Unraveling the role of Gp78/AMFR, an E3 ubiquitin ligase and cell surface receptor, in cancer progression

by

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ABSTRACT

Background/Aim:

This thesis focuses on the role of Gp78/AMFR in cancer progression by (1) investigating the relationship between the dual functions of Gp78/AMFR as a cell surface receptor and intracellular ubiquitin ligase in ERAD; (2) unraveling the post-translational modification (PTM) of Gp78/AMFR selectively targeted by the cancer marker 3F3A mAb and (3) the impact of the PTM on the ubiquitin ligase activity, localization and degradation pathway of Gp78/AMFR.

Results:

Using microRNA mediated gene silencing technology we showed that a significant reduction of total and cell surface expression of Gp78/AMFR in Gp78/AMFR knockdown HEK293 cells; associated with decreased degradation of the established substrates KAI1, a tumor metastasis suppressor and preventing extracellular AMF/PGI dampening of thapsigargin and ATP-evoked ER calcium release and tunicamycin and thapsigargin induced ER-stress and pre-apoptosis. Next, we demonstrated that 3F3A selectively recognizes dephosphorylated S538 of Gp78/AMFR. Furthermore, we showed that serum starvation induced S538 phosphorylation of Gp78/AMFR via a p38 MAPK signaling pathway. Intriguingly, Gp78/AMFR phosphomimetic mutant S538D prevented Gp78/AMFR-dependent degradation of mitofusin 1 and 2, large GTPases essential for mitochondrial fusion. Serum starvation reduced mitofusin degradation by wild-type Gp78/AMFR, but not dominant negative Gp78/AMFR S538A, an effect that was reversed by p38 MAPK inhibition. We also found that S538A Gp78/AMFR mutation promoted peripheral ER distribution, which is consistent with previous reports that Gp78/AMFR mediated ubiquitylation is initiated from the peripheral ER labelled by 3F3A mAb. In addition, we showed that S538D did not alter the turnover of Gp78/AMFR but did signal its proteasomal degradation. In contrast, Gp78/AMFR S538A sensitized Gp78/AMFR for lysosomal degradation, indicating that S538 phosphorylation is a critical determinant of the degradation pathway of this E3 ubiquitin ligase.
Conclusion:

By dissecting the epitope of the malignancy associated 3F3A mAb based on immunohistochemical analysis, this thesis represents the beginning of an understanding of the relationship between the surface cytokine receptor of AMF/PGI and ER-localized E3 ubiquitin ligase Gp78/AMFR. Moreover, it reveals the important role of serine phosphorylation in the regulation of the distribution, ubiquitin ligase activity and degradation pathway of Gp78/AMFR, thereby furthering our understanding of the mechanism of Gp78/AMFR promotion of cancer progression and metastasis.
PREFACE

Chapter 2 is based on two papers published in the Journal of Biological Chemistry entitled: “A role for KAI1 in promotion of cell proliferation and mammary gland hyperplasia by the gp78 ubiquitin ligase” (Joshi, B., Li, L., Nabi, I. R., 2010) and in Cell Death and Differentiation entitled: “Autocrine motility factor/phosphoglucose isomerase regulates ER stress and cell death through control of ER calcium release” (Fu, M., Li, L., Albrecht, T., Johnson, J. D., Kojic, L. D., Nabi, I. R., 2011). I am the second author in both papers and my contribution was genotyping the transgenic mice, testing and validating the specificity of multiple commercially available polyclonal and monoclonal antibodies to Gp78/AMFR, optimizing the Gp78/AMFR western blot and screening multiple cell lines to detect Gp78/AMFR expression, generating the Gp78/AMFR knockdown stable cell lines using microRNA technology and documenting the genotype change of Gp78/AMFR knockdown stable cell lines.

I performed most of the experiments presented in Chapter 3. Dr. Jay Shankar did the experiments presented in Figure 5 and Guang Gao did the western blot in Figure 4C. Dr. Bharat Joshi helped generate the GST-Gp78 WT plasmid. I wrote the manuscript under the supervision of Dr. Ivan Robert Nabi, and the manuscript entitled: “p38 MAPK phosphorylation of the Gp78 E3 ubiquitin ligase” (Li, L., Shankar, J., Gao, G., Joshi, B., Nabi, I.R., 2014) was submitted to the journal and is currently being revised by Guang Gao who has continued the project in the lab.

Chapter 4 was based on a manuscript (in preparation) entitled: “Phosphorylation of serine 538 of ubiquitin ligase Gp78 regulates its distribution in the endoplasmic reticulum and degradation pathway” (Li, L., Nabi, I.R., 2014). I performed all of the experiments in the paper and wrote the manuscript under the supervision of Dr. Ivan Robert Nabi. Dr. St Pierre Pascal helped to set up the experiment and quantification of Figure 3A.
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<tr>
<td>AAT</td>
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<td>CFTR</td>
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<td>Cytosolic E3 ubiquitin ligase C-terminal Hsp70 Interacting Protein</td>
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<td>CP</td>
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<td>HCQ</td>
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<tr>
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<td>Mitofusin 2</td>
</tr>
<tr>
<td>MKK6</td>
<td>MAPK kinase 6</td>
</tr>
<tr>
<td>MKP</td>
<td>MAPK Phosphatases</td>
</tr>
<tr>
<td>MLK</td>
<td>Mixed-Lineage Kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MNK</td>
<td>Mitogen-interacting Kinases</td>
</tr>
<tr>
<td>MSK</td>
<td>Mitogen-and Stress-activated Kinase</td>
</tr>
<tr>
<td>MSV</td>
<td>Moloney Sarcoma Virus</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>mammalian Target Of Rapamycin Complex 1</td>
</tr>
<tr>
<td>MVB</td>
<td>Multivesicular Bodies</td>
</tr>
<tr>
<td>NBR1</td>
<td>Neighbor of BRC1</td>
</tr>
<tr>
<td>NLK</td>
<td>neuroleukin</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 Upregulated Modulator of Apoptosis</td>
</tr>
<tr>
<td>OIS</td>
<td>Oncogene ras-Induced Senescence</td>
</tr>
<tr>
<td>p38IP</td>
<td>p38 α Interacting Protein</td>
</tr>
<tr>
<td>PACS-2</td>
<td>Phosphofurin Acidic Cluster Sorting protein 2</td>
</tr>
<tr>
<td>PAM</td>
<td>Plasma membrane-Associated Membrane</td>
</tr>
<tr>
<td>PARP-14</td>
<td>Poly (ADP-Ribose) Polymerase-14</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate Dehydrogenase</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein Disulphide Isomerase</td>
</tr>
<tr>
<td>PDK</td>
<td>Proline-Directed Kinase</td>
</tr>
<tr>
<td>PDPK</td>
<td>Proline-Directed Protein Kinase</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PERK</td>
<td>PKR (RNA-dependent protein kinase) like ER Kinase</td>
</tr>
<tr>
<td>PGI</td>
<td>Phosphoglucone Isomerase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-Kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma Membrane</td>
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<tr>
<td>PNGase</td>
<td>Peptide N-Glycanase</td>
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<td>PP1</td>
<td>Protein Phosphatase 1</td>
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<tr>
<td>PP2A</td>
<td>Protein Phosphatase 2A</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td>PP2C</td>
<td>protein phosphatase 2C</td>
</tr>
<tr>
<td>PRAK</td>
<td>p38-Regulated or Activated Kinase</td>
</tr>
<tr>
<td>Pru</td>
<td>Pleckstrin-like receptor for ubiquitin</td>
</tr>
<tr>
<td>PT</td>
<td>Pertussis Toxin</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-Translational Modification</td>
</tr>
<tr>
<td>PTP1B</td>
<td>tyrosine phosphatase 1B</td>
</tr>
<tr>
<td>PTP-SL</td>
<td>Protein Tyrosine Phosphatase SL</td>
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<tr>
<td>QC</td>
<td>Quality Control</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic Acid</td>
</tr>
<tr>
<td>RACK1</td>
<td>Receptor for Activated C-Kinase 1</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RCAN1</td>
<td>Regulator of Calcineurin I</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RING</td>
<td>Really Interesting New Gene</td>
</tr>
<tr>
<td>RK</td>
<td>Reactivating Kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RP</td>
<td>Regulatory Particles</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>S1P</td>
<td>Site-1 proteases</td>
</tr>
<tr>
<td>S2P</td>
<td>Site-2 proteases</td>
</tr>
<tr>
<td>SAP-1a</td>
<td>SRF Accessory Protein-1a</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress-Activated Protein Kinase</td>
</tr>
<tr>
<td>SCF</td>
<td>S-phase-kinase-associated-protein-1(SKP1)-Cullin-F-box</td>
</tr>
<tr>
<td>SER</td>
<td>Smooth Endoplasmic Reticulum</td>
</tr>
<tr>
<td>Sig-1R</td>
<td>Sigma-1 receptor</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>SP</td>
<td>Signal Peptidase</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum Response Factor</td>
</tr>
<tr>
<td>SRP</td>
<td>Signal-Recognition Particle</td>
</tr>
<tr>
<td>STEP</td>
<td>Striatal Enriched tyrosine Phosphatase</td>
</tr>
<tr>
<td>SVIP</td>
<td>Small VCP-Interacting Protein</td>
</tr>
<tr>
<td>sXBP1</td>
<td>shorter variant of XBP1</td>
</tr>
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<td>TAA</td>
<td>Tumor-Associated Antigen</td>
</tr>
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</tr>
<tr>
<td>TAK1</td>
<td>TGF-β-Activated-protein Kinase 1</td>
</tr>
<tr>
<td>TAO 1 and 2</td>
<td>Thousand And One amino acid 1 and 2</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic Acid</td>
</tr>
<tr>
<td>TCF</td>
<td>Ternary Complex Factor</td>
</tr>
<tr>
<td>TCR</td>
<td>T-Cell Receptor</td>
</tr>
<tr>
<td>TG</td>
<td>Thapsigargin</td>
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<tr>
<td>TGH</td>
<td>Triacylglycerol Hydrolase</td>
</tr>
<tr>
<td>TMD</td>
<td>Trans-Membrane Domain</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor alpha</td>
</tr>
<tr>
<td>TPL2</td>
<td>Tumor Progression Loci 2</td>
</tr>
<tr>
<td>TRAF2</td>
<td>Tumor necrosis factor Receptor-Associated Factor 2</td>
</tr>
<tr>
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<td>TRB3</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
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<td>Tunicamycin</td>
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<td>Ub</td>
<td>Ubiquitin</td>
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<td>UBA</td>
<td>Ubiquitin associate domain</td>
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<tr>
<td>UBA</td>
<td>Ubiquitin Associated domain</td>
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<tr>
<td>UBD</td>
<td>Ubiquitin-Binding Domain</td>
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<td>UBD</td>
<td>Ubiquitin Binding Domains</td>
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<tr>
<td>UBL</td>
<td>Ubiquitin-Like domain</td>
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<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>UBX</td>
<td>Ubiquitin-regulatory X domain</td>
</tr>
<tr>
<td>UGGT</td>
<td>UDP-Glucose/Glycoprotein Glucosyl Transferase</td>
</tr>
<tr>
<td>UIM</td>
<td>Ubiquitin-Interacting Motif</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase-type Plasminogen Activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>urokinase Plasminogen Activator Receptor</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin-Proteasome System</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-Dependent Anion Channel</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very-Low-Density Lipoprotein</td>
</tr>
<tr>
<td>XBP1</td>
<td>X box Binding Protein 1</td>
</tr>
<tr>
<td>XBP1u</td>
<td>unspliced form of XBP1</td>
</tr>
<tr>
<td>Zap70</td>
<td>ζ – chain-associated protein kinase</td>
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CHAPTER 1. Introduction

Gp78, an E3 ubiquitin ligase, plays an important role in endoplasmic reticulum-associated degradation (ERAD). Gp78 also functions as the receptor for autocrine motility factor (AMF, equivalent to the glycolytic enzyme phosphoglucose isomerase, PGI) at the cell surface, thereby also known as AMFR. AMF/PGI binding to cell surface Gp78/AMFR stimulates a signal transduction cascade that results in enhanced tumor cell motility and metastasis. AMF/PGI has been shown to be internalized to the smooth endoplasmic reticulum through a receptor-mediated, dynamin- and raft-dependent pathway. The Gp78/AMFR-specific 3F3A monoclonal antibody (mAb) recognizes Gp78/AMFR both at the cell surface and a subpopulation of Gp78/AMFR specific to mitochondria-associated smooth endoplasmic reticulum (SER) tubules whose association is regulated by free cytosolic calcium (Goetz et al., 2007; Wang et al., 2000). Importantly, 3F3A mAb mimics AMF stimulation of tumor cell motility in vitro and metastasis in vivo and competes with AMF for binding to Gp78/AMFR. However, the relationship between the surface cytokine receptor of AMF and ER-localized E3 ubiquitin ligase Gp78/AMFR remains unclear. The close association of Gp78/AMFR expression with malignancy, based on immunohistochemical analysis with the 3F3A mAb has been shown in a number of tumor types. Therefore, the aim of the study is to identify and characterize the cancer-specific 3F3A binding motif and thereby further understand the mechanisms of Gp78/AMFR promotion of cancer progression and metastasis.

By dissecting the epitope of the malignancy associated 3F3A mAb, this thesis represents the beginning of an understanding of the relationship between the surface cytokine receptor of AMF and ER-localized E3 ubiquitin ligase Gp78. Moreover, it reveals the important role of serine phosphorylation within the 3F3A mAb binding motif in the regulation of the distribution, ubiquitin ligase activity and degradation pathway of Gp78/AMFR, thereby furthering our understanding of the mechanism of Gp78/AMFR promotion of cancer progression and metastasis.
1.1 The Ten Hallmarks of Cancer

Cancer has become one of the leading killers around the world. In western populations, about 1 person in 5 is destined to die from one or another form of cancer (Weinberg, 2007). The treatment of cancer has achieved tremendous progress but development of drug resistance and metastasis are still big challenges. The hallmarks of cancer acquire various biological capabilities during the multistep development from normal cells to tumorigenic and eventually malignant cells. More than one decade ago, Dr. Douglas Hanahan and Dr. Robert A. Weinberg summarized six hallmarks of cancer, including sustaining proliferative signaling, avoidance of growth suppressor, resisting cell death, enabling replicative immortality, initiation and sustaining angiogenesis and activating invasion and metastasis (Hanahan and Weinberg, 2000). With rapid progress in cancer research in the last decade, four more emerging hallmarks and characteristics of cancer were added to the list: reprogramming cellular energetics, gain of genome instability and unlimited replicative potential, evasion of immune surveillance and creating a tumor microenvironment (Hanahan and Weinberg, 2011).

Scheme 1.1: The ten hallmarks of cancer.

Adapted from (Hanahan and Weinberg, 2011) and reproduced with permission of Elsevier.
1.2 The Role of AMF/PGI and Gp78/AMFR in Acquiring Hallmarks of Cancer

The autocrine motility factor (AMF) is identical to PGI, a glycolytic enzyme released from the cell via a non-classical secretory pathway that acts as a cytokine (Liotta et al., 1986). Gp78/AMFR is the cell surface receptor for the AMF cytokine (Silletti et al., 1991) that also functions as an E3 ubiquitin ligase involved in the ubiquitin-proteasome mediated ERAD. Both AMF/PGI and Gp78/AMFR have well-established roles in tumor progression. Binding to its ligand AMF at the cell surface, Gp78/AMFR stimulates a signal transduction cascade that results in enhanced tumor cell motility and metastasis as well as AMF internalization into smooth ER (Benlimame et al., 1998; Fairbank et al., 2009; Silletti et al., 1991; Tsutsumi et al., 2004; Watanabe et al., 1991a; Watanabe et al., 1991b). In the ER, it plays an important role as an E3 ubiquitin ligase in degradation of the tumor metastasis suppressor KAI1 and mitochondrial fusion proteins mitofusins via ERAD (Fu et al., 2013; Tsai et al., 2007).

AMF was originally identified from a human malignant melanoma cell line A2058 derived from a brain metastasis as a self-producing factor that was able to enhance directed and random migration (Liotta et al., 1986). AMF was genetically identified as a phosphohexose isomerase (PHI)/phosphoglucose isomerase (PGI), neuroleukin (NLK), a neurotrophic factor and maturation factor (MF), which mediates the differentiation of human myeloid leukemic cells (Gurney et al., 1986; Watanabe et al., 1996; Xu et al., 1996). Gp78/AMFR was originally purified from metastatic B16-F1 melanoma cells (Nabi and Raz, 1987; Nabi and Raz, 1988) and was subsequently identified as the receptor of AMF, and thereby named AMFR (Nabi et al., 1990; Nabi et al., 1991; Silletti et al., 1991). Later, based on the similarity of amino acid sequence, the gene encoding the AMFR was predicted to be an E3 ubiquitin ligase of RING finger family (Shimizu et al., 1999). Gp78/AMFR was the first E3 ubiquitin ligase found to be involved in ERAD in mammalian cells (Fang et al., 2001). A close association of Gp78/AMFR expression with malignancy, based on immunohistochemical analysis with the 3F3A mAb, has been described in a number of tumor types (Chiu et al., 2008). Importantly, the anti-Gp78/AMFR 3F3A mAb mimics the effect of AMF by stimulating cell motility in vitro and metastasis in vivo (Nabi et al., 1990; Watanabe et al., 1991b). Previous studies support roles for AMF/PGI and its receptor Gp78/AMFR in all of the ten mechanisms of cancer progression.
mentioned above except genome instability and mutation. Therefore, I will now summarize their roles in cancer progression according to the nine acquired hallmarks of cancer.

Scheme 1.2 The role of AMF/PGI and its receptor Gp78/AMFR in cancer progression.

AMF/PGI and its receptor Gp78/AMFR are involved in various cellular pathways associated with tumorigenesis and metastasis, including (1) glycolysis, (2) matrix remodeling, (3) ERAD and (4) receptor-mediated endocytosis. Image from (Fairbank et al., 2009). Reproduced by permission of the Royal Society of Chemistry (DOI: 10.1039/B820820B).
1.2.1 Sustaining proliferative signaling

Cancer cells produce growth factor ligands themselves, to which they can respond via the expression of cognate receptors, resulting in autocrine proliferative stimulation. AMF functions as cytokine by binding to its receptor Gp78/AMFR upon its secretion. Less than 1% of total cellular AMF/PGI is secreted when it is ectopically expressed (Lagana et al., 2000) and less than 15% and 5% of endogenous Gp78/AMFR is localized at the cell surface and caveolae, an invagination of the plasma membrane, respectively (Benlimame et al., 1998), suggesting that only a small portion of AMF/PGI is secreted to the outside of the cell and Gp78/AMFR transported to the cell surface. Indeed, early studies revealed AMF/PGI to be localized into tubular-like vesicles, diffusely distributed throughout the cytoplasm and not colocalized with any particular cytoskeletal network by immunohistochemical analysis (Niinaka et al., 1998). Moreover, confocal microscopic imaging showed a partial colocalization between AMF/PGI and its receptor, especially on the surface periphery of malignant cells (Niinaka et al., 1998). Intriguingly, both normal and neoplastic cells express the identical AMF/PGI gene product, however, overexpression associated with selective secretion of the protein was observed only in tumor cells, suggesting that its secretion by neoplastic cells is independent of mutation or alternative splicing (Dobashi et al., 2006; Niinaka et al., 1998). Indeed, aberrant secretion of AMF/PGI was observed in the blood and urine sample of patients with various cancers, suggesting a potential prognostic value (Baumann et al., 1990). Later it was found that AMF/PGI is secreted by tumor cells via a non-classical pathway that is regulated by phosphorylation at serine 185 by casein kinase II (CKII) (Haga et al., 2000) and ubiquitylation (Yanagawa et al., 2007). It has been shown that poly (ADP-ribose) polymerase-14 (PARP-14), a binding partner of AMF/PGI enhances its secretion by inhibiting ubiquitylation and degradation of AMF/PGI via the proteasome (Yanagawa et al., 2007). Interestingly, it was found that although non-phosphorylated protein species retain cytokine activity, phosphorylation results in a less active form of PGI by affecting the allosteric kinetic properties of the enzyme (Yanagawa et al., 2005). A further study demonstrated that EGF enhanced AMF secretion by CK-II mediated phosphorylation in HER2 positive breast cancer cells in a PI3K and ERK signaling dependent manner (Kho et al., 2014). On the other hand, the phosphorylation and secretion of AMF/PGI has been found to be negatively regulated by Insulin-like Growth Factor Binding Protein-3 (IGFBP-3) in a dose-dependent fashion (Mishra et al., 2004).
Receptor signaling can also be deregulated by elevating the levels of receptor proteins displayed at the cancer cell surface, rendering such cells hyper-responsive to otherwise limiting amounts of growth factor ligand. For example, our group has demonstrated that elevated surface expression of Gp78/AMFR in both Ras- and Abl-transformed NIH3T3 compared to parental NIH 3T3 cells which is associated with decreased expression of Cav1 and increased AMF/PGI uptake (Le et al., 2002). We also showed the increased surface expression of Gp78/AMFR in tumorigenic MCF7 and metastatic MDA231 and MDA435 breast cancer cell lines relative to dysplastic MCF 10A mammary epithelial cells (Kojic et al., 2007).

Overexpression of AMF/PGI induced cellular transformation and tumorigenicity in NIH-3T3 cells that was dependent upon the PI3K-Akt/PKB signaling pathway and associated with down-regulation of Cav1 (Tsutsumi et al., 2003a). AMF/PGI binding to its receptor AMFR/Gp78 resulted in increased cell proliferation by activating the PI3K/Akt signaling pathway (Haga et al., 2003). Dr. Soichi Tsutsumi also showed that ectopic overexpression of AMF/PGI in fibroblasts accelerates G1 to S cell cycle transition by up-regulation of cyclin D1, cyclin-dependent kinase (CDK) activity and hyperphosphorylation of retinoblastoma protein (Rb) as well as down-regulation of the cyclin-dependent kinase inhibitor p27Kip1 (Tsutsumi et al., 2003b).

1.2.2 Evading growth and metastasis suppressor

Accumulating studies have shown that cell-to-cell contact inhibition of cell proliferation in dense populations of normal cells is abolished in various types of cancer cells. The expression of cell surface Gp78/AMFR is down-regulated in contact-inhibited normal or non-metastatic cells but not transformed and metastatic carcinoma cells (Otto et al., 1994; Silletti et al., 1996; Silletti and Raz, 1993; Silletti et al., 1993). Moreover, up-regulation of Gp78/AMFR expression and down-regulation of E-cadherin, an intercellular adhesion molecule have been shown to be associated with more malignant carcinoma derived from human urinary bladder tissue (Silletti et al., 1993) and poor prognosis in the superficial bladder carcinoma patient group (Otto et al., 1994).

Using a lung metastasis model, Tsai showed that Gp78/AMFR is involved in the metastasis of an aggressive human sarcoma by ubiquitylation and degradation of tumor
suppressor KAI1/CD82. Overexpression of Gp78/AMFR and decreased expression of the metastasis suppressor KAI1 were shown in advanced stages of human tumor tissue by Tissue Microarray (Tsai et al., 2007). KAI1/CD82 is a tetraspanin and belongs to the family of metastasis suppressor genes and is believed to suppress metastasis by multiple mechanisms (Miranti, 2009; Tsai and Weissman, 2011a). These include decreasing cell surface levels of integrin and EGFR by accelerating their internalization, reducing the binding and proteolytic activation of pro-urokinase uPA through inducing the receptor uPAR redistribution into focal adhesions and the association with integrin (Liu and Zhang, 2006) and activation of apoptosis through oxidative stress (Schoenfeld et al., 2004). Our lab has also shown that transgenic mouse overexpression of Gp78/AMFR in mammary gland induces preneoplastic hyperplasia phenotype and downregulation of KAI1 expression. In contrast, Gp78 knockdown results in decreased degradation of KAI1 and cell proliferation. More interestingly, when KAI1 is knocked down by siRNA in the Gp78 knockdown stable cell line, the suppressed cell proliferation can be rescued, suggesting that Gp78/AMFR regulates cell proliferation by degrading its substrate KAI1 (Joshi et al., 2010).

1.2.3 Resisting cell death

13 forms of cell death have been characterized (Galluzzi et al., 2012); apoptosis is the most well-studied and mediated through at least three major pathways regulated from extrinsic signals via death receptors and intrinsic signals from both the mitochondria and the ER (Daniel et al., 2003). Early study showed that mouse fibroblast NIH3T3 cells transfected with AMF/PGI gene secreted AMF and were resistant to the induction of apoptosis evoked by serum deprivation in a PI3K-Akt signaling dependent manner (Tsutsumi et al., 2003a). Furthermore, AMF/PGI-transfected HT-1080 cells that originally secreted high levels of AMF was found to be resistant to mitomyein C-induced apoptosis due to the loss of expression of the apoptotic protease activating factor-1 (Apaf-1) and caspase-9 genes that encode for the proteins that form the “apoptosome” complex. In addition, the loss of the Apaf-1 and caspase-9 was recovered by PKC, PI3K and MAPK inhibitors but not G protein sensitive pertussis toxin (PT) by inhibiting the Gαi protein down stream signaling adenylate cyclase in AMF/PGI-transfected HT1080 cell line. However, only PKC inhibitor could rescue the expression of the Apaf-1 and caspase-9 in
mitomycin-C treated murine ascites Ehrlich cells, suggesting that tumor apoptotic resistance to chemotherapy induced by AMF/PGI is dependent on different signaling in different cell lines to regulate apoptosome formation (Haga et al., 2003). Moreover, AMF/PGI has been found to bind and inhibit IGFBP-3 induced apoptosis in T47D and MCF-7 breast cancer cell lines in a dose-dependent manner, suggesting a role of AMF/PGI in the anti-proliferative pro-apoptotic effects of IGFBP-3 (Mishra et al., 2004). Recent studies from our lab showed that AMF/PGI protects against ER stress-induced cell death via regulation of ER calcium release and PI3K/Akt signaling in a Gp78/AMFR dependent manner (Fu et al., 2010).

1.2.4 Enabling replicative immortality

In addition to apoptosis, senescence is one of the two barriers that cancer cells need to overcome to become immortal in order to form tumors (Hanahan and Weinberg, 2011). Cellular senescence is a state in which cells have irreversibly lost the ability to re-enter the active cell cycle but remain metabolically active. Senescence is characterized by a much larger flattened cytoplasm containing many vacuoles and expression of acidic senescence-associated β-galactosidase (Campisi, 2013; Provinciali et al., 2013; Rodier and Campisi, 2011). It has been shown that inhibition of AMF/PGI by siRNA sensitized human fibrosarcoma cells to cellular senescence induced by oxidative stress by up-regulation of superoxide dismutase (SOD) and p21 cyclin-dependent kinase inhibitor (Funasaka et al., 2007). In addition, Gp78/AMFR was shown to be involved in ubiquitination and degradation of KAI1/CD82 (Tsai et al., 2007) which has also been demonstrated to induce tumor cell senescence upon interaction with the endothelial cell surface protein Duffy Antigen Receptor for Chemokine protein (DARC) (Bandyopadhyay et al., 2006).

1.2.5 Inducing angiogenesis

The formation of capillary blood vessels, also termed as neovascularization, is essential to form a circulatory system at the embryonic phase and wound healing in the adult vertebrate. Solid tumors of more than several millimetres in diameter also rely on neovascularization for nutrition, oxygen supplies, metabolic wastes and carbon dioxide evacuation in order to grow
Tumor growth is accelerated with the induction of angiogenesis, enabling invasion into the surrounding host tissue and dissemination to distant organs. The correlation between high tumor vascular density and poor prognosis of clinical malignancy in numerous malignant tumors has been reported (Gasparini and Harris, 1995; Weidner et al., 1992). Neovascularization results from enzymatic degradation of the basement membrane, endothelial cell migration from pre-existing blood vessels and proliferation to extend the blood vessel into the tumor (Carmeliet and Jain, 2011). Tumor angiogenesis is promoted by angiogenic-stimulating factors such as vascular endothelial growth factor (VEGF) which acts through two tyrosine-phosphorylated trans-membrane high affinity receptors named Flt1/VEGFR-1 and KDR/Flk1/VEGFR-2 (de Vries et al., 1992; Millauer et al., 1993). The two VEGF receptors are almost undetectable in the vessels of healthy tissue but have been shown to be expressed preferentially in the proliferating endothelium of vessels lining and penetrating solid tumors (Hatva et al., 1995; Plate et al., 1992). It has been shown that AMF stimulation leads to 2-fold increased motility of human umbilical vein endothelial cells (HUVEC) by up-regulating Flt1/VEGFR-1 and AMFR/Gp78 expression via a PI3K and PKC-dependent signaling pathway (Funasaka et al., 2001; Funasaka et al., 2002a). However, AMF did not affect proliferation of HUVEC in vitro, consistent with previous reports that KDR/Flk1/VEGFR-2 expression is mainly responsible for endothelial cell proliferation while Flt1/VEGFR-1 is involved in endothelial cell migration (Kanno et al., 2000; Soker et al., 2001; Yanagawa et al., 2004). Moreover, AMF/PGI mixed with Matrigel in liquid form subcutaneously injected into mice has been found to promote capillary-like tube formation (Funasaka et al., 2001). In addition, AMF secreted from AMF/PGI gene transfected HT1080 in a diffusion chamber transplanted into a mouse dorsal sac promotes angiogenesis in vivo (Funasaka et al., 2002a). Furthermore, it has been shown that AMF/PGI up-regulates VEGF and AMFR/Gp78 expression in HT-1080 cells in an autocrine manner and AMFR/Gp78 in HUVEC in vitro in a paracrine manner as well as on the edge of newborn microvessels in vivo (Funasaka et al., 2001; Funasaka et al., 2002a; Funasaka et al., 2002b). These studies suggest that AMF not only acts as an autocrine factor to stimulate AMF producing tumor cell motility, but also induces endothelial cells motility in a paracrine manner to facilitate tumor angiogenesis, highlighting the interaction between the tumor and the host via the crosstalk between AMF/PGI-Gp78/AMFR and VEGF-VEGFR signaling pathways.
1.2.6 Activating invasion and metastasis

Malignant tumors are characterized by their unrestrained growth and invasion into surrounding host tissue. The multistep process of invasion and metastasis has been schematized as a sequence of discrete steps, often termed the “invasion-metastasis cascade”. This depiction envisions a succession of cell-biologic changes, beginning with local invasion of basement membrane by cancer cells, then migration of cancer cells into nearby blood and lymphatic vessels (intravasation) and transport of cancer cells through the blood and lymphatic vessels, followed by escape of cancer cells from the lumen of such vessels into the parenchyma of distant tissues (extravasation), the formation of small nodules of cancer cells in distant organs (micrometastasis), and finally the growth of micrometastatic lesions into macroscopic tumors (colonization) (Hanahan and Weinberg, 2011; Talmadge and Fidler, 2010).

To gain entrance into the microcirculation, tumor cells must degrade connective tissue extracellular matrix and basement membrane components (type IV collagen, laminin and fibronectin) that constitute barriers against invading tumor cells by secreting proteolytic enzymes and glycosidases (Funasaka and Raz, 2007). AMF/PGI has been shown to up-regulate Matrix Metalloproteinase-3 (MMP3) and MMP2 for degradation of matrix and cell invasion by activating the JNK and ERK MAPK signaling pathways (Haga et al., 2008; Torimura et al., 2001; Yu et al., 2004a).

The ability to locomote and migrate is fundamental to the acquisition of invasive and metastatic properties by tumor cells. Cell migration is a multistep process involving changes in the cytoskeleton, cell-substrates adhesions and the extracellular matrix which can be mechanistically broken down into four distinct steps: lamellipodium protrusion, new adhesion formation and turnover, cell body contraction and tail retraction by detachment from the substratum (Ridley, 2001). Protruding from the broad, sheet-like lamellipodia are spike-like structures termed filopodia that are thought to sense the extracellular environment and initiate the formation of focal adhesions by integrins (Ridley, 2001). AMF/PGI has been shown to stimulate integrin-mediated B16a cell adhesion, spreading, and invasion in a 12-lipoxygenases and PKC dependent manner (Timar et al., 1996). Furthermore, activation of Gp78/AMFR by AMF/PGI has been shown to induce an integrin phenotype characteristic of invasive/metastatic melanoma that involves upregulating beta-3 and downregulating beta-1 integrins at the cell surface, which is correlated to their in vivo spontaneous metastatic potential (Timar et al., 2002).
In contrast, AMF/PGI enhances hepatoma cell invasion by upregulation and activation of beta-1 integrin (Torimura et al., 2001). This suggests that integrins may play opposite roles in different cell types and cellular context.

In addition to integrins, Rho GTPases also play key roles in cell motility including affecting the different components of the cytoskeleton, cell-substrate adhesion and extracellular matrix remodeling (Ridley, 2001). AMF/PGI stimulation has been shown to result in cytoskeleton rearrangement in a time and dosage dependent manner in human melanoma cells by activating the Rho signaling pathway. The increased stress-fibre formation is concomitant with redistribution of RhoA to the leading edges of AMF/PGI-treated cells and up-regulation and activation of both RhoA and Rac1 expression but no apparent changes in the expression level or activation state of Cdc42 (Tsutsumi et al., 2002). In addition, a significant positive correlation between expression of Gp78/AMFR and small Rho-like GTPases RhoC, which is involved in the formation of actin stress fibers and focal adhesion contacts (Ridley, 2001), has been observed in hepatocellular carcinoma (HCC) tissues. Moreover, the expression level of GP78/AMFR highly correlated with increasing stage of HCC; with significant high recurrence/metastasis and shorter survival in HCC patients with high-expression of Gp78/AMFR compared with low expression of Gp78/AMFR group. This implicated that elevated expression of RhoC is probably required for the Gp78/AMFR signal transduction and Gp78/AMFR might be a potential prognostic marker for HCC patients (Wang et al., 2007). Further study showed that AMF induced melanoma cells to produce interleukin (IL)-8, a proinflammation cytokine whose principle roles in infection and inflammation appear to involve the recruitment and activation of circulating and tissue neutrophils to sites of tissue damage (Luscinskas et al., 1992). Indeed, IL-8 has been shown to stimulate haptotactic migration and regulate pathological angiogenesis, proliferation, and metastasis in a wide variety of cell type (Brat et al., 2005). The secreted IL-8 induced by AMF/PGI is mediated through ERK signaling dependent pathway and acted in an autocrine manner to promote the migration of early stage melanoma cells, whose migration was inhibited by a specific neutralizing antibody against IL-8. These results suggested that the induction of the migratory ability by AMF may have two pathways: one pathway indirectly stimulated cellular migration dependent on autocrine IL-8 production, and the other pathway directly stimulates cellular migration through the Rho and Rac1 pathways (Araki et al., 2009).
Lamellipodia formation and resulting cell motility are strongly stimulated by a number of growth factors and their cognate tyrosine kinase receptors. Heregulin beta1 (HRG), a combinatorial ligand for human epidermal growth factor receptor (EGFR) 3 and 4 has been demonstrated to be involved in up-regulation of AMF/PGI mRNA expression in a p42/44 MAPK and p38 MAPK signaling dependent manner in MCF-7 breast cancer cells, suggesting a potential role of AMF/PGI regulation by a growth factor in promoting motility and invasiveness of breast cancer cells (Talukder et al., 2000). Furthermore, it was found that overexpression of a leader sequence-fused AMF/PGI to enhance secretion via the classical secretion pathway results in enhanced breast cancer cells motility by altering actin organization, and stimulating transcription of β-catenin/TCF and activating protein 1 (AP1), a marker of motility and invasiveness. Silencing AMF/PGI expression by RNAi attenuated EGF-induced invasion by damping extracellular signal-regulated kinase (ERK) signaling while AMF overexpression overcomes EGFR inhibitor gefitinib inhibited invasion of breast cancer cells by activating HER2 in Akt and ERK signaling dependent manner. This suggests that AMF targeting can offer therapeutic benefit against the gefitinib-resistant phenomena (Kho et al., 2014).

Early studies showed that not only the surface expression but also recycling the Gp78/AMFR to the leading edge of motile cells in response to AMF/PGI are involved in control of cell motility during metastasis. Gp78/AMFR tubulovesicles colocalize with microtubules and extension of the tubulovesicular network to the cell periphery is dependent on the presence of intact microtubules. Gp78/AMFR labeled vesicles can be induced to translocate between the cell center and periphery. Binding of AMF/PGI to its receptor induces the internalization and transport of its receptor to the leading edge of stimulating pseudopodial protrusion and cell motility (Nabi et al., 1992). AMF/PGI also induces Gp78/AMFR internalization to intracellular tubulovesicles including lysosome and transport to the leading edge (Watanabe et al., 1993a). It has been shown that the protein-independent murine fibrosarcoma cells (Gc-4 PF) responded to the anti-Gp78/AMFR 3F3A mAb by 3-fold increased motility in vitro and 8- to 20-fold increased lung colonization in vivo, while the serum-dependent counterpart (Gc-4 SD) failed to respond to motile stimulation both in vitro and in vivo. Immuno-analysis revealed higher cell surface expression of Gp78/AMFR in the Gc-4 PF cells compared to Gc-4 SD cells although both cell lines secrete an equal amount of AMF to the culture media. The results suggested that protein-free culture of fibrosarcoma cells is associated with high response to AMF and with high expression of its receptor, and that autonomous motile regulation may play a role in tumor
dissemination (Watanabe et al., 1993b). In addition, decreased cell motility of murine melanoma cell lines triggered by 3F3A mAb due to retinoic acid (RA) treatment has been shown to be associated with inhibition of cell surface and intracellular expression of Gp78/AMFR in a dosage and time dependent manner (Lotan et al., 1992). Furthermore, AMF/PGI treatment induced up-regulation of surface expression and translocation of Gp78/AMFR from an intracellular perinuclear pool to tubulovesicles which extended to the cell periphery in the high-metastatic (K1735-M1) but not the low-metastatic variant (K1735-Cl.11) of melanoma cells exclusively through stimulation of endogenous expression of 12-lipoxygenase (Silletti et al., 1994). Further study showed that clathrin-mediated endocytosis of AMF/PGI and recycling of Gp78/AMFR to fibronectin is a limiting factor for NIH3T3 cell motility (Le et al., 2000).

The signaling following binding of AMF to its receptor Gp78/AMFR has been shown to be mediated by a pertussis toxin sensitive G protein, inositol phosphate production, phosphorylation the receptor, PKC mediated cytoskeletal protein rearrangement as well as production of lipid second messenger molecules such as 12-(S)-HETE (Nabi et al., 1990; Nabi et al., 1991; Timar et al., 1993; Timar et al., 1999). For example, AMF induced robust translocation of PKCα to the stress fibers and cortical actin, suggesting a critical role for this kinase in the generation of the motility signal (Timar et al., 1999). Another study showed that activation of Gp78/AMFR by AMF/PGI treatment in highly metastatic melanoma cells resulted in enhanced production of a lipoxygenase metabolite of arachidonic acid which affects the cytoskeletal architecture of murine melanoma cells transduction (Silletti et al., 1994; Timar et al., 1993). These results demonstrate that AMF/PGI and its receptor Gp78/AMFR promote the invasiveness and motility of tumor cells through a complicated networking. Indeed, the interaction between the tumor cells and the host microenvironment adds another layer of complexity in the signaling transduction that facilitates cancer metastasis, which will be discussed in further details in section 1.2.8.

1.2.7 Tumor–promoting inflammation

Based on the natural history of certain diseases and epidemiology studies, a strong association has been established between particular chronic inflammatory conditions and eventual tumor progression. Solid tumors require a stroma for their growth and recruit
macrophages to synthesize various essential growth factors that sustain proliferative signaling, survival factors that limit cell death, pro-angiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis that they do not have the capacity to produce. The microenvironment of the local host tissue appears to be an active participant in exchanging cytokines and enzymes with tumor cells that modify the local extracellular matrix, stimulate migration, and promote tumor angiogenesis, proliferation and survival (Schultz, 2003). Inflammation can contribute to multiple hallmark capabilities by supplying bioactive molecules to the tumor microenvironment including inductive signals that lead to activation of Epithelial-to-Mesenchymal transition (EMT) (Karnoub and Weinberg, 2006). EMT is a process that occurs during embryonic development (as stem cells differentiate into various components of the organism) and during cancer progression (as tumor cells gradually acquire a more motile phenotype). Cancer cells that undergo EMT, which entails an intricate series of changes in cell-cell contacts, cell-matrix interactions, and cell signaling, can gain the ability to invade, extravasate and potentially re-establish as a metastatic lesion in a lymph node or distant organ. During EMT, the epithelial phenotype, in which cells are bound together tightly, exhibit apical-basolateral cell polarity and attach to the underlying extracellular matrix, is replaced by a mesenchymal phenotype, characterized by loss of apical-basal polarity, an elongated cell shape, and individual cell migration into the matrix. In this process, loss of E-cadherin expression is specifically associated with loss of epithelial cell-cell adherens junctions. The EMT is also implicated in the regulation of cancer metastasis. Differentiated mesenchymal cells can spread into tissues surrounding the original tumor as well as separate from the tumor, invade blood and lymph vessels, and travel to new locations where they divide and form additional tumors (Hanahan and Weinberg, 2011).

