INSIGHT INTO ER QUALITY CONTROL MECHANISMS:

NOVEL CHARACTERIZATION OF THE E3 UBIQUITIN LIGASE

GP78/AUTOCRINE MOTILITY FACTOR RECEPTOR

AND THE GαS SUBUNIT IN THE ER

by

Maria Fairbank

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Abstract

Background/Aim:

This manuscript looks at ER Quality Control (ERQC) mechanisms and in particular, focuses on two cellular pathways: (1) the ER-associated degradation (ERAD) pathway and (2) the ER stress response. ERQC represents a complex assembly of pathways that are vital in maintaining proper cellular function and homeostasis, by helping the cell adapt to ER stress, prevent chronic imbalance in the ER and avoid many protein conformational diseases. Here, we investigate (1) a regulatory role for palmitoylation of the E3 ubiquitin ligase gp78/AMFR in the ERAD pathway; (2) the implication of G proteins in gp78/AMFR functions; and (3) the involvement of ER-localized Gαs in both substrate polyubiquitylation and ER stress.

Results:

The dynamic posttranslational modification, palmitoylation, is important for receptor stability and intracellular trafficking. Using metabolic radiolabeling and Acyl-Biotinyl Exchange Chemistry, in chapter 2, we show that the E3 ubiquitin ligase gp78/AMFR is palmitoylated within the catalytic RING finger motif, a domain that is responsible for its ubiquitin ligase activity. We also discuss the modulatory implication of gp78/AMFR palmitoylation, showing that palmitoylation disrupts the RING finger motif, regulates its ER distribution and enhances its turnover. Whether palmitoylation of E3 ubiquitin ligases is gp78/AMFR-specific or a general mechanism to control the activity of RING finger ubiquitin ligases remains to be determined.

Next, we look at the Gα subunit, a known component of the G protein-coupled receptor (GPCR) signal transduction pathway. In chapter 3, using immunoprecipitation and immunocytochemistry experiments, we report that the E3 ubiquitin ligase gp78/AMFR interacts with and recruits several G proteins to the ER, namely Gαi1 and Gαs. Thus, we reintroduce the possibility that gp78/AMFR is a novel ER-localized GPCR. The Gαs subunit is further discussed in chapter 4 where we characterize in detail its ER localization and its association with ERAD components, as well as we show a novel intracellular function, demonstrating the ability of Gαs to induce substrate polyubiquitylation and protect against ER stress.

Conclusion:

Together, these findings mark the beginning in understanding the physiological significance of (1) E3 ubiquitin ligase palmitoylation; (2) G protein binding to gp78/AMFR; (3) Gαs-mediated substrate polyubiquitylation and protection against ER stress, in ERQC mechanisms.

Preface

Biohazard Approval Certificate at the University of British Columbia was approved for containment level 2 (protocol number H07-0138).

Parts of chapter 1 (section 1.2 to (and including) section 1.2.5) have been published in (Fairbank et al., 2009) - Reproduced by permission of The Royal Society of Chemistry (http://dx.doi.org/10.1039/B820820B). Section 1.2.1 to (and including) section 1.2.3 were originally drafted by Pascal St-Pierre. Also, Schemes 1.2.1 and 1.2.2 were designed by Pascal St-Pierre.

The project in chapter 2 started in collaboration with Dr. Ala El-Husseini (deceased) and Kun Huang (Department of Psychiatry and the Brain Research Centre, University of British Columbia, Vancouver) performed all the metabolic radiolabeling experiments for Figure 2.1B-D and Figure A.1. A version of this chapter has been submitted for publication.

In chapter 4, the immunoblot presented in Figure 4.4B was done by Min Fu in the laboratory of Dr. Ivan R Nabi. We intend to submit a version of chapter 4 for publication once we obtain data from Dr. Thomas Simmen (University of Alberta).

List of Publications:

1. RING Finger Palmitoylation of the Endoplasmic Reticulum Gp78 E3 Ubiquitin Ligase. **Fairbank M**, Huang K, El-Husseini A, Nabi IR. Submitted (in progress).
2. Differential impact of caveolae and caveolin-1 scaffolds on the membrane raft proteome. Zheng YZ, Boscher C, Inder KL, **Fairbank M**, Loo D, Hill MM, Nabi IR, Foster LJ. Mol Cell Proteomics. 2011 Oct;10(10):M110.007146. Epub 2011 Jul 13.

   - I provided Figure 6.
3. The complex biology of autocrine motility factor/phosphoglucose isomerase (AMF/PGI) and its receptor, the gp78/AMFR E3 ubiquitin ligase. **Fairbank M**, St-Pierre P, Nabi IR. Mol Biosyst. 2009 Aug;5(8):793-801. Epub 2009 May 29. Review.

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CHAPTER 1 Introduction

Protein folding in the endoplasmic reticulum (ER) is carefully monitored by ER Quality Control (ERQC) mechanisms in which molecular chaperones evaluate substrate conformations and target proteins to their final destination (Ellgaard and Helenius, 2003). Most proteins need to attain a three-dimensional conformation for proper function, a process that can be highly error prone. Thus, misfolded, non-functional and misassembled proteins are directed to the endoplasmic reticulum-associated degradation (ERAD) pathway where aberrant proteins are recognized, targeted for retro-translocation to the cytoplasm and degraded by the ubiquitin-proteasome machinery (Nakatsukasa and Brodsky, 2008). The proper function of the ERAD pathway is vital in preventing misfolding-induced toxicity. Chronic imbalance of the ER and cellular homeostasis may lead to the development of serious pathologies such as antitrypsin deficiency and protein aggregation diseases (i.e. Parkinson's, Alzheimer's and Huntington's), and other protein conformational diseases (i.e. cystic fibrosis) (Coughlan and Brodsky, 2005). This manuscript introduces novel findings that expand our understanding of two vital cellular pathways involved in ERQC mechanisms, the ERAD pathway and the ER stress response.

The first protein that was studied is the E3 ubiquitin ligase gp78/AMFR, a membrane receptor that is mostly expressed in the ER and plays a significant role in protein degradation within the ERAD pathway. In chapter 2, we show that gp78/AMFR undergoes a posttranslational modification, palmitoylation within the catalytic RING finger domain that seems to modulate its intracellular distribution and its own degradation. This is the first report of palmitoylation of an E3 ubiquitin ligase and we propose that palmitoylation may be a general mechanism to regulate the activity of this ER protein family.

The second protein of interest is the Gαs subunit that is a known component of the G protein-coupled receptor (GPCR) signal transduction pathway. Here, we are interested in looking at Gαs functions that occur within other cellular compartments and are distinct from the plasma membrane. In chapter 3, we observe that gp78/AMFR can interact with different G protein subunits in the ER including Gαs, and we reintroduce the possibility that gp78/AMFR is a novel ER-localized GPCR. These findings suggest that Gαs interacts with protein(s) other than its effector adenylyl cyclase and may signal via a pathway that is independent from the plasma membrane. We expand this concept in chapter 4 where we demonstrate that Gαs is indeed localized to the ER and is involved in substrate polyubiquitylation via an ERAD protein complex. Moreover, we show that Gαs plays a protective role in the ER stress response.

Together, we describe for the first time, novel intracellular functions for this unique Gαs subunit.

These research projects mark the beginning in understanding the physiological significance of these new elements associated with ERQC mechanisms: (1) E3 ubiquitin ligase palmitoylation; (2) the association of G proteins with gp78/AMFR; (3) Gαs-mediated substrate polyubiquitylation in the ER; (4) the protective role of Gαs in the ER stress response.

1.1 The ERAD pathway

1.1.1 Molecular chaperones

Recognition and selection of ERAD substrates still remains unclear. Several aspects of this process have been unraveled and are briefly discussed. Soluble substrates are maintained in solution and resist aggregation because they are temporally bound to molecular chaperones that in turn help deliver target substrates from the ER to the proteasome, a process described as molecular chaperone-mediated selection. A similar mechanism exists for transmembrane proteins. Molecular chaperones belonging to the 70 kDa heat-shock protein (Hsp70) family temporarily bind to hydrophobic polypeptide motifs that are hidden in properly folded proteins, and when exposed in the unfolded state, may cause protein aggregation. The binding and release of Hsp70-family substrates is ATP-dependent and modulated by other proteins such as Hsp40-family co-chaperones and nucleotide exchange factors (NEFs). The Hsp70-Hsp40 chaperone system is essential in protein degradation since it also assists in the recruitment of E3 ubiquitin ligases and facilitates substrate polyubiquitylation of certain soluble and integral membrane substrates (Denic et al., 2006; Meacham et al., 2001; Mimura et al., 2008; Nakatsukasa et al., 2008). An immunoglobin binding protein BiP (known as GRP78), also an Hsp70 molecular chaperone, has been shown to associate with ERAD substrates (Nishikawa et al., 2001). However, it is unclear whether BiP binding and/or BiP-associated NEFs and/or Hsp40-family co-chaperones are necessary for all ERAD substrate recognition.

Other molecular chaperones include protein disulfide isomerases (PDI) (Gillece et al., 1999; Tsai et al., 2002) and ER-resident chaperone-like lectins in the Calnexin-Calreticulin cycle (Caramelo and Parodi, 2007). In mammals, the Calnexin-Calreticulin cycle is well-characterized within the ERQC pathway (Scheme 1.1.1) and involves the addition or removal of glucose residues to the N-linked glycan core of glycosylated proteins in the ER lumen. Degradation of misfolded glycoproteins is critical for proper cell function since accumulation following

treatment of an inhibitor for N-glycosylation, Tunicamycin, results in ER stress and induction of the unfolded protein response (UPR) (discussed in section 1.1.5). In the case of non-glycosylated proteins, substrate recognition is less clear. The homocysteine-inducible protein (HERP) is a membrane-associated cytoplasmic protein that has been shown to bind to non-glycosylated BiP substrates and to the 26S proteasome (Okuda-Shimizu and Hendershot, 2007). Based on these data, there seems to be a distinction in the degradation pathway between glycosylated and non-glycosylated proteins.

1.1.2 Substrate ubiquitylation

Most ERAD substrates are ubiquitylated prior to proteasome-dependent degradation in which ubiquitin, a 76 amino-acid peptide, is covalently attached to the ε-amino group of a lysine (Lys) residue via an isopeptide bond. The linkage often occurs through Lys48 and to trigger degradation of the ubiquitylated protein by the 26S proteasome, the ubiquitin chain must contain at least four ubiquitin subunits, forming a polyubiquitin chain (Pickart and Fushman, 2004) (discussed in section 1.1.4). Otherwise, substrates that are labeled with one or multiple ubiquitin subunits are targeted to different pathways and not surprisingly, degradation is only one of several outcomes for ubiquitin-tagged proteins (Scheme 1.1.2). Ubiquitin seems to play a vital role in cell surface receptor internalization and down-regulation (Hicke, 1999), and in other cases, it is implicated in non-proteolytic signaling that still remains poorly understood (Deng et al., 2000; Kaiser et al., 2000). The subcellular localization of the substrate and/or the number and the type of ubiquitin linkage are all important factors in determining the cellular fate of the target protein (Pickart, 2000). In the following sections, we will solely focus on ubiquitin-mediated proteasomal protein degradation.

1.1.3 Protein complexes involved in substrate polyubiquitylation

Protein polyubiquitylation involves the sequential activation of three classes of enzymes: the E1 ubiquitin-activating enzymes, the E2 ubiquitin-conjugating enzymes and the E3 ubiquitin ligases (Pickart, 2001) (Scheme 1.1.3). Ubiquitin is first conjugated to the E1 ubiquitin-activating enzyme (Haas and Rose, 1982), a highly efficient enzyme that binds two molecules of activated ubiquitin in an ATP-dependent conformation. The thiol-linked ubiquitin is then transferred from the E1 to the E2 ubiquitin-conjugating enzyme. The highly conserved cysteine residue within the active site of the E2 enzyme interacts with ubiquitin via a thioester linkage. Finally, the E3 ubiquitin ligase covalently attaches ubiquitin to the target protein. E3

ubiquitylation depends on both its binding to the E2 enzyme and to the substrate. Additional factors have been shown to affect the coupling of the E3 to the substrate and these include substrate phosphorylation (Feldman et al., 1997; Skowyra et al., 1997), reversible E3 posttranslational modification (Lahav-Baratz et al., 1995) and binding of small compounds to E3 enzymes (Turner et al., 2000). In some cases, an additional protein, the E4 ubiquitin-chain-extension enzyme, may facilitate in the ubiquitin-dependent proteolysis by binding to multiubiquitin chains (Koegl et al., 1999; Richly et al., 2005). In fact, certain E3 ubiquitin ligases can also act as E4 enzymes and catalyze both the initial ubiquitin ligation to substrate and subsequent ubiquitin-ubiquitin chain elongation (discussed in section 1.2.2). Thus, conjugation of ubiquitin to proteins is a highly selective and efficient process involving the coordination of large protein complexes.

E3 ubiquitin ligases are multi-spanning membrane proteins and can be categorized into three major classes: HECT, RING and U-box domain-containing proteins (Kostova et al., 2007). The HECT (homologous to E6-AP carboxy terminus) domain E3s contain a highly conserved cysteine within a 350-residue region that forms a thiol ester intermediate with the substrate during catalysis (Huibregtse et al., 1995). The HECT E3-substrate intermediate state is not seen with the other types of E3s. Further, the N-terminal has the ability to bind to the substrate while the C-terminal HECT domain directly transfers ubiquitin from the thioester bond to the substrate (reviewed in (Jackson et al., 2000)). Thus the HECT domain regulates E2 binding and selectively ubiquitylates substrates (Huibregtse et al., 1993; Schwarz et al., 1998).

The majority of E3 ubiquitin ligases belong to the RING (Really Interesting New Gene) and RING finger-like E3 enzymes (reviewed in (Deshaies, 1999; Page and Hieter, 1999)). The RING finger family is composed of several classes of ligases, these include SCF (Skp1, cullin, F-box), VBC (the von Hippel-Lindau (VHL)-Elongin B-Elongin C), APC (anaphase-promoting complex), and single protein RING finger E3 ubiquitin ligases (reviewed in (Jackson et al., 2000)). These enzymes are defined by a conserved 40-60 amino acid motif in which several carefully spaced cysteine and histidine residues stably interact with two zinc ions in a cross-brace structure. The association with and the spacing of the zinc ions are highly conserved and essential in maintaining the proper globular conformation and function (Borden, 2000; Eisenhaber et al., 2007) (Scheme 1.1.4). In addition to their role in ERAD, RING finger proteins are considered molecular scaffolds and are implicated in many other cellular processes (reviewed in (Joazeiro and Weissman, 2000)). In fact, the ring between ring fingers (RBR) proteins make

up a large and diverse group in which the RBR domain is among one of multiple domains within the protein and usually mediates protein-protein interactions (Eisenhaber et al., 2007).

Finally, the U-box domain enzymes (Patterson, 2002) achieve a similar conformation as the RING finger enzymes whereby the U-box domain adopts an analogous tertiary structure as the RING finger via electrostatic interactions instead of conserved cysteine residues (reviewed in (Aravind and Koonin, 2000)). As seen with RING finger E3 ubiquitin ligases, U-box domain E3s also transfer ubiquitin from the E2 to the substrate.

ERAD substrates can be degraded by more than a single E3 ubiquitin ligase and in turn, E3 ubiquitin ligases can complement each other's activities as seen with the degradation of the cystic fibrosis transmembrane regulator (CFTR) (Younger et al., 2006). Substrate recognition and specificity is also determined by the presence of motifs, known as ubiquitylation signals, imbedded within the primary structure of the substrate that is in turn recognized by E3 ubiquitin ligases (Laney and Hochstrasser, 1999). This is described as the N-end rule, in which the E3 enzyme recognizes substrates based on their N-terminal domain that consists of an N-terminal residue (the ubiquitylation signal) and an accessible lysine residue (the ubiquitylation site) (Chau et al., 1989). It remains unknown how many substrates undergo N-terminal ubiquitylation or the probability that a specific E3 enzyme is responsible for this type of ubiquitylation. Protein ubiquitylation is a complex posttranslational modification whereby variation in the length of the ubiquitin chain and ubiquitin-linkages seem to have distinct biological functions (Scheme 1.1.2). Thus, the dynamic and reversible nature of ubiquitin can be described as a versatile intracellular messenger that is not limited to protein degradation (Woelk et al., 2007).

1.1.4 Substrate targeting and proteasomal degradation

Following polyubiquitylation, some substrates are extracted from the membrane into the cytosol by a p97-dependent protein complex and later targeted to the proteasome for degradation. P97 (also known as VCP and Cdc48) is an hexameric AAA+ ATPase, an ATP-hydrolyzing enzyme that provides the driving force for ATP-dependent membrane extraction (Vij, 2008) (Scheme 1.1.3). The transfer of targeted proteins from the ER to the cytosol is dependent on ubiquitin chain elongation, interaction with the CUE domain (ubiquitin-binding domain) of an E3 ubiquitin ligase and p97/VCP/Cdc48 recruitment (Vembar and Brodsky, 2008). In mammals, the P97/VCP/Cdc48 complex has been shown to be recruited to the ER membrane by valosin-containing protein-interacting membrane protein (VIMP) (Neuber et al.,

2005; Ye et al., 2004) and to interact with the Gp78/AMFR, Der1 and Hrd1 complex (Denic et al., 2006; Gauss et al., 2006; Schuberth and Buchberger, 2005).

In addition to the p97/VCP/Cdc48 complex, there is evidence to support a retro-translocation event. Some studies describe the involvement of the Sec61 translocation channel (Pilon et al., 1997; Schmitz et al., 2000) as well as proteins from the Derlin family (i.e. Derlin-1) (Lilley and Ploegh, 2004; Wahlman et al., 2007; Ye et al., 2004). Others suggest that E3 ubiquitin ligases also function as retro-translocation channels (Zhong et al., 2004). As of yet, the identity of the retro-translocation channel is obscure and the process itself is currently not well-defined.

The 26S proteasome is a complex structure consisting of two 19S caps and a 20S catalytic core and its primary function is to recognize and degrade ER substrates (Raasi and Wolf, 2007) (Scheme 1.1.3). Each 19S cap is composed of 19 subunits containing many peripheral factors and ubiquitin receptors (Husnjak et al., 2008), and is responsible for the removal of ubiquitin from substrates via de-ubiquitylating enzymes (Dubs) (Amerik and Hochstrasser, 2004). An example of a studied ubiquitin receptor is ataxin-3 which also binds to several ERAD components including P97/VCP/Cdc48 and the Derlin-VIMP complex (Wang et al., 2006). In fact, P97/VCP/Cdc48 associates with the 19S cap (Verma et al., 2000) and it seems likely that both P97/VCP/Cdc48- and proteasome-dependent factors are linked prior to protein degradation. Examples of proteasome-dependent factors include the UBA- and UBL-domain ubiquitin-regulatory proteins (Raasi and Wolf, 2007). On the other hand, the 20S core, consisting of 28 subunits, is lined with enzymes that possess trypsin-like, chymotrypsin-like and post-glutamylpeptide hydrolyzing activities, that function to degrade ER misfolded proteins.

1.1.5 Cross-talk between ERAD and other pathways

Proper ER function is critical for efficient degradation and disposal of aberrant proteins. Otherwise, protein accumulation and/or aggregation can induce ER stress that in turn compromises ER homeostasis. Thus, several mechanisms have been developed by the cell to reduce ER stress by modulating ERAD function. The unfolded protein response (UPR), originally identified in yeast (Scheme 1.1.5), can be induced by accumulation of misfolded proteins in the ER and has been shown to be closely associated with ERAD function (Casagrande et al., 2000; Friedlander et al., 2000; Ng et al., 2000; Travers et al., 2000). In Eukaryotes, the UPR consists of three transducers: (1) the inositol-requiring protein-1 (IRE1), an ER-localized transmembrane Ser/Thr kinase and site-specific endoribonuclease that is

inactivated by BiP binding; (2) ER-stress-activated PERK, a transmembrane kinase that inhibits protein translation by phosphorylating the α-subunit of the eukaryotic translation initiation factor-2 (eIF2); (3) Activating Transcription Factor-6 (ATF6) that upregulates target genes in the nucleus through cleavage of ATF6-fragment transcription factor (Kimata et al., 2007). Thus, the UPR also reduces ER stress by affecting other factors, some of which include: (1) upregulation of lipid synthesis and expanding the volume of the ER; (2) upregulation of molecular chaperones and enzymes involved in posttranslational modifications; (3) reduction in protein translation and ER translocation (Pickart, 2001).

Under severe and irreversible ER stress conditions, the apoptotic pathway is activated via UPR transducers, namely IRE1 and PERK, (Barone et al., 1994; Urano et al., 2000) and seems to be mediated through the mitochondria (Rao et al., 2004). Several components involved in the apoptotic response have been described and these include the activation of the ER-membrane-localized caspase-12 that associates with TNF-receptor-associated factor-2 (TRAF2) and results in the activation of downstream effectors p38 and c-Jun N-terminal kinase (Nakagawa et al., 2000; Nishitoh et al., 1998; Urano et al., 2000; Yoneda et al., 2001). Another component includes the upregulation of C/EBP-homologous protein (CHOP), a transcriptional repressor that inhibits the expression of pro-survival BCL2 proteins. In addition to its role in apoptosis, CHOP also leads to cell-cycle arrest (Barone et al., 1994).

Based on these findings, it is evident that the interplay between ERAD and the UPR is critical in maintaining efficient protein degradation and minimizing ER stress. In fact, both pathways display complimentary roles during ERQC. Thus, ERAD is not an isolated process but, on the contrary, it is closely associated with and monitored by other cellular pathways. As of yet, mechanisms underlying ERAD-dependent molecular cross-talk remain poorly understood.

1.2 Autocrine motility factor receptor (gp78/AMFR)*

Since the mid 1980s, multiple groups have linked the expression of autocrine motility factor (AMF/PGI) and its receptor, autocrine motility factor receptor (gp78/AMFR), to increased metastasis development and poor prognosis in cancer patients (Chiu et al., 2008). Since then, extensive studies have been conducted on the function of both proteins in cancer cells as well as their physiological role in normal cells. AMF/PGI plays a dual role as a catalytic enzyme in the

* A version of section 1.2 was published in Fairbank, M., St-Pierre, P., and Nabi, I.R. (2009). The complex biology of autocrine motility factor/phosphoglucose isomerase (AMF/PGI) and its receptor, the gp78/AMFR E3 ubiquitin ligase. Mol Biosyst *5*, 793-801.

gluconeogenesis – glycolysis pathways and, when secreted, as a cytokine (Liotta et al., 1986; Watanabe et al., 1996). Its cytokine role has been linked to cell differentiation, survival and growth. AMF/PGI cytokine function in tumor cell motility is dependent on interaction with its receptor, gp78/AMFR. Gp78/AMFR, like its ligand, has multiple roles dictated by its cellular localization (Scheme 1.2.1). At the cell surface, gp78/AMFR is a cytokine receptor that stimulates cell motility upon AMF/PGI activation. It is also localized to an intracellular mitochondria associated smooth ER domain where it functions as an E3 ubiquitin ligase. The relationship between its cell surface signaling and intracellular ubiquitin ligase activities remains uncertain. However, gp78/AMFR function, as both cytokine receptor and ubiquitin ligase, is linked to metastasis development and increased invasiveness. The complex biology of AMF/PGI and its receptor, gp78/AMFR is reviewed in (Fairbank et al., 2009).

Gp78/AMFR was originally named gp78 after a glycoprotein of 78 kDa purified from metastatic B16-F1 melanoma cells (Nabi and Raz, 1987, 1988). It was subsequently identified as the receptor for AMF/PGI and called AMFR (Nabi et al., 1990; Silletti et al., 1991). A monoclonal antibody (mAB), called 3F3A, has been used to study gp78/AMFR distribution and its role in cell motility (Nabi et al., 1990). The 3F3A antibody was shown to be specific as it recognized transfected gp78/AMFR and showed a reduced signal following gp78/AMFR-specific siRNA treatment by immunofluorescence and immunoblotting (Goetz et al., 2007; Registre et al., 2004). Binding of the 3F3A mAB competes with AMF/PGI for gp78/AMFR binding and stimulates cell motility in a similar fashion to AMF/PGI (Kojic et al., 2007; Nabi et al., 1990). Gp78/AMFR was further confirmed as an AMF/PGI receptor based on a protein–protein binding assay in which purified AMF/PGI bound directly to immunopurified gp78/AMFR and on the ability of soluble gp78/AMFR to competitively inhibit AMF/PGI-stimulated cell motility (Silletti et al., 1991). *In vivo*, pre-treatment of B16-F1 cells with either polyclonal anti-gp78/AMFR or the 3F3A mAB followed by injection into the tail vein of syngeneic mice resulted in a two-fold increase in lung colonization (Nabi and Raz, 1987; Watanabe et al., 1991b). These results suggest that both 3F3A mAB and AMF/PGI bind to and activate gp78/AMFR at the same extracellular domain. However, while AMF/PGI is a natural ligand for gp78/AMFR, little is known about the structural basis of the interaction between AMF/PGI and gp78/AMFR.

1.2.1 Cloning and sequence analysis of gp78/AMFR*

The first sequence reported for human gp78/AMFR was in 1991 (Watanabe et al., 1991a) using expression cloning with the 3F3A antibody. The cDNA fragment identified was 1.9 kilobases long and gave an open reading frame (ORF) of 321 amino acids (GenBank accession number L35233). In 1999, a sequence from a mouse EST clone library was identified that was 74% homologous to the previous sequence but with a remarkably different ORF of 643 amino acids (GenBank accession number AA260491). The difference was due to four single base deletions and two single base insertions in the first published sequence. Isolation of a cDNA cloned from HeLa human cells confirmed the second reading frame with 94.7% homology to the mouse gene (Shimizu et al., 1999). Gp78/AMFR was first proposed to be an E3 ubiquitin ligase after sequence analysis identified a catalytic RING finger and CUE motif, responsible for ubiquitin ligase activity and ubiquitin binding, respectively (Ponting, 2000; Shimizu et al., 1999) (Scheme 1.2.2A). The RING finger domain is composed of eight conserved cysteines (Cys) and histidines (His) that coordinate two Zn ions in a ‘‘cross-braced’’ fashion (Scheme 1.1.4). This domain can either be classified as RING-H2 or RING-HC depending on whether position 5 is occupied by His (-H2) or Cys (-HC). Gp78/AMFR is a RING-H2 ubiquitin ligase with two His residues in position 4 and 5 and six Cys residues in positions 1–3, 6–8 (Scheme 1.1.4). The ubiquitin binding motif family CUE was identified by a database search for homologous sequences of the yeast protein Cue1p (Biederer et al., 1997; Ponting, 2000). The role of gp78/AMFR as an ubiquitin ligase has been confirmed experimentally and the RING finger, the CUE motif and an E2 binding site have all been shown to be essential to its E3 function (Chen et al., 2006; Fang et al., 2001; Li et al., 2007).

1.2.2 ER localization of gp78/AMFR and its role in ERAD

The cellular distribution of gp78/AMFR was assessed by immunoelectron microscopy using the 3F3A antibody. Gp78/AMFR was found on the plasma membrane in caveolae and on smooth ER tubules (Benlimame et al., 1998; Benlimame et al., 1995; Goetz et al., 2007; Wang et al., 1997; Wang et al., 2000), consistent with its role as a cell surface receptor and ubiquitin ligase involved in the ERAD pathway.

* A version of section 1.2.1 and 1.2.2 was published in Fairbank, M., St-Pierre, P., and Nabi, I.R. (2009). The complex biology of autocrine motility factor/phosphoglucose isomerase (AMF/PGI) and its receptor, the gp78/AMFR E3 ubiquitin ligase. Mol Biosyst *5*, 793-801.

Gp78/AMFR is a key component in the ERAD machinery, a process involving recognition of misfolded proteins, ubiquitylation, deglycosylation, retro-translocation to the cytosol and targeting to the proteasome (Meusser et al., 2005). Gp78/AMFR is amongst the best characterized ubiquitin ligases involved in this process (Ballar et al., 2006; Cao et al., 2007; Chen et al., 2006; Fang et al., 2001; Lee et al., 2006; Liang et al., 2003; Morito et al., 2008; Song et al., 2005; Tsai et al., 2007; Zhong et al., 2004) (Scheme 1.1.3). Ubiquitylation has been described as a three step process requiring the enzymes E1, E2 and E3 ((Hershko and Ciechanover, 1998); described in detail in section 1.1.3). However, an *in vitro* ubiquitylation assay using gp78/AMFR has changed the sequential model of ubiquitin chain elongation for a preassembled ubiquitin chain model. Gp78/AMFR can catalyze elongation of an ubiquitin chain on an E2 (in this case Ube2g2) and then transfer this preassembled chain to a substrate (in this case Herpc) (Li et al., 2007). This represents the first report that an ubiquitin chain is preassembled prior to its transfer to a substrate and further investigation is needed to assess if this is a general mechanism.

In addition to E3 ligase activity, gp78/AMFR also has been recently reported to have E4 ligase activity, in which gp78/AMFR elongates the ubiquitin chain of previously ubiquitylated substrates. Gp78/AMFR is involved in the recognition and degradation of mutant cystic fibrosis transmembrane regulator (CFTRD508). Gp78/AMFR binds to CFTRD508 via its ubiquitin binding domain CUE in a complex including p97, Derlin-1 and BAP31, the latter being implicated in ER export of membrane proteins (Carlson et al., 2006; Morito et al., 2008; Sun et al., 2006; Wang et al., 2008). Interestingly, silencing of RMA1 ubiquitin ligase prevents ubiquitylation of CFTRD508 by gp78/AMFR while knockdown of another E3 ubiquitin ligase involved in CFTRD508 ubiquitylation, namely CHIP, has no effect (Morito et al., 2008). These findings propose that gp78/AMFR can function as an E4 ligase in cooperation with other ubiquitin ligases.

Gp78/AMFR directly binds to the cytosolic p97 ATPase, also known as VCP (its yeast homologue is called CDC48), via its C-terminal domain (Ballar et al., 2006; Zhong et al., 2004). P97 is a hexameric ATPase with multiple cellular activities belonging to the AAA family that provides the ‘‘driving force’’ to extract ERAD substrates from the lipid bi-layer at the retro-translocation site (Ye et al., 2005). The gp78/AMFR-p97 interaction is also important for the recruitment of gp78/AMFR to the retro-translocation complex composed of the valosin-interacting membrane protein (VIMP), Derlin-1 and peptide N-glycanase (PNGase) (Li et al., 2006a; Ye et al., 2005; Ye et al., 2004). As shown by GST pull-down assay, gp78/AMFR does

not physically interact with any other member of the complex (Li et al., 2006a). Gp78/AMFR recruitment to the retro-translocation complex is regulated by small p97/VCP/Cdc48-interacting protein (SVIP); p97 binds SVIP and gp78/AMFR in a mutually exclusive manner such that SVIP sequesters the p97–Derlin-1 complex and prevents gp78/AMFR recruitment to the complex (Ballar et al., 2007).

Ufd1 is another cofactor that binds directly to gp78/AMFR and has been implicated in the regulation of its ubiquitin ligase activity (Cao et al., 2007). Ufd1 was previously identified in a complex with p97 and Npl4 that delivers the ubiquitylated substrates to the proteasome (Bays and Hampton, 2002; Meyer et al., 2002; Ye et al., 2001, 2003). It was proposed that this complex forms a ring-like structure providing the driving force to dislocate ERAD substrates across the membrane into the cytosol (DeLaBarre and Brunger, 2003; Pye et al., 2007). Although both proteins, gp78/AMFR and Ufd1, recruit p97, this happens in a mutually exclusive manner (Ballar et al., 2006). Ufd1 has two ubiquitin binding domains in its N-terminus for mono- and polyubiquitin (Park et al., 2005). Using the gp78/AMFR substrate HMG-CoA reductase, Cao *et al.* have determined that the monoubiquitin binding site of Ufd1 is essential to enhance the ubiquitin ligase activity of gp78/AMFR (Cao et al., 2007). Subsequently, Ufd1 affinity for polyubiquitin increases upon its recruitment to p97 (Park et al., 2005; Ye et al., 2003). This would promote the formation of polyubiquitylated substrates in complex with Ufd1–p97–Npl4 and increase proteasomal degradation.

Gp78/AMFR plays an important role in cholesterol homeostasis via the sterol-mediated ubiquitylation of HMG CoA reductase and its cofactor Insig-1 (Lee et al., 2006; Song et al., 2005). HMG-CoA reductase is involved in the reduction of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate, a critical step in cholesterol synthesis. In the presence of high sterol levels, mevalonate synthesis is no longer required and Insig-1 binds to HMG-CoA reductase and promotes the recruitment of gp78/AMFR-p97, resulting in HMG-CoA reductase degradation. It still remains to be determined if Insig-1 is degraded along with HMG-CoA reductase in the presence of sterol (Song et al., 2005). When the level of sterol is low, gp78/AMFR targets Insig-1 for proteasomal degradation, protecting HMG-CoA reductase and promoting cholesterol synthesis (Lee et al., 2006). Gp78/AMFR is also implicated in the degradation of apolipoprotein B100 (ApoB), the single protein component of atherogenic low and very low density lipoprotein (Liang et al., 2003). Interestingly, upon overexpression of gp78/AMFR, the intracellular level of ApoB protein remains the same but the secreted amount is greatly reduced. This suggests that gp78/AMFR selectively regulates the degradation of ApoB in the secretory pathway.

It was recently reported that KAI1, also called CD82, is a substrate of gp78/AMFR (Tsai et al., 2007) representing the first reported evidence that the ubiquitin ligase activity of gp78/AMFR is involved in metastasis development. KAI1 is a tetraspanin glycoprotein known as a metastasis suppressor. The loss of its expression is correlated with metastasis development in several cancers (Abe et al., 2008; Briese et al., 2008; Protzel et al., 2008; Xu et al., 2008; Yang et al., 2008). Knockdown of gp78/AMFR results in increased KAI1 expression and reduced morbidity in an experimental metastasis model. The CUE and RING domains are essential to restore metastasis development. Interestingly, replacement of the membrane spanning domains of gp78/AMFR, believed to be the AMF/PGI binding site, by a single pass transmembrane domain restored ubiquitylation and degradation of KAI1 and enhanced its metastatic potential (Tsai et al., 2007). This suggests that the gp78/AMFR E3 ligase function can affect tumor progression and metastasis development independently of its cytokine function. Gp78/AMFR is therefore a critical component of the ERAD machinery and the relationship of this ER-localized function to its role as the cell surface receptor for AMF/PGI signaling remains to be determined.

1.2.3 Mitochondrial association of the gp78/AMFR smooth ER domain*

By immunofluorescence labeling, the 3F3A mAb labels a subdomain of the ER in close association with mitochondria that shows little overlap with the calnexin, calreticulin and Sec61β-labeled central ER (Benlimame et al., 1995; Goetz et al., 2007; Wang et al., 2000). The 3F3A-labeled SER domain is distinct from the ER–Golgi intermediate compartment (ERGIC) and sensitive to morphology disruption by ilimaquinone (Benlimame et al., 1995; Wang et al., 1997). The 3F3A-labeled ER domain is also distinct from myc-reticulon4a and GFP-Sec61β, markers of the peripheral, tubular and saccular, perinuclear ER, respectively (Goetz et al., 2007; Voeltz et al., 2006). Upon overexpression, FLAG- and GFP-tagged gp78/AMFR are localized throughout the ER (Fang et al., 2001; Registre et al., 2004). 3F3A labeling defines a peripheral ER subcompartment that colocalizes with reticulon4a and associates with mitochondria, but does not label overexpressed, transfected gp78/AMFR localized in the central ER (Goetz et al., 2007). 3F3A labeling therefore defines a specific domain of the ER and, at least upon gp78/AMFR overexpression, a subpopulation of the gp78/AMFR pool. Interaction between ER and

* A version of section 1.2.3 was published in Fairbank, M., St-Pierre, P., and Nabi, I.R. (2009). The complex biology of autocrine motility factor/phosphoglucose isomerase (AMF/PGI) and its receptor, the gp78/AMFR E3 ubiquitin ligase. Mol Biosyst *5*, 793-801.

mitochondria is critical for intracellular calcium homeostasis (Montero et al., 2000; Rizzuto et al., 1993; Rizzuto et al., 1998; Simpson et al., 1997; Szabadkai et al., 2006). These contact sites allow local calcium released from the ER to be rapidly taken up by mitochondria stimulating their metabolism. Using permeabilized cells, cytosolic calcium concentrations under 100 nM favor dissociation of the 3F3A-labeled SER domain and mitochondria, while concentrations above 1 mM favor their close association (Wang et al., 2000). Similarly, inhibition of ER calcium uptake using Thapsigargin or ATP stimulation of the purinergic receptor both result in a temporary cytosolic calcium increase and reversible ER–mitochondria dissociation. In intact cells, SER is predominantly associated with mitochondria at calcium concentrations lower than 60 nM and increased calcium concentrations to physiological levels of 100–200 nM are associated with dissociation of these organelles (Goetz et al., 2007). Several Ca2+ channels, such as the inositol (1,4,5)-triphosphate receptor (IP3R) and the ryanodine receptor (RyR), have been shown to cluster at ER–mitochondria contact sites (Rizzuto et al., 1993; Ross et al., 1989). Gp78/AMFR and IP3R colocalize at these ER–mitochondria contact sites but when SER–mitochondria dissociation is induced, the 3F3A and IP3R-labeled ER dissociate from mitochondria into two distinct domains after 2 minutes and reassociate after 5 minutes. This suggests that multiple mechanisms regulate the association of distinct SER domains with mitochondria (Goetz et al., 2007). Interestingly, gonadotropinreleased hormone (GnRH) activation of IP3R induces recruitment of the ERAD machinery that peaks between 3 and 15 minutes post-stimulation. Gp78/AMFR co-immunoprecipitation with IP3R is increased after 3 minutes and peaks at 7 minutes post-stimulation (Pearce et al., 2007), which correlates with the reassociation of 3F3A and IP3R-rich ER domains (Goetz et al., 2007). The specific localization of gp78/AMFR to a mitochondria associated smooth ER domain implicates this ER domain localization in gp78/AMFR ERAD function as well as in other functions associated with mitochondria-associated ER, such as calcium homeostasis and apoptosis.

1.2.4 Interaction of gp78/AMFR with its ligand, AMF/PGI on the cell surface*

Autocrine Motility Factor (AMF, estimated size of 55 kDa and 64 kDa under nonreducing and reducing conditions, respectively) was purified from serum-free conditioned medium of human A2058 melanoma cells and first described as a tumor secreted cytokine that

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stimulates direct and random migration (Liotta et al., 1986). AMF exhibits sequence identity with a glycolytic enzyme, namely phosphoglucose isomerase (PGI) (also called phosphohexose isomerase (PHI) and glucose-6-phosphate isomerase (GPI)) that is expressed in all tissues and is vital to cellular metabolism in normal cells (Watanabe et al., 1996). PGI is a critical enzyme in glycolysis and gluconeogenesis that specifically catalyzes the interconversion of glucose 6-phosphate and fructose 6-phosphate. Throughout the dissertation, this protein will be referred to as AMF/PGI.

Factors mediating the binding of AMF/PGI to gp78/AMFR have been proposed to include the sugar binding and catalytic domains of AMF/PGI as well as gp78/AMFR glycosylation (Haga et al., 2006a). Gp78/AMFR was originally found to be O-glycosylated in B16-F1 cells (Nabi and Raz, 1987, 1988) and a putative N-linked glycosylation site is located at amino acid 595 (NKSS) (Shimizu et al., 1999). The exact function of gp78/AMFR glycosylation remains unclear; however, the putative N-linked sugar chain on gp78/AMFR has been proposed to be essential for AMF/PGI binding (Haga et al., 2006a). Mutation of the gp78/AMFR N-linked glycosylation site prevented interaction with AMF/PGI in a cross-linking experiment, and purified gp78/ AMFR treated with N-glycosidase F was unable to compete with the cell motility-stimulating activity of AMF (Haga et al., 2006a). However, gp78/AMFR has yet to be shown to be N-glycosylated and the putative N-glycosylation site is, on some gp78/AMFR topology models, predicted to be intracellular.

AMF/PGI can interact with proteins other than gp78/AMFR. Based on cross-linking and biotinylation experiments, AMF/PGI has been identified as a membrane-associated binding partner for insulin-like growth factor binding protein-3 (IGFBP-3, a protein that binds to and modulates insulin-like growth factors in the circulation), on the cell membrane of human breast cancer cells (Mishra et al., 2004). The binding of IGFBP-3 to AMF/PGI negatively regulates AMF/PGI activity, and also reduces its phosphorylation, secretion and translocation to the plasma membrane. IGFBP-3 decreases AMF/PGI binding to cell monolayers and inhibits AMF/PGI-induced cell migration of MCF-7 or T47D cells (Mishra et al., 2004). AMF/PGI also binds both fibronectin and heparin sulfate at acid pH (Lagana et al., 2005). This may represent a pH-dependent cytokine trap for this ubiquitous circulating protein.

Cellular interaction of AMF/PGI is highly complex and potentially mediated by more than a single receptor. This is highlighted in human acute monocytic leukemic cells lacking gp78/AMFR, where AMF/PGI does not promote cell locomotion but still acts as a maturation factor inducing differentiation, a cellular response mediated by an as yet unknown receptor

(Haga et al., 2006a). The cellular response to this cytokine is therefore highly regulated and may vary between different cell types and/or cancers.

1.2.5 Cell surface interaction of AMF/PGI and its receptor - Raft-dependent endocytosis of AMF/PGI and cell survival*

AMF/PGI can be internalized via two distinct pathways, raft-dependent endocytosis to the SER and clathrin-dependent endocytosis to multi-vesicular bodies (Benlimame et al., 1998; Le et al., 2000; Le et al., 2002; Le and Nabi, 2003). AMF/PGI is internalized to the SER through a receptor-mediated, dynamin and raft-dependent pathway that is negatively regulated by caveolin-1 (Cav1). Increased uptake of AMF/PGI upon reduction of Cav1 expression has been demonstrated in Rasor Abl-transformed NIH-3T3 cells (Le et al., 2002). Recently, our laboratory showed that in invasive breast cancer cells, Cav1 protein expression negatively regulates the uptake of AMF/PGI, while PI3K is a positive regulator of AMF/PGI endocytosis (Kojic et al., 2007). Overexpression of AMF/PGI in NIH-3T3 cells promotes transformation and survival that is dependent upon the PI3K–Akt signaling pathway and down-regulation of Cav1 (Tsutsumi et al., 2003). A highly significant correlation between gp78/AMFR and pAkt expression was observed in invasive breast cancers by tissue microarray analysis (TMA) (Kojic et al., 2007). Of particular interest, recent studies have shown that a paclitaxel conjugate of AMF/PGI is able to inhibit tumor cell proliferation and induce tumor regression upon intratumoral injection into mouse melanoma tumors (Kojic et al., 2008). This suggests that the PI3K-dependent uptake of AMF/PGI by gp78/AMFR might be associated with a pro-survival role in cancer and might also represent a promising route for drug delivery to tumor cells.

