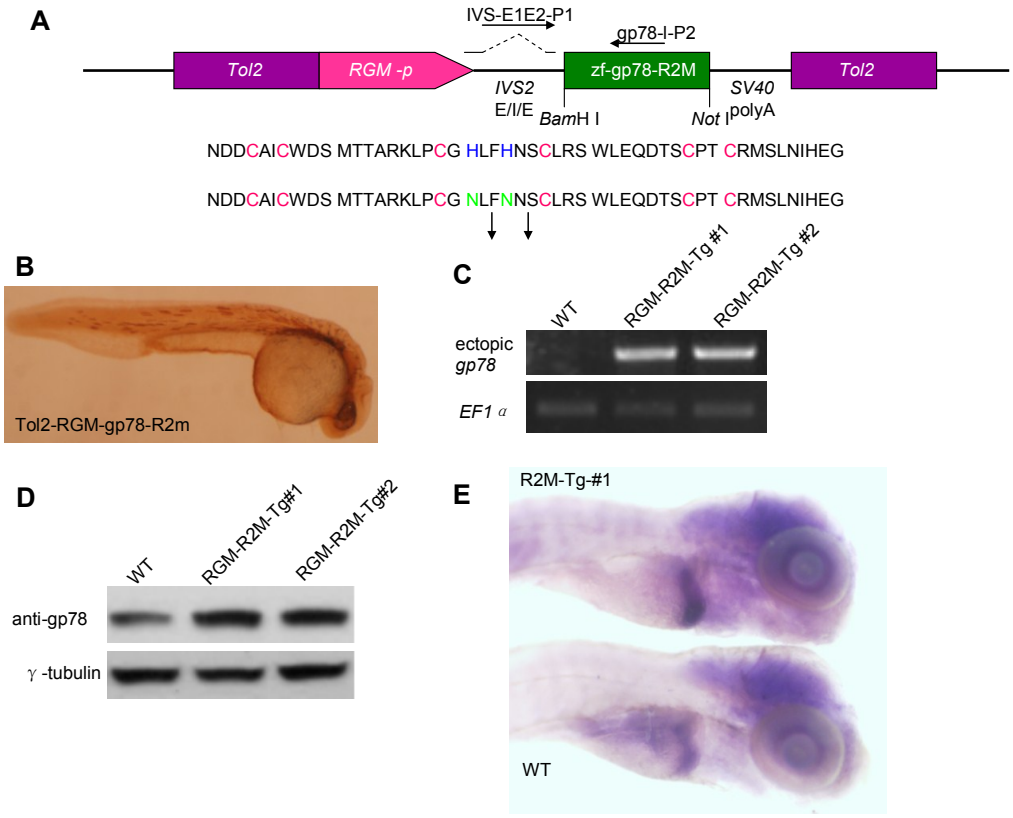

Fig. 12. Liver specific overexpression of gp78-R2M in transgenic fish.

(A) Schematic of the *Tol2-RGM:gp78-R2M*^{myc} construct. 2 critical histidine residues, H354 and H357 in the RING finger domain, were mutated to asparagine residues (N354 and N357) to generate the R2M mutant. **(B)** The *Tol2-RGM:gp78-R2M*^{myc} construct was injected into embryos at the 1- or 2-cell stage and the transient expression of gp78-R2M-myc was detected by whole mount anti-myc tag antibody staining. **(C)** Expression of the *gp78-R2M-myc* minigene was detected in the livers of adult fish of 2 *RGM:gp78-R2M*^{myc} transgenic lines by RT-PCR. **(D)** Overexpression of gp78-R2M in these 2 lines was confirmed by Western blot using the monoclonal antibody 1F1 and dissected livers. **(E)** Liver-specific overexpression of gp78-R2M in the 2 lines was also confirmed in 5.5 dpf larvae by whole mount *in situ* hybridization using a *gp78* antisense probe.

To examine the long-term effects of the overexpression of gp78-R2M on hepatic ER stress under physiologic conditions, livers dissected from 2-month-old adult wild-type and transgenic fish were subjected to RT-PCR for 2 ER stress markers *bip* and *chop*. No significant difference was observed at the transcript level of these 2 ER stress markers under physiologic conditions (**Fig. 13 A-D**). Next, to examine the hepatic ER stress levels under the stressed condition, 2-month-old adult zebrafish were treated with 2.5 μg/mL tunicamycin for 6 h. As expected, the overexpression of gp78-R2M rendered adult zebrafish more sensitive to tunicamycin-induced hepatic ER stress as shown by semi-quantitative RT-PCR (**Fig. 13 A-D**). The level of *bip* mRNA in the livers of tunicamycin treated R2M-transgenic fish was dramatically increased compared with that in tunicamycin treated wild-type fish. The difference in *chop* mRNA levels was not striking (**Fig. 13 A-D**). Consistently, we observed the enhanced protein level of Bip in R2M transfected HeLa cells treated with 2μg /mL of tunicamycin (**Fig. 13 O**).

4) Overexpression of gp78-R2M in the liver enhances the expression of Srebp target genes in response to ER stress

It has been demonstrated that ER stress activates the SREBP transcription factors, key regulators of fatty acid and cholesterol synthesis, in insulin- and alcohol-induced hepatic steatosis (Kammoun et al 2009). Since we observed enhanced sensitivity to hepatic ER stress in the adult R2M-transgenic fish in response to tunicamycin, we next checked the mRNA levels of Srebp target genes involved in the synthesis of fatty acids and cholesterol. RT-PCR analysis revealed that the mRNA levels of both Srebp-1c target genes *acc1*, *fatty acid desaturase* (*fads*) and *fatty acid*

synthase (*fasn*)) and Srebp-2 target genes, (*3-hydroxy-3-methylglutaryl coenzyme A reductase a* (*HMGCRa*), *3-hydroxy-3-methylglutaryl coenzyme A synthase 1* (*HMGCS1*)) were dramatically increased in the livers of R2M-transgenic fish compared with wild-type fish after tunicamycin challenge for 6 h (**Fig. 13 A, E-J**). Consistent with this, we also observed the upregulation of *Srebp1* and *Srebp2* transcripts in the livers of R2M-transgenic fish (**Fig. 13 A, K-L**). Importantly, we did not observe the enhanced expression of *cyp2e1*, a key enzyme in drug metabolism, in R2M-transgenic fish (**Fig. 13 A, M**), indicating the upregulation of Srebp target genes is not caused by increased uptake of tunicamycin.

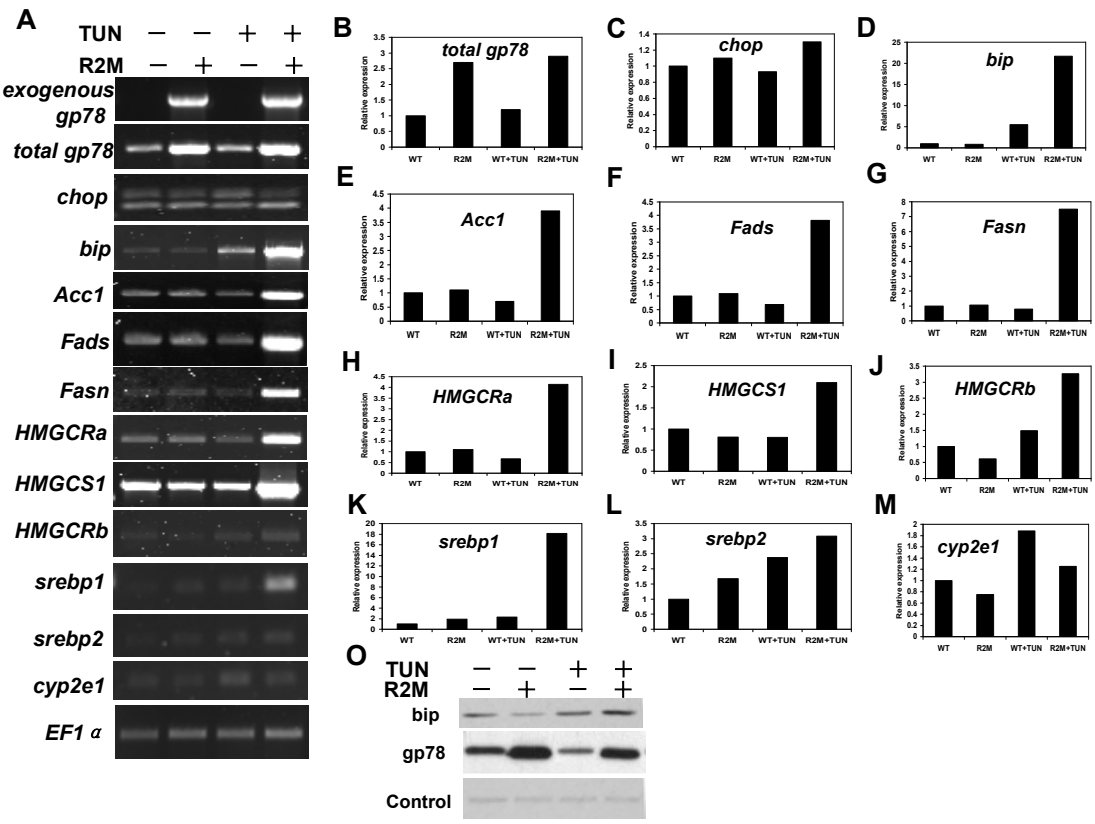

Fig. 13. Overexpression of the dominant-negative form of gp78 sensitizes hepatic ER stress and enhances the expression of Srebp target genes in response to ER stress.