Accumulating studies have indicated that certain tumors may acquire stromal support by inducing some of their own cancer cells to undergo various types of metamorphosis to produce stromal cell types rather than relying on recruited host cells to provide their functions (Hanahan and Weinberg, 2011). Earlier studies showed that loss of E-cadherin associated with increased Gp78/AMFR expression in bladder carcinomas results in a poor urothelial carcinomas outcome (Otto et al., 1997; Otto et al., 1994). Further study showed that upregulated Gp78/AMFR was associated with downregulated E-cadherin expression and increased cellular motility and loss of the polarized epithelial phenotype following cell transformation of epithelial MDCK cells by the invasive Moloney Sarcoma Virus (MSV) (Simard and Nabi, 1996). It has been shown that
overexpression of AMF in human pancreatic cancer cells enhances the liver metastasis associated with down-regulation of E-cadherin and up-regulation of SNAIL, a transcription factor that have been shown to orchestrate the EMT (Tsutumi et al., 2004). However, knocking down of AMF/PGI in HT-1080 human lung fibrosarcoma cells and mesenchymal human osteosarcoma cells prevents secretion of AMF/PGI and loss of tumorigenicity and pulmonary metastasis in nude mice by inducing mesenchymal-to-epithelial transition (MET) (Funasaka et al., 2007; Niinaka et al., 2010). Furthermore, it has been shown that ectopic expression of AMF/PGI induced EMT in MCF10A normal human breast epithelial cells, and inhibition of AMF/PGI expression triggered MET in aggressive mesenchymal-type human breast cancer MDA-MB-231 cells. These results suggest for the first time that AMF/PGI is a key gene to both EMT in the initiating steps of cancer metastasis and MET in the later stages of metastasis during breast cancer progression (Funasaka et al., 2009). Further study revealed that silencing of PGI/AMF expression in MDA-MB-231 cells led to overexpression of miR-200s, which was associated with reversal of EMT phenotype as shown by alterations in the relative expression of epithelial (E-cadherin) and mesenchymal (vimentin, ZEB1, ZEB2) markers and decreased aggressiveness (Ahmad et al., 2011).

AMF secreted from Ehrlich mouse ascites tumor cell lines has been shown to induce gaps in an endothelial or mesothelial monolayer from host peritoneal wall and diaphragm by upregulation of Gp78/AMFR expression and stimulating a cellular movement which results in accelerating ascites accumulation that could be dramatically reduced by anti-AMF antibody. This suggests that AMF is able to affect directly host tissue cells and causes hyperpermeability of microvasculature supplying peritoneal lining tissues and subsequent extravasation of a plasma-rich exudate that contribute to the progressive growth of tumors by loosening of the mesothelial cell sheet (Funasaka et al., 2002b). Therefore, it appears that the autocrine and paracrine signaling of AMF may contribute to both recruiting host cells and converting epithelial carcinoma cells into mesenchymal, fibroblast-like cancer cells that may well assume the duties of cancer-associated fibroblasts (CAFs) in some tumors to provide the tumor microenvironment.
1.2.8 Reprogramming energy metabolism

In addition to uncontrolled cell proliferation and motility, the energy metabolism of most cancer cells differs dramatically from that of normal cells which has been termed “aerobic glycolysis”, a trait first described nine decades ago by Otto Warburg and thereby also termed the “Warburg effect” (Bensinger and Christofk, 2012). Unlike normal cells that generate energy by oxidation of pyruvate in the citric acid cycle in mitochondria, most cancer cells produce energy by a high rate of glycolic catabolism to lactate in the cytosol even in the presence of sufficient oxygen (Bensinger and Christofk, 2012). Emerging evidence over the past decade has revealed that alterations of oncogenes and tumor suppressors that drive the cancer-promoting signals also drive the reprogrammed glucose metabolism in cancer cells, however, the underlying molecular basis remains largely unknown (Gao et al., 2012). It is well known that oxidative phosphorylation generates up to 36 ATPs upon complete oxidation of one molecule of glucose whereas glycolysis produces only 2 ATPs per molecule of glucose. The immediate question arises as to why cancer cells would switch to a much less efficient form of energy production. As a matter of fact, the increase in aerobic glycolysis which confers a selective growth advantage, thereby promotes unconstrained proliferation and invasion has been proposed to be a solution for the pre-cancer cells to adapt to the intermittent hypoxia found in pre-malignant lesions (Gatenby and Gillies, 2004).

AMF/PGI plays a key role in both glycolysis and gluconeogenesis by catalyzing the interconversion of glucose 6-phosphate and fructose 6-phosphate (Faik et al., 1988). It has been reported that AMF/PGI is induced by hypoxia in various cancer cell lines which is mediated by up-regulation of transcription factor hypoxia-inducible factor (HIF)-1α in a PI3K signaling dependent manner (Funasaka et al., 2005; Yoon et al., 2001). In addition, tumor cell motility is suppressed by inhibitors of AMF/PGI, suggesting that extracellular AMF plays an important role in enhanced tumor metastasis under hypoxia (Funasaka et al., 2005). However, given the link between the extracellular and intracellular AMF/PGI, the possibility that malignant tumor migration and hypermetabolism of glucose resulted from the increased expression and catalytic enzymatic activity of PGI induced by the hypoxia cannot be ruled out. Indeed, the catalytic domain of AMF/PGI has been shown to overlap the cytokine domain since inhibitors of the catalytic enzyme activity of AMF/PGI such as mannose 6-phosphate also inhibits the cytokine function of this protein in cancer cell motility (Watanabe et al., 1996).
In addition to macromolecular synthesis driven by reduced citric acid cycle activity (Vander Heiden et al., 2009), the Warburg effect also plays a primary role in maintaining an acidic pH of the tumor microenvironment by accumulation of lactic acid (Mazzio et al., 2012). Acid-induced conformation change of the tertiary structure of AMF/PGI was shown to result in dramatic increase of direct binding with extracellular matrix fibronectin (FN) independent of receptor at the cell surface which is different from the receptor-dependent binding with FN at neutral pH (Amraei et al., 2003; Lagana et al., 2005). In addition, at acid pH, AMF/PGI binds directly to heparin sulphate proteoglycan (HS) which also inhibits the endocytosis of AMF/PGI (Lagana et al., 2005). As mentioned in section 1.2.5, AMF/PGI stimulates angiogenesis via the paracrine stimulation of VEGFR-1/Flt-1 expression. In addition to AMF/PGI, VEGF has also been reported to exhibit pH dependent binding to FN (Amraei et al., 2003; Goerges and Nugent, 2003; Goerges and Nugent, 2004). Therefore, pH-dependent association with FN may serve to sequester angiogenic cytokines under conditions of hypoxia to concentrate proangiogenic factors at sites of tissue damage to promote angiogenesis towards the damaged region upon resolution of the tissue insult (Lagana et al., 2005). In summary, based on the above data, AMF/PGI not only may play important role in inducing Warburg effect but also be affected by the effect to promote cancer progression through enhancing cell motility and angiogenesis.

Accumulating studies demonstrated that PI3K signaling pathway is involved in Warburg effect in cancer cells in that this signaling pathway is linked to both glucose metabolism and growth control (Vander Heiden et al., 2009). In addition to directing available amino acids into protein synthesis via mTOR, PI3K pathway activation also renders cells dependent on high levels of glucose flux. Receptor mediated endocytosis of extracellular AMF/PGI has been shown to be up-regulated in invasive breast cancer cells in PI3K signaling dependent manner (Kojic et al., 2007). Recently, Gp78/AMFR has been demonstrated to be responsible for glucose intolerance and hyperlipidemia by showing an increased energy expenditure and utilization of glucose in liver-specific Gp78/AMFR knockout mice associated with dramatic elevated Fibroblast Growth Factor (FGF) 21 expression whose major role in glucose uptake in adipocytes has been well established (Kharitonenkov et al., 2005; Liu et al., 2012). Given the effect of the pH on receptor mediated endocytosis of AMF/PGI, it will be very interesting to investigate the link between Warburg effect and the role of Gp78/AMFR mediated uptake of AMF/PGI in response to PI3K signaling in regulation of glucose metabolism.
1.2.9 Evading immune destruction

It is well accepted that tumor cells thrive in an immunocompromised environment. In fact, tumors can create localized microenvironments where immune function is compromised by TGF-β (Wahl et al., 2004), IL-10 (Tsuruma et al., 1999) and FasL (Webb et al., 2002) mediated mechanisms to keep functional cytotoxic cells such as NK cells and macrophages at some distance. Therefore, immunotherapy has been successfully used for various tumors treatment combined with chemotherapy and radiotherapy.

By far the best known of the passive immunization treatment involves the humanized monoclonal antibody termed Herceptin, also called trastuzumab which reacts strongly with the EGFR-related protein human epidermal growth factor receptor (HER) 2 which is overexpressed in as many as 30% of the breast cancers (Slamon et al., 1987). Although Herceptin were originally developed by blocking growth factor receptor–mediated autocrine/paracrine proliferation, accumulating studies have demonstrated that HER2 targeted by Herceptin is indeed a tumor-associated antigen (TAA) (Hudis, 2007; Spector and Blackwell, 2009). The therapeutic activity of Herceptin critically depends on the involvement of FcγR-expressing lymphocytes mediated antibody-dependent cellular cytotoxicity (ADCC) in which, NK cells and macrophages kill the HER2 overexpressing breast cancer cells by binding the constant regions of IgG antibody molecules that coat the surface of Herceptin treated breast cancer cells through their Fcγ receptors (Clynes et al., 2000; Petricevic et al., 2013; Spiridon et al., 2004). Accumulating studies have shown that Herceptin mediated ADCC paly important role in growth inhibition of various cancer cells expressing HER2 (Collins et al., 2012; Kawaguchi et al., 2009; Petricevic et al., 2013). Indeed, Herceptin has become the standard of therapy for patients with HER2 expressing breast cancers, however, this drug only yields response rates of 12% to 35% when administered as a monotherapy (Petricevic et al., 2013). Moreover, many HER2-positive patients initially or eventually become resistant to this treatment (Arteaga et al., 2012; Kawaguchi et al., 2009). Recently, it was found that AMF can bind and induce HER2 cleavage which results in ectodomain-deleted p95HER2, a constitutively active form receptor by triggering HER2 phosphorylation and metalloprotease-mediated ectodomain shedding, which in turn activates phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling. Therefore, this signaling provides a mechanism of Herceptin resistance developed in patients with HER2 positive breast carcinoma (Kho et al., 2013). Because AMF/PGI mediated
binding and cleavage of HER2 might result in masking or losing the tumor-associated antigen HER2 and subsequent Herceptin induced ADCC, the HER2 expressing breast cancer cells treated by Herceptin may escape the immune surveillance by up-regulation of AMF/PGI. Indeed, Herceptin-mediated inhibition of AMF mRNA expression has been shown to be reversed by Heregulin (HRG), the combinatorial ligand of HER3 and HER4 (Talukder et al., 2000; Talukder et al., 2002). Therefore, this AMF/PGI mediated ablation of the ability of Herceptin to induce ADCC may offer another possible mechanism of Herceptin resistance developed in patients with HER2 positive breast carcinoma. Direct evidence showing that AMF/PGI and Gp78/AMFR function in tumor cell evasion of immune surveillance has not yet been reported as most studies of the role of AMF and Gp78/AMFR in tumorigenesis and metastasis used nude mice and SCID mice in which the immune system of the animal model has been compromised (Onishi et al., 2003; Timar et al., 2002; Tsai et al., 2007; Tsutsumi et al., 2003b; Tsutsumi et al., 2004). However, the fact that the patients with HER2 positive breast carcinoma developed Herceptin resistance provides a clue that AMF/PGI mediated binding and cleavage of HER2 might play a role in facilitating the breast carcinoma to escape from the immune surveillance.

In summary, given the role of AMF/PGI and Gp78/AMFR in acquiring the ten hallmarks of cancer, it is believed that blocking AMF/PGI and Gp78/AMFR mediated signaling can be beneficial to delay cancer progression and metastasis.

1.3 The Role of Gp78/AMFR in ERQC and ERAD

1.3.1 Protein quality control and ERQC

Proteins are usually co-translationally inserted into the ER through a narrow aqueous channel formed by the heterotrimeric Sec61 complex; which is coupled to early steps in protein maturation, including the addition of N-linked oligosaccharides, chaperone-mediated folding associated with specific disulfide bond formation and also sometimes complex assembly into functional receptors or channels (Tsai and Weissman, 2011b). Protein quality control, which is performed by intricate collaboration among chaperones and targeted protein degradation,
functions to minimize the level and toxicity of misfolded proteins in the cell. Indeed, around one third of the eukaryotic genome encodes membrane and secretory protein (Diehn et al., 2006), most of which are folded and assembled in the ER. ER quality control (ERQC) is an elaborate surveillance system to facilitate folding and modification of secretory and membrane proteins and eliminate terminally misfolded polypeptides through ERAD or autophagic degradation (Araki and Nagata, 2011). The process of glycosylated protein quality control, especially of N-glycosylated proteins, has been extensively studied relative to unglycosylated protein quality control mechanisms. The whole process of ERQC of N-glycosylated proteins can be roughly divided into five steps as shown in Scheme 1.3. (A) **Co-translational translocation.** A *de novo* synthesized polypeptide bearing the ER signal sequence is bound to a signal-recognition particle (SRP) which guides the nascent polypeptide/ribosome complex to the narrow Sec61 translocon channel through interaction with the SRP receptor in the ER membrane. As the polypeptide chain elongates, it passes through the translocon channel into the lumen of the ER where the signal sequence is degraded by the signal peptidase (SP). Folding of nascent polypeptides commences during translocation and is assisted by various chaperones residing in the ER lumen such as BiP/GRP78 of the Hsp70 family. In the ER, translocated proteins undergo modifications such as N-linked glycosylation in which the oligosaccharide is transferred from the dolichol donor onto asparagine residues of a specific sequence (Asn-X-Ser/Thr) of newly forming polypeptide chains catalyzed by the oligosaccharyl transferase (OST) complex. After that, the two outermost glucose residues are sequentially removed by glucosidases I and II (GI & GII). (B) **Posttranslational membrane insertion.** Tail anchored proteins translated in the cytosol have recently been shown to insert into the ER membrane with the aid of the Bat3 complex which recognizes the C-terminal single trans-membrane domain (TMD) of the protein and transfers the protein to the cytoplasmic chaperone TRC40/Get3 for targeting to the ER-membrane localized Get1/2 receptor. (C) **CNX/CRT cycle.** Once GI and GII have removed the two terminal glucose residues of the N-linked oligosaccharide, glycoproteins in the ER can be recognized by the lectin-chaperone calnexin (CNX) or its soluble luminal homolog calreticulin (CRT). The binding of CNX/CRT to unfolded, monoglycosylated glycoproteins allows such glycoproteins time to achieve their proper three-dimensional conformation by preventing them from aggregation and premature export from the ER. CNX also recruits the oxidoreductase ERp57 of PDI family to promote the proper disulfide-bond formation during the glycoprotein folding attempts. Then trimming of the terminal glucose residue by GII releases the polypeptide
from CNX/CRT. The folding state of the glycoprotein is monitored by the folding sensor UDP-glucose/glycoprotein glucosyl transferase (UGGT) which senses exposed hydrophobic regions on unfolded or misfolded glycoproteins. The misfolded protein is reglycosylated by UGGT and sent for another round of folding by interaction with CNX and ERp57. During this step, the glycoprotein is shuttled between the peripheral smooth ER and a juxtanuclear compartment termed ERQC compartment (ERQCC), possibly with the aid of CNX/CRT or Bap31 (this will be described in more detail in section 1.4.3 and section 1.4.4). (D) **ER exit.** The correctly folded glycoproteins that have achieved their native three-dimensional structures and passed the monitoring criteria of UGTT are released from the CNX/CRT cycle and packed by COPII vesicles for transport to their destination such as plasma membrane and various organelles of the cell via Golgi complex. (E) **Degradation pathway.** Terminally misfolded glycoproteins that cannot fold properly after several CNX/CRT folding cycles are delivered to be degraded through ERAD or autophagic degradation. Extensive trimming of the mannose of the glycoproteins is a stringent requirement for defective glycoproteins to be targeted to ERAD. Indeed, those misfolded proteins are probably confined into the ERQCC where ERManI and EDEM family proteins are enriched so that the mannose residues in their N-glycans are extensively trimmed allowing them to be recognized by the lectins OS9 and XTB3-B and delivered to the ERAD machinery for ubiquitylation by the ubiquitin ligases complexes such as HRD1 or GP78 and sent to proteasomal degradation in the cytosol (Araki and Nagata, 2011; Benyair et al., 2011; Leitman et al., 2013).
1.3.2 E3 ubiquitin ligases in ERAD

Ubiquitylation

Ubiquitylation is a post-translational modification of proteins in which the covalently attachment of the 76 residue polypeptide ubiquitin (Ub) to substrates serves as a molecular tag. It plays an important regulatory role in various critical cellular processes in eukaryotes including targeting of proteins for proteasomal and lysosomal degradation, DNA repair, transcription, and protein trafficking within the secretary and endocytic pathways (Ande et al., 2009). Ubiquitylation of proteins is a highly ordered cascade of enzymatic process through E1 Ub-activating enzymes, E2 Ub-conjugating enzymes and E3 Ub ligases as well as E4 Ub elongation
factors and deubiquitylation enzymes DUBs (Trempe, 2011; Tsai and Weissman, 2011b). E1 binds and activates the C-terminus of Ub in an ATP-dependent manner. Once Ub is activated, it is transferred to E2 and subsequently to E3 ligase which transfers Ub to a substrate. As a result, the carboxyl group of Gly76 of Ub forms an isopeptide bond with, most typically, the ε amine group of a lysine residue within substrates followed by additional Ubs added to the first using the terminal carboxylic group of glycine 76 on one Ub (G76) and lysine 48 (K48) of another Ub molecules, generating a chain of at least four or five Ubs, a process known as polyubiquitylation. In addition to the K48-linked polyubiquitin chain which serves as a signal for proteasomal degradation, Ub has another six lysines (K6, K11, K27, K29, K33, K63) that have been shown to form polyubiquitin chains, of which the K63-linked polyubiquitin chain has been well characterized in DNA repair and NF-κB signaling (Trempe, 2011; Xu et al., 2009). The seven lysines of ubiquitin provide for the formation of different three-dimensional structures by adopting different isopeptide chain linkages, all of which are represented in eukaryotic cells (Xu et al., 2009). In contrast to polyubiquitylation, single Ub molecule can be added to different lysine residues on the target protein so that the final stoichiometry is one Ub per lysine on one or multiple lysines of the target protein, a process known as monoubiquitylation or multiubiquitylation which affects protein trafficking, transcriptional regulation and DNA repair (Ramanathan and Ye, 2012). E4 elongation factors are responsible for elongation of short polyubiquitin chain whereas DUBs dismantle these chains by removing Ub from polyUb chains or specific substrates with various degrees of linkage selectivity, a process opposing ubiquitylation termed deubiquitylation (Trempe, 2011; Tsai and Weissman, 2011b). The human genome encodes two E1s, approximately 50 E2s, 600–1000 E3s and 100 DUBs (Ande et al., 2009; Ramanathan and Ye, 2012). The specific combination of E2 and E3 enzymes recruited to a substrate dictates the chain linkage type which provides versatility of target proteins modified with different modes of ubiquitylation for different biological process as summarized in Scheme 1.4. To discriminate these different structures, the human genome encodes more than 20 different types of ubiquitin-binding domains (UBDs) in over 200 proteins (Clague and Urbe, 2010; Dikic et al., 2009; Ikeda et al., 2010). In fact, to target proteins to different pathways and fates, it is important to generate the specificity between proteins containing a particular UBD and distinct 3D dimensional Ub linkage structures (Sadowski et al., 2012).
Scheme 1.4: Different structural diversity of ubiquitylation results in different substrates fates.

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The process of ERAD

ERAD is an important protein quality control mechanism to ensure misfolded, misassembled and non-functional proteins are degraded and to maintain efficient protein folding at steady state. ERAD is a highly coordinated process including recognition of misfolded protein, ubiquitylation, deglycosylation, retrotranslocation and degradation (Vembar and Brodsky, 2008) to guarantee aberrant proteins are destroyed and properly folded proteins are routed to their final destination. Much effort has been expanded towards identifying the proteolytic system in the ER using the unassembled components of the T cell antigen receptor and further details have been revealed since the 1980s (Klausner and Sitia, 1990; Tsai and Weissman, 2011b; Vembar and Brodsky, 2008). Firstly, misfolded proteins containing defective lesions localized at the cytosolic face or lumen of the ER or embedded in the membrane of the ER are recognized by ER chaperones and associated factors such as BiP, CNX, CRT and protein disulphide isomerases (PDIs). Then the substrates are partially translocated through a protein
conducting channel called the retrotranslocon which consists of Sec61 complex, Derlin-1 and transmembrane region of E3 ubiquitin ligases such as Hrd1. Thirdly, partially translocated substrates are tagged with polyubiquitin chain signals by ER-resident ubiquitin ligases E3 such as Gp78 and Hrd1 and their cognate ubiquitin conjugating E2 enzymes, a process called ubiquitylation, on the cytosolic face of the ER membrane as mentioned above. Fourthly, the polyubiquitylated substrates are further dislocated to the cytosolic face of the ER by the p97/VCP (Cdc48 in yeast) complex which is comprised of a hexamer of the AAA ATPase with ubiquitin binding activity and substrate-recruiting cofactors Ufd1 and Npl4 heterodimer. p97/VCP complex functions as a ratchet and provides the driving energy of retrotranslocation derived from ATP hydrolysis. More importantly, DUBs such as Ataxin-3 (OTU1 in yeast) and E4 elongation factors such as Ufd2 have been found to be associated with the p97/VCP complex and are involved in editing the polyubiquitin chains of the substrates to the optimal length. In addition, peptide N-glycanase (PNGase) which is involved in deglycosylation of substrates has also been shown to bind directly to p97/VCP complex. Finally, the dislocated substrates are delivered to the 26S barrel like proteasome which contains 19S regulatory cap and the 20S catalytic core particle. The 19S regulatory cap captures the ubiquitylated substrates, removes Ub chains from the substrates and recycles the dismantled Ub, unfolds the substrates and drive the substrates to the 20S catalytic core particle bearing the peptidase activities responsible for the degradation of substrates, which will be discussed in further detail in section 1.5.1 (Raasi and Wolf, 2007; Tsai and Weissman, 2011b; Vembar and Brodsky, 2008).

**E3 ubiquitin ligases in ERAD machineries**

Given the large number of genes encoding E3 ligases in human genome, it is not surprising to found that substrate specificity is conferred by E3 ligases combined with the specific E2 conjugating enzymes. E3 Ub ligases have been classified into two major families based on how the E3 facilitates Ub transfer from E2s to substrates. The largest class is RING (Really Interesting New Gene) domain E3s which contain a cysteine-rich tandem zinc finger motif of 40-60 amino acids while the minority are the HECT (Homologous to E6-AP C-Terminus) domain E3s which form an essential Ub intermediate prior to transferring Ub from the E2 enzymes to the substrates in contrast to RING ligases. Furthermore, the RING type E3s can be subdivided into monomeric ubiquitin ligases such as Gp78 and the multi-subunit E3s
assembled around Cullin or Cullin homology such as S-phase-kinase-associated-protein-1(SKP1)-Cullin-F-box (SCF) E3 complex (Ande et al., 2009; Vembar and Brodsky, 2008). E3 ligases are often assembled into large complexes with various components including chaperons, folding enzymes and deglycosylation and retrotranslocation enzymes, that coordinately work together to function in ERAD. Accumulating studies suggests that depending on the location of the lesions in the misfolded protein, different substrates are targeted to different ERAD machineries, in that proteins with lesions in the cytoplasmic, luminal and membrane-spanning domains follow the ERAD-C, ERAD-L and ERAD-M pathways, respectively. In yeast, two separate ERAD machineries named HRD1 and DOA10 complex organized around membrane-associated E3 ligases of RING finger family Hrd1 and Doa10 have been well studied. HRD1 is involved in degradation of misfolded proteins containing lesions in both the luminal and membrane-spanning domains of the ER (ERAD-L and ERAD-M) whereas DOA10 works on substrates with defective regions on the cytosolic side of the ER (ERAD-C) (Araki and Nagata, 2011; Kostova et al., 2007; Vembar and Brodsky, 2008). However, in mammalian cells, the distinctions between the pathways became blurred as higher eukaryotes possess a more elaborate repertoire of ERAD machineries including HRD1, RAM1, CHIP, GP78, TEB4 and TRC8, etc. that function redundantly to increase degradation efficiency and allow the cell to compensate for substrate overload in one or the other pathway (Tsai and Weissman, 2010; Vembar and Brodsky, 2008). For example, Hrd1, RAM1, CHIP and Gp78 have been shown to overlap in degradation of CFTRΔF508 (Ballar et al., 2010; Morito et al., 2008; Younger et al., 2006).

The ERAD machineries assembled around E3 ligases in yeast and mammalian cells share several similarities as shown for the HRD1 ligase complex in Scheme 1.5. In yeast, the cognate E2 conjugating enzyme Ubc7 associated with the core component of multi-transmembrane E3 ligase Hrd1 is Ube2j1 which bounds to Cue1 in the HRD1 complex while in mammalian cells, the cognate E2 conjugating enzyme is Ube2g2 in GP78 complex which contains Cue domain by itself. The mammalian homologues of lectin Yos9 in yeast are OS-9 and XTP3-B, locating in the luminal side of ER. The lectins function as adaptor proteins to recruit the nonglycosylated or glycosylated substrates in the ER lumen to the E3 ubiquitin ligases. Protein chaperone BiP, associated with misfolded proteins in mammalian cells and acting as an ER stress sensor in UPR is equivalent to the Hsp70 family protein Kar2 in yeast located in the lumen of the ER and recruited to the Hrd1 ligase complex by substrate receptor
Hrd3 (equivalent to SEL1L in mammals) anchored in the ER. In addition, Derlin-1, the mammalian homolog of Der1 in yeast, which spans the ER membrane four times, functions as a component of the retrotranslocation channel. Derlin is found to interact with both multi-transmembrane Hrd1 and Gp78 ubiquitin ligases via the double spanning membrane adaptor protein HERP in mammalian cells and Usa1 in yeast, respectively. Furthermore, the AAA ATPase complex p97, the homolog of Cdc48 complex associated with its co-factors Npl1 and Ufd1, serves to dislocate substrates from the luminal to the cytosolic side of the ER in mammalian cells. The complex is recruited to the Hrd1 ubiquitin ligases through Ubx2 which has two membrane spans with both N- and C-termini facing the cytosolic of ER or the p97/VIM binding motif of Gp78 ligase. More interestingly, the N-terminal of Ubx2 containing an UBA (Ubiquitin associate) domain and C-terminal compassing an UBX (ubiquitin-regulatory X) domain serves as binding sites of p97 complex, suggesting that multiple factors contribute to the recruitment of the p97 complex to the ubiquitin ligase in addition to the polyubiquitylated substrates. The interaction between the p97 complex and the cytoplasmic proteasomes which finally degrade the substrates depends on the interactions of multiple ubiquitin binding motifs present in both the p97 complex and the 19S regulatory cap of the proteasome with the polyubiquitylated substrates. It has been summarized by Raasi Shahri in 2007 (Raasi and Wolf, 2007) and will be discussed in further details in section 1.5.1 (Araki and Nagata, 2011; Benyair et al., 2011; Kostova et al., 2007; Vembar and Brodsky, 2008).
Scheme 1.5: ERQC and ERAD machinery in yeast and mammalian cells.

Image from (Benyair et al., 2011) and reproduced with permission from Elsevier (DOI: 10.1016/B978-0-12-386033-0.00005-0).
1.3.3 The functional domain and substrates of Gp78/AMFR in ERAD

The function of Gp78 as a RING-finger E3 ubiquitin ligase has been well characterized. Gp78 has multiple N-terminal transmembrane domains, thought to potentially form the retrotranslocation channel with Derlin-1 (Ye et al., 2005; Ye et al., 2004). The Gp78 C-terminal domain contains a catalytic RING finger, CUE motif, dimerization domain as well as E2 ubiquitin conjugation enzyme (Ube2g2) and p97 binding domains (Chen et al., 2006a; Fang et al., 2001; Li et al., 2009b). The RING finger domain, composed of six conserved cysteines and two histidines that coordinate two Zn ions in a “cross-braced” fashion, is responsible for catalyzing Ub transfer from the E2 ubiquitin conjugation enzyme to its substrates (Fang et al., 2003). The dimerization domain between the RING and CUE domains has been reported to facilitate the ubiquitin chain elongation on E2 ubiquitin conjugation enzyme, Ube2g2, in order to transfer pre-assembled polyubiquitin chain to its substrates cytosolic domain of human HERP (HERPc) (Li et al., 2009b). The G2BR binding domain dramatically increases binding affinity between Gp78 and Ube2g2 facilitating Gp78-mediated ubiquitin transfer from the E2 to its substrates (Das et al., 2009). The p97 binding domain directly binds the cytosolic p97 ATPase complex composed of p97, Small VCP-Interacting Protein (SVIP), Ufd1, Npl4 and PNGase (Li et al., 2005). p97 also known as VCP or CDC48, is a hexametric ATPase with multiple cellular activities belonging to the AAA family that provides the “driving force” to extract ERAD substrates from the lumen of the ER to the cytosol (Ballar et al., 2006; Ballar et al., 2007; Li et al., 2005; Ye et al., 2005; Zhong et al., 2004). The activity of Gp78 in the ERAD relies on various regulatory binding partners. It has been shown that Ufd1 (Cao et al., 2007), SVIP (Ballar et al., 2007) and its substrate Huntingtin (Yang et al., 2010) compete the p97 complex binding with Gp78 by sequestering p97 complex with other binding partners in a mutually exclusive manner.

Three different models of Gp78 mediated ubiquitylation of substrates have been proposed (Fairbank et al., 2009). Gp78 acts as E3 ubiquitin ligase in the “sequential model” where the E2 conjugated Ub is recruited and a poly-Ub chain is formed by sequential addition of Ub moieties and in the “preassembled model” where E3 ubiquitin ligase first preassembles a poly-Ub chain on an E2 conjugating enzyme and then transfers the preassembled Ub chain directly to the substrate. In addition, Gp78 functions as an E4 ubiquitin elongation factor in the
“elongation model” where a short poly-Ub chain is assembled on an ERAD substrate CFTRΔ508 by other E3 ubiquitin ligase RAM1 (Morito et al., 2008).

The substrates of Gp78 include T cell receptor subunit (CD3-delta, TCR alpha), the ApoB lipoprotein, Insig-1, HMG CoA reductase, the Z variant of α1-antitrypsin, mutant cystic fibrosis transmembrane conductance regulator (CFTRΔ5080), SOD1, Ataxin 3 and KAI1/CD82 (Kostova et al., 2007; Tsai et al., 2007; Ying et al., 2009). Dysfunction of Gp78 has been linked to lipid metabolism disorder, obesity, diabetes, cystic fibrosis, neurodegenerative diseases, and cancer (Lee et al., 2006; Liu et al., 2012; Morito et al., 2008; Song et al., 2005; Tsai et al., 2007; Vij et al., 2006; Ying et al., 2009). Recently, our lab identified novel substrates of Gp78, mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2), that play critical role in mitochondria fusion and mitophagy (Fu et al., 2013; Shankar et al., 2013).

1.3.4 ER stress and the UPR response

The ER is a central cellular organelle that serves crucial biosynthetic, sensing and signaling functions in eukaryotic cells. It maintains a tightly regulated oxidizing and calcium

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Scheme 1.6: Schematic representation of the E3 ubiquitin ligase Gp78 involved in mammalian ERAD.

Gp78 is predicted to span the ER membrane 5–6 times and interacts with the ERAD E2 Ube2g2 via the G2BR (Ube2g2 binding region) which is located between residues 579–600 which facilitates the interaction between the E2 and the RING domain (341–379). The ubiquitin binding CUE domain is located between 459–497 and is required for the binding the polyUb chain and the C-terminal p97/VCP binding site is responsible for recruiting the p97 complex.
rich environment for the proper folding of newly synthesized proteins destined for secretion, the cell surface or intracellular organelles and provides the cell with a calcium reservoir for signal transduction. This organelle is susceptible to a variety of endogenous and exogenous disturbances like calcium flux across the ER membrane, glucose starvation (reduced protein glycosylation), hypoxia (reduced formation of disulfide bonds) or defective protein secretion or degradation that interfere with proper protein folding. These insults will ultimately cause an imbalance between protein folding capacity and load, which provokes a cellular stress condition known as ER stress. In an attempt to re-establish ER homeostasis and restore ER functions, the ER responds to these physiological and pathological perturbations by activating an integrated intracellular signal transduction pathway, termed unfolded protein response (UPR) (Schroder and Kaufman, 2005). Three major arms of the UPR signaling pathway, PERK (PKR (RNA-dependent protein kinase) like ER kinase), IRE1 (inositol requiring 1) and ATF6 (activating transcription factor 6) are activated to relieve the ER stress by up-regulation of chaperones to facilitate protein folding, inhibiting general protein synthesis to reduce the protein load, and enhancing protein degradation by ERAD (Banhegyi et al., 2007; Chakrabarti et al., 2011; Kim et al., 2008b; Lai et al., 2007; Naidoo, 2009). The UPR occurs in 3 phases: adaptation, alarm and cell death. During adaptation, the UPR tries to reestablish folding homeostasis, however, if the duration and intensity of the ER stress are severe or protracted, the UPR induces a cellular alarm and finally triggers cell death (Chakrabarti et al., 2011; Kim et al., 2009).

PERK-eIF2α signaling

Upon accumulation of misfolded or unfolded protein in the ER, the PERK branch of UPR transduces both pro-survival and pro-apoptotic signals. PERK, a type I transmembrane protein anchoring in the ER, is composed of a stress sensor in the lumen of the ER and a protein kinase domain in the cytosolic face of the ER. Activation of PERK is initiated from dissociation of BiP from the N-terminus of PERK, followed by dimerization and autophosphorylation of the kinase domain. The kinase domain phosphorylates the α subunit of the eukaryotic initiation factor 2 (eIF2α) to attenuate cap-dependent global protein synthesis in order to reduce the protein-folding load. PERK activation also induces the expression of pro-survival genes such as cellular inhibitor of apoptosis (cIAP) and activation PI3K-Akt pathway during the adaption phase of UPR (Hamanaka et al., 2009). In addition, the activation of the PERK leads to the
induction of cap-independent ATF4 translation, a cAMP response element-binding transcription factor. ATF4 contains internal ribosome entry site (IRES) sequence in the 5’ untranslated regions that can bypass the translational block of phosphorylated eIF2α. ATF4 has been found to drive the expression of genes involved in amino acid transporter, protein secretion and glutathione biosynthesis (Harding et al., 2003). Moreover, ATF4 is involved in upregulation of LC3B, a key component of the autophagosomal membrane to facilitate autophagy (Rzymski et al., 2010). These mechanisms are initially protective and crucial for cell survival under stress conditions. However, under prolonged or excessive stress, the activation of PERK leads to upregulation of CCAAT/enhancer-binding protein (C/EBP)-homologous protein/growth arrest and DNA damage-inducible protein 153 (CHOP/GADD153), a transcription factor that plays an important role in transmitting pro-apoptotic signals (Zinszner et al., 1998). More interestingly, GADD34, the downstream effector of CHOP, is a binding partner of protein phosphatase 1 (PP1) which has been shown to negatively regulate the PERK signaling by direct de-phosphorylation of eIF2α (Novoa et al., 2001). Besides, p58IPK, has been shown to be involved in the negative feedback loop of PERK signaling by inhibition PERK kinase activity through direct binding to the cytosolic kinase domain of PERK (van Huizen et al., 2003; Yan et al., 2002). Indeed, p58IPK expression might mark the end of the adaptation phase of UPR and the beginning of the alarm and apoptosis phase of the ER stress response as p58IPK induction occurs several hours after PERK activation and eIF2α phosphorylation (Szegezdi et al., 2006).

**ATF6 signaling**

ATF6 is a bZIP (basic leucine zipper) ER transmembrane protein whose major role in ER stress is thought to be transcriptional regulation of pro-survival genes. ATF6 is activated by sequential cleavage by Site-1 and Site-2 proteases (S1P and S2P) in Golgi upon dissociation from BiP induced by ER stress, followed by translocation to the nucleus. Then it binds to ER stress elements (ERSE) to induce genes, such as chaperone proteins BiP and GRP94 (glucose regulated protein 94), PDI (protein disulfide isomerase), that are involved in protein folding and clearance as well as ER degradation-enhancing alpha-mannosidase-like protein 1 (EDEM1). In addition, ATF6 induces the expression of X box binding protein 1 (XBP1), which is further processed by IRE1α, as discussed below in more detail, linking ATF6 with pro-survival signaling emitted through IRE1 (Chakrabarti et al., 2011; Gorman et al., 2012; Jager et al.,
The cytoprotective role of ATF6 has been strengthened by the growing list of genes such as regulator of calcineurin I (RCAN1). RCAN1 sequesters calcinurin, a phosphatase involved in dephosphorylation of BAD (Bcl2-antagonist of cell death) and inhibition of anti-apoptotic activity of Bcl2 (Belmont et al., 2008). However, it has also been shown to be involved in inducing apoptosis by upregulation of CHOP (Yoshida et al., 2000) and downregulation of antiapoptotic protein MCL-1 (Morishima et al., 2011). The activity of ATF6 has been shown to be negatively regulated by binding to XBP1u, the unspliced form of XBP1, which leads to its proteasomal degradation during the recovery phase of UPR (Yoshida et al., 2009).

**IRE1 mediated “UPRosome”**

IRE1 is the most evolutionarily conserved ER stress receptor mediating UPR across species from yeast to mammalian cells. Indeed, unlike in mammalian cells where both PERK and ATF6 mediated signaling are involved in UPR, IRE1 mediated signaling is the only branch of UPR in response to ER stress in yeast (Chawla et al., 2011). Accumulating studies suggest that IRE1 is at the crossroad of cell survival-death decisions under prolonged ER stress by clustering and assembling various regulatory and adaptor proteins referred to as “UPRosome” (Hetz and Glimcher, 2009; Li et al., 2010). IRE1 is a type I ER transmembrane dual-activity enzyme, having both kinase and endoribonuclease activity (Lee et al., 2002). Activation of IRE1 resulting from either dissociation from BiP or direct interaction with misfolded proteins followed by homo-oligomerization and autophosphorylation, enables both its kinase and RNase activities to transduce signals simultaneously through two distinct signaling pathways. The endoribonuclease activity of IRE1 cleaves an intron from XBP1-mRNA which generate a frame shift shorter variant of XBP1 (sXBP1) that functions as a potent transcription factor and regulates the expression of a variety of genes involved in protein folding and ERAD (Chakrabarti et al., 2011; Jager et al., 2012). More interestingly, sXBP1 also induce the expression of p58IPK, a negative regulator of PERK activity, representing one of the crosstalk signaling pathways between the UPR branches as mentioned above (Yan et al., 2002). The kinase activity of IRE1 triggers the ASK1-MKK4/3-JNK/p38 MAPK signaling cascades by recruiting TRAF2 (Chakrabarti et al., 2011; Urano et al., 2000) and result in autophagy and apoptosis which will be addressed in further detail below.
IRE1 activity is regulated by several mechanisms including tyrosine phosphatase 1B (PTP1B), ASK1-interactive protein 1 (AIP1), members of HSP family of proteins as well as Bcl2 family proteins such as Bax inhibitor 1 (BI-1). It has been shown that lack of PTP1B dampened both IRE1 dependent XBP1 splicing and JNK activation in response to ER stressor (Gu et al., 2004). In addition, AIP1 has been shown to facilitate activation of IRE1-JNK/XBP1 signaling under ER stress by direct physical interaction with both IRE1 and TRAF2 (Luo et al., 2008). HSP72 have also been shown to promote the endoribonuclease activity of IRE1 by formation of a stable protein with the cytosolic domain of IRE1 (Gupta et al., 2010). In contrary to PTP1B, AIP1 and HSP72 which enhance IRE1 signaling, BI-1 is a negative regulator of IRE1 signaling by directly binding and inhibiting the endoribonuclease domain of IRE1 in early adaptive phase against ER stress (Lisbona et al., 2009). Besides, scaffolding protein receptor for activated C-kinase 1 (RACK1) and protein phosphatase 2A (PP2A) have also been shown to negatively regulate IRE1 activity by forming a complex containing IRE1, RACK1, and PP2A to promote dephosphorylation of IRE1 (Qiu et al., 2010). More interestingly, IRE1 has also been demonstrated to directly interact with proapoptotic protein BAX and BAK, providing a physical link between the UPR and members of the core apoptotic pathway (Hetz et al., 2006). Therefore, with various positive and negative regulators in the UPRosome, it appears that the control of IRE1 activity is crucial for the switch from cell survival to apoptotic cell death in response to ER stress.
ER stress induced autophagy and apoptosis

As mentioned above, ER stress triggers UPR mediated by three ER transmembrane receptors: PERK, ATF6 and IRE1 which occurs in 3 phases: adaptation, alarm and cell death. During adaptation, the UPR tries to reestablish folding homeostasis by up-regulation of chaperones to enhance protein folding capacity, inhibiting global protein synthesis and increasing the ERAD and autophagy to reduce the protein folding load. Autophagy functions as a compensatory system to remove aggregated misfolded proteins that cannot be degraded by
ERAD. If the duration and intensity of the ER stress are too severe, the UPR induces a cellular alarm as well as autophagy and apoptosis mediated cell death via mitochondria and ER dependent programs (Chakrabarti et al., 2011; Kim et al., 2009; Kim et al., 2008b). The PERK and ATF6 branches are thought to be activated before IRE1 (Szegezdi et al., 2006) to promote ER adaptation to misfolding, whereas IRE1 performs a dual role at late phases of UPR, transmitting both survival and pro-apoptotic signals. This ordering is consistent with the signals that each branch transduces. IRE1 transduce signals simultaneously through two distinct signaling axes by enabling both its endoribonuclease and kinase activities. As mentioned above, the endoribonuclease activity cleaves an intron from the XBP1mRNA and results in a frame shift variant (sXBP1) that functions as a potent transcription factor to regulate the expression of a variety of ER chaperones and ERAD related genes. However, the kinase activity drives apoptosis signal-regulating kinase 1 (ASK1) activation by interacting with tumor necrosis factor receptor-associated factor 2 (TRAF2) and subsequent downstream kinases MKK4 and MKK3 which in turn activate JNK and p38 MAPK respectively. JNK signaling activation has been shown to induce autophagy and apoptosis by activating the proapoptotic protein BIM (Lei and Davis, 2003; Putcha et al., 2003) and inhibition of Bcl2 activity (Wei et al., 2008a; Wei et al., 2008b; Yamamoto et al., 1999) by phosphorylation of various sites of both proteins. p38 MAPK activation induces BAX/BAK dependent apoptosis by inhibition of Bcl2 activity by phosphorylation of various sites of Bcl2 (De Chiara et al., 2006) and activation of the proapoptotic transcription factor CHOP (Wang and Ron, 1996). In addition to its post transcriptional activation by p38 MAPK, transcriptional up-regulation of CHOP can be induced through ATF6, ATF4 and XBP1 signaling triggered by ER stress (Oyadomari and Mori, 2004). CHOP is reported to promote cell death in part by reducing the expression of anti-apoptotic factor Bcl-2, and changing intracellular redox state through the induction of ERO1\(\alpha\), an ER oxidase, that promotes oxidative protein folding in the ER (Marciniak et al., 2004; McCullough et al., 2001).