AMF/PGI has been shown to have anti-apoptotic and pro-survival roles. Its stable overexpression in NIH-3T3 cells induces cellular transformation and resistance to apoptosis by serum starvation through a PI3K–Akt signaling pathway (Tsutsumi et al., 2003). Human fibrosarcoma HT-1080 cells secreting high levels of AMF/PGI are resistant to drug-induced apoptosis via the down-regulation of two essential pro-apoptotic proteins, namely apoptotic protease activating factor-1 (Apaf-1) and caspase-9 (Haga et al., 2003). To further investigate the apoptotic pathway following AMF/PGI–gp78/AMFR binding, HT-1080 cells were treated with inhibitors of protein kinase C (PKC), phosphatidylinositol-3 kinase (PI3K) and mitogen-

* A version of section 1.2.5 was published in Fairbank, M., St-Pierre, P., and Nabi, I.R. (2009). The complex biology of autocrine motility factor/phosphoglucose isomerase (AMF/PGI) and its receptor, the gp78/AMFR E3 ubiquitin ligase. Mol Biosyst *5*, 793-801.

activated protein kinase (MAPK), and through quantitative RT-PCR, it was observed that Apaf-1 and caspase-9 mRNA levels were restored and comparable to control cells (Haga et al., 2003). AMF/PGI has also been shown to protect melanoma cells from taxol-induced cell death (Kojic et al., 2008). In a recent study, AMF/PGI protection against Thapsigargin- and Tunicamycin-induced ER stress and apoptosis seems to dependent upon its receptor gp78/AMFR, is partially mediated by PI3K/Akt signaling and modulates ER calcium release (Fu et al., 2011). Together, these findings imply a novel role for the interaction between AMF/PGI and gp78/AMFR in the protection against ER stress and tumor cell survival. Overexpression and secretion of AMF/PGI may thereby contribute to tumor cell growth and survival in response to anti-cancer agents. The extent to which the pro-survival functions of AMF/PGI are mediated by its raft dependent delivery to the SER remains to be determined.

1.3 Protein lipid modifications

Covalent linkage of fatty acids to proteins is emerging as an important form of modification that can significantly alter both protein structure and function, as well as be a critical component in regulating many protein-protein and protein-membrane interactions. Also known as protein fatty acylation, target proteins can sequentially be modified with one or more lipids that consist of a single type or a combination of different types of lipid modifications. We will begin our discussion by describing the most common protein lipidations: prenylation, myristoylation and palmitoylation.

1.3.1 Protein prenylation

Protein prenylation is comprised of two types, namely farnesylation (15-carbon) and geranylgeranylation (20-carbon) that both result in the addition of an isoprenoid moiety to one or more cysteine residue(s) via a thioether bond located near or at the C-terminal domain of the target protein. Unlike other types of lipid modifications, prenylation is easily identified by a consensus sequence, the CAAX box [A is an aliphatic amino acid residue and X can either determine farnesylation (if X is Ala, Ser, Cys, Met or Gln) or geranylgeranylation (if X is Leu or Phe)](Maurer-Stroh and Eisenhaber, 2005). Protein farnesylation is catalyzed by the enzyme protein farnesyltransferase, and in the case of geranylgeranylation, two enzymes have been identified, protein geranylgeranlytransferase type I and geranylgeranlytransferase type II (Pereira-Leal et al., 2001). Protein prenylation of small GTPases such as Ras, Rho and Rab, has been studied and data suggest that small GTPases prenylation remains an important signaling

component that mediates their membrane trafficking and protein-protein interactions (An et al., 2003; Seabra et al., 2002).

1.3.2 N-myristoylation

The myristoylation reaction, specifically N-myristoylation, is well-characterized and described by the addition of myristate, a 14-carbon saturated fatty acid, to a glycine via an amide bond. In most cases, N-myristoylation is irreversible and catalyzed by the enzyme N-myristoyl transferase that targets proteins with the consensus sequence Met-Gly-X-X-X-Ser/Thr. Many groups of proteins containing the consensus motif have been shown to undergo N-myristoylation and these include guanine nucleotide binding proteins, protein kinases and phosphatases, membrane- and cytoskeletal-bound structural proteins, and viral proteins (reviewed in (Resh, 1999)).

N-myristoylation of proteins provides structural and enzymatic stability (eg. protein kinase A (Zheng et al., 1993)), as well as promotes membrane targeting and binding that often requires a secondary signal such as a cluster of basic amino acids (eg. Src (Buser et al., 1994; Murray et al., 1998; Sigal et al., 1994)) or a palmitate moiety (eg. p59fyn (Alland et al., 1994)). Despite its irreversible nature, N-myristoylation can dynamically be regulated by a mechanism described as ‘myristoyl swiches’ (Resh, 1999) in which N-myristoylated proteins can transition between conformations that either hide or expose myristate. These protein structural changes are regulated by factors such as ligand binding (eg. Arf-1; (Goldberg, 1998)), electrostatics (eg. MARCKS; (Thelen et al., 1991)) or proteolysis (eg. HIV-1 Gag; (Hermida-Matsumoto and Resh, 1999)).

1.3.3 Palmitoylation

For the remainder of section 1.3, we will focus on palmitoylation and specifically discuss sulfhydryl cysteine palmitoylation (or S-palmitoylation) of both soluble and membrane proteins. Unlike other protein lipid modifications, palmitoylation is a dynamic, reversible posttranslational modification. For this reason, this type of fatty acylation is becoming a critical mechanism in the cell that has been shown to rapidly regulate many cellular responses (Baekkeskov and Kanaani, 2009; Linder and Deschenes, 2007).

The enzymology of palmitoylation is less understood compared to other fatty acylation reactions such as myristoylation, but in almost all cases, palmitoylation of proteins occurs with the attachment of palmitate (16-carbon fatty acid) to the sulfhydryl group of one or more

cysteine(s) via a thioester linkage (this is called S-palmitoylation). Occasionally, N-palmitoylation can take place and this has been reported with human Sonic hedgehog in which palmitate is linked to the amino group of the N-terminal cysteine. Acting as a lipid tether, hedgehog palmitoylation proves to be a critical component for its function (Pepinsky et al., 1998). Even more rare is O-palmitoleoylation that occurs on a serine residue via an oxyester linkage. This has been documented with the murine Wtn-3a protein that undergoes S-palmitoyltion in addition to O-palmitoleoylation in which the latter is required for its proper protein secretion (Takada et al., 2006). Unlike myristoylation, palmitoylation sites are difficult to predict and palmitoyl proteins cannot be identified by a common palmitoylation motif. In fact, palmitoylation can occur on any cysteine residue and often the motif consists of multiple cysteines displaying different affinities for palmitate. This is observed with many membrane proteins such as several AMPA receptor subunits and the NMDA receptor, whereby two palmitoylated sites differentially regulate cell surface expression and receptor trafficking (Hayashi et al., 2005; Hayashi et al., 2009).

1.3.4 Approaches used to study protein palmitoylation

Partly due to the lack of reliable detection technologies, identification of palmitoyl proteins remains a challenging task. Site-directed mutagenesis is commonly used to identify and characterize palmitoylation sites whereby cysteine(s) of interest are substituted with either serine or alanine residues. This approach is useful however it is possible that the observed phenotype is caused by the replacement of cysteine residues in question rather than loss of palmitoylation (Dunphy and Linder, 1998).

The most common assay for protein palmitoylation is metabolic labeling with radiolabeled palmitate in which labeled palmitate, usually ^{3}H-palmitate, is added to the culture medium and the cells metabolically incorporate the labeled palmitate into palmtoylation motifs. Next, the palmitoylated proteins are purified and identified through SDS-PAGE. This technique identifies all types of palmitoylation however some limitations include: (1) the ability to only label live cells; (2) the use of tritiated palmitate that is more expensive and may not be accessible as compared to the use of a non-radioactive compound; (3) To detect a palmitoylation signal, high concentrations of substrate and/or long autoradiographic exposures are required.

An alternative method, developed by Drisdel and Green called Acyl-Biotinyl Exchange (ABE) chemistry (also called Biotin-BMCC labeling) (Drisdel and Green, 2004), has been used that only identifies S-palmitoylated proteins (Scheme 1.3.1). The first step is to block all pre-

existing, free sulfhydryl groups with N-ethylmaleimide (NEM). This is followed by cleavage of the thioester bond that covalently links the fatty acyl group to the cysteine residue with hydroxylamine (HAM) treatment. Finally, the cysteine residue with the free sulhydryl group is labeled with a thiol-specific reagent (such as a non-radioactive sulhydryl-specific compound containing biotin). The latter can be affinity-purified using streptavidin-agarose and identified through SDS-PAGE or proteomic mass spectrometry. Some advantages of ABE over metabolic labeling with tritiated palmitate include: (1) the ability to label non-living tissues; (2) its compatibility with mass spectrometry (Wan et al., 2007); and (3) a more sensitive and quantitative approach. Details of ABE chemistry are discussed in (Drisdel et al., 2006).

1.3.5 S-palmitoylation in neurons

A large number of palmitoylated proteins have been identified, mostly through large-scale profiling and proteomics analysis in both yeast (Roth et al., 2006a; Roth et al., 2006b) and mammalian cells (Martin and Cravatt, 2009). Using ABE proteomic technology, one recent study describes rat neural palmitoyl-proteomes (Kang et al., 2008). With this approach, the authors confirmed 68 known neuronal palmitoyl proteins and identified more than 200 new palmitoylated proteins in which palmitoylation of 21 candidate proteins were confirmed using other methods (Kang et al., 2008). This study is just one example that illustrates the abundance of palmitoyl proteins spread between multiple protein families; some of which include adhesion molecules, transporters, scaffolding proteins, neurotransmitter receptors and other vesicular trafficking proteins. Proteins known to be reversibly palmitoylated include members of the Src family of protein tyrosine kinases, Ras isoforms, G protein subunits and GPCRs, rhodopsin, and other neuronal proteins (PSD-95, SNAP-25, GAP-43) (Huang and El-Husseini, 2005; Resh, 2004). In fact, rhodopsin, the light-sensitive pigment in the rod cells of the eye, was one of the first neuronal palmitoyl protein identified (O'Brien and Zatz, 1984; Papac et al., 1992).

Palmitoyl proteins are not homogeneously distributed on the plasma membrane but rather concentrated either on the Golgi or in plasma membrane subdomains enriched in cholesterol and sphingomyelin (McCabe and Berthiaume, 2001). Termed lipid-raft domains, these specialized intracellular and plasma membrane domains are insoluble in non-ionic detergents and vital for neuronal development and function (Simons and Ikonen, 1997). Not exclusive to neurons, palmitoylation is an important mechanism for protein sorting (discussed in section 1.3.6) and one manner in which palmitoylation accelerates neurotransmitter response is by inducing ion channel clustering, likely within lipid-raft domains. Thus in the latter example, palmitoylation represents

a mechanism for neurons to regulate transmission and synaptic plasticity at postsynaptic sites. Finally, palmitoylation can modulate cell development and signaling by dynamically changing protein palmitoylation levels according to specific physiological stimuli (reviewed in (el-Husseini Ael and Bredt, 2002)). From the overwhelming list of palmitoyl proteins, it is unmistakable that palmitoylation plays a critical role in neuronal protein trafficking and function. In fact, most functions attributed to palmitoylation such as protein sorting and clustering in specialized membrane domains, are not restricted to neurons but commonly seen in many cell types.

1.3.6 Physiological significance of protein S-palmitoylation

S-palmitoylation mediates multiple regulatory functions of both soluble and membrane proteins (Charollais and Van Der Goot, 2009) and it is now becoming apparent that palmitoyl proteins are located in all cellular compartments. In fact, the subcellular site of palmitoylation seems to be unique to each substrate, ranging from the Golgi, ER and plasma membrane (Nadolski and Linder, 2007).

Many palmitoyl proteins are hydrophilic and in this case, S-palmitoylation likely acts as a membrane tether, targeting substrates to the plasma membrane and assuring proper membrane-localized function. In some cases, palmitoylation must be accompanied by another fatty acylation. This is seen with several Src family kinases and Gα subunits whereby myristoylation is followed by palmitoylation, encoded by the Met-Gly-Cys motif (Alland et al., 1994; Galbiati et al., 1999; van't Hof and Resh, 1997). The addition of multiple fatty acids to a protein enhances hydrophobicity, leading to rapid and more efficient binding to the membrane. This also applies to dually palmitoylated proteins (Shahinian and Silvius, 1995) whereby mutation of one palmitoylated cysteine can significantly reduce membrane binding and redirect the protein to another intracellular compartment (Wolven et al., 1997).

Membrane targeting can rapidly and reversibly be modulated by changes in protein palmitoylation states that are, in turn, closely coupled to signaling events. Plasma membrane targeting of Gαs is mediated by changes in its palmitoylation that seems to depend upon β-adrenergic receptor stimulation. Agonist stimulation leads to Gαs depalmitoylation and translocation from the plasma membrane to the cytosol. Once Gαs undergoes GTP hydrolysis, the protein changes conformation, reassociates with Gβγ and tethers to the plasma membrane via palmitoylation (Iiri et al., 1996; Wedegaertner and Bourne, 1994; Wedegaertner et al., 1996). The role of Gαs palmitoylation not only demonstrates the dynamic nature and signaling potential

of this fatty acyl modification but also its function as a scaffold, reinforcing protein-protein interactions while contributing to membrane targeting.

Palmitoylation affects many membrane proteins and this is exemplified by the abundant palmitoylation of GPCRs (Qanbar and Bouvier, 2003). Palmitoylation of membrane proteins takes place at many subcellular sites and in fact, compared to other types of fatty acylation, integral membrane proteins are emerging as substantial substrates. In some cases, palmitoylation acts as a targeting signal and enhances protein-protein interactions, being one of many factors that determines protein compartmentalization; palmitoyl proteins are targeted to specialized membrane microdomains such as lipid rafts and caveolae (discussed in section 1.3.5) (Lei et al., 2005; Prior et al., 2001; Webb et al., 2000). Thus, palmitoylation can act as a signal for protein trafficking between organelles and for protein sorting or clustering within membrane compartments (Greaves and Chamberlain, 2007). This is seen with the human δ opioid receptor that depends on palmitoylation for its efficient delivery to the plasma membrane (Petaja-Repo et al., 2006).

Due to its reversible nature, palmitoylation can regulate signaling efficiency. This is demonstrated with the adrenergic receptor that undergoes multiple palmitoylation/depalmitoylation cycles. Upon prolonged agonist stimulation, the β_2-adrenergic receptor undergoes depalmitoylation that is closely coupled to its phosphorylation by PKA, resulting in receptor desensitization (Loisel et al., 1996; Moffett et al., 2001). On the contrary, agonist-promoted depalmitoylation of the α_{2A}-adrenergic receptor has no effect on its desensitization but instead inhibits its down-regulation (Eason et al., 1994). Here, we show how the function of palmitoylation/depalmitoylation is unpredictable and appears to be receptor subtype-specific. Thus, structural changes implemented by palmitoylation can significantly change protein localization as well as its function within signaling pathway(s). In the case of GPCRs, palmitoylation alters receptor conformation, modifying G protein coupling, desensitization and trafficking (reviewed in (Qanbar and Bouvier, 2003)).

Palmitoylation has been shown to regulate protein turnover and stability. This is documented with several GPCRs such as the A1 adenosine receptor whereby receptor depalmitoylation promotes rapid degradation (Gao et al., 1999), and the chemokine receptor CCR5, whereby the absence of palmitoylation results in reduced plasma membrane expression and rapid proteolytic degradation (Percherancier et al., 2001). Even in the case of soluble proteins, palmitoylation accompanied by isoprenylation, are both required for RhoB degradation through the lysosomal pathway (Perez-Sala et al., 2009). There is additional evidence that

palmitoylation affects protein stability by protecting receptors from ubiquitylation. Palmitoylation of anthrax toxin receptor isoforms TEM8/CMG2 prevents premature internalization and targeting to lysosomes by segregating the isoforms away from their E3 ubiquitin ligases that in turn control receptor endocytosis and turnover (Abrami et al., 2006). In yeast, the endosomal SNARE Tlg1 is shown to be palmitoylated by Swf1, a member of the *S. cerevisiae* DHHC-CRD family (discussed in section 1.3.7); in the absence of palmitoylation, Tlg1 is recognized by the ubiquitin ligase Tul1 where it undergoes ubiquitylation, is targeted to multivesicular bodies and destined for degradation in the vacuolar compartment (Valdez-Taubas and Pelham, 2005). The mechanism responsible for increased protein ubiquitylation followed by degradation in the absence of palmitoylation is unclear; but in the case of Tlg1, the authors speculate that a conformational change occurs in the non-palmitoylated protein where lysines adjacent to the palmitoylation site become more accessible to E3 ubiquitin ligases (Valdez-Taubas and Pelham, 2005).

The idea that palmitoylation significantly alters protein conformation is not unusual and likely a common mechanism. The lipoprotein receptor-related protein LRP6, involved in canonical Wtn signaling, is palmitoylated in the ER and this posttranslational modification is thought to induce a tilt within its transmembrane domain that is required for ER exit and plasma membrane targeting. Otherwise, the palmitoylation-deficient LRP6 is retained in the ER via a monubiquitylation-dependent mechanism (Abrami et al., 2008). The relationship between palmitoylation and ubiquitylation is complex and it has been suggested, not only for LRP6, that palmitoylation of certain membrane proteins may even contribute to ER Quality Control. The yeast chitin synthase Chs3, a polytopic transmembrane protein, is palmitoylated by the palmitoyl acyl transferase Pfa4, whereby Chs3 palmitoylation seems to be vital for its ER export and in preventing accumulation of high-molecular mass aggregates in the ER (Lam et al., 2006). In summary, palmitoylation can be a factor in mediating protein ubiquitylation and degradation, however its functional consequence is often specific to the target protein and as of yet, there is no general mechanism describing the effect of palmitoylation in the protein degradation pathway.

1.3.7 Palmitoyl acyl transferases, DHHC enzymes

Another difficulty in studying palmitoylation is the occurrence of nonenzymatic protein palmitoylation that can take place on the sulfhydryl group of cysteines in the absence of any enzymes. Autoacylation has been observed with several Gα subunits (Duncan and Gilman, 1996). However, it is unlikely to be the general mechanism since nonenzymatic palmitoylation is too slow and cannot account for rapid palmitoylation of signaling proteins, as well as fails to support acyl chain selectivity.

The first two palmitoyl acyl transferases (PATs) were discovered in yeast *Saccharomyces cerevisiae* (Akr1; (Roth et al., 2002) and Erf2-Shr5/Erf4; (Lobo et al., 2002)). PATs share several transmembrane domains but most importantly, a 50 residue-long zinc finger-like sequence that eventually led to the identification of 23 isoforms in the human genome (Iwanaga et al., 2009). This motif consists of a highly conserved cysteine-rich domain (CRD) with a core Asp-His-His-Cys (DHHC) motif that has been shown to mediate its enzymatic activity; for this reason, PATs are often referred to as DHHC enzymes (Fukata et al., 2004; Keller et al., 2004; Roth et al., 2002; Smotrys and Linder, 2004). In yeast, proteomic analysis identified the DHHC family as the primary PATs responsible for protein palmitoylation (Roth et al., 2006a). As expected, the human isoforms have the ability to mediate addition of palmitoyl CoA to proteins and are described as enzymes responsible for protein S-palmitoylation (Fukata et al., 2006a; Iwanaga et al., 2009). Despite the fact that palmitoyl CoA is the preferred substrate, PATs also exhibit an affinity for myristoylated proteins and often, palmitoyl proteins are accompanied by other fatty acid linkages; these may include stearate, oleate and arachidonate (Casey, 1995). Occasionally, palmitoylation can even be replaced by other membrane targeting signals, one being myristoylation (Jones and Gutkind, 1998).

DHHC enzymes seem to be present in all tissue types and based on exogenous expression, these proteins localize to the ER, Golgi, endosomal vesicles and a few to the plasma membrane (Fukata et al., 2004; Ohno et al., 2006). Substrates have been identified using proteomic analysis in yeast (Roth et al., 2006a), however the degree of substrate specificity and PAT selectivity still remains unclear. Despite the fact that DHHC enzymes often have more than one target protein, they exhibit distinct substrate specificity (Fukata et al., 2006b; Huang et al., 2009). Screening analysis of 23 mammalian DHHC enzymes demonstrated that a number of substrates could be differentially palmitoylated by specific DHHC enzymes (Fang et al., 2006; Fukata et al., 2006a; Fukata et al., 2006b; Huang et al., 2009). These data demonstrate both the specificity and redundancy amongst DHHC enzymes that may have functional relevance if one

considers the differential expression profile of DHHC enzymes within various tissue types (Ohno et al., 2006).

Since palmitoyltion is a dynamic process, it is not surprising that protein depalmitoylation also requires enzymes. To date, only two acylprotein thioesterases have been identified. Acylprotein thioesterase-1 (APT1) was originally characterized as a lysophospholipase, despite its preference for palmitoylated proteins, and is able to remove palmitate from proteins found on the cytosolic side of membranes. APT1 has been described in the deplamitoylation of Gα subunits and Ras proteins (Duncan and Gilman, 1998). The other is protein palmitoylthioesterase-1 (PPT1) that is localized to lysosomes and has been shown to remove palmitate from substrates during protein degradation (Camp et al., 1994; Verkruyse and Hofmann, 1996).

Palmitoylation represents a ubiquitous posttranslational mechanism that dynamically modifies protein function. Similar to phosphorylation, both DHHC enzymes and acylprotein thioesterases convey the tightly regulated process of protein palmitoylation that is highly dependent upon the cellular environment.

1.3.8 Palmitoylation and human disease

Many proteins that rely on palmitoylation for localized function are important players in cellular signaling, cancer and synaptic transmission (discussed in sections 1.3.5 and 1.3.6). Disruption of this posttranslational modification can lead to development of disease as documented with palmitoylation of the oncogenic NRAS and leukemogenesis (Cuiffo and Ren, 2010). A number of DHHC enzymes have also been linked to cancer development and progression. Up-regulation of DHHC enzymes is detected in several human cancer cell types, and dysfunction in both PAT activity and protein palmitoylation has been associated with neurological diseases: (1) Alzheimer's disease (Meckler et al., 2010; Sidera et al., 2005); (2) Schizophrenia and mutations in the ZDHHC8 gene (Mukai et al., 2004); (3) Several forms of X-linked mental retardation, one of which is associated with mutations in the ZDHHC9 gene (Raymond et al., 2007; Ropers, 2006); (4) Mice with mutations in the ZDHHC13 gene develop severe disease pathology: cachexia, alopecia, osteoporosis, systematic amyloidosis and yield early death (Saleem et al., 2010).

Huntington disease (HD), a complex neurodegenerative disorder, involves a mutant form of the huntingtin (htt) protein that consists of an elongated polyglutamine tract and is subject to a different posttranslational profile, resulting in phenotypic features of HD (reviewed in detail by

(Ehrnhoefer et al., 2011)). Wild-type htt protein is palmitoylated at C214 by two neuronal DHHC enzymes, huntingtin interacting protein 14 (HIP14/ ZDHHC17) and HIP14L; unlike the mutant htt protein that is significantly less palmitoylated and shows reduced interaction with HIP14/DHHC17 (Singaraja et al., 2002). Further, mutations of the palmitoylation motif within wild-type htt protein lead to neuronal toxicity (Yanai et al., 2006). Thus, these data suggest that reduced palmitoylation of the mutant htt protein is one mechanism responsible for axonal trafficking defects and formation of insoluble aggregates, both features of HD (Huang et al., 2004). The importance of HIP14/DHHC17 is further highlighted in the Hip14-deficient mouse model that shares some features of HD and reinforces the close relationship between palmitoyltion of htt protein and HIP14/DHHC17 in the pathogenesis of HD (Huang et al., 2011; Singaraja et al., 2011). In addition to htt protein, HIP14/DHHC17 targets other synaptic proteins for palmitoylation and these include SNAP-25, GAD-65, PSD-95 and synaptotagmin 1 (Huang et al., 2004). Other studies have been conducted on HIP14/DHHC17 activity and demonstrate its ability to promote cellular transformation (Ducker et al., 2004).

In summary, PATs are now considered potential targets for new anti-cancer drugs and using cell-based screens, one study identified several PAT inhibitors that showed anti-proliferative activity and inhibited tumor growth *in vivo* (Ducker et al., 2006).

1.4 G proteins within the G protein-coupled receptor (GPCR) signaling pathway

GPCRs have a common membrane topology that consists of an amino-terminal extracellular domain, seven hydrophobic transmembrane domains, and a carboxyl-terminal intracellular domain (Kristiansen, 2004; Schioth and Fredriksson, 2005). GPCRs represent the largest class and the most diverse type of cell surface receptors (Kristiansen, 2004). In fact, GPCRs can be classified into five main families: Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2 and Secretin (also referred to as the GRAFS classification system)(Schioth and Fredriksson, 2005). GPCRs translate changes occurring within the extracellular environment into an intracellular response via heterotrimeric guanine-nucleotide binding proteins (G proteins) (Downes and Gautam, 1999; Downes et al., 1999; Neer, 1995) (Scheme 1.4.1). The signal transduction pathway is initiated by binding of a specific ligand to the receptor, followed by a conformational change of the receptor, the exchange of GDP for GTP on the Gα subunit, and a conformational change within (and possible dissociation of) the heterotrimeric G protein (Chidiac, 1998; Kjeldgaard et al., 1996). Both the GTP-bound Gα subunit and the Gβγ complex promote downstream signaling by stimulating a number of effector molecules, thereby activating

or inhibiting the production of various second messengers. Thus, G proteins regulate important cellular components such as metabolic enzymes, ion channels and the transcriptional machinery (Neves et al., 2002).

Gα proteins are segregated into four families based on sequence homology of the Gα subunit and on their effector interaction: (1) Gαs (stimulates adenylyl cyclase); (2) Gαi (inhibits adenylyl cyclase); (3) Gαq (stimulates phospholipase Cβ); (4) Gα12 (activates small GTPases i.e. Rho) (Downes and Gautam, 1999). The diversity within G protein subunits and the subtle variations in expression between tissues may be important in partly determining GPCR signal specificity (Downes and Gautam, 1999). Several GPCRs can elicit multiple signals and activate diverse subcellular pathways by adopting ligand-specific receptor active states. This can be described with 'the allosteric ternary complex model' (Samama et al., 1993), stating that receptors exist in two interconvertible allosteric states, R(inactive) and R*(active), whereby R* interacts with G proteins. The binding of an agonist shifts the equilibrium from R to R* and results in the formation of a high-affinity agonist-receptor (R*)-G protein ternary complex. In the case of antagonist binding, we can observe either no change in receptor constitutive activity as seen with neutral antagonist (equal high affinities for R and R* states) or a decrease in receptor constitutive activity as seen with inverse antagonist (higher affinity for R state). Thus, receptors can be differentially affected by both ligands and G proteins, depending on the shift within the R and R* equilibrium. For example, GPCRs may fluctuate between two inactive conformational states and bind to two different G protein subunits (Harding and Gong, 2004; Kenakin, 2001). For example, β_2-adrenergic receptor (AR) can activate both Gαs and Gαi signaling pathways in the heart. It was also observed that posttranslational modifications are one important factor in this process whereby PKA-mediated phosphorylation of β_2-AR switches the coupling preference of the receptor from Gαs to Gαi (Daaka et al., 1997; Xiao, 2001). The critical role that G proteins play in mediating cellular responses is evident, suggesting that disruption in G protein expression or function may result in significant clinical implications, such as endocrine diseases and oncogenic transformation (Levine, 1999; Radhika and Dhanasekaran, 2001).

Most of our discussion will focus on Gα subunits however, it is important to acknowledge the important contribution of the Gβγ complex in the signal transduction pathway. The Gβγ subunit is not limited to its interaction with GPCRs and Gα proteins, in fact it is able to modulate downstream responses by directly binding to a number of effectors. Examples of Gβγ effectors include inwardly rectifying potassium channels (GIRK) (Nakajima et al., 1996), N-type calcium channels (Ikeda, 1996), adenylyl cyclase isoforms (Sunahara and Taussig, 2002),

phospholipase C β2 and β3 isoforms (Camps et al., 1992; Park et al., 1993; Smrcka and Sternweis, 1993), G protein-coupled receptor kinase 2 (GRK2) (Pitcher et al., 1992) and phosphoinositide 3-kinase γ (PI3Kγ) (Stephens et al., 1994). Recently, other Gβγ binding partners have been identified and these include PDZ domain containing proteins (Li et al., 2006b) and guanine exchange factors (GEFs) for small G proteins (Mayeenuddin et al., 2006).

In the next section, we introduce evidence that the E3 ubiquitin ligase gp78/AMFR can be described as a putative GPCR in the ER (gp78/AMFR is discussed in section 1.2).

1.4.1 G-protein regulation of gp78/AMFR activity*

Most of the data suggesting that gp78/AMFR is associated with G proteins are derived from the use of the Bordella pertussis toxin, responsible for the inactivation of specific G proteins belonging to the Gαi and Gαo protein family (namely, Gαi1-3 and Gαo, respectively) (Kohn et al., 1990; Milligan et al., 1990; Nabi et al., 1990). Pre-treatment with pertussis toxin is able to inhibit AMF/PGI-promoted cell motility and IP3 production in A2058 human melanoma cells (Kohn et al., 1990), as well as motility induced by the anti-gp78/AMFR 3F3A mAB in B16-F1 cells (Nabi et al., 1990). Furthermore, AMF/PGI purified from HT-1080 human fibrosarcoma or B16-F1 murine melanoma cells is able to stimulate proliferation and locomotion of BALB/c 3T3-A31 murine fibroblasts via a paracrine motility response inhibited by pertussis toxin. In the same study, pertussis toxin repressed DNA synthesis and wound healing of 3T3-A31 murine fibroblasts following AMF/PGI stimulation (Silletti and Raz, 1993). Gp78/AMFR undergoes rapid phosphorylation at one or more serine (and/or tyrosine) residues following AMF/PGI stimulation, a process that can be significantly reduced by pertussis toxin preincubation (Silletti et al., 1998; Watanabe et al., 1991a). These data and others (Watanabe et al., 1991b) suggest the involvement of a pertussis-toxin sensitive G protein (the Gαi and/or Gαo subunits) in the signal transduction pathway downstream of AMF/PGI-stimulated cell motility via its receptor gp78/AMFR.

The topology of gp78/AMFR has not been clearly defined and is an area of controversy. It was speculated that gp78/AMFR only contains five transmembrane domains (Ponting, 2000), however based on hydrophobic analysis of the amino acid sequence, gp78/AMFR can also resemble a 6 or 7 transmembrane domain protein (Scheme 1.2.2B). Despite the fact that

* A version of section 1.4.1 was published in Fairbank, M., St-Pierre, P., and Nabi, I.R. (2009). The complex biology of autocrine motility factor/phosphoglucose isomerase (AMF/PGI) and its receptor, the gp78/AMFR E3 ubiquitin ligase. Mol Biosyst *5*, 793-801.

gp78/AMFR is not homologous to any other GPCR, the receptor shows significant structural similarity to a hypothetical GPCR in Caenorhabditis elegans, called F26E4.11 (Shimizu et al., 1999).

Receptor dimerization and the formation of oligomeric complexes is thought to be a requirement for GPCR signaling, ER trafficking and retention (Park et al., 2004; Salahpour et al., 2004). A putative leucine zipper at amino acids 262–284 of mouse gp78/AMFR, identified by sequence homology analysis (Shimizu et al., 1999), could potentially function as a protein dimerization domain (Alber, 1992). This raises the possibility that gp78/AMFR may exist as a homodimer, as for other GPCRs (Bulenger et al., 2005; Franco et al., 2007). Further study into the topology of gp78/AMFR is required to determine its structure and orientation within the plasma membrane as well as the basis for regulation of its activation by a pertussis-toxin sensitive G protein. Indeed, the contribution of G proteins to the localization and function of gp78/AMFR and whether gp78/AMFR directly binds to G proteins still remains to be determined.

1.4.2 Cell surface Gαs-cAMP-PKA signaling pathway

The Gαs class of G protein subunits comprises two genes (*Gnas* and *Gnal* genes) that encode either the ubiquitously expressed Gαs (45 kDa) or the guanine nucleotide binding protein, α stimulating, olfactory type, Gαolf (primarily localized to the olfactory epithelium and a few regions of the brain), respectively (Plagge et al., 2008). Moreover, an ‘extra large’ isoform of the Gαs protein (XLGαs, 52 kDa), first identified in PC12 cells (Kehlenbach et al., 1994), originates from the same *Gnas* gene but is transcribed from an alternative promoter/first exon, resulting in a much longer N-terminal domain (reviewed in (Plagge et al., 2008)).

Following ligand-GPCR binding at the cell surface, the Gαs subunit activates the large transmembrane effector adenylyl cyclase (encoded by nine different mammalian genes, termed type I-IX) on the cytosolic face of the plasma membrane and stimulates the production of the ubiquitous second messenger cyclic adenosine monophosphate (cAMP) (Scheme 1.4.1). The physiological role of cAMP has been extensively studied and the foremost, but not the only, cAMP effector is the cAMP-dependent protein kinase A (PKA) (Taylor et al., 1992). Other proteins directly activated by cAMP include Epac (de Rooij et al., 1998) and cAMP-regulated ion channels (Kaupp and Seifert, 2002). The PKA holoenzyme is a heterotetramer composed of two regulatory and two catalytic subunits. The mechanism by which cAMP amplifies the cell surface signal is through binding to the regulatory subunit of PKA that leads to the dissociation

and release of its active catalytic subunit, responsible for the phosphorylation of many target proteins on serine and threonine residues. Some proteins that undergo PKA-dependent phosphorylation include the transcription factor CREB (Shaywitz and Greenberg, 1999) and protein phosphatase 2A (Usui et al., 1998) (Scheme 1.4.1).

Thus, it is not surprising that cAMP-PKA signaling is vital in multiple physiological processes, some of which include cellular differentiation, metabolism, growth, development, synaptic transmission and ion channel function (McKnight et al., 1998; Montminy, 1997; Taylor et al., 1992). In Eukaryotic cells, there are several PKA holoenzyme isoforms that acquire distinctive expression and distribution patterns. In fact, studies have shown that the regulatory subunits are a critical factor in defining PKA protein structure and biological functions (Taylor et al., 1992). As a result, different properties associated with PKA isoforms may account for, at least in part, the large number of biological responses associated with extracellular signals and adenylyl cyclase activation.

1.4.3 cAMP signaling associated with mitochondria

Mitochondria, the site of energy production, is closely coupled to metabolic demands and enables the cell to adapt oxidative respiration to the extracellular environment via signaling events generated at the cell surface. In Eukaryotic cells, PKA is concentrated on membranes and organelles, such as the mitochondria, through its interaction with a complex called A-Kinase-Anchor-Proteins (AKAPs) (Jarnaess and Tasken, 2007). AKAPs not only show differential binding affinities for PKA isoforms but also act as multiprotein scaffolds able to simultaneously bind and anchor signal transduction proteins (Michel and Scott, 2002). This leads to the compartmentalization of PKA, as well as other receptors and cyclases, that together enhance the specificity and efficiency of PKA-mediated events by (1) targeting PKA to specific substrates and (2) promoting cross-talk between different signaling pathways (Tasken and Aandahl, 2004). Likewise, another mechanism used to propagate specific PKA signaling events via PKA-AKAP complexes, involves the maintenance of cAMP microdomains and gradients; this is primarily achieved through the regulation of cAMP inactivation by phosphodiesterases (PDEs) (reviewed in (Jarnaess and Tasken, 2007)).

In the case of the mitochondria, spatial and temporal regulation of cAMP signals by the AKAP complex is critical in achieving highly co-ordinated pathways and establishing tightly controlled mitochondrial responses to hormone stimulation (Feliciello et al., 2005). Thus, AKAPs play a fundamental role in mammalian mitochondrial physiology that includes the

regulation of mitochondrial dynamics and signaling to the ubiquitin-proteasome pathway (Carlucci et al., 2008). The relationship between cAMP, AKAP and the proteasome pathway may be an important regulatory mechanism of mitochondrial dynamics and oxidative metabolism. Briefly, Mitol, a mitochondrial E3 ubiquitin ligase that localizes to the outer mitochondrial membrane, promotes ubiquitin-dependent degradation of two proteins via the proteasome: (1) dynamin-related protein 1 (Drp1; mitochondrial fission) and (2) mitofusin 1 (Mfn1; mitochondrial fusion). In the current model, ligand-mediated stimulation of a GPCR on the plasma membrane activates and increases the production of cAMP, which in turn binds to and activates PKA that enables phosphorylation and inactivation of Drp1, resulting in mitochondria fusion and cell survival (described in (Cribbs and Strack, 2007).

Gαs has been shown to modulate the apoptotic response of several cancer cells (Burchett, 2000) and some studies insist on a protective role of Gαs in cell death, in which cell viability is prolonged via the cAMP/PKA signaling pathway (Cho et al., 2011; Martin et al., 2005). In this example, locally activated PKA can phosphorylate the mitochondrial substrate, the proapoptotic BAD protein (a BH3-proapoptotic Bcl-2 family member) that is a key component in the mitochondrial apoptotic pathway. Phosphorylation of BAD by PKA inhibits apoptosis by blocking the association of BAD to the anti-apoptotic Bcl-2 homologues and results in cell survival (Harada et al., 1999). However, the effect of Gαs in apoptosis seems to be inconsistent and highly dependent upon the receptor type, the cell line used and the experimental model (Hsiung et al., 2008; Insel et al., 2011). A recent study demonstrated that by impairing Gβγ binding, Gαz is 'mislocalized' to the mitochondria (Fishburn et al., 2000). These findings confirm that Gβγ binding is necessary to specifically target Gαz to the plasma membrane, as well as introduce the possibility that G proteins may also be localized to the mitochondria under certain conditions, able to directly associate with mitochondrial proteins and participate in local signaling events.

1.4.4 Gαs signaling in other intracellular compartments

Some G protein subunits have been observed to rapidly shuttle between the plasma membrane and the cytosol, and in some cases, their cytoplasmic localization seems to increase upon G protein activation. GPCR activation following isoproterenol or cholera toxin treatments results in the translocation of Gαs from the plasma membrane to the cytosol (Levis and Bourne, 1992; Wedegaertner and Bourne, 1994; Wedegaertner et al., 1996; Yu and Rasenick, 2002). However, the mechanism underlying Gαs release from the plasma membrane remains unclear.

Factors such as Gαs palmitoylation (Tsutsumi et al., 2009; Wedegaertner and Bourne, 1994) and binding of Gβγ (Chisari et al., 2007; Dupre et al., 2009; Marrari et al., 2007) both seem to be vital for the cytosolic distribution of Gαs (Iiri et al., 1996). Further, the notion that Gαs translocates from the plasma membrane independently from its receptor has been previously documented with β_2AR (Allen et al., 2005), and the possibility that Gαs interacts with other effectors at different cellular sites has also been proposed.

Not limited to the cell surface, heterotrimeric G proteins have been found on membranes of intracellular compartments and recently, it is becoming apparent that Gαs is also involved in functions that are not associated with the adenylyl cyclase/cAMP signaling pathway (Donati et al., 2001; Miura et al., 2001; Rybin et al., 2000). Gαs directly interacts with tubulin and the microtubule cytoskeleton ((Sarma et al., 2003); reviewed in (Dave et al., 2009)). Despite the fact that other G protein subunits are also capable of binding tubulin, Gαs is the only Gα subunit that is known to internalize following receptor activation via caveolae/lipid raft-derived vesicles, where it interacts with the plus end of microtubules (rich in tubulin-GTP), most likely within lipid raft microdomains. The consequence of tubulin-GTP hydrolysis by Gαs is an increase in microtubule dynamics and a decrease in microtubule stability (Allen et al., 2005). Thus, G protein regulation of microtubule stability is becoming an important mechanism in neuronal differentiation, process outgrowth and plasticity (reviewed in (Dave et al., 2009)).

The involvement of Gαs in membrane trafficking along endocytic and secretory pathways has been previously examined (Bomsel and Mostov, 1992; Helms, 1995; Nurnberg and Ahnert-Hilger, 1996; Stow and Heimann, 1998). Studies began with the observation that GTPγS (a non-hydrolyzable analogue of GTP) and AlF_4^- (complex of aluminum and fluoride that mimics the γ phosphate of GTP by binding to the GDP-bound form of G proteins) both inhibit various stages of the endocytic and secretory pathways (Mayorga et al., 1989; Melancon et al., 1987). Recently, it is becoming apparent that whether G proteins stimulate or inhibit vesicular transport, this process is highly specific to the G protein subtype within the specific cell model system. In epithelial cells, Gαs is shown to specifically mediate apical protein transport whereby inhibition of Gαs prevents transport of haemagglutinin from the *trans*-Golgi network to the apical cell surface, but has no effect on transport between the endoplasmic reticulum and the Golgi (Pimplikar and Simons, 1993). Further, in the rat exocrine pancreas that is specialized in protein secretion, both Gαi3 and Gαq/11 are widely distributed across multiple Golgi fractions, while Gαs is mostly restricted to the *trans*-Golgi network (affects vesicular budding) and Gβ is

only detected on the plasma membrane (Denker et al., 1996). These data not only illustrate that Gα subunits have distinctive distributions within Golgi membranes but that they are no longer bound to Gβ, suggesting that these free Gα subunits are likely active in the GTP-bound conformation and/or associated with unidentified protein(s) that substitute for the Gβγ subunit. Finally, Gαs has also been shown to participate in the process of fusion between endosomes in an *in vitro* system (Colombo et al., 1994). In fact, several G protein subunits (Gαs, Gαi3 and Gβ) as well as the effector adenylyl cyclase, have been detected in rat liver endosomes (Van Dyke, 2004). These data reinforce the concept that endosomes are not only cargo vessels, but can undergo signaling events as observed with activated insulin receptors (Balbis et al., 2000). Thus, these signal components (including G proteins) most likely traffic into hepatocytes on endosome membranes, generating a signaling cascade that is spatially distinct from the plasma membrane.