(A-M) 2-month-old adult wild-type and transgenic fish were treated with or without 2.5 μg/mL tunicamycin for 6 h. Livers were dissected and subjected to semi-quantitative RT-PCR for the ER stress markers *bip* and *chop*, the Srebp-1c target genes *acc1*, *fads*, and *fasn*, the Srebp-2 target genes *hmgcra*, and *hmgcs1*, *srebp1*, *srebp2,* and *cyp2e1*. No significant differences were observed at the transcript levels for *bip* and *chop* under the treated and untreated conditions. However, the overexpression of gp78-R2M enhanced the expression of the ER stress markers *bip* and *chop* in response to tunicamycin treatment. This was accompanied by the enhanced expression of the Srebp1 target genes *acc1*, *fads*, and *fasn*, and the Srebp-2

target genes, *hmgcra*, and *hmgcs1 as* well as increased *srebp1* and *srebp2* transcripts, but not increased *cyp2e1* transcripts. **(O)** We consistently observed enhanced protein levels of Bip in R2M transfected HeLa cells treated with 2 μg /mL of tunicamycin.

5) Overexpression of gp78-wt in the liver renders the liver less sensitive to tunicamycin-induced hepatic ER stress

To further test the liver specific function of gp78 in regulation of hepatic ER stress and lipid metabolism, transgenic fish lines expressing gp78-wt specifically in the liver were generated (**Fig. 14**). Next we asked whether the specific overexpression of gp78-wt in the liver renders the liver less sensitive to tunicamycin-induced ER stress. Again, we detected the ectopic expression of the transgene by RT-PCR and confirmed its overexpression by Western blot using livers dissected from adult fish livers and whole mount *in situ* hybridization (**Fig. 14 B-D**).

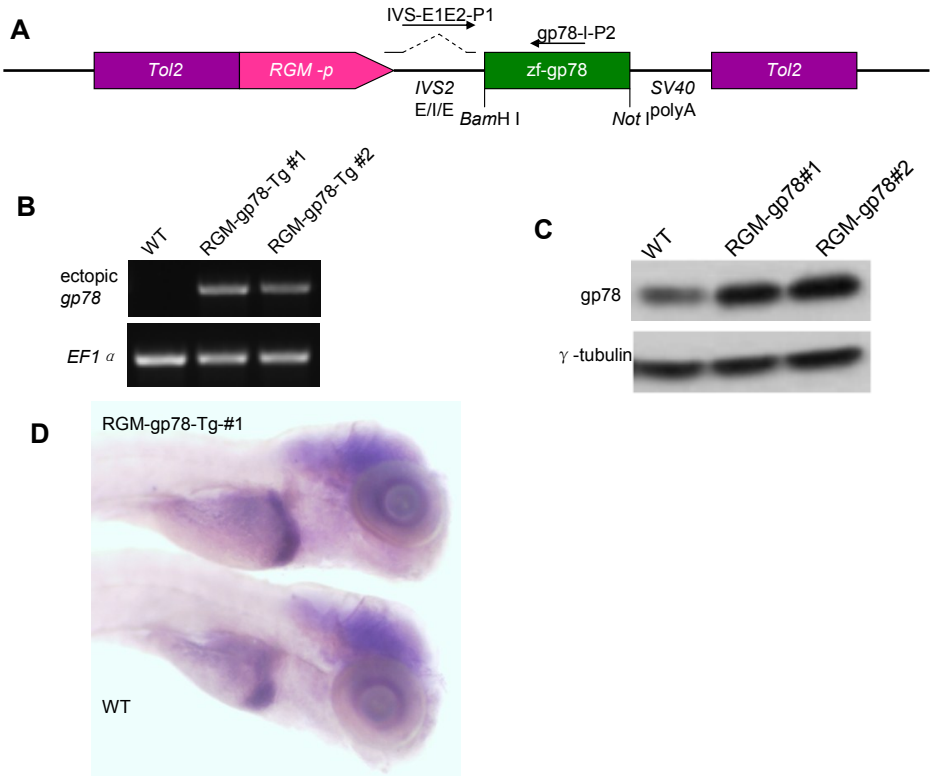

Fig. 14. Liver specific overexpression of gp78-wt in transgenic fish.

(A) Schematic of the *Tol2-RGM:gp78-wt*^{myc} construct. **(B)** Expression of the *gp78-wt-myc* minigene was detected in the livers of adult fish of 2 *RGM:gp78-wt*^{myc} transgenic lines by RT-PCR. **(C)** Overexpression of gp78-wt in these 2 lines was confirmed by Western blot using the monoclonal antibody 1F1 on dissected livers. **(D)** Liver specific overexpression of gp78-wt in the 2 lines was also confirmed in 5.5 dpf larvae by whole mount *in situ* hybridization using the *gp78* antisense probe.

To examine the long-term effects of the overexpression of gp78-wt on hepatic ER stress, 2-month-old adult zebrafish were subjected to treatment with 2.5 μg/mL tunicamycin for 6 h. As expected, the overexpression of gp78-wt rendered zebrafish less sensitive to tunicamycin-induced hepatic ER stress as shown by semi-quantitative RT-PCR (**Fig. 15 C-D**). The increased level of *bip* mRNA in the livers of tunicamycin treated fish was attenuated in *RGM:gp78-wt*^{myc} transgenic fish (**Fig. 15 D**), although the *chop* mRNA levels were slightly increased (**Fig. 15 C**). Consistently, no change in expression of Bip protein was observed between non-transfected and R2M-transfected HeLa cells treated with 2μg /mL of tunicamycin (**Fig. 15 O**).

6) Overexpression of gp78-wt in the liver did not enhance the expression of Srebp target genes in response to ER stress

To further test whether the overexpression of gp78-wt affects the expression of Srebp target genes in response to tunicamycin-induced ER stress, we determined the mRNA levels of Srebp target genes by RT-PCR analysis. The results revealed that the mRNA levels of both the Srebp target genes, *acc1*, *fads* and *fasn*, and Srebp-2 target genes, *hmgcra*, *hmgcs1*, were not affected (**Fig. 15 A, E-J**). Consistent with this, neither *srebp1* nor *srebp2* transcript levels were affected (**Fig. 15 A, K-L**).