Caspase-12, specifically localized at ER and synthesized as an inactive proenzyme, has also been shown to be involved in ER stress mediated apoptosis. Both calpain dependent cleavage (Nakagawa and Yuan, 2000) and IRE1/TRAF2 complex recruitment mediated self-cleavage of caspase-12 have been shown to promote the activation of caspase-12 (Yoneda et al., 2001). Activation of caspases-12 leads to caspase-9 and caspase-3 activation cascade which is independent of mitochondrial cytochrome c release and subsequent Apaf-1 pathway (Morishima
et al., 2002; Rao et al., 2002). However, as the caspase-12 gene has acquired deleterious mutations that prevent the expression of a functional protein, the biological significance in humans remains elusive (Breckenridge et al., 2003a). In addition, calcium exchange between the ER and mitochondria also contribute to cell death which will be addressed in further details in section 1.4.2.

Scheme 1.8: UPR events and connection to the cell death machinery.

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1.4 The ER Domains and Functions

1.4.1 The ER domains and distinct functions

The ER is the largest organelle in most cell types extending from the nuclear envelope to the plasma membrane, constituting more than half of the total membrane of an average animal cell and occupying more than 10% of the total cell volume (Alberts and NCBI Bookshelf, 2002). Though the ER is characterized as a continuous membrane system, executing diverse cellular functions including calcium homeostasis, lipid synthesis, protein folding and degradation, more and more specialized ER domains such as mitochondria-associated membrane (MAM), the ERQC compartment (ERQCC) and the plasma membrane-associated membrane (PAM) have been identified (Leitman et al., 2013; Lynes and Simmen, 2011). The ER has been classically divided into rough and smooth ER based on morphology by electron microscopy and function more than 6 decades ago (Dallner et al., 1963; Palade and Siekevitz, 1956). More recently, the ER has been divided into two distinct domains based on the live cell imaging: juxtanuclear central ER sheets including nuclear envelope and peripheral network of membrane tubules. In the past decades, how the distinct domains of the ER are shaped has been well characterized. The low curvature ER sheets composed of two membranes aligned in a parallel way are maintained by the coiled-coil membrane protein Climp-63 (cytoskeleton-linking membrane protein of 63 kDa) and an ER-localized, ribosome-interacting protein p180 (Shibata et al., 2010). The individual Climp proteins located on opposite sides of the sheets flatten the membranes and stabilize the luminal space at 30-50nm (Shibata et al., 2010). Reticulon and DP1/Yop1p family proteins contain at least two hydrophobic transmembrane domains, thus forming a hairpin like wedge structure in the ER membrane, inducing a positive curvature of the ER tubules and also stable homo-oligomers in the ER, thereby forming a scaffold that stabilizes the tubular shape (Shibata et al., 2009; Shibata et al., 2008). Moreover, the Dynamin-like GTPases, atlastins, interact with reticulon complementing their functions by promoting the homotypic fusion of ER tubules and catalyzing the branching of ER tubules (Hu et al., 2009). Recent studies have revealed that overexpression of reticulons result in tubular ER expansion whereas knockdown of reticulons gives rise to sheet-like ER increase. In addition, the absence of atlastin leads to fragmented, unbranched smooth ER, while their over-expression promotes sheet-like ER (Hu et al., 2009; Voeltz et al., 2006). Therefore, the expression level of
the two key proteins, reticulons/DP1 and atlastin, may drive the formation of the sheet-like and tubular structures of the ER and may be responsible for the generation of the functional rough and smooth ER (Hu et al., 2011; Shibata et al., 2010).

The rough ER, characterized by the presence of ribosomes and translocation machineries, mediates the synthesis of secretary and membrane proteins while the smooth ER, devoid of ribosomes, is rich in enzymes involved in drug detoxification such as TPNH-cytochrome c reductase (Orrenius et al., 1965), epoxide hydrolase (Galteau et al., 1985) and cytochrome P450 CYPs (Szczesna-Skorupa and Kemper, 2008). Forms fit function. Indeed, the tubular and sheet-like ER is in a dynamic change based on the physiologic demand of the cell. For example, during the differentiation of B cells into plasma cells, the rough ER expands concomitant with an increased production of proteins involved in the production of secretary of proteins (van Anken et al., 2003). Similarly, in the presence of high amounts of ethanol, the smooth ER increases in size, coinciding with an elevated production of smooth ER-associated detoxifying enzyme cytochrome P450 2E1 (Takahashi et al., 1993). Not only does ER morphology undergo dynamic changing states, but also proteins localized in the ER have to be precisely targeted to the specific domain within the ER to execute their function. For example, the peripheral smooth ER distribution of triacylglycerol hydrolase (TGH), an enzyme involved in the mobilization of triacylglycerol, is altered when its ER retention signal is mutated from HIEL to a classical KDEL sequence. Consequently, the mutated KDEL-TGH becomes inefficient in the mobilization of lipids and subsequent assembly of very-low-density lipoprotein (VLDL) particles compared to the HIEL-TGH. This demonstrates that the precise subcellular localization of proteins within the ER is critical to their biological roles (Gilham et al., 2005).
**Scheme 1.9: The ER sheets and tubules.**

Based on live cell imaging, the ER has been classified into central, juxtanuclear ER sheets defined by Sec61β, found in the rough ER and the nuclear envelope, and polygonal tubular peripheral smooth ER generated by the reticulon protein family. The low curvature ER sheets composed of two membranes aligned in a parallel way are maintained by the coiled-coil membrane protein Climp63 and the ER-localized, ribosome-interacting protein p180. Reticulon and DP1/Yop1p family proteins induce a positive curvature of the ER tubules and stabilize the tubular shape. The Dynamin-like GTPases atlastins promotes the homotypic fusion of ER tubules and catalyzes the branching of ER tubules by interacting with reticulons. Image from (Park and Blackstone, 2010) and reproduced with permission from JOHN WILEY AND SONS (DOI: 10.1038/embor.2010.92).

1.4.2 MAM and calcium signal induced cell death

Mitochondria associated ER membrane (MAM) is a well characterized ER domain. Indeed, as much as 20% of the mitochondria surface is in association with the ER, suggesting a very important role in the dynamic and highly regulated communication between the two organelles (Rizzuto et al., 1998). Electron microscopy and fluorescence microscopy studies have shown that the width of the close contact sites between ER and mitochondria is a mere 10-25
nm (Csordas et al., 2006; Hayashi et al., 2009; Perkins et al., 1997; Rizzuto et al., 2009). The physical linkage of the ER and mitochondria interface ensures the fast non-vesicular mediated lipid transfer and calcium exchange between the two organelles and hence functions as a major cellular signaling hub that controls cellular metabolism and cell death (de Brito and Scorrano, 2010; Grimm, 2012; Rowland and Voeltz, 2012). The close association of the two organelles is held together by several protein tethering complexes. One of the physical bridges between ER and mitochondria in mammalian cells is GRP75 (Glucose-Related Protein 75), a cytosolic chaperone that links IP3R on the ER to VDAC (Voltage-Dependent Anion Channel) on the outer membrane of the mitochondria which facilitate the calcium transfer between the two organelles (Szabadkai et al., 2006). Another tether that is formed in the MAM is the smooth ER protein BAP31 that interact with mitochondrial fission protein Fission 1 homologue (Fis1), which leads to recruitment of procaspase-8 to the MAM during onset of apoptosis (Iwasawa et al., 2011). It has also been proposed that ER-resident mitofusin 2 couples ER with mitochondria by forming homotypic and heterotypic complexes with mitochondrial-resident mitofusin 1 or 2 (Mfn1/2) to facilitate efficient mitochondrial calcium uptake (de Brito and Scorrano, 2008a). However, other study using electron microscopy showed that loss of Mfn2 increases ER-mitochondria juxtaposition, suggesting Mfn2 may not play a critical role in tethering mitochondria to the ER (Cosson et al., 2012). In addition, an ER-localized calcium-sensitive and ligand-operated receptor chaperone Sigma-1 receptor (Sig-1R) is another potential link that may preserve communication at the MAM during ER stress. It dissociates from BiP upon ER calcium depletion or IP3R stimulation, leading to prolonged calcium signaling into mitochondria via IP3Rs (Hayashi and Su, 2007; Marriott et al., 2012).

Under resting conditions, mitochondrial uptake of calcium released from the ER is needed to activate the mitochondrial enzyme pyruvate dehydrogenase (PDH) to drive the tricarboxylic acid (TCA) cycle by providing sufficient reducing equivalents to support oxidative phosphorylation (Cardenas et al., 2010). The disruption of the ER-mitochondria interface has been shown in various kinds of pathological conditions including Alzheimer's disease (Hedskog et al., 2013; Schon and Area-Gomez, 2010), cerebral ischemia (Ouyang and Giffard, 2012), type 2 diabetes mellitus (Johnson et al., 2012; Leem and Koh, 2012) and malignant tumors (Akl and Bultynck, 2013; Pinton et al., 2011). Accumulating evidence indicates that during the adaptive phase of ER stress, ER and mitochondria coupling is increased to reestablish cellular homeostasis by enhancing the calcium exchange between the two organelles (Bravo et al., 2011;
Csordas et al., 2006). It is well established that ER stress results in apoptotic cell death by disruption of mitochondrial calcium homeostasis (Leem and Koh, 2012). However, it has also been shown that rapid calcium transfer from the ER to mitochondrial triggers the mitochondrial apoptotic pathways (Boehning et al., 2003) since calcium overload leads to a large production of ROS and a loss of mitochondrial membrane potential (Laude and Simpson, 2009). Therefore, a number of studies have shown that the perturbation of ER-mitochondria contacts by depleting tethering complexes leads to delayed apoptosis progression due to decreased transfer of calcium from the ER to mitochondria (Akl and Bultynck, 2013; Grimm, 2012; Johnson et al., 2012; Pinton et al., 2011). Indeed, calcium release from the ER through 1,4,5-triphosphate receptor (IP3R) and the Ryanodine receptor (RyR), the two well-characterized ER calcium channels, triggers both mitochondria-dependent and independent apoptosis. For example, as mentioned above, the increase of calcium in the cytosol triggers the activation of calpain, resulting in cleavage and activation of proapoptotic factor caspase-12 (Nakagawa and Yuan, 2000) and BAX (Wood et al., 1998). Additionally, the increase of calcium in the cytosol also triggers the activation of calcium protein phosphatase calcineurin which leads to dephosphorylation and activation of BAD (Wang et al., 1999) as well as activation of the calcium-dependent transcription factor MEF2 which can up-regulate TR3/Nur77, an orphan member of the steroid receptors, that induces cytochrome c release by translocation from the nuclear and binding to mitochondria (Li et al., 2000). More importantly, mitochondrial uptake of calcium released from juxtaposed ER induces opening of the mitochondrial permeability transition pore by regulation of expression of pro- and antiapoptotic Bcl-2 family proteins (Antonsson and Martinou, 2000; Hajnoczky et al., 2000). Reciprocally, Bcl-2 functions as negative regulator of calcium exchange between ER and mitochondria, antagonizing the positive roles of BAX/BAK and BH-3 only proteins such as BIK, BIM and NOXA(Cheng et al., 2001) in Ca^{2+}-dependent cross link between the two organelles (Breckenridge et al., 2003a). The integral membrane protein of the endoplasmic reticulum BAP31 is recruited to MAM by interaction with mitochondria fission protein Fis1 and cleaved into pro-apoptotic p20BAP31 by caspase-8. The subsequent recruitment of procaspase 8L, the novel procaspase-8 isoform, to the Fis1-Bap31 platform upon prolonged ER stress establishes a feedback loop by releasing calcium from the ER that activates the mitochondria for apoptosis (Breckenridge et al., 2003b; Iwasawa et al., 2011).

Several chaperones and oxidoreductases involved in protein quality control have been shown to play critical roles in regulation the calcium signaling by modulation of calcium
channels/pumps and MAM conformation. For example, ER-resident oxidase Ero1α has been shown to relocate to the MAM and stimulate IP3R activity upon ER stress and calcium release from the ER which triggers mitochondrial mediated apoptosis by activating calcium/calmodulin-dependent protein kinase II (CaMKII)(Gilady et al., 2010; Li et al., 2009a). In contrast, ERp44 has been shown to directly inhibit the activity of IP3R type 1 (IP3R1) by interacting with the luminal loop of the channel in a pH, redox state and Ca^{2+} concentration dependent manner (Higo et al., 2005). Protein folding chaperone BiP has also been demonstrated to positively regulate of IP3R activity during ER stress by facilitating assembly of tetrametric IP3R channels (Higo et al., 2010). Furthermore, another ER oxidoreductase ERp57 has been demonstrated to reduce the frequency of calcium oscillations enhanced by SERCA 2b by interacting with the CRT binding loop of SERCA2b in a Ca^{2+} dependent manner (Li and Camacho, 2004). More interestingly, the activity of ER Ca^{2+} pump SERCA2b has been shown to be inhibited or activated by CNX, depending on the posttranslational modification status of CNX, that is recruited by cytosolic sorting protein PACS-2 to the MAM, which will be discussed in further details in section 1.4.4 and section 1.4.5 (Giorgi et al., 2009; Lynes et al., 2012; Myhill et al., 2008; Roderick et al., 2000).

In summary, MAM enriched for ER chaperones, oxidoreductases and Ca^{2+} channels/pumps, appears to be a processing hub that integrates signaling information derived from Ca^{2+}, redox and protein folding and thereby creating an ideal environment for oxidative protein folding and regulation of Ca^{2+} signaling (Araki and Nagata, 2011).
1.4.3 ERQCC and its function in protein quality control

The ERQC Compartment (ERQCC) is a membrane-enclosed compartment localized in the juxtanuclear domain of the ER. Upon proteasome inhibition, unfolded or terminally misfolded secretory proteins, ER-folding and quality control proteins (CNX, CRT) as well as ERAD components (Derlin-1, Sec61β, Hrd1, p97/VCP, ubiquitin, EDEM1, ERManI, Bap31) are segregated in the ERQCC which is distinct from the ER-Golgi Intermediate Compartment (ERGIC) (Frenkel et al., 2004; Groisman et al., 2011; Kamhi-Nesher et al., 2001; Kondratyev et al., 2007; Schmitz et al., 2004; Spiliotis et al., 2002; Wakana et al., 2008; Walter and Ron, 2011; Wang et al., 2008; Ye et al., 2003). In contrast, some of the factors that mediate protein folding remain localized in the peripheral ER even during ER stress. For example, UDP-glucose: glycoprotein glucosyltransferase (GT) and glucosidase II (GII) do not accumulate in the ERQCC in response to ER stress (Benyair et al., 2011; Cannon and Helenius, 1999; Coe and
Michalak, 2010; D’Alessio et al., 2010; Kamhi-Nesher et al., 2001). As mentioned in section 1.3.1, GT functions as the folding sensor in the CNX/ CRT cycle by reglycosylating N-glycans on incompletely folded glycoproteins for their binding to CNX/CRT while GII removes terminal glucose residues from N-glycans, preventing reassociation of glycoproteins to CNX. Similarly, the oxidoreductases ERp57, PDI (Frenkel et al., 2004; Lucocq et al., 1986) and ER chaperone HSP70 BiP which is involved in protein folding and activation of the UPR (Walter and Ron, 2011) are also excluded from the ERQCC (Kamhi-Nesher et al., 2001; Kondratyev et al., 2007). However, mannosidases ERManI and EDEM1 as well as lectin OS9 have been shown to be constitutively localized in the ERQCC even in the absence of misfolded proteins (Avezov et al., 2008; Ron et al., 2011).

It has been shown that extensive mannose trimming by ERManI and EDEM1 are required for misfolded N-glycosylated protein delivery to ERQCC for degradation. Therefore, compartmentalization of ERAD machinery and protein folding machinery is crucial for maintenance of protein homeostasis since it provides a solution to distinguish between the folding intermediates and terminally misfolded polypeptides in the ER. The non-native folding intermediates need to be protected from unwanted recognition by ERAD machinery that could prematurely interrupt ongoing folding cycles whereas the terminally misfolded proteins need to be efficiently cleared from the ER to prevent toxicity trigged by protein aggregates. Indeed, the relocation of proteins to this membrane-enclosed ERQCC involves microtubule-dependent trafficking (Kamhi-Nesher et al., 2001; Spiliotis et al., 2002; Wakana et al., 2008). During the process, CNX/ CRT and Bap31 might function in the shuttling of misfolded proteins from the peripheral ER to the ERQCC (Wakana et al., 2008; Wang et al., 2008). Given the localization of the protein folding machinery and folding sensor, one can imagine that through the CNX/CRT cycle, slow-folding and misfolded glycoproteins would be segregated from the peripheral ER to the ERQCC and then cycle back for deglycosylation, reglycosylation, and a new folding attempt (Leitman et al., 2013). All in all, some early quality control factors remain in the peripheral ER which is segregated from the late quality control. When misfolded proteins accumulate, ERAD components are recruited to the ERQCC upon demand, enabling separation of the processes of folding and targeting to degradation in space and time.
1.4.4 ER resident protein and intra-ER trafficking

The retention and retrieval mechanism

Membrane proteins can be retained in the ER based on either direct retention or retrieval mechanisms (Rothman and Wieland, 1996). In the retention model, proteins are restricted to the ER whereas in the retrieval model, they are transported out of the ER to a post-ER compartment and returned to the ER by a receptor-mediated process in a microtubule-dependent manner. Therefore, the retention and retrieval models can be distinguished by their microtubule-dependence. Proteins that are directly retained in the ER do not redistribute after treatment with microtubule inhibitors whereas proteins localized in the ER by the retrieval mechanism do (Hsu et al., 1991).

ER-resident proteins which involve receptor mediated retrieval from post-ER compartments are defined by a short sequence of targeting signals present in those proteins. The best studied are BiP (Pelham, 1990) and PDI (Cosson and Letourneur, 1994) which contain the KDEL motif involving the KDEL receptor mediated retrieval or KKXX motif and binding to COPI coat proteins, respectively. However, the mechanisms for direct retention of ER proteins have remained elusive and until now a true ER retention motif has not been identified. However, it has long been proposed that ER retention is achieved by actively preventing transport of resident membrane proteins from the so-called ER exit sites (ERESs), a specialized regions of the ER where properly folded and assembled membrane-bound soluble proteins are concentrated and incorporated into COPII-coated vesicular carriers (Barlowe, 2003). How this can be conferred is poorly understood. However, the properties of the transmembrane domain, self-oligomerization, and binding to large immobile complexes or networks that are excluded from transport vesicles and hence unable to enter these ERESs have been proposed (Neve and Ingelman-Sundberg, 2008; Rothman and Wieland, 1996).

ER stress induced protein trafficking between ERQCC and MAM

As mentioned before, some components of ERAD machinery such as ERMan1, EDEM1 and OS9 (Avezov et al., 2008; Ron et al., 2011) are constitutively localized in the ERQCC while
others, such as CNX and CRT, distribute throughout peripheral regions of the ER in the absence of misfolded proteins. Still others like Hrd1, Gp78, EDEM1, Derlin-1, Sec61β and p97 concentrate at the ERQCC upon misfolded protein accumulation, making the ERQCC a possible staging ground for efficient degradation (Kondratyev et al., 2007; St-Pierre et al., 2012). Protein folding chaperones CNX and CRT have been shown to be recruited to ERQCC with misfolded proteins upon ER stress to facilitate degradation of misfolded proteins (Frenkel et al., 2004; Kamhi-Nesher et al., 2001). In contrast, the BiP chaperone remains in the peripheral ER upon ER stress. However, upon misfolded protein accumulation, the UPR sensor PERK and IRE1 are recruited to the ERQC, allowing these sensors to physically segregate from BiP and resulting in activation of downstream UPR programs (Kondratyev et al., 2007). More interestingly, it has been demonstrated that overexpression of a constitutive phosphorylation mimic eIF2α (S51D) mutant promotes the compartmentalization of ERAD machinery whereas dominant negative PERK or unphosphorylatable mutant of eIF2α (S51A) prevents it. This suggests that phosphorylation of eIF2α by PERK is not only necessary but also sufficient for recruitment of ERAD machinery components and misfolded proteins to the ERQCC and may represent a positive feedback mechanism to amplify and maintain the UPR program (Kondratyev et al., 2007). Subsequent study suggested that membrane-bound protein Homocysteine-induced ER Protein (HERP), a component of ERAD machinery induced by the PERK/eIF2a-ATF4 pathway, to be responsible for protein recruitment to the ERQCC (Benyair et al., 2011; Leitman et al., 2014). Based on these studies, it is tempting to speculate that in addition to attenuating protein synthesis and reducing the burden of misfolded proteins, eIF2α phosphorylation upon ER stress might play another cytoprotective role by segregating misfolded proteins and ERAD machineries in the ERQCC to enhance the efficiency of the degradation process and promote cell homeostasis and survival (Benyair et al., 2011).

CNX trafficking between the ERQCC and MAM is regulated by post translational modifications in response to ER stress. Previously, it was shown that CNX is targeted to the rough ER and interacts more strongly with ribosomes, affecting glycoprotein folding when it is phosphorylated by protein kinases CK2 at S534/S544 and proline-directed kinase at S563 (Chevet et al., 1999). A further study demonstrated that under misfolding conditions, ERK1 MAPK enhanced the phosphorylation of CNX at S563, which coincided with a prolonged association of a partially misfolded glycoprotein α1-antitrypsin (AAT) in the ER. In contrast, when CNX was not phosphorylated at S563 in the presence of the MEK1 inhibitor PD98059,
enhanced secretion of AAT was observed, implicating decreased associated of non-phosphorylated CNX with client glycoproteins (Cameron et al., 2009; Chevet et al., 2010). Indeed, under conditions of protein misfolding, MEK1-induced activation of ERK1 has been shown to be enhanced, leading to the activation of the UPR via an IRE1α-dependent mechanism (Nguyen et al., 2004). Recently, it was demonstrated that depalmitoylated CNX performs its chaperone function in quality control by interacting with the oxidoreductase ERp57 in an ER stress dependent mechanism. In contrast, palmitoylation of CNX promotes its trafficking to the MAM where it interacts with ER calcium pump SERCA2b to determine ER Ca^{2+} content. Reciprocally, Ca^{2+} exchange between the ER and mitochondria induced palmitoylation of CNX (Lynes et al., 2012). Indeed, previously it has been demonstrated that interaction of CNX with SERCA2b results in inhibition of intracellular calcium oscillations in a xenopus oocytes model dependent on S562 phosphorylation which is a putatively mediated by protein kinase C(PKC) /proline-directed kinase (PDK) phosphorylation. On the other hand, dephosphorylation of S562 of CNX induced by IP3 mediated Ca^{2+} release resulted in significantly reduced interaction with SERCA2b, suggesting that S562 phosphorylation of CNX acts as a molecular switch that affects calcium signaling in the ER by modulating the interaction of the chaperone with ER Ca^{2+} pump SERCA2b (Roderick et al., 2000). Interestingly, upon CNX dephosphorylation at protein kinase CK-2 sites S554/564, a considerable portion of CNX is localized to tubular, smooth ER and the MAM with the assistance of PACS-2 (phosphofurin acidic cluster sorting protein 2), a protein that regulates ER MAM-localized lipid-synthesizing enzymes (Gilady et al., 2010; Myhill et al., 2008). This correlates with a preferential distribution of CNX to the MAM under resting conditions, or the rough ER and ERQCC following ER stress (Chevet et al., 1999; Myhill et al., 2008; Roderick et al., 2000). Later it was found that phosphorylated S565 and S584 of CNX, which controls PACS-2 binding and interaction with SERCA2b, undergoes dephosphorylation upon prolonged ER stress treatment (Tunicamycin, 16 hours) (Lynes et al., 2012). Therefore, it appears that phosphorylation and palmitoylation of CNX in an ER stress dependent mechanism is the switch that regulates CNX trafficking between the ERQCC where it functions as chaperon in protein folding and the MAM where it acts as regulator of Ca^{2+} signaling (Lynes et al., 2012; Lynes et al., 2013).

In summary, the ERQCC acts as a staging ground for misfolded protein degradation at the initial adaption stage of the UPR by segregating misfolded proteins with ERAD machinery while it is the MAM in which apoptosis is triggered upon prolonged stress by a signaling
pathway. How this information is conveyed to the MAM to initiate an apoptotic response upon prolonged ER stress remains elusive. However, the cycling of CNX and Bap31 between the ERQC (Frenkel et al., 2004; Kamhi-Nesher et al., 2001; Wakana et al., 2008) and MAM (Lynes et al., 2012; Myhill et al., 2008; Simmen et al., 2005) may provide a clue. Given the role of ERQC in ERAD and MAM in initiating apoptosis, the dynamics of movement of CNX and Bap31 between the different compartments might link early (adaptation phase) and late UPR stage (alarm and cell death phases) during ER stress (Benyair et al., 2011).

1.4.5 ERAD tuning and compartmentation of ERAD factors in the ERQC

ERAD tuning

The newly synthesized proteins have to be properly folded before targeting to their final destination. Even for correct gene products, a significant fraction ranging from 30% to much less never attains the native structure (Schubert et al., 2000; Vabulas and Hartl, 2005). Those unfolded or misfolded polypeptides are cleared from the ER lumen and most of them are ubiquitylated and degraded by proteasomes, a series of process collectively defined as ERAD as mentioned in section 1.3.2 (Brodsky, 2012; Reggiori et al., 2011). When the capacity of folding proteins in the ER lumen is persistently saturated or exceeded under stress conditions, three arms of ER stress sensor, PERK, IRE1α and ATF6α will be activated to initiate the UPR programs resulting in up-regulation of chaperones to facilitate protein folding, enhancing protein degradation by ERAD and inhibiting general protein synthesis to reduce the protein loading (Hattori et al., 2009; Kim et al., 2008b; Lai et al., 2007; Tsai and Weissman, 2010). However, UPR activation has a latency period of several hours due to the requirement of signal transduction from the ER to the nucleus for activation of transcriptional and translational programs (Pincus and Walter, 2012; Walter and Ron, 2011). Moreover, the recoveries from a stress rely on activation of poorly understood mechanisms that must remove the excess of chaperones produced during the ER stress phase and reduce the size of the expanded ER triggered by UPR. Therefore, a more rapid and readily reversible response is needed in response to weak and/or short lasting variations in ER cargo load. Indeed, ERAD tuning, a process involving the regulated degradation of ERAD factors to determine the constitutive level of the
ERAD activity and to adapt it to variations in misfolded protein load has been proposed. The concept of ERAD tuning is based on observations that several ERAD factors are constitutively segregated from the ER and subjected to fast turnover and the half-life of those ERAD factors might be regulated by luminal expression of misfolded proteins (Merulla et al., 2013).

Immuno-electron microscopy reveals that the ERAD tuning vesicles, also known as EDEMsomes, have a diameter of about 150 nm and may swell to up to 1 µm upon cell exposure to lysosomotropic agents, which prevents their fusion with acidic degradative organelles (Bernasconi et al., 2012b; Cali et al., 2008). The ER-derived ERAD tuning vesicles lack a recognizable coat which is distinct from the conventional secretory vesicles which display a COP-II coat at their surface and autophagosomes which contain lipidated LC3-II (see section 1.5.2 for detail). These vesicles contain the ERAD factors EDEM1 and OS9 in the lumen and type I ER protein SEL1L as well as nonlipidated LC3-I. Indeed, LC3-I is non-covalently associated with the limiting membrane and can be separated from lighter secretory organelles and autophagosomes by isopycnic density gradients. The SEL1L and LC3-I complex has been found to serve as ERAD tuning receptors to deliver ERAD factors into the vesicles and dispose of EDEM1 and OS9 in acidic organelles for degradation. However, it remains to be established whether LC3-I binds the cytosolic tail of SEL1L directly or indirectly through an adaptor associated with the transmembrane or the cytosolic domains of SEL1L. When misfolded proteins are present, SEL1L is engaged with multimeric retrotranslocation machinery built around E3 ubiquitin ligases, whereas when misfolded polypeptides are absent, at least part of the SEL1L is disengaged from the dislocation machinery and associates with the luminal ERAD factors and deliver them into the ERAD tuning vesicles which leads to the disposal of EDEM and OS9 in the lysosome. In this way, misfolded polypeptides regulate the ERAD activity by tightly controlling the turnover of these crucial ERAD regulators.

Therefore, constitutive and rapid removal of ERAD regulators is important for efficient protein folding and disposal of unwanted proteins at steady state. Dysregulated ERAD activity caused by insufficient luminal content of ERAD factors may result in accumulation of misfolded proteins and toxic depositions before activation of UPR induced by ER stress. In contrast, dysregulated ERAD activity caused by excessive luminal content of ERAD factors may lead to premature interruption of ongoing folding attempts such that proteins that would have attained functional structures are inappropriately destroyed thus causing loss-of-function
phenotypes or disorders such as cystic fibrosis due to the folding defective polypeptides CFTRΔF508 (Merulla et al., 2013). Indeed, this loss-of-function defects could be rescued by targeting with therapeutic agents that affect the ERAD tuning to offer mutated polypeptides wider time windows to eventually attain their native functional structure.

Scheme 1.1: ERAD tuning.

Image originally published from (Bernasconi and Molinari, 2011) and reproduced with permission from Elsevier (DOI: 10.1016/j.ceb.2010.10.002).

**Compartmentation of ERAD factor in the ERQCC**

As mentioned in section 1.4.3, the ER lumen where protein folding occurs contains two classes of non-native polypeptides: *de novo* synthesized polypeptides undergoing folding cycles that have not attained the native structure and terminally misfolded components that must be cleared from the folding compartment. Cellular quality control machineries have to distinguish between the two classes of non-native polypeptides to prevent premature degradation of actively folding intermediates due to early exposure to the ERAD machinery and avoid proteotoxicity.
caused by export of misfolded proteins out of the ER. Otherwise, nascent polypeptide chains might nonspecifically associate with misfolded proteins exposing hydrophobic patches. In this respect, compartmentalization might provide an ideal solution. Indeed, trimming of mannose residues by mannosidase ERManI is obligatory steps for removing the glycoprotein from reglycosylation and CNX/CRT binding cycles and trapping substrates in the ERQC for ERAD (Avezov et al., 2008). Accordingly, ERManI is constitutively localized in the ERQC which leads to a high local concentration of ERManI, required for its function in extensive mannose trimming since in vitro study has shown that ERManI removes only one specific mannose residue at low concentrations while it can excise up to four mannose residues at very high concentrations (Avezov et al., 2008). However, another group of study has demonstrated that overexpression of mannosidase EDEM1 overrides the extensive trimming requirement for delivering ERAD substrates to lectin OS9 which has been shown to be constitutively localized in the ERQC, without the requirement of misfolded protein accumulation for ERAD (Ron et al., 2011). Therefore, the localization and expression level of EDEM1 has to be tightly regulated to assure the folding intermediates can be preserved but the terminally misfolded polypeptides can be efficiently shuttled to the ERAD machinery for destruction.

It has been shown that EDEM1 concentrates in the ERQC where ERManI and ERAD machinery components are localized together with the ERAD substrate upon proteasomal inhibition (Ron et al., 2011). ERManI (Termine et al., 2009; Wu et al., 2007), EDEM1 (Cali et al., 2008; Gauss et al., 2011; Le Fourn et al., 2009; Reggiori et al., 2010) and OS9 (Bernasconi et al., 2012a; Reggiori et al., 2010) are constitutively localized in the ERQC and rapidly removed from the ER in unstressed cells by lysosome mediated degradation. However, many other ERAD regulators such as HERP (Bernasconi et al., 2013; Hori et al., 2004; Miura et al., 2010), SEL1L (Cattaneo et al., 2011; Mueller et al., 2006) and Gp78 (Ballar et al., 2010; Shmueli et al., 2009) also showed fast turnover and are degraded by the ubiquitin proteasome system.

Indeed, cumulative studies highlight the role of misfolded protein in directly determining ERAD machinery assembly and activity by various mechanisms: (1) competing with heterologous ubiquitylation and self-ubiquitylation of E3 ubiquitin ligases which has been shown to be constitutively cleared from the ER membrane (Weissman et al., 2011); (2) maintenance of the integrity of functional ERAD complexes and of superamolecular dislocation
machineries; (3) inhibition of the segregation of ERAD factors in ER-derived vesicles or in the ER subdomains; (4) promoting recruitment of 26S proteasomes at the ER membrane by interference with the polyubiquitylation of the membrane protein JAMP (Bernasconi et al., 2013).

It has been shown that concentration of misfolded proteins and ERAD machinery in the ERQCC enhanced the efficiency of the degradation process (Kondratyev et al., 2007). Hrd1 has been shown to be recruited to the ERQCC through the PERK-eIF2a branch of UPR by interaction with HERP, a master organizer of the compartmentalization of the ERAD machinery (Kny et al., 2011; Leitman et al., 2014). The double spanning membrane adaptor protein HERP in mammalian cells (homologue of Usa1 in yeast) has been found to be necessary and sufficient for Hrd1 and ERAD substrate accumulate in the ERQCC (Leitman et al., 2014). Indeed, misfolded protein accumulation at the ERQCC dependent on protein levels and not proteasome inhibition. In resting cells, transient accumulation of unfolded protein molecules probably also results in temporary activation of the PERK pathway and induction of HERP at low routine levels of ER load, leading to dynamic protein recruitment to the pericentriolar ERQC. In other words, some level of UPR activation and HERP induction must occur under so-called physiological “unstressed” conditions and is a prerequisite for protein recruitment to the ERQCC and for ERAD. Retrotranslocation complexes might be transiently assembled only as needed. The association of HERP with the substrates increases strongly in the presence of MG132 whereas interaction of the substrates with CNX drastic decreased over time upon proteasome inhibition (Leitman et al., 2014; Lynes et al., 2013). Therefore, assembly and segregation of ERAD machinery in the subdomain of ER have been shown to be an adaptive and dynamic organization process using a system-level strategy that integrates proteomics, functional genomics and the transcriptional response to ER stress (Christianson et al., 2012).

Taken together, fine tuning the ERAD activity and compartmentalization of ERAD machinery based on the variations in misfolded protein load by regulating the turnover rate and localization of ERAD factors are more rapid and cost-effective to regulate ERAD capacity to maintain cellular proteostasis and thereby having a protective role.
1.5 Protein Degradation Pathway

All proteins in eukaryotic cells are continually being degraded and replaced. Cellular homeostasis, which is needed for the cells to survive, requires a well-controlled balance in protein turnover, which was thought of as a passive cellular process and remained a neglected research area until the discovery of the lysosomal and, later, the proteasomal protein degradation machineries (Ciechanover, 2013; Ohsumi, 2006). It has been well accepted that not only unfolded and misfolded or unassembled and damaged proteins undergo degradation; but highly regulated proteins also require degradation to maintain optimal level according to the demand of the cells. Protein turnover is a highly complex, temporally controlled, tightly regulated and highly specific process. Moreover, the specific degradation process for individual protein can vary under different conditions. Indeed, protein degradation is as important as transcription and translation in regulation of protein expression level. In contrast to gene expression, which usually requires the nuclear trafficking of transcriptional factors and initiation of assembly of translational machinery, regulation of protein degradation is a relatively fast and non-reversible mechanism to control, through regulated expression of critical regulatory proteins, various cellular process such as cell cycle, cell motility, signal transduction, receptor-mediated endocytosis and protein quality control (Ciechanover, 2013). Therefore, it is not surprising that dysregulation of the pathway have been implicated in the pathogenesis of many diseases such as neurodegeneration, immune system disorders and malignancies with a multitude of substrates targeted and processes involved. As a result, more and more research labs and pharmaceutical companies have been focusing on targeting the proteolytic system to develop novel drugs to treat those disorders since the first one of them is successively developed and already on the market (see section 1.5.1) (Ciechanover, 2013).

Degradation of intracellular proteins in the cells occurs by proteasomes, lysosomes and other cytosolic and organellar proteases (Fuertes et al., 2003a), of which proteasomes and lysosomes are the two major degradation pathways. The lysosome was originally considered the principle site of degradation of cellular proteins before the discovery of the ubiquitin-proteasome degradation pathway. However, it is very hard to use the lysosome mediated degradation to explain three observations. One of them is that half-lives of most cellular proteins are insensitive to alkalization of the lysosomes where degradation relies on the action of resident acid-dependent proteases and the others are requirement of metabolic energy and the varying
stability of different proteins (Ciechanover, 2013; Clague and Urbe, 2010). These observations finally lead to the discovery the proteasome mediated degradation in the late 1970s and early 1980s, which, in collaboration with the sophisticated ubiquitin system, is referred to as the UPS (ubiquitin-proteasome system) and is found to play a critical role in maintaining cellular homeostasis by clearing toxic proteins and controlling the half-lives of regulatory proteins and enzymes (Goldberg, 2003; Hershko, 2005a; Hershko, 2005b; Hershko and Ciechanover, 1998).

1.5.1 Proteasome mediated protein degradation

Proteins that are degraded by the proteasome are usually modified with a polyubiquitin chain for the selection of target proteins. As mentioned in section 1.3.2, proteins tagged with chains of four or more ubiquitins of various topologies except K63 linkage polyUb chain are recognized by the 26S proteasome for degradation (Jacobson et al., 2009; Xu et al., 2009). The proteasome is a large complex located in the nucleus and the cytoplasm which consists of a hollow-barrel shaped 20S catalytic core particle (20S complex, CP) and a pair of 19S regulatory particles (19S complex, RP) flanked on the two sides as caps as shown in Scheme 1.12. The 20S proteasome is made up of four rings of seven proteins that stacked together, forming a cylindrical core structure with a narrow pore that can only allow unfolded or partially folded polypeptide chains to pass through. The two identical outer α rings are comprised of 7 subunits form the gate of the protease chamber; the two identical β inner rings are also comprised of 7 subunits and bear the tryptic, chymotryptic, and peptidyl-glutamyl peptidase activity residing on the interior surface of the rings. The target protein must enter the central pore before it is degraded. The two 19S RP caps are made up of lids at the pore opening which contain deubiquitylating enzyme Rpn11 and base complex that consists of ATPase subunits (Rpt1 to Rpt6). Rpn11 edits the polyubiquitin chain of the substrates for selectively recognition of substrates and recycling the ubiquitin monomers. The base complex provides the energy to facilitate access of the target proteins to the 20S CP by unfolding the substrates and opening the catalytic channel. Furthermore, Rpn10 subunit, an ubiquitin receptor that links the base with the lid of the 19S RPs, combined with Rpn1, were identified as substrate docking sites at the proteasome by interacting with Rad23 and Dsk. Both Rad23 and Dsk contain Ubiquitin Associated (UBA) domain and Ubiquitin-Like (UBL) domain and act as substrate delivery
cofactors that are associated with the hexametric AAA-ATPase p97/Cdc48 complex as mentioned in section 1.3.2. (Li and Li, 2011; Raasi and Wolf, 2007; Voutsadakis, 2012).

Scheme. 1.12. The ubiquitin-proteasome system.

Image from (Raasi and Wolf, 2007) and reproduced with permission from Elsevier (DOI: 10.1016/j.semcdb.2007.09.008).

Approximately one-third of all proteins in eukaryotes are targeted to the secretory pathway. As discussed in section 1.3.3 and 1.4.3, due to spontaneous errors during transcription and translation, genetic mutations, toxic compounds and cellular stress, those misfolded or unfolded proteins that cannot pass the ERQC checkpoint along the secretory pathway must be destroyed. Moreover, the unassembled subunits of multimeric proteins are degraded by proteasome, a process called ERAD as mentioned in section 1.3.2 (Vembar and Brodsky, 2008).
In addition, some short lived regulatory proteins and enzymes are also subjected to ubiquitin-proteasome mediated degradation in response to external and internal physiological stimuli (Li and Li, 2011). More interestingly, the half-lives of some components of ERAD machineries are also controlled by UPS which has been discussed in section 1.4.5. Therefore, dysfunction of UPS results in a lot of pathological conditions including neurodegenerative diseases, immune-inflammatory disorders, cardiovascular diseases and cancer. Indeed, the complete clinical responses of the first proteasome inhibitor bortezomib (Velcade, Millennium Pharmaceuticals), in patients with refractory or rapidly advancing multiple myeloma, approved by FDA in US more than a decade ago, has demonstrated the therapeutic potential of intervention in the UPS in a variety of malignancies (Adams and Kauffman, 2004; Bedford et al., 2011b).

1.5.2 Lysosome mediated protein degradation

Lysosomes, discovered by Christian de Duve more than six decades ago, are vacuolar structures that contain a diverse array of hydrolase enzymes which function optimally at an acidic pH. The major function of the organelles is to break down exogenous particles and proteins (a process known as heterophagy including endocytosis, pinocytosis and phagocytosis depending on the cargoes that are targeted to the lysosomes) as well as cellular organelles and endogenous proteins (a process called autophagy including micro-, macro- and chaperon-mediated autophagy, CMA). Proteins are therefore delivered to lysosomes for degradation either by endocytosis from outside the cell (endosome-lysosome pathway) or by autophagy within the cell (autophagy-lysosome system, ALS) (Harada, 2011). To prevent the random destruction of cellular components, this structure is separated from the cytosol by a limiting membrane. It has been generally accepted that both lysosomes and proteasomes are involved in degradation of misfolded/dysfunctional proteins; however, autophagy-mediated lysosome pathway degrades long-lived proteins whereas the proteasome degrades short-lived proteins (Chen and Yin, 2011).