The Gαs subunit is linked to the trafficking and down-regulation of the epidermal growth factor (EGF) receptor (Zheng et al., 2004), promoting ligand-dependent degradation of EGF receptors. In addition to Gαs, this process also seems to require Gαs binding of its GTPase activating protein (GAP) RGS-Px1 and the hepatocyte growth-factor-regulated tyrosine kinase substrate (Hrs) (Zheng et al., 2004). The extent of Gαs in receptor degradation is unclear or whether Gαs is important for degradation of other receptors. However, it is possible that Gαs acts with Hrs and represents a general mechanism for degradation of several receptors. In fact, Hrs is responsible for the degradation of other GPCRs such as the chemokine receptor CXCR4 (Marchese et al., 2003) and the δ opioid receptor (Hislop 2004); in the latter studies, the role for Gαs is unknown.

Stable interactions between receptors and G proteins independent of receptor activation have been reported to exist in the ER. Using Bioluminescence Resonance Energy Transfer (BRET) in living cells and coimmunoprecipitation experiments, Rebois *at al.* demonstrated that stable complexes exist between G proteins and effectors; among several examples, the authors describe Gαs as being persistently associated with its effector adenylyl cyclase and unaffected by isoproterenol treatment (Rebois et al., 2006). Thus it is likely that the pre-assembled signaling complex between receptors, G proteins and effectors occurs prior to membrane targeting and shortly after biosynthesis within the ER (David et al., 2006; Dupre et al., 2007; Robitaille et al., 2009). Other studies imply that these stable G protein complexes formed in the ER may not only lead to G protein-receptor specificity on the cell surface but may provide rapid signaling at other sites such as the nucleus and the ER (Boivin et al., 2008; Wang et al., 2007). For example, GPR30, a GPCR that is exclusively targeted to the ER, is a functional receptor for estrogen

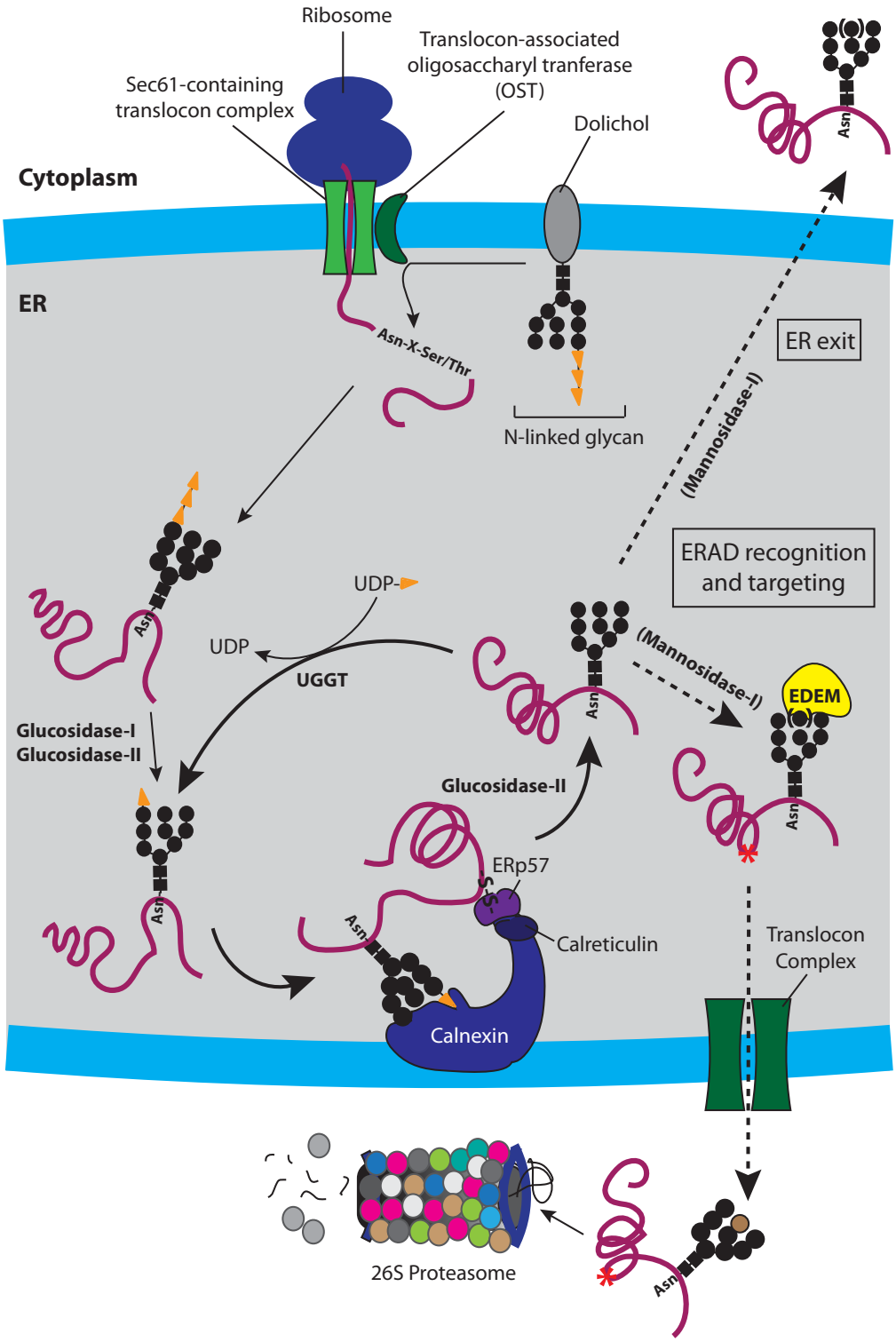
(Revankar et al., 2005). The notion that GPCRs and G proteins must directly traffic to and are functionally limited to the plasma membrane has recently been challenged. It is becoming more apparent that both GPCRs and G proteins may participate in signaling events within other intracellular compartments.

1.5 Objectives of the research manuscript

ER Quality Control mechanisms are a critical component in the cell and for this reason, in the following chapters, I would like to deepen our understanding by introducing new elements that I think play an important function within this pathway. I will begin by discussing RING finger S-palmitoylation of the E3 ubiquitin ligase gp78/AMFR and its physiological implication in the ERAD pathway. Next, I will show that gp78/AMFR is a putative GPCR and associates with several G proteins in the ER. Finally, I will focus on the Gαs subunit and outline its role in substrate polyubiquitylation and protection against the ER stress response.

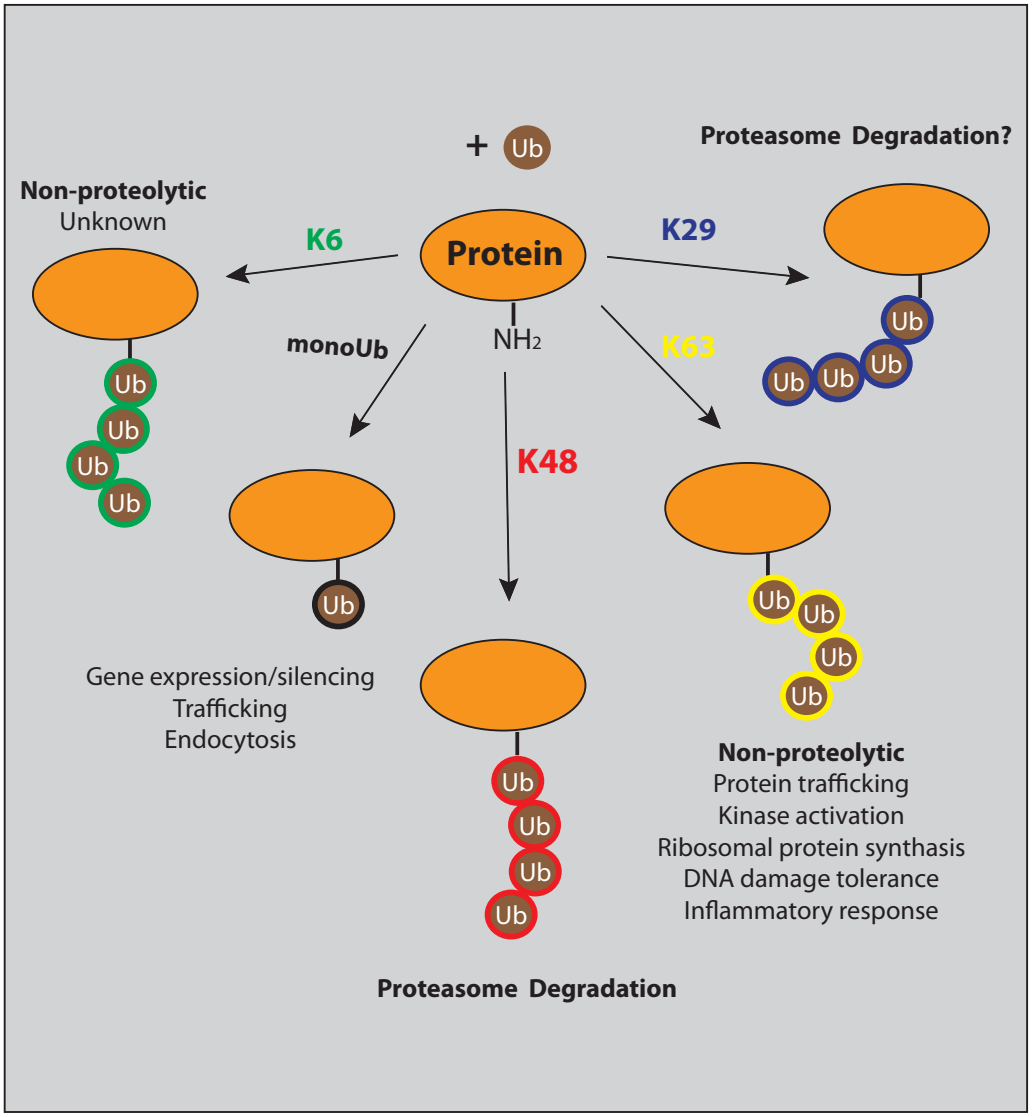
Scheme 1.1.1 The Calnexin-Calreticulin cycle

Many proteins in the ER undergo N-linked glycosylation whereby glycans are cotranslationally transferred from dolichol to the substrate via the OST complex. Enzymes, glucosidase-I and II remove two terminal glucose molecules and the remaining monoglucosylated glycoprotein is recognized by and interacts with lectin chaperones, calnexin and calreticulin that both assist in the folding of proteins with monoglucosylated N-linked glycans. A protein disulphide isomerase, ERp57, is found within this complex and catalyses disulphide bonds with the bound glycoprotein. The glycoprotein remains within the Calnexin-Calreticulin cycle until it achieves its native conformation and reentry into the folding cycle occurs by the reglucosylation of the glycoprotein by UDP-glucose:glycoprotein glucosyltransferase (UGGT). Finally, the glycoprotein is released from the ER following the removal of the last glucose (by glucosidase-II) and can also be demannosylated (by ER mannosidase I; mannose in parentheses). In the case of permanently misfolded glycoproteins, the substrate is targeted by ERAD components and undergoes proteasome-dependent degradation in the cytosol. EDEM: ER degradation-enhancing α-mannosidase-like lectins. Adopted from (Vembar and Brodsky, 2008) and (Ellgaard and Helenius, 2003).


Ribosome
Translocon-associated oligosaccharyl tranferase (OST)
Sec61-containing translocon complex
Dolichol
Cytoplasm
ER
Asn-X-Ser/Thr
N-linked glycan
ER exit
(Mannosidase-I)
ERAD recognition and targeting
UDP-
UDP
UGGT
Glucosidase-I
Glucosidase-II
(Mannosidase-I)
EDEM
Glucosidase-II
ERp57
Calreticulin
Calnexin
Translocon Complex
26S Proteasome
Asn

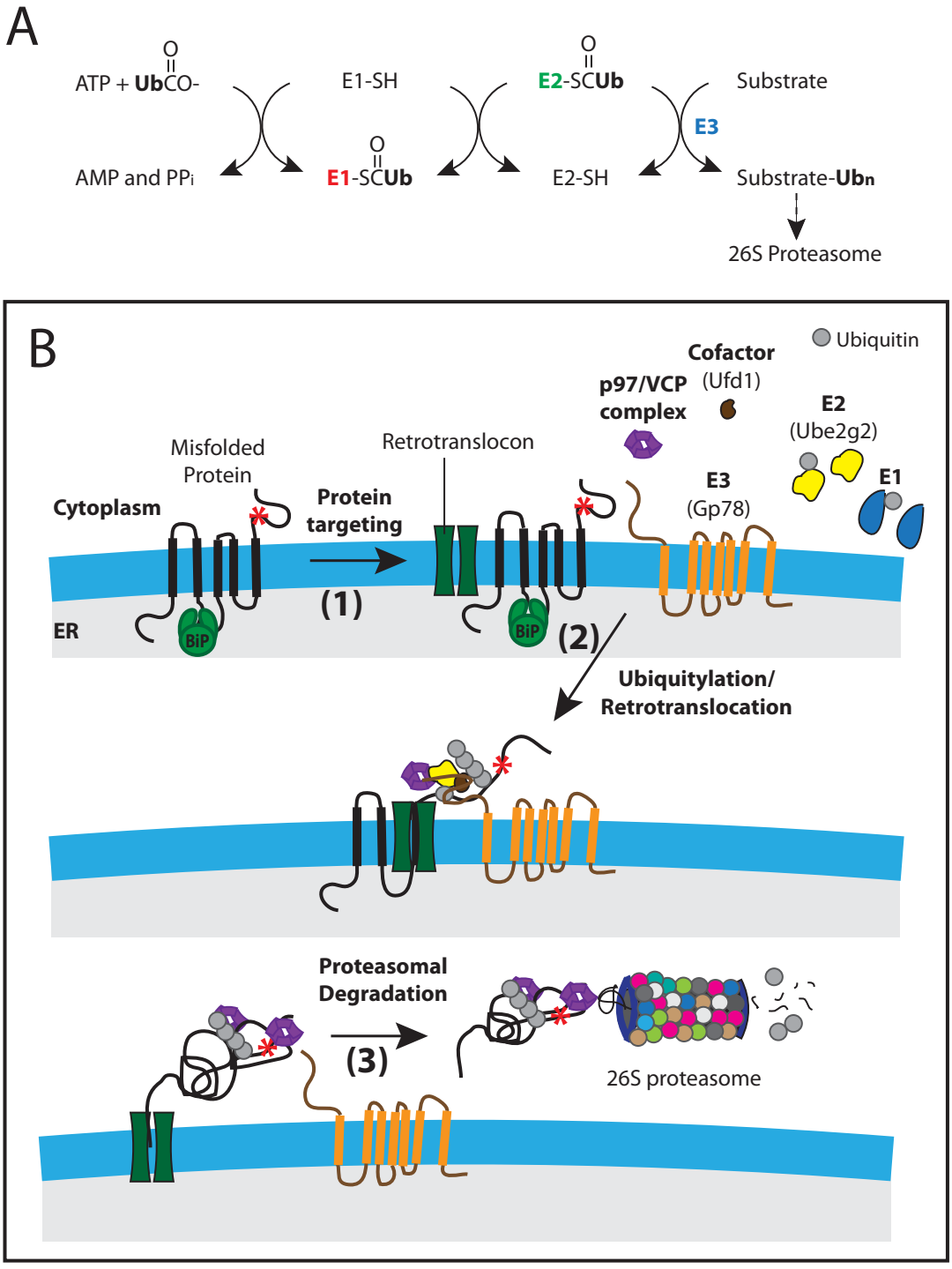
Scheme 1.1.2 Intracellular functions of protein ubiquitylation

Ubiquitin (Ub), a 76 amino-acid peptide, attaches to lysine residues in substrates and affects their fate within the cell. However, ubiquitin contains several lysine residues itself and the type of covalent linkage via lysines within the ubiquitin chain is an important determining factor of its function once bound to the substrate (covalent linkages are represented by color rings around ubiquitin). Adopted from (Pickart and Fushman, 2004).



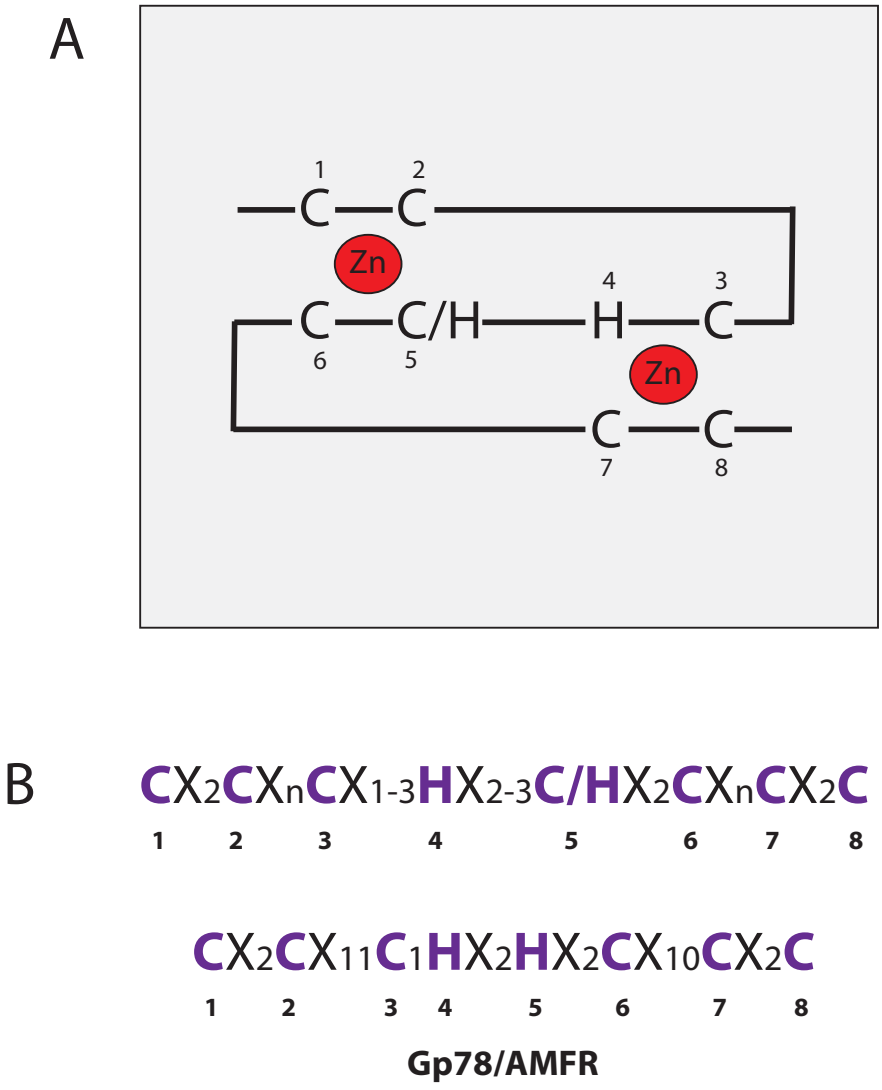
Scheme 1.1.3 Protein polyubiquitylation in the ERAD pathway

(A) The enzymatic pathway of ubiquitin conjugation to substrate via E1, E2 and E3 enzymes. Ubiquitin (Ub) with carboxy group. (B) Protein degradation in the ER involves large complexes and here we describe the main events in ERAD using the E3 ubiquitin ligase gp78/AMFR. (1) The misfolded membrane protein is targeted for degradation by interaction with molecular chaperones (such as BiP) that have been shown to recruit E3 ubiquitin ligases and facilitate substrate polyubiquitylation of certain proteins. (2) In addition to its catalytic RING finger motif and its ubiquitin-binding Cue domain, gp78/AMFR interacts with many other components essential for its ubiquitin ligase activity. Some of which include the cofactor Ufd1, the E2 Ube2g2 and the p97/VCP/Cdc48 complex. Polyubiquitylation of the misfolded protein may occur simultaneously with its retro-translocation from the ER to the cytosol (via the retrotranslocon). (3) The p97/VCP/Cdc48 complex provides the driving force for ATP-dependent membrane extraction and targets the polyubiquitylated protein to the 26S proteasome where it is recognized and degraded.


A
ATP + UbCO-
AMP and PPi
E1-SH
E1-SCUb
E2-SCUb
E2-SH
Substrate
E3
Substrate-Ubn
26S Proteasome
B
Ubiquitin
Cofactor
(Ufd1)
p97/VCP
complex
E2
(Ube2g2)
E1
E3
(Gp78)
Misfolded
Protein
Retrotranslocon
Cytoplasm
Protein
targeting
(1)
ER
BiP
BiP
(2)
Ubiquitylation/
Retrotranslocation
Proteasomal
Degradation
(3)
26S proteasome

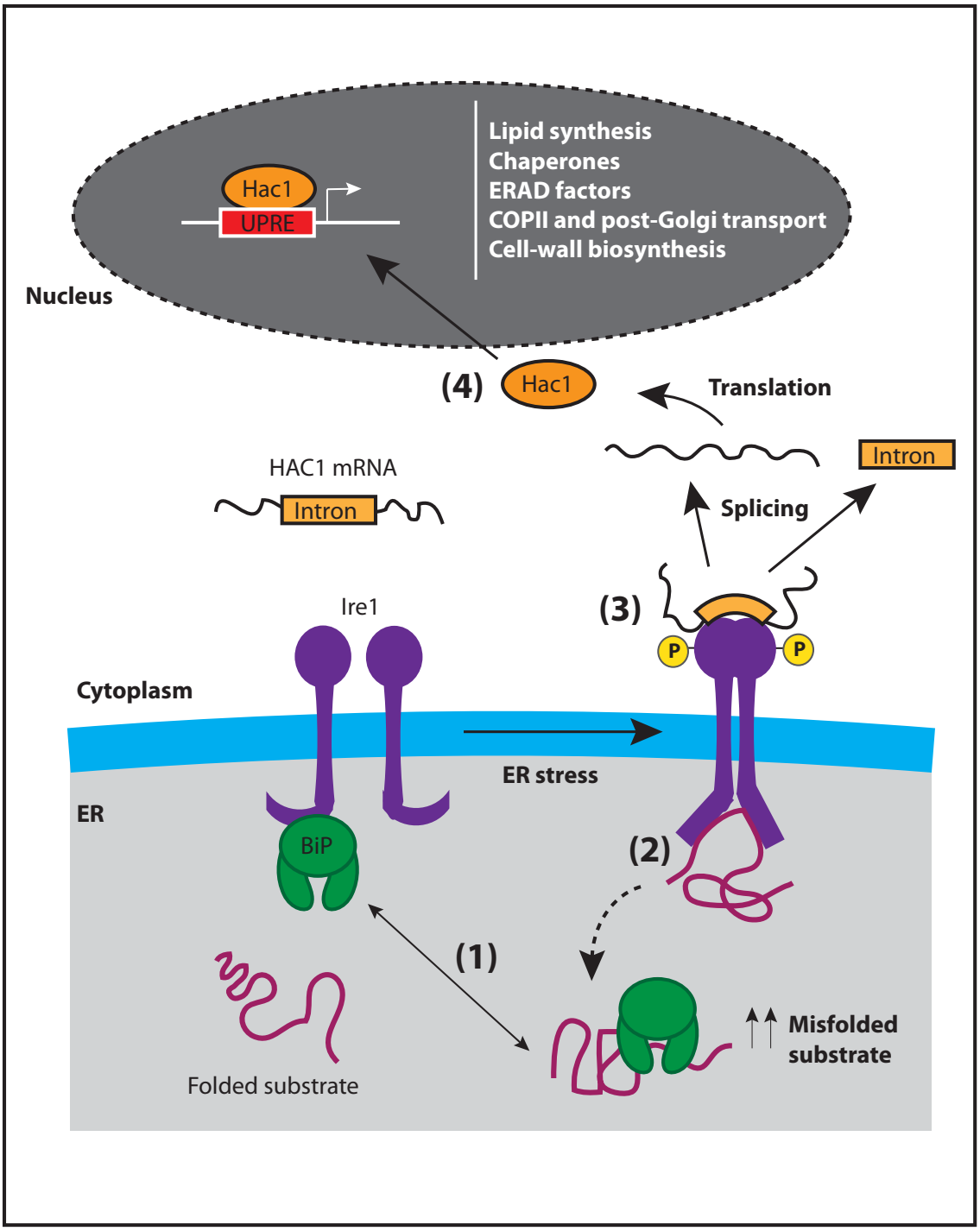
Scheme 1.1.4 Structure of the RING finger

(A) Illustration of the conserved RING finger motif in a cross-brace structure that is mediated by its interaction with two zinc ions. (B) Components that define the RING finger motif. Many E3s have a histidine instead of a cysteine in the fifth position and are referred to as RING-H2 fingers. Gp78/AMFR is an example of a RING-H2 ubiquitin ligase with two histidine residues in position 4 and 5 and six cysteine residues.



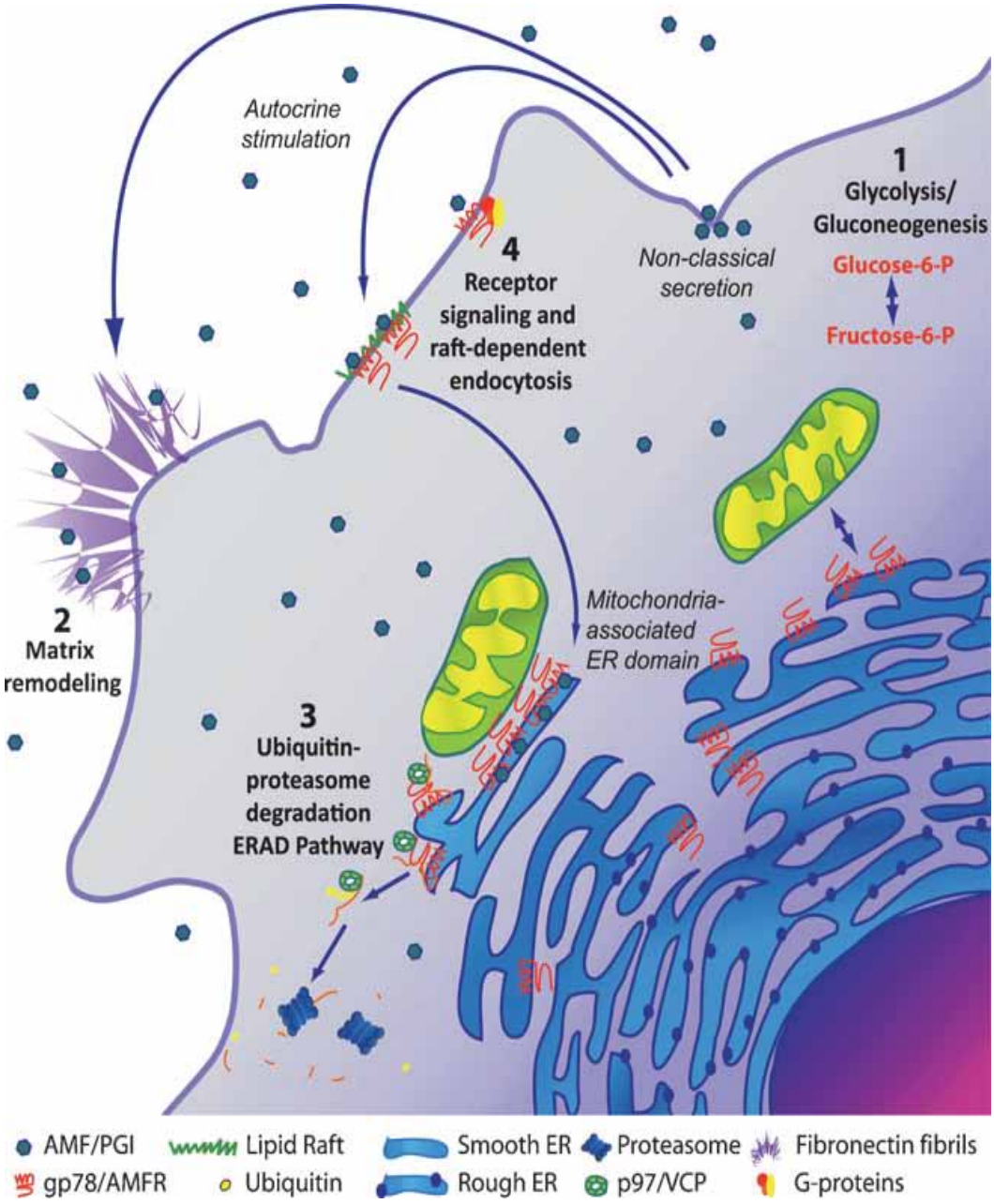
Scheme 1.1.5 The UPR pathway

Accumulation of misfolded proteins can trigger the unfolded protein response (UPR). In yeast, the UPR transducer is an ER-localized transmembrane Ser/Thr kinase and endoribonuclease, called inositol-requiring protein-1 (Ire1). Under normal conditions, Ire1 is maintained in an inactive state by binding to immunoglobulin binding protein (BiP). (1) Following ER stress, BiP preferentially binds misfolded proteins and releases Ire1 resulting in its activation. (2) Ire1 dimerizes and directly binds misfolded proteins within a peptide-binding pocket. (3) Ire1 dimerization also results in the transphosphorylation of its cytoplasmic domain, triggering its endoribonuclease activity that splices an intron in the mRNA of an UPR transcriptional activator called Hac1 (homologous to ATG6/CREB). The resulting mRNA is religated and translated into the protein Hac1. (4) Hac1 translocates into the nucleus and binds to UPR elements (UPRE) and other promoters of target genes, upregulating their expression. COPII: coatomer protein complex-II; ERAD: ER-associated degradation. Adopted from (Vembar and Brodsky, 2008).


Lipid synthesis
Chaperones
ERAD factors
COPII and post-Golgi transport
Cell-wall biosynthesis
Hac1
UPRE
Nucleus
(4)
Hac1
Translation
Intron
HAC1 mRNA
Intron
Splicing
Ire1
(3)
P
P
Cytoplasm
ER stress
ER
BiP
(2)
(1)
Misfolded
substrate
Folded substrate

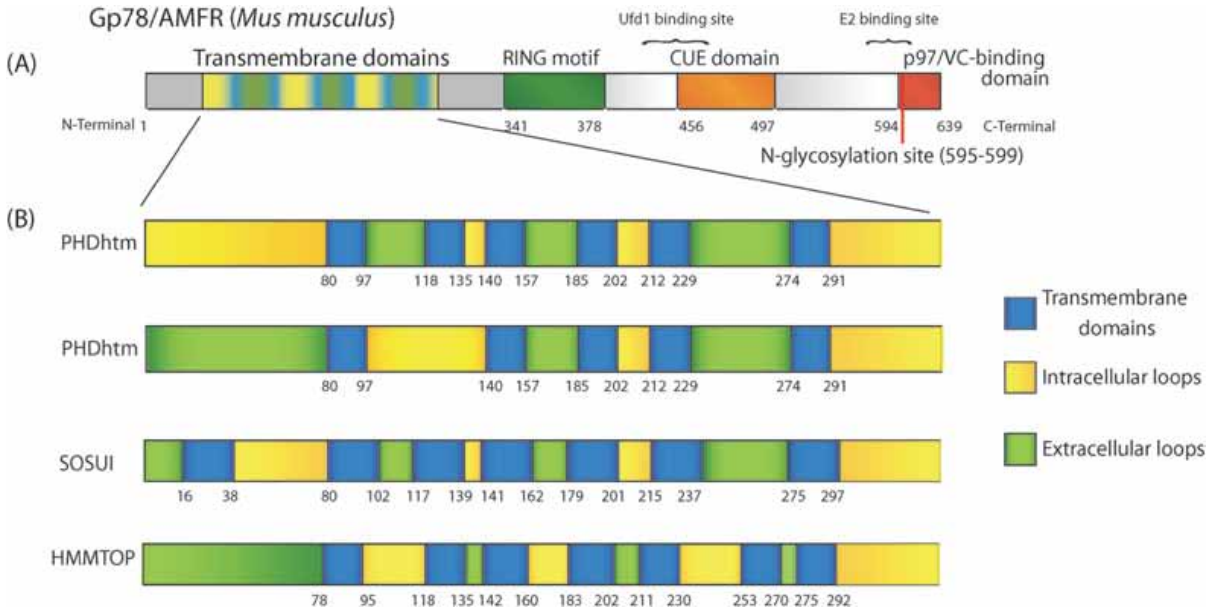
Scheme 1.2.1 Gp78/AMFR and its ligand AMF/PGI

Both AMF/PGI and its receptor gp78/AMFR are involved in various cellular pathways linked to metastasis development. These include (1) glycolysis; (2) matrix remodeling; (3) ER-associated degradation; (4) receptor signaling and endocytosis. Image from (Fairbank et al., 2009) - Reproduced by permission of The Royal Society of Chemistry (http://dx.doi.org/10.1039/B820820B).



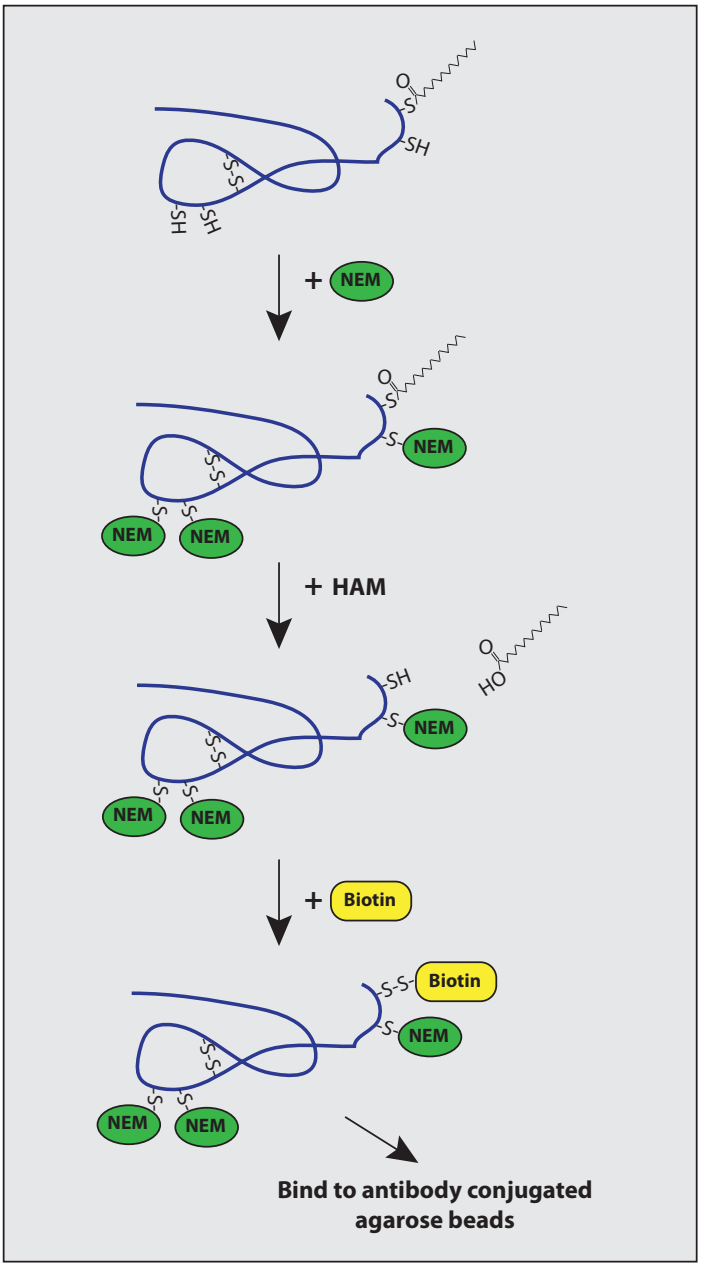
Scheme 1.2.2 Gp78/AMFR structure and topology

(A) The cytosolic C-terminal tail of gp78/AMFR includes a RING finger motif, responsible for ubiquitin ligase activity, an ubiquitin binding CUE motif that partially overlaps with the binding site of the cofactor Ufd1, an E2 binding domain and a p97/VCP/Cdc48 binding domain. (B) The topology and number of transmembrane domains of gp78/AMFR is still a matter of debate. The transmembrane domains presented here have been predicted using different online software: PHD (www.predictprotein.org), SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/) and HMMTOP (http://www.enzim.hu/ hmmtop/). Image from (Fairbank et al., 2009) - Reproduced by permission of The Royal Society of Chemistry (http://dx.doi.org/10.1039/B820820B).



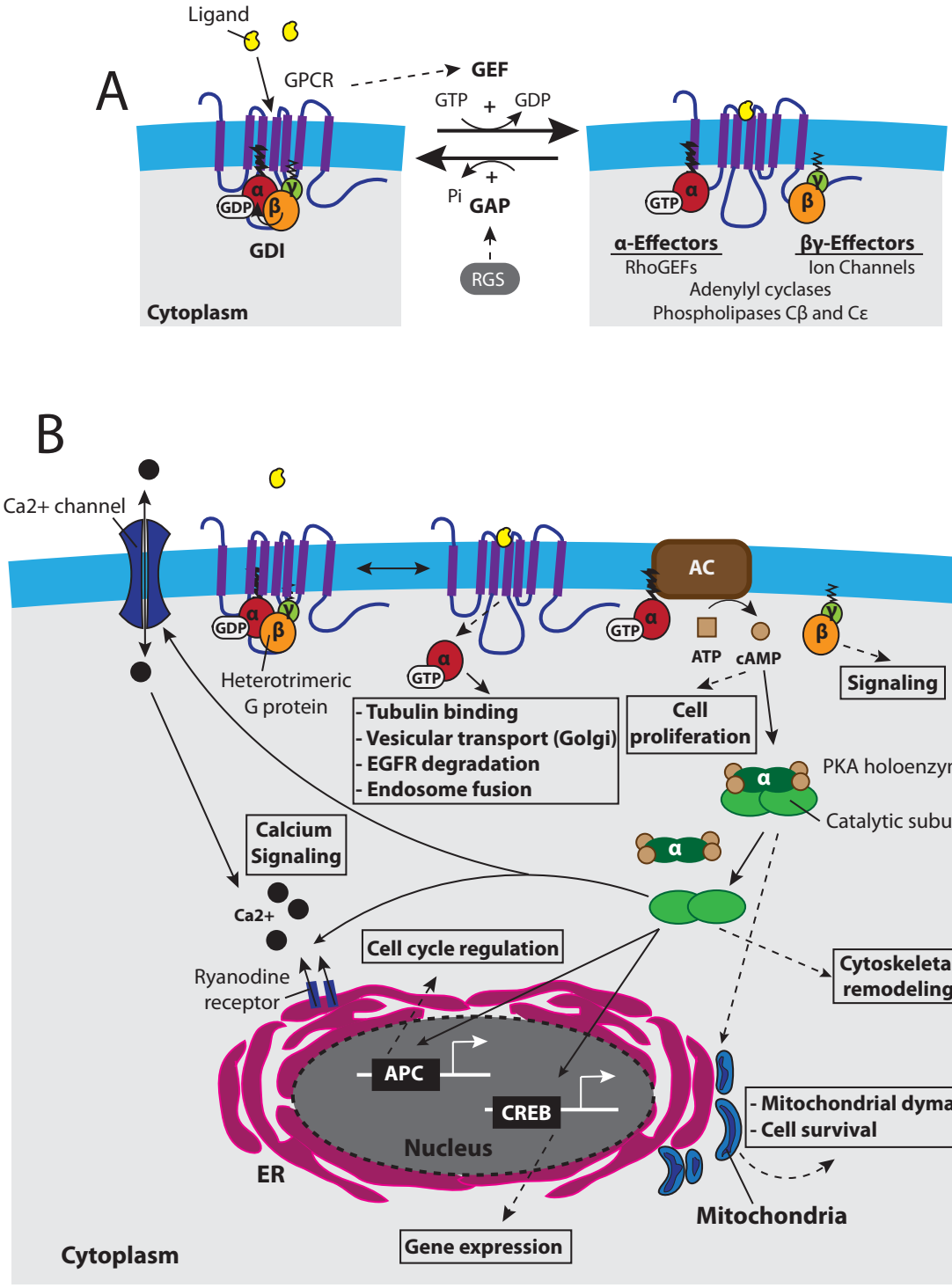
Scheme 1.3.1 The Acyl-Biotinyl Exchange (ABE) chemistry

First, all pre-existing, free sulfhydryl groups are blocked with N-ethylmaleimide (NEM). The thioester bond that covalently links the fatty acyl group to the cysteine residue is cleaved with hydroxylamine (HAM) treatment. The cysteine residue with the free sulhydryl group is labeled with a thiol-specific reagent (such as a non-radioactive sulhydryl-specific compound containing biotin). Finally, the latter can be affinity-purified using streptavidin-agarose and identified through SDS-PAGE or proteomic mass spectrometry. This assay is also referred to as Biotin-BMCC labeling.



Scheme 1.4.1 The Gαs signaling pathway

(A) Standard model for GPCR-mediated activation. In the absence of ligand, the seven-transmembrane GPCR is coupled to a heterotrimetic G protein complex. Gβγ dimer enhances Gα coupling to the receptor and prevents the release of GDP from Gα, acting as a guanine nucleotide dissociation inhibitor (GDI). Following ligand binding, the GPCR acts as a guanine nucleotide exchange factor (GEF) for Gα, resulting in GDP release, binding of GTP and dissociation of Gβγ. Both GTP-bound Gα and Gβγ subunits modulate the activity of many effectors. Regulators of G-protein signaling (RGS) proteins enhance the hydrolysis of GTP to GDP on the Gα subunit, acting as a GTPase-accelerating protein (GAP). (B) Summary of signaling events initiated by ligand-receptor binding that lead to the activation of adenylyl cyclase (AC) and production of cytosolic cAMP via the plasma membrane-localized Gαs subunit. Binding of cAMP to the catalytic subunit of PKA results in its activation and phosphorylation of target proteins that alter pathways within many cellular compartments. Moreover, Gαs translocates from the plasma membrane to the cytosol following receptor activation and is likely involved in other cellular pathways.