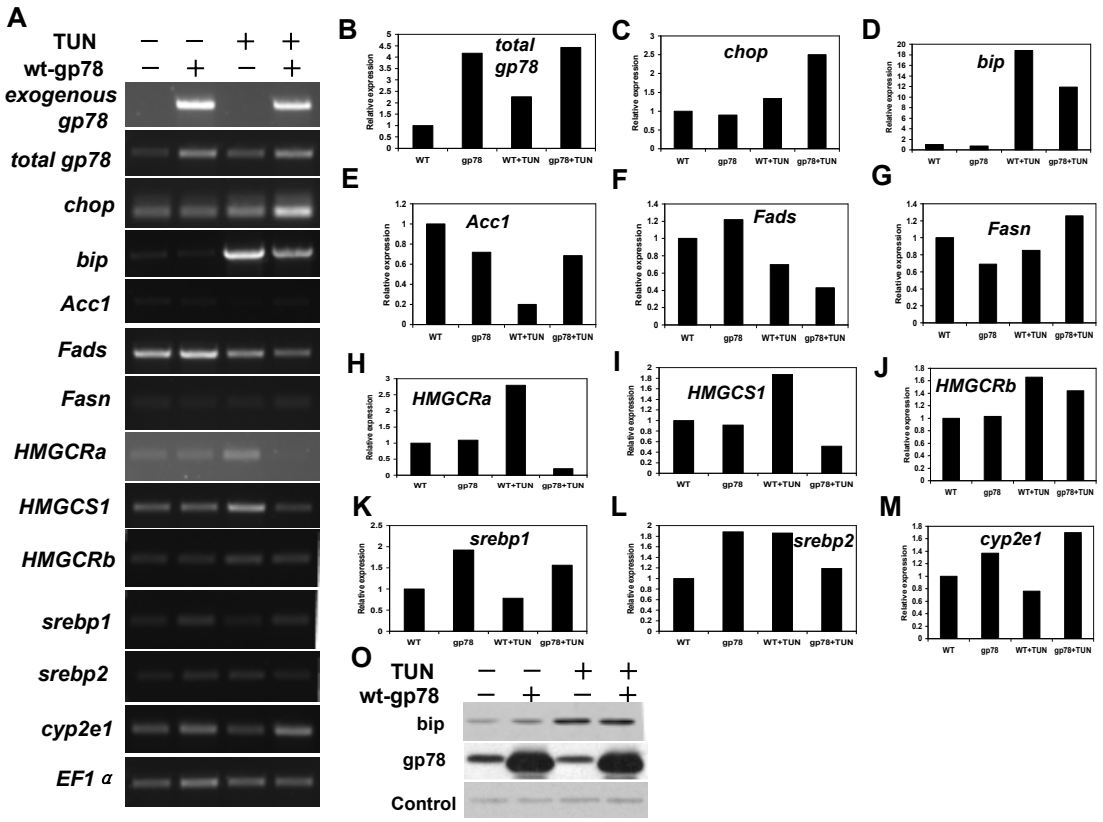

Fig. 15. Overexpression of gp78-wt renders less sensitive to hepatic ER stress and does not enhance the expression of Srebp target genes in response to ER stress.

(A-M) 2-month-old adult wild-type and gp78-wt transgenic fish were treated with or without 2.5 μg/mL tunicamycin for 6 h. Livers were dissected and subjected to semi-quantitative RT-PCR for the ER stress markers *bip* and *chop*, the Srebp-1c target genes *acc1*, *fads,* and *fasn*, the Srebp-2 target genes, *hmgcra*, *hmgcs1*, *srebp1*, *srebp2*, and *cyp2e1*. No significant differences were observed at the transcript levels of *bip* and *chop* under the treated and untreated conditions. The overexpression of gp78-R2M neither enhanced the expression of the ER stress markers *bip* and *chop* in response to tunicamycin treatment, nor enhanced the expression of the Srebp1 target genes *acc1*, *fads* and *fasn*, and the Srebp-2 target genes, *hmgcra*, *hmgcs1*, nor increased *srebp1*

increased *srebp1* and *srebp2* transcript levels. **(O)** We consistently observed a lack of enhancement of the protein levels of Bip in gp78-wt transfected HeLa cells treated with 2 μg/mL of tunicamycin.

Discussion

In this study we analyzed the function of gp78 in hepatic ER stress in zhebrafish. We have demonstrated that gp78 plays a key role in the regulation of hepatic ER stress and lipid metabolism. Genetic manipulation of gp78 expression through morpholino knockdown or the overexpression of dominant negative gp78 does not affect hepatic ER stress under physiologic conditions but renders the hepatocytes more sensitive to ER stress in both larval and adult zebrafish. Moreover, our results indicate a potential role for gp78 in the regulation of Srebp cleavage induced by ER stress.

1) gp78 and UPR

It has been demonstrated that HRD1, a gp78 homolog, is upregulated by ER stress and protects against ER stress-induced apoptosis by accelerating the degradation of misfolded proteins (Kaneko, et al 2007, Allen et al 2004). Recently, it has been shown that *HRD1* promoter carries a functional unfolded protein response element (UPRE) to which XBP1 binds directly (Yamamoto et al 2008). However, whether *gp78* carries a UPRE, and which branches of the UPR are responsible for gp78 to protect cells against ER stress, remains to be determined. Nevertheless, we demonstrated the upregulation of *gp78*, *p97*, and *derlin1* upon tunicamycin treatment in our model system, although we do not rule out the possibility that other E3 ligases are upregulated and that the upregulation of *p97* and *derlin1* coordinately enhances the ERAD activity of other E3 ligases. It is also possible that different E3 ligases function in different tissues upon the activation of the UPR pathways. However, since most studies have been performed in cultured cells, the tissue-specific functions of

the various E3 ligases remain unknown. Nevertheless, we demonstrated the significance of g78 in the UPR pathway and in hepatic function *in vivo* in the present study.

2) gp78 and ER stress

We have demonstrated that the knockdown of gp78 or the overexpression of gp78-wt or gp78-R2M does not induce hepatic ER stress under physiologic conditions. This result is consistent with a previous study showing that the overexpression of wild-type or mutant Hrd1 or gp78 alone could not induce massive ER stress in cultured cells (Bernardi et al 2010,). Interestingly, Bip, Hsp70 and p97 levels were not altered upon gp78 knockdown (Fisher et al 2011). In contrast, another study showed that the knockdown of gp78 caused mutant SOD1-induced ER stress (Ying et al 2009). In HEK293 cells in which ER stress was induced by the co-transfection of mutant SOD-1, the knockdown of gp78 increased the level of Bip, whereas the overexpression of gp78 decreased the level of Bip (Ying et al 2009). It should be noted that the ERAD-mediated degradation of SOD-1 is primarily driven by gp78 and is only slighted affected by Hrd1 (Ying et al 2009). Given that gp78 is the primary ubiquitin ligase that targets SOD-1 for degradation, the knockdown of gp78 will result in the accumulation of mutant SOD-1 and the induction of ER stress. These results are consistent with our finding that gp78 functions under stress conditions but not under physiologic conditions.

3) gp78 and hepatic lipid metabolism

We have demonstrated that gp78 may play a role in protecting against hepatic steatosis and fatty liver disease. Recently, through studies using cultured cells, it has

become clear that gp78 is involved in targeting several hepatic proteins responsible for lipid metabolism for degradation through the ERAD pathway. For example, gp78 regulates the degradation of HMG CoA reductase, a rate-limiting enzyme in cholesterol biosynthesis in the liver, under high cholesterol conditions (Song et al 2005, Jo et al 2010) and the secretion of ApoB-100, a protein mainly synthesized in the liver, in cultured cells (Liang et al 2003, Fisher et al 2011). Although some studies suggest that a high fat diet induces hepatic ER stress and thus causes hepatic steatosis, it is not clear whether such regulation works through the activation of the UPR induced by ER stress (Li et al 2012). The Srebp pathway is the key pathway responsible for lipogenesis in the liver, and here we showed that the overexpression of dominant-negative gp78 (gp78-R2M) renders hepatocytes more sensitive to tunicamycin-induced ER stress and thus enhances the upregulation of Srebp target genes, suggesting a link between ERAD, UPR, and lipid metabolism.

Bibliography

Adachi Y, Bradford BU, Gao W, Bojes HK, Thurman RG. 1994. Inactivation of Kupffer cells prevents early alcohol-induced liver injury. *Hepatology* 20(2):453-60.

Allen JR, Nguyen LX, Sargent KE, Lipson KL, Hackett A, et al. 2004. *Biochem Biophys Res Commun* 324(1):166-70.

Ashlock MA, Olson ER. 2011. Therapeutics development for cystic fibrosis: a successful model for a multisystem genetic disease. *Annu Rev Med* 62:107-25.

Ballar P, Ors AU, Yang H, Fang S. 2010. Differential regulation of CFTRDeltaF508 degradation by ubiquitin ligases gp78 and Hrd1. *Int J Biochem Cell Biol* 42(1):167-73.

Ballar P, Pabuccuoglu A, Kose FA. 2011. Different p97/VCP complexes function in retrotranslocation step of mammalian ER-associated degradation (ERAD). *Int J Biochem Cell Biol* 43(4):613-21.