Once inside the lysosome, proteins are broken down by a combination of proteases, with the characteristic properties of soluble lysosomal hydrolases consisting of a mixture of endopeptidases and exopeptidases. These lysosomal hydrolases act in concert to produce a mixture of amino acids and dipeptides, which are transported across the lysosomal membrane into the cytosol by a combination of diffusion and carrier-mediated transport (Winchester,
Lysosome mediated protein degradation is inhibited by directly neutralizing the H\(^+\) ions (weak base chloroquine and ammonium chloride) or dissipating the H\(^+\) gradient across the lysosomal membrane by treatment with the acid ionophore X537A, increasing intralysosomal pH and inhibiting the lysosomal proteases that function optimally at an acidic pH (Ciechanover, 2005).

Scheme 1.13 The digestive process mediated by the lysosome.

Image published originally in (Ciechanover, 2005) and reproduced with permission from Nature Publishing Group (doi:10.1038/sj.cdd.4401692).
Autophagy-lysosome pathway

Autophagy is an intra-cellular degradation and recycling system to sustain cellular anabolic needs during times of nutrient or energy deprivation. This self-eating system is divided into three classes of pathways: macroautophagy, chaperone-mediated autophagy and microautophagy based on the substrate-to lysosome delivery mechanism used (Mizushima et al., 2008).

Macroautophagy, the prototype of autophagy, begins with the de novo synthesis of isolation membranes which engulf cytoplasmic constituents into double-membrane vesicles called autophagosomes and ends with fusion of vesicles with lysosomes for final degradation of the sequestered cytoplasm including long-lived or damaged proteins and organelles as well as other macromolecules. Fusion of autophagosomes with lysosomes to form autolysosomes triggers the breakdown of the inner autophagosomal membrane followed by degradation of the contents by lysosomal hydrolases (Chen and Yin, 2011; Roberts and Deretic, 2008). The process of macroautophagy is divided into four steps: initiation, nucleation, maturation and fusion with lysosomes which are mediated by a series of proteins encoded by autophagy-related genes (ATGs) (Doppalapudi et al., 2012). The initiation step (step 1) of macroautophagy is structurally defined as the generation of the crescent-shaped membranous structure called isolation membrane (IM) or phagophore. Studies have shown that de novo synthesis of IM for the formation of autophagosomes recruits lipids from several organelles including the ER (Hayashi-Nishino et al., 2009), mitochondria (Yu et al., 2010) and plasma membrane associated with clathrin coated structures (Ravikumar et al., 2010a; Ravikumar et al., 2010b), and from the mitochondria-associated membrane (MAM) domains (Hailey et al., 2010; Hamasaki et al., 2013a; Hamasaki et al., 2013b). Indeed, the initiation of autophagy is regulated by mTORC1 complex (mammalian Target Of Rapamycin Complex1), an important component of a network that senses the nutrient state of the cell and accordingly controls the levels of anabolism and catabolism to maintain homeostasis via class I of PI3K/Akt pathway (Yu et al., 2010). The nucleation (step 2) of autophagosome is negatively regulated by Bcl2 by binding to Beclin-1/Atg6, one of the components of the nucleation complex, and positively regulated by the class III PI3K, known as Vsp34 by recruiting a component of ubiquitin-like conjugation system LC3 (light chain of the microtubule-associated protein 1/Atg8) which plays a critical role in elongation, curvature and closure of the autophagosome, a process called maturation (step 3).
The autophagosome elongation mechanism depends on two Ub-like conjugation systems. A multimeric complex formed from the first conjugation system consisting of Atg5-Atg12/Atg16 is recruited to the phagophore and may function as an E3-like enzyme to facilitate the Ub-like protein LC3/Atg8 conjugated to phosphatidylethanolamine (PE). In this second conjugation system, the unconjugated form of LC-3/Atg8 (LC3-I) is cleaved by Atg4 in the cytosol, whereas the conjugated form (LC3-II) targets the autophagosomal membrane. This association of LC3/Atg8 to the autophagosome is considered important for the membrane elongation of the phagophore and the eventual enclosure of the membrane to form the autophagosome in that the conjugated form of LC3 can mediate tethering and hemifusion of lipid membrane. This suggests that it constitutes the driving force for autophagosome expansion by tethering delivered lipid membrane components and facilitating their incorporation into the expanding autophagosome (Nakatogawa et al., 2007). The Atg5-Atg12/Atg16 complex may be recycled, whereas LC3/Atg8 stays on the membrane until it is degraded by the lysosome (Chen and Yin, 2011).

The fusion step (step 4) is transportation of the autophagosome along microtubules to fuse with endosomes or lysosomes, which is mediated by dynein. These form double-membraned vesicles that contain cytoplasmic components by electron micrograph and their presence is regarded as a gold standard for the presence of autophagosomes inside cells. Upon fusion with the lysosome, autophagosome contents are degraded and the resultant products, including amino acid and lipids, are released for re-utilization in cellular metabolism. Inhibition of macroautophagy can be achieved through microtubule disrupting agents such as nocodazole, inhibition a specific vacuolar H^+ ATPase to suppression of lysosomal acidification by Bafilomycin A and other weak alkaline agents such as chloroquine (CQ) and hydroxychloroquine (HCQ) as mentioned before (Benbrook and Long, 2012).

In contrast to the non-selective degradation process induced by starvation, a nutrient-independent basal autophagy imposes intracellular Quality Control (QC) by selective delivery of aberrant protein aggregates and damaged organelles modified by ubiquitin to the inside of the developing autophagosome before fusion with the lysosome, a process called selective autophagy, also known as quality control autophagy (QC autophagy). Analogous to the proteasome, where ubiquitylated cargo is delivered by Ub receptors, autophagic clearance of protein aggregates involves Ub-binding adaptor proteins such as p62, NBR1 (Neighbor of BRC1), HDAC6 (Histone DeACetylase 6) (Johansen and Lamark, 2011; Kirkin et al., 2009c; Komatsu and Ichimura, 2010; Lee et al., 2010). Indeed, depending on the pattern of
ubiquitylation, the cargo proteins are sorted to the proteasome or autophagosome for degradation, which will be discussed in further detail in section 1.5.3 and 1.5.4.

**Chaperone-mediated autophagy (CMA)** targets specific cytosolic proteins bearing a general sequence of amino acid KFFERQ or a sequence that can be made to mimic KFFERQ through post-translational modifications into the lysosome for rapid degradation (Dice, 2007). It is estimated that at least 30% of cytosolic proteins contain a related KFFERQ. These proteins can be recognized and trapped by the molecular chaperone heat shock cognate protein of 70 kDa (Hsc70/HSPA8) and translocated into the lysosomal lumen through the interaction with lysosomal receptor Lysosome-Associated Membrane Protein type 2A (LAMP-2A). At the lysosome, the transported protein is pushed through the translocon comprising of multimer of LAMP-2A and unfolded by a complex of other chaperones inside the lysosome which interact with Hsc70 whose stability is tightly controlled by the pH of the lumen (Doppalapudi et al., 2012). **Microautophagy** occurs at basal levels and can be induced by nitrogen starvation or mitochondrial damage, as for macroautophagy and CMA. However, microautophagy appears to work synergistically with the other two forms of autophagy in contrast to the antagonism of macroautophagy by CMA (Doppalapudi et al., 2012).

**Endolysosome Degradation**

The endosome mediated lysosome pathway has been shown to be responsible for not only quantitative regulation of native plasma membrane proteins but also qualitative maintenance of conformational defective integral membrane proteins that escape the ERQC. The process of disposal of conformationally damaged integral membrane proteins along the late secretory and endocytic pathways in post ER compartments is called post-ERQC or peripheral QC (MacGurn et al., 2012). In recent years, to maintain homeostasis of integral membrane proteins, those membrane proteins with limited or delayed conformational defects that escaped from the ER were either retrieved from the cis-Golgi compartment back to the ER or targeted from the trans-Golgi network by vesicular transport carriers to early endosomes and finally degraded by the lysosome (Arvan et al., 2002; Houck and Cyr, 2012; Okiyoneda et al., 2011). The global conformational defects of these plasma membrane proteins may be attributed to impaired targeting to the CNX/CRT chaperon cycle at the ER (Lederkremer, 2009), and/or
direct structural destabilization of the native fold in a chaperone-independent manner (Glozman et al., 2009; Hanson et al., 2009). For example, the rapid degradation of the glycosylation-deficient CFTR from the PM was induced by a combination of these mechanisms (Glozman et al., 2009).

Indeed, the process of down-regulation of native functional plasma membrane proteins and quality control of conformational defective transmembrane proteins confined to the cell surface share similar signal sorting mechanism and cellular machinery (MacGurn et al., 2012; Okiyoneda et al., 2011). Like the ERAD and selective macroautophagy mediated degradation, the complex series of the quality and quantity maintenance systems for PM proteins are intimately controlled by ubiquitylation, which generally acts as a sorting signal that is recognized, captured and delivered to a specific cellular destination via proteins containing ubiquitin-binding domains. In fact, it is at the endosome where the fate of transmembrane proteins is decided: some are recycled back to the trans-Golgi network or to the plasma membrane, and others are sorted for lysosome-mediated degradation pathway. Poly-Ub and multiple-mono-Ub can serve as efficient endocytic and lysosomal targeting signals, these posttranslational modifications are exploited for the regulated disposal of both native and non-native PM proteins in mammalian cells (Rotin and Staub, 2011; Traub and Lukacs, 2007). PM proteins are sorted to the endosomal subdomains by adaptor protein Epsins/Eps and Eps15 family proteins which recognize both K63-linked and K48-linked poly-Ub chain and interact with ubiquitin through tandem ubiquitin-interacting motif (UIM) domains via clathrin dependent or clathrin independent, lipid raft-dependent endocytosis (Barriere et al., 2006; Kazazic et al., 2009; Liu and Chang, 2006; Polo et al., 2002; Rotin and Staub, 2011; Sigismund et al., 2005; Traub and Lukacs, 2007). Ub-cargoes are then recognized and captured by Ub-binding domains of the endosomal sorting complexes required for transport 0 complex (ESCRT-0), a tetramer (two heterodimer composed of Hrs and STAM1/2) which could bind up to 10 distinct ubiquitin moieties. Following ESCRT-0 complex mediated cargo sorting, the limiting membrane of the early endosome buds into the lumen, forming intraluminal vesicles (ILVs) loaded with cargoes, which leads to the multivesicular appearance of late endosomes, also known as multivesicular bodies (MVBs), which then fuse with lysosomes, depositing their contents into the lumen of the lysosome where cargoes are degraded and their amino acids recycled (Ciechanover, 2013).
Recently identified components of the PM QC recognition and effector machineries responsible for ubiquitylation and lysosomal degradation of conformationally damaged PM proteins uncovered striking similarities to and differences from that of the ERQC machinery (Okiyoneda et al., 2011). Using proteomic analysis and phenotypic siRNA screening, the components of ubiquitylation machinery involved in conformationally defective PM protein degradation via the peripheral QC system has been identified, including cytosolic E3 ubiquitin ligase C-terminal Hsp70 interacting protein (CHIP), as well as chaperones Hsp90 and Hsc70 (Apaja et al., 2010). Co-chaperone Hsc70 may link the chaperone-PM protein complex to Ub-binding adaptors to the internalization and lysosomal sorting machinery through its Ub-like domain (Okiyoneda et al., 2010).

Therefore, similar principles may govern the recognition of structurally defective proteins at different cellular locations. This redundancy of parallel and complementary peripheral QC pathways appears to enhance the recognition flexibility and fidelity of the QC system to triage a significant fraction of newly synthesized proteins at the ER and cytoplasm.

1.5.3 Ubiquitin sorting signals of proteasome and lysosome mediated degradation pathways

Ubiquitin (Ub), also known as ATP-dependent Proteolysis Factor 1 (APF-1) (Ciechanover, 2013), is a common signal in the targeting of substrates to the two major protein degradation pathways in mammalian cells: the proteasome and the lysosome which includes both endosomal and autophagosome mediated degradation pathways (Ciechanover, 2013; Clague and Urbe, 2010). As mentioned in section 1.3.2, Ub has 7 lysines (K6, K11, K27, K29, K33, K48, and K63) and the N-terminal of Ub that can form various topologies of polyUb chains (including Ub chain length and linkage type). In addition, single or multiple Ub (Ubs) can attach to the substrates, resulting in monoubiquitylation and multiubiquitylation. Accordingly, the proteasome, autophagosome and endosomal-lysosome system also contain various receptors and adaptors possessing a variety of ubiquitin binding domains (UBDs) to recognize different Ub signals. Therefore, the Ub tagging signals and the Ub binding motifs provide an excellent system to route different substrates to different degradation pathways.
As mentioned in section 1.5.1, the 26S proteasome is composed of a 20S core particle containing multiple proteolytic sites and a 19S regulatory particle that governs access to the core. A diverse set of ubiquitin receptors such as Rpn10 and Rpn13 which contain UIM (ubiquitin-interacting motif) domains and Pru (Pleckstrin-like receptor for ubiquitin) domains respectively directly dock ubiquitylated substrates onto the proteasome at its 19S regulatory particle through ubiquitin binding domains (Clague and Urbe, 2010). Alternatively, ubiquitylated substrates can be delivered to the proteasome by adaptor proteins such as Rad23 and Dsk2 which contain one or two ubiquitin associated (UBA) domains that bind polyUb chains and a UBL (ubiquitin like) domain that interacts with the proteasome. In contrast to Rpn10 and Rpn13 which themselves serve as subunits of 19S regulatory particle of the proteasome, Rad23 and Dsk2 function as a shuttle that guide ubiquitylated substrates from the ATPase p97/Cdc48 complex to proteasomes for degradation. More interestingly, the mammalian regulatory particle has been shown to be associated with a variety of E3 ubiquitin ligases, E4 elongation factors and DUBs that specifically modify the different linkage of Ub chains to provide for a proof-reading mechanism. This facilitates some substrates escape degradation by dissociating them from the proteasome and also allows ubiquitin salvage from the proteasome to be recycled to maintain the cellular ubiquitin pool (Finley, 2009). Indeed, the ubiquitin tags of substrates are in a highly dynamic state. For instance, DUBs Ubc6 and Uch37 trim the Ub chain from the distal end to allow the substrate to escape from the proteasome mediated degradation whereas the ubiquitin ligase Hul5 elongates the Ub chain to counteract the DUBs’ activity to allow enough time for the proteasome to select substrates for degradation. Once the substrate is finally committed for degradation, a subunit of 19S RP of proteasome Rpn1 cleaves off the entire Ub chain of proteins by hydrolyzing the isopeptide bond between the lysine in the substrate and the first ubiquitin moiety in the tag to rescue the ubiquitin and also prevent clogging of the proteasome (Schrader et al., 2009).

It is well established that proteins that are degraded by the proteasome are usually modified with a lysine 48 (K48)-linked polyubiquitin chain consisting of at least four conjoined ubiquitin molecules. The affinity of unanchored K48 linked polyUb chains for the proteasome increases more than 100-fold from di- to tetra-ubiquitin and less steeply thereafter (Thrower et al., 2000). Moreover, K6, K11, K29 but not K63 linked polyUb chain has been shown in vivo to be accumulate upon 26S proteasome dysfunction using quantitative mass spectrometry of ubiquitin linkage-specific signature peptides (Bedford et al., 2011a). Indeed, cellular K63-linked
chains have been shown to have less proteasomal accessibility compared with K48 chains in that proteasome-bound K63 linked chains are more rapidly deubiquitylated, which could cause inefficient degradation of K63 conjugates by proteasome (Jacobson et al., 2009). Structurally, K48 linked ubiquitin chains adopt a closed conformation with extensive contacts between two neighbouring ubiquitin monomers whereas K63 linked ubiquitin chains form an extended conformation like a string of beads. It has been revealed that the K63-linked polyubiquitin chain is the only exception amongst all unconventional ubiquitin linkage to be incapable of targeting proteins for proteasome degradation (Xu et al., 2009). The wholly different ubiquitin topologies between K48 linked and K63 linked ubiquitin chain may explain why cells use these distinct structures to target different protein degradation pathway (Lim and Lim, 2011).

In contrast, K63-linked polyUb chain of protein aggregates has been shown to be responsible for clearance of protein aggregates by lysosome, a process called selective autophagy by recruitment of p62 or NBR1 (BRCA1 gene 1), adaptors that couple K63-linked ubiquitylated substrates to the preautophagosomal membrane rich in LC-3/Atg8 through its ubiquitin-interacting domain (UBA) and LC3-interacting region (LIR motif) (Johansen and Lamark, 2011; Kirkin et al., 2009c; Komatsu and Ichimura, 2010). More interestingly, although LC3/Atg8 family proteins do not share any primary sequence homology with Ub, they structurally resemble Ub and are classified as Ubiquitin Like proteins (UBLs). In addition, LC3/Atg8 is also conjugated to their substrates which are required for autophagosome maturation (Kirkin et al., 2009c). Another adaptor protein HDAC6 which does not contain the LIR motif has been demonstrated to facilitate the transport of aggregated protein cargo to dynein motors to form aggresomes, juxtanuclear inclusion bodies, by virtue of its ability to bind both ubiquitylated misfolded proteins and dynein motors (Kawaguchi et al., 2003). Moreover, HDAC6 has also been found to promote autophagosome-lysosome fusion via recruitment of actin remodeling machinery to ubiquitylated protein aggregates for selective clearance of misfolded proteins and damaged mitochondria (Lee et al., 2010). Therefore, HDAC6 influenced both the recruitment of ubiquitylated proteins to the aggregates and the lysosomal dynamics (Kirkin et al., 2009c). Interestingly, all the three ubiquitin binding autophagy receptors described above, i.e. p62, NBR1 and HDAC6, show preference for K63-linked polyUb chain compared to K48-linked polyUb, supporting that this form of ubiquitin modification acts as a cargo selection signal for the clearance of aggregated proteins by autophagic degradation (Kirkin et al., 2009a; Kirkin et al., 2009b; Olzmann et al., 2007; Tan et al., 2008).
Although polyUb chains are most frequently associated with proteolytic degradation, recent study has shown that monoubiquitylation of substrates is sufficient to deliver substrates to autophagosomes in a p62-dependent manner in mammalian cells using a lysine-less mutant of ubiquitin fused with red fluorescent protein (Kim et al., 2008c). However, the low binding affinity between the p62 UBA domain and monoUb suggests that other factors such as substrate/receptor oligomerization might play a role in selective degradation of monoubiquitylated cargo by autophagy (Kirkin et al., 2009c). Indeed, it has been demonstrated that monoubiquitylation of α-synuclein, a critical player in Parkinson’s disease leads to the aggregation of α-synuclein and inclusion body formation within dopaminergic cells (Engelender, 2008). The tendency of misfolded proteins to become aggregated would not only preclude their degradation by the proteasome, whose function might be impaired by clogging its narrow catalytic pore, but also serve as seeds to recruit adaptor proteins such as p62 and NBR1 for directing to the autophagosome for degradation (Engelender, 2008; Lamark et al., 2009; Lim and Lim, 2011).

Many endocytic cargoes including both internalized normal proteins and defective integral membrane proteins that escape the ERQC into the endolysosomal degradation pathway are subjected to monoubiquitylation or K63-linked polyubiquitylation in vivo (Duncan et al., 2006; Hoeller and Dikic, 2010; Lauwers et al., 2010; MacGurn et al., 2012; Paiva et al., 2009; Polo et al., 2002; Vina-Vilaseca and Sorkin, 2010). It has been demonstrated that proteins fused to a single Ub moiety were efficiently delivered to the MVB lumen, strongly indicating that a single Ub is sufficient for sorting to MVBs (Stringer and Piper, 2011). However, another study showed that monoubiquitylation is sufficient for initial internalization of protein from the cell surface but that efficient sorting to the multivesicular body (MVB) by the ESCRT machinery requires K63-linked polyubiquitin (Lauwers et al., 2009). A third study made the finding more solid by showing that the binding affinity of intact ESCRT-0 to K63-linked tetra-ubiquitin is 50 times tighter to monoubiquitin (Ren and Hurley, 2010). Interestingly, a recent study demonstrated that K63 linkages are not required for MVB sorting of cargo. In addition to K63-linked poly-Ub chains, K11-, K29-, and K48- linked poly-Ub chains can also be recognized as internalization and lysosomal sorting signals, suggesting that ESCRTs do not specifically recognize the K63 linkage (Boname et al., 2010). Indeed, K63-linked tetra-ubiquitin has been shown to bind 2-fold more tightly to intact human ESCRT-0 than K48-linked tetra-ubiquitin (Ren and Hurley, 2010), consistent with the emerging model that an overlapping set of Ub-
chains can participate in proteasome-dependent and lysosomal-dependent protein degradation with variable efficiency (Boname et al., 2010; Xu et al., 2009).

Similar to the proteasome which associates with both E3 ubiquitin ligases and DUBs, the ESCORT machinery has also been found to associate with various E3 ubiquitin ligases and DUBs that can either rescue a substrate or hasten its demise. For example, in yeast, both E3 ubiquitin ligase Rsp5 and DUB Ubp2 have been shown to be localized to endosomal structures and interact with ESCRT-0 components and are involved in editing the K63-linked ubiquitin chains of substrates thereby influencing cargo fate on the endosome (Kee et al., 2005; Kee et al., 2006; Ren et al., 2007; Wang et al., 2001).

Given the proposed role of different linked ubiquitylation in the routing of intracellular proteins, it is not surprising to find that the same proteins that are modified with different type of ubiquitin chain undergo different degradation pathways. For example, ENaC (epithelial sodium channel), a plasma membrane protein which plays a crucial role in Na\(^+\) transport and fluid reabsorption in the kidney, lung and colon and whose dysfunction leads to Liddle’s syndrome and pseudohypoaldosteronism type I, has been shown to be degraded by a dual degradation pathway; K48 linked polyubiquitylated ENaC is degraded by the proteasome whereas monoubiquitylated ENaC is trafficked to the endosome and degraded in the lysosome. Similarly, tau, \(\alpha\)-synuclein and SOD1 mutants, which play a critical role in neurodegenerative diseases, are also subjected to both proteasome and autophagy-lysosome mediated degradation pathways depending on the ubiquitylation type (Engelender, 2008; Shin et al., 2005b; Tan et al., 2008; Wang and Mandelkow, 2012).

Although it is clear that proteins may be degraded via both proteasome and lysosome systems, the existence and nature of the presumed molecular switch that mediates the choice between these two pathways remains to be resolved. It has been found that the capability of E3 ubiquitin ligase CHIP and parkin to form both K48-linked and K63-linked ubiquitin chains relies on the ubiquitin-conjugating enzymes E2 partners they interact with. For example, interacting with UbcH5a, CHIP generates K48-linked ubiquitin chains whereas engaging Ubc13/Uev1a, the only E2 known to date to mediate the exclusive assembly of K63-linked polyUb chains, CHIP results in K63-linked polyUb chains (Lim and Lim, 2011; Zhang et al., 2005). This ability of CHIP to bind Ubc13 and other E2s would potentially allow the enzyme to mediate degradation decisions between lysosomal and proteasomal pathways, as appears to be
the case with α-synuclein, whose CHIP-mediated K63-linked ubiquitylation targets the aggregation-prone protein for autophagic clearance (Shin et al., 2005b). Like CHIP, parkin can also bind different E2 partners to mediate the formation of distinct topology of ubiquitin chains. For instance, parkin interacts with UbcH7 or H8 to catalyze the formation of K48-linked degradation associated ubiquitin chains, while parkin-mediated K63-linked polyubiquitylation of synphilin-1 promotes its sequestration into inclusion bodies as well as its subsequent clearance by autophagy. Similarly, it has been demonstrated that K63 ubiquitin modification of misfolded DJ-1 by parkin couples the proteins to the dynein motor complex via HDAC6 adaptor, thereby, enhancing its recruitment into aggresomes although misfolded DJ-1 is usually rapidly degraded by the proteasome. Indeed, the generation of DJ-positive aggresomes by parkin-mediated K63 ubiquitylation appears to occur only during periods of proteasomal stress, which supports the existence of an ubiquitin-associated molecular switch that mediates cellular triage between proteasomal and lysosomal degradation under different conditions including various stresses (Lim and Lim, 2011). Although how the multi-catalytic parkin enzyme chooses its E2 partners to suit various cellular circumstances is presently not well understood, one could assume that its affinity for Ubc13 will increase in the presence of proteasome dysfunction. More interestingly, the capacity of parkin to divert misfolded proteins away from a compromised proteasome may explain its extraordinary ability to preserve proteasome function under various conditions of proteolytic stress (Lim and Lim, 2011).

In summary, the factors that direct a substrate toward a particular route of degradation likely include ubiquitin chain length and linkage type, which may favour interaction with particular receptors or confer differential susceptibility to ubiquitin ligase and DUB activity associated each pathway. This adaptable nature of ubiquitin signals for targeting proteins to different degradation pathway under different physiological and pathological conditions could reflect the extensive cellular processes that are tightly regulated by specific signaling events.

1.5.4 Interplay between the proteasome and lysosome mediated degradation

From above discussion, it is not too hard to find that the significant difference between the proteasome and lysosome degradation pathway with respect to their substrates (the activity of UPS is restricted to functional or misfolded soluble proteins whereas lysosome mediated
degradation is broader including not only soluble proteins, but also protein complexes, oligomers and aggregates), machinery structure (lysosome has a vesicular structure while the enzymatic reactions of the UPS occur directly in the cytosol), localization (lysosome is localized in the cytosol whereas proteasome resides in both cytosol and nuclear), specificity, kinetics, and control (Korolchuk et al., 2010). Therefore, for a long time, the two degradation pathways were regarded as independent of each other with few or no points of intersection. However, the view started to change recently based on the finding of reliance of ubiquitylation by both proteasome and lysosome mediated degradation pathways. Indeed accumulating studies have shown that perturbations in the flux through either pathway affect the activity of the other system (Korolchuk et al., 2010).

It has been reported that impairment of the UPS leads to enhanced autophagic degradation, which provides a compensatory mechanism for cell survival, allowing cells to reduce the burden of accumulated UPS substrates under ER stress via UPR signaling (Ding et al., 2007b). Indeed, two independent studies have revealed the role of transcription factor ATF4 in upregulation of autophagy genes ATG5, ATG7 and LC3/Atg8. ATF4 mediated upregulation of ATG5 and ATG7 is dependent on phosphorylation of eIF2α (Zhu et al., 2010) whereas upregulation of LC3/Atg8 is independent of PERK- eIF2α signaling (Milani et al., 2009) upon proteasome inhibition. In addition, Wei et al. has shown that starvation induced autophagy is due to disrupting autophagy-inhibitory interaction of Bcl-2 with Beclin-1/Atg6 by phosphorylation of Bcl-2 via IRE1-JNK signaling (Wei et al., 2008a). Moreover, IRE1-JNK pathway is also required for the lipid conjugation of LC3 in autophagy induced by proteasome inhibitors (Chen and Yin, 2011). Furthermore, the ER stress-associated Ca^{2+} release from the ER into the cytosol can also trigger autophagy by inhibition of mTORC1 through activating CaMKKβ (Calmodulin-dependent kinase-β)-AMPK (AMP activated protein kinase) pathway (Hoyer-Hansen and Jaattela, 2007). HDAC has also been identified as a necessary factor for enhancing delivery of substrates to the autophagic machinery for degradation when the UPS is impaired in a fly neurodegenerative disease model (Pandey et al., 2007a; Pandey et al., 2007b). The compensatory response of autophagy induced by proteasome inhibition also comes from the study showing that the p97/VCP which coordinated the proteasome-dependent ERAD is implicated in maturation of ubiquitin-containing autophagosomes following proteasome inhibition (Tresse et al., 2010).
On the other hand, however, autophagy impairment has been demonstrated to result in decreased UPS flux due to the competition binding of ubiquitylated proteins by autophagic adaptor p62 (Korolchuk et al., 2009). As mentioned in section 1.5.3, ubiquitylated proteins undergo degradation via one of the two major routes: the proteasome or the lysosome. Classically, K48-linked polyUb chain allows recognition of the proteolytic substrate by UBD-containing proteasomal receptors, whereas K63-linked chains and monoubiquitylation marked cargoes may be preferentially targeted to the lysosomal degradation pathway. Although Ub-binding autophagy receptor p62 has been reported to have preference for the K63-linked Ub chains, it has been shown to compete for ubiquitylated cargo with the classical proteasomal receptors like p97/VCP complex. Inhibition of autophagy and accumulation of p62 slowed down degradation of regular proteasomal substrates by delaying their delivery to the proteasome's proteases, most likely due to the excessive interaction between stabilized oligomeric p62 and K48-linked Ub-conjugated substrates (Korolchuk et al., 2009). More interestingly, p62 has also been suggested to directly interact with the proteasome (Seibenhener et al., 2004), suggesting autophagy receptors may have a more direct role in proteasomal degradation of ubiquitylated proteins.

In addition, the relative contribution of degradation pathways may vary greatly under different conditions. Quantitative mass spectrometry revealed that in most cases of cells cultured under stress-free conditions, proteasomal degradation predominates whereas under amino acid starved conditions, autophagy mediated lysosomal degradation was identified as the main compensatory degradation route due to the degradation of proteasome (Kristensen et al., 2008). Intriguingly, rates of degradation of many proteins were shown to change with changing physiological conditions, such as availability of nutrients or hormones and pathophysiological conditions such as starvation or re-supplementation of nutrients (Ciechanover, 2013; Fuertes et al., 2003a; Fuertes et al., 2003b). For example, CYP2E1, an ER anchored cytochrome P450, responsible for the biotransformation of clinical relevant drugs, exhibits biphasic turnover. CYP2E1 substrate complexation converts it into a stable slow-turnover species (t1/2, 37h), degraded largely via autophagic lysosomal degradation whereas substrate decomplexation withdrawal results in a fast turnover species (t1/2, 7h), that incurs ERAD dependent proteasome degradation, probably by regulation of the phosphorylation and ubiquitylation of the protein (Wang et al., 2010a).
As mentioned before, both p97/VCP and p62 have been shown to be involved in proteasome and lysosome mediated degradation pathways (Seibenhener et al., 2004; Tresse et al., 2010). Moreover, the E3 ubiquitin ligases Parkin and CHIP that can target proteins to the proteasome have been shown to be implicated in generating a tag for autophagic degradation of proteins. Therefore, those factors may provide the physical link between both proteasome and lysosome mediated degradation pathways. Ubiquitin chain length and linkage type and the location, etc. may determine which degradation pathway is responsible for degradation of a particular substrate due to the subcellular localization of specific E3 ligases in combination with a high local concentration of ubiquitin binding proteins, which may favour interaction with either the proteasome or the lysosome machinery (Clague and Urbe, 2010).

1.6 p38 MAPK Signaling Pathway

p38 MAPK signaling is one of the three conventional branches of MAPK (Mitogen Activated Protein Kinases) pathways as shown in Scheme 1.14. Unlike the ERK (Extracellular signal-Regulated Kinases) MAPKs activated by growth factors, p38 MAPKs, similar to the JNKs (Jun N-terminal kinases), are activated by environmental and genotoxic stresses and inflammatory cytokines (Cuadrado and Nebreda, 2010). Thereby both the p38 and JNK MAPK signaling pathways are termed Stress-Activated Protein Kinase (SAPK) pathways (Cargnello and Roux, 2011; Roux and Blenis, 2004).

Although each MAPK has unique characteristics, each family of MAPKs is composed of a set of three evolutionarily conserved protein modules which consist of a three sequentially acting kinase cascade: a MAPK, a MAPK kinase (MAPKK, MAP2K), and a MAPKK kinase (MAPKKK, MAP3K). Upon activation by an upstream component, a serine-threonine kinase MAP3K leads to the phosphorylation and activation of a dual specificity kinase MAP2K, which in turn stimulates MAPK activity via dual phosphorylation on threonine and tyrosine residues within a flexible loop termed the activation loop or phosphorylation lip located close to the kinase active site. Once activated, MAPKs phosphorylate target substrates, on serine or threonine residues followed by proline, therefore known as proline-directed protein kinases (PDPK) (Roux and Blenis, 2004).
Different kinds of stimuli promote the activation of 3 conventional and 2 atypical MAPKs which in turn lead to activation of 5 subgroups of MAPKAPKs including RSK, MSK, MNK, MK2/3, and MK5. Image from (Cargnello and Roux, 2011) and reproduced with permission from American Society for Microbiology.

1.6.1 Activation of p38 MAPK signaling pathway

Since p38α was found simultaneously by four independent groups in 1994 (thereby also termed as Cytokine-Suppressive anti-inflammatory drug-Binding Protein, CSBP; Homologue of *Saccharomyces cerevisiae* Hog1, hHOG1; Reactivating Kinase, RK and SAPK2) (Freshney et al., 1994; Han et al., 1994; Lee et al., 1994; Rouse et al., 1994), four p38 MAPK isoforms have been identified which share approximately 60% identity in their overall amino acid sequence and 90% identity within the kinase domains. The four p38 MAPKs are encoded by different genes and have different tissue expression patterns, with p38α (MAPK14, SAPK2a,) and p38β
(MAPK11, SAPK2b) (Jiang et al., 1996) being ubiquitously expressed in most cell lines and tissues, whereas p38γ (MAPK12, SAPK3, ERK6) (Cuenda et al., 1997; Li et al., 1996) and p38δ (MAPK13, SAPK4) (Goedert et al., 1997) seem to be expressed in a more restricted expression patterns, for example, p38γ in skeletal and cardiac muscle and p38δ in testes, pancreas and small intestine (Kumar et al., 1997; Marber et al., 2011). p38α and p38β are closely related proteins that could have overlapping functions. p38α is expressed at highly abundant levels in most cell types whereas p38β seems to be expressed at very low levels and its contribution to p38 MAPK signaling is not clear. Moreover, several alternatively spliced isoforms of p38α (Mxi2, Exip, CSBP1) have also been reported; however, the functional differences of the sliced isoforms of p38α are poorly characterized (Cargnello and Roux, 2011; Cuadrado and Nebreda, 2010; Wagner and Nebreda, 2009). Similarly, p38β also has another alternative form p38β2 (SAPK2b2), which differs by the lack of the additional 8 amino acid insertion unique to p38β (Kumar et al., 1997; Stein et al., 1997). In addition to the notable differences in tissue expression among these isoforms, the upstream activators and downstream effectors and sensitivity to chemical inhibitors are also different despite their high sequence homology. However, the specific function of individual isoforms in physiological and pathological processes has yet to be clearly defined. Indeed, the specific isoform of p38 MAPK knockout mice demonstrated that genetic ablation of p38α results in embryonic lethality at embryonic day 10.5-11.5 due to defective placental development, abnormal angiogenesis in the embryo and the yolk sac whereas knockout of single p38β, p38γ, p38δ genes, or double ablation of p38γ and p38δ results in viable and fertile mice with no apparent phenotype, indicating that p38α is the only isoform that is critical for mouse development. In addition, it has been shown that ablation of one isoform of p38 does not affect the expression or activity of the other isoforms (Aouadi et al., 2006). In this thesis, if it is not particularly pointed out, p38 MAPK usually refers to p38α and p38β due to their similar sensitivity to chemical inhibitors and overlapping functions.

As for the other MAPKs, activation of p38 MAPKs requires dual phosphorylation of one threonine and one tyrosine residue within a Threonine-Glycine-Tyrosine (TGY) tripeptide sequence (Wilson et al., 1996) in the activation loop. The dual-specificity MAPK2K, MKK3 and MKK6, are thought to be the major and highly specific protein kinases responsible for p38 MAPK activation (Cuadrado and Nebreda, 2010; Raingeaud et al., 1996). MKK6 activates all of the four p38 isoforms whereas MKK3 is somewhat more selective, as it preferentially phosphorylates the p38α, p38δ and p38γ isoforms but not p38β (Alonso et al., 2000; Enslen et
al., 1998). Furthermore, MKK4, an activator of the JNK pathway, has been shown to possess some activity toward p38α activation under the direction of select stimuli (Brancho et al., 2003). Due to variations in MAP2K expression levels among different cell types, the relative contribution of different MAPK2Ks to activation of p38 MAPK family members depends on the cell type, but also on the stress stimulus. The specificity in p38 activation is thought to result from the formation of functional complexes between MKK3/6 and different p38 isoforms and from the selective recognition of the activation loop of p38 isoforms by MKK3/6.

In addition to the canonical activation pathway discussed above, p38α MAPK can be activated by another two alternative auto-phosphorylation mechanisms. Autophosphorylation of the TGY activation loop is achieved by phosphorylation of tyrosine 323 by the TCR-proximal tyrosine kinase Zap70 (ζ-chain-associated protein kinase) in T-lymphocytes upon T-cell receptor (TCR) engagement (Salvador et al., 2005). The other mechanism involves a scaffold protein known as TGF-β-activated protein kinase 1 binding protein 1 (TAB1) which has been shown to directly activate p38 MAPK during myocardial ischemia without interacting with the upstream MAPK3K, TGF-β-activated-protein kinase 1 (TAK1) which is capable of activating MKK3 and MKK6 (Marber et al., 2011). In addition, TAB1 is a p38 substrate, suggesting that it may also regulate p38 activity by acting as a component of a closed feedback loop.

As mentioned above, MKK3 and MKK6 are activated by a plethora of MAPK3Ks, including TAK1, ASK1 (Apoptosis signal-regulating kinase 1), MEKK (MAPK/ERK kinase kinase) 3 and 4, MLK (Mixed-lineage kinase) 3, TPL (Tumor Progression Loci) 2, TAO (Thousand And One amino acid) 1 and 2 (Cargnello and Roux, 2011; Cuadrado and Nebreda, 2010; Cuevas et al., 2007). ASK1 will be discussed in further detail in section 1.6.4. Regulation upstream of the cascade of MAP3Ks is even more complex, involving phosphorylation by STE20 family kinases and binding of small GTP-binding proteins of the Rho family as well as ubiquitylation-based mechanisms. Therefore, the diversity of MAP3Ks and their regulatory mechanisms provide the ability to respond to many different stimuli and to integrate p38 MAPK activation with other signaling pathways (Cuevas et al., 2007).
1.6.2 Inactivation and inhibitors of p38 MAPKs

It is critical to regulate the intensity and duration of activity of p38 MAPKs to achieve specific outcomes in that transient phosphorylation can be associated with growth factor induced cell survival whereas sustained phosphorylation is frequently associated with induction of apoptosis (Coulthard et al., 2009). The duration of the kinase catalytic activity of p38 signaling is controlled by the phosphatases that dephosphorylate serine/threonine or tyrosine residues within the activation loop of p38 MAPKs. Those phosphatases include generic serine/threonine specific phosphatases (such as protein phosphatase 2A (PP2A) and PP2C) and tyrosine specific phosphatases (such as STriatal Enriched tyrosine Phosphatase (STEP), haemopoietic tyrosine phosphatase (HePTP) and Protein Tyrosine Phosphatase SL (PTP-SL)) as well as Dual Specificity Phosphatases (DUSPs, or MAPK Phosphatases/MKPs) (such as MKPs 1, 4, 5 and 7) that dephosphorylate phosphoserine/phosphothreonine and phosphotyrosine residues simultaneously (Cuadrado and Nebreda, 2010). Multiple cellular systems have demonstrated that MAPK activity correlates inversely with expression of a MAPK phosphatase, that is, induction of a MAPK phosphatase is paralleled by a decrease in the extent of MAPK activity. In addition to the correlative studies with endogenous MAPK phosphatase, ectopic expression of exogenously introduced MAPK phosphatase expression results in changes in the extent and timing of MAPK activation. For example, induction of MKP-1 in U937 leukemic cells conditionally expressing MKP-1 from the human metallothionein IIa promoter resulted in a loss of phorbol 12-myristate 13-acetate PMA-inducible p38 MAPK activity (Franklin and Kraft, 1997). As the expression of the dual specific MAPK phosphatases is generally not constitutive but is dependent on new transcription and translation, rapid MAPK dephosphorylation is usually mediated by the generic serine/threonine specific or tyrosine specific phosphatases. The activity of the generic phosphatases would lead to the formation of monophosphorylated or unphosphorylated forms of p38α MAPK, which shows 10-20 fold less or completely loss of catalytic activity (Zhang et al., 2008). However, in most cases, inactivation of MAPKs requires DUSPs to dephosphorylate both phosphoserine/threonine and phosphotyrosine residues, particularly after sustained MAPK activation that leads to new gene transcription. It has been found that MAP kinase phosphatases are commonly induced in response to the same stimuli that activate MAPK themselves, creating a negative feedback loop that tightly regulates activity of p38 MAPKs. Not unexpectedly, MAPK activation itself is often an important component in the regulation of MAPK phosphatase induction. However, relatively few examples have been
reported for specific loss of MAPK phosphatase function because of the potential redundancy, suggesting that loss of a single phosphatase may not have dramatic effects in a dynamic system. Perhaps combinations of MAPK phosphatase gene disruptions will inform about function as well as cross-regulation. For example, two tyrosine phosphatases encoded by PTP2 and PTP3 have been found to play important roles in down-regulating the activity of the basal as well as the induced activity of Hog1p after increased osmolarity, a homologue of mammalian p38 MAPK in *Saccharomyces cerevisiae* (Wurgler-Murphy et al., 1997). In addition, the level of MKP-1 induced by both hyperosmolarity in hepatoma cells and p38 activator anisomycin in mesangial cells is in a p38 MAPK dependent manner; while p38 MAPK inhibitor SB203580 reduces the induced expression of MKP-1 (Bokemeyer et al., 1997; Schliess et al., 1998).