A
Ligand
GPCR
GEF
GTP + GDP
Pi + GAP
RGS
GDI
Cytoplasm
α-Effectors
RhoGEFs
Adenylyl cyclases
Phospholipases Cβ and Cε
βγ-Effectors
Ion Channels
B
Ca2+ channel
AC
ATP
cAMP
Signaling
Heterotrimeric
G protein
- Tubulin binding
- Vesicular transport (Golgi)
- EGFR degradation
- Endosome fusion
Cell
proliferation
PKA holoenzyme
Catalytic subunit
Calcium
Signaling
Ca2+
Cell cycle regulation
Ryanodine
receptor
Cytoskeletal
remodeling
APC
CREB
Nucleus
ER
- Mitochondrial dymamics
- Cell survival
Mitochondria
Gene expression
Cytoplasm

CHAPTER 2 RING Finger Palmitoylation of the Endoplasmic Reticulum Gp78/AMFR E3 Ubiquitin Ligase*

2.1 Summary

Palmitoylation, a dynamic posttranslational modification, in which a lipid palmitate binds to cysteine residue(s) is important for receptor stability and intracellular trafficking. Using metabolic labeling with ^{3}H-palmitate and Acyl-Biotinyl Exchange chemistry (Biotin-BMCC labeling), we found that gp78/AMFR, an E3 ubiquitin ligase within the endoplasmic reticulum-associated degradation (ERAD) pathway, is palmitoylated in Cos7 cells. Site-directed mutagenesis showed that Flag-tagged gp78/AMFR undergoes sulfhydryl cysteine palmitoylation (S-palmitoylation) on multiple cysteines within the RING finger motif, the domain responsible for its ubiquitin ligase activity. Mutation of one or a combination of six RING finger cysteines partially reduced gp78/AMFR palmitoylation, while mutation of all six cysteines significantly inhibited gp78/AMFR palmitoylation. Screening of 19 palmitoyl acyl transferases (PATs) identified five that increased gp78/AMFR RING finger palmitoylation, of which one, DHHC6, consistently enhanced gp78/AMFR palmitoylation and was ER localized. While RING finger mutation and inhibition of palmitoylation with 2-bromopalmitate restricted gp78/AMFR to the central ER, Myc-DHHC6 overexpression induced the distribution of Flag-gp78/AMFR from the central ER to the peripheral ER. Moreover, DHHC6-mediated palmitoylation enhanced the proteasomal degradation of wild-type gp78/AMFR but not the RING finger mutant (C352S) that remained stable. Palmitoylation of gp78/AMFR therefore disrupts the RING finger motif, regulates its ER distribution and enhances its turnover. Whether palmitoylation of E3 ubiquitin ligases is gp78/AMFR-specific or a general mechanism to control the activity of RING finger ubiquitin ligases remains to be determined.

* A version of chapter 2 has been submitted for publication.

2.2 Introduction

Targeted protein proteolysis is an important quality control mechanism that temporally regulates protein levels under physiological conditions. In eukaryotic cells, this is accomplished by the addition of a specific covalent modification, a chain of polyubiquitin that binds to and targets functionally abnormal or misfolded proteins for retrotranslocation from the ER to the cytosol and for proteasome-dependent degradation. This process, known as the endoplasmic reticulum-associated degradation (ERAD) pathway, involves a multi-component system: (1) ubiquitin activation (via E1 ubiquitin-activating enzymes); (2) conjugation to a carrier (via E2 ubiquitin-conjugating enzymes); and (3) transfer and binding of ubiquitin to the target protein (via E3 ubiquitin ligases). E3 ubiquitin ligases are important in substrate recognition, as well as binding to both target protein and the E2 ubiquitin-conjugating enzyme (Meusser et al., 2005; Vembar and Brodsky, 2008).

Gp78/AMFR is a well-studied E3 ubiquitin ligase in the ERAD pathway. Several gp78/AMFR substrates associated with pathological processes have been identified such as α1-antitrypsin, CFTRΔF508 and Kai-1 (a tumor metastasis suppressor protein) (Joshi et al., 2010; Morito et al., 2008; Shen et al., 2006; Tsai et al., 2007). Other gp78/AMFR substrates undergo degradation in response to physiological changes including inositol-phosphate receptor (IP3R) (Pearce et al., 2007), apolipoprotein B100 (Liang et al., 2003), the unassembled subunits of the T-cell receptors CD3δ and TCRα (Chen et al., 2006; Fang et al., 2001), HMG-CoA reductase (Cao et al., 2007) and Insig-1 (Lee et al., 2006; Song et al., 2005). Gp78/AMFR is a multi-spanning transmembrane receptor whose C-terminal domain includes several motifs necessary for promoting ubiquitylation events (Chen et al., 2006). A key motif involved in modulating its ubiquitin ligase activity is the RING finger that consists of a conserved series of cysteine and histidine residues that bind two zinc atoms in a unique ‘cross-brace’ arrangement. The specific structural conformation of the RING finger is a critical component of its function (Freemont, 2000).

Sulfhydryl cysteine palmitoylation (or S-palmitoylation) of soluble and membrane proteins is one type of fatty acylation that involves the addition of palmitate, a 16-carbon saturated fatty acid, to specific cysteine residues via a thioester linkage. A large number of palmitoylated proteins have been identified (Resh, 2006), including many neuronal proteins (el-Husseini Ael and Bredt, 2002) and G protein-coupled receptors (GPCRs) (Qanbar and Bouvier, 2003). Palmitoylation, similar to phosphorylation, is a dynamic, reversible posttranslational

modification that regulates cellular responses to different stimuli (Baekkeskov and Kanaani, 2009; Resh, 1999). Palmitoylation affects signaling pathways by altering receptor trafficking, function and stability (Huang and El-Husseini, 2005; Linder and Deschenes, 2007).

Palmitoyl acyl transferases (PATs), belonging to the DHHC protein family, share several transmembrane domains and have recently been characterized as enzymes that mediate the addition of palmitoyl CoA to proteins (Iwanaga et al., 2009). Their activity has been shown to occur via a highly conserved cysteine-rich domain (CRD) with a core Asp-His-His-Cys (DHHC) motif (Fukata et al., 2004; Keller et al., 2004; Roth et al., 2002; Smotrys and Linder, 2004). In the human genome, there are 23 PATs (also called DHHC enzymes), present in all tissue types; based on exogenous expression, most DHHC enzymes localize to the ER, Golgi and endosomal vesicles, while a few localize to the plasma membrane (Fukata et al., 2004; Ohno et al., 2006). Substrates have been identified for this family of proteins using proteomic analysis (Roth et al., 2006a), however the degree of substrate specificity and PAT selectivity still remains unclear.

Here, we report palmitoylation of gp78/AMFR within the RING finger motif and show for the first time, palmitoylation of a novel class of ER proteins, E3 ubiquitin ligases. Screening of 19 DHHC enzymes identified 5 DHHC enzymes that target gp78/AMFR for palmitoylation. One of these, ER localized DHHC6, induced the distribution of gp78/AMFR to the peripheral ER and enhanced its proteasomal degradation. Overall, these findings describe a novel posttranslational modification of a key ubiquitin ligase in the ERAD pathway.

2.3 Materials and methods

2.3.1 Plasmids and constructs

PSD95-GFP and PSD95 C3/5S-GFP as previously described (Topinka and Bredt, 1998) was kindly provided by Alaa El-Husseini (University of British Columbia). All 19 DHHC expression plasmids are previously described in (Ohno et al., 2006) and were provided by Michael R. Hayden and Alaa El-Husseini (University of British Columbia). Myc-Rtn4a (GrandPre et al., 2000) and GFP-Sec61β (Voeltz et al., 2006) expression plasmids were kindly provided by Gia Voeltz (University of Colorado). HA-Ub wild-type was a gift from Anthony Morielli (University of Vermont). Flag-gp78/AMFR wild-type was inserted into pcDNA 3.1 (+) as described (Registre et al., 2004). The Flag-gp78/AMFR N-terminal transmembrane domain construct was generated by substituting L330 for a stop codon. The C-terminal gp78-GFP

construct was generated by inserting the C-terminal fragment of gp78 (from E300 to T639) in frame at BamHI and ECORI restriction sites into the pEGFP-N1 backbone plasmid [Clontech]. The termination codon within the gp78 sequence was removed to incorporate a C-terminal GFP-tag. Flag-gp78/AMFR-IRES-GFP wild-type and Flag-gp78/AMFR C352S–IRES-GFP RING finger mutant were prepared by subcloning their pcDNA clones into the pIRES2-GFP backbone plasmid [Clontech, #6029-1] at ECORI and BamHI restriction sites. The Flag-gp78/AMFR N-terminal transmembrane domain mutants (C88/89A, C194/195A, C200/201A, C194/195/200/201A and ΔC) and the Flag-gp78/AMFR RING finger mutants (C337/340A, C352S, C360A, C371/374A, ΔC RING) were all generated by the Quickchange Mutagenesis Kit (Stratagene). The sequence of all mutated DNA constructs was confirmed.

2.3.2 Antibodies

Anti-Flag M2, anti-βactin and anti-calnexin were purchased from Sigma, anti-Myc Tag (clone 4A6) from Millipore, and anti-GFP from Synaptic Systems and anti-HA from Neomarker. The rat IgM 3F3A monoclonal antibody against gp78/AMFR is described in (Nabi et al., 1990). Fluorescently conjugated secondary antibodies for immunocytochemistry were purchased from Invitrogen. For immunoblotting experiments, HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch. For the Odyssey Infrared Imaging System (Li-COR Biosciences), a fluorescently conjugated secondary antibody, anti-mouse IRDye 800 antibody, was purchased from Rockland and streptavidin-cy5 was purchased from Invitrogen.

2.3.3 Cell culture and transfection

Cos7 cells were maintained in DMEM containing 10% fetal bovine serum (FBS, MediCorp.), and supplemented with 0.2 mM L-Glutamine, 10 μg/ml Penicillin, 10 μg/ml Streptomycin, vitamins and non-essential amino acids (all supplements were purchased from Invitrogen). For transient transfection of plasmid DNA into Cos7 cells, Effectene Transfection Reagent (Qiagen) was used as described by the manufacturer. At 24 to 48 h post-transfection, cells were used as outlined in each experiment. The drugs used were 2-bromohexadecanoic acid (2-bromopalmitate [2-BP]) (Fluka) and MG132 (Sigma).

2.3.4 Metabolic labeling

Cells were seeded on 10 cm petri dishes and 24 h post-transfection, cells were metabolically labeled with [9,10-^{3}H(N)]palmitic acid (5mCi/ml; Perkin Elmer Life Sciences) for 3 h during which [^{3}H]palmitoyl-CoA was synthesized enzymatically. Next, cells were harvested and the protein of interest was immunoprecipiated. Tritiated palmitoyl proteins were detected by placing film onto the PVDF membrane and exposing for one week at -80°C.

2.3.5 Immunoprecipitation and immunoblotting

For anti-Flag immunoprecipitation, Cos7 cells were rinsed and manually collected in cold phosphate-buffered saline (PBS). Cell pellets were resuspended in ice-cold lysis buffer A [20 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, 0.1% SDS and 1 mg/ml protease inhibitor cocktail (Roche)]. Following a 30 minute rotation at 4°C, 1% Triton X-100 was added to each sample and incubated for an additional 30 minutes. Lysates were passed through a 25G syringe 6 times and the insoluble material was removed by centrifugation. Supernatants were incubated overnight with anti-Flag coupled with agar beads (Sigma) that have been pre-blocked with 1% BSA (1 h at 4°C). Beads were then washed 3 times with wash buffer A [20 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, 0.2% BSA], 3 times with wash buffer B [20 mM Tris-HCl pH 8, 500 mM NaCl, 0.5% Triton X-100, 0.2% BSA] and one time with wash buffer C [50 mM Tris-HCl pH 8]. For anti-GFP immunoprecipitation, cell pellets were resuspended in ice-cold lysis buffer B [50 mM Tris-HCl pH 7, 150 mM NaCl, 1% Triton X-100] and precleared by incubation with protein A+G agar beads (GE Healthcare) for 45 minutes at 4°C with rocking. Precleared samples were immunoprecipitated with anti-GFP antibody (3 μg, rabbit) conjugated with protein A+G agar beads. For both immunoprecipitations, proteins in cell lysates and immunoprecipitates were denatured by boiling in SDS sample buffer, and later separated by SDS-PAGE, transferred onto PVDF membranes (Pall Corporation), blocked with 5% nonfat milk and probed with appropriate antibodies. Proteins were visualized either by Bioflex film (Clonex Corporation) or the Odyssey Infrared Imaging System (Li-COR Biosciences). To measure protein turnover, 24 h post-transfection, Cos7 cells were treated for 6 h with 5 μM or 30 μM MG132 and later, cells were collected for anti-Flag immunoprecipitation and immunoblotting as described above.

2.3.6 Biotin-BMCC labeling

This method was adapted from (Drisdel and Green, 2004). During the anti-Flag and anti-GFP immunoprecipitations, supernatants were supplemented with 50 mM N-ethylmaleimide (NEM, Sigma). Later, beads were incubated with non-stringent buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 1% Triton X-100] supplemented with 10 mM NEM for 10 min on ice, followed by one fast wash with stringent buffer [50 mM Tris-HCl pH7.4, 0.5 M NaCl, 5 mM EDTA, 0.02% NaN_3, 0.1% SDS] and 3 additional washes with non-stringent buffer. Each sample was divided into two portions for treatment with or without (as a control) 1 M hydroxylamine (HAM, Sigma) in non-stringent buffer (pH 7.4) for 1 h at room temperature. Subsequently, all samples were incubated with 1 μM EZ-link Biotin-BMCC (biotin-N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide biotinylation; Fisher Scientific) in non-stringent buffer (pH 6.2) for 1 h at 4°C to label reactive cysteine residues. Following SDS-PAGE and transfer to PVDF membranes, the Odyssey Infrared Imaging System was used to visualize Biotin-BMCC-labeled Flag-gp78/AMFR (probed with streptavidin-cy5 antibody) and total Flag-gp78/AMFR (probed with anti-Flag antibody and anti-mouse IRDye 800 antibody).

2.3.7 Immunofluorescence labeling

Cells were grown on glass cover slips and 24 h post-transfection, cells were fixed with pre-cooled (-80°C) methanol:acetone (80:20) for 15 minutes at -20°C. Then, cells were washed 3 times for 15 minutes with PBS supplemented with 1 mM $CaCl_2$ and 10 mM $MgCl_2$ (PBS-CM). Cells were first blocked with PBS-CM containing 1% Bovine Serum Albumin (BSA) (blocking solution) for 15 minutes and all the antibody incubations were performed in blocking solution. Cells were then incubated with primary antibodies for 30 minutes, rinsed 3 times for 2 minutes with blocking solution, incubated with the corresponding secondary antibodies for an additional 30 minutes, and then rinsed three times for 10 minutes with blocking solution. Cover slips were mounted on microscope slides with Celvol 205: Polyvinyl Alcohol (Celanese Chemicals Ltd.) and imaged with the 100x Planapochromatic objective of a Fluoview 1000 confocal laser scanning microscope (Olympus).

2.3.8 Statistical analysis

For immunoblot analysis, densitometry was done using the Scion image analysis (NIH Image) and results are expressed as Mean +/- S.E.M. Statistical comparisons between groups

were performed by using a One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests (GraphPad PRISM).

2.3.9 Confocal image analysis

A number of approaches are used to assess colocalization between fluorescently labeled cellular markers. These include Pearson's correlation coefficient (R_r), overlap coefficient according to Manders (R), overlap coefficients k_1 and k_2, colocalization coefficients M_1 and M_2 and colocalization coefficients m_1 and m_2 (Zinchuk et al., 2007). Pearson's coefficient is one approach that we used to describe subcellular colocalization between proteins in two-dimensional images from at least three independent experiments and this coefficient was calculated using ImagePro analysis software (Media Cybernetics). Pearson's coefficient assesses similarities between shapes and ignores signal intensity. It ranges from 1 to -1, with 1 representing complete positive correlation, -1 for a negative correlation and 0 for no correlation (Bolte and Cordelieres, 2006). Negative values of Pearson's coefficient should be carefully interpreted and reanalyzed using the overlap coefficient according to Manders (values range from 0 to 1). This is especially relevant when two fluorochromes display different intensities (Zinchuk et al., 2007). Some general limitations associated with colocalization analysis include (1) high levels of background noise that should be corrected by thresholding labeling intensity and/or deconvolving the image. Excessive background within acquired images can compromise the reliability of the colocalization estimate; and (2) cross-talk between fluorophores that can be minimized with sequential scanning of the images (Bolte and Cordelieres, 2006; Zinchuk et al., 2007).

We also used another approach to quantify the localization of gp78/AMFR within the central ER (as opposed to both the central and peripheral ER) and instead of looking at the Pearson's coefficient, we were able to monitor changes in the amount of central ER-localized gp78/AMFR (based on its intensity). Again, all analysis was done using ImagePro analysis software (Media Cybernetics) and the data represent at least three independent experiments. The central ER was identified by either labeling for endogenous calnexin or expressing GFP-Sec61β (St. Pierre et al., 2011; Voeltz et al., 2006). The nucleus was excluded from all images and a mask of the central ER was obtained by thresholding labeling intensity of either calnexin or GFP-Sec61β surrounding the nuclei (excluding the cell periphery). The mask was used to determine the percentage of Flag-gp78/AMFR localized in the central ER. We measured the ratio

between the anti-Flag labeling intensity within a mask of the central ER and the total Flag-gp78/AMFR intensity of the cell.

2.4 Results

2.4.1 Gp78/AMFR is palmitoylated in the RING finger motif

Gp78/AMFR contains a total of 15 cysteine residues: 9 cysteines distributed between the transmembrane domains and the remaining 6 cysteines all localized within the RING finger domain (Fig. 2.1A). To study protein palmitoylation, we used metabolic labeling with tritiated palmitate [^{3}H-palmitate] and as a positive control, we show that the neuronal protein postsynaptic density-95 (PSD95-GFP) undergoes palmitoylation. PSD95-GFP is a well-documented palmitoyl protein and the palmitoylation site has been localized to two cysteine residues (C3 and C5) in its NH_2 terminus (Craven et al., 1999; Topinka and Bredt, 1998), shown with the PSD95 C3/5S-GFP mutant that is no longer palmitoylated (Figure 2.1D). Next, to study gp78 palmitoylation, we expressed three gp78/AMFR constructs in Cos7 cells: full-length Flag-gp78/AMFR (FL), Flag-tagged N-terminal transmembrane domain (TMD) and GFP-tagged C-terminal domain (C-Term). Following metabolic labeling with radiolabeled ^{3}H-palmitate, all three constructs were palmitoylated (Fig. 2.1B - 2.1C). It became apparent that this approach was identifying more than one type of palmitoylation, specifically in the case of the N-terminal transmembrane domain that remained palmitoylated despite mutation of all 9 cysteines into alanines (Fig. A.1). Identification of palmitoylated residue(s) remains challenging as there are no strict consensus sites and the 'motifs' vary significantly between proteins (Bijlmakers and Marsh, 2003; Charollais and Van Der Goot, 2009). A palmitoyl acyl transferase, Hhat, was shown to attach a palmitate via an amide linkage to the N-terminal cysteine of Sonic Hedgehog (Buglino and Resh, 2008). A similar amide linkage of a palmitate to the N-terminal Flag tag of Flag-gp78/AMFR could explain the ^{3}H-palmitate labeling of Flag-gp78/AMFR TMD.

To specifically determine whether gp78/AMFR is S-palmitoylated, we used Acyl-Biotinyl Exchange chemistry or the Biotin-BMCC labeling that specifically labels proteins that undergo cysteine palmitoylation, called S-palmitoylation (Drisdel and Green, 2004). The Biotin-BMCC method labeled full-length Flag-gp78/AMFR (FL) as well as full-length Flag-gp78/AMFR lacking N-terminal cysteines (FL ΔC TMD) but not the N-terminal transmembrane domain gp78/AMFR construct (TMD) (Fig. 2.1E - 2.1F). This confirms our previous observation

that the N-terminal transmembrane domain of gp78/AMFR undergoes a non-conventional palmitoylation and suggests that S-palmitoylation is confined to the C-terminal RING finger cysteines.

Using the Biotin-BMCC labeling and site-directed mutagenesis, we characterized palmitoylation of RING finger cysteines in full-length Flag-gp78/AMFR. Mutations of individual cysteines or combinations of two or three cysteines all significantly reduced gp78/AMFR palmitoylation. However, the palmitoylation signal was only lost when every cysteine was substituted with an alanine (Fig. 2.2). This suggests that the RING finger cysteines all show affinity for S-palmitoylation.

A RING finger mutant that contains an inhibitory point mutation in the third cysteine (C352S) shows significantly reduced ability to generate polyubiquitylated substrate when co-transfected with HA-tagged ubiquitin (HA-Ub) (St. Pierre et al., 2011). We used this assay to test the ability of the different RING finger mutants to polyubiquitylate substrates in the *in vitro* ubiquitin ligase activity assay. We found that each single cysteine mutation or a combination of two-three mutations significantly reduced the ubiquitylated protein smear, and the cysteine-free RING finger mutant (ΔC RING) possessed little to no activity (Fig. 2.3). This suggests that all the cysteines play a role in RING finger function and palmitoylation of one or more RING finger cysteines should therefore decrease gp78/AMFR ubiquitin ligase activity. However, we should also take into account that cysteine mutagenesis alone likely disrupts zinc ion binding within the RING finger, inhibiting its ubiquitin ligase activity independently of cysteine palmitoylation. Progressive substitution of cysteines for alanines shows a 50% loss in the ability of gp78/AMFR to polyubiquitylate substrates (Fig. 2.3) and this inhibition of activity can also be explained by the loss of binding to one (or two) zinc ion(s) which would induce changes within the RING finger structure and directly affect its function. One approach to maintain zinc ion binding within the RING finger and prevent S-palmitoylation is to mutate cysteines into histidines, instead of alanines. An NMR study showed that histidine binds strongly to zinc in addition to copper and iron at pH 7.4 (Nair et al., 2010). But with the current data, we are unable to distinguish between the effect of palmitoylation on RING finger activity and the structural consequence of cysteine mutations affecting zinc ion binding. In a study of membrane-associated guanylate kinase proteins, the authors showed that differential subcellular localization and function of these proteins are attributed to differences within their N-terminal cysteines that either undergo palmitoylation or zinc ion binding (El-Husseini et al., 2000b). It was demonstrated that these two

factors are mutually exclusive, showing that N-terminal palmitoylation of both PSD-95 and PSD-93 contributes to their predominant localization to postsynaptic sites, while SAP-102 is not palmitoylated and binds zinc via a cluster of four cysteines and three histidine residues, a region that is homologous to RING finger and LIM domains. Zinc binding to SAP-102 is shown to mediate its localization to both axons and dendrites in neurons (El-Husseini et al., 2000b). In the case of gp78/AMFR, the relationship between cysteine palmitoylation and zinc ion binding within the RING finger is unclear and further investigation is required to describe the interplay of these two factors on the structure of the RING finger and gp78/AMFR ubiquitin ligase activity.

We then investigated modulation of gp78/AMFR palmitoylation by exogenously co-expressing palmitoyl acyl transferases (PATs), also known as DHHC enzymes. Using the Biotin-BMCC labeling, we screened 19 DHHC enzymes and identified 5 DHHC enzymes (DHHC2, DHHC6, DHHC11, DHHC13 (HIP14L) and DHHC24) that increase gp78/AMFR palmitoylation by at least 1.5 fold (Fig. 2.4A - 2.4B). In Cos7 cells, these DHHC enzymes vary in their subcellular distribution: DHHC2 localizes mostly to the plasma membrane, DHHC13 is predominantly found in the Golgi, while DHHC6, DHHC11 and DHHC24 are all expressed in the ER (Fig. A.2). We decided to further explore the role of one DHHC enzyme, DHHC6, based on its ER localization and its ability to consistently modulate gp78/AMFR palmitoylation in the Biotin-BMCC labeling. To confirm that DHHC6 targets cysteines within the RING finger motif, we co-expressed the cysteine-free RING finger Flag-gp78/AMFR mutant (ΔC RING) with Myc-DHHC6 in the Biotin-BMCC labeling and showed that in the absence of all RING finger cysteines, Flag-gp78/AMFR palmitoylation is not enhanced by DHHC6 (Fig. 2.4C).

2.4.2 Gp78/AMFR palmitoylation regulates gp78/AMFR ER distribution and stability

Next, we investigated the effect of DHHC6 on the distribution of gp78/AMFR in Cos7 cells and found that in the presence of Myc-DHHC6, Flag-gp78/AMFR had a lower intensity in the central ER as defined by the central ER marker GFP-Sec61β, and remained abundant in the periphery of the cell. This gp78/AMFR redistribution was not observed with DHHC19, another DHHC enzyme that is found in the ER (Fig. 2.5A) but does not modulate gp78/AMFR palmitoylation (Fig. 2.4). As previously reported for the C352S RING finger mutation (St. Pierre et al., 2011), the cysteine-free RING finger Flag-gp78/AMFR mutant (ΔC RING) was concentrated in the central ER, a distribution that was not affected by expression of Myc-

DHHC6 (Fig. 2.5A and Fig. A.3). In the presence of Myc-DHHC6, Flag-gp78/AMFR redistributed to the peripheral ER where it colocalized extensively with 3F3A anti-gp78/AMFR labeling (Fig. 2.5C) that is highly specific for the smooth ER (Benlimame et al., 1998; Benlimame et al., 1995). This suggests that inefficient palmitoylation upon overexpression of Flag-gp78/AMFR may restrict its delivery to the peripheral smooth ER and result in its accumulation in the central ER.

To confirm a role for palmitoylation in peripheral gp78/AMFR distribution, we studied the effect of gp78/AMFR depalmitoylation by treating Cos7 cells with a broad inhibitor of palmitoylation, 2-bromopalmitate (2-BP). In the Biotin-BMCC labeling, we showed that a 6 h treatment with 2-BP significantly reduces gp78/AMFR palmitoylation by 40% (Fig. 2.6A). The same 2-BP treatment results in Flag-gp78/AMFR accumulation in the GFP-Sec61β-defined central ER and reverts the enhanced peripheral distribution of Flag-gp78/AMFR in the presence of DHHC6 (Fig. 2.6B). Thus, gp78/AMFR palmitoylation promotes gp78/AMFR distribution to the peripheral ER.

To investigate in more detail the functional implication of gp78/AMFR palmitoylation, we assessed gp78/AMFR stability by generating IRES-GFP vectors that express either Flag-gp78/AMFR or Flag-gp78/AMFR C352S RING finger mutant, and loss of Flag-gp78/AMFR protein was determined relative to GFP that serves as an internal control of plasmid expression. The presence of DHHC6 significantly reduces wild-type gp78/AMFR protein levels but not that of the RING finger mutant (Fig 2.7). As observed here for a single point mutation (C352S) within the RING finger motif of gp78/AMFR, mutations within the RING finger motif that disrupt ubiquitin ligase activity have been shown to enhance stability of gp78/AMFR (Shmueli et al., 2009). The selective effect of Myc-DHHC6 co-transfection on the stability of Flag-gp78/AMFR-IRES-GFP, but not Flag-gp78/AMFR C352S-IRES-GFP RING finger mutant, suggests that gp78/AMFR RING finger palmitoylation is specifically affecting gp78/AMFR stability. Gp78/AMFR is subject to ubiquitin-mediated proteasomal degradation by Hrd1 (Shmueli et al., 2009). In the present study, proteasome inhibition with MG132 prevented the DHHC-induced reduction in gp78/AMFR protein (Fig. 2.7) suggesting that palmitoylation of gp78/AMFR by DHHC6 targets gp78/AMFR for degradation via the proteasome. Palmitoylation-dependent distribution of g78 to the peripheral ER would therefore appear to be associated with its proteasome-mediated degradation.

2.5 Discussion

This study describes, for the first time, palmitoylation of the RING finger motif of an E3 ubiquitin ligase. Gp78/AMFR is an E3 ubiquitin ligase in ERAD that can be palmitoylated by DHHC6, an ER localized PAT. We show that palmitoylation promotes the distribution of gp78/AMFR to the peripheral ER, the site of gp78/AMFR ubiquitin ligase activity (St. Pierre et al., 2011), as well as gp78/AMFR turnover. Palmitoylation of the gp78/AMFR RING finger motif may affect gp78/AMFR substrate degradation in the ERAD pathway by disrupting the RING finger motif but also by regulating gp78/AMFR expression levels. Thus, we introduce a novel role for palmitoylation as a regulator of the expression and activity of an ubiquitin ligase in ERAD.

Using two methods for detecting protein palmitoylation, metabolic labeling and the Biotin-BMCC labeling, we found that full-length Flag-gp78/AMFR is S-palmitoylated in the RING finger domain on multiple cysteines (Fig. 2.1). Using site-directed mutagenesis, we failed to identify a preferentially palmitoylated cysteine since all mutants displayed a 40-60% decrease in palmitoylation, with the exception of the cysteine-free RING finger mutant (Fig. 2.2). These data reinforce the concept that palmitoylation can occur on more than one cysteine residue within membrane proteins, as seen with several AMPA receptor subunits and the NMDA receptor, whereby two palmitoylated sites differentially regulate cell surface expression and receptor trafficking (Hayashi et al., 2005; Hayashi et al., 2009). The N-terminal Flag-tagged transmembrane domain of gp78/AMFR also undergoes unconventional palmitoylation on an unknown residue. However, since the construct tested lacks the functional C-terminal domain, it is uncertain whether the same N-terminal palmitoylation occurs in the full-length protein.

In yeast, proteomic analysis identified the DHHC family of proteins as the primary PATs responsible for mediating most protein palmitoylation (Fukata et al., 2006a; Roth et al., 2006a). In mammalian cells, the subcellular localization of DHHC enzymes varies and includes the ER, Golgi, endosomal vesicles and the plasma membrane (Ohno et al., 2006). Despite the fact that DHHC enzymes often have more than one substrate, they can exhibit distinct substrate specificity (Fukata et al., 2006a; Huang et al., 2009). Concurrently, screening analysis of 23 mammalian DHHC enzymes demonstrated that a number of substrates could be differentially palmitoylated by specific DHHC enzymes (Fang et al., 2006; Fukata et al., 2006a; Huang et al., 2010). This is consistent with our observation that Flag-gp78/AMFR is preferentially palmitoylated by a selection of DHHC enzymes (DHHC2, DHHC6, DHHC11, DHHC13 and

DHHC24) (Fig. 2.4). Our studies are based on co-transfection experiments of the DHHC enzymes with Flag-gp78/AMFR such that PAT expression levels may vary. This may account for the observed variability between experiments and may also limit our ability to detect gp78/AMFR palmitoylation due to expression of other endogenous DHHC enzymes. Moreover, expression of a single DHHC enzyme in Cos7 cells may promote gp78/AMFR palmitoylation that does not occur under physiological conditions. This can be due to the abundance of the exogenous DHHC enzyme within the cell and its ability to palmitoylate more than one substrate. In this model, the site of gp78/AMFR palmitoylation may take place beyond the primary cellular compartment and/or occur via a DHHC enzyme with a lower affinity for gp78/AMFR. This possibility is partly supported by the finding that gp78/AMFR is palmitoylated by five DHHC enzymes, all localized to different compartments (ER, Golgi and plasma membrane). However, it is also possible that gp78/AMFR undergoes palmitoylation at all these cellular sites. Another approach in identifying DHHC enzyme(s) responsible for gp78/AMFR palmitoylation is to look at endogenous DHHC enzymes and determine their expression levels within the cell line in question. Next, we can use siRNA transfection to inhibit the expression of the most abundant DHHC enzymes in the goal of detecting changes in both exogenous/endogenous gp78/AMFR palmitoylation levels. This method supplies another means to observe the effects of gp78/AMFR palmitoylation on protein trafficking and function however it is likely that gp78/AMFR will still be targeted by multiple DHHC enzymes that may vary with different cell lines.

In the study, we describe the functional role of only one DHHC enzyme, DHHC6. However, we have performed confocal image analysis using all 5 DHHC enzymes and found that all target DHHC enzymes promoted a similar, but not as significant, redistribution of gp78/AMFR as seen with DHHC6 (data not shown). At the same time, studies done with DHHC19 that neither increases gp78/AMFR palmitoylation nor induces the peripheral ER redistribution of gp78/AMFR (Fig. 2.5), represent a control for DHHC function. Overall, these data are indicative of both specificity and redundancy amongst DHHC enzymes that may have functional relevance if one considers the differential expression of DHHC enzymes in various tissue types (Ohno et al., 2006).

By electron microscopy, the ER can be separated into smooth and rough, ribosome studded domains. These have more recently been shown to correspond, in cultured cells, to the peripheral, tubular ER and central, saccular ER. Formation of peripheral ER tubules is based on expression of the curvature-stabilizing reticulons and DP1/Yop1p proteins; these proteins also

contribute to curvature at sheet edges while coiled-coil membrane proteins act as spacers to form ribosome-studded central ER sheets (Shibata et al., 2010). However, mechanisms that control protein distribution between these domains remain poorly understood. We show here that gp78/AMFR palmitoylation targets the protein to the peripheral ER (Fig. 2.5). Mutation of RING finger cysteines (Fig. A.3) and inhibition of palmitoylation with 2-BP (Fig. 2.6B) both restrict gp78/AMFR to the central ER. Enhanced gp78/AMFR palmitoylation due to DHHC6 overexpression induces an increased peripheral ER distribution of the protein. RING finger palmitoylation therefore appears to enhance the peripheral ER distribution of gp78/AMFR.

Gp78/AMFR was localized by electron microscopy with the 3F3A anti-gp78/AMFR antibody to a smooth ER domain and shown to be excluded from the rough ER (Benlimame et al., 1998; Benlimame et al., 1995; Wang et al., 1997). The 3F3A-labeled smooth ER domain was subsequently shown to associate with mitochondria and upon gp78/AMFR overexpression to extend to the reticulon-positive peripheral ER (Goetz et al., 2007; Wang et al., 2000). Furthermore, consistent with the recently reported ER tubule sliding on stable microtubules (Friedman et al., 2010), 3F3A-labeled smooth ER tubules have been shown to associate with stable detyrosinated microtubules (Wang et al., 1997). This is indicative of equivalence between the peripheral and smooth ER (Shibata et al., 2010) and suggests that 3F3A labeling is a specific marker of the smooth ER. The 3F3A antibody selectively labels the peripheral ER, even upon overexpression of Flag-gp78/AMFR that accumulates in the central ER (Goetz et al., 2007). The basis for the selective 3F3A recognition of peripheral, smooth ER localized gp78/AMFR remains to be determined. However, the ability of exogenous Myc-DHHC6 to increase the peripheral ER distribution of Flag-gp78/AMFR suggests that the central ER accumulation of Flag-gp78/AMFR may be a consequence of insufficient palmitoylation of the overexpressed protein. This argues that endogenous gp78/AMFR is predominantly localized to the smooth ER, a distribution that is apparently dependent on efficient palmitoylation of the RING finger domain of the protein.

Palmitoylation-induced gp78/AMFR redistribution to the peripheral ER is therefore associated with its proteasomal degradation. HRD1, an ER ubiquitin ligase, has been shown to target gp78 for ubiquitin-mediated, proteasome-dependent degradation (Ballar et al., 2007; Fang et al., 2001; Shmueli et al., 2009). Indeed, RING finger mutants that show reduced palmitoylation (Fig. 2.2) also remain more stable (Fig. 2.7; (Amemiya et al., 2008; Wu et al., 2004)) and increased gp78/AMFR palmitoylation due to DHHC6 enhances its proteasomal degradation (Fig. 2.7). In fact, palmitoylation has been previously shown to affect protein

stability by protecting receptors from ubiquitylation (Abrami et al., 2006; Valdez-Taubas and Pelham, 2005). In the case of two GPCRs, A1 adenosine receptor and chemokine receptor CCR5, the regulation of protein turnover and stability is dependent upon their palmitoylation state (Gao et al., 1999; Percherancier et al., 2001). RING finger palmitoylation, by both inhibiting gp78/AMFR ubiquitin ligase activity and targeting the protein for degradation, therefore represents an important regulator of gp78/AMFR ERAD activity. Since palmitoylation is highly dynamic, RING finger palmitoylation may represent a novel mechanism for the regulation of the activity, stability and cellular distribution of this ERAD-associated ubiquitin ligase. Thus, RING finger palmitoylation could have significant implications for the activity of other E3 ubiquitin ligases and their ERAD substrates, ultimately affecting homeostatic and pathological processes. Whether RING finger palmitoylation is gp78/AMFR-specific or a general mechanism to control the activity of RING finger ubiquitin ligases still remains to be determined.*

* This work was initiated as a collaboration with Alaa El-Husseini, since deceased, and we are grateful for his insight and support. We also thank Michael Hayden for his help in completing this study. Supported by the Canadian Institutes for Heath Research (CIHR MT-15132) and a Michael Smith Foundation for Health Research Trainee award to MF.

Figure 2.1 Gp78/AMFR is S-palmitoylated in the C-terminal domain and undergoes non-conventional palmitoylation in its N-terminal transmembrane domain

A. Schematic of mouse gp78/AMFR constructs used in the metabolic radiolabeling assay: Full-length (FL) Flag-tagged gp78/AMFR; N-terminal Flag-tagged transmembrane domain (TMD) of gp78/AMFR; GFP-tagged C-terminal domain (C-Term) of gp78/AMFR. (*) point to the location of cysteine residues. B–D. Cos7 cells were transiently transfected with pcDNA3.1 vector control or gp78/AMFR constructs, or PSD95-GFP (positive control) and PSD95 C3/5S-GFP (negative control) constructs. Following metabolic labeling with ^{3}H-palmitate, cell lysates were incubated with anti-Flag beads (B) or anti-GFP beads (C and D). Films were exposed for 1 week to detect palmitoylation and anti-Flag or anti-GFP immunoblots were done (1% input). Data are representative of three independent experiments. E-F. Gp78/AMFR constructs [FL Flag-tagged gp78/AMFR; FL Flag-tagged gp78/AMFR absent of all 9 cysteines in the transmembrane domain (ΔC TMD); truncated N-terminal Flag-tagged gp78/AMFR transmembrane domain (TMD)] were tested for palmitoylation in the Biotin-BMCC labeling. Briefly, following anti-Flag immunoprecipitation, beads were treated with 1 M hydroxylamine (HAM) to cleave Cys-palmitoyl thioester linkages and subsequently, with a sulfhydryl-specific labeling compound, 1 μM Biotin-BMCC, to detect the palmitoylated cysteine residue(s). Immunoblots were probed with fluorescently labeled streptavidin-cy5 (to detect Biotin-BMCC-labeled proteins) and anti-Flag antibody followed by IRDye 800 mouse antibody (to detect Flag-tagged total proteins), and later imaged with the Odyssey Infrared Imaging System. The immunoblots are representative of three independent experiments.

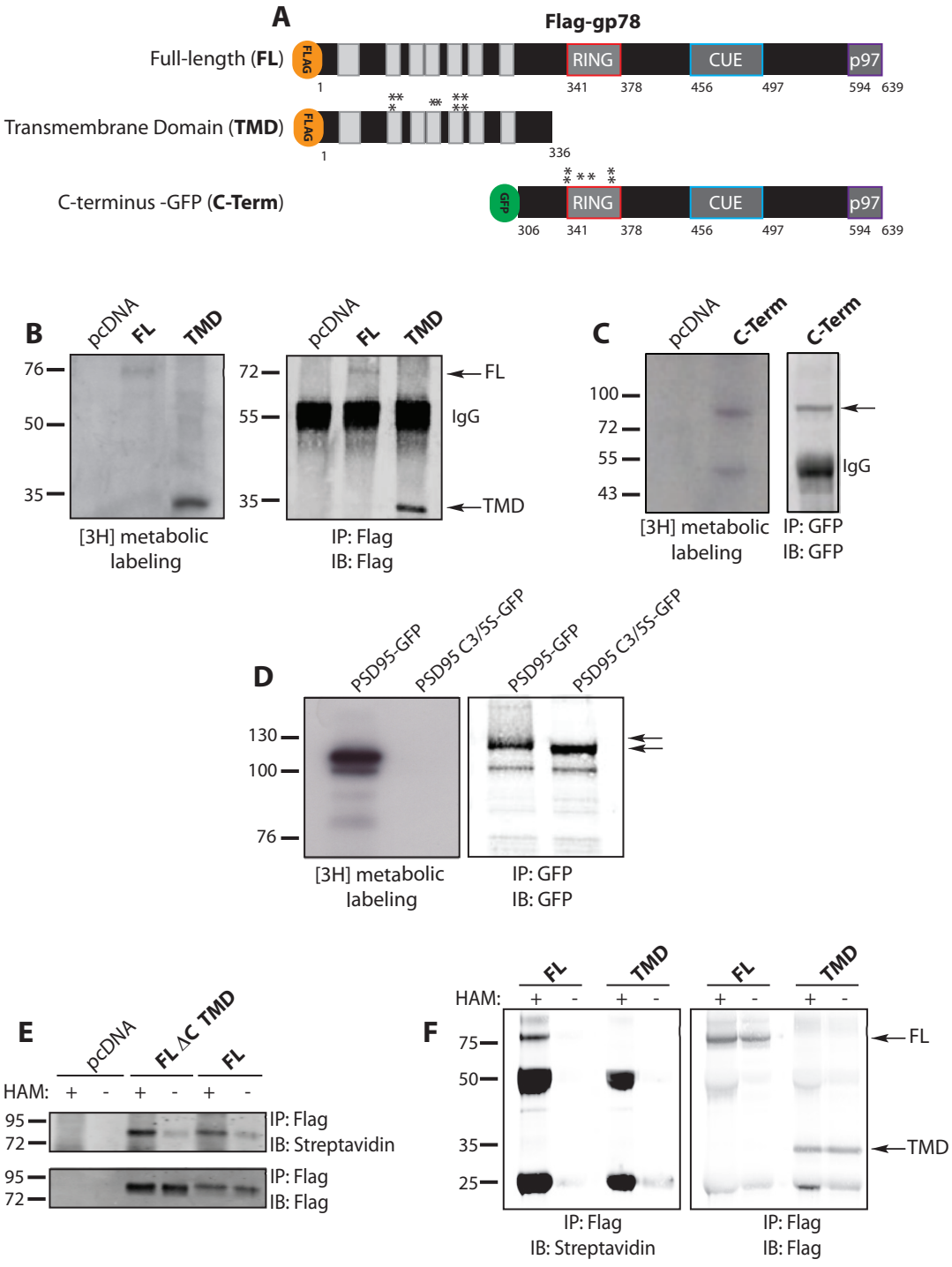

A
Flag-gp78
Full-length (FL)
Transmembrane Domain (TMD)
C-terminus -GFP (C-Term)
FLAG
GFP
RING
CUE
p97
B
pcDNA
FL
TMD
[3H] metabolic labeling
IP: Flag
IB: Flag
IgG
C
C-Term
IP: GFP
IB: GFP
D
PSD95-GFP
PSD95 C3/5S-GFP
E
FL ΔC TMD
HAM:
IP: Flag
IB: Streptavidin
F

Figure 2.2 Gp78/AMFR is S-palmitoylated on cysteines within the C-terminal RING finger domain

A. The C-terminal of gp78/AMFR contains 6 cysteines all located within a RING finger motif that is responsible for its ubiquitin ligase activity. Additional domains include an ubiquitin binding CUE motif and a p97/VCP/Cdc48 binding domain. B and C. Using site-directed mutagenesis, cysteines in the RING finger motif were systematically substituted for alanines (C337, C340, C360, C371, C374) or a serine (C352) in full-length Flag-tagged gp78/AMFR, generating a cysteine-free RING finger mutant (ΔC RING). 8 mutant constructs were tested for palmitoylation in the Biotin-BMCC labeling as described previously. Immunoblots were probed with fluorescently labeled streptavidin-cy5 (to detect Biotin-BMCC-labeled proteins) and anti-Flag antibody followed by IRDye 800 mouse antibody (to detect Flag-tagged total proteins), and later imaged with the Odyssey Infrared Imaging System. D. Band densities were first normalized with respect to protein levels from anti-Flag immunoblots, and then each mutant was normalized to wild-type gp78/AMFR. Mean ± S.E.M.; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to wild-type. $^{\#}P < 0.05$ compared to the cysteine-free RING finger mutant. The immunoblots are representative of four to six experiments.