Ballar P, Shen Y, Yang H, Fang S. 2006. The role of a novel p97/valosin-containing protein-interacting motif of gp78 in endoplasmic reticulum-associated degradation. *J Biol Chem* 281(46):35359-68.

Ballar P, Zhong Y, Nagahama M, Tagaya M, Shen Y, et al. 2007. Identification of SVIP as an endogenous inhibitor of endoplasmic reticulum-associated degradation. *J Biol Chem* 282(47):33908-14.

Bär PR, Amelink GJ. 1997. Protection against muscle damage exerted by oestrogen: hormonal or antioxidant action? *Biochem Soc Trans* 25(1):50-4.

Basseri S, Austin RC. 2008. ER stress and lipogenesis: a slippery slope toward hepatic steatosis. *Dev Cell* 15(6):795-6.

Basseri S, Austin RC. 2012. Endoplasmic reticulum stress and lipid metabolism: mechanisms and therapeutic potential. *Biochem Res Int* 2012:841362.

Bays NW, Gardner RG, Seelig LP, Joazeiro CA, Hampton RY. 2001. Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. *Nat Cell Biol* 3(1):24-9.

Bays NW, Hampton RY. 2002. Cdc48-Ufd1-Npl4: stuck in the middle with Ub. *Curr Biol* 12(10):R366-71.

Behrends C, Harper JW. 2011. *Nat Struct Mol Biol* 18(5):520-8.

Bengoechea-Alonso MT, Ericsson J. 2007. SREBP in signal transduction: cholesterol metabolism and beyond. *Curr Opin Cell Biol* 19(2):215-22.

Ben-Saadon R, Zaaroor D, Ziv T, Ciechanover A. 2006. The polycomb protein Ring1B generates self atypical mixed ubiquitin chains required for its *in vitro* histone H2A ligase activity. *Mol Cell* 24(5):701-11.

Bernardi KM, Forster ML, Lencer WI, Tsai B. 2008. Derlin-1 facilitates the retro-translocation of cholera toxin. *Mol Biol Cell* 19(3):877-84.

Bernardi KM, Williams JM, Kikkert M, van Voorden S, Wiertz EJ, et al. 2010. The E3 ubiquitin ligases Hrd1 and gp78 bind to and promote cholera toxin retro-translocation. *Mol Biol Cell* 21(1):140-51.

Bian YH, Xu C, Li J, Xu J, Zhang H, et al. 2011. Development of a transgenic zebrafish model expressing GFP in the notochord, somite and liver directed by the hfe2 gene promoter. *Transgenic Res* 20(4):787-98.

Biederer T, Volkwein C, Sommer T. 1997. Role of Cue1p in ubiquitination and degradation at the ER surface. *Science* 278(5344):1806-9.

Bobrovnikova-Marjon E, Hatzivassiliou G, Grigoriadou C, Romero M, Cavener DR, et al. 2008. PERK-dependent regulation of lipogenesis during mouse mammary gland development and adipocyte differentiation. *Proc Natl Acad Sci U S A* 105(42):16314-9.

Bommiasamy H, Back SH, Fagone P, Lee K, Meshinchi S, et al. 2009. ATF6alpha induces XBP1-independent expansion of the endoplasmic reticulum. *J Cell Sci* 122(Pt 10):1626-36.

Bordallo J, Plemper RK, Finger A, Wolf DH.1998. Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. *Mol Biol Cell* 9(1):209-22.

Bordallo J, Wolf DH. 1999. A RING-H2 finger motif is essential for the function of Der3/Hrd1 in endoplasmic reticulum associated protein degradation in the yeast Saccharomyces cerevisiae. *FEBS Lett* 448(2-3):244-8.

Cao J, Wang J, Qi W, Miao HH, Wang J, et al. 2007. Ufd1 is a cofactor of gp78 and plays a key role in cholesterol metabolism by regulating the stability of HMG-CoA reductase. *Cell Metab* 6(2):115-28.

Cardozo AK, Ortis F, Storling J, Feng YM, Rasschaert J, et al. 2005. Cytokines downregulate the sarcoendoplasmic reticulum pump Ca2+ ATPase 2b and

deplete endoplasmic reticulum Ca2+, leading to induction of endoplasmic reticulum stress in pancreatic beta-cells. *Diabetes* 54(2):452-61.

Carvalho P, Goder V, Rapoport TA. 2006. Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell* 126(2):361-73.

Carvalho P, Stanley AM, Rapoport TA. 2010. Retrotranslocation of a misfolded luminal ER protein by the ubiquitin-ligase Hrd1p. *Cell* 143(4):579-91.

Chen B, Mariano J, Tsai YC, Chan AH, Cohen M, et al. 2006. The activity of a human endoplasmic reticulum-associated degradation E3, gp78, requires its Cue domain, RING finger, and an E2-binding site. *Proc Natl Acad Sci U S A* 103(2):341-6.

Chen G, Liang G, Ou J, Goldstein JL, Brown MS. 2004. Central role for liver X receptor in insulin-mediated activation of Srebp-1c transcription and stimulation of fatty acid synthesis in liver. *Proc Natl Acad Sci U S A* 101(31):11245-50.

Cinaroglu A, Gao C, Imrie D, Sadler KC. 2011. Activating transcription factor 6 plays protective and pathological roles in steatosis due to endoplasmic reticulum stress in zebrafish. *Hepatology* 54(2):495-508.

Crabb DW, Liangpunsakul S. 2006. Alcohol and lipid metabolism. *J Gastroenterol Hepatol* 21 Suppl 3:S56-60.

Colgan SM, Tang D, Werstuck GH, Austin RC. 2007. Endoplasmic reticulum stress causes the activation of sterol regulatory element binding protein-2. *Int J Biochem Cell Biol* 39(10):1843-51.

Das R, Mariano J, Tsai YC, Kalathur RC, Kostova Z, et al. 2009. Allosteric activation of E2-RING finger-mediated ubiquitylation by a structurally defined specific E2-binding region of gp78. *Mol Cell* 34(6):674-85.

Davies SP, Carling D, Munday MR, Hardie DG. 1992. Diurnal rhythm of phosphorylation of rat liver acetyl-CoA carboxylase by the AMP-activated protein kinase, demonstrated using freeze-clamping. Effects of high fat diets. *Eur J Biochem* 203(3):615-23.

Deak PM, Wolf DH. 2001. Membrane topology and function of Der3/Hrd1p as a ubiquitin-protein ligase (E3) involved in endoplasmic reticulum degradation. *J Biol Chem* 276(14):10663-9.

Denic V, Quan EM, Weissman JS. 2006. A luminal surveillance complex that selects misfolded glycoproteins for ER-associated degradation. *Cell* 126(2):349-59.

Dikic I, Wakatsuki S, Walters KJ. 2009. Ubiquitin-binding domains - from structures to functions. *Nat Rev Mol Cell Biol* 10(10):659-71.

Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, et al. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest* 15(5):1343-51.

on Y, McCammon JM, Miller JC, Faraji F, Ngo C, et al. 2008. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat Biotechnol* 26(6):702-8.

Du SJ, Dienhart M. 2001. Gli2 mediation of hedgehog signals in slow muscle induction in zebrafish. *Differentiation* 67(3):84-91.

Duennwald ML, Lindquist S. 2008. Impaired ERAD and ER stress are early and specific events in polyglutamine toxicity. *Genes Dev* 22(23):3308-19.

Ellgaard L, Helenius A. 2003. Quality control in the endoplasmic reticulum. *Nat Rev Mol Cell Biol* 4(3):181-91.

Endo K, Shirai A, Furukawa M, Yoshizaki T. 2006. Prognostic value of cell motility activation factors in patients with tongue squamous cell carcinoma. *Hum Pathol* 37(8):1111-6.

Fairbank M, St-Pierre P, Nabi IR. 2009. The complex biology of autocrine motility factor/phosphoglucose isomerase (AMF/PGI) and its receptor, the gp78/AMFR E3 ubiquitin ligase. *Mol Biosyst* 5(8):793-801.

Fang S, Ferrone M, Yang C, Jensen JP, Tiwari S, et al. 2001. The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. *Proc Natl Acad Sci U S A* 98(25):14422-7.

Fang S, Weissman AM. 2004. A field guide to ubiquitylation. *Cell Mol Life Sci* 61(13):1546-61.

Farhat MY, Lavigne MC, Ramwell PW. 1996. The vascular protective effects of estrogen. *FASEB J* 10(5):615-24.