In addition to the direct dephosphorylation of the dual phosphorylation sites of p38 MAPKs to inactivate p38 MAPKs, another two negative regulatory mechanisms to control the activity of p38 MAPKs have also been reported. One involves the G-protein coupled Receptor Kinase-2 (GRK2) that can prevent MKK6-induced phosphorylation and activation of p38 MAPK by phosphorylation on a residue in its docking groove (T123) (Peregrin et al., 2006). The other is mediated by the p38 MAPK downstream substrate TAB-1. Once phosphorylated by p38 MAPK upon stimulation by cytokines such as TNFα or IL-1, TAB-1 decreases the activity of TAK-1, an upstream activator of p38 MAPK, thereby forming a negative feedback loop to inhibit the p38 MAPK activity (Cheung et al., 2003; Schindler et al., 2007).

X-ray crystallography has revealed that the structure of p38 MAPKs consists of two distinct lobes, creating a deep channel between the two domains where potential substrates may bind. The N-terminal domain forms a binding pocket for ATP and the C-terminal domain contains the catalytic residues involved in the transfer of the γ phosphate to the protein substrate. When p38 MAPK is in the non-dual phosphorlylated, inactive conformation, the activating loop mentioned above is thought to reside in the peptide-binding channel that lies in the cleft between the N- and C-terminal lobes of the kinase. In addition, misalignment of these lobes prevents the cooperation between the N-terminal lobe and the C-terminal lobe, imperative to the binding and stabilization of ATP. However, dual phosphorylation events induced by the MKK is thought to cause the activation loop to refold and move out of the peptide-binding channel. This movement is then thought to exert a “crank-handle” effect on the overall tertiary structure of the kinase reorienting the N-and C-terminal lobes towards one another, enabling the cooperation necessary.
for ATP binding and restoring the active conformation of the catalytic spine of the kinase. Thus, based on structure and function, ATP binding occurs after, and not before, dual phosphorylation of the activation loop (Marber et al., 2011). The dual phosphorylation induced conformational reorganization relieves steric blocking and stabilizes the activation loop in an open and extended conformation, facilitating substrate binding.

Most ATP-competitive p38 MAPK inhibitors bind with similar affinity to both activated and non-activated kinases (Schindler et al., 2007). The pyridinyl imidazole SB203580, selectively inhibits the kinase activity of p38α and p38β MAPKs but not p38γ and p38δ MAPKs by acting as a competitive inhibitor of ATP binding (Young et al., 1997). The X-ray crystal structures of p38 MAPK-SB203580 complex revealed that the three-dimensional structure of p38β is highly similar overall to that of p38α; however, there are some differences in the relative orientation of the N- and C-terminal domains that causes a reduction in the size of the p38β ATP-binding pocket. This difference in size between the two pockets could perhaps be exploited to achieve selectivity of ATP-competitive inhibitory compounds (Patel et al., 2009; Wrobleski and Doweyko, 2005).

1.6.3 Function of p38 MAPK signaling pathway

p38 MAPKs are activated by exogenous cellular stresses and proinflammatory cytokines. Consistent with its designation as a stress activated protein kinase, p38 MAPKs are activated by a number of chemical, heat, pH, osmotic shock, hypoxia, ischemia, oxidative stress, ultraviolet, ionizing radiation, and growth factor withdrawal, high or low glucose, photodynamic therapy, lipopolysaccharides (LPS) as well as various cytokine stimuli such as interleukin (IL)-1, tumor necrosis factor alpha (TNFα). In addition to environmental stresses, p38 MAPKs have also been shown to be activated by intracellular stresses including ER stress arising from the overloaded protein in the ER which will be discussed in greater detail in section 1.6.4.

The substrates of p38 MAPKs include protein kinases, transcription factors, enzymes, and cytoskeletal proteins. The downstream kinases that are activated by p38 MAPK are members of MAPK activated kinases (MAPKAPKs) family which belong to the calcium/calmodulin-dependent protein kinase (CAMK) family including Mitogen-interacting Kinases (MNKs) and Mitogen-and Stress-activated Kinases (MSKs) and MAPK-activated
protein Kinases (MKs). p38 MAPKs modulate mRNA stability, protein translation, chromatin remodeling and transcription factor activity through phosphorylation and activation of MK2/3 (McLaughlin et al., 1996; Rouse et al., 1994), MNK1(Knauf et al., 2001; Waskiewicz et al., 1997) and MSK1/2 (Deak et al., 1998; McCoy et al., 2007; Vermeulen et al., 2009), that represent an additional enzymatic amplification step in the MAPK catalytic cascade (Cargnello and Roux, 2011; Roux and Blenis, 2004). In addition, p38 dependent cytokine production (Han et al., 1994; Lee et al., 1994; Neininger et al., 2002; Winzen et al., 1999), actin remodeling, cell migration (Hedges et al., 1999; Menon et al., 2009; Rousseau et al., 1997), and cell cycle control (MacCorkle and Tan, 2005; Manke et al., 2005; Weber et al., 2005) occurs primarily through MK2/3 activation (Gaestel, 2006; Ronkina et al., 2008).

In addition to phosphorylation of downstream kinases, p38 MAPKs directly phosphorylate various transcription factors, including ATF1, 2 and 6, Elk1, and CHOP, p53, C/EBPα, myocyte enhance factor 2A/2C (MEF-2A/2C), SRF (Serum Response Factor) Accessory Protein (SAP-1a) and regulate their activity (Cuadrado and Nebreda, 2010). Moreover, a core network of 16 transcription factors has also been recently proposed to mediate the regulation by p38α of human squamous carcinoma cell quiescence (Adam et al., 2009).

Furthermore, p38 MAPK signaling is also emerging as an important modulator of protein turnover. For example, rapid activation of the p38 MAPK pathway in response to UV radiation enhances the damaged-DNA-binding complex 2 (DDB2) ubiquitylation and degradation via target protein phosphorylation, which facilitates nuclear excision repair factor XPC assembly in DNA damage sites in response to UV-induced DNA lesions (Zhao et al., 2008). Another example is phosphorylation of FLIPs short isoform of FILCE (Fas-associated death domain-like IL-1β converting enzyme-inhibitory protein) by ASK1-p38 MAPK signaling. Indeed, ROS triggered by bacteria infection facilitates the interaction between phosphorylated FLIP and E3 ubiquitin ligase c-Cbl which leads to proteasome degradation of FLIP and in turn allows procaspase 8 to interact with FADD to initiate apoptosis (Kundu et al., 2009). A third example is p18Hamlet protein, a transcription co-activator that recruits p53 to promoters, is phosphorylated by p38α MAPK, which increases its stability and hence enhances transcription of several p53-dependent genes such as Noxa and PUMA involved in apoptosis (Cuadrado et al., 2007). Furthermore, the RING finger ubiquitin ligase Siah2 has also been shown to be
phosphorylated by p38 MAPKs which lead to its stabilization and in turn down-regulation of PHD3, which controls the stability of HIF-1alpha (Khurana et al., 2006).

**Scheme 1.15: The p38 MAPK signaling cascades.**

Different stimuli such as various environmental stresses, inflammatory cytokines and growth factors can activate p38 MAPKs. The signals are sequentially transduced through the kinase cascade MAPKK kinases (MAPKKK, MAP3K), MAPK kinases (MAPKK, MAP2K), and various isoforms of p38 MAPKs to downstream substrates including protein kinases, cytosolic substrates, transcription factors and chromatin remodelers to cause a wide variety of cellular functions. Image reproduced from (Cuadrado and Nebreda, 2010) with permission from Portland Press Ltd (DOI:10.1042/BJ201000323).
1.6.4 The role of p38 MAPK signaling pathway in cell death and survival

p38 MAPK signaling pathways have been involved in regulation of both cell death and survival. Indeed, p38 MAPK pathway promotes both apoptosis and autophagy mediated cell death. It has been shown that p38 MAPK signaling pathway is implicated in apoptosis not only via extrinsic Fas ligand mediated apoptosis pathway in differentiated PC12 cells triggered by NGF withdrawal (Le-Niculescu et al., 1999) but also through intrinsic mitochondrial mediated apoptosis pathway in various human cancer cell lines including ovarian carcinoma CA-OV-3 cells treated by retinoid CD337 (Holmes et al., 2003), cervical carcinoma Hela cells treated with nocodazole, a microtubule-interfering chemotherapeutic drug (Deacon et al., 2003), T cell lymphoma Jurkat cells and non-small cell lung cancer NCI H460 cells treated with phytosphingosine (Park et al., 2003).

The mechanism of p38 MAPK signaling mediated apoptosis, particularly via the reactive oxygen species (ROS) sensor Apoptosis Signal-regulating Kinase 1 (ASK1), has been well established. ASK1 is the only one of 20 identified MAP3Ks that contains a thioredoxin-binding region and promotes apoptosis signal-regulating kinase specificity in response to the intracellular redox state. ASK1 is activated in response to both intracellular and extracellular signals including oxidant stress, ATP (Noguchi et al., 2008) and death receptor ligands (such as TNFα, Fas ligand) (Chang et al., 1998; Ichijo et al., 1997). More recent studies have revealed new mechanisms by which ASK1 is activated in response to various stimuli, such as Ca^{2+} signaling (Takeda et al., 2004), G-protein coupled receptor (GPCR) signaling and ER stress (Matsukawa et al., 2004; Matsuzawa et al., 2002; Nishitoh et al., 2008; Nishitoh et al., 2002). ASK1 has a threonine kinase domain in the middle of the molecule and two coiled-coil domains in the N- and C-terminal which are important for ASK1 activation by homo-oligomerization leading to auto- and/or cross-phosphorylation as well as a 14-3-3 binding motif in the C-terminal that binds 14-3-3, a negative regulator of ASK1 activity in an oxidative stress dependent manner. Activation of ASK1 is tightly regulated by phosphorylation of the threonine residue within the activation loop in the kinase domain of ASK1. When the cell is at rest, ASK1 forms a homo-oligomer via its C-terminal coiled-coil domain and the N-terminal is bound to the redox sensitive protein thioredoxin (Trx), maintaining the inactive state. Exposure of cells to ROS generated in response to cellular stresses, cytokine stimulation and LPS oxidizes thioredoxin, resulting in dissociation from ASK1 and thus releasing ASK1 from inhibition
In addition, using gel filtration column chromatography, ASK1 has been shown to constitutively form a high molecular mass complex, designated the ASK1 signalosome, through its C-terminal coiled-coil domain. Upon dissociation of Trx from ASK1 by ROS, the ASK1 signalosome forms a higher molecular mass complex by reciprocally recruiting at least two TNF receptor-associated factor (TRAF) family proteins, TRAF2 and TRAF6, which appear to stabilize the complex and enhance the activating phosphorylation of ASK1 (Nishitoh et al., 1998). In mammalian cells, TRAF2 is not only an essential molecule in oxidative stress-induced activation of ASK1 but also a pivotal component that links ER-resident molecules sensing ER stress to the cytosolic ASK1-MAPKs pathways (Nishitoh et al., 2002; Urano et al., 2000) of which the details of the role in ER stress will be discussed in further details below. This “activated ASK1 signalosome” induces the activation both the MKK4/MKK7-JNK and MKK3/MKK6-p38 MAPK downstream pathways (Homma et al., 2009; Matsuzawa and Ichijo, 2008; Sekine et al., 2006). Over-expression of wild type or the constitutively active ASK1 results in mitochondria-dependent apoptosis (Ichijo et al., 1997; Kanamoto et al., 2000; Saitoh et al., 1998) by releasing cytochrome c, activating caspase 3/9 and reduction of antiapoptotic activity of Bcl-2 via phosphorylation, indicating that ASK1 constitutes a pivotal signaling pathway in various types of stress-induced apoptosis (Hatai et al., 2000; Nishitoh et al., 2002; Yamamoto et al., 1999). Fas-binding protein Daxx activated by death receptor Fas and TNFα can also activate ASK1-JNK/p38 signaling pathway (Chang et al., 1998; Tobiume et al., 2001). Indeed, phosphorylated and polyubiquitylated Daxx induced by TNFα via ASK1 has been shown to activate sustained ASK1-JNK signaling and apoptosis in a positive feedback manner (Fukuyo et al., 2009; Kitamura et al., 2009). Contrary to its role in apoptosis, ASK1 has been reported to be involved in cell differentiation and survival in a rat pheochromocytoma cell line PC12 in response to serum starvation in a p38 MAPK dependent manner, suggesting that ASK1 has a broad range of biological activities other than stress-induced apoptosis depending on cellular context and/or cell types (Takeda et al., 2000). Therefore, the variety of stresses that activate ASK1 as mentioned above are closely linked to physiological phenomena in the control of cell fate, and the resultant apoptosis is implicated in the pathophysiology of a broad range of human diseases.

In addition to inducing apoptosis, evidence is also emerging for a role of p38 in autophagy. However, similar to the role of p38 in both cell death and survival, studies have shown that p38 signaling is involved in both positive and negative regulation of autophagy.
depending on the cell type and stimuli. For example, activation of p38 by osmotic stress in rat hepatocytes results in inhibition of autophagic proteolysis by controlling autophagosome formation (Haussinger et al., 1999; vom Dahl et al., 2001); on the other hand, prolonged blockade of p38 by inhibitor SB202190 specific for p38α/β kinases leads to autophagic cell death in colorectal cancer cells (Comes et al., 2007). The molecular mechanisms of how p38 regulates autophagy have yet to be elucidated. Previous data has shown that inhibition of p38 leads to an induction of autophagy initially, but that prolonged p38 blockade results in autophagic cell death in colorectal cancer cells (Simone, 2007). Indeed, p38α MAPK was found to inhibit the autophagic lysosomal degradation pathway by interfering with the intracellular trafficking of the transmembrane protein Atg9, most probably via competition with p38IP (p38 α Interacting Protein) which is required to bind to Atg9 to facilitate autophagosome formation induced by starvation (Webber and Tooze, 2010a; Webber and Tooze, 2010b). It would, therefore, be beneficial to the cell to have a negative feedback mechanism by which p38α is reactivated after sufficient rounds of mAtg9 trafficking and protein degradation, preventing unwanted autophagic cell death. As autophagy is emerging as an important pathway in health and disease, further understanding the mechanism by which p38 is able to exert its influence on the autophagic pathway and mAtg9 trafficking in different cell types will be important for understanding potential points at which we can intervene and either inhibit or upregulate autophagy (Webber and Tooze, 2010a).

p38 MAPKs were generally believed to be a kinase that primarily mediated cell death; however, accumulating studies also show that activation of p38 MAPK signaling can enhance cell survival. For example, p38 is required for the proliferation of haematopoietic cells induced by granulocyte colony-stimulating factor (G-CSF) (Rausch and Marshall, 1999) and of various cancer cells such as rat chondrosarcoma cells (Halawani et al., 2004) and melanoma cells (Recio and Merlino, 2002). Moreover, p38 MAPK contributes to resistance to chemotherapeutic DNA-methylating drugs by facilitating DNA mismatch repair and inducing G2/M cell cycle arrest in glioma cells (Hirose et al., 2003). In addition, activation of p38 protects primary neonatal rat ventricular myocytes from anisomycin-induced apoptosis (Zechner et al., 1998). Another mechanism of p38 mediated cell survival involves the direct phosphorylation and inactivation of GSK3β followed by the accumulation of β-catenin primarily in the brain and thymocytes (Thornton et al., 2008). More interestingly, p38 MAPK also inhibits autophagosome formation
in various colon cancer cell lines and thereby, inhibits colon cancer cell death (Comes et al., 2007; Simone, 2007).

Furthermore, the components of downstream of p38 MAPK signaling are also involved in cell survival. For example, MSK1-mediated phosphorylation of histone H3 at Ser10 was found to be required for tumor promoter-induced cell transformation (Kim et al., 2008a). In addition, MSK have been suggested to phosphorylate the proapoptotic protein Bad induced by UVB radiation in a p38 MAPK dependent manner, which results in dissociation of Bad from Bcl-X(L) and in turn promotion of cell survival (She et al., 2002). Expression of the constitutively active form of MNK1 has been shown to promote tumorigenesis in a mouse lymphoma model primarily by suppressing apoptosis through regulation of expression of the antiapoptotic protein Mcl-1 (Wendel et al., 2007). MK2 has been shown to promote the G2/M checkpoint in response to UV irradiation by directly phosphorylating dual specificity phosphatase CDC25B/C and thereby promoting CDC25 binding to 14-3-3 proteins to exit from the cell cycle (Manke et al., 2005). In addition, MK2 has also been found to dampen the extent and duration of the p53 response to stress by phosphorylation and activation of HDM2, a RING family E3 ubiquitin ligase that targets p53 for degradation (Weber et al., 2005).

In summary, the role of p38 MAPK in cell survival and cell death mediated by apoptosis and autophagy, due to various cellular stresses, is cell context and cell type specific as well as signal intensity and duration dependent.

1.6.5 The role of p38 MAPK signaling pathway in cancer progression

In addition to their well-established function in inflammation and the immune response, accumulating studies have revealed that p38 MAPKs play important roles in cell differentiation, cell survival, apoptosis, angiogenesis and cell migration, suggesting that p38 MAPK signaling pathway could be of therapeutic benefit in cancer therapy. Indeed, various activators of p38 MAPK signaling pathway have been identified as tumor suppressors, including p38 upstream kinases MKK4 (Robinson et al., 2003), MKK6 (Hickson et al., 2006), MKK3 (Ellinger-Ziegelbauer et al., 1999) whereas negative regulators of p38 MAPK signaling such as phosphatase Wip1/PPMID and DUSP26/ MAP kinase phosphatase-8 have been identified as oncogenes (Belova et al., 2005; Bulavin et al., 2002; Li et al., 2002; Yu et al., 2007). In support
of the tumor suppressor function of p38α, several components of the p38 MAPK pathway including p38 isoforms themselves are mutated in human tumors (Greenman et al., 2007). Moreover, down-regulation of p38α, MAKK6 (Iyoda et al., 2003; Ventura et al., 2007), MKK4 (Robinson et al., 2003) and overexpression of Wip1 (Bulavin et al., 2002; Rauta et al., 2006) and Glutathione S-transferase Mu GSTM1 and 2, inhibitors of the MAP3K ASK1 (Dolado et al., 2007) have been shown in various human cancers.

p38 signaling pathway functions as a negative regulator of the cell cycle by promoting G1/S and G2/M checkpoint arrest via various mechanisms including direct phosphorylation and activation of p53 and retinoma protein (pRb), down-regulation of cyclin D, phosphorylation and inhibition of Cdc25A and Cdc25B phosphatases and up-regulation of cyclin-dependent kinases (CDKs) inhibitors such as p16 and p21 (Bulavin and Fornace, 2004; Cuenda and Rousseau, 2007; Thornton and Rincon, 2009). Furthermore, p38 MAPK has been shown to regulate cell-cell contact inhibition in non-transformed fibroblasts through accumulation of p27 Kip1 CDK inhibitor, a known effector of contact inhibition (Faust et al., 2005) and through downregulation of the epidermal growth factor receptor EGFR-stabilizing protein Sprouty2 (Spry2) (Swat et al., 2009). In addition to the function of the p38 MAPK in restraining uncontrolled cell proliferation by promoting cell cycle arrest and cell-cell contact inhibition, the role of p38 MAPK in inducing mitochondria-dependent apoptosis specifically in response to reactive oxygen species (ROS) through ASK1-MKK3/4/6 pathway has been well established (Dolado et al., 2007; Kennedy et al., 2007), which has been discussed in further detail in section 1.6.4. Interestingly, p38 MAPK also has a crucial role in Oncogene ras-Induced Senescence (OIS), the permanent growth-arrest of cells by functioning in the downstream of Raf-1-MEK-ERK signaling pathway. The p38 MAPK mediated OIS sequentially activates PRAK (p38-Regulated or Activated Kinase) and p53 as a result of ROS accumulation (Han and Sun, 2007).

In contrast to the well-established tumor suppressor role of the p38 MAPK pathway, some publications also provide evidence supporting the tumor-promoting potential of this pathway through regulation of angiogenesis, tumor growth, invasion and metastasis as well as generating tumor microenvironment by modulating the inflammation and immune response. Studies have shown that p38 MAPK is not only involved in the increased production of angiogenetic factor VEGF by promoting mRNA stability in response to hypoxia (Pages et al., 2000) but is also required for VEGFR mediated cell motility resulting from enhanced actin
polymerization (Rousseau et al., 2000). In addition, p38 MAPK also plays a role in controlling ECM remodeling by up-regulation of matrix metalloproteases MMP-9 (Ringshausen et al., 2004; Simon et al., 2001) and MMP-2 (Huang et al., 2005; Shin et al., 2005a; Xu et al., 2006), suggesting a potential role of p38 signaling in promoting tumor cell invasion and metastasis.

1.6.6 The potential of p38 MAPKs as therapeutic targets

Consistent with its various substrates involved in pro-inflammatory cytokine production, p38 MAPKs play critical role in immune and inflammatory responses. p38 MAPK signaling has been shown to be associated with several cytokine-dependent inflammatory diseases, including rheumatoid arthritis, Crohn’s disease, psoriasis and asthma as well as pain and cardiovascular disease such as myocardial infarction and acute coronary syndrome (Schindler et al., 2007) (Coulthard et al., 2009). Indeed, p38 MAPKs have been a popular target for the design of anti-inflammatory drugs since the mid-1990s. Inhibitors of p38 MAPKs, most designed to occupy the ATP-binding site, have been one of the most intensively studied classes of therapies for the treatment of inflammatory pathologies. Most p38 MAPK inhibitors block the activity of both the p38α and p38 β MAPKs. However, p38α MAPK seems to be the major isoform involved in the immune and inflammatory response as p38β knockout mice did not show defective LPS-induced cytokine production and delayed development of joint and bowel inflammation when crossing with TNF overexpressing mice (Beardmore et al., 2005; Schindler et al., 2007).

Several of the compounds that have proven efficacy in preclinical models and good pharmacological properties entered Phase I or II clinical trials for several different pathological conditions. However, many trials have been stopped due to decreased efficacy resulting from chronic inhibition or unacceptable neurological, gastrointestinal and/or cardiovascular toxicities. The main reasons for the decreased efficacy and systemic toxicity is likely due to activation of negative feedback loops such as TAB1 mediated regulatory mechanism known to control p38 activity mentioned in section 1.6.1 and 1.6.2, the diversity of tissue- and pathology-specific functions of p38MAPK as well as crosstalk with other intracellular regulatory pathways that interact, inhibit or regulate p38 MAPK by phosphorylation or dephosphorylation, limiting its clinical potential. For example, inhibition of p38 MAPK could stop feedback loops that suppress activity of upstream regulatory kinase such as TAK1, leading to activation of other pro-inflammatory pathways such as JNK (Wagner and Nebreda, 2009).
Although targeting p38 MAPKs creates many challenges for therapeutic benefit, the cell-specific activity of p38 MAPK is very precise and tightly regulated, which is one of the reasons why this pathway remains attractive for the design of novel therapeutics. To overcome those problems, developing drugs targeting to upstream kinases or downstream substrates or specific regulators of p38MAPK rather than directly targeting p38 MAPK or its isoforms might be a more attractive strategy to exploit this pathway for therapeutic benefit. Such an approach might be less toxic if the targeted regulators or substrates do not interact in feedback and crosstalk loops. For example, MKK6 was found to suppress metastatic colonization in human ovarian carcinoma (Hickson et al., 2006) but promote cell survival in cultured cardiomyocytes (Martindale et al., 2005), suggesting cell-type-specific effects of this signaling pathway. Therefore, to inform the development of new classes of compounds that might exploit p38 MAPK signaling in the future, it is crucial to better understand the biology underlying the toxicity observed in trials using direct inhibition of this pathway. It is important to carefully dissect the roles of specific p38 MAPK isoforms in the physiology of normal cells and in the pathogenesis of disease, and how they control and are integrated with other regulatory pathways. This knowledge should identify which p38MAPK isoforms, substrates or regulators will be the best targets in specific disease settings for the development of novel therapeutics with high efficacy but acceptable toxicity (Coulthard et al., 2009; Marber et al., 2011; Schindler et al., 2007).

1.7 Objectives of the Research Manuscript

The close association of Gp78/AMFR expression with malignancy, based on immunohistochemical analysis with the 3F3A mAb which recognizes both the cell surface and ER-localized Gp78/AMFR has been shown in a number of tumor types. Given the potential benefit of blocking the Gp78/AMFR mediated signaling as a target for cancer therapy, in the following chapters, I will focus on the role of Gp78/AMFR in cancer progression by (1) investigating the relationship between the dual functions of Gp78/AMFR as a cell surface receptor and intracellular ubiquitin ligase in ERAD; (2) unraveling the phosphorylation site of Gp78/AMFR selectively targeted by the cancer marker 3F3A mAb via p38 MAPK signaling.
and (3) the impact of the phosphorylation site within 3F3A binding motif on the ubiquitin ligase activity, localization and degradation pathway of Gp78/AMFR.
CHAPTER 2. Gp78/AMFR microRNA Mediated Gene Silencing Dampens Both Cell Surface and ER Localized Gp78/AMFR Functions

Gp78 is a key E3 ubiquitin ligase involved in endoplasmic reticulum-associated degradation (ERAD). Gp78 also functions as the receptor for autocrine motility factor (AMF, equivalent to the glycolytic enzyme phosphoglucose isomerase, PGI) at the cell surface. AMF/PGI binding to cell surface of Gp78/AMFR stimulates a signal transduction cascade that results in enhanced tumor cell motility and metastasis (Fairbank et al., 2009). AMF is internalized to the smooth ER via a receptor mediated raft dependent-pathway (Le et al., 2002), however, the relationship between the surface cytokine receptor of AMF and ER-localized ubiquitin ligase activity of Gp78/AMFR remains unclear.

Using microRNA mediated gene silencing technology, stable Gp78/AMFR knockdown HEK293 cell lines were generated. Western blot and Fluorescence-Activated Cell Sorting (FACS) analysis showed a significant reduction of total and cell surface expression of Gp78/AMFR, respectively. Gp78/AMFR knockdown results in decreased degradation of an established substrate KAI1, a tumor metastasis suppressor associated with reduced cell proliferation. However, when KAI1 is knocked down by siRNA in the Gp78/AMFR knockdown stable cell line, the suppressed cell proliferation can be rescued. This result demonstrates that ERAD function of Gp78/AMFR is diminished in Gp78/AMFR knockdown cell lines. Moreover, the cytokine receptor function has also been demonstrated to be compromised in Gp78/AMFR knockdown stable cell lines by showing that: (1) extracellular AMF/PGI decreases thapsigargin and ATP-evoked ER Ca$^{2+}$ release in non-targeting control cells whereas AMF/PGI stimulation has no significant effect on Gp78/AMFR knockdown stable cell lines; (2) extracellular AMF/PGI mediated protection against tunicamycin and thapsigargin induced ER-stress and pre-apoptosis is lost when Gp78/AMFR is knocked down. These results suggest that Gp78/AMFR microRNA mediated gene silencing dampens both cell surface and ER localized Gp78/AMFR functions.
2.1 Introduction

Gp78, also called Autocrine Motility Factor Receptor (AMFR) is an E3 ubiquitin ligase localized to the endoplasmic reticulum (ER) and plays an important role in ER-associated degradation (ERAD). Gp78 also functions at the cell surface as the receptor of Autocrine Motility Factor (AMF, equivalent to the glycolytic enzyme phosphoglucone isomerase, PGI). A close association of Gp78/AMFR expression with malignancy, based on immunohistochemical analysis with the 3F3A monoclonal antibody (mAb), has been described in a number of tumor types (Chiu et al., 2008). The anti-Gp78/AMFR 3F3A mAb was generated against membrane proteins from B16-F1 melanoma cells treated with neuraminidase and purified by lactose elution from a Peanut Agglutinin Affinity (PNA) sepharose column (Nabi and Raz, 1987). It labels both the cell surface and mitochondria-associated smooth domains of the ER (SER) tubules (Benlimame et al., 1995; Goetz and Nabi, 2006; Wang et al., 2000). Importantly, the anti-Gp78/AMFR 3F3A mAb mimics the effect of AMF by stimulating cell motility in vitro and metastasis in vivo (Nabi et al., 1990). AMF is internalized to the SER via a receptor mediated raft dependent-pathway (Le et al., 2002), however, the relationship between the surface cytokine receptor of AMF and ER-localized ubiquitin ligase activity of Gp78/AMFR remains unclear.

Gp78/AMFR was identified as one of the 189 most frequently mutated genes in breast and colon cancers (Sjoblom et al., 2006). Gp78/AMFR expression correlates with aggressive tumor biology and poor outcome for malignancies of the lung, tongue, esophagus, stomach, colon, rectum, liver, breast, thymus and skin (Chiu et al., 2008). Notably, in bladder, colorectal, gastric, skin and esophageal cancers (Hirono et al., 1996; Maruyama et al., 1995; Nagai et al., 1996; Nakamori et al., 1994; Otto et al.), Gp78/AMFR is either not expressed or expressed at significantly reduced levels in adjacent normal tissue. Substrates of Gp78/AMFR E3 ubiquitin ligase activity include CD3-delta (Zhong et al., 2004), the T cell receptor (Fang et al., 2001), ApoB lipoprotein (Liang et al., 2003), HMG CoA reductase (Song et al., 2005), Cystic Fibrosis Transmembrane conductance Regulator (CFTR) (Morito et al., 2008), and the metastasis suppressor KAI1(CD82) (Tsai et al., 2007). KAI1 is a tetraspanin whose expression is lost in advanced stages of many human malignancies; however, the mechanism of action of this suppressor remains unknown (Jackson et al., 2005; Sridhar and Miranti, 2006). The identification of KAI1 as a Gp78/AMFR ERAD substrate functionally links Gp78/AMFR ubiquitin ligase activity in ERAD to its established role in metastasis (Tsai et al., 2007).
AMF/PGI is a glycolytic enzyme that also functions as an extracellular cytokine following secretion by a non-classical mechanism. Intriguingly, both normal and neoplastic cells express the identical AMF gene product, however, overexpression associated with selective secretion of the protein was observed only in tumor cells, suggesting that its secretion by neoplastic cells is independent of mutation or alternative splicing (Dobashi et al., 2006; Niinaka et al., 1998). Indeed, aberrant secretion of AMF was observed in blood and urine samples of patients with various cancers, suggesting a potential prognostic value (Baumann et al., 1990). Accumulating studies has shown that AMF/PGI binding to cell surface Gp78/AMFR stimulates a signal transduction cascade that results in enhanced tumor cell motility and metastasis (Fairbank et al., 2009). In addition, AMF/PGI overexpression in NIH-3T3 cells has been shown to induce cellular transformation and tumorigenicity, as well as tumor cell survival by PI3K/Akt signaling (Tsutsumi et al., 2003a).

Induced in response to hypoxia, nutrient deprivation and low pH of the tumor microenvironment, ER stress contributes to tumor cell survival and can be required for tumor growth; indeed, expression of various ER chaperones, including BiP, is upregulated in various cancers (Moenner et al., 2007). Both inhibition of UPR, resulting in a limited ability of the cell to deal with the stressful environment, or enhancing stress and overloading the UPR represents a possible approach to drive cancer cells towards apoptosis (Healy et al., 2009). However, effectiveness of any such anti-cancer strategy will be dependent on understanding the mechanisms underlying cancer cell resistance to ER stress induction. In the event of prolonged or severe ER stress that is not resolved, the UPR switches to initiation of apoptosis (Szegedzi et al., 2006). Certain components of the UPR are strongly linked to induction of ER stress-induced apoptosis. In this regard, CHOP is induced by all three arms of the UPR through XBP1, ATF4 and ATF6 transcription factors, and is required in many instances for ER stress-induced apoptosis (Zinszner et al., 1998). ER stress is also associated with the release of ER Ca\(^{2+}\), whose uptake by mitochondria is a critical regulator of cellular Ca\(^{2+}\) homeostasis and of the mitochondrial apoptosis pathway (Hajnoczky et al., 2006; Pinton et al., 2008). Indeed, prolonged ER stress conditions cause a slow but sustained increase in mitochondrial matrix free Ca\(^{2+}\), that upon reaching a critical threshold is one of the strongest inducers of pro-apoptotic mitochondrial membrane permeabilization (Deniaud et al., 2008).
In this study, we showed that Gp78/AMFR knockdown by microRNA mediated gene silencing results in significant reduction of both total and cell surface expression of Gp78/AMFR associated with decreased degradation of the established substrates KAI1 and cell proliferation. Furthermore, the ability of extracellular AMF/PGI to protect against tunicamycin and thapsigargin induced ER stress is compromised in Gp78/AMFR knockdown stable cell lines. This suggests that Gp78/AMFR microRNA mediated gene silencing dampens both cell surface and ER-localized Gp78/AMFR functions, which may thereby further provide insight into the mechanism by which Gp78/AMFR promotes cancer progression and metastasis.

2.2 Materials and Methods

2.2.1 Antibodies and chemicals

The rat IgM 3F3A mAb against Gp78/AMFR was as described (Nabi et al., 1990). Antibodies to CHOP and cleaved caspase 3 were from Cell Signaling. Rabbit anti-KAI1 (sc-1087) was purchased from Santa Cruz Biotechnology (TX, USA). Mouse β-actin antibody was from Sigma. HRP conjugated secondary antibodies to mouse, rabbit IgG and cross-absorbed secondary antibodies to rat IgM were from Molecular Probes. Rhod-2 AM and pluronic F-127 (PAc) were from Invitrogen. AMF/PGI (P9544), tunicamycin, thapsigargin, ATP, Fura-2 AM, MTT reagent (Thiazolyl Blue Tetrazolium Bromide) and other reagents were from Sigma.

2.2.2 Cell culture, transfection and cell treatment

HEK293 cells were grown in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 25 mM HEPES buffer at 37°C in a humid atmosphere (5% CO2 and 95% air) and transfected using Effectene transfection reagent (Qiagen, Germany) according to the manufacture’s protocol. Non-specific, non-targeting control (D-001810-10-05) as well as human CD82 (KAI1) (L-003901-00) specific siRNAs pools were purchased from Dharmacon. Control and KAI1 specific siRNA smart pools were transiently transfected in to HEK293 stable cell lines using Lipofectamine 2000 (Invitrogen Corporation, USA) following the protocol described before (Joshi et al., 2008). KAI1 knockdown was assessed by western blotting. For AMF/PGI pretreatment, cells were starved overnight and
incubated with fresh media containing AMF/PGI for 8 hours. Cells were subsequently treated with tunicamycin and thapsigargin for 24 hours in media containing AMF/PGI.

2.2.3 Generation of Gp78/AMFR knockdown stable cell lines

To generate stable Gp78/AMFR knockdown cell lines, pre-miRNA sequences for Gp78/AMFR and non-targeting control miRNAs were cloned into pcDNA6.2-GW/+EmGFP-miR (Invitrogen Corporation, USA). The microRNA nucleotide sequences targeting human Gp78/AMFR (GenBank Accession number: NM_001144.4) were as follows:

- miR1: TGCTGTGAATATGGTGCATGAGGTCCGTTTTGGCCACTGACTGACGGACCTCACACCATATTCA
- miR2: TGCTGTAAACGCAGCTGACAATAAGATGGTTTTGGCCACTGACTGACCATCTTTACAGCTGCGTTA
- miR3: TGCTGTATTACAGCTGACCACTGACTGACCACATCTTTACAGCTGCGTTA
- miR4: TGCTGTACATGATGTCAGCTGACTGACCACATCTTTACAGCTGCGTTA

HEK293 cells were transfected with control or Gp78/AMFR miRNA vectors and selected with 5 µg/ml blasticidin. Clones were sorted by flow cytometry and Gp78/AMFR downregulation confirmed by immunoblotting.

2.2.4 Lysate preparation and western blotting

For western blotting, cells were grown to 80% confluency, washed in cold PBS and cell lysates, prepared as described previously (Kojic et al., 2007). Briefly, cells were lysed with buffer containing 20mM Tris pH 7.6, 0.5% NP-40, 250mM NaCl, 3mM EGTA and 3mM EDTA. Equal amounts of cell lysates were separated on SDS-PAGE, transferred to nitrocellulose membranes and western blotted for target proteins (Gp78, KAI1, CHOP, cleaved caspase 3 and β-Actin) followed by appropriate HRP-conjugated secondary antibodies. Chemiluminescence was revealed using ECL (GE Healthcare Bio-Sciences Corp., USA) and densitometry performed using Quantity One V4.62 (Bio-Rad Laboratories, USA) or Scion software.
2.2.5 Gp78/AMFR surface expression

Cell surface Gp78/AMFR was labeled with the 3F3A anti-Gp78/AMFR antibody at 4°C followed by Alexa-647 anti-rat IgM secondary antibody and propidium iodide to identify dead cells by flow cytometry, as previously described (Kojic et al., 2007).

2.2.6 MTT assay

Cell proliferation assays were performed as described previously (Joshi et al., 2008). Briefly, 10,000 cells were seeded on 96 well plates for 48-72 hours, then 1:10 diluted MTT reagent from 5 mg/ml stock in DMEM medium were added to the cells, after that, the cells were allowed to grow in a CO2 incubator in the dark for 4-5 hours. Medium was decanted, precipitates of metabolite (Formazan) dissolved in 200μl DMSO and absorbance measured at 570nm. Background absorbance in the presence of DMSO was subtracted, normalized and subjected to statistical analysis. For siRNA knockdown experiments, cells were transiently transfected with control and target specific siRNAs in 96 well plates and incubated for 48 hours prior to performing the MTT assay.

2.2.7 Calcium detection

To monitor cytosolic or mitochondrial Ca\(^{2+}\) dynamics, cells were loaded with 5 μM Fura-2 AM or Rhod-2 AM in fresh media supplied with 0.02% PAc to help disperse AM ester for 45 or 30 min, respectively. Cells were perfused with normal buffer and images acquired with the 40× UApo objective of an inverted Olympus IX71 microscope. Fura-2 was excited at 340 and 380 nm with a PTI high speed random access monochromator (Photon Technology International, Inc., USA) and emission detected at 510 nm. Rhod-2 was excited at 545 nm and emission detected at 575 nm. Images were analyzed using InVivo Analyzer V3.0 software (Media Cybernetics Inc., USA).

2.2.8 Statistical analyses

Data are presented as mean ± SEM. One-way ANOVA and Student's t-test were used for group paired observations. Differences were considered statistically significant when p <0.05.
2.3 Results

2.3.1 Gp78/AMFR knockdown reduces both total and cell surface expression of Gp78/AMFR.

The cell surface receptor for AMF/PGI, Gp78/AMFR, is localized both at the cell surface and in the ER where it functions as an E3 ubiquitin ligase in ERAD. To determine the relationship between the surface cytokine receptor of AMF and ER-localized ubiquitin ligase activity of Gp78/AMFR, we generated stable Gp78/AMFR specific microRNA transfected HEK293 cells (miR2 and miR3) that show a 50-60% reduction in Gp78/AMFR expression compared with non-targeting miRNA control transfected HEK293 cells (NTC1 and NTC2) by western blot analysis (Figure 2.1A). Using flow cytometry, we found that cell surface Gp78/AMFR expression showed 60-80% reduction in microRNA transfected HEK293 cells (miR2 and miR3) compared with non-targeting miRNA control transfected HEK293 cells (NTC1 and NTC2) (Figure 2.1B).

2.3.2 ERAD function of Gp78/AMFR is diminished in Gp78/AMFR knockdown cells.

To better define the function of Gp78/AMFR as an E3 ubiquitin ligase in the ERAD, we used stable Gp78/AMFR knockdown HEK293 cell lines to study the role of Gp78/AMFR in degradation of the established substrate KAI1 as a regulator of cell proliferation. By western blot, stable Gp78/AMFR knockdown cells showed elevated levels of KAI1 as well as reduced NAD(P)H-dependent cellular oxidoreductase enzyme activity by MTT assay, suggesting reduced cell proliferation relative to the scrambled miRNA line (Figure 2.2A, B). Interestingly, when these stable cell lines were subjected to transient KAI1 knockdown by siRNA, the suppressed enzyme activity can be rescued only in the Gp78/AMFR knockdown line (Figure 2.2C, D). Gp78 therefore might promote cell proliferation via degradation of KAI1 defining a mechanistic link between this E3 ubiquitin ligase and its substrate in controlling cell proliferation. This result demonstrates that ERAD function of Gp78/AMFR is diminished in Gp78/AMFR knockdown cell lines.
2.3.3 AMF/PGI does not protect against TUN and TG induced ER stress response and apoptosis in Gp78/AMFR knockdown cells

To study the role of Gp78/AMFR as a cytokine receptor, Gp78/AMFR knockdown stable cell lines were also used. Incubation of non-targeting control HEK293 cells (NTC1 and NTC2) with 2 μg/ml tunicamycin (TUN), an inhibitor of N-glycosylation, or 3 μM thapsigargin (TG), a SERCA inhibitor that prevents ER Ca\(^{2+}\) uptake, results in increased expression levels of CHOP, one of the major components of the ER stress-mediated apoptosis pathway. However, pretreatment of the cells with 25 μg/ml AMF/PGI for 8 hours prevented increased CHOP expression following treatment with either TUN or TG (Figure 2.3A, B). AMF/PGI therefore protects against TUN and TG induced ER stress. Prolonged TUN and TG treatment to 24 hours also results in upregulation of cytosolic cleaved caspase-3, consistent with the occurrence of apoptotic death in cells subjected to chronic ER stress. In contrast, AMF/PGI pre-treatment prevented TUN and TG-induced caspase-3 cleavage suggesting that AMF/PGI-mediated protection from ER stress is caspase-3 dependent and occurs through the mitochondrial pathway (Figure 2.3A, B). However, in Gp78/AMFR-specific microRNA transfected HEK293 cells (miR2 and miR3), AMF/PGI had no effect on ER stress-induced CHOP and cleaved Caspase-3 expression (Figure 2.3A, B). This indicates that AMF/PGI protection against ER stress and associated apoptosis is mediated by Gp78/AMFR.