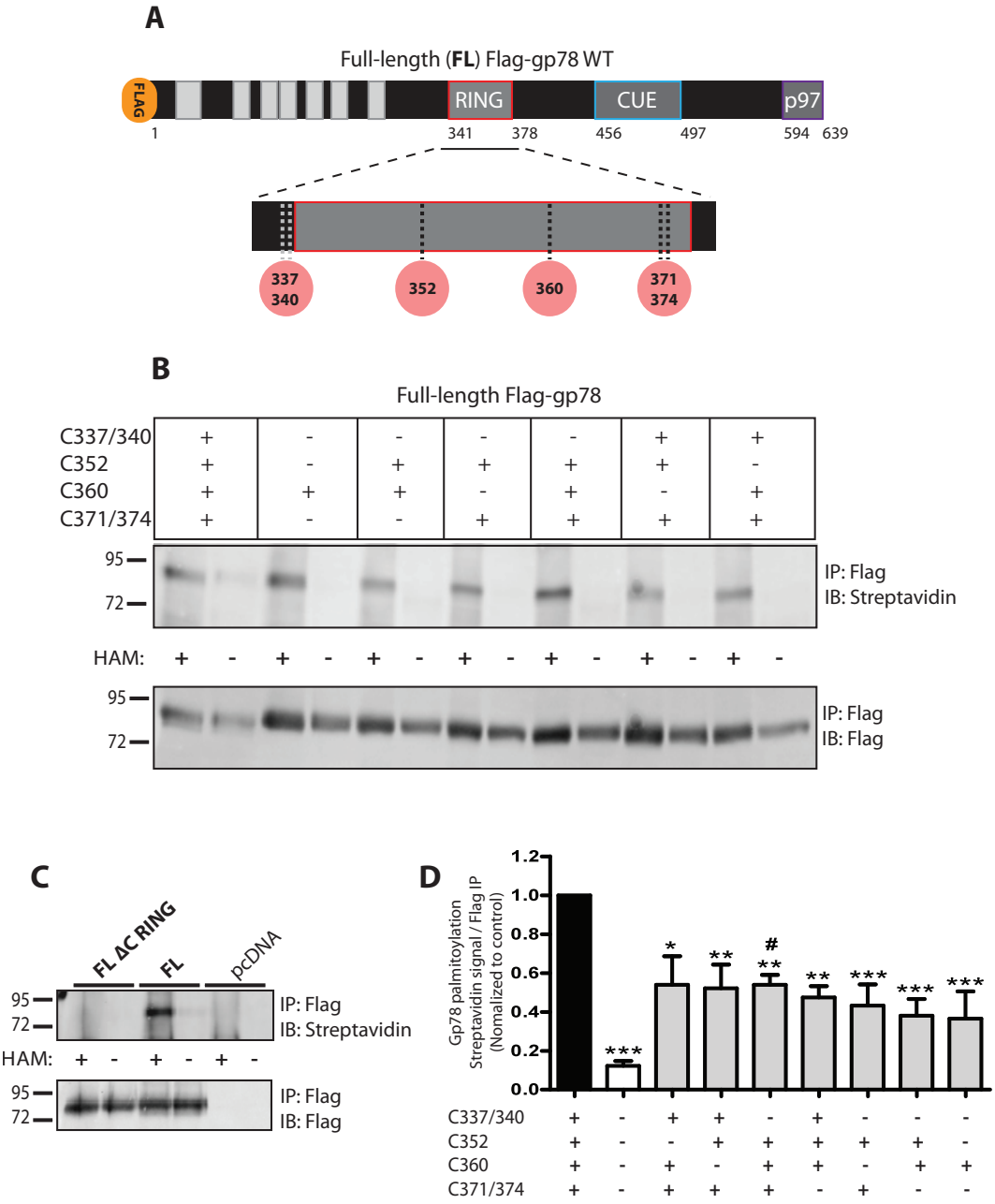

A
Full-length (FL) Flag-gp78 WT
FLAG
RING
CUE
p97
1
341
378
456
497
594
639
337
340
352
360
371
374
B
Full-length Flag-gp78
C337/340
C352
C360
C371/374
95
72
IP: Flag
IB: Streptavidin
HAM:
IP: Flag
IB: Flag
C
FL ΔC RING
FL
pcDNA
IP: Flag
IB: Streptavidin
IP: Flag
IB: Flag
D
Gp78 palmitoylation
Streptavidin signal / Flag IP
(Nomalized to control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C337/340
C352
C360
C371/374

Figure 2.3 Gp78/AMFR RING finger cysteine mutants show reduced ability to polyubiquitylate substrates compared to wild-type

A. In the *in vitro* ubiquitin ligase activity assay, Cos7 cells were transiently cotransfected with HA-tagged polyubiqutin (HA-Ub wt) and one of seven Flag-gp78/AMFR RING finger cysteine mutants. Cell lysates were collected for anti-Flag immunoprecipitation and immunoblots were probed for anti-HA, anti-actin and anti-Flag. B. Band densities of the polyubiquitylated substrate smear generated by each construct were first normalized to their anti-Flag protein levels, and then to wild-type gp78/AMFR. Mean ± S.E.M.; $^{*}P < 0.05$. The immunoblot is representative of three experiments.

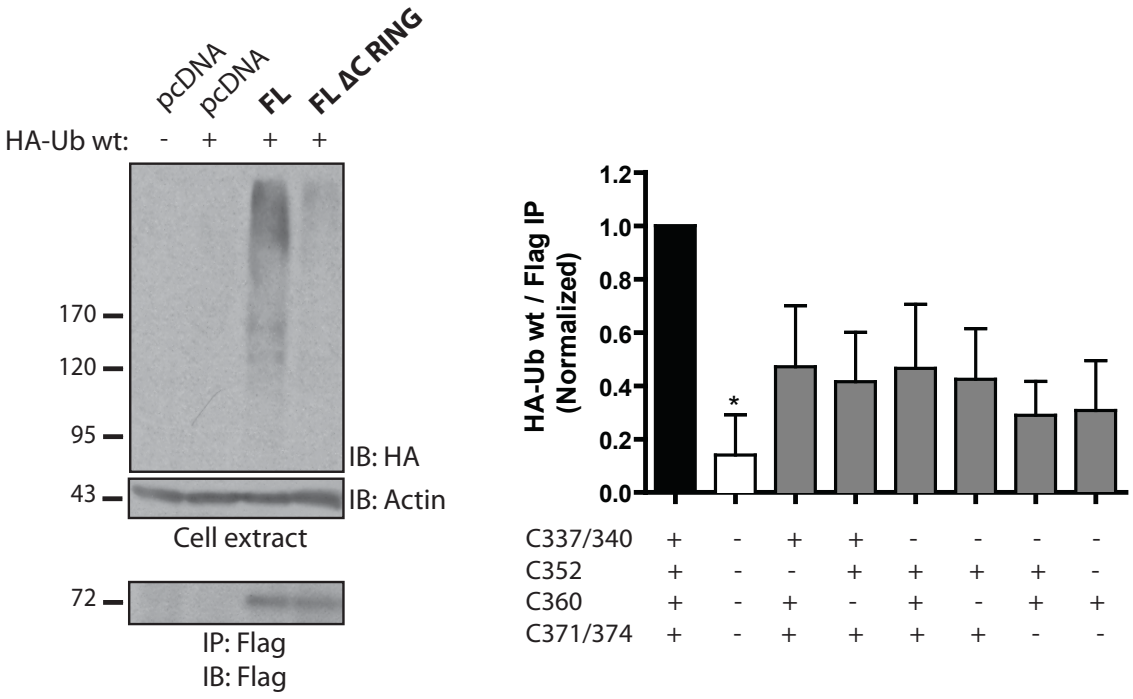


Figure 2.4 Gp78/AMFR is S-palmitoylated by several DHHC enzymes within the RING finger motif

A. Cos7 cells were transiently transfected with either full-length Flag-gp78/AMFR alone or with a Myc-tagged DHHC enzyme. Following anti-Flag immunoprecipitation, palmitoylation of Flag-gp78/AMFR was determined by the Biotin-BMCC labeling. Immunoblots were probed with fluorescently labeled streptavidin-cy5 (to detect biotin labeled proteins) and anti-Flag antibody followed by IRDye 800 mouse antibody (to detect Flag-tagged total proteins), and later imaged with the Odyssey Infrared Imaging System. B. Quantification of gp78/AMFR palmitoylation by individual DHHC enzymes as labeled by streptavidin-cy5 was first normalized to protein levels from anti-Flag immunoblots, and then to wild-type gp78/AMFR alone. C. Cos7 cells were transfected with the cysteine-free RING finger mutant (Flag-gp78/AMFR ΔC RING), in the presence and absence of Myc-DHHC6. Following anti-Flag immunoprecipitation and the Biotin-BMCC labeling, the Flag-gp78/AMFR ΔC RING palmitoylation signal was compared to wild-type gp78/AMFR alone. Mean ± S.E.M.; $^{***}P < 0.001$. The immunoblots are representative of three to five experiments.

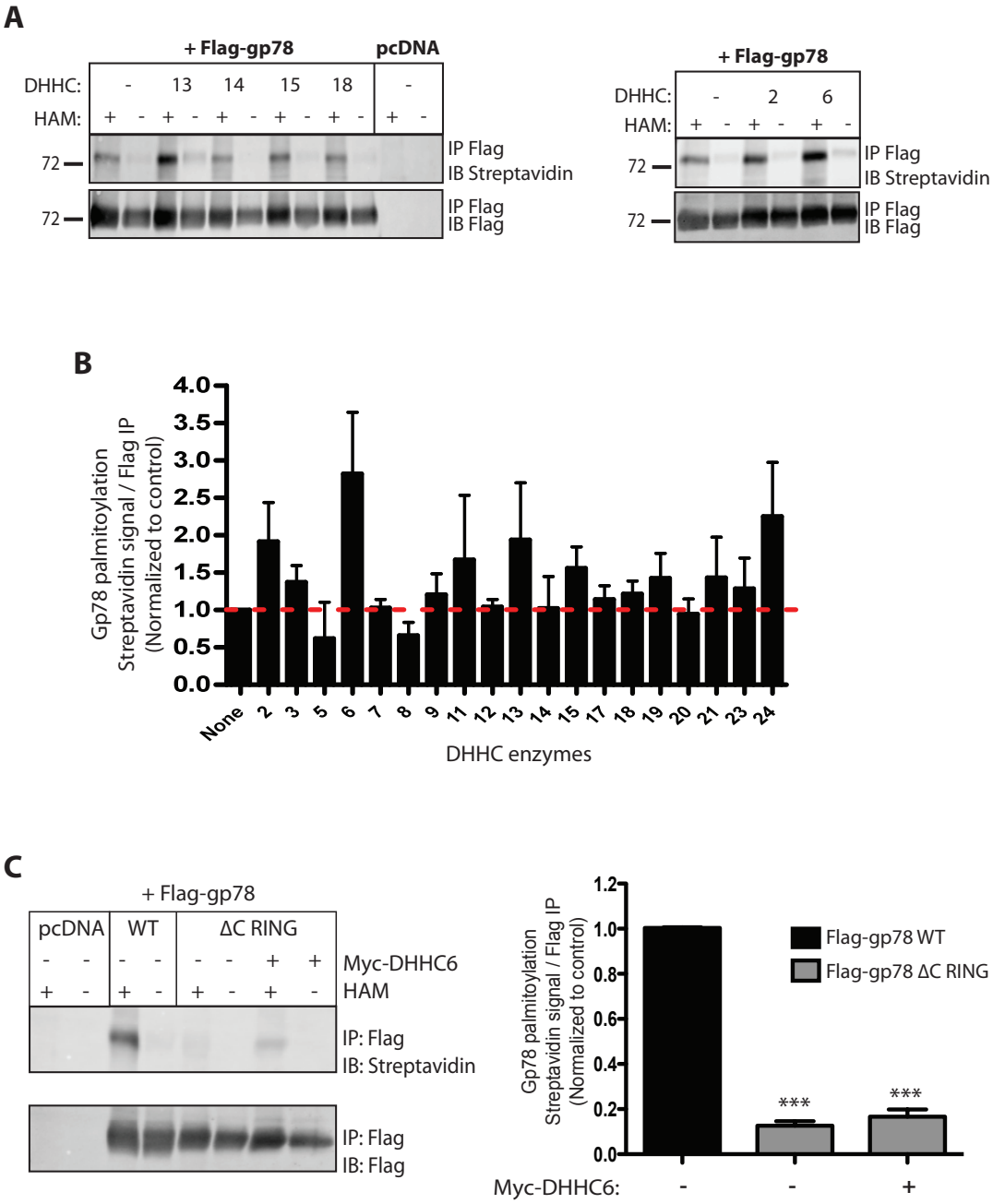

A
+ Flag-gp78
pcDNA
DHHC:
HAM:
72
IP Flag
IB Streptavidin
IP Flag
IB Flag
B
Gp78 palmitoylation
Streptavidin signal / Flag IP
(Normalized to control)
DHHC enzymes
C
pcDNA
WT
ΔC RING
Myc-DHHC6
HAM
IP: Flag
IB: Streptavidin
IB: Flag
Flag-gp78 WT
Flag-gp78 ΔC RING
***
Myc-DHHC6:

Figure 2.5 Gp78/AMFR RING finger palmitoylation by DHHC6 enhances its peripheral ER distribution

A. Cos7 cells were transiently transfected with a central ER marker (GFP-Sec61β) and one Myc-tagged DHHC enzyme alone (Myc-DHHC6 or Myc-DHHC19) or in the presence of either Flag-gp78/AMFR wild-type (WT) or the cysteine-free RING finger mutant (Flag-gp78/AMFR ΔC RING). Cells were fixed with methanol/acetone and images were acquired with the confocal microscope. Arrows indicate protein accumulation in the central ER. B. The percent intensity of Flag-gp78/AMFR (with and without DHHC enzyme) in the central ER was determined by using GFP-Sec61β as a mask. A One-way ANOVA followed by Tukey's multiple comparison tests was done. Mean ± S.E.M.; $^{*}P < 0.05$ compared to Flag-gp78/AMFR ΔC RING. 22-42 cells (for each condition) were imaged from five to six independent experiments. Scale bar = 20 micrometers; C. Cos7 cells were transiently transfected with Flag-gp78/AMFR WT in the presence and absence of Myc-DHHC6. The peripheral ER was identified by labeling endogenous gp78/AMFR with the 3F3A anti-gp78/AMFR antibody. Cells were fixed with methanol/acetone and images were acquired with the confocal microscope. Colocalization between proteins was assessed using Pearson's coefficient. Mean ± S.E.M.; An unpaired *t* test was done ($^{**}P < 0.01$). 18-24 cells (for each condition) were imaged from six independent experiments. Scale bar = 20 micrometers; zoom scale bar = 0.3 micrometers.

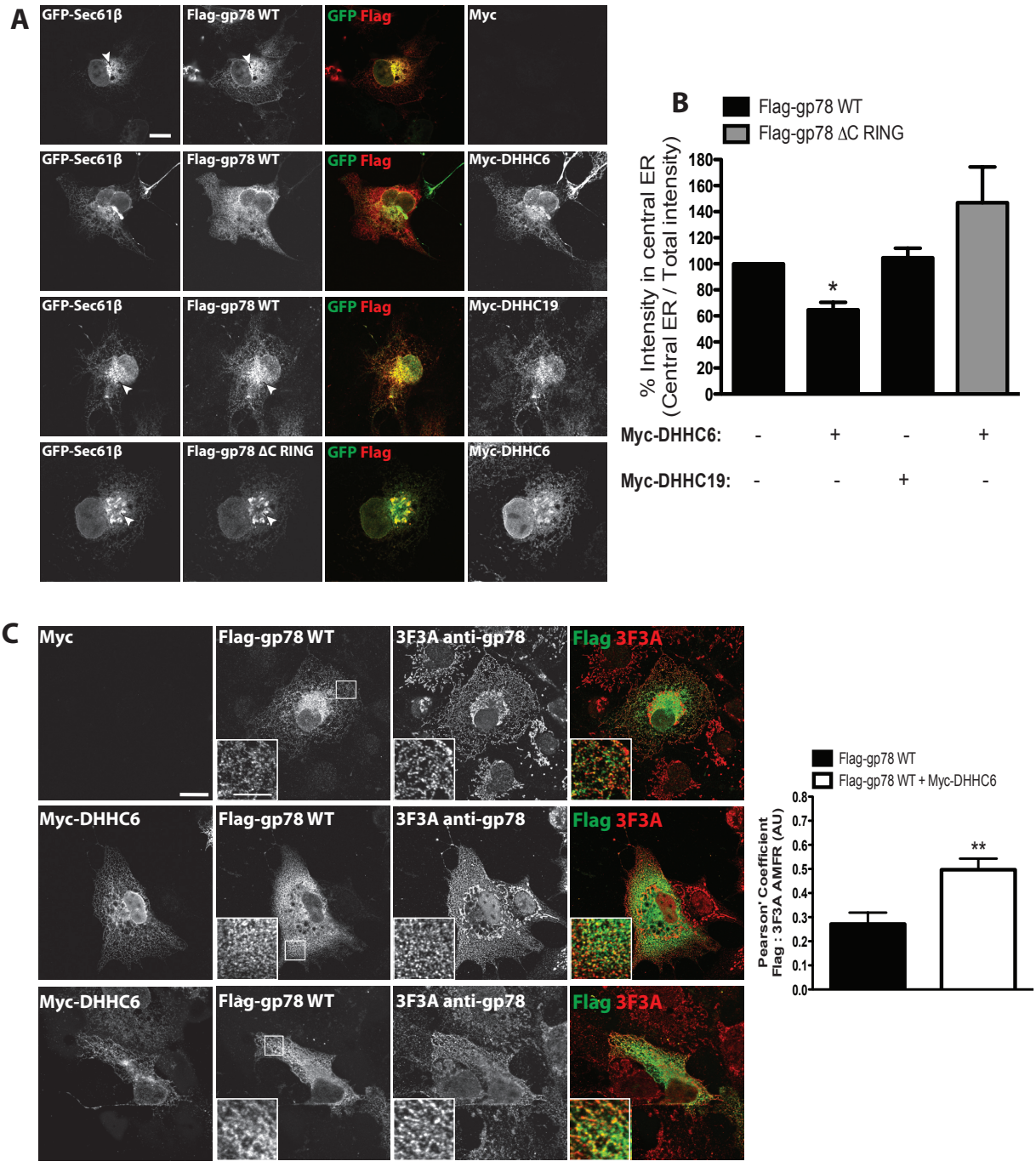

A
GFP-Sec61β
Flag-gp78 WT
GFP Flag
Myc
Myc-DHHC6
Myc-DHHC19
Flag-gp78 ΔC RING
B
Flag-gp78 WT
Flag-gp78 ΔC RING
% Intensity in central ER
(Central ER / Total intensity)
180
160
140
120
100
80
60
40
20
0
*
Myc-DHHC6: - + - +
Myc-DHHC19: - - + -
C
Myc
Flag-gp78 WT
3F3A anti-gp78
Flag 3F3A
Myc-DHHC6
Flag-gp78 WT + Myc-DHHC6
Pearson' Coefficient
Flag : 3F3A AMFR (AU)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**

Figure 2.6 Inhibition of palmitoylation by 2-bromopalmitate restricts gp78/AMFR to the central ER

A. The Biotin-BMCC labeling was used to determine the level of Flag-gp78/AMFR palmitoylation in Cos7 cells after a 6 h treatment with a solvent (DMSO) or a broad inhibitor of palmitoylation, 2-bromopalmitate (100 nM 2-BP). Following anti-Flag immunoprecipitation, immunoblots were probed with fluorescently labeled streptavidin-cy5 (to detect Biotin-BMCC-labeled proteins) and anti-Flag antibody followed by IRDye 800 mouse antibody (to detect Flag-tagged total proteins), and later imaged with the Odyssey Infrared Imaging System. Quantification of gp78/AMFR palmitoylation as labeled by streptavidin-cy5 was first normalized to protein levels from anti-Flag immunoblots, and then to wild-type gp78/AMFR alone. Mean ± S.E.M.; An unpaired *t* test was done ($^{*}P < 0.05$). The immunoblot is representative of three independent experiments. B. Cos7 cells were transiently cotransfected with the central ER marker GFP-Sec61β and Flag-gp78/AMFR WT in the presence and absence of Myc-DHHC6. 24 h following transfection, cells were treated for 6 h with 100 nM 2-BP and fixed with methanol/acetone. Images were acquired with the confocal microscope. Arrows indicate protein accumulation in the central ER. The percent intensity of Flag-gp78/AMFR in the central ER was determined by using GFP-Sec61β as a mask. A One-way ANOVA followed by Tukey's multiple comparison tests was done. Mean ± S.E.M.; $^{*}P < 0.05$ compared to Flag-gp78/AMFR WT. Scale bar = 20 micrometers. 27-41 cells (for each condition) were imaged from five to six independent experiments.

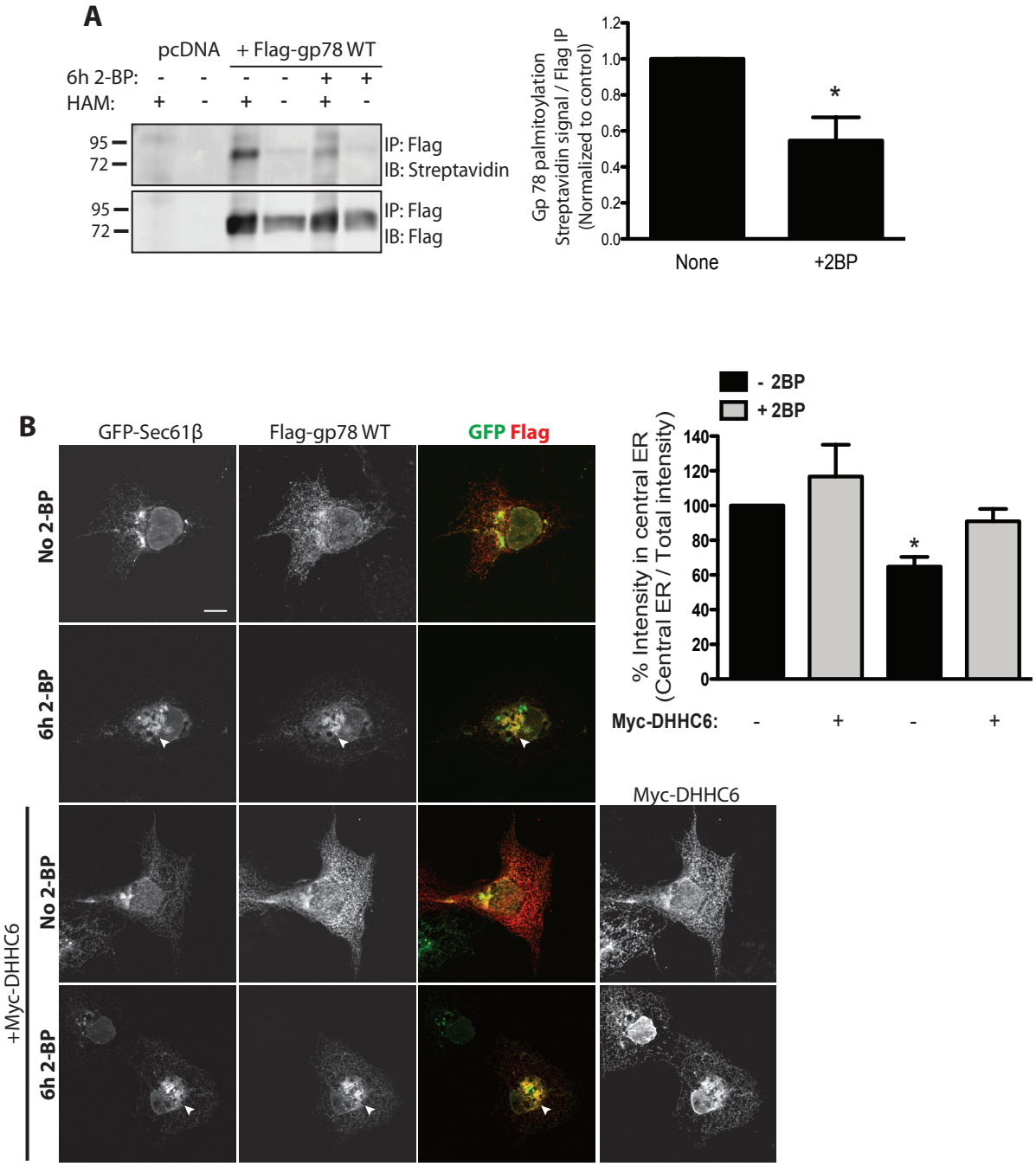
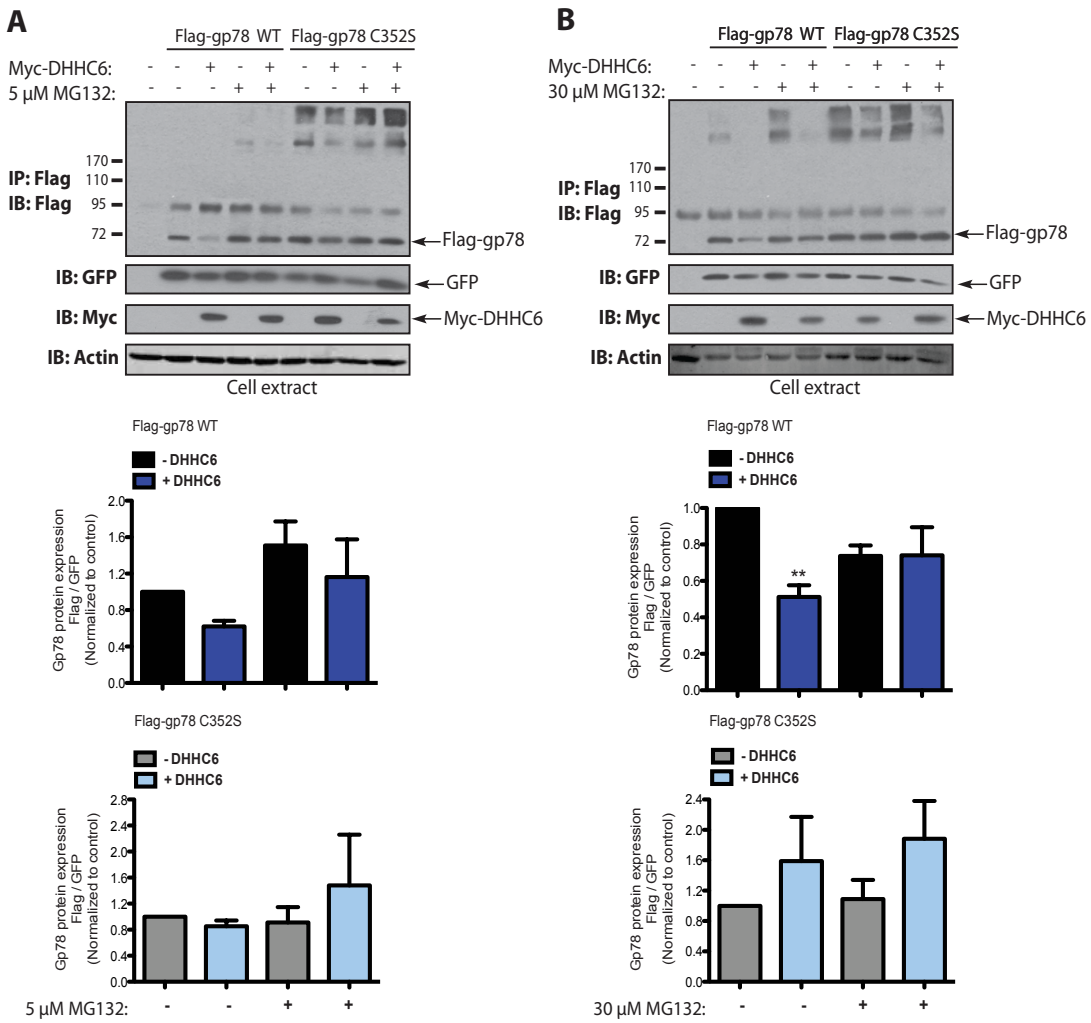

A
pcDNA
+ Flag-gp78 WT
6h 2-BP:
HAM:
95
72
IP: Flag
IB: Streptavidin
IP: Flag
IB: Flag
Gp 78 palmitoylation
Streptavidin signal / Flag IP
(Normalized to control)
None
+2BP
B
GFP-Sec61β
Flag-gp78 WT
GFP Flag
No 2-BP
6h 2-BP
+Myc-DHHC6
Myc-DHHC6
- 2BP
+ 2BP
% Intensity in central ER
(Central ER / Total intensity)
Myc-DHHC6:

Figure 2.7 Palmitoylation of the RING finger motif by DHHC6 targets gp78/AMFR for proteasomal degradation

Cos7 cells were cotransfected with either Flag-gp78/AMFR-IRES-GFP wild-type or Flag-gp78/AMFR C352S–IRES-GFP RING finger mutant in the presence and absence of Myc-DHHC6. 48 h post-transfection, cells were treated for 6 h with either (A) 5 μM or (B) 30 μM MG132 and lysates underwent anti-Flag immunoprecipitation and immunoblotting. Flag-gp78/AMFR protein levels were first normalized to GFP expression in cell extracts, and then to Flag-gp78/AMFR without DHHC6 in the anti-Flag immunoprecipitation. Mean ± S.E.M.; $^{**}P < 0.01$ compared to Flag-gp78/AMFR WT – Myc-DHHC6. The immunoblots are representative of three to five independent experiments.



CHAPTER 3 The E3 Ubiquitin Ligase Gp78/AMFR Recruits G Proteins to the ER

3.1 Summary

G protein-coupled receptors (GPCRs) are seven transmembrane-spanning proteins that are well-known for their ability to translate changes from the extracellular environment into an intracellular response via heterotrimeric guanine-nucleotide binding proteins (G proteins). GPCRs are abundant on the plasma membrane however they can also localize to other intracellular membrane compartments, where they participate in diverse signaling pathways. Here, we show in Cos7 cells that Flag-gp78/AMFR, an E3 ubiquitin ligase and a putative GPCR, coimmunoprecipitates in a complex with different GFP-tagged G protein subunits (Gαi1, Gαs, Gβ1, Gγ2). Moreover, all the G protein subunits colocalize with Flag-gp78/AMFR in the ER, including GFP-Gαi1 that is recruited from the plasma membrane to the ER in Flag-gp78/AMFR overexpressing cells. A similar G protein interaction is observed with the N-terminal transmembrane domain of Flag-gp78/AMFR in both coimmunoprecipitation and immunocytochemistry experiments. Finally, we investigated a role for G proteins in the ER-associated degradation (ERAD) pathway by expressing a Flag-gp78/AMFR C352S RING finger mutant that is deficient in its ubiquitin ligase activity. Looking at protein distribution in Cos7 cells, the GFP-Gαs subunit is the only G protein that shows significantly less recruitment to the ER by the Flag-gp78/AMFR RING finger mutant. However, GFP-Gαs remains associated with the Flag-gp78/AMFR RING finger mutant in the anti-Flag immunoprecipitation, raising the question of whether Gαs is directly interacting with gp78/AMFR or recruited indirectly by another protein to the same ER complex. Taken together, our data suggest a novel role for G proteins in the ERAD pathway and explore the possibility that an E3 ubiquitin ligase can be identified as a GPCR in the ER via its association with two opposing G proteins, Gαi1 and Gαs.

3.2 Introduction

Protein folding in the endoplasmic reticulum (ER) is carefully monitored by ER Quality Control (ERQC) mechanisms (Ellgaard and Helenius, 2003). Misfolded, non-functional and misassembled proteins are directed to the endoplasmic reticulum-associated degradation (ERAD) pathway where they are recognized, targeted for retrotranslocation to the cytoplasm and degraded by the ubiquitin-proteasome machinery (Nakatsukasa and Brodsky, 2008). Most ERAD substrates are ubiquitylated prior to proteasome-dependent degradation and require E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases. Following polyubiquitylation, substrates are extracted from the membrane by a p97/VCP/Cdc48 - dependent protein complex and targeted to the proteasome. The transfer of targeted proteins from the ER to the cytosol is dependent upon ubiquitin chain elongation, interaction with the CUE domain (ubiquitin-binding domain) of an E3 ubiquitin ligase and p97/VCP/Cdc48 recruitment (Pickart, 2001; Vembar and Brodsky, 2008). Thus, the ERAD pathway is complex and vital in preventing misfolding-induced toxicity (Coughlan and Brodsky, 2005), in which cellular stress can disrupt ER function and homeostasis resulting in accumulation of non-functional proteins within the ER.

E3 ubiquitin ligases are an integral component of a large protein complex essential for the proper function of the ERAD pathway. Two mammalian E3s, gp78 and Hrd1, have been found to be the principal E3 ligases associated with ERAD and may even cooperate with each other to degrade their substrates (Christianson et al., 2012; Kostova et al., 2007). Gp78 (also known as the Autocrine Motility Factor Receptor, AMFR) was first characterized as an E3 ubiquitin ligase based on its conserved C-terminal domain that includes the CUE motif, responsible for ubiquitin binding, and the catalytic RING finger domain that mediates its ubiquitin ligase activity (Ponting, 2000; Shimizu et al., 1999). However, gp78/AMFR is not restricted to the ER and a small proportion is found on the cell surface where it acts as a cytokine receptor and stimulates cell motility upon binding to its ligand, the Autocrine Motility Factor (AMF)/Phosphoglucose Isomerase (PGI) (AMF/PGI) (Fairbank et al., 2009). The expression of both AMF/PGI and gp78/AMFR has been associated with increased metastasis development and poor prognosis in cancer patients (Chiu et al., 2008). Yet,

little is known about the mechanism underlying the AMF/PGI - gp78/AMFR protein interaction and factors such as AMF/PGI sugar binding and catalytic domains, and gp78/AMFR glycosylation have all been proposed to mediate their binding (Haga et al., 2006a). Interestingly, there is evidence to suggest the involvement of a pertussis-toxin sensitive G protein in the signal transduction pathway downstream of AMF/PGI-stimulated cell motility via its receptor gp78/AMFR (Stracke et al., 1987). These data and others are derived from the use of the Bordella pertussis toxin that is responsible for the inactivation of specific G proteins belonging to the Gαi and Gαo protein family (namely, Gαi1-3 and Gαo, respectively) (Kohn et al., 1990; Milligan et al., 1990; Nabi et al., 1990). Pretreatment with pertussis toxin was shown to inhibit motility induced by the anti-gp78/AMFR 3F3A mAB in B16-F1 cells (Nabi et al., 1990), as well as significantly reduce AMF/PGI-promoted cell motility and IP3 production in A2058 human melanoma cells (Kohn et al., 1990). Moreover, pertussis toxin inhibited proliferation and locomotion of BALB/c 3T3-A31 murine fibroblasts in the presence of purified AMF/PGI from HT-1080 human fibrosarcoma or B16-F1 murine melanoma cells (Silletti and Raz, 1993). As of yet, gp78/AMFR topology remains undetermined but the receptor shows significant structural similarity to a hypothetical GPCR in Caenorhabditis elegans, called F26E4.11 (Shimizu et al., 1999). Based on different online softwares and hydrophobic analysis of its amino acid sequence, gp78/AMFR may contain five or six or seven transmembrane domains (Fairbank et al., 2009; Ponting, 2000), raising the possibility that it may be a putative GPCR.

GPCRs are the largest class and the most diverse type of cell surface receptors, consisting of an amino-terminal extracellular domain, seven hydrophobic transmembrane domains, and a carboxyl-terminal intracellular domain (Kristiansen, 2004; Schioth and Fredriksson, 2005). The signal transduction pathway is initiated by binding of a specific ligand to the receptor and both the GTP-bound Gα subunit and the Gβγ complex promote downstream signaling by stimulating diverse effector molecules. Here, we begin to characterize gp78/AMFR as a putative GPCR and demonstrate its ability to interact with and recruit multiple G protein subunits to the ER via its N-terminal transmembrane domain. Thus, we speculate that G proteins, in particular Gαs, may possess a function in the ER and represent novel components in the ERAD pathway.

3.3 Materials and methods

3.3.1 Plasmids and constructs

Wild-type Flag-gp78/AMFR was inserted into pcDNA 3.1 (+) as described in (Registre et al., 2004). Flag-gp78/AMFR RING finger mutant (C352S) was done using site-directed mutagenesis and characterized in (St. Pierre et al., 2011). The Flag-gp78/AMFR N-terminal transmembrane domain construct was generated by substituting L330 for a stop codon. GFP-Gαs expression plasmid was kindly provided by Dr. Mark M. Rasenick (University of Illinois). GFP-Gαi1, GFP-Gβ1 and GFP-Gγ2 were a gift from Dr. Terence Hebert (McGill University). The pEGFP expression vector was purchased from Clontech.

3.3.2 Antibodies

Anti-Flag M2, anti-calnexin and anti-βactin were purchased from Sigma. Anti-GFP was obtained from Synaptic Systems, anti-HA from Neomarker and anti-GM130 from Abcam. Fluorescently conjugated secondary antibodies (highly cross-absorbed Alexa488, Alexa568 and Alexa647) for immunocytochemistry were purchased from Invitrogen. HRP-conjugated IgG secondary antibodies were purchased from Jackson Immunoresearch.

3.3.3 Cell culture and transfection

Cos7 cells were maintained in DMEM containing 10% fetal bovine serum (FBS, MediCorp.), and supplemented with 0.2 mM L-Glutamine, 10 μg/ml Penicillin, 10 μg/ml Streptomycin, vitamins and non-essential amino acids (all supplements were purchased from Invitrogen). For transient transfection of plasmid DNA, Effectene transfection reagent (Qiagen) was used as described by the manufacturer. The general inhibitor of palmitoylation 2-bromohexadecanoic acid (2-bromopalmitate [2-BP]) (Fluka) was used.

3.3.4 Immunoprecipitation and immunoblotting

For anti-Flag immunoprecipitation, Cos7 cells were rinsed and manually collected in cold phosphate-buffered saline (PBS). Cell pellets were resuspended in ice-cold lysis

buffer A [20 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, 0.1% SDS and 1 mg/ml protease inhibitor cocktail (Roche)]. Following a 30 minute rotation at 4°C, 1% Triton X-100 was added to each sample and incubated for an additional 30 minutes. Lysates were passed through a 25G syringe 6 times and the insoluble material was removed by centrifugation. Supernatants were incubated overnight with anti-Flag coupled with agar beads (Sigma) that have been pre-blocked with 1% BSA (1 h at 4°C). Beads were then washed 3 times with wash buffer A [20 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, 0.2% BSA], 3 times with wash buffer B [20 mM Tris-HCl pH 8, 500 mM NaCl, 0.5% Triton X-100, 0.2% BSA] and one time with wash buffer C [50 mM Tris-HCl pH 8]. For anti-GFP immunoprecipitation, cell pellets were resuspended in ice-cold lysis buffer B [50 mM Tris-HCl pH 7, 150 mM NaCl, 1% Triton X-100] and precleared by incubation with protein A+G agar beads (GE Healthcare) for 45 minutes at 4°C with rocking. Precleared samples were immunoprecipitated with anti-GFP antibody (3 μg, rabbit) conjugated with protein A+G agar beads. For both immunoprecipitations, proteins in cell lysates and immunoprecipitates were denatured by boiling in SDS sample buffer, and later separated by SDS-PAGE, transferred onto PVDF membranes (Pall Corporation), blocked with 5% nonfat milk and probed with appropriate antibodies. Proteins were visualized either by Bioflex film (Clonex Corporation) or the Odyssey Infrared Imaging System (Li-COR Biosciences).

3.3.5 Immunocytochemistry and imaging

Cells were grown on glass cover slips and 24 h post-transfection, cells were fixed with pre-cooled (-80°C) methanol:acetone (80:20%) for 15 minutes at -20°C. Cells were washed 3 times for 15 minutes with PBS supplemented with 1 mM $CaCl_2$ and 10 mM $MgCl_2$ (PBS-CM). Cells were first blocked with PBS-CM containing 1% Bovine Serum Albumin (BSA) (blocking solution) for 15 minutes and all the antibody incubations were performed in blocking solution. Cells were then incubated with primary antibodies for 30 minutes, rinsed 3 times for 2 minutes with blocking solution, incubated with the corresponding species-specific fluorescently-conjugated secondary antibodies for an additional 30 minutes, and finally rinsed three times for 10 minutes with blocking solution. Cover slips were mounted on microscope slides with Celvol 205 (Polyvinyl

alcohol, Celanese Chemicals Ltd.) and imaged with the 100x (NA 1.4) plan apochromatic objective of a Fluoview 1000 confocal laser scanning microscope (Olympus).

3.3.6 Statistical analysis

For fluorescence quantification experiments, colocalization between two proteins is expressed by Pearson's correlation measurements from at least three independent experiments and was calculated using the ImagePro analysis software (Media Cybernetics). Refer to section 2.3.9 for more details.