Feitsma H, Cuppen E. 2008. Zebrafish as a cancer model. *Mol Cancer Res* 6(5):685-94.

Fisher EA, Khanna NA, McLeod RS. 2011. Ubiquitination regulates the assembly of VLDL in HepG2 cells and is the committing step of the apoB-100 ERAD pathway. *J Lipid Res* 52(6):1170-80.

Fisher EA, Lapierre LR, Junkins RD, McLeod RS. 2008. The AAA-ATPase p97 facilitates degradation of apolipoprotein B by the ubiquitin-proteasome pathway. *J Lipid Res* 49(10):2149-60.

Flynn EJ 3rd, Trent CM, Rawls JF. 2009. Ontogeny and nutritional control of adipogenesis in zebrafish (Danio rerio). *J Lipid Res* 50(8):1641-52.

Foufelle F, Ferré P. 2002. New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. *Biochem J* 366(Pt 2):377-91.

Fu M, Li L, Albrecht T, Johnson JD, Kojic LD, et al. 2011. Autocrine motility factor/phosphoglucose isomerase regulates ER stress and cell death through control of ER calcium release. *Cell Death Differ* 18(6):1057-70.

Fuchs CD, Claudel T, Kumari P, Haemmerle G, Pollheimer MJ, et al. 2012. Absence of adipose triglyceride lipase protects from hepatic endoplasmic reticulum stress in mice. *Hepatology* doi: 10.1002/hep.25601. [Epub ahead of print]

Ginsberg HN. 1995. Synthesis and secretion of apolipoprotein B from cultured liver cells. *Curr Opin Lipidol* 6(5):275-80.

Glaumann H, Bergstrand A, Ericsson JL. 1975. Studies on the synthesis and intracellular transport of lipoprotein particles in rat liver. *J Cell Biol* 64(2):356-77.

Goldstein JL, DeBose-Boyd RA, Brown MS. 2006. Protein sensors for membrane sterols. *Cell* 124(1):35-46.

Greenblatt EJ, Olzmann JA, Kopito RR. 2011. Derlin-1 is a rhomboid pseudoprotease required for the dislocation of mutant α-1 antitrypsin from the endoplasmic reticulum. *Nat Struct Mol Biol* 18(10):1147-52.

Gregor MF, Yang L, Fabbrini E, Mohammed BS, Eagon JC, et al. 2009. Endoplasmic reticulum stress is reduced in tissues of obese subjects after weight loss. *Diabetes* 58(3):693-700.

Guan HP, Goldstein JL, Brown MS, Liang G. 2009. Accelerated fatty acid oxidation in muscle averts fasting-induced hepatic steatosis in SJL/J mice. *J Biol Chem* 84(36):24644-52.

Hampton RY. 2002. ER-associated degradation in protein quality control and cellular regulation. *Curr Opin Cell Biol* 14(4):476-82.

Hampton RY, Gardner RG, Rine J. 1996. Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Mol Biol Cell* 7(12):2029-44.

Hänzelmann P, Schindelin H. 2011. The structural and functional basis of the p97/valosin-containing protein (VCP)-interacting motif (VIM): mutually exclusive binding of cofactors to the N-terminal domain of p97. *J Biol Chem* 286(44):38679-90.

Hastings GA, Coleman TA, Haudenschild CC, Stefansson S, Smith EP, et al. 1997. Neuroserpin, a brain-associated inhibitor of tissue plasminogen activator is localized primarily in neurons. Implications for the regulation of motor learning and neuronal survival. *J Biol Chem* 272(52):33062-7.

He L, Simmen FA, Ronis MJ, Badger TM. 2004. *J Biol Chem* 279(27):28113-21.

Hebert DN, Bernasconi R, Molinari M. 2010. ERAD substrates: which way out? *Semin Cell Dev Biol* 21(5):526-32.

Hebert DN, Foellmer B, Helenius A. 1995. Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell* 81(3):425-33.

Hegarty BD, Bobard A, Hainault I, Ferré P, Bossard P, et al. 2005. Distinct roles of insulin and liver X receptor in the induction and cleavage of sterol regulatory element-binding protein-1c. *Proc Natl Acad Sci U S A* 102(3):791-6.

Heo JM, Livnat-Levanon N, Taylor EB, Jones KT, Dephoure N, et al. 2010. A stress-responsive system for mitochondrial protein degradation. *Mol Cell* 40(3):465-80.

Hirano Y, Yoshida M, Shimizu M, Sato R.2001. Direct demonstration of rapid degradation of nuclear sterol regulatory element-binding proteins by the ubiquitin-proteasome pathway. *J Biol Chem* 276(39):36431-7.

Hölttä-Vuori M, Salo VT, Nyberg L, Brackmann C, Enejder A, et al. 2010. Zebrafish: gaining popularity in lipid research. *Biochem J* 429(2):235-42.

Hölttä-Vuori M, Uronen RL, Repakova J, Salonen E, Vattulainen I, et al. 2008. BODIPY-cholesterol: a new tool to visualize sterol trafficking in living cells and organisms. *Traffic* 9(11):1839-49.

Horton JD, Bashmakov Y, Shimomura I, Shimano H. 1998. Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice. *Proc Natl Acad Sci U S A* 95(11):5987-92.

Horton JD, Shimomura I, Ikemoto S, Bashmakov Y, Hammer RE. 2003. Overexpression of sterol regulatory element-binding protein-1a in mouse

adipose tissue produces adipocyte hypertrophy, increased fatty acid secretion, and fatty liver. *J Biol Chem* 278(38):36652-60.

Hosokawa N, Wada I, Nagasawa K, Moriyama T, Okawa K, et al. 2008. Human XTP3-B forms an endoplasmic reticulum quality control scaffold with the HRD1-SEL1L ubiquitin ligase complex and BiP. *J Biol Chem* 283(30):20914-24.

Howarth DL, Vacaru AM, Tsedensodnom O, Mormone E, Nieto N, et al. 2012. Alcohol disrupts endoplasmic reticulum function and protein secretion in hepatocytes. *Alcohol Clin Exp Res* 36(1):14-23.

Imai Y, Soda M, Takahashi R. 2000. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J Biol Chem* 275(46):35661-4.

Imuro Y, Gallucci RM, Luster MI, Kono H, Thurman RG. 1997. Antibodies to tumor necrosis factor alfa attenuate hepatic necrosis and inflammation caused by chronic exposure to ethanol in the rat. *Hepatology* 26(6):1530-7.

Ji C, Chan C, and Kaplowitz N. 2006. Predominant role of sterol response element binding proteins (SREBP) lipogenic pathways in hepatic steatosis in the murine intragastric ethanol feeding model. *J Hepatol* 45(5):717-24.

Ji C, Kaplowitz N, Lau MY, Kao E, Petrovic LM, et al. 2011. Liver-specific loss of glucose-regulated protein 78 perturbs the unfolded protein response and exacerbates a spectrum of liver diseases in mice. *Hepatology* 54(1):229-39.

Ji C, Mehrian-Shai R, Chan C, Hsu YH, Kaplowitz N. 2005. Role of CHOP in hepatic apoptosis in the murine model of intragastric ethanol feeding. *Alcohol Clin Exp Res* 29(8):1496-503.

Jo Y, Debose-Boyd RA. 2010. Control of cholesterol synthesis through regulated ER-associated degradation of HMG CoA reductase. *Crit Rev Biochem Mol Biol* 45(3):185-98.

Jo Y, Sguigna PV, DeBose-Boyd RA. 2011. Membrane-associated ubiquitin ligase complex containing gp78 mediates sterol-accelerated degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J Biol Chem* 286(17):15022-31.

Jones KS, Alimov AP, Rilo HL, Jandacek RJ, Woollett LA, et al. 2008. A high throughput live transparent animal bioassay to identify non-toxic small molecules or genes that regulate vertebrate fat metabolism for obesity drug development. *Nutr Metab (Lond)* 5:23.

Joshi B, Li L, Nabi IR. 2010. A role for KAI1 in promotion of cell proliferation and mammary gland hyperplasia by the gp78 ubiquitin ligase. *J Biol Chem* 285(12):8830-9.

Kammoun HL, Chabanon H, Hainault I, Luquet S, Magnan C, et al. 2009. GRP78 expression inhibits insulin and ER stress-induced SREBP-1c activation and reduces hepatic steatosis in mice. *J Clin Invest* 119(5):1201-15.