2.3.4 AMF/PGI regulation of TG and ATP-evoked cytosolic Ca\(^{2+}\) response is dependent on cytokine receptor function of Gp78/AMFR

The stable Gp78/AMFR specific microRNA transfected HEK293 cells were also used to assess the effect of AMF/PGI ER Ca\(^{2+}\) release induced by ER stressors (Figure 2.4). While extracellular AMF/PGI treatment reduced the TG and TUN induced \([\text{Ca}^{2+}]_{\text{cyt}}\) transient in non-targeted control miRNA transfected cells, it did not impact on TG and TUN induced \([\text{Ca}^{2+}]_{\text{cyt}}\) release in Gp78/AMFR-specific miRNA2 and miRNA3 transfected cells compared with non-targeted control HEK293 cells (NTC1 and NTC2) (Figure 2.4A). Interestingly, both peak amplitude and area under the curve (AUC) of the TG and TUN induced \([\text{Ca}^{2+}]_{\text{cyt}}\) release were significantly elevated in Gp78/AMFR-specific miRNA transfected cells relative to non-targeted controls (Figure 2.4A). Moreover, we determined whether AMF/PGI treatment also impacts on \([\text{Ca}^{2+}]_{\text{cyt}}\) released from IP3R-mediated ER stores. ATP was used to evoke an IP3-mediated
[Ca\textsuperscript{2+}]\textsubscript{cyt} transient (Fig. 4B). The peak amplitude of the ATP-evoked [Ca\textsuperscript{2+}]\textsubscript{cyt} response was increased significantly in Gp78/AMFR knockdown HEK293 cells (miR2 and miR3) compared with non-targeted control NTC1 and NTC2 cells. [Ca\textsuperscript{2+}]\textsubscript{cyt} responses to ATP stimulation was decreased significantly upon AMF/PGI treatment in control NTC1 and NTC2 cells but not in Gp78/AMFR knockdown cells (miR2 and miR3). AMF/PGI treatment attenuated the peak amplitude of ATP-evoked [Ca\textsuperscript{2+}]\textsubscript{cyt} in NTC1 and NTC2 control cells but not in miR2 and miR3 Gp78/AMFR knockdown cells (Figure 2.4B). Gp78/AMFR expression levels are therefore inversely associated with the extent of IP3-sensitive ER Ca\textsuperscript{2+} stores and Gp78/AMFR-dependent AMF/PGI stimulation further reduces ER Ca\textsuperscript{2+} release upon IP3R activation. These results indicated that the cytokine receptor function is compromised in Gp78/AMFR knockdown stable cell lines.

2.4 Discussion

Gp78/AMFR has been localized to both the plasma membrane and SER by using the Gp78/AMFR-specific 3F3A mAb that competes with AMF/PGI for cell surface binding (Fairbank et al., 2009). Here, we show that upon Gp78/AMFR knockdown, expression of the Gp78/AMFR ubiquitin ligase substrate KAI1 is increased and responsible for reduced proliferation of these Gp78/AMFR knockdown cells. Moreover, Gp78/AMFR knockdown cells present reduced cell surface 3F3A labeling and extracellular AMF/PGI no longer prevents ER stress-induced expression of CHOP and cleaved caspase-3. In addition, although AMF/PGI treatment dramatically attenuated the peak amplitude of ATP-evoked [Ca\textsuperscript{2+}]\textsubscript{cyt} in non-targeting control cells, it had no significant effect on the peak amplitude of ATP-evoked [Ca\textsuperscript{2+}]\textsubscript{cyt} in Gp78/AMFR microRNA stably transfected knockdown cells. Therefore, loss of Gp78/AMFR in these cells affects both its ubiquitin ligase activity and its cell surface receptor function mediating AMF/PGI pro-survival activity.

KAI1 has been characterized as a metastasis suppressor, however its mechanism of action remains largely unknown (Jackson et al., 2005; Miranti, 2009). Downregulation of KAI1 expression has been widely reported in tumor promotion and distant metastases in various cancers supporting its proposed anti-metastatic function. Reduced or abrogated expression of KAI1 is linked to elevated tumor cell migration, invasion and proliferation in various
carcinomas and tumors (Choi et al., 2009; Christgen et al., 2008; Ruseva et al., 2009). In pancreatic cancer cells, KAI1 overexpression inhibits cell proliferation, colony formation and abrogates anchorage independent growth through cell cycle arrest (Guo et al., 2005). KAI1 physically interacts with the endothelial cell surface protein, DARC (also known as gp-Fy), and promotes inhibition of cell proliferation in vascular endothelial cells (Bandyopadhyay et al., 2006). KAI1 is an established ERAD substrate of Gp78/AMFR and the inverse relationship between Gp78/AMFR and KAI1 expression was observed in sarcoma (Tsai et al., 2007) and HEK293 cells upon Gp78/AMFR knockdown. The ability to rescue the reduced proliferation of Gp78/AMFR knockdown cells, but not Gp78/AMFR expressing cells, by reducing KAI1 levels with siRNA provides direct evidence that Gp78/AMFR-mediated degradation of KAI1 regulates cell proliferation.

Previous reports have described a role for AMF/PGI in protection against apoptosis and cell death (Haga et al., 2003; Tsutsumi et al., 2003a). The AMF/PGI receptor, Gp78/AMFR, is an ER membrane anchored E3 ubiquitin ligase that is a key component of the ERAD machinery involved in ubiquitylation of ER proteins (Fang et al., 2001; Song et al., 2005), thereby suggesting a possible role in protecting cells against ER stress. In this study, we demonstrate that AMF/PGI protects against ER stress and associated apoptosis by the intermediary of its receptor, Gp78/AMFR. The release of Ca\(^{2+}\) from ER and local interactions between the ER and mitochondria facilitating the transfer of Ca\(^{2+}\) from the ER to mitochondria represent important mechanisms of apoptosis regulation (Pinton et al., 2008).

The Gp78/AMFR positive SER domain is closely associated with mitochondria (MAM) and this interaction is regulated by [Ca\(^{2+}\)]\(_{\text{cyt}}\) (Goetz et al., 2007; Wang et al., 2000). In response to TG and TUN, AMF/PGI reduced the peak amplitude although not total (AUC) elevation of [Ca\(^{2+}\)]\(_{\text{cyt}}\) and importantly affected the rate of Ca\(^{2+}\) uptake to mitochondria. AMF/PGI effect on [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation was not however affected by mitochondrial uncoupling, suggesting that it is not related to mitochondrial association of the Gp78/AMFR labeled SER domain (Goetz et al., 2007; Wang et al., 2000). AMF/PGI interaction with Gp78/AMFR, therefore, regulates ER Ca\(^{2+}\) release upon ER stress. TGF-β treatment has been shown to reduce the rate, but not the amplitude, of the [Ca\(^{2+}\)]\(_{\text{cyt}}\) response and completely depress mitochondrial Ca\(^{2+}\) uptake because of downregulation of IP3R (Pacher et al., 2008). Regulation of ER Ca\(^{2+}\) release by AMF/PGI defines a novel role for an extracellular cytokine in regulation of cellular Ca\(^{2+}\) homeostasis.
Overexpression and secretion of AMF/PGI by tumor cells induce autocrine stimulation of tumor cell motility, cell transformation and tumorigenicity (Tsutsumi et al., 2003a) and elevated circulating AMF/PGI levels are found in the serum of cancer patients (Baumann et al., 1988; Bodansky, 1954). AMF/PGI protects against chemotherapeutic agents in vitro as well as in implanted tumors where it protects against paclitaxel mediated cell death (Haga et al., 2003; Kojic et al., 2008). AMF/PGI interaction with Gp78/AMFR may therefore contribute to tumor cell survival and resistance to chemotherapy by limiting the ER stress response of tumor cells growing in the harsh conditions of the tumor microenvironment.

2.5 Figure Legends

**Figure 2.1. MicroRNA knockdown of Gp78/AMFR in HEK293 cells**

(A) Western blot analysis of total expression of Gp78/AMFR from whole cell lysates in microRNA mediated Gp78/AMFR gene silencing stable HEK293 cell line (miR2, miR3, miR4, miR5) and non-targeting control 1 (NTC1) and non-targeting control 2 (NTC2) transfected HEK293 cell line. β-actin is the loading control. Bar graph are presented as mean ± SEM, n=3.

(B) miR2, miR3, NTC1 and NTC2 cells were surface labeled with the 3F3A anti-Gp78/AMFR antibody at 4°C and Alexa-647 anti-rat IgM secondary antibody and detected by flow cytometry. Bar graph shows percentage of the 3F3A anti-Gp78/AMFR positive cells (data are presented as mean ± SEM, n=3). Data reproduced from (Fu et al., 2010) with permission of Nature Publishing Group (doi: 10.1038/cdd.2010.181).
Figure 2.2. KAI1 mediates Gp78/AMFR regulation of cell proliferation.

(A) HEK293 stable cell lines expressing control (Scr=Scrambled) and Gp78 targeting miRNA were western blotted for Gp78 (3F3A rat IgM), KAI1 and β-actin. Quantification of Gp78 and KAI1 levels relative to β-actin is presented as a bar graph (n=3; ±SEM). Gp78 knockdown results in increased KAI1 expression level but have no effect on β-actin expression levels. (B) The MTT assay was used to detect the NAD(P)H-dependent cellular oxidoreductase enzyme activity in Gp78 knockdown stable cell line (Gp78 miRNA) and non-targeting control cell line (Scr miRNA) (n=3; ±SEM; *p< 0.05). (C) HEK293 stable cell lines expressing control (Scr=Scrambled) and Gp78 targeting miRNA were transfected with control or KAI1-specific siRNA and probed by western blot for KAI1 and β-actin. (D) MTT assay was performed on HEK293 stable cell lines expressing control (Scr=Scrambled) or Gp78 targeting miRNA transfected with either control or KAI1-specific siRNA. KAI1 siRNA increased cell proliferation only in Gp78 miRNA stable HEK293 knockdown cells (n=3; ±SEM; *p< 0.05). Data reproduced from (Joshi et al., 2010) with permission of American Society for Biochemistry and Molecular Biology (doi: 10.1074/jbc.M109.074344).
Figure 2.3. AMF/PGI does not protect against TUN and TG induced ER stress response and apoptosis in Gp78/AMFR knockdown cells

Cos7 cells either untreated (CTL) or pretreated with 24 µg/ml AMF/PGI (+AMF/PGI) for 8 hours prior to addition of either 2 µg/ml TUN or 3 µM TG for 24 hours were immunoblotted with antibodies to CHOP and cleaved caspase-3 in Gp78/AMFR microRNA (miR2 and miR3) or non-targeting control (NTC1 and NTC2) transfected HEK293 cells. Graphs show quantification of CHOP (top) and cleaved caspase-3 (bottom) relative to β-actin bands by densitometry (n=3, ±SEM; *p<0.05; **p<0.01 relative to CTL cells). Data reproduced from (Fu et al., 2010) with permission of Nature Publishing Group (doi: 10.1038/cdd.2010.181).
Figure 2.4. AMF/PGI regulation of TG and ATP-evoked cytosolic Ca\(^{2+}\) response is dependent on Gp78/AMFR expression

(A) Representative recordings of TG-evoked cytosolic Ca\(^{2+}\) transients recorded by Fura-2 ratio (F\(_{340}/F_{380}\)) in NTC1, NTC2, miR2 and miR3 cells with (blue) or without (red) AMF/PGI pretreatment (24 µg/ml; 8 hours), as indicated. Bar graphs show cytosolic Ca\(^{2+}\) peak amplitude and area under curve response to TG stimulation in NTC1, NTC2, miR2 and miR3 cells with (blue) or without (red) AMF/PGI treatment (mean±SEM; 100-150 responding cells; *p<0.05, **p<0.01 relative to CTL cells). (B) Representative recordings of ATP-evoked cytosolic Ca\(^{2+}\) transients recorded by Fura-2 ratio (F\(_{340}/F_{380}\)) in NTC1, NTC2, miR2 and miR3 cells with (blue) or without (red) AMF/PGI pretreatment (24 µg/ml; 8 hours), as indicated. Bar graphs show cytosolic Ca\(^{2+}\) peak amplitude and area under curve response to ATP stimulation in NTC1, NTC2, miR2 and miR3 cells with (blue) or without (red) AMF/PGI treatment. (mean±SEM; 100-150 responding cells; *p<0.05, **p<0.01 relative to CTL cells). Data reproduced from (Fu et al., 2010) with permission of Nature Publishing Group (doi: 10.1038/cdd.2010.181).
CHAPTER 3. p38 MAPK Phosphorylation of the Gp78 E3 Ubiquitin Ligase

Gp78 is a key E3 ubiquitin ligase in endoplasmic reticulum-associated degradation (ERAD). We have localized the conformation-specific epitope of the Gp78-specific 3F3A monoclonal antibody (mAb) to the C-terminal portion of Gp78. Progressive C-terminal deletion mapped the epitope of 3F3A and a commercial anti-Gp78 C-terminal specific mAb (anti-Gp78C) to an eight amino acid (533-541) motif whose deletion prevents binding of both antibodies to Gp78. The shared epitope forms part of a highly conserved 41 amino acid region containing 14-3-3 and WW binding domains and overlapping known Gp78 serine phosphorylation sites, including S538 within a p38 MAPK consensus phosphorylation site. Phosphomimetic S538D mutation prevents binding of 3F3A, but not anti-Gp78C, to Flag-Gp78 and bacterially-expressed GST-tagged Gp78. Alkaline phosphatase treatment of immunoprecipitated Flag-Gp78 selectively increases 3F3A binding to Gp78 but not non-phosphorylatable Gp78 S538A. 3F3A binding is reduced by phosphatase inhibition with NaF and by serum starvation, with the latter reversed by the p38 MAPK inhibitor SB203580. MKK6 activation of p38 MAPK reduces 3F3A recognition of Flag-Gp78. p38 MAPK-dependent phosphorylation of S538 therefore inhibits 3F3A binding to Gp78. In HT-1080 fibrosarcoma cells, SB203580 inhibition of p38 MAPK increased 3F3A western blot recognition of endogenous Gp78. Gp78 S538D mutation prevented Gp78-dependent degradation of mitofusins. Serum starvation reduced mitofusin degradation by wild-type Gp78, but not Gp78 S538A, an effect that was reversed by p38 MAPK inhibition with SB203580. Stress-induced p38 MAPK-dependent phosphorylation of Gp78 S538 therefore represents a novel mechanism of regulation of this key E3 ubiquitin ligase mediated degradation of mitofusins.

3.1 Introduction

Gp78 is a key E3 ubiquitin ligase in the endoplasmic reticulum-associated degradation (ERAD) pathway (Christianson et al., 2012), that targets misfolded and physiological substrates for ubiquitylation and subsequent degradation by the proteasome. Gp78 substrates include T cell receptor subunit (CD3-delta, TCR alpha), ApoB lipoprotein, Insig-1, HMG CoA reductase, the Z variant of α1-antitrypsin, mutant cystic fibrosis transmembrane conductance regulator
(CFTRΔ508), SOD1, Ataxin 3 and the metastasis suppressor KAI1/CD82 linking Gp78 ERAD activity to lipid metabolism disorders, cystic fibrosis, neurodegenerative diseases and cancer (Fang et al., 2001; Kostova et al., 2007; Tsai et al., 2007; Ying et al., 2009). Increased expression of Gp78 and degradation of KAI1 is associated with preneoplastic hyperplasia in a transgenic breast cancer model (Joshi et al., 2010) and sarcoma metastasis (Tsai et al., 2007). More recently, we showed that Gp78 promoted mitochondrial fission and, upon mitochondrial depolarization, mitophagy via degradation of the mitochondrial fusion proteins mitofusins (Fu et al., 2013).

Gp78 was originally identified as a cancer-associated glycoprotein of 78 kDa and subsequently as the receptor for autocrine motility factor (AMFR) (Nabi and Raz, 1987; Nabi and Raz, 1988; Nabi et al., 1990; Silletti et al., 1991). The 3F3A anti-Gp78 monoclonal antibody (3F3A) localized Gp78 to both the cell surface and the mitochondria-associated smooth endoplasmic reticulum (Benlimame et al., 1998; Benlimame et al., 1995; Wang et al., 1997). More recently, the 3F3A mAb was shown to selectively recognize Gp78 in the peripheral ER where Gp78-dependent ubiquitylation is initiated, suggesting that it recognizes a conformationally active sub-population of Gp78 (St-Pierre et al., 2012). Immunohistochemistry with the 3F3A mAb has been extensively used to define an association between Gp78 expression and tumor malignancy (Chiu et al., 2008). However, the Gp78 motif recognized by the 3F3A mAb has yet to be identified.

Gp78 contains an N-terminal multi-transmembrane domain and a C-terminal domain that contains a catalytic RING finger domain, CUE motif, dimerization domain and E2 ubiquitin conjugation enzyme (Ube2g2) and p97 binding domains (Chen et al., 2006a; Fang et al., 2001; Li, 2009 #35). Epitope mapping of the 3F3A antibody has now defined a novel p38 MAPK S538 phosphorylation site, downstream of the CUE motif and upstream of the E2 binding domain of Gp78 that controls the E3 ubiquitin ligase mediated degradation of mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2).
3.2 Materials and Methods

3.2.1 Antibodies and chemicals

The rat IgM 3F3A mAb against Gp78 was as described (Nabi et al., 1990). Antibodies to Flag and β-actin antibody were from Sigma, to Gp78C and mitofusin 1 from Abcam, to GST from Cell Signaling, to GFP from Synaptic Systems GmbH (Germany), and to KAI1 and mitofusin 2 from Santa Cruz Biotechnology (TX, USA). HRP conjugated secondary antibodies to mouse and rabbit IgG were from Molecular Probes and to rat IgM from Jackson ImmunoResearch. SB203580 was from Calbiochem. NaF and other reagents were from Sigma.

3.2.2 Cell culture and transfection

Cos7 and HEK293 cells were grown in DMEM and HT-1080 cells in RPMI 1640 supplemented with 10% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine and 25 mM HEPES buffer at 37°C in a humid atmosphere (5% CO2 and 95% air) and transfected using Lipofectamine transfection reagent (Invitrogen Corporation, USA) according to the manufacture’s protocol. For drug treatment, cells were incubated with 5.0 mM NaF or 30µM SB203580 for 4 hours in the presence or absence of serum, as indicated.

3.2.3 Constructs and site directed mutagenesis

Flag-Gp78 constructs were as described (Registre et al., 2004; St-Pierre et al., 2012). GST fused C-terminal of Gp78 was prepared using pGEX-4T backbone plasmid from GE Healthcare. Briefly, a full length FLAG tagged mouse Gp78 cloned in pCDNA-3.1 (used as a template) and a set of primers (GST-cAMFR-BH1-F: 5’GC GGA TCC CCG CCC CCG ATC CGT CGA CAC AAG AAT TAT TTG 3’, GST-cAMFR-NotI-R: 5’AGT CGG CCG CTC CTA GGT TGT CCG TTG CCT CTG 3’) was used to PCR generate C-terminal Gp78 (encoding for amino acids: 309-639). The amplified DNA product was restriction digested and ligated into pGEX-4T plasmid at Bam-HI-Not-I sites, sequence verified and transformed into E. Coli BL21 (DE3) pLysS strain in order to produce GST fused Gp78C recombinant protein. GST-Gp78C protein expression was induced by 1mM IPTG at 37°C for 4 hours. Constitutively active form of MKK6 was provided by Dr. Michael Underhill (UBC). Site-directed mutagenesis was carried
out according to the protocol recommended by QuikChange Site-Directed Mutagenesis Kit (Stratagene).

### 3.2.4 Western blots and dot blots

For dot blots, bacterial lysates were prepared in 1X loading buffer (50 mM Tris pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% v/v glycerol, 100 mM β-mercaptoethanol) and then equal amounts of GST-Gp78 bacterial lysates were blotted onto nitrocellulose membrane and probed with 3F3A, anti-Gp78C or anti-GST antibodies. Chemiluminescence was revealed using ECL (GE Healthcare Bio-Sciences Corp., USA) and densitometry performed using Scion software. Western blots were as described (Shankar et al., 2013).

### 3.2.5 Immunoprecipitation

Cells were solubilized for 1 hour with lysis buffer containing 20mM Tris pH 7.4, 1% TritonX-100, 125mM NaCl, 3mM EDTA, 3mM EGTA, 2mM DTT, 0.5mM PMSF, 0.1% SDS, 3.0mM Na₃VO₄, 2.5mM NaF, 2mM tetra-Na pyrophosphate, 25mM β-glycerophosphate disodium salt hydrate, supplemented with protease inhibitor and PhosSTOP phosphatase inhibitor cocktail tablets (Roche). Cell lysates were passed through a syringe at least 15 times to disrupt the pellets, cleared by ultracentrifugation and incubated with Flag M2 beads (Sigma) overnight at 4°C. Flag beads were washed once with lysis buffer, three times with 150mM NaCl, 20mM Tris, pH8.0, 5mM EDTA, 0.5% Triton X-100, 0.1% SDS, 0.2% BSA; three times with 500mM NaCl, 20mM Tris, pH8.0, 0.5% Triton X-100, 0.2% BSA; and once with 50mM Tris pH8.0. All the washing buffers contained 2.5mM NaF and 0.1mM Na₃VO₄ except for CIAP assay. Where indicated, Flag-Gp78 immunoprecipitates were incubated with 50 unit of calf intestine alkaline phosphatase (New England Biolabs) at 37°C for 1 hour.

### 3.2.6 Statistical analyses

Data are presented as mean ± SEM. One-way ANOVA and Student's t-test were used for group paired observations. Differences were considered statistically significant when p <0.05.
3.3 Results

3.3.1 The 3F3A and anti-Gp78C epitopes map to a region between amino acids 533 and 541 of Gp78.

To identify the epitope recognized by the 3F3A mAb, we first truncated full length Gp78 into Flag-tagged N-terminal Gp78 comprising the multispansing membrane domains of Gp78 (Flag-Gp78N) and GFP-tagged C-terminal Gp78 (GFP-Gp78C) (Figure 3.1A). We then expressed full length Flag-Gp78, Flag-Gp78N as well as RING finger (C356S; Flag-RINGmut), CUE domain (M467F, F468S, P469S; Flag-CUEmut) and p97 deletion (Flag-Gp78Δp97) mutants of Gp78 (St-Pierre et al., 2012) in Cos7 cells. Both the 3F3A and anti-Gp78C mAbs recognized immunoprecipitated Flag-Gp78, Flag-RINGmut, Flab-CUEmut and Flag-Δp97 but not Flag-Gp78N (Figure 3.1B). Following anti-GFP immunoprecipitation, both 3F3A and anti-Gp78Cter monoclonal antibodies recognized GFP-Gp78C but not free GFP (Figure 3.1C). The 3F3A epitope is therefore localized in the C-terminal domain of Gp78.

We then generated progressive Flag-tagged C-terminal deletion mutants of Gp78 (Figure 3.2A) and tested 3F3A and anti-Gp78C reactivity with the Flag immunoprecipitated proteins by immunoblot. C-terminal truncation of Gp78 to amino acid 541 but not 500 was recognized by both 3F3A and anti-Gp78C antibodies mapping their epitopes to a region between amino acids 500-541 (Figure 3.2B), downstream of the CUE domain (amino acids 432-497) and upstream of the G2BR E2 binding domain (amino acids 574-590). This region contains four putative serine phosphorylation sites (S505, S512, S518, S538) identified by large scale mass spectrometry serine phosphorylation analysis (21-25) and predicted by PhosphoMotif finder [http://www.hprd.org/PhosphoMotif_finder] to be putative phosphorylation regulated 14-3-3 and WW binding domains (Figure 3.2A). To further refine the antibody binding motif, we generated additional deletion mutants between amino acids 510-541 based on those phosphorylation sites (Figure 3.2A). This set of Gp78 C-terminal deletion mutants mapped the 3F3A and anti-Gp78C mAb binding region to a segment of 8 amino acids between amino acids 533 and 541 (Figure 3.2B). Finally, an internal deletion mutant lacking residues 533-541 (Δ3F3A) abrogated binding by both the 3F3A and anti-Gp78C mAbs localizing the epitope for these two Gp78 antibodies to the same 8 amino acid motif (Figure 3.2C). This suggests that this
8 amino acid segment recognized by both the 3F3A rat and anti-Gp78C mouse monoclonal antibodies may represent an immunodominant motif in the Gp78 protein.

3.3.2 3F3A mAb selectively recognizes dephosphorylated S538 of Gp78.

The binding region of both 3F3A and anti-Gp78 mAbs is a hydrophobic 8 amino acid motif, PLDLSPRL, containing one acidic (D), one basic (R), one hydrophilic (S) and 5 hydrophobic amino acids (P, L) that is predicted to form a coiled-coil structure and contains a putative serine phosphorylation regulated WW binding motif. The multi-alignment of 12 different species of Gp78 available from Genbank showed that this motif is highly conserved with all species, except bird (including chicken), containing the putative phosphorylation site at serine 538 of Gp78 (Figure 3.3A).

The antigenic epitope prediction program [http://immunax.dfci.harvard.edu/Tools/] identified serine 538 and aspartate 536 as amino acids critical for antigenicity within this motif. Based on this prediction, we generated non-phosphorylatable S538A and phosphomimetic S538D and S538E mutants as well as a D536A mutant. The anti-Gp78C mAb recognizes S538A, S538D, S538E and D536A mutants, however 3F3A recognizes only S538A and not D536A, S538D or S358E (Figure 3.3B). This suggests that 3F3A binding, but not that of anti-Gp78C, is selectively prevented by phosphorylation of serine 538 (S538). To further validate the binding difference between the two antibodies, we GST-tagged the Gp78 C-terminal region (GST-Gp78C) containing the 3F3A deletion (GST-Gp78CΔ3F3A) and serine 538 phosphorylation mutants (GST-Gp78C-S538A, Gp78C-S538D and S538E). Dot blot analysis showed that both antibodies recognized GST-Gp78C and GST-Gp78C-S538A but not free GST or Gp78C-Δ3F3A. Recognition of phosphomimetic Gp78C-S538D and S538E mutants was selectively lost for 3F3A and retained for anti-Gp78C mAb (Figure 3.3C). These results suggest that the 3F3A, but not Gp78C, mAb selectively recognizes dephosphorylated Gp78 at serine 538. To confirm these findings, we used in vitro alkaline phosphatase treatment of Flag-Gp78 immunoprecipitates. Treatment with calf intestine alkaline phosphatase (CIAP) resulted in a 2.5 fold increase in the 3F3A/FLAG ratio of wild-type Flag-Gp78 compared with untreated samples and did not significantly change the extent of 3F3A recognition of the Flag-Gp78S538A mutant (Figure 3.3D). Mass spectrometry also validates the phosphorylation site at serine 538 of Gp78.
This confirms that the 3F3A mAb selectively recognizes dephosphorylated S538 of Gp78.

3.3.3 Serum deprivation stimulates S538 phosphorylation of Gp78 via p38 MAPK signaling

As seen in Figure 3.4A, serum starvation dramatically reduced 3F3A recognition of Flag-Gp78, indicating increased S538 phosphorylation. Treatment of the cells with NaF, a broad serine phosphatase inhibitor, suppressed the elevated 3F3A/Flag ratio in the presence of serum confirming the sensitivity of 3F3A binding to Gp78 phosphorylation. Serum starvation activates the cellular stress response and p38 MAPK signaling and treatment of cells with the p38 MAPK inhibitor SB203580 restored the elevated 3F3A/FLAG ratio in the absence of serum. To confirm a role for p38 MAPK signaling in S538 Gp78 phosphorylation, we co-transfected Cos7 cells with Flag-Gp78 and constitutively active MKK6, an upstream p38MAPK signaling activator, in the absence or presence of SB203580. Co-transfection of Flag-Gp78 with MKK6 resulted in a decreased 3F3A/FLAG ratio, which was restored by SB203580 treatment (Figure 3.4B). These results suggest that serum deprivation stimulates phosphorylation of Gp78 at serine 538 via p38 MAPK signaling.

To test whether endogenous Gp78 is also phosphorylated by p38 MAPK on S538, we treated HT-1080 cells, that express elevated levels of endogenous Gp78 (St-Pierre et al., 2012) with SB203580 and western blotted lysates for 3F3A and anti-Gp78C. As seen in Figure 3.4C, SB203580 inhibition of p38 MAPK increased recognition of endogenous Gp78 by 3F3A but not by anti-Gp78C.

3.3.4 Serine 538 phosphorylation of Gp78 prevents its degradation of the mitofusins

3F3A mAb that selective recognizes dephosphorylated S538 of Gp78 has been shown to label the endogenous Gp78 that localized in the smooth ER domain associated with mitochondria (Benlimame et al., 1998; Benlimame et al., 1995; Goetz et al., 2007; Wang et al., 1997; Wang et al., 2000). The integral GTPases mitofusin (Mfn) 1 and 2, located on the outer mitochondrial membrane (OMM) play an essential role in mitochondrial morphology by
controlling the fusion of two adjacent mitochondria through formation of homotypic or heterotypic complexes (Santel, 2006). We recently identified Mfn1 and Mfn2 are novel substrates of Gp78 (Fu et al., 2013). Therefore, we tested whether Gp78 S538 phosphorylation regulates Gp78 mediated degradation of Mfn1 and Mfn2 and we found that Gp78 overexpression induces their degradation. However, while the Gp78 S538A mutant retained the ability to degrade Mfn1 and Mfn2, the S538D mutant was unable to degrade these two proteins (Figure 3.5A). Furthermore, serum starvation prevented the ability of Gp78 to target the mitofusins for degradation, an effect that was reversed by SB203580 and therefore due to p38 MAPK-dependent phosphorylation of Gp78 (Figure 3.5B). Serum starvation and/or p38MAPK inhibition did not affect mitofusin levels in untransfected or Gp78 S538A transfected cells and is therefore mediated by S538 phosphorylation of Gp78 (Figure 3.5). The 3F3A binding site is therefore identified as a serine phosphorylation site that is a critical regulator of this E3 ubiquitin ligase in degradation of mitofusins.

3.4 Discussion

The ubiquitin ligase activity of Gp78 depends on C-terminal RING finger, CUE and E2 binding domain (Chen et al., 2006a). Indeed, the N-terminal transmembrane domains of Gp78 appear to be dispensable for its ubiquitin ligase activity as a truncated form of Gp78 expressing a single transmembrane domain and the complete C-terminal tail effectively rescues Gp78 knockdown HT-1080 cancer cells, reducing KAI1 levels and restoring metastatic ability in a RING finger and CUE domain dependent manner (Tsai et al., 2007). The G2BR E2 binding domain motif induces allosteric changes in the E2 ubiquitin conjugating enzyme Ube2g2, increasing its affinity for Gp78 RING finger domain by almost 50-fold, increasing the efficiency of ubiquitin transfer from the active site of Ube2g2 to substrates in the presence of G2BR (Das et al., 2009). Moreover, a hydrophobic motif (amino acids 415-443) located downstream of the RING finger domain (amino acid 337-378) and partially overlapping with CUE domain (amino acid 432-494) promotes Gp78 oligomerization that can interact with multiple Ube2g2s (Li et al., 2009b). This therefore suggests the RING finger, dimerization motif and CUE domain mediate interaction with a downstream G2BR domain that is adjacent to the p97 binding motif. We define here a highly conserved proline rich 41 amino acid motif (amino acid 500-541)
containing a phosphorylation regulated WW binding domain (S538) located between the CUE and G2BR domains of Gp78. This motif contains additional serine phosphorylation sites within two other WW binding domains (S512 and S518) as well as a 14-3-3 binding motif (S505) (Fig. 2A). Therefore, p38 MAPK-dependent phosphorylation of S538 within this domain inhibits Gp78 mediated Mfn1/2 degradation defining a novel regulatory domain of Gp78 activity.

Recognition of the active, S538 non-phosphorylated form of Gp78 is consistent with selective recognition by 3F3A of the peripheral ER localized Gp78, the ER domain to which we have localized initiation of Gp78 ubiquitin ligase activity (St-Pierre et al., 2012). Close association of Gp78 expression with malignancy of various tumor types has been shown based on immunohistochemical analysis with the 3F3A monoclonal antibody (Chiu et al., 2008). Specificity of 3F3A for an active form of Gp78 in degradation of mitofusins suggests that it is Gp78 ubiquitin ligase activity that is specifically associated with tumor malignancy. Indeed, Gp78 ubiquitin ligase targeting of the metastasis suppressor KAI1 promoted metastasis of fibrosarcoma HT-1080 cells (Tsai et al., 2007). Similarly, transgenic overexpression of Gp78 in mammary gland induced hyperplasia, also via KAI1 degradation (Joshi et al., 2010).

The demonstration here that p38 MAPK mediated phosphorylation controls Gp78 mediated degradation of Mfn1/2 defines a distinct signaling mechanism that regulates the activity of this key E3 ligase. p38 MAPK is activated downstream of the ER stress response element PERK (Liang et al., 2006; Zhang et al., 2010) and mediates the protective response against ER stress through a number of mechanisms, including activation of the ER stress sensors ATF6α, upregulation of BiP/GRP78, activation of Akt, as well as down-regulation of TRB3 expression, probably by phosphorylation and decreased transactivation activity of pro-apoptotic transcription factor CHOP/GADD153 (Egawa et al., 2011; Ranganathan et al., 2006; Seimon et al., 2009; Zou et al., 2009). However, p38 MAPK also has pro-apoptotic functions via the ER stress response signaling IRE1-TRAF2-ASK1-MKK6 pathway, inducing BAX/BAK dependent apoptosis by inhibition of anti-apoptotic Bcl2 activity through phosphorylation of Bcl2 and activation of CHOP/GADD153 (De Chiara et al., 2006; Maytin et al., 2001; Oyadomari and Mori, 2004; Wang and Ron, 1996). Therefore, the role of p38 MAPK in cell survival and cell death, due to various cellular stresses, including ER stress, is cell context and cell type specific as well as signal intensity and duration dependent (Coulthard et al., 2009; Cuadrado and Nebreda, 2010; Karunakaran et al., 2008; Seimon et al., 2009; Wagner and Nebreda, 2009).
However, the role of Gp78 in p38 MAPK mediated signaling in ER stress remains uncertain. Interestingly, the Gp78 cell surface ligand, autocrine motility factor (AMF), was recently shown to protect against ER stress induced cell death by regulating ER calcium release in a Gp78 dependent manner (Fu et al., 2011). However, AMF selectively prevent Gp78 targeting of mitochondrial substrates mitofusins, and thereby promote mitochondrial fusion but not KAI1 (Shankar et al., 2013) suggesting that AMF might act via p38 MAPK-dependent S538 phosphorylation of Gp78. p38 MAPK-dependent S538 phosphorylation therefore represents a novel inhibitory mechanism of Gp78 ligase activity whose regulation of substrate degradation may represent a novel mediator of the p38 MAPK stress response. However, it remains to be tested whether S538 phosphorylation of Gp78 trigged by p38 MAPK signaling inhibits Gp78-mediated degradation of other substrates.

3.5 Figure Legends

**Figure 3.1. 3F3A and anti-Gp78C mAbs recognize the C-terminal of Gp78.**

(A) Schematic representation of the functional domain of Gp78 and the FLAG tagged and GFP tagged Gp78 used for immunoprecipitation in B and C. (B) Cos7 cells were untransfected (Cos7 NT) or transfected with empty vector (PC3 EV), FLAG-tagged wild type of Gp78 (Flag-Gp78-WT), CUE domain mutant Gp78 (Flag-Gp78-CUEmut), RING finger domain mutant Gp78 (Flag-Gp78-RINGmut), P97 binding domain deletion mutant Gp78 (Flag-Gp78-Δp97), C-terminal deletion mutant Gp78 (Flag-Gp78-N7) and immunoprecipitated with FLAG beads and probed with 3F3A mAb (upper panel) or Gp78 C-terminal targeting mAb, Gp78C (middle panel) or anti-FLAG mAb (lower panel). (C) Cos7 cells were untransfected (Cos7 NT) or transfected with EGFP empty vector (EGFP), GFP-tagged N-terminal deletion mutant Gp78 (GFP-Gp78C) and immunoprecipitated with anti-GFP Ab and probed with 3F3A mAb (upper panel) or Gp78C mAb (middle panel) or anti-GFP Ab (lower panel). The 10% input of total cell lysates of Cos7 transfected with EGFP empty vector (10% EGFP) or GFP-tagged N-terminal deletion mutant Gp78 (10% GFP-Gp78C) were controls.
Figure 3.2. 3F3A and anti-Gp78C mAbs map to the same 8 amino acid epitope.

(A) Schematic representation of the FLAG tagged C-terminal progressive deletion mutants of Gp78 used for immunoprecipitation in B and C. (B/C) Cos7 cells were untransfected (Cos7 NT) or transfected with empty vector (PC3 EV), FLAG tagged full length (FCA-639), C-terminal progressive deletion mutants of Gp78 (FCA-590, FCA-541, FCA-500, FCA-424, FCA-381, FCA328, FCA-510, FCA-517, FCA-526 FCA-530, FCA-533) or amino acid 533-541 deletion mutant (FCA-Δ3F3A) and immunoprecipitated with FLAG beads and probed with 3F3A mAb or Gp78 C-terminal targeting mAb, gp78C or anti-FLAG mAb.
Figure 3.3. 3F3A mAb, but not anti-Gp78C, selectively recognizes dephosphorylated S538 Gp78.

(A) Multi-alignment of different species of Gp78 showing the conservation of 3F3A binding site. (B) Cos7 cells were untransfected (Cos7 NT) or transfected with empty vector (PC3 EV), FLAG -tagged wild type (WT), phosphorylation dominant negative mutant (S538A ) and phosphorylation mimetic mutant (S538D, S538E), antigenic mutant (D536A), amino acid 533-541 deletion mutant (Δ3F3A) of Gp78 and immunoprecipitated with FLAG beads and probed with 3F3A mAb or Gp78 C-terminal targeting mAb(αGp78C) or anti-FLAG (αFLAG) mAb (C) 3F3A mAb recognizes dephosphorylated Gp78 by site directed mutagenesis in prokaryotic cells. GST-tagged empty vector (GST-EV) or C-terminal of Gp78 wild type (GST-Gp78C-WT), phosphorylation dominant negative mutant (S538A ) and phosphorylation mimetic mutant (S538D, S538E), amino acid 533-541 deletion mutant (Δ3F3A) of Gp78 C-terminal expression were uninduced (N) or induced by IPTG (I), total bacterial lysates were dot blotted onto membrane and probed by 3F3A mAb, αGp78C mAb or αGST mAb. (D) Flag-tagged wild type of Gp78 (Flag-Gp78-WT) or phosphorylation dominant negative mutant (Flag-Gp78-WT S538A) from Cos7 cells were immunoprecipitated and followed by untreatment or treatment with Calf Intestine Phosphatase (CIAP) and probed with 3F3A mAb, αGp78C or αFLAG mAb. Bar graph: mean ± SEM, n=3.
D

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<tr>
<th>Flag-Gp78-WT</th>
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<td>CIAP: -</td>
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**Western Blotting**

78KD
- IP: αFLAG
- IB: 3F3A

78KD
- IP: αFLAG
- IB: αGp78C

78KD
- IP: αFLAG
- IB: αFLAG

**Graphs**

- Untreated
- +CIAP

Normalized Ratio 3F3A/Flag

Normalized Ratio Gp78C/Flag

*P<0.05

NS
Figure 3.4. Serum deprivation stimulates p38 MAPK dependent S538 phosphorylation.

(A) Cos7 cells were transfected with FLAG tagged wild type of Gp78 for 14.5 hours and followed by non-treatment (NT), or treatment with 5 mM NaF or 30μM SB203580 (SB) for 4 hours in the presence or absence of serum. FLAG tagged wild type of Gp78 were immunoprecipitated with FLAG beads and probed with 3F3A mAb or αFLAG mAb. Bar graph: mean ± SEM, n=4. (B) Cos7 cells were transfected with FLAG tagged wild type of Gp78 alone or co-transfect with constitutive active MKK6 for 14.5 hours and followed by treatment with 30 μM SB203580 (SB) for 4 hours in the presence of serum. The rest of procedure is the same as (A). Bar graph: mean ± SEM, n=3. (C) HT-1080 cell were treated with or without 30μM SB203580 for 4 hours and then western blotted for 3F3A, αGp78C and β-actin. Bar graph: mean ± SEM, n=3. Differences considered statistically significant when *p<0.05.
Figure 3.5. Gp78 S538 phosphorylation inhibits its ability to degrade mitofusins.