3.4 Results

3.4.1 Gp78/AMFR is found in a complex with G protein subunits

Gp78/AMFR is a multi-spanning transmembrane receptor whose E3 ubiquitin ligase activity has been well-described in the ERAD pathway and shown to be mediated by its C-terminal domain (Chen et al., 2006) (Fig. 3.1A). However, the topology of its transmembrane domain is undefined and there is evidence that gp78/AMFR may be associated with a pertussis-toxin sensitive G protein downstream of its ligand AMF/PGI on the cell surface (Fairbank et al., 2009; Nabi et al., 1990). Thus, we began to look at its interaction with four GFP-tagged G proteins (GFP-Gαi1, GFP-Gαs, GFP-Gβ1 and GFP-Gγ2) in Cos7 cells. We observe in the anti-Flag immunoprecipitation that all GFP-G protein subunits have a similar ability to interact with full-length Flag-gp78/AMFR (Fig. 3.1B). Moreover, G protein binding occurs in the absence of the C-terminal domain of Flag-gp78/AMFR and is mediated by its N-terminal transmembrane domain (Fig. 3.1B). Concurrently, we also performed an anti-GFP immunoprecipitation and under these conditions, we observe that GFP-Gαs is the only G protein that associates with full-length Flag-gp78/AMFR and the Flag-tagged N-terminal transmembrane domain of gp78/AMFR no longer binds to any GFP-G proteins (Fig. 3.1C), contrary to the result observed in the anti-Flag immunoprecipitation (Fig. 3.1B). It is unclear whether gp78/AMFR directly interacts with any of the G proteins and we are unable to characterize G protein binding to gp78/AMFR using cell lysate immunoprecipitation experiments. Together, these data imply that gp78/AMFR exists in a complex with at

least two Gα subunits and the Gβγ subunit, in which Gαs seems to be the most consistent gp78/AMFR binding partner.

3.4.2 Gp78/AMFR interacts with G proteins in the ER domain

Full-length Flag-gp78/AMFR alone is localized to the ER as labeled by the anti-calnexin antibody (Fig. 3.2A), while the N-terminal transmembrane domain that lacks the C-terminus is retained in the Golgi as marked by the anti-GM130 antibody (Fig. 3.2B). Moreover, treatment with the broad inhibitor of palmitoylation 2-bromopalmitate, induces translocation of the N-terminal transmembrane domain of Flag-gp78/AMFR from the Golgi to the calnexin-labeled ER (Fig. 3.2C). These data reinforce the observation in chapter 2 that the N-terminal transmembrane domain of Flag-gp78/AMFR undergoes palmitoylation (Fig. 2.1 and Fig. A.1) and here, we show that this posttranslational modification can alter its intracellular distribution. The significance of this finding has yet to be determined and perhaps the N-terminal transmembrane domain construct can later be used as a tool to gain insight into gp78/AMFR protein trafficking.

To look at the distribution of G proteins with gp78/AMFR, we coexpressed either full-length or the N-terminal transmembrane domain (TMD) of Flag-gp78/AMFR with each GFP-G protein and fixed the cells with a very cold methanol/acetone mixture (discussed in section 4.4.1). We used Pearson's coefficient as a measure of colocalization between Flag-gp78/AMFR and each GFP-G protein subunit expressed in Cos7 cells. Both full-length and the TMD of Flag-gp78/AMFR extensively colocalize with all four GFP-G proteins (Gαi1, Gαs, Gβ1, Gγ2) within the ER domain (Fig. 3.4C). We focused on the two GFP-Gα subunits, since GFP-Gβ1 and GFP-Gγ2 were expressed independently and we were unable to look at the complete GFP-Gβγ complex with Flag-gp78/AMFR. In the presence of full-length Flag-gp78/AMFR, GFP-Gαi1 that is normally present on the plasma membrane (Fig. 3.3A) translocates to and is highly enriched in the calnexin-labeled ER, where it colocalizes with Flag-gp78/AMFR (Fig. 3.3B). Similarly, GFP-Gαi1 is recruited by the TMD of Flag-gp78/AMFR but in this case, both proteins are primarily colocalized in the Golgi compartment (Fig. 3.3C). Finally, we observe that GFP-Gαs is also closely associated with both full-length and the TMD of Flag-gp78/AMFR in the calnexin-labeled ER (Fig. 3.4A - 3.4B). Thus, GFP-

Gαi1 and GFP-Gαs do not change the distribution of Flag-gp78/AMFR but are themselves recruited to the ER domain where they remain in close contact with Flag-gp78/AMFR via its N-terminal transmembrane domain. These findings are consistent with the anti-Flag immunoprecipitation experiment (Fig. 3.1A), supporting that gp78/AMFR is indeed associated with two Gα subunits. As of yet, the functional consequence of the different G protein interactions with gp78/AMFR remains unknown.

3.4.3 GFP-Gαs is only partially recruited by the full-length Flag-gp78/AMFR RING finger mutant to the central ER

Consistent with previous findings (Fairbank et al., 2009; Nabi et al., 1990), we report that gp78/AMFR colocalizes with the pertussis-toxin sensitive Gαi1 subunit in the ER (Fig. 3.3). In addition, we also show that gp78/AMFR colocalizes with the stimulatory subunit Gαs (Fig. 3.4) and based on the latter, we decided to investigate whether the Gαs ER distribution is dependent upon gp78/AMFR ubiquitin ligase activity. We used a Flag-gp78/AMFR RING finger mutant construct that contains an inhibitory point mutation in the third cysteine (C352S) and this mutation has been shown to significantly reduce its ubiquitin ligase activity (St. Pierre et al., 2011). Compared to wild-type Flag-gp78/AMFR, GFP-Gαs shows a significantly lower colocalization coefficient with the Flag-gp78/AMFR RING finger mutant, indicating that it is no longer being recruited to the ER and its protein association is partly dependent on gp78/AMFR ubiquitin ligase activity (Fig. 3.5A). This phenomenon seems to be specific to GFP-Gαs and is not observed with GFP-Gβ1 that remains colocalized with both wild-type Flag-gp78/AMFR and the Flag-gp78/AMFR RING finger mutant (Fig. 3.5B). The mechanism describing the observed difference in Gαs distribution is unclear, however GFP-Gαs still remains associated with both wild-type Flag-gp78/AMFR and the Flag-gp78/AMFR RING finger mutant in the anti-Flag immunoprecipitation (Fig. 3.5C). As discussed above, it is unclear whether Gαs binds directly to Flag-gp78/AMFR but rather, that both proteins are found in the same ER complex. It is possible that the Flag-gp78/AMFR RING finger mutant disrupts the ERAD complex and leads to the dissociation of Gαs from either gp78/AMFR itself or an adaptor protein that is unable to bind to the Flag-gp78/AMFR RING finger mutant. This change would not be detected in the anti-Flag

immunoprecipitation but is supported by the confocal images whereby Gαs appears to display less colocalization with the Flag-gp78/AMFR RING finger mutant (Fig. 3.5A). In light of these findings, we can only speculate a role for Gαs in the ERAD pathway that may dependent on gp78/AMFR or another ERAD protein.

3.5 Discussion

Previous studies have suggested that gp78/AMFR is coupled to a pertussis-toxin sensitive G protein in the signal transduction pathway downstream of AMF/PGI-stimulated cell motility via its receptor gp78/AMFR (also discussed in section 1.4.1). Here, we demonstrate that Flag-gp78/AMFR interacts with GFP-Gαi1 in the anti-Flag immunoprecipitation (Fig. 3.1A). Moreover, both full-length and the N-terminal transmembrane domain of Flag-gp78/AMFR are able to recruit GFP-Gαi1 from the plasma membrane to the ER (Fig. 3.3). In each case, this protein interaction occurs in the absence of receptor activation and thus may act independently from the adenylyl cyclase pathway. This has been demonstrated in the human melanoma cell line A2058 whereby pertussis toxin inhibited AMF-induced motility in a time- and dose-dependent manner in the absence of adenylyl cyclase activation (Stracke et al., 1987). The mechanism underlying the gp78/AMFR - Gαi1 complex and most importantly, the significance of Gαi1 recruitment by gp78/AMFR to the ER remains unknown. Nevertheless, these findings are in agreement with previous studies, supporting the idea that gp78/AMFR is coupled to a pertussis-toxin sensitive G protein such as the Gαi1 subunit. As of now, we can only speculate that Gαi1 is implicated in the cell surface expression of gp78/AMFR and the signal transduction pathway downstream of AMF/PGI binding. To further investigate the possibility that Gαi proteins regulate gp78/AMFR cell surface expression, we can label cells with the 3F3A anti-gp78/AMFR monoclonal antibody and use cell surface fluorescence-activated cell sorting (FACS) analysis (Kojic et al., 2007). With this approach, we are able to study how the absence of Gαi (via transient siRNA transfection) alters both gp78/AMFR cell surface expression and FITC-AMF/PGI endocytosis (discussed in sections 1.2.4 and 1.2.5).

In addition to Gαi1, gp78/AMFR associates with a second Gα subunit, Gαs. In fact, the interaction between Flag-gp78/AMFR and GFP-Gαs is consistently observed in

both anti-Flag and anti-GFP immunoprecipitations (Fig. 3.1), which is not the case with GFP-Gαi1. It is difficult to discuss whether Gαi1 binds directly to gp78/AMFR since the lack of association between GFP-Gαi1 and Flag-gp78/AMFR in the anti-GFP immunoprecipitation may be an indication of lower protein affinity or indirect binding. In the current study, we show that gp78/AMFR binds to both Gαi1 and Gαs, however it is unknown whether binding of endogenous AMF/PGI is a critical factor in mediating this protein interaction. One approach to look at receptor activation following ligand binding is to assess gp78/AMFR binding to constitutively active Gα proteins. The issue of receptor activation should be addressed, since treatment with the ligand AMF/PGI may alter gp78/AMFR conformation and enhance Gαi1 binding, thus revealing a difference between Gαi1 and Gαs in terms of their placement within the gp78/AMFR signaling pathway.

The implication of Gαs in gp78/AMFR function remains unclear, however this interaction may depend upon the catalytic RING finger domain. Here, we observe significantly reduced ER colocalization between GFP-Gαs and the Flag-gp78/AMFR RING finger mutant compared to wild-type (Fig. 3.5A), despite the fact that both proteins remain in a complex in the anti-Flag immunoprecipitation (Fig. 3.5C). These findings imply that gp78/AMFR ubiquitin ligase activity affects its association with Gαs, whether it is direct or mediated by an unidentified adaptor protein. Thus, this is the first report that Gαs may be a component in the ERAD pathway and this possibility is further explored in chapter 4.

We show that GFP-Gαs associates with full-length Flag-gp78/AMFR in Cos7 cells and as seen with GFP-Gαi1, this interaction appears to be mediated by the transmembrane domain of Flag-gp78/AMFR (Fig. 3.4). This is not only evident in the anti-Flag immunoprecipitation (Fig. 3.1B), but also in the immunocytochemistry experiments whereby the transmembrane domain of Flag-gp78/AMFR recruits both GFP-Gαi1 and GFP-Gαs to the Golgi (Fig. 3.3C and Fig. 3.4B). The Golgi distribution of the N-terminal transmembrane domain of Flag-gp78/AMFR may supply a clue into gp78/AMFR trafficking, in particular a role for palmitoylation. Here, we show that following treatment with a broad inhibitor of palmitoylation 2-bromopalmitate, the N-terminal transmembrane domain of gp78/AMFR translocates from the Golgi to the

calnexin-labeled ER, suggesting that palmitoylation sequesters the protein in the Golgi compartment (Fig. 3.2). However, the palmitoylation of the N-terminal transmembrane domain remains uncharacterized (discussed in chapter 2) and the significance of its localization remains unclear, since we cannot exclude the possibility that the Golgi distribution is due to accumulation of a misfolded, non-functional truncated protein. As discussed in section 1.3, many GPCRs undergo palmitoylation and in chapter 2, we show that gp78/AMFR possesses two types of palmitoylation, the first localized within the transmembrane domain and the other in the RING finger motif. Thus, we cannot ignore the relationship between palmitoylation and G protein binding, whereby palmitoylation may affect Gα binding to gp78/AMFR. To determine whether RING finger S-palmitoylation mediates G protein binding, we can look at the interaction between GFP-Gαi1/Gαs subunits and the full-length RING ΔC Flag-gp78/AMFR construct (deficient in S-palmitoylation) using an anti-Flag immunoprecipitation. Assuming that the protein interaction between gp78/AMFR and Gα is direct, we can speculate that Gα will show reduced affinity for the RING ΔC Flag-gp78/AMFR construct. This would further reinforce that gp78/AMFR is an ER-localized GPCR in which palmitoylation is a mechanism that modulates (and selects for) G protein binding, perhaps switching its function towards a cytokine receptor on the cell surface. As of now, we can report that two Gα subunits associate with gp78/AMFR via its transmembrane domain, and this is in agreement with other studies, showing that G proteins bind to the intracellular loops within the transmembrane domains of GPCRs (Kostenis et al., 1998).

To address the controversy surrounding the identity of gp78/AMFR as an ER-localized GPCR, it is necessary to first determine whether gp78/AMFR directly binds to any Gα subunit and future studies should consist of *in vitro* direct protein-protein binding assays. One possible approach is to use purified HIS-tagged Gαi1/Gαs proteins from bacteria and isolate membranes from Sf9 insect cells that have been infected with baculovirus encoding c-myc-tagged gp78/AMFR receptor as described in (Cladman and Chidiac, 2002). For the protein-protein binding assay, we can detect the interaction between myc-gp78/AMFR and the HIS-Gα subunit by performing an anti-HIS immunoprecipitation, followed by an anti-myc immunoblot. Moreover, G proteins can be activated with the addition of [$AlCl_3$, $MgCl_2$ and NaF] into the lysis buffer. These

compounds induce a conformation (Gα-GDP-AlF^-_4) thought to mimic the transitional state of Gα subunit bound to the gamma phosphate of GTP (i.e. activated Gα protein) (Tesmer et al., 1997). This approach will determine whether gp78/AMFR has a higher affinity for the GDP-AlF^-_4 activated form of Gα proteins, suggesting that gp78/AMFR binding to Gα may be dependent upon its ligand AMF/PGI. Together, these data will finally characterize the ability of gp78/AMFR to bind to one or more Gα subunit(s). We can only speculate that differential Gαi1 and Gαs binding may be a determining factor in modulating gp78/AMFR activity and intracellular distribution, specifically between its cell surface interaction with AMF/PGI and its ERAD function within the ER (Fairbank et al., 2009).

In summary, we demonstrate that gp78/AMFR can interact with more than one G protein. In fact, this is seen with several GPCRs that fluctuate between two inactive conformational states and bind to two different G protein subunits (Harding and Gong, 2004; Kenakin, 2001). The α2-adrenergic receptor (α2-AR) can activate both Gαs and Gαi signaling pathways in the heart in which PKA-mediated phosphorylation of α2-AR switches the coupling preference of the receptor from Gαs to Gαi (Daaka et al., 1997; Xiao, 2001). Thus, GPCRs can elicit multiple signals and activate diverse subcellular pathways by adopting ligand-specific receptor active states. In the case of gp78/AMFR, we can speculate that the differential Gαi1 and Gαs binding may be a determining factor in modulating its activity and distribution (Fairbank et al., 2009). As discussed in section 1.4, GPCRs consist of diverse types of receptors but one common feature is their topology. In the case of gp78/AMFR, it does not show homology to any other GPCR and does not contains an 'E/DRY' motif governing receptor conformation and G protein coupling/recognition (Rovati et al., 2007). Further, the number of transmembrane domains has yet to be determined. It was suggested that gp78/AMFR only contains five transmembrane domains (Ponting, 2000), however based on hydrophobic analysis of the amino acid sequence, gp78/AMFR can also resemble a 6 or 7 transmembrane domain protein (Scheme 1.2.2B). Thus, gp78/AMFR may represent a non-conventional GPCR or may bind to a GPCR both at the cell surface and in the ER. As of yet, the impact of G proteins on gp78/AMFR function is under investigation and remains controversial.

Figure 3.1 Flag-gp78/AMFR is in a protein complex with several GFP-G protein subunits

A. Schematic of gp78/AMFR constructs used in the immunoprecipitation experiments. B-C. Cos7 cells were transiently cotransfected with different GFP-G protein subunits (Gαi1, Gαs, Gβ1, Gγ2) and either pcDNA3.1 vector (control for B) or GFP alone (control for C) or full-length Flag-gp78/AMFR wild-type or the N-terminal Flag-tagged transmembrane domain of gp78/AMFR (TMD). 24 h post-transfection, cells were collected, lysed and incubated with anti-Flag beads (B) or anti-GFP beads (C) for immunoprecipitation (IP). Immunoblots for anti-GFP or anti-Flag were done respectively to assess protein-protein interactions and IP efficiency. The immunoblots are representative of three independent experiments.

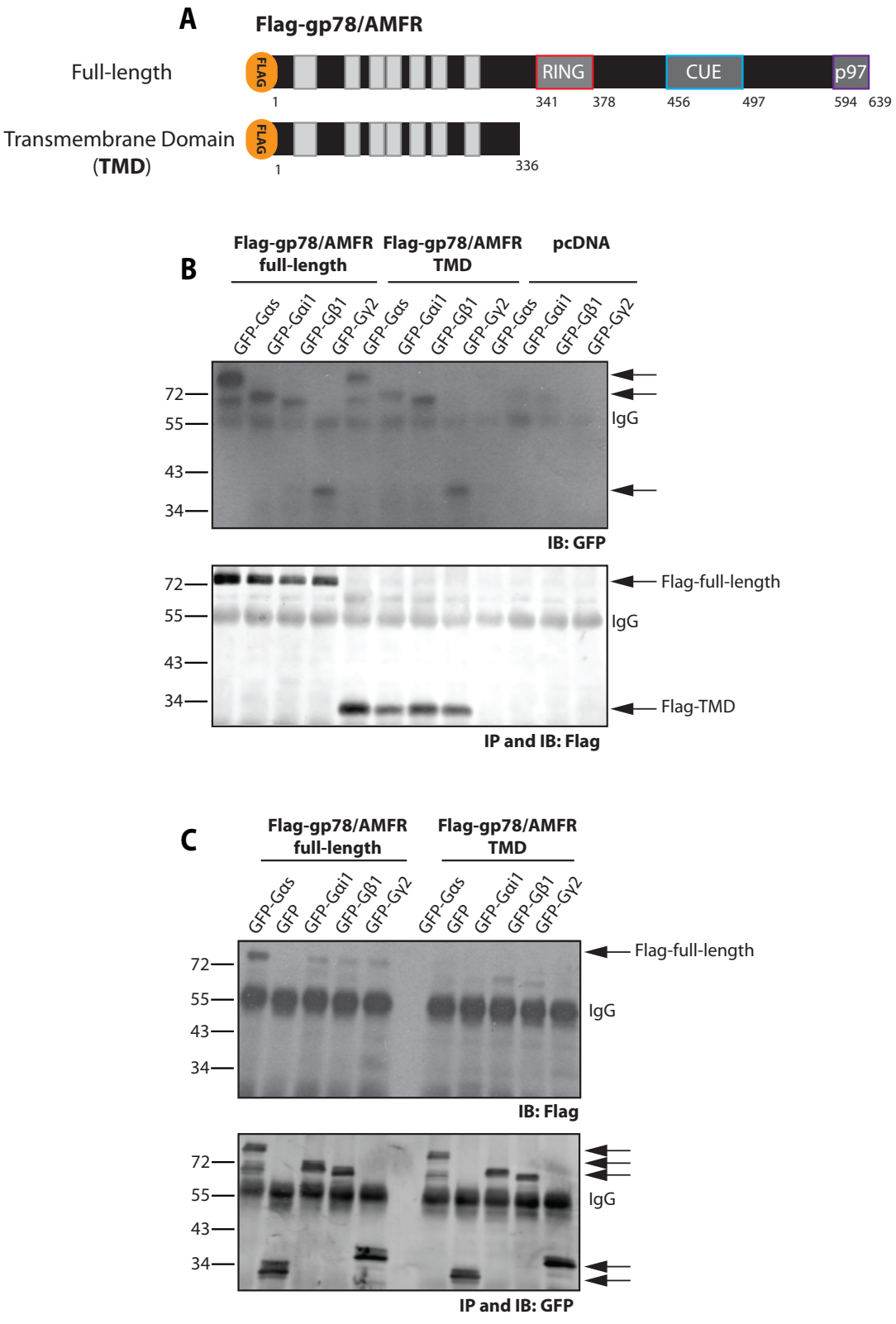

A
Flag-gp78/AMFR
Full-length
FLAG
RING
CUE
p97
1
341
378
456
497
594
639
Transmembrane Domain
(TMD)
FLAG
1
336
B
Flag-gp78/AMFR
full-length
Flag-gp78/AMFR
TMD
pcDNA
GFP-Gαs
GFP-Gαi1
GFP-Gβ1
GFP-Gγ2
72
55
43
34
IgG
IB: GFP
Flag-full-length
Flag-TMD
IP and IB: Flag
C
Flag-gp78/AMFR
full-length
Flag-gp78/AMFR
TMD
GFP-Gαs
GFP
GFP-Gαi1
GFP-Gβ1
GFP-Gγ2
Flag-full-length
IgG
IB: Flag
IP and IB: GFP

Figure 3.2 Full-length Flag-gp78/AMFR, and not the N-terminal transmembrane domain, is localized to the calnexin-labeled ER

A. Cos7 cells were transiently transfected with Flag-tagged gp78/AMFR full-length (FL) and the ER was identified with the anti-calnexin antibody. B-C. Cos7 cells expressing the N-terminal transmembrane domain (TMD) Flag-gp78/AMFR construct were labeled for the Golgi with the anti-GM130 antibody and the ER with the anti-calnexin antibody. 24 h post-transfection, cells were treated for 6 h with the general inhibitor of palmitoylation 2-bromopalmitate (2-BP) and in all cases, cells were fixed with methanol/acetone. Images were acquired with the confocal microscope. 25 – 30 cells (for each condition) were imaged in three independent experiments. Scale bar = 20 micrometers.

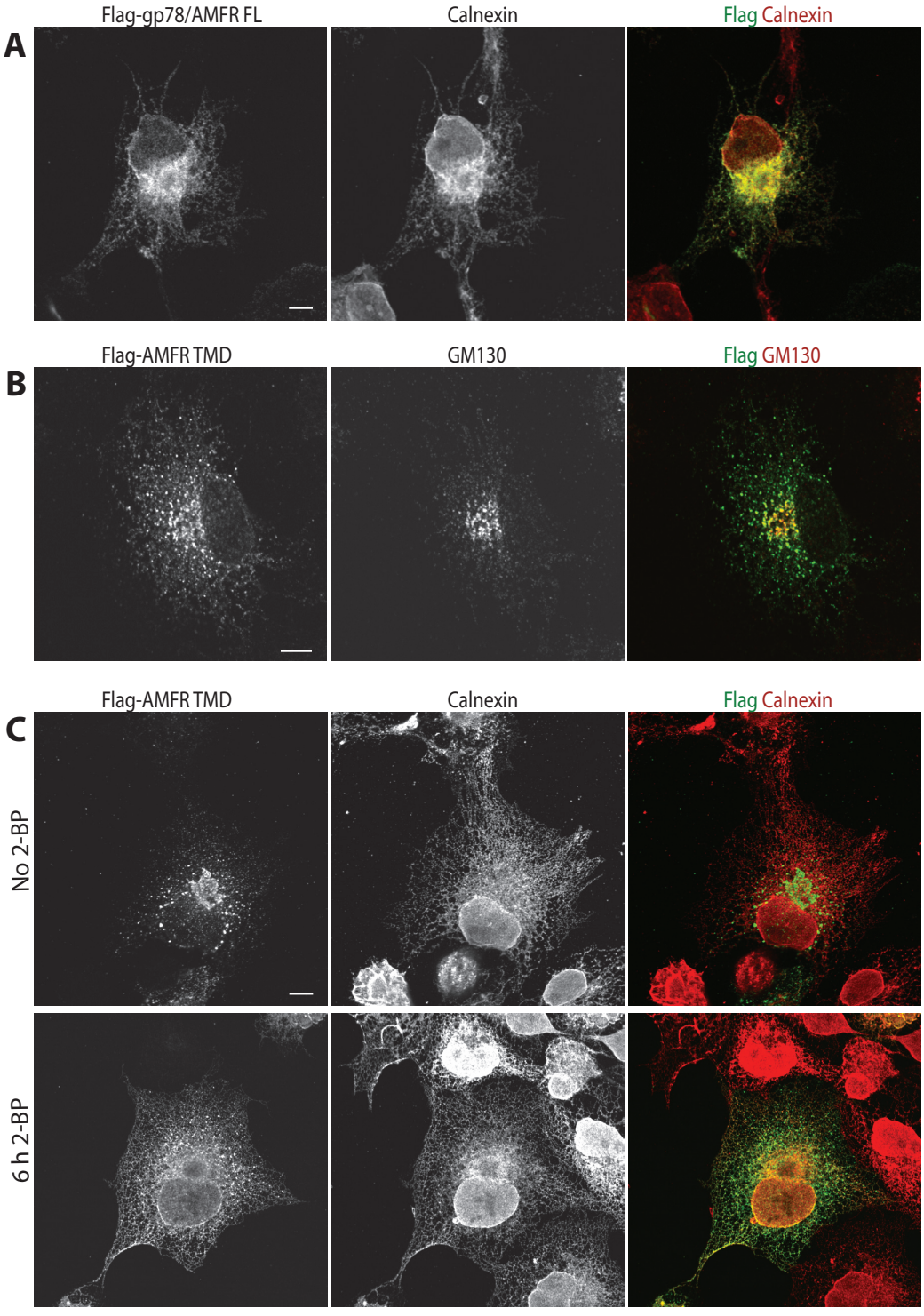

A
Flag-gp78/AMFR FL
Calnexin
Flag Calnexin
B
Flag-AMFR TMD
GM130
Flag GM130
C
Flag-AMFR TMD
Calnexin
Flag Calnexin
No 2-BP
6 h 2-BP

Figure 3.3 Flag-gp78/AMFR recruits GFP-Gαi1 to the ER

Cos7 cells were transiently transfected with GFP-Gαi1 alone (A) or in combination with full-length (FL) Flag-gp78/AMFR wild-type (B) or the N-terminal Flag-tagged transmembrane domain (TMD) of gp78/AMFR (C). Cells were fixed with methanol/acetone and labeled for the ER with the anti-calnexin antibody. Images were acquired with the confocal microscope. Colocalization between GFP-Gαi1 and calnexin was assessed using Pearson's coefficient (Mean ± S.E.M). An unpaired *t* test was done and the P value is reported on the graph comparing GFP-Gαi1 alone to its coexpression with either Flag-gp78/AMFR FL or Flag-gp78/AMFR TMD. Approximately 20-30 cells (for each condition) were imaged in six independent experiments. Scale bar = 20 micrometers; zoom scale bar = 0.3 micrometers.

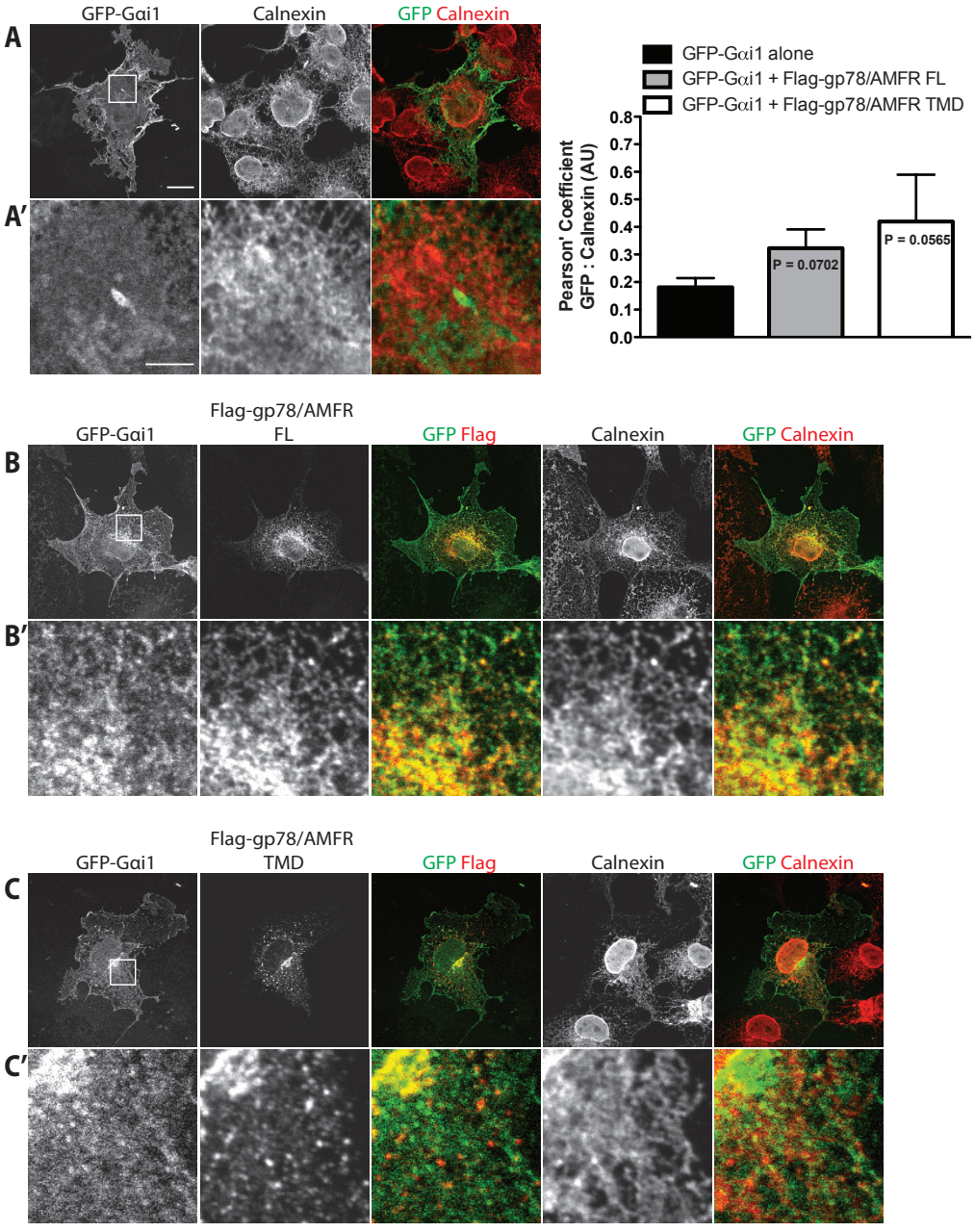

GFP-Gαi1
Calnexin
GFP Calnexin
A
A'
GFP-Gαi1 alone
GFP-Gαi1 + Flag-gp78/AMFR FL
GFP-Gαi1 + Flag-gp78/AMFR TMD
Pearson' Coefficient GFP : Calnexin (AU)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
P = 0.0702
P = 0.0565
Flag-gp78/AMFR FL
GFP-Gαi1
GFP Flag
Calnexin
GFP Calnexin
B
B'
Flag-gp78/AMFR TMD
GFP-Gαi1
GFP Flag
Calnexin
GFP Calnexin
C
C'

Figure 3.4 Flag-gp78/AMFR colocalizes with GFP-Gαs and other G proteins in the ER

Cos7 cells were transiently cotransfected with GFP-Gαs and either full-length (FL) Flag-gp78/AMFR wild-type (A, A') or the N-terminal Flag-tagged transmembrane domain of gp78/AMFR (TMD) (B, B'). 24 h post-transfection, all cells were fixed with methanol/acetone and images were acquired with the confocal microscope. Approximately 50 cells (for each condition) were imaged in six independent experiments. Scale bar = 20 micrometers; zoom scale bar = 0.3 micrometers. C. Colocalization between different G protein subunits and either Flag-gp78/AMFR FL or Flag-gp78/AMFR TMD was determined by Pearson's coefficient. A One-way ANOVA followed by Tukey's multiple comparison tests was done. Mean ± S.E.M.; $^{\#}P < 0.05$ compared to Flag-gp78/AMFR FL + Gγ2; $^{**}P < 0.01$ compared to Flag-gp78/AMFR TMD + Gγ2; the graph represents six to eleven independent experiments.

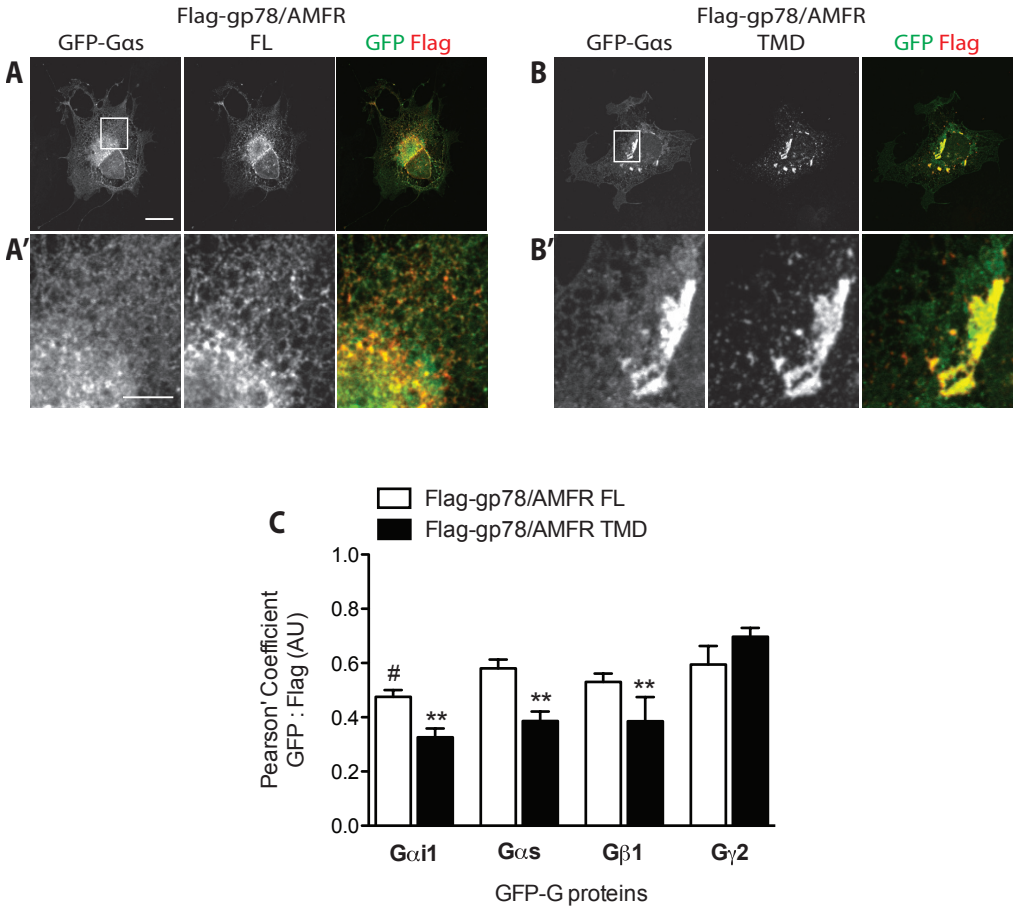
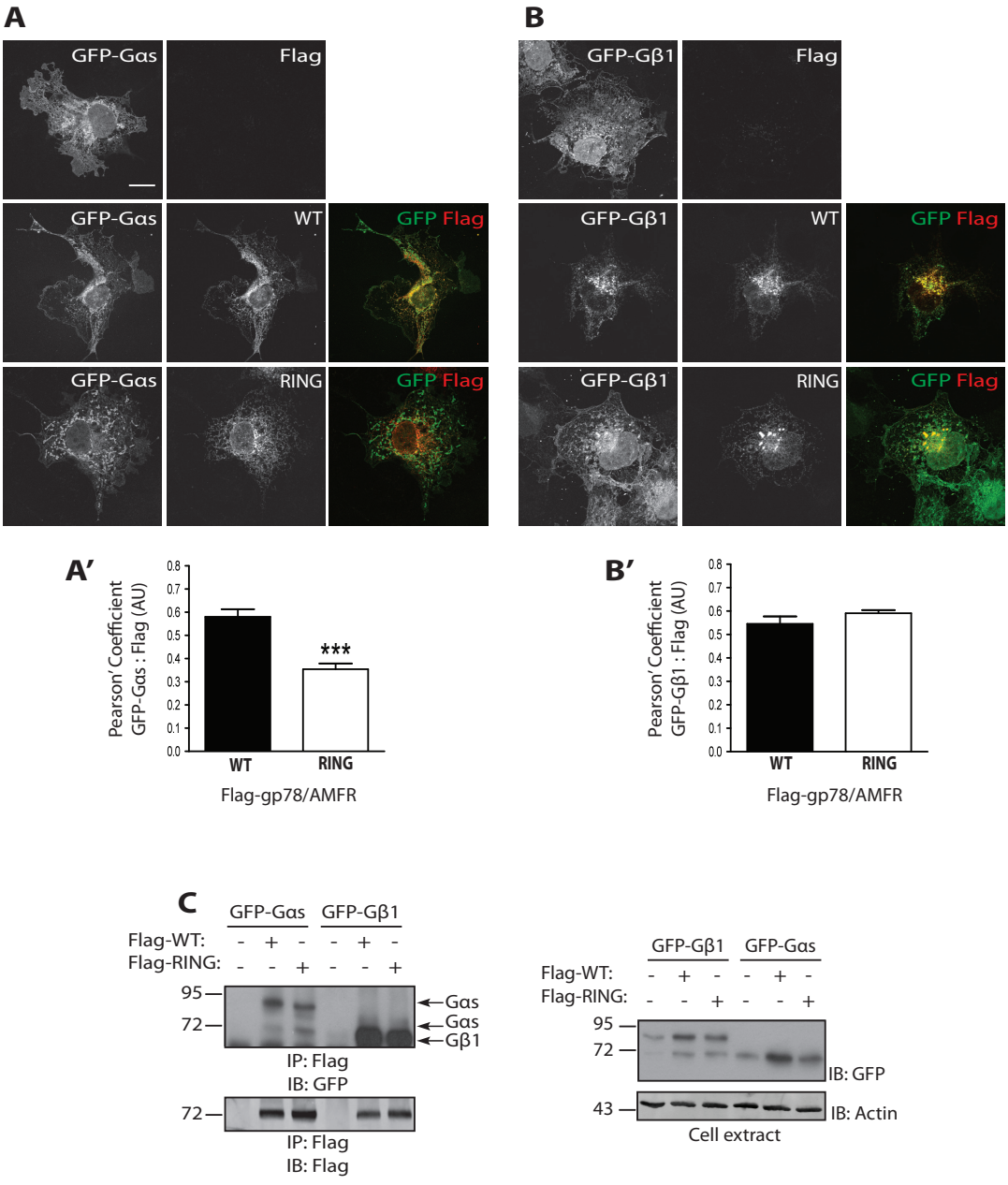


Figure 3.5 GFP-Gαs is only recruited by full-length Flag-gp78/AMFR wild-type, and not a RING finger mutant, to the central ER

Cos7 cells were transiently transfected with either GFP-Gαs (A) or GFP-Gβ1 (B) in the presence of Flag-gp78/AMFR wild-type (WT) or a RING finger mutant (C352S). Cells were fixed with methanol/acetone and images were acquired with the confocal microscope. Approximately 30-40 cells (for each condition) were imaged in three to five independent experiments. Scale bar = 20 micrometers; zoom scale bar = 0.3 micrometers. Colocalization between proteins was assessed using Pearson's coefficient. An unpaired *t* test was done. Mean ± S.E.M.; $^{***}P < 0.001$. C. Cos7 cells were cotransfected with one GFP-G protein subunit (GFP-Gαs or GFP-Gβ1) and either pcDNA3.1 vector control or Flag-gp78/AMFR WT or Flag-gp78/AMFR RING mutant. 24 h following transfection, cells were lysed and subjected to anti-Flag immunoprecipitation (IP). Protein-protein interactions were detected with immunoblots probed for anti-GFP and later for anti-Flag. Cell extracts from IP samples were collected and probed with anti-GFP to verify G protein expression. The immunoblot represents three independent experiments.


A
GFP-Gαs
Flag
GFP-Gαs
WT
GFP Flag
GFP-Gαs
RING
GFP Flag
B
GFP-Gβ1
Flag
GFP-Gβ1
WT
GFP Flag
GFP-Gβ1
RING
GFP Flag
A′
Pearson′Coefficient
GFP-Gαs : Flag (AU)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
WT
RING
Flag-gp78/AMFR
B′
Pearson′Coefficient
GFP-Gβ1 : Flag (AU)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
WT
RING
Flag-gp78/AMFR
C
GFP-Gαs
GFP-Gβ1
Flag-WT: - + - - + -
Flag-RING: - - + - - +
95
72
Gαs
Gαs
Gβ1
IP: Flag
IB: GFP
72
IP: Flag
IB: Flag
GFP-Gβ1
GFP-Gαs
Flag-WT: - + - - + -
Flag-RING: - - + - - +
95
72
IB: GFP
43
IB: Actin
Cell extract

CHAPTER 4 The Gαs Subunit is Localized to the ER where it Mediates Substrate Polyubiquitylation and Protects against Tunicamycin-Induced ER Stress

4.1 Summary

Gαs, the stimulatory subunit of the heterotrimeric G protein complex, has been well-characterized in the G protein-coupled receptor (GPCR) signal transduction pathway, where it activates the effector adenylyl cyclase at the plasma membrane and promotes production of the second messenger cAMP. However, there is increasing evidence that G protein subunits are able to redistribute from the plasma membrane to intracellular compartments where they participate in other signaling events. Here, we report three G protein subunits (GFP-Gαs, GFP-Gβ1, GFP-Gγ2) that are all localized to the ER, and we begin to characterize in detail the intracellular distribution of Gαs in Cos7 cells. Apart from its cell surface expression, we find that Gαs colocalizes with several ER markers that include both the central and tubular ER, and is able to promote substrate polyubiquitylation in the *in vitro* ubiquitin ligase activity assay. As of yet, the mechanism(s) involved in Gαs-mediated substrate polyubiquitylation is unclear but seems to exclude the E3 ubiquitin ligase gp78/AMFR. Based on immunoprecipitation and immunocytochemistry data, Gαs may act through a JNK1-associated membrane protein (JAMP) that is another component of the ER-associated degradation (ERAD) pathway and a potential ER-localized GPCR. Moreover, Gαs plays a protective role in the TUN-induced ER stress response whereby exogenous GFP-Gαs significantly reduces expression of BiP and CHOP proteins, while the absence of endogenous Gαs alone enhances ER stress that seems to be partially mediated by the adenylyl cyclase/cAMP pathway. Taken together, this is the first report that reveals Gαs in the ER where it is likely to possess distinct functions within novel, yet undescribed, ER protein complex(es) beyond the traditional GPCR signal transduction pathway.