Kaneko M, Yasui S, Niinuma Y, Arai K, Omura T, et al. 2007. A different pathway in the endoplasmic reticulum stress-induced expression of human HRD1 and SEL1 genes. *FEBS Lett* 581(28):5355-60.

Kaufman CK, White RM, Zon L. 2009. Chemical genetic screening in the zebrafish embryo. *Nat Protoc* 4(10):1422-32.

Kharroubi I, Ladrière L, Cardozo AK, Dogusan Z, Cnop M, et al. 2004. Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress. *Endocrinology* 145(11):5087-96.

Kikkert M, Hassink G, Wiertz E. 2005. The role of the ubiquitination machinery in dislocation and degradation of endoplasmic reticulum proteins. *Curr Top Microbiol Immunol* 300:57-93.

Kim JB, Sarraf P, Wright M, Yao KM, Mueller E, et al. 1998. Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *J Clin Invest* 101(1):1-9.

Kim SM, Acharya P, Engel JC, Correia MA. 2010. Liver cytochrome P450 3A ubiquitination *in vivo* by gp78/autocrine motility factor receptor and C terminus of Hsp70-interacting protein (CHIP) E3 ubiquitin ligases: physiological and pharmacological relevance. *J Biol Chem* 285(46):35866-77.

Kostova Z, Mariano J, Scholz S, Koenig C, Weissman AM. 2009. A Ubc7p-binding domain in Cue1p activates ER-associated protein degradation. *J Cell Sci* 122(Pt 9):1374-81.

Kostova Z, Tsai YC, Weissman AM. 2007. Ubiquitin ligases, critical mediators of endoplasmic reticulum-associated degradation. *Semin Cell Dev Biol* 18(6):770-9.

Le May C, Pineau T, Bigot K, Kohl C, Girard J, et al. 2000. *FEBS Lett* 475(3):163-6.

Lee AH, Scapa EF, Cohen DE, Glimcher LH. 2008. Regulation of hepatic lipogenesis by the transcription factor XBP1. *Science* 320(5882):1492-6.

Lee JN, Song B, DeBose-Boyd RA, Ye J. 2006. Sterol-regulated degradation of Insig-1 mediated by the membrane-bound ubiquitin ligase gp78. *J Biol Chem* 281(51):39308-15.

Lee JN, Ye J. 2004. Proteolytic activation of sterol regulatory element-binding protein induced by cellular stress through depletion of Insig-1. *J Biol Chem* 279(43):45257-65.

Lee S, Tsai FT. 2005. Molecular chaperones in protein quality control. *J Biochem Mol Biol* 38(3):259-65.

Lemoine M, Ratziu V, Kim M, Maachi M, Wendum D, et al. 2009. *Liver Int* 29(9):1431-8.

Lewis GF, Carpentier A, Adeli K, Giacca A. 2002. Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. *Endocr Rev* 3(2):201-29.

Li G, Zhao G, Schindelin H, Lennarz WJ. 2008. Tyrosine phosphorylation of ATPase p97 regulates its activity during ERAD. *Biochem Biophys Res Commun* 375(2):247-51.

Li G, Zhou X, Zhao G, Schindelin H, Lennarz WJ. 2005. Multiple modes of interaction of the deglycosylation enzyme, mouse peptide N-glycanase, with the proteasome. *Proc Natl Acad Sci U S A* 102(44):15809-14.

Li H, Xu J, Bian YH, Rotllant P, Shen T, et al. 2011. Smyd1b_tv1, a key regulator of sarcomere assembly, is localized on the M-line of skeletal muscle fibers. *PLoS One* 6(12):e28524.

Li J, Huang J, Li JS, Chen H, Huang K, et al. 2012. Accumulation of endoplasmic reticulum stress and lipogenesis in the liver through generational effects of high fat diets. *J Hepatol* 56(4):900-7.

Li W, Tu D, Brunger AT, Ye Y. 2007. A ubiquitin ligase transfers preformed polyubiquitin chains from a conjugating enzyme to a substrate. *Nature* 446(7133):333-7.

Li W, Tu D, Li L, Wollert T, Ghirlando R, et al. 2009. Mechanistic insights into active site-associated polyubiquitination by the ubiquitin-conjugating enzyme Ube2g2. *Proc Natl Acad Sci U S A* 106(10):3722-7.

Li YX, Yang HT, Zdanowicz M, Sicklick JK, Qi Y, et al. 2007. Fetal alcohol exposure impairs Hedgehog cholesterol modification and signaling. *Lab Invest* 87(3):231-40.

Liang JS, Kim T, Fang S, Yamaguchi J, Weissman AM, et al. 2003. Overexpression of the tumor autocrine motility factor receptor Gp78, a ubiquitin protein ligase, results in increased ubiquitinylation and decreased secretion of apolipoprotein B100 in HepG2 cells. *J Biol Chem* 278(26):23984-8.

Liang J, Yin C, Doong H, Fang S, Peterhoff C, et al. 2006. Characterization of erasin (UBXD2): a new ER protein that promotes ER-associated protein degradation. *J Cell Sci* 119(Pt 19):4011-24.

Lieschke GJ, Currie PD. 2007. Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* 8(5):353-67.

Lilley BN, Ploegh HL. 2004. A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* 429(6994):834-40.

Lilley BN, Ploegh HL. 2005. Multiprotein complexes that link dislocation, ubiquitination, and extraction of misfolded proteins from the endoplasmic reticulum membrane. *Proc Natl Acad Sci U S A* 102(40):14296-301.

Lin JH, Walter P, Yen TS. 2008. Endoplasmic reticulum stress in disease pathogenesis. *Annu Rev Pathol* 3:399-425.

Lindholm D, Wootz H, Korhonen L. 2006. ER stress and neurodegenerative diseases. *Cell Death Differ* 13(3):385-92.

Lorick KL, Jensen JP, Fang S, Ong AM, Hatakeyama S, et al. 1999. RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Natl Acad Sci U S A* 96(20):11364-9.

Malhotra JD, Kaufman RJ. 2007. The endoplasmic reticulum and the unfolded protein response. *Semin Cell Dev Biol* 18(6):716-31.

Marche K, Danel T, Bordet R.2011. Fetal alcohol-induced hyperactivity is reversed by treatment with the PPARα agonist fenofibrate in a rat model. *Psychopharmacology (Berl)* 214(1):285-96.

Meusser B, Hirsch C, Jarosch E, Sommer T. 2005. ERAD: the long road to destruction. *Nat Cell Biol* 7(8):766-72.

Meyer HH, Shorter JG, Seemann J, Pappin D, Warren G. 2000. A complex of mammalian ufd1 and npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. *EMBO J* 19(10):2181-92.

Miranda E, Römisch K, Lomas DA. 2004. Mutants of neuroserpin that cause dementia accumulate as polymers within the endoplasmic reticulum. *J Biol Chem* 279(27):28283-91.

Morito D, Hirao K, Oda Y, Hosokawa N, Tokunaga F, et al. 2008. Gp78 cooperates with RMA1 in endoplasmic reticulum-associated degradation of CFTRDeltaF508. *Mol Biol Cell* 19(4):1328-36.

Mueller B, Klemm EJ, Spooner E, Claessen JH, Ploegh HL. 2008. *Proc Natl Acad Sci U S A* 105(34):12325-30.

Nabi IR, Raz A. 1987. Cell shape modulation alters glycosylation of a metastatic melanoma cell-surface antigen. *Int J Cancer* 40(3):396-402.

Nabi IR, Watanabe H, Raz A. 1990. Identification of B16-F1 melanoma autocrine motility-like factor receptor. *Cancer Res* 50(2):409-14.

Nadav E, Shmueli A, Barr H, Gonen H, Ciechanover A, et al. 2003. A novel mammalian endoplasmic reticulum ubiquitin ligase homologous to the yeast Hrd1. *Biochem Biophys Res Commun* 303(1):91-7.

Nasevicius A, Ekker SC. 2000. Effective targeted gene 'knockdown' in zebrafish. *Nat Genet* 26(2):216-20.

Nielsen LB, Bartels ED, Bollano E. 2002. Overexpression of apolipoprotein B in the heart impedes cardiac triglyceride accumulation and development of cardiac dysfunction in diabetic mice. *J Biol Chem* 277(30):27014-20.