(A) Cos7 and HEK293 cells untransfected or were transfected with FLAG tagged wild type Gp78, RING finger mutant, Flag-Gp78-S538A and Flag-Gp78-S538D and lysate prepared. Lysates were analyzed by western blot for MFN1, MFN2 and β-actin expression or immunoprecipitated with anti-Flag beads and immunoprecipitates blotted with anti-Flag mAb. (B) Lysates of untransfected Cos7 cells, or transfected with FLAG tagged wild type Gp78 and Flag-Gp78-S538A for 14.5 hours and either non-treated (NT) or treated with 30μM SB203580 (SB) for 4 hours in the presence or absence of serum were analyzed by western blot for MFN1, MFN2, and β-actin.
CHAPTER 4. Serine 538 Phosphorylation of Gp78 Ubiquitin Ligase Regulates its Distribution in the ER and Degradation Pathways

The endoplasmic reticulum (ER) is a multifunctional organelle involved in various cellular functions including Ca\textsuperscript{2+} homeostasis, lipid and protein metabolism. Morphologically, it has been divided into two distinct domains, juxtanuclear central rough ER sheets and peripheral smooth membrane tubules. Gp78 is a key multi-transmembrane E3 ubiquitin ligase anchoring in the ER and plays a central role in ERAD in mammalian cells (Fang et al., 2001). The Gp78-specific 3F3A monoclonal antibody (mAb), does not label central ER localized FLAG-tagged Gp78 and is restricted to the peripheral smooth ER domain where Gp78 substrate ubiquitylation is initiated. Unlike the 3F3A mAb, a commercially available mAb raised against the Gp78 C-terminal tail (Gp78C), labels both the Sec61β-GFP defined sheet-like central ER and the myc-Reticulon defined tubular, peripheral ER. Previously, 3F3A mAb has been characterized to selectively recognize the S538 dephosphorylated pool of Gp78 whereas Gp78C mAb recognizes both dephosphorylated and phosphorylated S538 of Gp78. Consistent with 3F3A labeling of the peripheral ER, we found that dominant negative S538A Gp78 mutation promoted peripheral ER distribution; while phosphomimetic S538D mutant was concentrated in the central ER, implying that based on its S538 phosphorylation state, Gp78 can be targeted for different subdomain of ER. In addition, we also found that phosphomimetic S538D mutation did not alter the turnover of Gp78 but did facilitate routing it for proteasomal degradation pathway. In contrast, non-phosphorylatable Gp78 S538A mutation sensitized Gp78 for lysosomal degradation, indicating that Gp78 can be targeted for degradation via both proteasomal and lysosomal pathways and that S538 phosphorylation is a critical determinant of the degradation pathway of this E3 ubiquitin ligase. We previously found that p38 MAPK mediated S538 phosphorylation controls Gp78 mediated degradation of mitofusins. Taken together, the study implies that dephosphorylated S538 Gp78 selectively recognized by 3F3A mAb, preferentially localizes in the peripheral ER and is routed to the lysosome-mediated degradation pathway. In contrast, S538 phosphorylation which promotes central ER localization of Gp78 prevents Gp78 mediated degradation of mitofusins but facilitates its own proteasome dependent degradation. Therefore it appears that by modulating the phosphorylation status of S538, Gp78 controls its ER distribution and degradation pathways.
4.1 Introduction

The ER is a continuous membrane system, executing diverse cellular functions including Ca^{2+} homeostasis, lipid and protein synthesis and metabolism. Classically, the ER has been divided into rough and smooth ER according to their morphology based on the electronic microscopy and functions. More recently, expression levels of two key protein families, reticulons/DP1 and atlastins, have been shown to drive the formation of the sheet-like, juxtanuclear rough ER and the tubular structures of the peripheral smooth ER (Shibata et al., 2006). Specialized ER domains include organelle-associated ER domains such as mitochondrial associated ER (MAM) as well as the ER quality control (ERQC) compartment (ERQCC), a central ER domain where the ERAD occurred that plays a critical role in protein targeting to the proteasome (Araki and Nagata, 2011; Lynes and Simmen, 2011).

Over the last years, a significant progress has been made in our understanding of how the degradation of ubiquitylated substrates is regulated. However, little is known how the turnover of the E3 ubiquitin ligases that is responsible for the ubiquitylation of the substrates is modulated. Gp78 are the first characterized E3 involved in ERAD in mammalian cells (Fang et al., 2001; Shmueli et al., 2009), however, the detailed regulatory mechanisms that control Gp78 degradation remain enigmatic. Recent study has shown that HRD1 dislocation machinery which is composed of Derlin-1/2, VIMP, p97 and HERP is characterized by half-lives exceeding 30 hrs (Cambridge et al., 2011; Iida et al., 2011). Endogenous Hrd1 is a relatively stable protein whose stability is dependent on interaction with its binding partner SEL1L, a short half-life adaptor protein involved in ERAD (Kikkert et al., 2004; Sun et al., 2014). Similarly, the stability of yeast counterpart Hrd1p was also found to be dependent on its stoichiometric relationship with its binding partner Hrd3, the SEL1L homologue in mammalian cells (Iida et al., 2011). In contrast, Gp78 shows rapid turnover with a half-life around 3 hours (Fang et al., 2001). Previous studies of Gp78 degradation reported the rapid autoubiquitylation of C-terminal GFP tagged Gp78 in a RING finger dependent manner (Fang et al., 2001) and that Hrd1 targets Gp78 for degradation through a RING finger independent pathway (Ballar et al., 2010; Shmueli et al., 2009) to the proteasome. A further study demonstrated that endogenous Hrd1 negatively regulates Gp78 ERAD function in facilitating CFTRΔF508, ATZ and CD3δ degradation by directly interacting with Gp78 and eventually decreasing Gp78 levels (Ballar et al., 2010; Ballar et al., 2011). Targeting of Gp78 for proteasomal degradation is dependent on the RING finger
domain of Hrd1, but this targeting is unidirectional as silencing Gp78 does not change the turnover of Hrd1. As both Gp78 and Hrd1 have been shown to specifically bind to PDI and Derlin-1 to promote retro-translocation of cholera toxin, this suggests that Hrd1 may recruit Gp78 to its ERAD machinery for degradation (Bernardi et al., 2010; Ye et al., 2005).

We previously showed that the Gp78-specific 3F3A monoclonal antibody (mAb) does not label central ER localized FLAG-tagged Gp78 and is restricted to the peripheral smooth ER domain where Gp78 substrate ubiquitylation is initiated (St-Pierre et al., 2012). This suggested that the 3F3A antibody recognizes a conformationally modified subpopulation of Gp78 that is distributed to the smooth ER, including the peripheral ER as well as the mitochondria-associated smooth endoplasmic reticulum (SER) tubules to which Gp78 has previously been localized (Benlimame et al., 1995; Goetz and Nabi, 2006; Wang et al., 2000). More interestingly, we recently identified that 3F3A selectively recognizes the dephosphorylated S538 of Gp78 by mapping the epitope of the 3F3A mAb and showed that this phosphorylation site regulates the Gp78 mediated degradation of Mfn1 and Mfn2. In this study, we found that dephosphorylation S538 promotes Gp78 localization in the peripheral ER and sensitizes the lysosome-mediated degradation of Gp78. In contrast, phosphorylation S538 enhances Gp78 localization in the central ER and facilitates the entry of itself to the proteasome degradation pathway. This suggests that the phosphorylation status of Gp78 S538 impacts its ER distribution and degradation via either lysosomal or proteasomal mediated pathways.

4.2 Materials and Methods

4.2.1 Antibodies and chemicals

The rat IgM 3F3A mAb against Gp78 was as described (Nabi et al., 1990) and mouse anti-Gp78C monoclonal antibody was from Abcam. Antibodies to Flag, β-actin, Derlin-1 were from Sigma, to HA from Neomarker and to GFP from Synaptic Systems GmbH (Germany). Rabbit mitofilin polyclonal antibody and anti-Myc (clone 4A6) were from Novus and Millipore, respectively. HRP conjugated secondary antibodies to mouse and rabbit IgG were from Molecular Probes. Alexa Fluor- 647 Goat anti-rat IgM and HRP-conjugated rat IgM were from Jackson ImmunoResearch. All fluorescent secondary antibodies except the Alexa Fluor- 647
Goat anti-Rat IgM were purchased from Invitrogen. Cycloheximide, leupeptin, MG132 and other reagents were from Sigma.

4.2.2 Cell culture and transfection

Cos7 cells were grown in DMEM (Sigma) supplemented with 10% FBS (FBS, Medicorp), 10 IU/ml penicillin, 10 µg/ml streptomycin, 0.2 mM L-glutamine, vitamins and non-essential amino acids (all supplements were purchased from Invitrogen) at 37°C in a humid atmosphere (5% CO2 and 95% air) and transfected using Effectene (Qiagen) or Lipofectamine transfection reagent (Invitrogen Corporation, USA) according to the manufacturers' protocol. For drug treatment, cells were incubated with 30 µM MG132 or 30µM leupeptin for the indicated time in the presence or absence of serum.

4.2.3 Constructs and site directed mutagenesis

Flag-Gp78, Flag-Gp78-IRES-GFP constructs were as described (Registre et al., 2004); (St Pierre and Nabi, 2012). Constitutively active MKK6 (caMKK6) were kindly provided by Dr. Michael Underhill (University of British Columbia). HA-Ubmono (K29, 48, 36R) or HA-Ubwt expression plasmid were obtained from Tony Morielli (University of Vermont). Site-directed mutagenesis was carried out according to the protocol recommended by QuikChange Site-Directed Mutagenesis Kit (Stratagene).

4.2.4 Immunofluorescence and image quantification

Cells grown on glass cover slips were fixed 24 hours post-transfection with pre-cooled (-80°C) methanol: acetone (80:20% v/v) for 15 minutes at -20°C. Then cells were extensively washed with PBS-CM (PBS supplemented with 1 mM CaCl2 and 10 mM MgCl2). Then cells were blocked with PBS-CM containing 1% BSA (blocking solution) for at least 30 minutes. Cells were then incubated with primary antibodies dissolved in blocking solution for 1 hour, rinsed 3 times for 2 minutes with blocking solution, incubated with the corresponding species-specific fluorescently conjugated secondary antibodies for an additional 30 minutes, followed by 3 times rinse for 10 minutes with PBS. Finally, cover slips were mounted with Celvol 205: Polyvinyl Alcohol (Celanese Chemical Ltd.) and images were acquired with the 100X plan...
apochromatic objective of a Fluoview 1000 confocal laser scanning microscope (Olympus Canada, Markham, ON, Canada). Quantification of the percentage of Flag-Gp78 WT and various mutants localized to the central ER was obtained by measuring the Flag labeling intensity overlapping with a mask of GFP-Sec61β (threshold 5000-65535) over the total Flag intensity signal using ImagePro image analysis software (Media Cybernetics, Bethesda, MD, USA). This method has been used and published before (Fairbank et al., 2012; St Pierre and Nabi, 2012).

4.2.5 Western blotting and immunoprecipitation

Cells were solubilized for 1 hour with lysis buffer containing 20mM Tris pH 7.4, 1% TritonX-100, 125mM NaCl, 3mM EDTA, 3mM EGTA, 2mM DTT, 0.5mM PMSF, 0.1% SDS, 3.0mM Na3VO4, 2.5mM NaF, 2mM tetra-Na pyrophosphate, 25mM β-glycerophosphate disodium salt hydrate, supplemented with protease inhibitor (cOmplete minitab from Roche) and PhosSTOP phosphatase inhibitor cocktail tablets (Roche). Cell lysates were passed through a 27¹/₂ gauge syringe at least 15 times to disrupt the pellets, cleared by ultracentrifugation and incubated with Flag M2 beads (Sigma) overnight at 4°C. Flag beads were washed once with lysis buffer, three times with 150mM NaCl, 20mM Tris, pH8.0, 5mM EDTA, 0.5% Triton X-100, 0.1% SDS, 0.2% BSA; 3X buffer containing 500mM NaCl, 20mM Tris, pH8.0, 0.5% Triton X-100, 0.2% BSA; and 1X buffer containing 50mM Tris pH8.0. All of the washing buffer containing 2.5mM NaF and 0.1mM Na3VO4. For co-immunoprecipitation, cells were lysed with lysis buffer except excluding 2mM DTT and washed with washing buffer containing 50mM Tris, pH7.4, 100mM NaCl, 0.5% TritonX-100, 2.5mM NaF and 0.1mM Na3VO4 for 5 times (Fang et al., 2001). Then equal amounts of cell lysates were separated on either 8% or 12% SDS-PAGE gels, transferred to nitrocellulose (for direct western blot) or PVDF (for immunoprecipitation) membranes and immunoblotted with antibodies to 3F3A, Gp78C, GFP or β-actin followed by appropriate HRP-conjugated secondary antibodies. Chemiluminescence was revealed using ECL (GE Healthcare Bio-Sciences Corp., USA) and densitometry performed using Scion software.
4.2.6 Protein turnover assay

Cos7 cells were transfected with WT, S538A, S538D or RING finger mutant Gp78-IRES-GFP plasmids. After 3.5 hours transfection, cells were equally split into 4 dishes and cultured for another 15 hours. Then the transfected cells were incubated with 50 μg/ml cycloheximide and harvested at 0, 2, 4, 8 hours after treatment. The Gp78 protein expression level was determined by the intensity of Flag band normalized by GFP and β-actin level. For Gp78 degradation rate after proteasome blocking, cells were pretreated with 30 μM MG132 for 1 hour and then incubated with 50μg/ml cycloheximide in the presence of 30 μM MG132.

4.2.7 In vivo ubiquitylation of Gp78

Cos7 cells were co-transfected with either HA-Ub mono or HA-Ub WT and Flag-WT, S538A, S538D mutant plasmids for 18.5 hours followed by treatment with MG132 for 8 hours. Cells were lysed with lysis buffer containing 25mM Tris pH 8.0, 20mM N-ethylmaleimide, 2mM EDTA, 50 μM PNGB, 0.5mM PMSF, 1% TritonX-100, 140mM NaCl supplemented with protease inhibitor (complete minitab from Roche). Then Flag-Gp78 was immunoprecipitated by Flag beads and extensively washed as described as before then probed with Flag and HA antibodies. This method was adopted from Allan M. Weissman Lab (Shmueli et al., 2009).

4.2.8 Statistical analyses

Data are presented as mean ± SEM. One-way ANOVA and Student’s t-test was used for group paired observations. Differences were considered statistically significant when p <0.05.

4.3 Results

4.3.1 Dephosphorylated S538 of Gp78 preferentially localizes in the peripheral ER

Recent studies based on the live cell imaging, have classified the ER into the central, juxtanuclear ER sheets defined by Sec61β which was found in the rough ER and the nuclear envelope and polygonal tubular peripheral smooth ER generated by reticulon protein family
Electron microscopy has localized 3F3A-labeled Gp78 to the smooth ER, including the mitochondria-associated ER, a distribution that is also evident by confocal microscopy (Benlimame et al., 1998; Benlimame et al., 1995; Goetz et al., 2007; Wang et al., 1997; Wang et al., 2000). However, other antibodies have localized Gp78 throughout the ER, a distribution that parallels that of transfected Flag-tagged Gp78 (Fang et al., 2001; Goetz et al., 2007; Registre et al., 2004). We therefore labeled Cos7 cells with both the 3F3A anti-Gp78 as well as a commercial monoclonal antibody raised against a Gp78 C-terminal peptide (referred to as anti-Gp78C). In untransfected cells, 3F3A labeling was restricted to the mitochondria-associated ER where it colocalized with anti-Gp78C labeling; the latter also extended throughout the ER where it showed extensive colocalization with the central ER marker Derlin-1 which has been found in Gp78 complexes and involved in retrotranslocation of ubiquitylated substrates but minimal colocalization with the 3F3A mAb labelled peripheral ER (St-Pierre et al., 2012) (Figure 4.1A, B). In Flag-Gp78 transfected cells, 3F3A labeling is restricted to the peripheral ER while anti-Gp78C labeling extends throughout the ER, including the central ER and nuclear membrane. Sec61β-GFP, which has been shown to be extensively colocalized with Derlin-1 and Derlin-2 (St-Pierre et al., 2012), shows a more perinuclear ER distribution in Flag-Gp78 transfected cells. Labeling with both Gp78 antibodies overlaps with the peripheral ER marker reticulin but only anti-Gp78C associates with the more central ER localized Sec61β-GFP (Figure 4.1C). Previously we mapped the epitope of the 3F3A and Gp78C monoclonal antibodies and found that 3F3A mAb selectively recognizes S538 dephosphorylated Gp78 whereas Gp78C mAb recognizes both the dephosphorylated and phosphorylated S538 Gp78 (Chapter 3). This suggests that a S538 dephosphorylated sub-population of Gp78 preferentially localizes to the smooth ER.

We then transfected Cos7 cells with Flag-Gp78 and labeled with either 3F3A or Gp78C mAb together with FLAG and acquired images at equivalent settings for Flag labeling in order to control for the degree of Flag-Gp78 expression per cell. Comparison of the relative increase in 3F3A or anti-Gp78C labeling density in transfected cells relative to adjacent untransfected cells provided a measure relative expression of the epitopes of the two antibodies in the Flag-Gp78 transfected cells (Figure. 4.2A). In contrast to the 10-fold increase in 3F3A labeling, anti-Gp78C labeling increased more than 200 fold in transfected cells relative to non-transfected cells. In order to further examine the difference between the labeling of the two antibodies, we performed a competition experiment. As shown in Figure 4.2B, in Flag-Gp78 transfected Cos7
cells, when labeling 3F3A mAb only, the peripheral ER network labeling dramatically increased relative to the adjacent untransfected cells, which is consistent with previous results. However, if the cells were first labeled with saturating amounts of Gp78C mAb and then labeled with 3F3A mAb, the enhanced peripheral ER labeling by 3F3A mAb completely disappeared, suggesting that Gp78C mAb blocked the 3F3A mAb labeling in Flag-Gp78 transfected Cos7 cells. In contrast, when labeling Gp78C mAb only, both the peripheral and central ER network labeling dramatically increased relative to the adjacent untransfected cells. However, when labeling 3F3A mAb first and then labeling Gp78C mAb in Flag-Gp78 transfected Cos7 cells, the Gp78C labeling was not blocked by 3F3A mAb. This result is consistent with the higher binding affinity of Gp78C mAb to Gp78 compared with 3F3A mAb (Figure 4.2A). Taken together, this result confirmed that the two antibodies bind to the same epitope and that 3F3A mAb selectively labels the peripheral ER network. This suggests that while Gp78 is localized throughout the ER, 3F3A-labeled dephosphorylated Gp78 S538 preferentially localizes in the peripheral (smooth) ER.

To test whether S538 phosphorylation of Gp78 could explain the selective labeling of the peripheral ER by 3F3A, we determined the ER distribution of Flag-Gp78 WT, S538A and S538D as well as RING finger mutant in relation to the central ER marker Sec61β-GFP (Figure 4.3A) and peripheral ER marker Myc-Rtn4 (Figure 4.3B). As observed for Flag-Gp78RINGmut (Goetz et al., 2007), Flag-Gp78 S538D was predominantly localized to the Sec61-GFP labeled central ER and showed reduced association with peripheral ER tubules labeled for Rtn4a. In contrast, the S538A mutant extended beyond the Sec61-GFP labeled central ER and exhibited extensive colocalization with Rtn4a. This suggests that S538 dephosphorylation promotes peripheral ER distribution of Gp78 and forms the basis for the selective localization of 3F3A to the peripheral ER.

We found that serum starvation enhanced the degradation rate of Gp78 by two fold as shown in Figure 4.4B. In order to determine the impact of stress triggered by p38 MAPK signaling pathway in Gp78 turnover, we performed the cycloheximide chase experiment by co-transfecting COS7 cells with caMKK6, a constitutive activator of p38 MAPK pathway and Gp78WT or S538A mutant. We found that Gp78 S538A mutation dramatically stabilizes the turnover of Gp78 (Figure 4.4C). These observations prompt us to examine whether this phosphorylation site change the turnover of Gp78.
4.3.2 S538 phosphorylation of Gp78 does not alter its turnover but does facilitate the proteasome dependent degradation of Gp78

Stable expression level of E3 ubiquitin ligases is critical for efficient protein folding and aberrant protein removal from the ER to maintain the ER homeostasis. Previous studies have shown that Gp78 exhibits rapid turnover with a half-life of around 3 hours by targeting itself through a RING finger dependent proteasome pathway using C-terminal GFP tagged Gp78 (Fang et al., 2001). Another study showed that Hrd1, another RING finger E3 ubiquitin ligase, targets Gp78 for degradation through a Gp78 RING finger independent pathway via the proteasome (Shmueli et al., 2009). More interestingly, it has been demonstrated that brief ER stress results in Gp78 stabilization probably by inhibiting Hrd1 ubiquitylation (Shen et al., 2007b). Previously, we showed that p38 MAPK signaling pathway induced the phosphorylation of Gp78 at serine 538 prevented Gp78 mediated degradation of mitofusins (Chapter 3). We therefore decided to test whether S538 phosphorylation impacts Gp78 stability.

Previously, study the half-life of Gp78 used GFP tagged C-terminal Gp78 (Fang et al., 2001). However, we decided to use the Flag-tagged full length Gp78 to perform the experiment because the GFP tag has been shown to result in loss of functionality and altered ER distribution of Gp78 (St Pierre and Nabi, 2012). Protein turnover assay is traditionally based on a pulse-chase approach, whereby cellular proteins are pulse radiolabeled by an isotopically marked amino acid and loss of radiolabeled protein followed over time (Roberts and Deretic, 2008). We developed a non-radioactive assay to determine the impact of S538 phosphorylation on Gp78 turnover, based on an Internal Ribosome Entry Site (IRES) containing bicistronic vector that permits the translation of two open reading frames from one messenger RNA. The IRES element, a sequence derived from the encephalomyocarditis virus (ECMV), allows the simultaneous expression of two proteins separately from the same RNA transcript. Therefore, we inserted the recombinant protein of interest (e.g. Flag-Gp78) into one reading frame and as a transfection efficiency control GFP into the second reading frame. MG132 has been shown to activate the CMV promoter resulting in higher transcription level of Gp78 at least in part by stabilizing transcription factors AP1 (Alvarez-Castelao et al., 2009). Using this construct, we can normalize the transcriptional level of Gp78 alteration by MG132 treatment with the GFP that is co-transcribed in the same IRES vector. Therefore, the use of the IRES bicistronic vector for protein turnover provides a useful method to quantitatively monitor the impact of post
translational modifications on protein turnover without using the radiolabeled amino acid incorporation based pulse-chase assay.

To study whether S538 phosphorylation impacts Gp78 stability, COS7 cells were transfected with wild-type Flag-Gp78 or Flag-Gp78 S538A, S538D or Ring finger mutants and chased with cycloheximide to inhibit protein synthesis for 0, 2, 4 and 8 hours to measure the rate of protein degradation. In addition, to achieve the same transfection efficiency, we transfected cells in the same dish then equally split cells into four dishes post transfection for 3.5 hours before we performed cycloheximide chase to inhibit protein synthesis. Consistent with previous reports (Fang et al., 2001), Flag-Gp78 WT exhibited about a 3 hour half-life while the Ring finger mutant was highly stabilized and exhibited minimal turnover in the 8 hour time period studied. However, neither phosphomimetic S538D nor dominant-negative S538A mutants showed altered turnover compared to wild type Gp78 (Figure 4.4).

4.3.3 S538 phosphorylation of Gp78 facilitates the proteasome dependent degradation of Gp78

To study the impact of S538 phosphorylation on the Gp78 proteasomal mediated degradation, we transfected COS7 cells with Flag-Gp78 WT or Flag-Gp78 S538A, S538D or Ring finger mutants and then pretreated cells with MG132 for 1 hour to block the proteasome followed by chase with cycloheximide in the presence of MG132 to inhibit both protein synthesis and proteasomal mediated degradation for 0, 2, 4 and 8 hours to measure protein degradation rate. Results showed that inhibition of proteasomal degradation with MG132 partially stabilized wild-type Gp78 and phosphomimetic Gp78-S538D but did not affect the degradation rate of Gp78-S538A (Figure 4.5). This suggests that S538 phosphorylation of Gp78 is required for its proteasomal degradation. The fact that Gp78 S538A is still efficiently targeted for degradation suggests that other degradation pathways are responsible for turnover of non-phosphorylated S538 Gp78.

4.3.4 S538A mutation sensitizes the lysosome dependent degradation of Gp78

The lysosome and proteasome are the two major pathways of protein degradation. Gp78 has been found localizing in the vesicles and tubular structures based on the Gp78 specific
monoclonal antibody 3F3A that selectively the dephosphorylated S538 of Gp78 by electron microscopy (Benlimame et al., 1995). In addition, AMF has been found to be internalized to the multivesicular body (MVB) via receptor Gp78 dependent pathway (Benlimame et al., 1998; Le et al., 2000). To test whether Gp78-S538A preferentially undergoes lysosomal degradation, we transfected COS7 cells with Gp78-IRES-GFP WT, S538A or S538D mutant plasmids and treated cells with leupeptin for 8 hours to block the lysosomal pathway. In the presence of leupeptin, levels of the S538A Gp78 mutant doubled whereas levels of wild-type Gp78 and the S538D did not change, suggesting that dephosphorylation of Gp78 S538 enhances its entry to the lysosomal degradation pathway (Figure 4.6A). In order to determine the impact of the S538 phosphorylation on Gp78 expression, we transiently transfected COS7 cells with Flag-Gp78 WT for different times and used the 3F3A mAb and Gp78C mAb as controls to determine the percentage of dephosphorylated Gp78 in the total population of Gp78. Results showed that the proportion of dephosphorylated Gp78 increased with transfection time, indicating that the cells might control the degradation pathway of Gp78 by modulating the phosphorylation status of S538 of Gp78 (Figure 4.6B).

4.4 Discussion

Here we show that S538 phosphorylation of Gp78 does not change its rate of turnover. However, Gp78 S538 dephosphorylation promotes Gp78 peripheral ER distribution and lysosome mediated degradation while phosphorylation of S538 of Gp78 is associated with its central ER distribution and proteasome dependent degradation.

4.4.1 Dephosphorylation of S538 of Gp78 promotes its distribution in the peripheral ER.

The ER is characterized as a continuous membrane system, executing diverse cellular functions including Ca^{2+} homeostasis, lipid and protein synthesis and metabolism, and more and more specialized ER domains such as mitochondria-associated membrane (MAM), the ER quality control compartment (ERQCC) has been characterized (Leitman et al., 2013; Lynes and Simmen, 2011). More recent studies based on the live cell imaging, have classified the ER into the central, juxtanuclear ER sheets defined by Sec61β which was found in the rough ER and the
nuclear envelope and polygonal tubular peripheral smooth ER generated by reticulon protein family (Shibata et al., 2006). Mitochondria associated ER membrane (MAM) is a distinct smooth ER subdomain that is associated with mitochondria where the close contact site between ER and mitochondria is about mere 10-25 nm (Csordas et al., 2006; Hayashi et al., 2009; Perkins et al., 1997; Rizzuto et al., 2009). The physical linkage of the ER and mitochondria interface ensures the fast non-vesicular mediated lipid transfer and Ca\(^{2+}\) exchange between the two organelles and hence functions as a major cellular signaling hub that controls cellular metabolism and cell death (de Brito and Scorrano, 2010; Grimm, 2012; Rowland and Voeltz, 2012). ERQCC is a membrane-enclosed compartment localizing in the juxtanuclear domain of ER where unfolded or terminally misfolded secretary proteins and ER-folding and quality control (CNX, CRT) as well as ERAD components (Derlin-1,Sec61β, Hrd1, p97/VCP, ubiquitin, EDEM1, ERManI, Bap31) are segregated upon proteasome inhibition (Frenkel et al., 2004; Groisman et al., 2011; Kamhi-Nesher et al., 2001; Kondratyev et al., 2007; Schmitz et al., 2004; Spiliotis et al., 2002; Wakana et al., 2008; Walter and Ron, 2011; Wang et al., 2008; Ye et al., 2003).

3F3A mAb has been shown to label the endogenous Gp78 that localized in the smooth ER domains associated with mitochondria, which extend to the peripheral ER overlapping with reticulon upon Gp78 overexpression (Benlimame et al., 1998; Benlimame et al., 1995; Goetz et al., 2007; Wang et al., 1997; Wang et al., 2000). In this study, we showed that: (1) Gp78C mAb, which recognizes both S538 phosphorylated and dephosphorylated Gp78 partially co-localizes with 3F3A mAb which selectively recognizes the S538 dephosphorylated Gp78; (2) 3F3A mAb only labels myc-reticulon defined peripheral ER but does not label the Sec61β-GFP defined central ER, whereas Gp78C mAb labeled both central and peripheral ER in Gp78 overexpressing cells; (3) Gp78C mAb blocked the 3F3A labeling in the peripheral ER in Gp78 overexpression cells; and (4) S538A mutant Gp78 shows significantly higher peripheral ER distribution compared to S538D mutant. Those results suggest that S538 phosphorylation regulates Gp78 distribution in the ER which is quite similar to the lectin chaperone calnexin (CNX), a transmembrane protein, whose shuttle between the MAM and ERQCC is controlled by phosphorylation as described in section 1.4.4. More recently, it was demonstrated that depalmitoylated CNX performs its chaperone function in quality control by interacting with the oxidoreductase ERp57 in an ER stress dependent mechanism. In contrast, palmitoylation of CNX promotes its trafficking to the MAM where it interacts with ER Ca\(^{2+}\) pump SERCA2b to
determine ER Ca\(^{2+}\) content and the regulation of ER-mitochondria Ca\(^{2+}\) crosstalk (Lynes et al., 2012). Therefore, it appears that phosphorylation and palmitoylation of CNX in an ER stress dependent manner is the switch that regulates CNX trafficking between ERQC where it functions as chaperon in protein folding and MAM where it acts as regulator of Ca\(^{2+}\) signaling (Lynes et al., 2012; Lynes et al., 2013).

Similar to CNX, S-palmitoylation of Gp78 within its RING finger domain by DHCC6 has been reported to result in peripheral ER distribution of the protein (Fairbank et al., 2012). In addition, we previously have reported that CUE domain of Gp78 restricts monoubiquitylated substrates in the peripheral of the ER whereas ubiquitin chain elongation of its substrates and interaction with p97 opposes the effect (St-Pierre et al., 2012). Given that Gp78 targets itself for proteasomal degradation in a RING finger dependent manner, it is not surprising to observe that higher proportion of Gp78 S538A, preferentially locating in the peripheral ER undergoes monoubiquitylation compared to S538D mutant which is more overlapping with central ER marker (Figure 4.7). As a matter of fact, a novel cytosolic protein folding quality control mechanism has been proposed in which monoubiquitylation acts as an ER retention signal to allow folding of newly synthesized membrane proteins. In this model, correct protein folding would lead to deubiquitylation while protein misfolding or misassembly would result in polyubiquitin chain extension and proteasomal degradation due to the recent finding of the biogenesis of lipoprotein receptor-related protein-6 (LRP6), a type-I membrane protein involved in Wnt signaling (Feldman and van der Goot, 2009). It has been found that S-palmitoylation on a juxtamembrane-cysteine residue of LRP6 is required for efficient transport of this protein from the ER to the plasma membrane whereas the palmitoylation -deficient mutant, which is monoubiquitylated in a juxtamembrane-lysine residue, is retained in the ER by either through the interaction with an ER resident ubiquitin-binding protein or through masking of an ER exit signal. However, mutation of the single lysine which abolished the monoubiquitylation of LRP6 resulted in the protein delivery to the plasma membrane, highlighting the importance of monoubiquitylation in the ER retention of the protein (Abrami et al., 2008). In the present study, we showed that phosphorylation of Gp78 S538 is involved in regulation of its distribution within ER, however, we could not exclude the other possibility that the distribution change of Gp78 within the ER is due to the effect that phosphorylation interplays with other posttranslational modifications of Gp78 such as palmitoylation and monoubiquitylation.
4.4.2 Stress signaling controls segregation of Gp78 in the central juxtanuclear ER

As mentioned in section 1.4.4 and section 1.4.5, some components of ERAD machinery such as ERManI, EDEM1 and OS9 (Avezov et al., 2008; Ron et al., 2011) are constitutively localized in the ERQCC while others, such as CNX and CRT, distribute throughout peripheral regions of the ER in the absence of misfolded proteins. Still others like Hrd1, Derlin-1, Sec61β and p97 concentrate at the ERQCC upon misfolded protein accumulation, making the ERQCC a possible staging ground for efficient degradation (Kondratyev et al., 2007). Protein folding chaperones CNX and CRT have been shown to be recruited to ERQCC with misfolded proteins upon ER stress to facilitate degradation of misfolded proteins (Frenkel et al., 2004; Kamhi-Nesher et al., 2001). A further study demonstrated that under misfolding conditions, ERK1 MAPK enhanced the phosphorylation of CNX at S563, which coincided with a prolonged association of a CNX client glycoprotein α1-antitrypsin (AAT), while an enhanced secretion of AAT was observed when CNX was not phosphorylated at the S563 in the presence of the MEK1 inhibitor PD98059, suggesting decreased association of non-phosphorylated CNX at ERK1 site with client glycoprotein. Indeed, under conditions of protein misfolding MEK1-induced activation of ERK1 has been shown to be enhanced leading to the activation of the UPR via IRE1α-dependent mechanism (Nguyen et al., 2004). Therefore, ERK1-mediated phosphorylation of CNX at S563, leading to prolonged retention of partially misfolded glycoprotein in the ER not only provides the first evidence of a phosphorylation-based regulation of ER quality control but also represent a low energy cost mechanism for regulation ER homeostasis independently of transcriptional activation (Cameron et al., 2009; Chevet et al., 2010).

In resting cells, transient accumulation of unfolded protein molecules probably also cause dynamic protein recruitment to the pericentriolar ERQCC by temporary activation of the PERK pathway under so-called physiological “unstressed” conditions. Hrd1 has been shown to target Gp78 for proteasome mediated degradation independent of the ubiquitin ligase activity of Gp78 which is underscored by the decreased level of Insig-1, a Gp78 specific substrate (Shmueli et al., 2009). Another study has also demonstrated that endogenous Hrd1 negatively regulates Gp78 ERAD function in facilitating CFTRΔF508, ATZ and CD3δ degradation by directly interacting with Gp78 and eventually decreasing Gp78 levels (Ballar et al., 2010; Ballar
et al., 2011). Hrd1 has been demonstrated to be co-immunoprecipitated with Gp78 which is dependent of intact CUE domain of Gp78 (Ballar et al., 2010). Indeed, Hrd1 has been shown to be recruited to the ERQC through the PERK-eIF2a branch of UPR by interaction with Homocysteine-induced ER Protein (HERP), a master organizer of the compartmentalization of the ERAD machinery (Kny et al., 2011; Leitman et al., 2014). The double spanning membrane adaptor protein HERP in mammalian cells (homologue of Usa1 in yeast) has been found to be necessary and sufficient for Hrd1 and ERAD substrate accumulate in the ERQC (Leitman et al., 2014). More importantly, cytosolic domain of human HERP (HERPc) has been shown to be ubiquitylated by Gp78 in vitro (Li et al., 2009b). Given the accumulating studies on the crosstalk between ER and p38 MAPK mediated stress signaling, it is intriguing to speculate that p38 MAPK mediated stress pathway which triggered the S538 phosphorylation of Gp78 promotes the segregation of Gp78 in the juxtanuclear central ER for proteasome degradation by promoting its interaction with Hrd1 or HERP. Therefore, the impact of S538 phosphorylation of Gp78 induced by stress on interaction with Hrd1 or HERP deserves further investigation.

4.4.3 Possible mechanism involved in Gp78 distribution in the peripheral and central ER.

As mentioned in section 1.4.4, the membrane proteins retained in the ER rely on the binding with receptors or immobile complex localized in the subdomain of the ER according to retention or retrieval mechanisms (Rothman and Wieland, 1996). In the retention model, proteins are restricted to the ER whereas in the retrieval model, they are transported out of the ER to a post-ER compartment and returned to the ER by a receptor-mediated process in a microtubules dependent manner. Therefore, the difference between the retention and retrieval can be distinguished by the effect of disruption of microtubules. Proteins that are directly retained in the ER do not redistribute after treatment with microtubular inhibitors whereas proteins localized in the ER by the retrieval mechanism do (Hsu et al., 1991). We did not observe obvious change of distribution of Gp78 after a microtubular inhibitor Brefeldin A (BFA) treatment (data not shown), suggesting that retrieval mechanism does not play a critical role in the ER localization of Gp78.

It has long been proposed that ER retention is achieved by actively preventing transport of resident membrane proteins from the so-called ER exit sites (ERESs), a specialized regions of
the ER where properly folded and assembled membrane-bound soluble proteins are concentrated and incorporated into COPII-coated vesicular carriers (Barlowe, 2003). How this can be conferred is poorly understood but the properties of the transmembrane domain as well as self-oligomerization and binding to large immobile complexes or networks that are excluded from transport vesicles and hence unable to enter these ERESs have been proposed (Neve and Ingelman-Sundberg, 2008; Rothman and Wieland, 1996). Gp78 not only contains multiple transmembrane domains in the N-terminal and at least two oligomerization domains but also forms a large complex in the ER, all of which might contribute to the ER retention of the protein. The multiple transmembrane domain in the N-terminal of Gp78, thought to potentially form the retrotranslocation channel with Derlin-1 (Wahlman et al., 2007; Ye et al., 2005; Ye et al., 2004) which is recruited to the ERQCC upon ER stress by forming a larger complex, together with HRD1-SEL1L and p97/VCP (Groisman et al., 2011; Kondratyev et al., 2007; Ye et al., 2003). The Gp78 C-terminal domain contains a catalytic RING finger, CUE motif, dimerization domain as well as E2 ubiquitin conjugation enzyme (Ube2g2) and p97 binding domains (Chen et al., 2006a; Fang et al., 2001; Li et al., 2009b). Gp78 that lacks the C-terminal preserved its ER localization network but formed a lot of aggregate structures, suggesting that N-terminal of multi-transmembrane of Gp78 contribute to the ER retention of the protein (data not shown). However, it is also possible that the Gp78 that lacks the C-terminal is misfolded and retains in the ER. The CUE domain of Gp78 has been shown to restrict Gp78 to the peripheral ER and slowed down the motility of Gp78 by interacting with ubiquitin of the substrates (St-Pierre et al., 2012). The p97 binding domain of Gp78 is indispensable for recruiting Gp78 to the ERQCC upon proteasome inhibition (St-Pierre et al., 2012). It has been shown that silencing of p97 led to dispersal of ubiquitin-associated aggregates throughout the ER even upon ER stress (Wojcik et al., 2004). Therefore, p97 served as an adaptor protein that links Gp78 with components of the retro-translocon complex including Derlin-1, VIMP and PNGase (Ballar et al., 2007; Groisman et al., 2011; Kondratyev et al., 2007; Li et al., 2006; Ye et al., 2005).

Differential localization of Gp78 in the subdomain of the ER due to the S538 phosphorylation status of Gp78 may be due to domain-specific complex interactions. Prediction based on the computer analysis has shown that the 3F3A binding motif is a phosphorylation regulated WW binding domain (S538). Its upstream vicinity contains one 14-3-3 binding motif (S505) and two other WW binding domain (S512 and S518) regulated by phosphorylation. It has been well established that both 14-3-3 (O'Kelly et al., 2002) and WW binding domain
containing proteins are responsible for protein trafficking such as Nedd4-2 in targeting epithelial Na⁺ channel (ENaC) to lysosome via endocytosis (Snyder, 2009). 14-3-3 β has been shown to be involved in forward transport of the major histocompatibility antigen class II-associated invariant chain Lip35 by suppression of the binding of the protein with retrograde transporter COPI in a phosphorylation-dependent mutually exclusive manner to overcome the ER retention signals (O'Kelly et al., 2002). Therefore one could envision that phosphorylation of S538 of Gp78 results in conformational changes of the Gp78, which recruits the unknown WW binding domain containing protein(s) or 14-3-3 proteins that leads to the exposure or masking of the ER localization signal. Consequently, the localization of the Gp78 within the ER will change due to the altered binding of Gp78 to ER receptors or binding partners localized in a specific domain of the ER.

Further studies will be required to understand the retention of Gp78 in the smooth ER by identification of the binding partners of 3F3A motif of Gp78 utilizing proteomics based strategy. Bap31, which has been implicated in diverse membrane proteins sorting in the ER (Wakana et al., 2008) is one of the candidates that might be responsible for peripheral ER distribution of Gp78 S538A mutant since it has been shown that Bap31 promoted retro-translocation of CFTRΔF508 by interaction with Sec61 translocons and Derlin-1 complex and all of those components has been shown to be interact with Gp78 (Wang et al., 2008). Moreover, Bap31 was found to cycle between ER subdomains, including the rough ER, the ERQC and the MAMs (Iwasawa et al., 2011; Myhill et al., 2008; Wakana et al., 2008), suggesting it might play a role as mediator of Gp78 shuttling between the subdomains of the ER. For example, Bap31 has been reported to be involved in targeting cytochrome P450 (CYPs), a large family of well-conserved integral membrane proteins that metabolize a variety of both endogenous and exogenous compounds to the smooth ER while newly synthesized and misfolded or inactivated mature CYPs are localized in the rough ER by binding with Sec61β (Szczesna-Skorupa and Kemper, 2008). Sec61β is one of the components of the translocation and retro-translocation channel involved in protein synthesis and quality control play a role in recruiting Gp78 to the ERQC (Li et al., 2005). In this study, we found that S538D mutant showed significantly higher overlapping with Sec61β-GFP based on immunofluorescence, compared to S538A Gp78. However, the interaction between Sec61β-GFP and Gp78 wild type and various mutants did not seem dramatically change according to co-immunoprecipitation assay (Figure 4.8), suggesting that recruitment of phosphomimetic S538D of Gp78 mutant to the central ER is not due to the
higher binding affinity between Gp78 and Sec61β. However, the binding affinity between S538 mutants of Gp78 and p97 or Derlin-1 deserves further investigation.

### 4.4.4 Proteasome and lysosome mediated dual degradation pathways of Gp78

A well-controlled balance in protein turnover is required for cellular homeostasis. In addition to unfolded and misfolded or unassembled and damaged proteins undergo degradation; highly regulated proteins also require degradation to maintain optimal level according to the demand of the cells. The proteasome and lysosome are the two major systems for protein degradation in eukaryotes (Fuertes et al., 2003b). The proteasome is responsible for one third of protein degradation and the lysosome is involved in plasma membrane protein degradation. It is generally accepted that short-lived proteins, which consist of most regulatory and abnormal proteins, are degraded by the ubiquitin/proteasome system, while long-lived proteins are degraded in the lysosome via autophagy (Ahlberg et al., 1985; Fuertes et al., 2003b; Henell et al., 1987). However, more and more research showed that it is not a universal rule and some proteins undergo dual degradation pathway (Roxrud et al., 2009; Wang and Mandelkow, 2012). Indeed, the relative contribution of degradation pathways may vary greatly under different conditions. Quantitative mass spectrometry revealed that in most cases of cells cultured under stress-free conditions, proteasomal degradation predominates whereas under amino acid starved conditions, autophagy mediated lysosomal degradation was identified as the main compensatory degradation route due to the degradation of proteasome (Kristensen et al., 2008). Intriguingly, rates of degradation of many proteins were shown to change with changing physiological conditions, such as availability of nutrients or hormones and pathophysiological conditions such as starvation or re-supplementation of nutrients (Ciechanover, 2013; Fuertes et al., 2003a; Fuertes et al., 2003b). For example, CYP2E1, an ER anchored cytochrome P450, responsible for the biotransformation of clinical relevant drugs, exhibits biphasic turnover. CYP2E1 substrate complexation converts it into a stable slow-turnover species (t1/2, 37h), degraded largely via autophagic lysosomal degradation whereas substrate decomplexation withdrawal results in a fast turnover species (t1/2, 7h), that incurs ERAD dependent proteasome degradation, probably by regulation of the phosphorylation and ubiquitylation of the protein (Wang et al., 2010a). Another example is EGF-induced degradation of EGFR which is thought to be initiated by phosphorylation of tyrosine 1045 of the receptor followed by binding of Cbl
adaptor proteins and ubiquitylation of the receptor. It is well documented that EGFR is processed from the cell surface via internalization leading to rapid lysosomal degradation; however, the proteasome has also been proposed to regulate EGFR degradation (Lipkowitz, 2003). Previous study reported that Gp78 undergoes proteasome mediated degradation, however, in this study we showed that Gp78 undergoes dual degradation pathways which are regulated by the phosphorylation status of S538 of Gp78.