4.2 Introduction

GPCRs, the largest class of cell surface receptors, translate changes occurring within the extracellular environment into an intracellular response via heterotrimeric guanine-nucleotide binding proteins (G proteins) that are composed of α, β and γ subunits (Downes and Gautam, 1999; Kristiansen, 2004; Neer, 1995; Schioth and Fredriksson, 2005). The signal transduction pathway is initiated by binding of a specific ligand to a receptor, followed by a conformational change of the receptor, the exchange of GDP for GTP on the Gα subunit, and a conformational change within (and possible dissociation of) the heterotrimeric G protein (Chidiac, 1998; Kjeldgaard et al., 1996). Both the GTP-bound Gα subunit and the Gβγ complex promote downstream signaling by stimulating a number of effector molecules, thereby activating or inhibiting the production of various second messengers. Thus, G proteins regulate a wide range of cellular components such as metabolic enzymes, ion channels and the transcriptional machinery (Neves et al., 2002).

During signaling events, the stimulatory subunit of heterotrimeric G proteins, Gαs activates the effector adenylyl cyclase on the cytosolic face of the plasma membrane and stimulates the production of the second messenger cyclic adenosine monophosphate (cAMP). The signal transduction pathway associated with the plasma membrane has been well-characterized. However, several G protein subunits have the ability to redistribute from the plasma membrane to intracellular compartments where they are implicated in other signaling pathways but their mechanism of action is still poorly understood (Sarma et al., 2003). Studies have shown that Gαs is enriched in endocytic vesicles (Van Dyke, 2004), the *trans*-Golgi network (Denker et al., 1996) and can interact with proteins other than adenylyl cyclase including tubulin and the microtubule cytoskeleton in neuronal cells (Sarma et al., 2003). Thus, novel intracellular Gαs functions have been identified and these include regulation of endosome fusion (Colombo et al., 1994), apical transport in liver epithelia (Pimplikar and Simons, 1993), as well as down-regulation of the epidermal growth factor (EGF) receptor (Zheng et al., 2004).

The endoplasmic reticulum (ER) is implicated in many cellular functions and under normal physiological conditions, these include protein folding and posttranslational modifications of secretory and membrane proteins (Ellgaard and Helenius, 2003). In the

event where ER function is compromised and leads to accumulation of unfolded and/or misfolded proteins, the cell responds by triggering the unfolded protein response (UPR) (Bernales et al., 2006; Papp et al., 2010). The UPR consists of a series of events that includes (1) inhibition of global protein synthesis, (2) increased expression of ER chaperone proteins such as BiP (known as glucose regulated protein 78, GRP78) that facilitate in correct folding of nascent proteins, and (3) induction of the ERAD pathway that degrades misfolded proteins through the proteasome. In the case of prolonged and/or severe ER stress, the UPR induces another response, ER stress-induced apoptosis and involves proteins such as CHOP (Zinszner et al., 1998) to promote cell death.

Here, we report that the Gαs subunit is not only present on the cell surface but is highly localized to the ER, where it is able to promote substrate polyubiquitylation in the peripheral ER, independent of the E3 ubiquitin ligase gp78/AMFR. Moreover, we show that Gαs is the preferred G protein-binding partner for another ERAD protein that is also a predicted GPCR, a JNK1-associated membrane protein (JAMP). Finally, we find that exogenous GFP-Gαs plays a protective role in the Tunicamycin (TUN)-induced ER stress response. Conversely, Gαs knockdown alone induces ER stress that is comparable to Tunicamycin or Thapsigargin treatments, and this response appears to be partially mediated by the adenylyl cyclase/cAMP signaling pathway. Overall, these findings introduce functions for Gαs in the ER, describing for the first time, its involvement in ER protein degradation and ER stress.

4.3 Materials and methods

4.3.1 Plasmids and constructs

GFP-Gαs expression plasmid was kindly provided by Dr. Mark M. Rasenick (University of Illinois). GFP-αi1, GFP-Gβ1 and GFP-Gγ2 were a gift from Dr. Terence Hebert (McGill University). Flag-JAMP was kindly provided by Ze'ev A. Ronai (Burnham Institute for Medical Research), Myc-Rtn4a by Gia Voeltz (University of Colorado) and HA-Ub wt by Anthony Morielli (University of Vermont). The pEGFP expression vector was purchased from Clontech. Wild-type Flag-gp78/AMFR was inserted into pcDNA 3.1 (+) as described in (Registre et al., 2004).

4.3.2 Antibodies

Anti-calnexin, anti-BiP, anti-Flag M2 and anti-βactin were purchased from Sigma. Anti-Gαs was purchased from Calbiochem, anti-GFP from Synaptic Systems, anti-HA from Neomarker, anti-CHOP from Cell Signaling, anti-Serca2A from Affinity BioReagents and anti-ERp57 from Santa Cruz. The rat IgM 3F3A monoclonal antibody against gp78/AMFR is described in (Nabi et al., 1990). Fluorescently conjugated secondary antibodies (highly cross-absorbed Alexa488, Alexa568 and Alexa647) for immunocytochemistry were acquired from Invitrogen. HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch. IRDye 800 mouse antibody, a fluorescently conjugated secondary antibody used for immunoblotting, was acquired from Rockland.

4.3.3 Cell culture and transfection

Cos7 and HEK293 cells were maintained in DMEM containing 10% fetal bovine serum (FBS, MediCorp.), and supplemented with 0.2 mM L-Glutamine, 10 μg/ml Penicillin, 10 μg/ml Streptomycin, vitamins and non-essential amino acids (all supplements were purchased from Invitrogen). For transient transfection of plasmid DNA into Cos7 cells, Effectene transfection reagent (Qiagen) was used as described by the manufacturer. Validated ON-TARGET plus SMARTpool small interfering RNAs (siRNA) for human GNAS (Gαs) and control siRNAs were purchased from Dharmacon. Lipofectamine 2000 (Invitrogen) was used for transient siRNA transfections into HEK293 cells as described by the manufacturer. For the *in vitro* ubiquitin ligase activity assay, 24 h post-siRNA transfection, HEK293 cells were transfected with plasmid DNA as specified in experiment. To inhibit the proteasome, cells were treated with 30 μM MG132 (Sigma) for 6 h. For the ER stress experiment, 24 h following siRNA transfection, HEK293 cells were starved overnight and treated with fresh media containing 2 μg/ml Tunicamycin (TUN, Sigma) or 3 μM Thapsigargan (TG, Sigma) or 10 μM Forskalin (FSK, Tocris Bioscience) for 8 h. For TUN- and TG-induced ER stress pretreatments, starved cells were incubated with fresh media containing 10 μM Forskalin for 30 min. Stable HT1080 gp78/AMFR knockdown cell line was generated and maintained as described in (Fu et al., 2011).

4.3.4 Immunoprecipitation and immunoblotting

For anti-Flag immunoprecipitation, Cos7 cells were rinsed and manually collected in cold phosphate-buffered saline (PBS). Cell pellets were resuspended in ice-cold lysis buffer A [20 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, 0.1% SDS and 1 mg/ml protease inhibitor cocktail (Roche)]. Following a 30 minute rotation at 4°C, 1% Triton X-100 was added to each sample and incubated for an additional 30 minutes. Lysates were passed through a 25G syringe 6 times and the insoluble material was removed by centrifugation. Supernatants were incubated overnight with anti-Flag coupled with agar beads (Sigma) that have been pre-blocked with 1% BSA (1 h at 4°C). Beads were then washed 3 times with wash buffer A [20 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, 0.2% BSA], 3 times with wash buffer B [20 mM Tris-HCl pH 8, 500 mM NaCl, 0.5% Triton X-100, 0.2% BSA] and one time with wash buffer C [50 mM Tris-HCl pH 8]. Proteins in cell lysates and immunoprecipitates were denatured by boiling in SDS sample buffer, and later separated by SDS-PAGE, transferred onto PVDF membranes (Pall Corporation), blocked with 5% nonfat milk and probed with appropriate antibodies. Proteins were visualized either by Bioflex film (Clonex Corporation) or the Odyssey Infrared Imaging System (Li-COR Biosciences).

4.3.5 Immunocytochemistry and imaging

Cells were grown on glass cover slips and 24 h post-transfection, cells were fixed with pre-cooled (-80°C) methanol:acetone (80:20%) for 15 minutes at -20°C. Then, cells were washed 3 times for 15 minutes with PBS supplemented with 1 mM $CaCl_2$ and 10 mM $MgCl_2$ (PBS-CM). Cells were first blocked with PBS-CM containing 1% Bovine Serum Albumin (BSA) (blocking solution) for 15 minutes. Cells were then incubated with primary antibodies for 30 minutes, rinsed 3 times for 2 minutes with blocking solution, incubated with the corresponding species-specific fluorescently conjugated secondary antibodies for an additional 30 minutes, and finally rinsed three times for 10 minutes with blocking solution. All antibody incubations were performed in blocking solution. Cover slips were mounted on microscope slides with Celvol 205: Polyvinyl Alcohol (Celanese Chemicals Ltd.) and imaged with the 100x (NA 1.4) plan

apochromatic objective of a Fluoview 1000 confocal laser scanning microscope (Olympus).

4.3.6 Statistical analysis

For immunoblot analysis, densitometry was done using the Scion image analysis (NIH Image) and results are expressed as Mean +/- S.E.M. Statistical comparisons between groups were performed by a One-way ANOVA followed by Tukey's multiple comparison tests (GraphPad PRISM). For fluorescence quantification experiments, colocalization between two proteins is expressed by Pearson's correlation measurements from at least three independent experiments and was calculated using the ImagePro analysis software (Media Cybernetics). Refer to section 2.3.9 for more details.

4.4 Results

4.4.1 Subcellular distribution of G protein subunits in Cos7 cells

To study the intracellular distribution of G proteins, we expressed several GFP-tagged G protein subunits in Cos7 cells and prior to labeling, we fixed the cells with a very cold methanol/acetone mixture, a fixation method that simultaneously dehydrates proteins leading to their precipitation or condensation and permeabilizes the cell by extracting membrane lipids. The methanol/acetone mixture was pre-cooled to -80°C and the rapid freezing of the cells has been shown to preserve dynamic ER structures better than paraformaldehyde fixation. In contrast, the paraformaldehyde fixation method cross-links proteins and DNA with little extraction (cells are later permeabilized with Triton-X) and is likely to better preserve cell structures. However, paraformaldehyde fixation can also chemically destroy binding sites and prevent antibody access, and in our case, paraformaldehyde fixation makes it more difficult to visualize G proteins within the ER due to their abundance in the cytoplasm. For these reasons, we decided to use methanol/acetone fixation to look at G proteins within ER structures and membranes by extracting the majority of the strong cytoplasmic labeling. We observe that both GFP-Gαs and GFP-Gαi1 are abundant on the plasma membrane (Fig. 4.1A, indicated by arrows), while GFP-Gβ1 shows reduced plasma membrane expression and GFP-Gγ2 is

primarily localized to the Golgi. Since GFP-Gβ1 and GFP-Gγ2 were expressed alone, it is likely that a fraction of the GFP-Gβ1 and GFP-Gγ2 distribution reflects uncoupled G proteins, caused by depletion of their respective endogenous Gγ and Gβ protein binding partner. We labeled the ER by using the anti-calnexin antibody and found that three G protein subunits (GFP-Gαs, GFP-Gβ1, GFP-Gγ2) are concentrated in the calnexin-labeled ER, with the exception of GFP-Gαi1 that is primarily expressed on the plasma membrane (Fig. 4.1). In the case of GFP-Gγ2, the colocalization between GFP-Gγ2 and calnexin is similar to GFP-Gαs and GFP-Gβ1 subunits, but GFP-Gγ2 is clearly expressed at a lower intensity in the ER, a factor that is ignored by the Pearson's coefficient (Fig. 4.1B).

Based on the observation in chapter 3 that Gαs associates with the E3 ubiquitin ligase gp78/AMFR in the ER (Fig. 3.1 and Fig. 3.4), we decided to characterize in detail the ER localization of Gαs by using a series of ER markers. We labeled the tubular ER by expressing Myc-reticulon (Myc-Rtn4a) and assessed the central ER by looking at the endogenous calcium(2+)-ATPase Serca2A and the protein disulfide isomerase ERp57. Consistent with Fig. 4.1, GFP-Gαs is enriched in both the central and tubular smooth ER (Fig. 4.2). Thus, these findings reinforce the idea that certain G protein subunits are localized to membranes other than the plasma membrane and, within these intracellular membrane compartments, G proteins may actively participate in signaling pathways.

4.4.2 Gαs promotes substrate polyubiquitylation in the peripheral ER

To look at substrate polyubiquitylation, we used the *in vitro* ubiquitin ligase activity assay in which formation of an HA smear represents accumulation of polyubiquitylated substrates. This is seen when HA-ubiquitin wild-type (HA-Ub wt) was coexpressed with Flag-gp78/AMFR, a well-characterized E3 ubiquitin ligase in the ERAD pathway (Fig. 4.3A). Surprisingly, a similar HA smear is observed with the expression of GFP-Gαs alone, and not with either GFP-Gαi1 or GFP-Gβ subunits (Fig. 4.3A). In addition to the *in vitro* ubiquitin ligase activity assay, we characterized the distribution of GFP-Gαs with HA-Ub wt in Cos7 cells. We find that GFP-Gαs and HA-Ub wt colocalize in the cell periphery, partially within the smooth tubular peripheral ER as marked by Myc-Rtn4a expression (Fig. 4.3B - 4.3C). This phenotype seems to be

unique to GFP-Gαs and is not observed with any other G protein, including the Gαi1 subunit (Fig. 4.3B). These data introduce, for the first time, a unique role for GFP-Gαs in the protein degradation pathway and suggest that Gαs-mediated substrate polyubiquitylation may initiate and take place in the peripheral ER, as observed with gp78/AMFR (St. Pierre et al., 2011).

In the absence of proteasome inhibition, polyubiquitylated substrates are rapidly degraded and are unable to accumulate in the central ER, an ER domain thought to be the site of proteasomal targeting as previously reported for the ER Quality Control pathway (Kamhi-Nesher et al., 2001). Previously, it has been shown with the expression of gp78/AMFR that following treatment with a proteasomal inhibitor MG132, polyubiquitylated substrates accumulate in the central ER, suggesting that gp78/AMFR-mediated polyubiquitylated substrate degradation takes place via the proteasome in the central ER (St. Pierre et al., 2011). To investigate the involvement of Gαs in proteasomal degradation, we treated cells with MG132 and unlike gp78/AMFR, in the presence of GFP-Gαs, MG132 treatment appeared to have no effect on the central and peripheral distribution of polyubiquitylated substrates and only resulted in a slight increase in the polyubiquitylated protein smear detected in the *in vitro* ubiquitin ligase activity assay (Fig. A.4). There is a possibility that Gαs itself is being degraded by the proteasome but as of yet, this model has not been tested. Together, these findings imply that Gαs may inhibit the proteasome and/or sequester polyubiquitylated proteins to the peripheral ER, perhaps acting as a modulator of the ERAD pathway.

Based on findings in chapter 3 and the ability of Gαs to promote substrate polyubiquitylation (Fig. 4.3), we investigated the role of Gαs in gp78/AMFR ubiquitin ligase activity by using the *in vitro* ubiquitin ligase activity assay. In the absence of endogenous Gαs, we observe no effect on gp78/AMFR ubiquitin ligase activity (Fig. 4.4A) and similarly, Gαs-mediated substrate polyubiquitylation appears unaffected in stable gp78/AMFR knockdown HT1080 cells (Fig. 4.4B). Together, these data suggest that Gαs promotes substrate polyubiquitylation independently of the E3 ubiquitin ligase gp78/AMFR despite being recruited by and localized within the same ER protein complex (Fig. 3.1 and Fig. 3.4 and Fig. 3.5).

Next, we decided to look at a seven-transmembrane protein, a Jun N-terminal Kinase 1 (JNK1) - associated membrane protein (JAMP) that was identified through binding to another E3 ubiquitin ligase, RNF5 (also known as RMA1) in the ER. Here, we observe that Flag-JAMP coimmunoprecipitates specifically with GFP-Gαs (Fig. 4.5A) and is found in the calnexin-labeled ER (Fig. 4.5B), where it extensively colocalizes with GFP-Gαs (Fig. 4.5C - 4.5D). Thus, it is possible that the observed Gαs substrate polyubiquitylation is mediated by JAMP. As of yet, there is no evidence to support this phenomenon and the significance of the JAMP-Gαs protein interaction requires further investigation. Overall, this study reveals a novel function for the Gαs subunit in the ER and describes its ability to promote substrate polyubiquitylation that may depend upon its direct association with one or more ERAD proteins. As of yet, the mechanism underlying Gαs-mediated substrate polyubiquitylation in the ER remains elusive.

4.4.3 Gαs protects against TUN-induced ER stress

To investigate the role of Gαs in ER stress, we overexpressed GFP-Gαs and treated HEK293 cells with Tunicamycin (TUN), an inhibitor of N-acetylglucosamine transferases. We monitored the ER stress response by looking at the expression profile of two ER stress markers, BiP and CHOP. We observe that in the presence of GFP-Gαs, the TUN-induced ER stress response is inhibited, as seen with reduced BiP and CHOP protein levels (Fig. 4.6). In the case where endogenous Gαs expression is significantly reduced following siRNA transfection, we detect the opposite response and find that Gαs knockdown promotes ER stress to the same levels as seen with TUN treatment and another ER stressor Thapsigargin (TG, an inhibitor of Ca^{2+} ion pump proteins of intracellular membranes) (Fig. 4.7). These findings introduce another role for Gαs in the ER, however more work needs to be done to define the mechanism(s) by which Gαs protects against the ER stress response.

Next, we investigated whether Gαs acts through the traditional signal transduction pathway that involves the effector adenylyl cyclase. We treated cells with forskalin (FSK), a reagent that directly activates adenylyl cyclase independently of Gαs and stimulates production of the second messenger cAMP. In control siRNA lysates, we observe an increase in BiP expression following FSK treatment alone that was slightly

enhanced with TUN (Fig. 4.7), suggesting that TUN-induced ER stress can be achieved via an alternative cAMP-dependent pathway. However, the FSK-induced BiP response was only slightly reduced in the Gαs siRNA lysates, as compared to FSK-treated control siRNA and untreated Gαs siRNA lysates. It is possible that the protective effect of Gαs in ER stress may only be partially dependent on adenylyl cyclase activation and that Gαs may interact with another protein and/or cross-talk to a different signaling pathway. Overall, we describe for the first time, a function for Gαs in ER stress that may not follow the well-characterized Gαs signal transduction pathway.

4.5 Discussion

Gαs, a subunit of the heterotrimeric G protein complex, is localized to the plasma membrane where it activates its effector adenylyl cyclase and results in the production of cAMP during the GPCR signal transduction pathway. However, Gαs is not limited by its interaction with adenylyl cyclase and has been shown to associate with proteins beyond the plasma membrane, one example being tubulin and the microtubule cytoskeleton in neuronal cells (Sarma et al., 2003). Thus, Gαs (and other G proteins) has the potential to redistribute from the plasma membrane to intracellular compartments where it is implicated in signaling pathways. Studies have been conducted on the role of G proteins in membrane trafficking (Denker et al., 1996) and in the endocytic and secretory pathways (Bomsel and Mostov, 1992; Helms, 1995; Nurnberg and Ahnert-Hilger, 1996; Stow and Heimann, 1998). Moreover, G proteins have been detected within the nucleus (Crouch et al., 2000), the mitochondria (Fishburn et al., 1999) and the cytosol where, in some cases, their cytoplasmic localization seems to increase upon G protein activation. For example, GPCR activation following isoproterenol or cholera toxin treatments has been shown to promote translocation of Gαs from the plasma membrane to the cytosol (Levis and Bourne, 1992; Wedegaertner and Bourne, 1994; Wedegaertner et al., 1996; Yu and Rasenick, 2002). Here, we document, for the first time, the intracellular localization of GFP-Gαs in Cos7 cells and show that apart from its cell surface distribution, GFP-Gαs is highly localized to the ER domain (Fig. 4.1 and 4.2). In addition, we looked at the distribution of endogenous Gαs in Cos7 cells and not surprisingly, Gαs is abundant on the plasma membrane but a significant portion of the

protein is detected on intracellular membrane structures that exclude the cytoplasm and resemble mitochondria and the ER (data not shown). In subsequent experiments, we show that both exogenous and endogenous proteins are localized to both the mitochondria as labeled by the OxPhosV antibody and the mitochondria-associated membrane (MAM) as labeled by the anti-3F3A AMFR antibody (data not shown). The latter antibody labels a subpopulation of gp78/AMFR that is exclusively localized to the MAM of the smooth ER (Benlimame et al., 1998; Benlimame et al., 1995; Wang et al., 1997) (gp78/AMFR is reviewed in (St Pierre and Nabi, 2012)). Finally, we performed an Optiprep Gradient Fractionation* on Gαs and as expected, we observe that endogenous Gαs is primarily found in the first two fractions that correspond to the plasma membrane (defined by E-cadherin), the cytosol (defined by actin) and the *cis*-Golgi (defined by βCOP) (data not shown). However, approximately 10% of Gαs is found in fraction 6 that marks the ER (defined by calnexin), the mitochondria (defined by complex II) and the mitochondria-associated membrane (MAM; defined by both FACL4 and calnexin) (data not shown). A similar distribution is seen with GFP-Gαs but in this case, the majority of the protein is found in fraction 6, instead of the first two fractions as seen with endogenous Gαs (data not shown). These data are in agreement with our confocal images and the observation that GFP-Gαs is highly localized to the ER in Cos7 cells as compared to endogenous Gαs. Based on these findings, it is possible that Gαs directly participates in signaling pathways within the MAM, the mitochondria and the ER. Unfortunately, we are unable to distinguish between the ER and the mitochondria in both the confocal images and the Optiprep Gradient Fractionation, making it impossible to determine whether Gαs is enriched in the MAM or the mitochondria or both. Protocols have been developed to efficiently isolate ER, MAM and mitochondria fractions (Bozidis et al., 2007) and in future experiments, it will be critical to characterize in detail the localization of Gαs within all these intracellular compartments. Recently, a technique has been published able to obtain highly purified MAM and mitochondria from rat liver and cultured cells, by using the crude mitochondrial fraction. Here, the authors show that they

* The Optiprep Gradient Fractionation was performed by Emily Lynes in Dr. Thomas Simmen's laboratory (University of Alberta).

are able to isolate a pure mitochondria fraction that excludes the ER, nuclear and other non-mitochondrial markers (Wieckowski et al., 2009).

Some G protein subunits have been observed to rapidly shuttle between the plasma membrane and the cytosol. The mechanism involved in the release of Gαs from the plasma membrane is still unclear, however posttranslational modifications of G proteins seem to be one important mechanism in modulating their distribution, activity and protein binding affinities (Chen and Manning, 2001). Recently, Gαs palmitoylation has been shown to dynamically regulate GPCR signaling, whereby palmitoylation enhances Gαs plasma membrane localization (Tsutsumi et al., 2009) and upon receptor activation, Gαs undergoes depalmitoylation (Wedegaertner and Bourne, 1994). In other cases, plasma membrane targeting also requires a complex with Gβγ (Chisari et al., 2007; Dupre et al., 2009; Marrari et al., 2007). It has been shown that active GTP-bound Gαs dissociates from Gβγ and undergoes depalmitoylation, resulting in receptor-mediated translocation to the cytosol (Iiri et al., 1996). Recently, palmitoylation has also been found to enrich ER membrane proteins such as the transmembrane thioredoxin family protein TMX and calnexin to the MAM. In fact, calnexin was shown to shuttle between the rough ER and the MAM, and its localization was determined by its palmitoylation state (Lynes et al., 2011). Based on this finding, in addition to targeting Gαs to the plasma membrane, Gαs palmitoylation may also be a determining factor for its MAM enrichment as observed in Cos7 cells (data not shown).

In the present study, we found that both GFP-Gαs and GFP-Gβ1 are highly concentrated in the ER (Fig. 4.1). In fact, stable interactions between receptors and G proteins independent of receptor activation have been shown to exist in the ER during protein biosynthesis and maturation. These ER complexes are formed prior to membrane targeting and have been previously described for Gβγ and its effectors, the Kir3 family of inwardly rectifying potassium channels and adenylyl cyclase (David et al., 2006; Dupre et al., 2007; Rebois et al., 2006; Robitaille et al., 2009). Other studies imply that these stable G proteins complexes formed in the ER may not only lead to G protein-receptor specificity on the cell surface but may provide rapid signaling at other sites (Boivin et al., 2008). As of now, the palmitoylation state of Gαs and its association with Gβγ in the ER remain unknown. We can only hypothesize that ER-associated Gαs is

depalmitoylated, free from Gβγ and able to promote signaling events through interaction(s) with other protein partners. The concept that Gαs translocates from the plasma membrane independently from its receptor, as seen with β_2AR (Allen et al., 2005), and interacts with other effectors at various cellular sites, has been demonstrated in several studies but still requires further investigation (Donati et al., 2001; Miura et al., 2001; Rybin et al., 2000).

Localized to the ER, GFP-Gαs expression induces substrate polyubiquitylation that was defined by the formation of an HA smear in the *in vitro* ubiquitin ligase activity assay. This is not observed with any other G protein subunit and the Gαs-induced HA smear is similar to the well-characterized E3 ubiquitin ligase, gp78/AMFR (Fig. 4.3A). Further, GFP-Gαs and not GFP-Gαi1, extensively colocalizes with wild-type HA-ubiquitin in the peripheral ER (Fig. 4.3B – 4.3C). The observed Gαs-mediated substrate polyubiquitylation seems to be independent of gp78/AMFR (Fig. 4.4) and the proteasome, demonstrated by the lack of polyubiquitylated substrate accumulation in the central ER following proteasome inhibition with MG132 (Fig. A.4; (St. Pierre et al., 2011)). It is possible that Gαs inhibits the proteasome and/or sequesters components of the ERAD protein complex, resulting in accumulation of polyubiquitylated substrates in the peripheral ER and not the central ER. As of yet, the Gαs protein-binding partners responsible for the observed substrate polyubiquitylation in the ER remain uncertain. However, we examined the possible involvement of another ERAD member that is also a potential GPCR in the ER, namely JAMP. Here, we show that Flag-JAMP preferentially associates with GFP-Gαs in the anti-Flag immunoprecipitation and both proteins colocalize within the calnexin-labeled ER in Cos7 cells (Fig. 4.5). Our data support previous findings that JAMP is primarily found within membrane fractions in HeLa cells (Kadoya et al., 2005) and in addition to its plasma membrane, lysosome and cytosolic distributions, JAMP is also localized to the ER in both C2C12 and HeLa cells (Kadoya et al., 2005; Tcherpakov et al., 2008). Most importantly, JAMP is present in the same ERAD protein complex as RNF5 and gp78/AMFR (Morito et al., 2008; Tcherpakov et al., 2008; Tcherpakov et al., 2009; Younger et al., 2006). In fact, JAMP activity is modulated by RNF5 that promotes its ubiquitylation and disrupts its scaffolding ability without altering stability. Thus, JAMP ubiquitylation leads to accumulation of misfolded

proteins, a phenomenon that was observed during GFP-Gαs expression (Fig. 4.3). Regulation of ERAD components is critical and most likely involves cross-talk between different cellular mechanisms (Shen et al., 2007). Under these experimental conditions, we are unable to define with certainty the direct Gαs binding partner(s), however we can hypothesize that accumulation of polyubiquitylated substrates observed with GFP-Gαs expression may be the result of JAMP ubiquitylation by RNF5 and its inability to efficiently clear misfolded proteins. Thus, Gαs may be part of the recognition complex involved in ERAD control, whereby association of Gαs with JAMP promotes a conformational change within the receptor and enhances its ubiquitylation by RNF5. Taken together, these data implicate Gαs in ER protein degradation as a novel component of the ERAD machinery. Furthermore, these findings are in agreement with a previous study that describes Gαs promoted ligand-dependent degradation of EGF receptors via binding of its GTPase activating protein (GAP) RGS-Px1 and the hepatocyte growth-factor-regulated tyrosine kinase substrate (Hrs) (Zheng et al., 2004). Hrs is also responsible for the degradation of other GPCRs including the chemokine receptor CXCR4 (Marchese et al., 2003) and the delta opioid receptor (Hislop 2004), however the involvement of Gαs in these processes remains undetermined.

Recently, a functional role for the Gβγ complex in the ER has been described in another model system, *Arabidopsis*. The authors showed that the majority of Gβ was localized in the ER and cofractionated with the membrane-associated ER luminal protein, BiP, suggesting that Gβ signaling is involved in UPR-associated cell death, independent of the Gα protein (Wang et al., 2007). In our HEK293 cell model system, we report that Gαs protects against ER stress and observe that exogenous GFP-Gαs significantly hinders expression of BiP (Fig. 4.6), while in the absence of endogenous Gαs, ER stress takes place without TUN treatment (Fig. 4.7). Since siRNA can cause non-targeted, non-specific effects by affecting other pathways, additional negative controls should be considered to confirm the ER stress response following Gαs siRNA transfection. Examples include the addition of (1) a 'mock' control (i.e. another unrelated protein) to determine whether siRNA transfection is affecting overall cell function; (2) a non-targeting negative control siRNA to confirm that any observed decrease in gene expression levels is related to a sequence-specific siRNA event; (3) a rescue experiment

whereby GFP-Gαs is reintroduced following Gαs siRNA transfection; as well as assessing for (4) cell viability (eg. Trypan blue staining) to determine degree of toxicity induced by transfection. In addition to evaluating Gαs protein expression, we can assess Gαs knockdown by a reduction in the amount of Gαs mRNA via real time RT-PCR.

The mechanism of action still remains unclear but raises the question of whether Gαs is mediating the ER stress response via stable interaction with its effector adenylyl cyclase or through a novel protein complex in the ER. In control lysates, we find a partial ER stress response following activation of adenylyl cyclase with forskalin in the absence of TUN that was slightly lower in Gαs siRNA lysates (Fig. 4.7). Thus, it is likely that the protective effect of Gαs in ER stress may partially depend on adenylyl cyclase activation but requires interaction with additional protein(s) from different signaling pathways. As mentioned above, JAMP acts as an adaptor in the ERAD and proteasome pathway (Tcherpakov et al., 2008), but it can also increase and prolong JNK1 activity in stress-induced apoptosis (Kadoya et al., 2005). A similar response was observed in a jamp-1 deleted C. elegans strain that exhibited basal ER stress and was hypersensitive to TUN-induced ER stress (Tcherpakov et al., 2008). Based on these data, we can only speculate that JAMP is coupled to Gαs and both proteins protect against TUN-induced ER stress. Currently, the involvement of Gαs in JAMP function is undefined and further studies also need to be conducted to characterize the role of the Gαs-adenylyl cyclase complex, cAMP production and Ca^{2+} levels in Gαs-mediated protection against ER stress.

Gαs has also been shown to modulate the apoptotic response of several cancer cells (Burchett, 2000), however the cAMP/PKA signaling pathway in cell death and survival appears to depend upon the receptor, cell type, and experimental models (Hsiung et al., 2008; Insel et al., 2011). One study in HeLa cervical cancer cells demonstrated that constitutively active Gαs can inhibit cisplatin-induced apoptosis and increase expression of the X-linked inhibitor of apoptosis protein (XIAP) in a cAMP-dependent manner (Cho et al., 2011). In another study, the PKA catalytic subunit was shown to phosphorylate and inactivate Drp1, resulting in mitochondria fusion and cell survival (Cribbs and Strack, 2007). In the current model, following GPCR activation, Gαs remains associated with the plasma membrane and indirectly participates in the apoptotic response via activation of its effector adenylyl cyclase. Based on our observation that Gαs is not only localized to

the plasma membrane but in the ER (Fig. 4.1 and Fig. 4.2), we speculate that Gαs directly acts within the ER domain in close proximity to the mitochondria, perhaps via another complex with gp78/AMFR (discussed in chapter 3). In fact, the ligand of the gp78/AMFR receptor, namely the Autocrine Motility Factor/Phosphoglucose Isomerase (AMF/PGI), has recently been shown to protect against the ER stress response and promote cell survival through the gp78/AMFR receptor and ER calcium release (Fu et al., 2011). Here, GFP-Gαs mimics the anti-apoptotic effect of AMF/PGI and results in a significantly lower expression of the ER stress-induced apoptosis protein CHOP following TUN treatment (Fig. 4.6). Additional apoptotic markers need to be tested and the relationship between AMF/PGI, gp78/AMFR and Gαs in the ER stress response remains to be determined.

Overall, these data propose that a portion of Gαs remains in the ER where it is involved in both substrate polyubiquitylation and protection against TUN-induced ER stress. Whether these two processes are linked and occur through the same Gαs protein-protein interaction(s) remain unknown. However, the significance of this study is the potential of a novel Gαs signaling pathway in the ER that may be distinct from the plasma membrane.*

* Supported by the Canadian Institutes for Heath Research (CIHR MT-15132) and a Michael Smith Foundation for Health Research Trainee award to MF.

Figure 4.1 Distribution of GFP-G protein subunits in Cos7 cells

A. Cos7 cells were transiently transfected with different GFP-tagged G protein subunits (Gαi1, Gαs, Gβ1, Gγ2). 24 h post-transfection, cells were fixed with methanol/acetone and labeled for the ER using the anti-calnexin antibody. Images were acquired with the confocal microscope. Arrows point to the G protein cell surface expression. Scale bar = 20 micrometers; Zoom scale bar = 0.3 micrometers. B. To quantify G protein ER distribution, the Pearson's coefficient was assessed between each G protein subunit and anti-calnexin labeling. A One-way ANOVA followed by Tukey's multiple comparison tests was done. Mean ± S.E.M.; $^{*}P < 0.05$. The graph represents three to five independent experiments (approximately 30-50 cells for each condition).

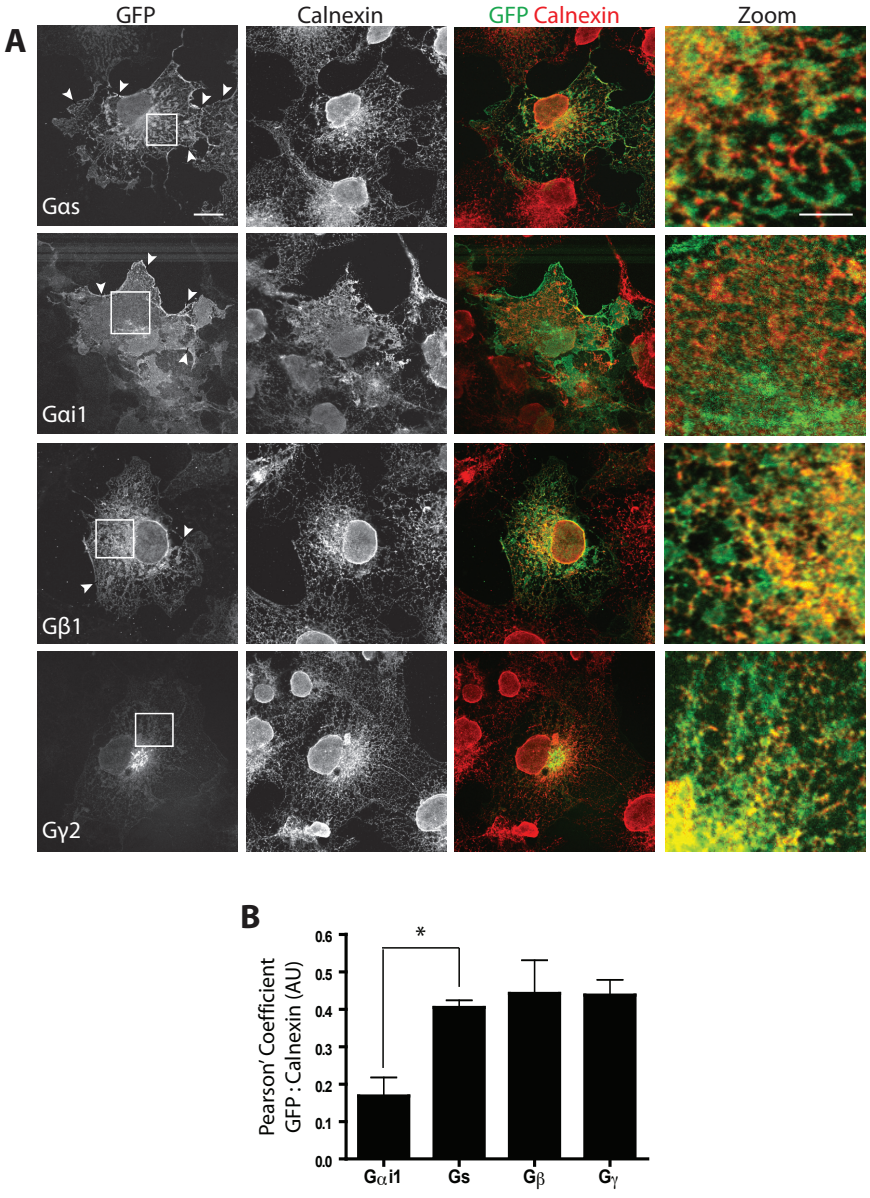

A
GFP
Calnexin
GFP Calnexin
Zoom
Gαs
Gαi1
Gβ1
Gγ2
B
Pearson' Coefficient
GFP : Calnexin (AU)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Gαi1
Gs
Gβ
Gγ

Figure 4.2 The GFP-Gαs subunit is partially localized in the ER

The GFP-tagged Gαs subunit was exogenously expressed in Cos7 cells and 24 h post-transfection, cells were fixed with methanol/acetone. The ER was identified using different protein markers. The tubular smooth ER was determined by expression of Myc-tagged reticulon (Myc-Rtn4a) (A). The central ER was marked by endogenous anti-Serca2A [calcium(2+)-ATPase] labeling (B) and endogenous anti-ERp57 [protein disulfide isomerase] labeling (C). All images were acquired with the confocal microscope. Arrows point to the G protein cell surface expression. Colocalization between GFP-Gαs and each ER marker was assessed using Pearson's coefficient (Mean ± S.E.M; the Pearson's coefficient is reported below). Approximately 10-13 cells (for each condition) were imaged in two independent experiments. Scale bar = 20 micrometers, zoom scale bar = 0.3 micrometers.

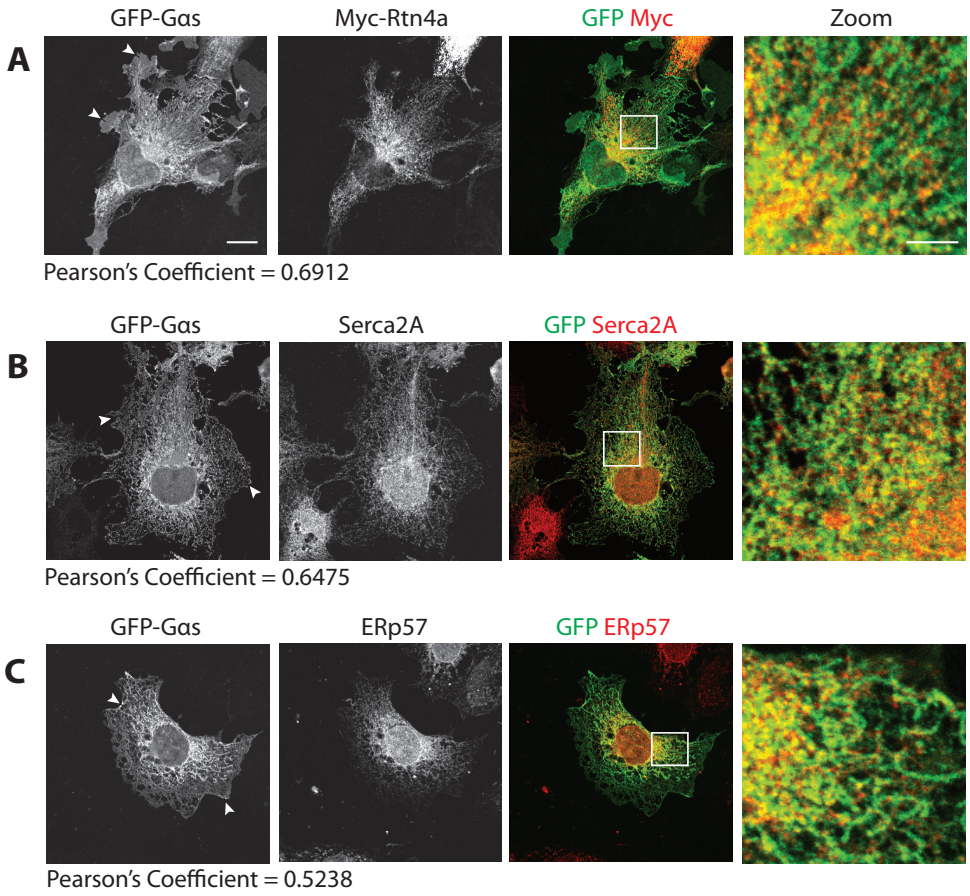


Figure 4.3 GFP-Gαs promotes substrate polyubiquitylation and colocalizes with HA-ubiquitin in the peripheral ER

A. The *in vitro* ubiquitin ligase activity assay. Cos7 cells were transiently cotransfected with HA-tagged ubiquitin wild-type (HA-Ub wt) and one GFP-tagged G protein subunit (Gαi1, Gαs, Gβ1) or Flag-tagged gp78/AMFR (positive control). Cell lysates were collected for anti-Flag immunoprecipitation and immunoblots were probed for anti-HA, anti-GFP, anti-actin and anti-Flag. The immunoblot represents three independent experiments. B-C. Cos7 cells were transiently cotransfected with HA-Ub wt and either GFP-Gαs or GFP-Gαi1. The smooth tubular ER was determined by Myc-tagged reticulon (Myc-Rtn4a) expression. 24 h post-transfection, all cells were fixed with methanol/acetone and images were acquired with the confocal microscope. Colocalization between proteins was assessed using Pearson's coefficient (Mean ± S.E.M; $^{***}P < 0.001$). Approximately 30 cells (for each condition) were imaged in four to five independent experiments. Scale bar = 20 micrometers; zoom scale bar = 0.3 micrometers.