Ostedgaard LS, Rogers CS, Dong Q, Randak CO, Vermeer DW, et al. 2007. Processing and function of CFTR-DeltaF508 are species-dependent. *Proc Natl Acad Sci U S A* 104(39):15370-5.

Ota T, Gayet C, Ginsberg HN. Inhibition of apolipoprotein B100 secretion by lipid-induced hepatic endoplasmic reticulum stress in rodents. *J Clin Invest* 18(1):316-32.

Oyadomari S, Harding HP, Zhang Y, Oyadomari M, Ron D. 2008. Dephosphorylation of translation initiation factor 2alpha enhances glucose tolerance and attenuates hepatosteatosis in mice. *Cell Metab* 7(6):520-32.

Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, et al. 2006. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* 313(5790):1137-40.

Parmar PK, Coates LC, Pearson JF, Hill RM, Birch NP. 2002. Neuroserpin regulates neurite outgrowth in nerve growth factor-treated PC12 cells. *J Neurochem* 82(6):1406-15.

Passeri MJ, Cinaroglu A, Gao C, Sadler KC. 2009. Hepatic steatosis in response to acute alcohol exposure in zebrafish requires sterol regulatory element binding protein activation. *Hepatology* 9(2):443-52.

Pearce MM, Wang Y, Kelley GG, Wojcikiewicz RJ. 2007. SPFH2 mediates the endoplasmic reticulum-associated degradation of inositol 1,4,5-trisphosphate receptors and other substrates in mammalian cells. *J Biol Chem* 282(28):20104-15.

Peer CJ, Sissung TM, Figg WD. 2011. CHIP and gp78-mediated ubiquitination of CYP3A4: Implications for the pharmacology of anticancer agents. *Cancer Biol Ther* 11(6):549-51.

Perlmutter DH. 2002. Liver injury in alpha1-antitrypsin deficiency: an aggregated protein induces mitochondrial injury. *J Clin Invest* 110(11):1579-83.

Polyzos SA, Kountouras J, Zavos C. 2009. Nonalcoholic fatty liver disease: the pathogenetic roles of insulin resistance and adipocytokines. *Curr Mol Med* 9(3):299-314.

Punga T, Bengoechea-Alonso MT, Ericsson J. 2006. Phosphorylation and ubiquitination of the transcription factor sterol regulatory element-binding protein-1 in response to DNA binding. *J Biol Chem* 281(35):25278-86.

Qiu W, Su Q, Rutledge AC, Zhang J, Adeli K. 2009. Glucosamine-induced endoplasmic reticulum stress attenuates apolipoprotein B100 synthesis via PERK signaling. *J Lipid Res* 50(9):1814-23.

Ravid T, Hochstrasser M. 2007. Autoregulation of an E2 enzyme by ubiquitin-chain assembly on its catalytic residue. *Nat Cell Biol* 9(4):422-7.

Reid PC, Sakashita N, Sugii S, et al. 2004. A novel cholesterol stain reveals early neuronal cholesterol accumulation in the Niemann-Pick type C1 mouse brain. *J Lipid Res* 45(3):582-91.

Reimers MJ, Flockton AR, Tanguay RL. 2004. Ethanol- and acetaldehyde-mediated developmental toxicity in zebrafish. *Neurotoxicol Teratol* 26(6):769-81.

Reimold AM, Etkin A, Clauss I, Perkins A, Friend DS, et al. 2000. An essential role in liver development for transcription factor XBP-1. *Genes Dev* 14(2):152-7.

Ruddock LW, Molinari M. 2006. N-glycan processing in ER quality control. *J Cell Sci* 119(Pt 21):4373-80.

Rutkowski DT, Kaufman RJ. 2004. A trip to the ER: coping with stress. *Trends Cell Biol* 14(1):20-8.

Rutkowski DT, Wu J, Back SH, Callaghan MU, Ferris SP, et al. 2008. UPR pathways combine to prevent hepatic steatosis caused by ER stress-mediated suppression of transcriptional master regulators. *Dev Cell* 15(6):829-40.

Sander JD, Cade L, Khayter C, Reyon D, Peterson RT, et al. 2011. Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nat Biotechnol* 29(8):697-8.

Sanyal AJ, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, et al. 2001. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 20(5):1183-92.

Sasagawa Y, Yamanaka K, Ogura T. 2007. ER E3 ubiquitin ligase HRD-1 and its specific partner chaperone BiP play important roles in ERAD and developmental growth in Caenorhabditis elegans. *Genes Cells* 12(9):1063-73.

Schekman R. 2004. Cell biology: a channel for protein waste. *Nature* 429(6994):817-8.

Schröder M, Kaufman RJ. 2005. The mammalian unfolded protein response. *Annu Rev Biochem* 74:739-89.

Schubert U, Antón LC, Gibbs J, Norbury CC, Yewdell JW, et al. 2000. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* 404(6779):770-4.

Schulze A, Standera S, Buerger E, Kikkert M, van Voorden S, et al. 2005. The ubiquitin-domain protein HERP forms a complex with components of the endoplasmic reticulum associated degradation pathway. *J Mol Biol* 354(5):1021-7.

Schwartz AL, Ciechanover A. 2009. Targeting proteins for destruction by the ubiquitin system: implications for human pathobiology. *Annu Rev Pharmacol Toxicol* 49:73-96.

Sever N, Song BL, Yabe D, Goldstein JL, Brown MS, et al. 2003. Insig-dependent ubiquitination and degradation of mammalian 3-hydroxy-3-methylglutaryl-CoA reductase stimulated by sterols and geranylgeraniol. *J Biol Chem* 278(52):52479-90.

Shen Y, Ballar P, Apostolou A, Doong H, Fang S. 2007. ER stress differentially regulates the stabilities of ERAD ubiquitin ligases and their substrates. *Biochem Biophys Res Commun* 352(4):919-24.

Shen Y, Ballar P, Fang S. 2006. Ubiquitin ligase gp78 increases solubility and facilitates degradation of the Z variant of alpha-1-antitrypsin. *Biochem Biophys Res Commun* 349(4):1285-93.

Shimomura I, Bashmakov Y, Ikemoto S, Horton JD, Brown MS, et al. 1999. Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. *Proc Natl Acad Sci U S A* 96(24):13656-61.

Shmueli A, Tsai YC, Yang M, Braun MA, Weissman AM. 2009. Targeting of gp78 for ubiquitin-mediated proteasomal degradation by Hrd1: cross-talk between E3s in the endoplasmic reticulum. *Biochem Biophys Res Commun* 390(3):758-62.

Silletti S, Watanabe H, Hogan V, Nabi IR, Raz A. 1991. Purification of B16-F1 melanoma autocrine motility factor and its receptor. *Cancer Res* 51(13):3507-11.

Soetandyo N, Ye Y. 2010. The p97 ATPase dislocates MHC class I heavy chain in US2-expressing cells via a Ufd1-Npl4-independent mechanism. *J Biol Chem* 285(42):32352-9.

Sommer T, Jentsch S. 1993. A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. *Nature* 365(6442):176-9.

Song BL, Sever N, DeBose-Boyd RA. 2005. Gp78, a membrane-anchored ubiquitin ligase, associates with Insig-1 and couples sterol-regulated ubiquitination to degradation of HMG CoA reductase. *Mol Cell* 19(6):829-40.

Sriburi R, Jackowski S, Mori K, Brewer JW. 2004. XBP1: a link between the unfolded protein response, lipid biosynthesis, and biogenesis of the endoplasmic reticulum. *J Cell Biol* 167(1):35-41.

Stapf C, Cartwright E, Bycroft M, Hofmann K, Buchberger A. 2011. The general definition of the p97/valosin-containing protein (VCP)-interacting motif (VIM) delineates a new family of p97 cofactors. *J Biol Chem* 286(44):38670-8.

Stoletov K, Fang L, Choi SH, Hartvigsen K, Hansen LF, et al. 2009. Vascular lipid accumulation, lipoprotein oxidation, and macrophage lipid uptake in hypercholesterolemic zebrafish. *Circ Res* 104(8):952-60.

Sun F, Zhang R, Gong X, Geng X, Drain PF, et al. 2006. Derlin-1 promotes the efficient degradation of the cystic fibrosis transmembrane conductance regulator (CFTR) and CFTR folding mutants. *J Biol Chem* 281(48):36856-63.

Sundqvist A, Bengoechea-Alonso MT, Ye X, Lukiyanchuk V, Jin J, et al. 2005. Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCF(Fbw7). *Cell Metab* 1(6):379-91.