We previously have demonstrated that Gp78 mediated ubiquitylation of substrates initiated in the peripheral ER (St-Pierre et al., 2012) and phosphorylation S538 of Gp78 compromised efficient degradation of its substrates Mfn1/2. Here we showed that non-phosphorylated S538A of Gp78 preferentially located in the peripheral ER and enhanced its entry to the lysosome mediated degradation pathway. On the other hand, phosphorylated Gp78 S538D is primarily distributed in the central ER where ERAD compartment resides and directs its proteasome dependent degradation. More interestingly, p38α MAPK has been found to inhibit the lysosomal degradation pathway of autophagy by interfering with the intracellular trafficking of the transmembrane protein Atg9 most probably via competition with interaction with p38α Interacting Protein (p38IP). p38IP is required to bind to Atg9 to facilitate its trafficking and autophagosome formation induced by starvation (Webber and Tooze, 2010a; Webber and Tooze, 2010b). Given that Gp78 has been shown to target itself for degradation in a RING finger dependent mechanism (Fang et al., 2001), we might speculate that phosphorylation S538 of Gp78 functions as a switch to autoubiquitylation/deliver itself rather than its substrates for degradation due to the competition between substrates and E3 ubiquitin ligase itself. In contrast, dephosphorylation of S538 of Gp78 might favor its substrates rather than itself delivered to the ERAD machinery followed by degradation in the proteasome.

Recent studies have shown that intramembrane proteases such as RHBDL4, an ubiquitin-binding rhomboid protease located in the ER, are involved in degradation of ER integral proteins by cleavage of select proteins in their transmembrane domains leading to apparently luminal proteolytic intermediates (Fleig et al., 2012; Tsai and Weissman, 2012). Intriguingly, we found that with longer transient expression of Flag-Gp78, the more the proportion of S538 dephosphorylated Gp78 accumulated in the cells. And although how dephosphorylated S538 Gp78 is routed to the lysosome is still unknown, autophagy, ERAD tuning vesicles (Bernasconi et al., 2012a; Bernasconi and Molinari, 2011) and the endosome-
mediated degradation pathway deserve further investigation. Indeed, our lab demonstrated that recruitment of LC3 to a Gp78/CNX labeled ER domain that is closely associated with depolarized mitochondria (Fu et al., 2013). Electron microscopy studies with the 3F3A mAb which selectively recognizes S538 dephosphorylated Gp78 localized Gp78 to the smooth mitochondria-associated ER domains (Benlimame et al., 1998; Benlimame et al., 1995; Goetz et al., 2007; Wang et al., 1997; Wang et al., 2000) which has been shown to be the site of autophagosome formation (Hamasaki et al., 2013a), supporting Gp78 S538A undergoes autophagy and ERAD tuning vesicles mediated degradation. In addition, 3F3A mAb labeled Gp78 has also been shown to be localized in the plasma membrane, tubular and vesicular structures (Benlimame et al., 1998; Benlimame et al., 1995; Le et al., 2000). Furthermore, AMF/Gp78 complex has been found to be internalized to the smooth ER and multivesicular body (MVB) via both clathrin and non-caveolar, dynamin-dependent, raft-mediated pathway (Benlimame et al., 1998; Kojic et al., 2007; Kojic et al., 2008; Le et al., 2000; Le et al., 2002; Shankar et al., 2013). Therefore, the endosome-lysosome mediated degradation pathway may also possible to be involved in mediating the degradation of the non-phosphorylated S538 of Gp78.

4.4.5 Stress signaling controls Gp78 turnover

Gp78 has been shown to undergo rapid turnover with half-life around 3 hours (Fang et al., 2001). Indeed, brief ER stress of 1 hour tunicamycin treatment has been demonstrated to stabilize Gp78 protein, enhance ERAD degradation of CD3δ and inhibit Hrd1 ubiquitylation (Yang et al., 2007). However, prolonged ER stress by 4 hour of tunicamycin treatment dramatically up-regulates Hrd1 (Kikkert et al., 2004). Therefore stabilization of E3 ubiquitin ligase Gp78 may represent an acute mechanism to increase the efficiency of ERAD at the early stage of ER stress whereas degradation of Gp78 by Hrd1 probably functions to exaggerate ER stress at the late stage of UPR to trigger apoptosis. Although under resting conditions, dephosphorylation of S538 of Gp78 does not impact the rate of Gp78 turnover, constitutively activating p38 MAPK mediated stress signaling pathway by co-transfection Flag-Gp78 WT or S538A mutant with caMKK6 were observed to dramatically stabilized Gp78 turnover when S538 of Gp78 is dephosphorylated. Moreover we observed that serum deprivation increased Gp78 degradation (data not shown). This suggests that S538 phosphorylation of Gp78 enhances
its turnover in response to p38 MAPK mediated stress signaling. Importantly, in addition to S538, five putative serine phosphorylation sites (S505, S512, S518, S527, S538) of Gp78 have been identified by large scale mass spectrometry serine phosphorylation analysis (Dephoure et al., 2008; Huttlin et al., 2010; Munton et al., 2007; Thingholm et al., 2008; Trost et al., 2009) and their role in regulation of Gp78 activity and stability remains to be determined.

In addition to regulation of the transcription, translation by modulation of mRNA stability, translation initiation factor complexes, chromatin remodeling and transcription factors p38 MAPK signaling is emerging as an important modulator of protein turnover (Cuadrado et al., 2007; Khurana et al., 2006; Kundu et al., 2009; Zhao et al., 2008). Accumulating studies have shown the crosstalk between p38 MAPK and ER stress signaling pathway (Hetz, 2012). Given that p38 MAPK signaling is trigged at late stage of ER stress by activating the kinase activity of IRE1 which has been shown to be involved in inducing apoptosis during prolonged ER stress via the IRE1-TRAF2-ASK1 pathway (Chakrabarti et al., 2011), it is intriguing to speculate that Gp78 S538 phosphorylation by p38 MAPK facilitates the UPR switch from the adaption phase to apoptosis by shutting down the ERAD pathway by enhancing degradation of itself and inhibition of its ERAD activity.

Taken together, this study represents a more rapid and cost-effective way to regulate ERAD capacity to maintain cellular proteostasis by fine tuning the E3 ubiquitin ligase activity and compartmentalization of Gp78 based on both intracellular and extracellular stress stimulus through regulating the degradation and localization of Gp78.

4.5 Figure Legends

Figure 4.1. 3F3A and Gp78C mAb labels different population of both endogenous and overexpressed Gp78.

(A) Cos7 cells were fixed with methanol/acetone and immunofluorescently labeled with Gp78C (green), 3F3A mAb (red) and mitofilin (blue) and images were acquired with the confocal microscope. (B) Cos7 cells were fixed with methanol/acetone and immunofluorescently labeled with Gp78C (red), and Derlin-1 (green) and images were acquired with the confocal microscope. (C) Cos7 cells were transiently co-transfected with FLAG-tagged wild type of
Gp78 with a central ER marker (Sec61β-GFP) or peripheral ER marker (Myc-Rtn4) and fixed with methanol/acetone and then labelled with Flag and 3F3A or Gp78C mAb together with Myc for peripheral ER marker or autofluorescence of GFP for central ER marker and images were acquired with the confocal microscope. Scale bar=20µm, inset scale bar=5 µm.
Figure 4.2. Gp78C mAb competes the 3F3A peripheral labeling in Gp78 overexpression cells.

(A) Cos7 cells were transiently transfected with FLAG-tagged wild type of Gp78 and fixed with methanol/acetone and then labelled with Flag and 3F3A or Gp78C mAb. The fields that were chosen include both the transfected and non-transfected cells. Images were acquired using the same setting for the Flag channel but adjust the dynamic range for the channel of 3F3A or Gp78C labeling. Then the total intensity of both transfected (T) and un-transfected (NT) cells for Flag and 3F3A or Gp78C labeling was quantified by the ImagePro software. The fold of increased labeling in transfected cells compared to the untransfected cells were determined after dividing the total intensity of Flag to normalize the expression level of Gp78. More than 30 images from each set of labeling were acquired from three independent experiments and bar graph are presented as mean ± SEM. Scale bar=20µm. (B) Cos7 cells were transiently transfected with FLAG-tagged wild type of Gp78 and fixed with methanol/acetone and then labelled with Flag and 3F3A or Gp78C mAb alone or first labeled with Gp78C or 3F3A and then sequentially labeled 3F3A or Gp78C. Images were acquired with the confocal microscope and the total area of the tubules labeled by either 3F3A or Gp78C (staining area) and the total area of the cells (total area) were quantified using the ImagePro software. The percentage of the staining area relative to the total area of the cell was determined to compare the difference between the 3F3A labeling alone and the sequential labeling with Gp78C mAb (blocking antibody) and then 3F3A mAb or reversely. 52 cells were analysed for each conditions from three independent experiments (mean ± SEM). Scale bar =20µm.
Figure 4.3. S538 phosphorylation of Gp78 regulate its ER distribution

(A) Cos7 cells were transiently co-transfected with FLAG-tagged wild type and various mutants of Gp78 with a central ER marker (Sec61β-GFP) and fixed with methanol/acetone and then labelled with Flag and imaged were acquired with confocal microscope. The percent intensity of Flag-Gp78 in the central ER was determined by using Sec61β-GFP as a mask. One-way ANOVA was used to statistical analysis of the difference among different constructs followed by Student’s t-test for two group comparison (**P<0.0001). For each condition more than 30 cells were analysed from three independent experiments (mean ± SEM). (B) Cos7 cells were transiently co-transfected with FLAG-tagged wild type and various mutants of Gp78 with peripheral ER marker (Myc-Rtn4) and fixed with methanol/acetone and then labelled with Flag (green) and Myc (red) and images were acquired with confocal microscope. Representative imaging were presented. Scale bar =20µm; Inset scale bar= 5µm.
A

FLAG-Gp78 WT  Sec61-GFP  FLAG/Sec61-GFP

FLAG-Gp78 S538A  Sec61-GFP  FLAG/Sec61-GFP

FLAG-Gp78 S538D  Sec61-GFP  FLAG/Sec61-GFP

![Image showing fluorescence microscopy results of different constructs](image)

**FLAG intensity overlapping with Sec61-b%**

- **Gp78-WT**
- **Gp78-S538A**
- **Gp78-S538D**
- **Gp78-dF3A**
- **Gp78-RF**

***P < 0.0001***
Figure 4.4. S538 phosphorylation of Gp78 does not alter its turnover.

Cos7 cells were transfected with FLAG-Gp78-IRES-GFP wild type (WT) or phosphorylation dominant negative mutant (S538A) or phosphorylation mimetic mutant (S538D) as well as Ring finger mutant for 3.5 hours and then Cos7 cells were equally split into 4 dishes and cultured for another 15 hours, followed by 0, 2, 4, 8 hours of 50 µg/ml cycloheximide chase. Gp78 were immunoprecipitated with FLAG beads and probed with anti-FLAG Ab. Half-life of the wild type of Gp78 or S538A, S538D and Ring finger mutants were determined by quantification of FLAG signal normalized by GFP and β-actin expression (mean ± SEM; n=3).
**Figure 4.5. S538D mutation facilitates the proteasome degradation of Gp78**

Cos7 cells were transfected with FLAG-Gp78-IRES-GFP WT, S538A, S538D or Ring finger mutants for 3.5 hours and then Cos7 cells were equally split into 4 dishes and cultured for another 14 hours, and the treat with 30 µM MG132 followed by 0, 2, 4, 8 hours of 50 µg/ml cycloheximide chase in the presence of 30 µM MG132. Gp78 were immunoprecipitated with FLAG beads and probed with FLAG Ab. Half-life of the wild type of Gp78 or S538A, S538D, Ring finger mutants were determined by quantification of FLAG signal normalized by GFP and β-actin expression (mean ± SEM; n=3).
Figure 4.6. S538A mutation sensitizes the lysosome degradation of Gp78

(A) Cos7 cells were transfected with FLAG-Gp78-IRES-GFP WT, S538A or S538D mutant for 14.5 hours followed by treatment with 60 µM Leupeptin to block the lysosome for 8 hours. Then Gp78 were immunoprecipitated with FLAG beads and probed with FLAG Ab and Gp78 expression levels determined by quantification of FLAG signal normalized by GFP and β-actin expression (mean ± SEM; n=3). (B) Increased S538 dephosphorylation with elongated expression time. Cos7 cells were transfected with FLAG-Gp78WT for 14.5hr, 16.5hr, 18.5hr or 20.5hr then Gp78 was immunoprecipitated with FLAG beads and probed with FLAG, 3F3A and anti-Gp78C Ab (mean ± SEM; n=3).
Figure 4.7. Gp78 is monoubiquitylated and S538A mutation enhances the monoubiquitylation of Gp78

Cos7 cells were either co-transfected HA-monoUb (K29, 48, 63R) or HA-Ub WT with FLAG-Gp78 WT, S538A or S538D mutant for 14.5 hours followed by treatment with 30 μM MG132 to block the proteasome for additional 8 hours. Then Gp78 were immunoprecipitated with FLAG beads and probed with anti-HA and anti-Flag Ab. Transfection of Flag-Gp78 WT or mutants with empty vector as control.
Figure 4.8. Gp78 S538 phosphorylation does not change its binding affinity with Sec61β-GFP

Cos7 cells were cotransfected with Sec61β-GFP and FLAG-Gp78-IRES-GFP WT, Δ3F3A, Ring finger, S538A, S538D mutant for 22.5 hours. Then cells were immunoprecipitated with Flag and probed with anti-Flag and anti-GFP Ab.
CHAPTER 5. Conclusion and Perspectives

5.1 Conclusion

In this dissertation, we linked the function of Gp78/AMFR as the cytokine AMF/PGI receptor at the cell surface and E3 ubiquitin ligase in the ER using microRNA mediated gene silencing technology (Chapter 2). More importantly, we unraveled the role of dephosphorylation of S538 of Gp78/AMFR, the motif recognized by the cancer marker 3F3A mAb, in: 1) Gp78/AMFR mediated degradation of mitochondrial fusion factors Mfn1/2 (Chapter 3); 2) distribution of Gp78/AMFR to the peripheral ER; and 3) Gp78/AMFR targeting to lysosomal degradation pathways (Chapter 4).

Scheme 5.1: Proposed working model of Gp78/AMFR in ERAD regulated by S538 phosphorylation.
These research findings have answered some unresolved questions lingered in the field for more than two decades since the development of 3F3A mAb (Nabi and Raz, 1987; Nabi and Raz, 1988) which first led to the discovery of the receptor of AMF (Nabi et al., 1990; Silletti et al., 1991), later was widely used to detect various cancers and finally was found that the gene encoding the AMFR was predicted to be an E3 ubiquitin ligase of RING finger family (Shimizu et al., 1999). However, it also remained and raised even more unsolved questions as listed below.

5.2 Perspectives

5.2.1 Identification of motifs that mediate the functional interaction between AMF and Gp78/AMFR at the cell surface.

It has been accepted that AMF is the natural ligand of Gp78/AMFR; however, little is known about the mechanism underlining the protein interaction between AMF and Gp78/AMFR. X-ray crystallography revealed that AMF/PGI exists as a dimer and the active site of the enzyme is localized to the cleft between the two PGI monomers (Arsenieva et al., 2002; Sun et al., 1999). However, the motifs responsible for AMF/PGI cytokine activity remained to be determined. Based on multiple site-directed mutagenesis of AMF/PGI domains, it was found that the sugar substrate recognition and enzymatic catalytic activity of AMF/PGI is structural overlap of the regions responsible for the cytokine activity of AMF/PGI (Tanaka et al., 2006; Tanaka et al., 2002). In addition, Gp78/AMFR N-glycosylation localized on the C-terminal of Gp78/AMFR is also essential for binding of AMF/PGI to Gp78/AMFR since Gp78/AMFR lacking the N-glycan chain is unable to interact with AMF/PGI in a cross-linking experiment. Moreover, Gp78/AMFR treated with N-glycosidase F to remove the sugar chain, is unable to compete with AMF/PGI in a cell motility assay (Haga et al., 2006). Based on these data, the Haga group proposed a biphasic AMF/PGI and Gp78/AMFR dimmers binding model in which the interaction between AMF/PGI and Gp78/AMFR sugar chain binding may be the first low-
affinity binding phase whereas the C-terminal of AMF/PGI mediated interaction with the core protein of the Gp78/AMFR extracellular domain is the second high-affinity binding phase. However, the location of the core extracellular domain is still unknown.

Both functional and biochemistry studies have shown that 3F3A mAb competes the binding between AMF/PGI and Gp78/AMFR. Those assays include that: 1) 3F3A mAb induced cell motility in a similar fashion to the AMF in vitro by phagokinetic track motility assay (Nabi et al., 1990); 2) 3F3A stimulated lung colonization in vivo in experimental pulmonary metastasis model (Watanabe et al., 1991a); 3) Neutralization assay showed that enhanced motility induced by either AMF treatment or 3F3A mAb was inhibited in presence of affinity purified Gp78/AMFR (Silletti et al., 1991); 4) Competitive inhibition assay showed reduced 3F3A mAb binding to Gp78/AMFR in the presence of AMF-containing conditioned medium (Nabi et al., 1990); 5) 125I-labeled purified AMF from conditioned medium and 3F3A mAb affinity column purified Gp78/AMFR from B16-F1 cell extracts showed direct protein-protein interaction (Silletti et al., 1991); 6) Cell surface binding of 3F3A mAb was efficiently competed by preincubation of the breast cancer cells with AMF/PGI (Kojic et al., 2007). These results suggest that both 3F3A mAb and AMF/PGI bind and activate Gp78/AMFR at a similar or overlapping extracellular domain. Therefore, identification of the 3F3A binding domain of Gp78/AMFR would elucidate the relationship between the surface cytokine receptor of AMF and ER-localized ubiquitin ligase activity of Gp78/AMFR.

In chapter 1 we showed that knocking down Gp78/AMFR in the HEK293 cell line results in decreased expression of both cell surface and total Gp78/AMFR associated with suppressed function of Gp78/AMFR as both cytokine receptor and E3 ubiquitin ligase. As 3F3A mAb mimics AMF stimulation of tumor cell motility in vitro and metastasis in vivo and competes with AMF/PGI for binding to Gp78/AMFR (Kojic et al., 2007; Nabi et al., 1990), it would be rational to test whether the 3F3A mAb and AMF/PGI share the same binding motif with Gp78/AMFR. To do that, purified 3F3A mAb and His-tagged AMF/PGI interaction with GST tagged C-terminal Gp78/AMFR WT and 3F3A epitope mutants (S538A and S538D) can be tested in vitro by Isothermal Titration Calorimetry (ITC), a technique used to determine the thermodynamic parameters of protein-protein interactions. The ability of the 10-40 amino acid peptides encompassing the 3F3A binding site to compete with Gp78/AMF and Gp78/3F3A interaction can then be tested. If so, further studies including whether the peptides compete with
AMF raft-dependent endocytosis, AMF regulation of ER calcium release and AMF cytoprotection from ER stress and regulation of smooth ER and mitochondrial interaction (Fu et al., 2010; Kojic et al., 2007; Shankar et al., 2013) can be carried out to further define the relationship between the dual functions of Gp78/AMFR as both cytokine receptor at the cell surface and E3 ubiquitin ligase in the ER.

5.2.2 The role of S538 phosphorylation in regulation of Gp78/AMFR ubiquitin ligase activity mediated by AMF/PGI endocytosis

AMF/PGI has been shown to be internalized to the smooth endoplasmic reticulum through a receptor-mediated, dynamin- and raft-dependent pathway that is negatively regulated by Caveolin-1 (Cav1). Cav1 is believed to negatively regulate uptake of AMF/PGI by stabilizing caveolae at the plasma membrane or by sequestering key components of raft-dependent uptake such as cholesterol and dynamin (Lajoie and Nabi, 2007; Nabi and Le, 2003). It has been demonstrated that in Ras- or Ab1-transformed NIH-3T3 cells in which Cav1 expression was reduced, AMF/PGI uptake increased compared with NIH-3T3 parental cells (Le et al., 2002). In addition, our laboratory demonstrated that PI3K positively regulates AMF/PGI endocytosis in invasive breast cancer cells (Kojic et al., 2007). More interestingly, it has also been shown that paclitaxel conjugated AMF/PGI is able to inhibit tumor cell proliferation and suppress tumor progression in both B16-F1 and K1735-M1 mouse melanoma models upon intratumoral injection, suggesting that AMF/PGI uptake by Gp78/AMFR might be a promising drug delivery route to tumor cells (Kojic et al., 2008).

The function of Gp78/AMFR as an E3 ubiquitin ligase in ERAD and cytokine receptor of AMF/PGI has been well characterized; however, the relationship between the dual roles of Gp78/AMFR plays in both the cell surface and the ER remains to be enigmatic. Recent studies from our lab have shed first light on how extracellular AMF/PGI regulated the activity of ER-localized Gp78/AMFR. It was found that raft-dependent internalization of AMF/PGI promotes Rac-PI3K signaling which feeds back to enhance AMF/PGI endocytosis. Indeed, receptor-mediated endocytosis-dependent activation of Rac1 has recently been linked to stimulation of endothelial and tumor cell motility, angiogenesis and oncogenicity (Joffre et al., 2011; Wang et al., 2010b). Intriguingly, endocytosis of AMF/PGI selectively inhibits the ability of Gp78/AMFR to target the mitofusins Mfn1/2 for degradation, thereby preventing Gp78/AMFR-
dependent mitochondrial fission. However, AMF/PGI does not appear to prevent Gp78-dependent degradation of tumor suppressor KAI1, suggesting that AMF does not generally inhibit Gp78/AMFR ERAD activity (Shankar et al., 2013). On the other hand, S538 phosphorylation abrogates Gp78/AMFR degradation of Mfn1/2. However, it remains to be tested whether S538 phosphorylation of Gp78/AMFR inhibits its degradation of other known substrates. It also needs to be determined whether extracellular AMF/PGI affects the phosphorylation of S538 of Gp78/AMFR in normal, non-invasive and invasive cancer cell lines. This will reveal whether AMF endocytosis and S538 phosphorylation of Gp78/AMFR are connected and provide an intriguing mechanism whereby the raft-dependent endocytosis of the cell surface ligand of an ER-localized E3 ubiquitin ligase impacts on tumor progression and metastasis.

5.2.3 Identification of the binding partners of 3F3A motif in Gp78/AMFR

In this study we showed that phosphorylation of S538 of Gp78/AMFR within the 3F3A binding motif abrogates Gp78/AMFR mediated degradation of mitofusins but targets itself for proteasome mediated degradation. This suggests that S538 phosphorylation might play a role in distinguishing between Gp78 autoubiquitylation and Gp78 mediated ubiquitylation of substrates. Previous studies have shown that Gp78/AMFR forms a complex with polyubiquitin chains, E2 ubiquitin conjugation enzymes and retrotranslocation channel components through its CUE, G2BR and p97 binding domains. The bioinformatics analysis suggests that the 3F3A binding site is distinct from any known motif and is predicted to be a phosphorylation regulated WW domain binding site. WW domain containing proteins are known to play very important role in protein trafficking, cell signaling and forming complex by acting as adaptor protein (Shearwin-Whyatt et al., 2006). In order to identify which proteins interact with the 3F3A motif, GST pull down (with WT and S538A, S538D mutant C-terminal Gp78/AMFR domains) (Chen et al., 2006b) can be employed and the identity of binding partners revealed by comparative proteomics. Interactions can be validated by coimmunoprecipitation with Flag-tagged Gp78/AMFR and endogenous Gp78/AMFR and the functional role of any identified interacting proteins assessed by siRNA knockdown. The list of the candidate binding partners will provide important clues about the pathways that are responsible for Gp78/AMFR degradation and the kinases and phosphatases that directly regulate Gp78/AMFR phosphorylation. In addition, more
components of Gp78/AMFR complex that regulate the ERAD and novel E2s, DUBs and substrates of Gp78/AMFR might be discovered.

5.2.4 The role of p38 MAPK mediated Gp78/AMFR phosphorylation in regulation of autophagy

In mammalian cells, the roles of autophagy are far more complex than in yeast cells where the major role of autophagy is to degrade long-lived proteins and recycle the basic building blocks of amino acids and lipids to adjust the metabolic machinery in response to varying energy sources and thereby ensuring survival of cells undergoing nutrient restriction. In addition, the crucial energy supply of autophagy during nutritional deprivation has been shown to play a key role in cell survival by scavenging intracellular energy potential when external sources are unavailable (Lum et al., 2005; Yu et al., 2008). Therefore, it is well-established that autophagy is a prosurvival mechanism (Lum et al., 2005). However, cell death triggered by autophagy has been observed for quite a long time and it has been a controversial whether autophagic cell death is a failed attempt to rescue cells from stress. It was not until 2004 that the controversy was ended by two groups who demonstrated conclusively that autophagy can be a cell death mechanism (Shimizu et al., 2004; Yu et al., 2004b). Although the molecular mechanisms of autophagic cell death are still not clear, autophagic cell death is an accepted notion in the field as accumulating studies have reported to the topic and substantial mechanisms have been revealed (Gozuacik and Kimchi, 2007). Autophagy seems to cause cell death via multiple mechanisms including over-digestion of cellular contents, selective degradation of cytoprotective effectors and triggering of existing apoptotic machinery (Chen and Yin, 2011). For example, one study demonstrated that autophagic cell death is induced via JNK pathway in a Atg7 and Beclin-1/Atg6-dependent manner upon inhibition of constitutively active caspase 8 by selective autophagic degradation of a key enzyme that neutralizes intracellular reactive oxygen species (ROS), catalase; resulting in ROS accumulation, lipid oxidation and plasma membrane damage (Yu et al., 2004b).

Indeed, the contradictory role of autophagy in cell survival and death can depend on factors such as the level of autophagy and the status of the cell. It was found that in response to ER-stress, autophagy plays a protective role in transformed cancer cells but incudes cell death in normal or nontransformed cells (Ding et al., 2007a). The mechanism of how autophagy
regulates cell death in different conditions has been studied. One study showed that overexpression of wild type of beclin-1/Atg6 which can bind to Bcl-2, a negative regulator of autophagy, induces autophagy whereas overexpression of a beclin-1/Atg6 mutant lacking the Bcl-2 binding domain results in excessive autophagy and autophagic cell death (Pattingre et al., 2005). This autophagic cell death presumably caused through a model of exaggerated engulfment of the cytoplasm is supported by additional evidence showing that physiological levels of autophagy promote survival during starvation, however, insufficient or excessive levels of autophagy contribute to death in C. elegans (Kang et al., 2007). Another group proposed a model that after a short period of nutrient deprivation, a small amount of Bcl-2 is phosphorylated and dissociated from the Bcl-2-Beclin 1 complex, but not from the Bcl-2-Bax complex which promotes cell survival by activating autophagy; after long hours of nutrient deprivation when Bcl-2 phosphorylation reaches maximal levels, phosphorylated Bcl-2 triggers cell death by disrupting the Bcl-2-BAX complex (Wei et al., 2008b).

Similarly, mitophagy is also involved in both cell survival and cell death. Rapid isolation and selective removal of damaged mitochondria by mitophagy is crucial to cell survival by preventing activation of the apoptosis cascade and providing a crucial “grace period” for cell recover in times of stress. On the contrary, mitophagy may also play a role in inducing cell death by excessive removal of mitochondria which leads to energy deprivation (Yu et al., 2008). Indeed, although knocking out both Uth1p, an outer mitochondrial membrane protein involved in mitochondrial biogenesis and aging (Camougrand et al., 2004), and Aup1p, a yeast mitochondrial protein phosphatase homolog (Tal et al., 2007), induces mitophagy in yeast, deletion of the former causes cell death whereas deletion of the latter promotes cell survival. Therefore, the disparate outcomes may be a reflection of the roles of mitophagy in cell fate decisions (Yu et al., 2008). Recently, our lab reported that Gp78/AMFR induces mitochondrial fragmentation and mitophagy upon mitochondria depolarization which is mediated by degradation of the mitochondrial fusion factor Mfn1 (Fu et al., 2013). In chapter 3 we showed that S538 phosphorylation of Gp78/AMFR triggered by p38MAPK signaling compromised the ability of Gp78/AMFR to degrade Mfn1 whereas dephosphorylation of S538 enables Gp78/AMFR degradation of Mfn1. This suggests that p38 MAPK signaling might control mitophagy by modulation of the phosphorylation status of Gp78/AMFR given that p38 MAPK has been shown to inhibit autophagy and autophagosome formation (Corcelle et al., 2007; vom Dahl et al., 2001). Therefore, whether phosphorylation of Gp78/AMFR S538 results in reduced
mitophagy in response to mitochondrial depolarization in normal, transformed and cancer cells is worthy of study and might identify a novel mechanism for p38 MAPK mediated tumor suppression through regulation of mitophagy.

5.2.5 The Role of p38 MAPK mediated S538 phosphorylation of Gp78/AMFR in the ER stress response

As mentioned in section 1.3.4, ER stress triggers UPR to ameliorate the accumulation of unfolded proteins in the ER. However, if the duration and intensity of the ER stress are severe or protracted, the UPR induces a cellular alarm and mitochondria dependent apoptosis program (Chakrabarti et al., 2011; Kim et al., 2009). The three branches of UPR are activated and inactivated in different stage in response to ER stress, transmitting both survival and pro-apoptotic signals. As mentioned in section 3.4, p38 MAPK is involved in ER stress induced apoptosis through IRE1-TRAF2-ASK1-MKK6 signaling (De Chiara et al., 2006; Maytin et al., 2001; Oyadomari and Mori, 2004; Wang and Ron, 1996). On the other hand, p38MAPK is activated downstream of the ER stress response element PERK (Liang et al., 2006; Zhang et al., 2010) and mediates the protective response against ER stress through a number of mechanisms (Egawa et al., 2011; Ranganathan et al., 2006; Seimon et al., 2009; Zou et al., 2009). Therefore, the role of p38 MAPK in cell survival and cell death, due to various cellular stresses, including ER stress, is cell context and cell type specific as well as signal intensity and duration dependent (Coulthard et al., 2009; Cuadrado and Nebreda, 2010; Karunakaran et al., 2008; Seimon et al., 2009; Wagner and Nebreda, 2009).

We demonstrated in this study that S538 phosphorylation of Gp78/AMFR compromised degradation of its substrates Mfn1/2 but facilitated recruitment of Gp78/AMFR to the ERQC for proteasome-mediated degradation. The latter will exacerbate ER stress by reducing Gp78/AMFR-dependent ERAD, overloading ER-associated misfolded proteins and eventually leading to cell death. In contrast, S538 dephosphorylation enhanced Gp78/AMFR distribution to the peripheral ER enabling ubiquitylation of substrates and prevented the proteasome-mediated degradation of Gp78/AMFR thereby reducing the accumulation of unfolded proteins in the ER. This suggests that S538 phosphorylation of Gp78/AMFR might function as a switch for the cell to decide when to commit suicide either by apoptosis or autophagic cell death. Therefore, it is important to monitor the phosphorylation status of S538 of Gp78/AMFR and p38 MAPK
signaling during different phase of ER stress and compare it with the dynamic response of three arms of UPR and cell death induced by different ER stressors such as tunicamycin (inhibiting N-linked glycosylation), thapsigargin (an inhibitor of SERCA pump to disrupt ER calcium store), A-23187 (a calcium ionophore), DTT (impairing disulfide bond formation), BFA (blocking ER to Golgi transport) or MG132, lactacystin (inhibitors of 26S proteasome to block protein degradation), hydrogen peroxide (inducing oxidative stress) and glucose deprivation (Breckenridge et al., 2003a; Kim et al., 2008b). This will allow us to determine when and how the ER stress induced Gp78/AMFR S538 phosphorylation mediated by p38 MAPK switch from transmitting survival signal to cell death signal by comparing the cell response to different ER stressor in time course and correlated to the threes arms of UPR signaling.

5.2.6 The role of Gp78/AMFR S538 phosphorylation in regulation of mitochondria and ER association

The 3F3A mAb has been shown to label a subdomain of smooth ER whose association with mitochondria is favored by low cytosolic Ca\(^{2+}\) concentration and disrupted by high Ca\(^{2+}\) concentration (Goetz et al., 2007; Wang et al., 2000). We also reported that cytoprotection against thapsigargin (TG) and tunicamycin (TN) induced ER stress by AMF/PGI is mediated by its receptor Gp78/AMFR through regulation of ER Ca\(^{2+}\) release. Accumulating studies have described crosstalk between the p38 MAPK and ER stress mediated signaling as mentioned in section 1.3.4 and section 3.4. Therefore, it is tempting to speculate that cellular stress activates the p38 MAPK signaling pathway, which in turn triggers the phosphorylation of Gp78/AMFR S538, reducing MAM localized Gp78/AMFR binding with mitochondria localized Mfn1/2, concomitant with decreased ER-mitochondria association. However, it might also result in increased association of ER with mitochondria due to decreased S538 phosphorylated Gp78/AMFR mediated degradation of Mfn1/2 at a later stage of UPR.

As mentioned in section 1.4.2, the disruption of ER-mitochondria interface has been shown in malignant tumors (Akl and Bultynck, 2013; Pinton et al., 2011). Indeed, accumulating evidence indicate that during the adaptive phase of ER stress, ER and mitochondria coupling are increased to reestablish the cellular homeostasis by enhancing the Ca\(^{2+}\) exchange between the two organelles (Bravo et al., 2011; Csordas et al., 2006). It is well established that ER stress results in apoptotic cell death by disruption of mitochondrial Ca\(^{2+}\) homeostasis (Leem and Koh,
However, the perturbation of ER-mitochondria contacts by depleting tethering complexes leads to delayed apoptosis progression due to decreased transfer of Ca\(^{2+}\) from the ER to mitochondria (Akl and Bultynck, 2013; Grimm, 2012; Johnson et al., 2012; Pinton et al., 2011). Given that 3F3A is a cancer marker, it would be interesting to screen more cancer and non-cancer cell line to reveal the link between the Gp78 S538 dephosphorylation and ER-mitochondria association in dealing with the two organelles Ca\(^{2+}\) communication in response to ER stress and association with the role of dephosphorylated S538 of Gp78/AMFR recognized by the 3F3A in tumorigenesis.

5.2.7 The role of S538 phosphorylation of Gp78/AMFR mediated by p38 MAPK signaling in tumor suppression

The integral GTPases mitofusin (Mfn) 1 and 2, located on the outer mitochondrial membrane (OMM) play an essential role in mitochondrial morphology by controlling the fusion of two adjacent mitochondria through formation of homotypic or heterotypic complexes (Santel, 2006). Although Mfn1 and Mfn2 are highly homologous, studies have shown that they are not entirely redundant in that the primary role of Mfn1 is in the regulation of the docking and fusion of the mitochondria, whereas Mfn2 participates in multiple signaling pathways not restricted to the regulation of mitochondrial fusion (de Brito and Scorrano, 2008b). Mfn2 is also present in the ER, especially enriched at mitochondria associated membranes (MAM) where it tethers ER and mitochondria, regulates ER shape and mitochondria uptake of Ca\(^{2+}\) released by the ER (de Brito and Scorrano, 2008a). More importantly, Mfn2, also known as hyperplasia suppressor gene (HSG), was originally found to suppress proliferation of vascular smooth muscle cells (VSMCs) by inhibition of ERK/MAPK signaling and inducing subsequent cell-cycle arrest (Chen et al., 2004). In addition, Mfn2 also mediates oxidative stress induced apoptosis of VSMCs mainly through inhibition of PI3K-Akt signaling and resultant activation of the intrinsic mitochondrial apoptotic pathway (Guo et al., 2007; Shen et al., 2007a). Furthermore, Mfn2 also binds and inhibits the proto-oncogene Ras that controls proliferation, cell cycle and morphology (de Brito and Scorrano, 2009). The anti-proliferation effect is independent of its function in mitochondria fusion since deletion of the p21 (Ras) signature motif in the N-terminal region of Mfn2, but not the mitochondrial targeting domain, abolished HSG-induced growth arrest (Chen et al., 2004).
Down-regulation of Mfn2 expression has been found in various tumors and hyperproliferative cells including urinary bladder carcinoma (Jin et al., 2011), hepatocellular carcinoma (HCC) (Qu et al., 2010; Wang et al., 2012a) as well as vascular smooth muscle cells (VSMCs) (Chen et al., 2004). Ectopic expression of Mfn2 has been shown to induce cell cycle arrest, apoptosis in the lung cancer cell line A549, the colon cancer cell line HT-29, (Wu et al., 2008) the urinary bladder carcinoma cell line T24 (Jin et al., 2011) and the breast cancer cell line MCF7 (Xia et al., 2007). Overexpression of Mfn2 also significantly suppressed the growth of subcutaneous tumors in nude mice both ex vivo and in vivo for both lung cancer cell line A549 and the colon cancer cell line HT-29 (Wu et al., 2008). Up-regulation of Mfn2 has also been shown to sensitize A549, HT-29 (Wu et al., 2008) and MCF7 (Xia et al., 2007) to chemotherapy and radiation. In addition, imbalance of Drp-1/Mfn-2 expression, especially decreased expression of Mfn2, promotes a state of mitochondrial fission that has been observed in both human lung cancer cell lines and lung tumor tissue samples from patients. Drp1 (Dynamin-related protein 1), also known as Dynamin-1-like protein in humans is a GTPase that regulates mitochondrial fission. Overexpression of Mfn-2 or knockdown of Drp-1 results in a dramatic increase in spontaneous apoptosis, reduction of cancer cell proliferation and tumor growth regression, implicating a critical role of Mfn2 as tumor suppressor gene and potential tumor target gene (Rehman et al., 2012).

The expression of Mfn2 is regulated at both the transcriptional and posttranslational levels. Previous research demonstrated that Mfn2 is a novel direct target of p53 that exerts apoptotic effects via the mitochondrial apoptotic pathway (Wang et al., 2012b). Recently, it was demonstrated that JNK phosphorylation of Mfn2 in response to cellular stress results in recruitment of the E3 ubiquitin ligase Huwel that mediates degradation of Mfn2 leading to mitochondrial fragmentation and apoptosis (Leboucher et al., 2012). Interestingly, our lab showed that Gp78/AMFR mediated the degradation of Mfn2 via the ubiquitin-proteasome degradation pathway (Fu et al., 2013; Shankar et al., 2013). In this thesis we showed that dephosphorylation of Gp78 S538 results in degradation of Mfn2. In contrast, p38 MAPK dependent phosphorylation of Gp78 S538 upon serum starvation prevents the degradation of Mfn2. This may explain the close association between strong 3F3A mAb staining in IHC and advanced tumor stage and poor prognosis in various cancer types (Chiu et al., 2008). However, it remains to be tested whether decreased degradation of Mfn2 due to Gp78/AMFR S538 phosphorylation mediated by p38 MAPK signaling directly contributes to cancer cell apoptosis.
induced by mitochondrial Ca\(^{2+}\) uptake caused by ER-mitochondria associated as shown in Scheme 5.2.

**Scheme 5.2: Proposed working model of S538 phosphorylation of Gp78/AMFR mediated by p38 MAPK signaling in tumor suppression.**

The mechanisms of Mfn2 mediated tumor suppression include inhibition of cell proliferation and apoptosis mediated by ERK signaling, PI3K-Akt signaling and Ca\(^{2+}\) uptake by mitochondria from the ER via the MAM, respectively. The stability of Mfn2 is controlled by Gp78/AMFR whose ERAD activity in degradation of Mfn2 is regulated by phosphorylation status of S538 via p38 MAPK signaling. The active form of Gp78/AMFR, which is selectively recognized by cancer marker 3F3A mAb, switches to inactive form in degradation of Mfn2 upon S538 phosphorylation mediated by p38 MAPK signaling. The stabilized Mfn2, therefore, results in tumor suppression through induction of mitochondrial mediated apoptosis.

In summary, this thesis focuses on dissecting the cancer marker 3F3A mAb binding motif of Gp78/AMFR to unravel the relationship between the cell surface expression of AMFR as cytokine receptor and ER localized Gp78 as E3 ubiquitin ligase. We found that knockdown of
Gp78/AMFR results in reduced cell surface and total expression of Gp78/AMFR associated with compromised cytokine function and E3 ubiquitin ligase activity. Moreover, we uncovered that 3F3A mAb selectively recognizes dephosphorylated S538 of Gp78/AMFR which is the active form in degradation of Mfn1/2 whereas phosphorylated S538 Gp78 which is the inactive form of Gp78 in degradation of Mfn1/2 is unable to bind 3F3A mAb. In addition, dephosphorylated S538 of Gp78/AMFR is preferentially localized in the peripheral ER and facilitates its entry into the lysosomal degradation pathway. In contrast, S538 phosphorylated Gp78/AMFR is concentrated in the central ER where it signals its own proteasomal degradation. Finally, we resolved some key issues in the field related to the development of the 3F3A mAb as a major cancer marker in the 1980’s, although more questions, especially the site of interaction between AM/PGI and Gp78/AMFR, remains to be answered.
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