Szegezdi E, Logue SE, Gorman AM, Samali A. 2006. Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep* 7(9):880-5.

Takahashi A, Motomura K, Kato T, Yoshikawa T, Nakagawa Y, et al. 2005. Transgenic mice overexpressing nuclear SREBP-1c in pancreatic beta-cells. *Diabetes* 4(2):492-9.

Tan X, Rotllant J, Li H, De Deyne P, Du SJ. 2006. SmyD1, a histone methyltransferase, is required for myofibril organization and muscle contraction in zebrafish embryos. *Proc Natl Acad Sci U S A* 103(8):2713-8.

Tarantino G, Conca P, Pasanisi F, Ariello M, Mastrolia M, et al. 2009. Could inflammatory markers help diagnose nonalcoholic steatohepatitis? *Eur J Gastroenterol Hepatol* 21(5):504-11.

Termine DJ, Moremen KW, Sifers RN. 2009. The mammalian UPR boosts glycoprotein ERAD by suppressing the proteolytic downregulation of ER mannosidase I. *J Cell Sci* 122(Pt 7):976-84.

Thakur PC, Stuckenholz C, Rivera MR, Davison JM, Yao JK, et al. 2011. Lack of de novo phosphatidylinositol synthesis leads to endoplasmic reticulum stress and hepatic steatosis in cdipt-deficient zebrafish. *Hepatology* 54(2):452-62.

Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, et al. 2000. Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* 101(3):249-58.

Tsai B, Ye Y, Rapoport TA. 2002. Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. *Nat Rev Mol Cell Biol* 3(4):246-55.

Tsai YC, Mendoza A, Mariano JM, Zhou M, Kostova Z, et al. 2007. The ubiquitin ligase gp78 promotes sarcoma metastasis by targeting KAI1 for degradation. *Nat Med* 13(12):1504-9.

Urasaki A, Morvan G, Kawakami K. 2006. Functional dissection of the Tol2 transposable element identified the minimal cis-sequence and a highly repetitive sequence in the subterminal region essential for transposition. *Genetics* 174(2):639-49.

Vembar SS, Brodsky JL. 2008. One step at a time: endoplasmic reticulum-associated degradation. *Nat Rev Mol Cell Biol* 9(12):944-57.

Vij N, Fang S, Zeitlin PL. 2006. Selective inhibition of endoplasmic reticulum-associated degradation rescues DeltaF508-cystic fibrosis transmembrane regulator and suppresses interleukin-8 levels: therapeutic implications. *J Biol Chem* 281(25):17369-78.

Wang B, Heath-Engel H, Zhang D, Nguyen N, Thomas DY, et al. 2008. BAP31 interacts with Sec61 translocons and promotes retrotranslocation of CFTRDeltaF508 via the derlin-1 complex. *Cell* 133(6):1080-92.

Wang D, Wei Y, Pagliassotti MJ. 2006. Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology* 47(2):943-51.

Wang Q, Liu Y, Soetandyo N, Baek K, Hegde R, et al. 2011. A ubiquitin ligase-associated chaperone holdase maintains polypeptides in soluble states for proteasome degradation. *Mol Cell* 42(6):758-70.

Wang Y, Ballar P, Zhong Y, Zhang X, Liu C, et al. 2011. SVIP induces localization of p97/VCP to the plasma and lysosomal membranes and regulates autophagy. *PLoS One* 6(8):e24478.

Wang Y, Guan S, Acharya P, Koop DR, Liu Y, et al. 2011. Ubiquitin-dependent proteasomal degradation of human liver cytochrome P450 2E1: identification of sites targeted for phosphorylation and ubiquitination. *J Biol Chem* 286(11):9443-56.

Wei Y, Wang D, Topczewski F, Pagliassotti MJ. 2006. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. *Am J Physiol Endocrinol Metab* 91(2):E275-81.

Weissman AM. 2001. Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* 2(3):169-78.

Weissman AM, Shabek N, Ciechanover A. 2011. The predator becomes the prey: regulating the ubiquitin system by ubiquitylation and degradation. *Nat Rev Mol Cell Biol* 12(9):605-20.

Wiertz EJ, Tortorella D, Bogyo M, Yu J, Mothes W, et al. 1996. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature* 384(6608):432-8.

Winget JM, Mayor T. 2010. The diversity of ubiquitin recognition: hot spots and varied specificity. *Mol Cell* 38(5):627-35.

Yamamoto K, Suzuki N, Wada T, Okada T, Yoshida H, et al. 2008. Human HRD1 promoter carries a functional unfolded protein response element to which XBP1 but not ATF6 directly binds. *J Biochem* 144(4):477-86.

Yamamoto K, Takahara K, Oyadomari S, Okada T, Sato T, et al. 2010. Induction of liver steatosis and lipid droplet formation in ATF6alpha-knockout mice

burdened with pharmacological endoplasmic reticulum stress. *Mol Biol Cell* 21(17):2975-86.

Yang H, Liu C, Zhong Y, Luo S, Monteiro MJ, et al. 2010. Huntingtin interacts with the Cue domain of gp78 and inhibits gp78 binding to ubiquitin and p97/VCP. *PLoS ONE* 5(1): e8905. doi:10.1371/journal.pone.0008905.

Yang H, Zhong X, Ballar P, Luo S, Shen Y, et al. 2007. Ubiquitin ligase Hrd1 enhances the degradation and suppresses the toxicity of polyglutamine-expanded huntingtin. *Exp Cell Res* 313(3):538-50.

Yang Y, Ju D, Zhang M, Yang G. 2009. Chronic high dose of insulin stimulates lipolysis in porcine adipocytes. *Sheng Wu Gong Cheng Xue Bao* 5(1):16-22.

Yagishita N, Ohneda K, Amano T, Yamasaki S, Sugiura A, et al. 2005. Essential role of synoviolin in embryogenesis. *J Biol Chem* 280(9):7909-16.

Ye Y. 2005. The role of the ubiquitin-proteasome system in ER quality control. *Essays Biochem* 41:99-112.

Ye Y, Meyer HH, Rapoport TA. 2003. Function of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. *J Cell Biol* 162(1):71-84.

Ye Y, Rape M. 2009. Building ubiquitin chains: E2 enzymes at work. *Nat Rev Mol Cell Biol* 10(11):755-64.

Ye Y, Shibata Y, Kikkert M, van Voorden S, Wiertz E, et al. 2005. Recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane. *Proc Natl Acad Sci U S A* 102(40):14132-8.

Ye Y, Shibata Y, Yun C, Ron D, Rapoport TA. 2004. A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* 429(6994):841-7.

Yin HQ, Kim M, Kim JH, Kong G, Kang KS, et al. 2007. Differential gene expression and lipid metabolism in fatty liver induced by acute ethanol treatment in mice. *Toxicol Appl Pharmacol* 23(3):225-33.

Ying Z, Wang H, Fan H, Wang G. 2011. The endoplasmic reticulum (ER)-associated degradation system regulates aggregation and degradation of mutant neuroserpin. *J Biol Chem* 286(23):20835-44.

Ying Z, Wang H, Fan H, Zhu X, Zhou J, et al. 2009. Gp78, an ER associated E3, promotes SOD1 and ataxin-3 degradation. *Hum Mol Genet* 18(22):4268-81.

Younger JM, Chen L, Ren HY, Rosser MF, Turnbull EL, et al. 2006. Sequential quality-control checkpoints triage misfolded cystic fibrosis transmembrane conductance regulator. *Cell* 126(3):571-82.

Zhong X, Shen Y, Ballar P, Apostolou A, Agami R, et al. 2004. AAA ATPase p97/valosin-containing protein interacts with gp78, a ubiquitin ligase for endoplasmic reticulum-associated degradation. *J Biol Chem* 279(44):45676-84.

Zhong Y, Wang Y, Yang H, Ballar P, Lee JG, et al. 2011. Importin beta interacts with the endoplasmic reticulum-associated degradation machinery and promotes ubiquitination and degradation of mutant alpha1-antitrypsin. *J Biol Chem* 286(39):33921-30.

Zhou QG, Zhou M, Hou FF, Peng X. 2009. Asymmetrical dimethylarginine triggers lipolysis and inflammatory response via induction of endoplasmic reticulum stress in cultured adipocytes. *Am J Physiol Endocrinol Metab* 96(4):E869-78.