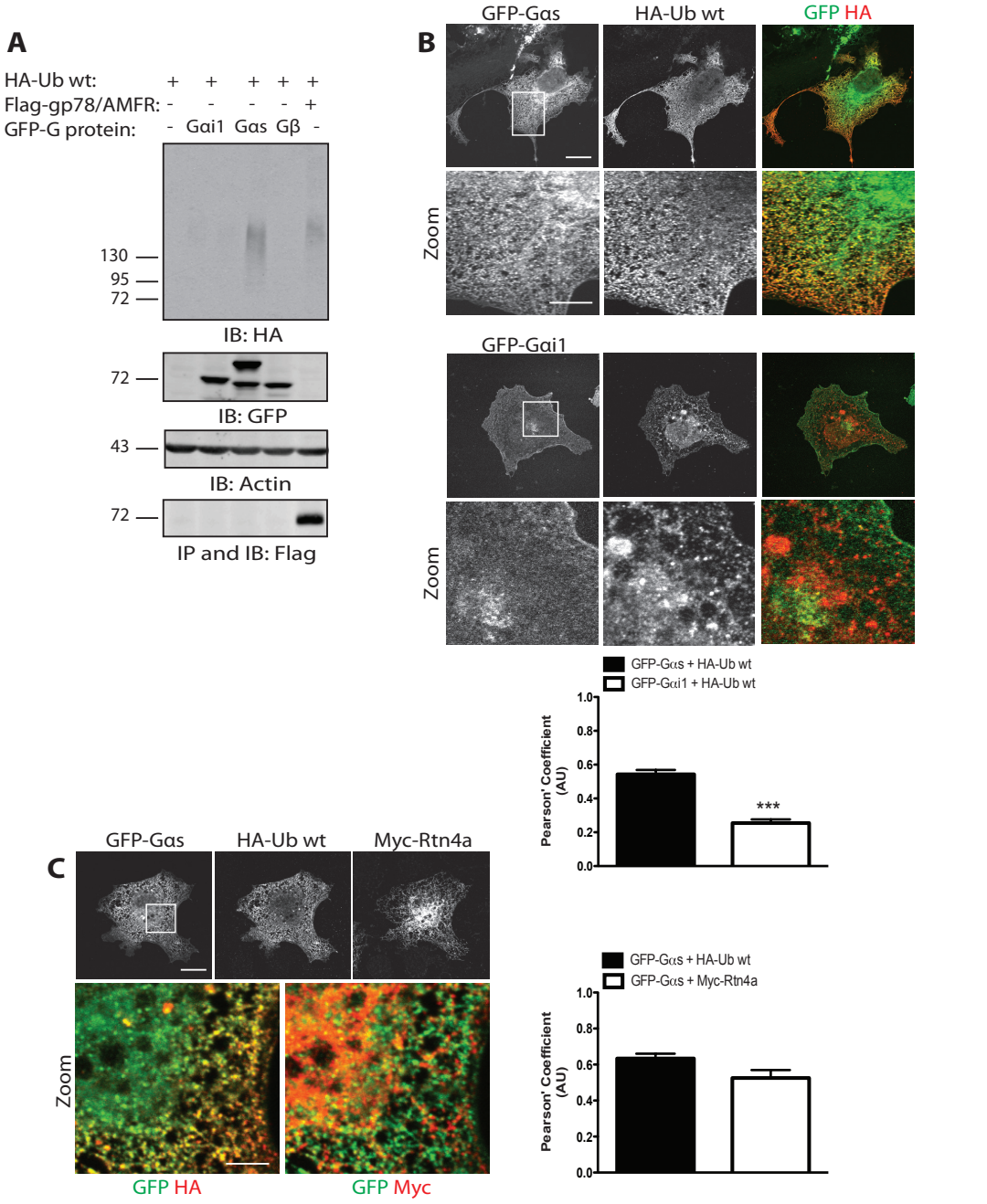
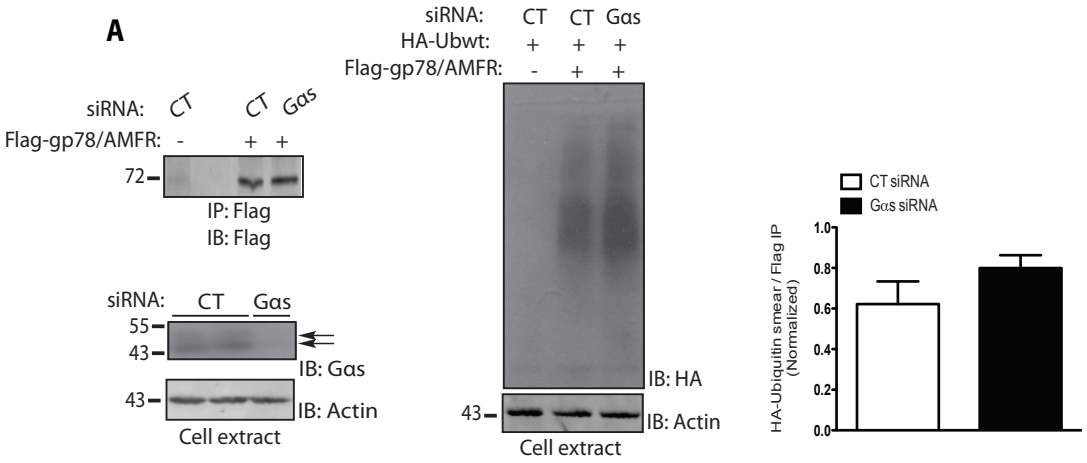

A
HA-Ub wt: + + + + +
Flag-gp78/AMFR: - - - - +
GFP-G protein: - Gαi1 Gαs Gβ -
130
95
72
IB: HA
72
IB: GFP
43
IB: Actin
72
IP and IB: Flag
B
GFP-Gαs
HA-Ub wt
GFP HA
Zoom
GFP-Gαi1
Zoom
GFP-Gαs + HA-Ub wt
GFP-Gαi1 + HA-Ub wt
Pearson' Coefficient (AU)
1.0
0.8
0.6
0.4
0.2
0.0
***
C
GFP-Gαs
HA-Ub wt
Myc-Rtn4a
Zoom
GFP HA
GFP Myc
GFP-Gαs + HA-Ub wt
GFP-Gαs + Myc-Rtn4a
Pearson' Coefficient (AU)
1.0
0.8
0.6
0.4
0.2
0.0

Figure 4.4 GFP-Gαs and Flag-gp78/AMFR polyubiquitylate substrates via distinct mechanisms

A. In the *in vitro* ubiquitin ligase activity assay, HEK293 cells were first transiently transfected with either control (CT) or Gαs siRNA. 24 h later, cells were cotransfected with HA-Ub wt alone (negative control) or with Flag-gp78/AMFR. Immunoblots of total cell extracts were probed for anti-HA, anti-actin and anti-Gαs, while gp78/AMFR expression was detected in the anti-Flag immmunoprecipitation (IP). Formation of polyubiquitylated substrates by Flag-gp78/AMFR in the presence and absence of Gαs was determined with the ratio between the HA smear and anti-Flag IP. The graph represents three independent experiments. B. HA-Ub wt alone (negative control) or with GFP-Gαs were transfected into stable HT1080 non-specific (N.S) control or gp78/AMFR knockdown cell line. Endogenous gp78/AMFR was labeled with 3F3A anti-gp78/AMFR monoclonal antibody in total cell extracts. The *in vitro* ubiquitin ligase activity assay was used to determine formation of polyubiquitylated substrates by GFP-Gαs. The immunoblot is representative of two independent experiments.


A
siRNA: CT CT Gαs
Flag-gp78/AMFR: - + +
72
IP: Flag
IB: Flag
siRNA: CT Gαs
55
43
IB: Gαs
43
IB: Actin
Cell extract
siRNA: CT CT Gαs
HA-Ubwt: + + +
Flag-gp78/AMFR: - + +
IB: HA
43
IB: Actin
Cell extract
CT siRNA
Gαs siRNA
HA-Ubiquitin smear / Flag IP (Normalized)
1.0
0.8
0.6
0.4
0.2
0.0

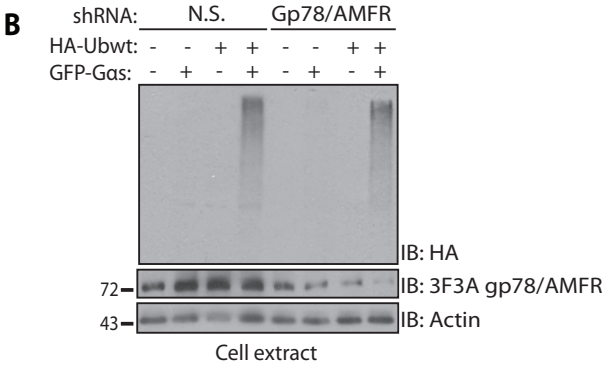

B
shRNA: N.S. Gp78/AMFR
HA-Ubwt: - - + + - - + +
GFP-Gαs: - + - + - + - +
IB: HA
72
IB: 3F3A gp78/AMFR
43
IB: Actin
Cell extract

Figure 4.5 GFP-Gαs and Flag-JAMP share the same protein complex and colocalize in the calnexin-labeled ER

A. Cos7 cells were transiently cotransfected with Flag-tagged JAMP and either pcDNA3.1 vector control or GFP (negative control) or different GFP-G protein subunits (Gαi1, Gαs, Gβ1). 24 h post-transfection, cells were collected, lysed and incubated with anti-Flag beads for immunoprecipitation. Total cell extracts were probed for anti-GFP to determine total G protein expression. The immunoblot is representative of three independent experiments. B and C. Cos7 cells were transiently transfected with Flag-JAMP alone (B) or with GFP-Gαs (C), and later labeled for the ER with the anti-calnexin antibody. Cells were fixed with methanol/acetone and images were acquired with the confocal microscope. Scale bar = 20 micrometers; zoom scale bar = 0.3 micrometers. D. Colocalization between proteins was determined by Pearson's coefficient. Mean ± S.E.M.; $^{**}P < 0.01$. The graph represents three independent experiments (the number of cells are indicated in each column).

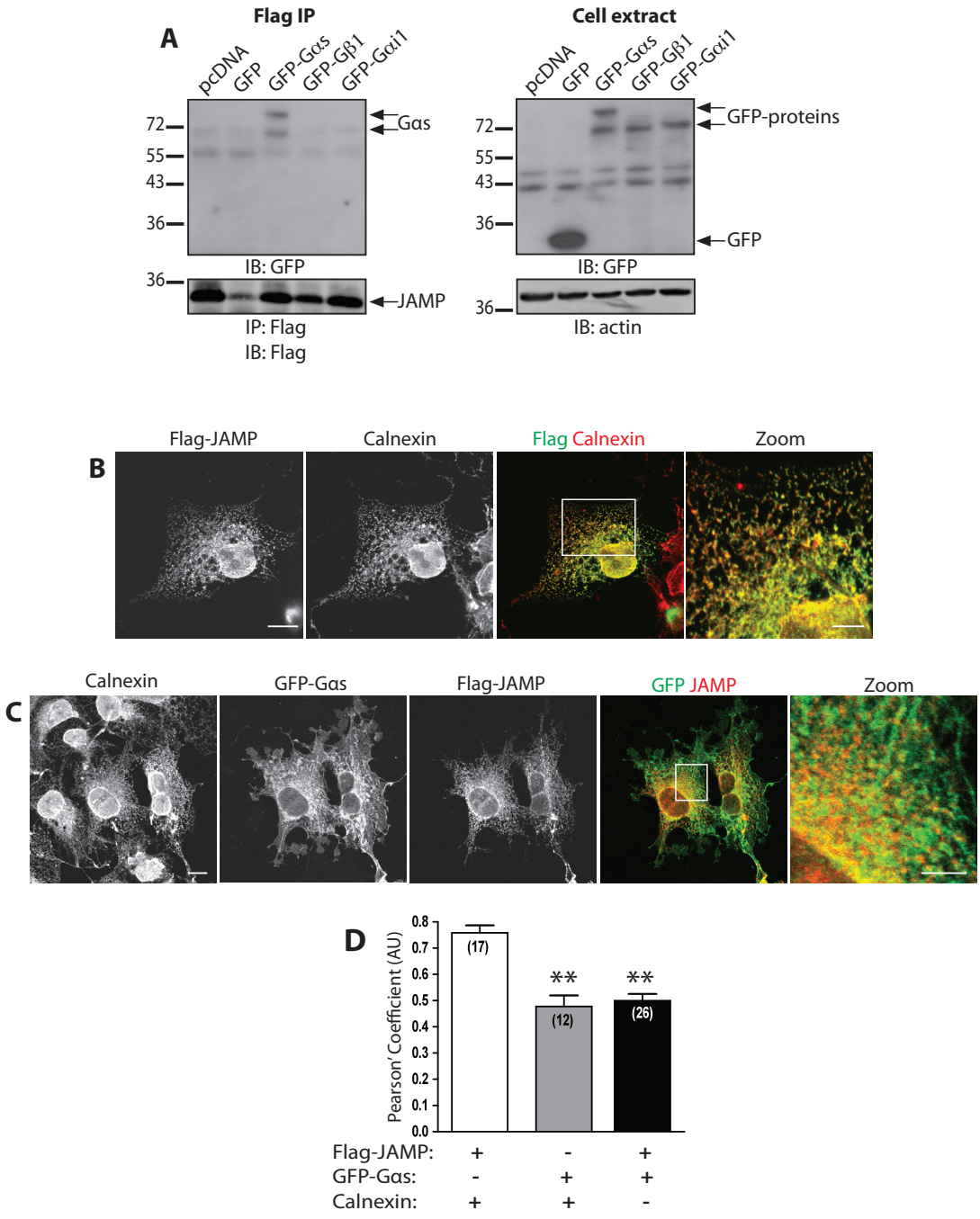

A
Flag IP
pcDNA
GFP
GFP-Gαs
GFP-Gβ1
GFP-Gαi1
72
55
43
36
Gαs
IB: GFP
36
JAMP
IP: Flag
IB: Flag
Cell extract
pcDNA
GFP
GFP-Gαs
GFP-Gβ1
GFP-Gαi1
72
55
43
36
GFP-proteins
GFP
IB: GFP
36
IB: actin
B
Flag-JAMP
Calnexin
Flag Calnexin
Zoom
C
Calnexin
GFP-Gαs
Flag-JAMP
GFP JAMP
Zoom
D
Pearson' Coefficient (AU)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
(17)
**
(12)
**
(26)
Flag-JAMP: + - +
GFP-Gαs: - + +
Calnexin: + + -

Figure 4.6 GFP-Gαs plays a protective role in the TUN-induced ER stress response
24 h post-transfection with GFP-Gαs, HEK293 cells were incubated overnight with serum-free DMEM. To promote the ER stress response, cells were treated for 8 h with Tunicamycin (2 µg/ml TUN) and immunoblots were probed for anti-BiP and anti-CHOP. The ER stress response was quantified based on total BiP and CHOP protein levels in cell extracts that were first normalized to actin expression and later, all conditions were normalized to the non-treated pcDNA control. A One-way ANOVA followed by Tukey's multiple comparison tests was done. Mean ± S.E.M.; $^{*}P < 0.05$. The immunoblot represents five independent experiments.

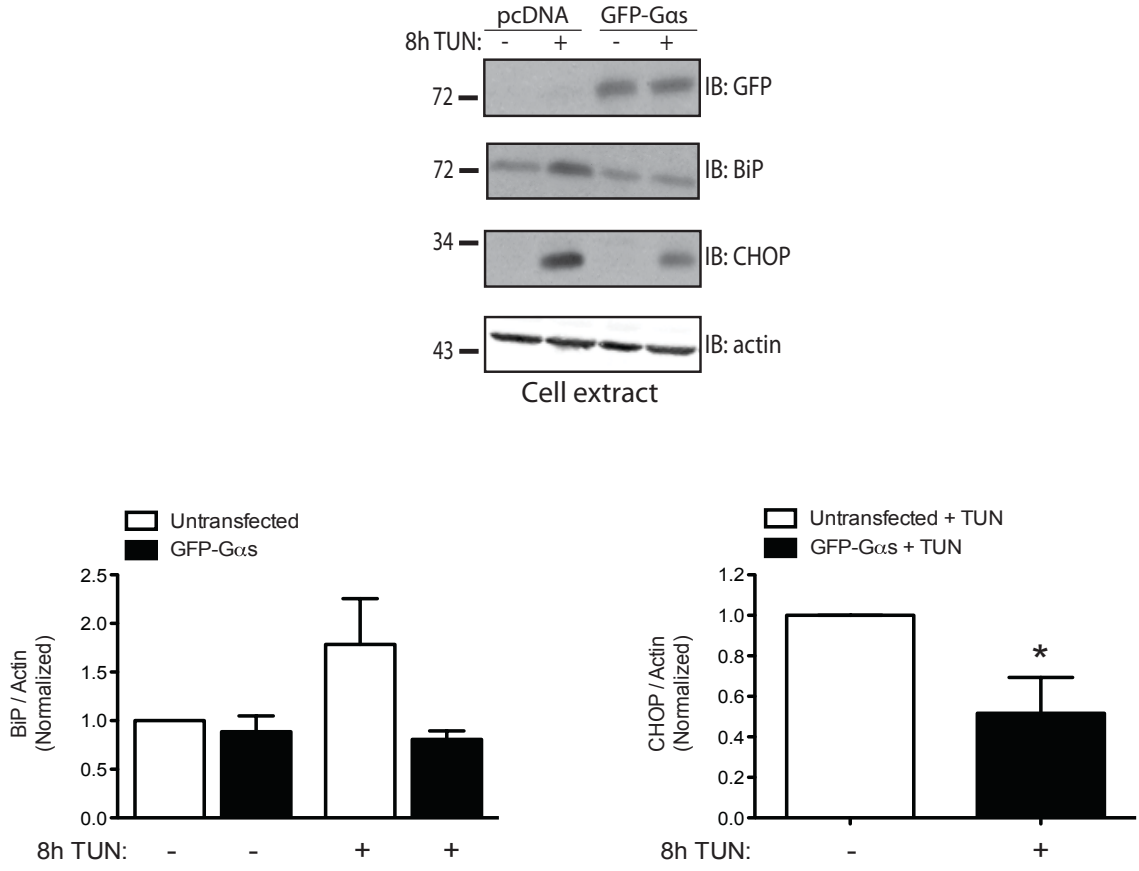
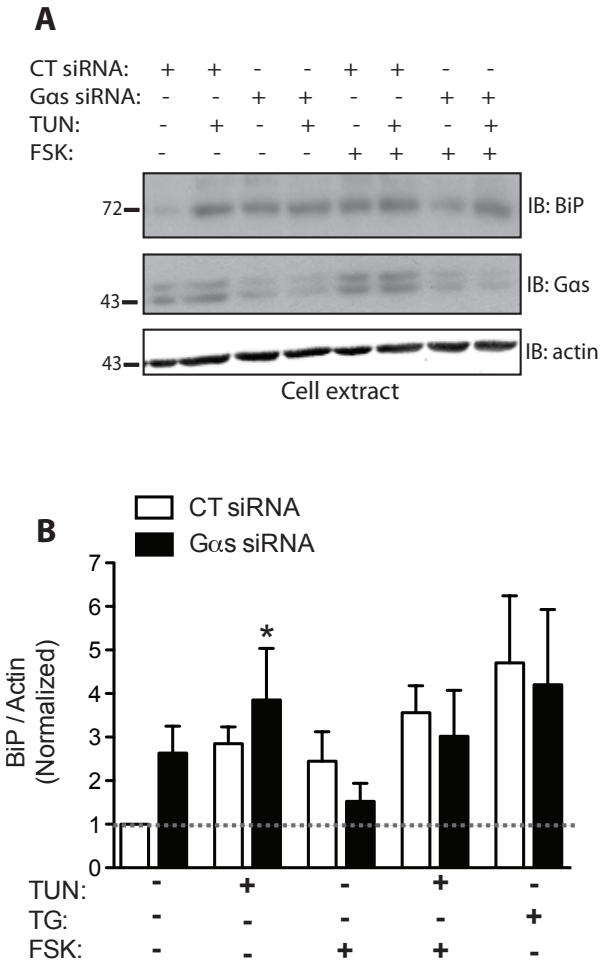


Figure 4.7 Gαs knockdown induces an ER stress response

A. HEK293 cells were transiently transfected with either control (CT) siRNA or siGαs siRNA. 24 h post-transfection, cells were incubated overnight with serum-free DMEM and later treated for 8 h with Tunicamycin (2 µg/ml TUN) or Thapsigargin (5 µM TG) to promote an ER stress response. Similarly, cells were either treated for 8 h with Forskolin (FSK, 10µM) alone, or pretreated for 30 min with FSK prior to an 8 h TUN treatment. B. The ER stress response was quantified using total BiP protein levels that were first normalized to actin expression and later, to the non-treated pcDNA control. A One-way ANOVA followed by Tukey's multiple comparison tests was done. Mean ± S.E.M.; $^{*}P < 0.05$ compared to control. The data represent three to fourteen independent experiments.



CHAPTER 5 Discussion

5.1 Novel components in ER Quality Control mechanisms

In this body of work, we studied in detail aspects of the ER Quality Control mechanisms, a pathway used by the cell to maintain homeostasis and protect itself against ER stress and in severe cases apoptosis. ER Quality Control is not a simple pathway but involves many protein complexes and an intricate web of synchronized events, some of which include molecular chaperones, the Calnexin-Calreticulin cycle, the UPR and the ERAD pathway. To preserve proper function and high efficiency, proteins within these pathways need to be regulated themselves, allowing the cell to quickly adapt to its extracellular environment. An example to illustrate this phenomenon is ubiquitylation of E3 ubiquitin ligases that allows self-regulation of their activity in the ERAD pathway (de Bie and Ciechanover, 2011; Weissman et al., 2011).

5.2 Perspective on RING finger S-palmitoylation

In chapter 2, we investigated the E3 ubiquitin ligase gp78/AMFR and discovered a dynamic form of palmitoylation (S-palmitoylation) within its catalytic RING finger domain that is responsible for its ubiquitin ligase activity. This is the first documented account of RING finger palmitoylation and the presence of palmitate likely tethers the RING finger domain to the membrane. It raises the possibility that palmitoylation of E3 enzymes, similar to ubiquitylation, may represent another important regulatory mechanism. As seen with gp78/AMFR, RING finger palmitoylation may function to negatively modulate E3 ubiquitin ligase activity by both enhancing its protein turnover and changing its cellular distribution. It is clear that more functional studies need to be conducted in order to define the details of this mechanism and most importantly, to investigate whether palmitoylation of the RING finger is unique to gp78/AMFR or affects the function of other E3 enzymes (i.e. RNF5/RMA1, Hrd1). Finally, we cannot neglect a possible interplay between E3 ubiquitylation and palmitoylation that has been previously documented with the Wnt signaling protein LRP6. Here, the authors show that LRP6 palmitoylation is required for its ER exit and protection against monoubiquitylation-dependent ER retention, suggesting that both modifications

contribute to the proper function of LRP6 at the cell surface (Abrami et al., 2008). In the case of gp78/AMFR, it has the ability to direct its own degradation as well as it is targeted for proteasomal degradation (Fang et al., 2001) by another E3 enzyme Hrd1 (Ballar et al., 2010; Shmueli et al., 2009). Here, the impact of gp78/AMFR palmitoylation on its ubiquitylation and degradation is unknown and as with LRP6, the relationship between palmitoylation and ubiquitylation may be important in determining its intracellular localization and stability.

The study of gp78/AMFR palmitoylation was conducted on exogenously expressed proteins that proved to be a powerful tool in describing the palmitoylation motif and the effect of DHHC6-mediated gp78/AMFR palmitoylation. In particular, identification of S-palmitoylation within the RING finger was very challenging and we were unable to find an individual cysteine with a higher affinity for palmitate. Instead, all six cysteines within the RING finger were able to undergo palmitoylation. In these experiments, we used site-directed mutagenesis that was critical in confirming gp78/AMFR RING finger palmitoylation but one limitation was that we were unable to use the RING finger mutants for functional studies since it is impossible to distinguish between the effect of (1) cysteine palmitoylation within the RING finger and (2) a disruption in the primary structure of the RING finger induced by cysteine mutagenesis. Next, we can focus on endogenous gp78/AMFR and strengthen our findings by showing that S-palmitoylation of gp78/AMFR takes place in both exogenously expressed and endogenous gp78/AMFR.

As mentioned above, there is a need to investigate the functional role of RING finger palmitoylation and one approach would be to look at both Flag-tagged and endogenous gp78/AMFR following a transient knockdown of one or more DHHC enzymes that have been identified to target gp78/AMFR (Fig. 2.4). As described in chapter 2, Myc-DHHC6 is a strong candidate however, it is important to verify the endogenous expression levels of each DHHC enzyme in the cell line of choice, since all five Myc-DHHC enzymes have the ability to palmitoylate gp78/AMFR and the DHHC enzyme(s) with the highest expression level(s) would be used in the siRNA experiment. With this approach, we are likely to avoid DHHC enzyme redundancy and study more accurately the effect of palmitoylation on gp78/AMFR function. In the absence of one or

more DHHC enzymes, we anticipate a significant reduction in gp78/AMFR palmitoylation confirming that in fact, gp78/AMFR is specifically targeted by DHHC enzymes and not subject to non-enzymatic palmitoylation (discussed below). Also, by inhibiting gp78/AMFR palmitoylation with the absence of endogenous DHHC enzymes, we hypothesize that gp78/AMFR will be expressed throughout the ER and highly stable showing increased protein levels because it will no longer be targeted for degradation by the proteasome. To further demonstrate that non-palmitoylated gp78/AMFR undergoes slower protein turnover, we can first treat cells with cycloheximide to block novel protein synthesis and later with the proteasome inhibitor MG132. Under these conditions, we predict that gp78/AMFR protein levels will be initially higher and remain more stable in the DHHC siRNA lysates compared to the control siRNA lysates where gp78/AMFR protein expression will gradually increase following prolonged MG132 treatments. The same experiment can be done with exogenous Myc-DHHC6 and in this case, by enhancing gp78/AMFR palmitoylation, we anticipate a decrease in protein stability and an increase in protein turnover (supporting data from Fig. 2.7).

In addition to protein stability, we can also look at the role of gp78/AMFR palmitoylation in terms of its ubiquitin ligase activity. Based on previous data that palmitoylation increases gp78/AMFR protein turnover, we hypothesize that RING finger S-palmitoylation will likely inhibit substrate degradation. Using the DHHC knockdown experiment described above, we can monitor E3 ubiquitin ligase activity by looking at: (1) a smear for total substrate polyubiquitylation in the *in vitro* ubiquitin ligase activity assay (described in chapter 2) and (2) protein expression levels of specific gp78/AMFR substrates (e.g. Kai-1, α1-antitrypsin, and CFTRΔF508). As of yet, it is unknown whether gp78/AMFR ubiquitin ligase activity is restricted to the peripheral smooth ER or whether its function extends to the plasma membrane. It is also unknown whether the ligand AMF/PGI is involved in gp78/AMFR ubiquitin ligase activity, as a positive or negative regulator. Gp78/AMFR palmitoylation may be the determining factor in modulating its function as an E3 ubiquitin ligase at different cellular sites. This is supported by the finding that gp78/AMFR is palmitoylated by several DHHC enzymes localized to the ER, the Golgi and the plasma membrane (Fig. 2.4 and Fig. A.2).

There is also the possibility that palmitoylation itself inhibits gp78/AMFR activity by altering its conformation and even selecting for different protein binding partners. To study this possibility further, we can use a Proteomics' approach that would lead to a large-scale study of all proteins that interact with gp78/AMFR and potentially detect differences in protein binding partners following induction of gp78/AMFR palmitoylation. In this experiment, we compare gp78/AMFR in the presence and absence of Myc-DHHC6 under different conditions: (1) Flag-gp78/AMFR alone; (2) Flag-gp78/AMFR + Myc-DHHC6; (3) HA-ubiquitin wild-type + Flag-gp78/AMFR; (4) HA-monoubiquitin + Flag-gp78/AMFR; (5) HA-ubiquitin wild-type + Flag-gp78/AMFR + Myc-DHHC6; (6) HA-monoubiquitin + Flag-gp78/AMFR + Myc-DHHC6. As an additional control, we can compare wild-type gp78/AMFR to the palmitoylation-deficient RING finger mutant that has little to no ubiquitin ligase activity (Fig. 2.3) and is not palmitoylated by DHHC6 (Fig. 2.4). Here, we are looking at the effect of palmitoylation on both gp78/AMFR protein binding partners (1-2) and gp78/AMFR polyubiquitylated substrates (3,5). We can exclude non-specific monoubiquitylated proteins to distinguish between initiation of ubiquitylation and polyubiquitin chain extension by using $K^{29,48,63}R$-Ubiquitin (HA-monoubiquitin) that prevents polyubiquitin chain elongation (4,6). The distinction between gp78/AMFR protein binding partners (1,2) and ubiquitylated substrates (3,5) may supply clues on whether palmitoylation alters gp78/AMFR conformation by selecting for different protein binding partners and/or inhibits its ubiquitin ligase activity by reducing affinity for substrates.

5.3 Gp78/AMFR undergoes palmitoylation in the N-terminal transmembrane domain

The observation that gp78 is not only S-palmitoylated within the RING finger but also has a non-conventional palmitoylation site (perhaps N-palmitoylation or O-palmitoylation) in its N-terminal transmembrane domain, was discovered by two methods: (1) In the metabolic radiolabeling experiment, the absence of all cysteines in the N-terminal transmembrane domain of gp78/AMFR construct (no C-terminus domain) did not remove the palmitoylation signal; this was not S-palmitoylation since it failed to be detected in the Biotin-BMCC labeling (Fig. 2.1E and Fig. A.1); (2) the full-length

cysteine-free RING finger Flag-gp78/AMFR mutant (ΔC RING) was no longer S-palmitoylated in the Biotin-BMCC labeling despite the presence of all the N-terminal cysteines within the transmembrane domain (Fig. 2.2). As of now, it is unclear whether the N-terminal palmitoylation occurs within the Flag tag or is indeed specific to the transmembrane domain of gp78/AMFR. The presence of this additional palmitoylation site is intriguing and probably merits further investigation however, it would be a highly challenging project, considering the current limitations in our ability to identify palmitoylation motifs: (1) no clear consensus sequence and in this case, specific residue; (2) time consuming and requires the use of metabolic radiolabeling; (3) to exclude Flag tag palmitoylation, we can substitute the Flag tag for a GFP tag; (4) systematic truncation of the N-terminal domain within the full-length RING ΔC Flag-gp78/AMFR construct to identify the palmitoylated region; (5) Alternatively, mass spectrometry can be used to locate the other type of palmitoylation using the full-length RING ΔC Flag-gp78/AMFR construct, however this may be problematic with the very low transfection efficiency of Flag-gp78/AMFR.

5.4 Contribution of DHHC enzymes in the palmitoylation reaction

The contribution of specific enzymes in mediating protein palmitoylation is challenged as some investigators argue that palmitoylation takes place non-enzymatically in cells (Duncan and Gilman, 1996). Historically, palmitoylation was thought to occur in the absence of enzymes and non-enzymatic palmitoylation has previously been observed *in vitro* (Bano et al., 1998; O'Brien et al., 1987). This is further supported by the observation that thiolases, an enzyme that catalyzes the hydrolysis of palmitoyl-CoA derivatives, are able to non-specifically transfer palmitoyl-CoA to the sulphydryl group of a cysteine. As discussed previously, it is becoming more apparent that enzymes, namely PATs, are responsible for the majority of protein palmitoylation and enzymatic palmitoylation is likely the primary mechanism, based on several observations: (1) the kinetics of palmitoylation are rapid and consistent with an enzymatic reaction; (2) Sequence specificity has been observed with many palmitoyl proteins and disruption of residues surrounding the target cysteine only inhibits palmitoylation in cells but not non-enzymatic palmitoylation *in vitro* (Resh, 1999); (3) Acyl-CoA-binding proteins (ACBP)

have been shown to inhibit non-enzymatic palmitoylation and maintain low acyl-CoA concentrations in the cell (Leventis et al., 1997).

In the case of gp78/AMFR, it is unclear how palmitoylation of the N-terminal transmembrane domain occurs and whether this process is an example of non-enzymatic palmitoylation. It is likely that the N-terminal transmembrane domain undergoes O-palmitoleoylation that occurs on a serine residue and has been documented with the murine Wtn-3a protein (Takada et al., 2006). On the other hand, we have strong evidence to support that S-palmitoylation within the RING finger domain is modulated by at least one DHHC enzyme, DHHC6, and appears to be a dynamic process that can be reversed by treatment with the general inhibitor of palmitoylation 2-bromopalmitate. Moreover, the RING finger palmitoylation site is preceded by a cluster of hydrophobic/basic residues, which is seen in other palmitoylated proteins such as PSD95 (El-Husseini et al., 2000a).

5.5 How does S-palmitoylation affect gp78/AMFR topology?

Palmitoylation has been shown to alter the conformation of receptors that in turn changes their distribution, enhances their degradation and even affects their function (discussed in section 1.3.6). Alterations in membrane topology can be achieved by different mechanisms, in addition to posttranslational modifications such as palmitoylation, and is a determining factor in protein function. GPCR activation is highly dependent on its stucture whereby agonist-induced conformational changes followed by ligand binding results in the rearrangement of transmembrane domains, activation of the G protein complex and β−arrestin binding prior to receptor internalization (Deupi and Standfuss, 2011). GPCRs employ multiple signaling pathways and the binding of PDZ adaptor proteins that function as scaffolds able to assemble multiprotein complexes, have been shown to be an important factor in determining cell-specific signaling and GPCR trafficking (Romero et al., 2011).

There is controversy surrounding the topology of gp78/AMFR that is partly due to its complex biological functions as a cytokine receptor on the cell surface and as an E3 ubiquitin ligase in the ERAD pathway (described in section 1.2; Scheme 1.2.1). Modulation in gp78/AMFR conformation may be a critical factor in determining its

distinctive intracellular functions. Based on computational studies, gp78/AMFR is a transmembrane receptor that has been shown to contain five or six or seven membrane-spanning domains. The interpretation is highly dependent on the type of analysis used (Scheme 1.2.2; (Fairbank et al., 2009)). In chapter 3, we discuss the possibility that gp78/AMFR is an ER-localized GPCR since it interacts with and recruits two Gα proteins (Gαi1 and Gαs) to the ER domain. Thus, it is possible that gp78/AMFR is one of the first non-conventional GPCRs localized to the ER. In addition to the ambiguity surrounding its transmembrane domains, the orientation of its N- and C-terminus is also unclear. One study proposed that the C-terminal region of gp78/AMFR is located on the extracellular side of the plasma membrane, and that the AMF/PGI-gp78/AMFR interaction is dependent on an N-glyco sugar chain and dimerization of the receptor via the leucine zipper (Haga et al., 2006b; Shimizu et al., 1999). Here, we have insufficient data to confirm the topology of gp78/AMFR, but we can speculate that gp78/AMFR is a six transmembrane receptor in which both the N-terminus and the C-terminus are oriented towards the cytosol (Scheme 5.5.1). Using an N-terminal tetracysteine tag, we are able to visualize gp78/AMFR by live cell imaging on the ER membrane whereby the tetracysteine tag fluorescences once bound to a FlAsH dye (an arsenic-based compound), suggesting that the N-terminus is found in the cytoplasm (St. Pierre et al., 2011). Evidence supporting the cytoplasmic orientation of the C-terminus includes palmitoylation of the RING finger by a DHHC enzyme and its interaction with many cytosolic ERAD factors (such as p97/VCP/Cdc48 (Ballar et al., 2006; Zhong et al., 2004)) on the ER membrane (Scheme 1.1.3).

The topology of gp78/AMFR may be different in the ER and on the plasma membrane. Here, we propose that palmitoylation is one mechanism that may modulate the conformation of gp78/AMFR and thereby impact on its function as a cytokine receptor on the cell surface and as an E3 ubiquitin ligase in the ERAD pathway (Scheme 5.5.1). In the current study, we show that RING finger palmitoylation occurs in the ER via a DHHC enzyme and enhances gp78/AMFR peripheral ER distribution (Fig. 2.5). S-palmitoylation results in the tethering of the RING finger domain to the membrane and the formation of an additional intracellular loop. This change within the palmitoylated

RING finger may be sufficient to inhibit interactions with ERAD factors and enable novel protein contacts in the ER.

5.6 Cell surface expression of gp78/AMFR and palmitoylation

We show in chapter 2 that in the presence of Myc-DHHC6, gp78/AMFR is redistributed to the smooth peripheral ER labeled by the 3F3A gp78/AMFR antibody and depleted in the central ER, the site of proteasomal degradation (Levine and Rabouille, 2005; St. Pierre et al., 2011). To further test this hypothesis, we can treat cells with the proteasome inhibitor MG132 and anticipate a significant accumulation of gp78/AMFR in the central ER.

As mentioned previously, a small portion of gp78/AMFR is normally expressed on the cell surface and we speculate that palmitoylation plays an additional role, in that it is able to target gp78/AMFR beyond the peripheral ER to the plasma membrane. In this context, gp78/AMFR palmitoylation may induce a conformational change that in turn enhances the receptor's affinity for proteins other than ERAD components (i.e. Gα proteins). These novel protein-protein interactions may assist in its translocation to the peripheral ER and the plasma membrane, where it binds to its ligand AMF/PGI and functions as a cytokine receptor on the cell surface (Scheme 5.5.1). Thus, S-palmitoylation may display a unique function for gp78/AMFR and represent the first step of many in targeting gp78/AMFR to the cell surface.

This hypothesis is very attractive but as of yet, there is no data to support this model. In the present study, we failed to detect an enhanced plasma membrane expression of gp78/AMFR in the presence of DHHC enzyme in confocal images. However, it is likely that a more sensitive approach is needed to detect subtle changes in gp78/AMFR plasma membrane expression, such as a cell surface biotinylation assay. Using this approach, we can begin to investigate the role of palmitoylation in gp78/AMFR cell surface expression by selectively biotinylating Flag-gp78/AMFR on the cell surface of living cells and followed by an anti-Flag immunoprecipitation, we can probe the membrane for anti-streptavidin to determine the pool of gp78/AMFR localized to the plasma membrane, compared to the total protein (probed with the anti-Flag antibody). In this way, we are able to monitor fluctuations in gp78/AMFR cell surface

expression that occur in the presence of expressed Myc-DHHC6 or in the absence of endogenous DHHC enzyme(s) or following treatment with 2-bromopalmitate.

5.7 Gα proteins in the ER?

In chapter 3, we show that two Gα proteins associate with gp78/AMFR in the ER, independently of receptor activation. Binding between gp78/AMFR and G proteins has not been characterized in detail and it is unknown whether this protein interaction is even direct. Further, conditions allowing gp78/AMFR to preferentially select one Gα protein (Gαi1 vs. Gαs) are unknown and may include factors such as ligand binding and palmitoylation. As discussed in section 1.4.1, previous data suggesting that gp78/AMFR is a putative GPCR are all derived from pertussis toxin treatments. Briefly, pertussis toxin is a bacterial toxin that catalyzes the ADP-ribosylation of a cysteine residue located near the carboxyl-terminal end of Gα belonging to the Gαi and Gαo protein family (Murayama and Ui, 1983). Thus, pertussis toxin treatment affects adenylyl cyclase signaling by altering the structure of Gαi/o and preventing its receptor coupling (Ribeiro-Neto and Rodbell, 1989). Here, it is important to acknowledge that pertussis toxin also releases the Gβγ subunit from Gαi/o and the presence of free Gβγ may be a significant factor in the gp78/AMFR signaling pathway.

Looking at gp78/AMFR G protein binding, we discover novel functions for the Gαs subunit in the ER, namely its ability to induce substrate polyubiquitylation and protect against the ER stress response. As of yet, our understanding is incomplete and more work needs to be done to define the mechanism(s), specifically how Gαs is involved in these pathways and whether these two events occur via the same Gαs protein complex. In future studies, the involvement of Gαs in the ERAD pathway should be determined in terms of its effect on other E3 enzymes such as RNF5/RMA1 and Hrd1, since our data suggest that Gαs is partly dependent upon gp78/AMFR ubiquitin ligase activity (Fig. 3.5 and Fig. 4.4). In chapter 4, we discuss the possibility that Gαs acts via another putative GPCR in the ER, the JNK-associated membrane protein (JAMP). JAMP was identified as an adaptor protein in ERAD (Morito et al., 2008; Tcherpakov et al., 2008) and a component in stress-induced apoptosis (Kadoya et al., 2005). Here, we show that JAMP preferentially associates with the Gαs subunit (Fig. 4.5). Thus, we speculate

that the observed substrate polyubiquitylation and protection against ER-stress during GFP-Gαs expression are both mediated by binding of Gαs to JAMP. However, additional studies need to be done to confirm direct binding of these proteins. Most importantly, the effect of Gαs on JNK signaling in ER stress as well as changes in JAMP ubiquitylation and activity in the presence of GFP-Gαs, also need to be considered.

In addition to its plasma membrane and ER localization, we have preliminary data to show that both GFP-Gαs and endogenous Gαs are closely associated with mitochondria. In fact, Dr. Simmen's laboratory (University of Alberta) demonstrated via Optiprep and Percoll gradient fractionation (described in (Lynes et al., 2011)) that Gαs is localized to the mitochondria-associated membrane (MAM), a domain of the ER that is critical in coupling the ER to the mitochondria (data not shown). The observed mitochondrial-ER distribution seems to be unique to Gαs and reinforces the concept that Gαs plays a more direct function within the ER.*

5.8 Summing-up

The research accomplished in this manuscript proposes a role for palmitoylation and G proteins in ER Quality Control mechanisms. In particular, we investigate (1) a regulatory role for palmitoylation of the E3 ubiquitin ligase gp78/AMFR in the ERAD pathway; (2) the implication of G proteins in gp78/AMFR functions; and (3) the involvement of ER-localized Gαs in both substrate polyubiquitylation and ER stress. As discussed above, these findings mark the beginning in our understanding of these new factors and additional work needs to be done to identify the mechanisms in question.

In chapter 2, we show that the E3 ubiquitin ligase gp78/AMFR is palmitoylated within the catalytic RING finger motif, responsible for its ubiquitin ligase activity, and propose a modulatory function for gp78/AMFR palmitoylation, whereby palmitoylation regulates its ER distribution and enhances its protein turnover. It still remains unknown whether palmitoylation of E3 ubiquitin ligases is gp78/AMFR-specific or a general mechanism to control the activity of RING finger ubiquitin ligases. Moreover, additional functional studies need to be conducted to fully characterize the effect of gp78/AMFR

* We plan to submit a version of chapter 4 for publication and include data from Dr. Simmen's laboratory.

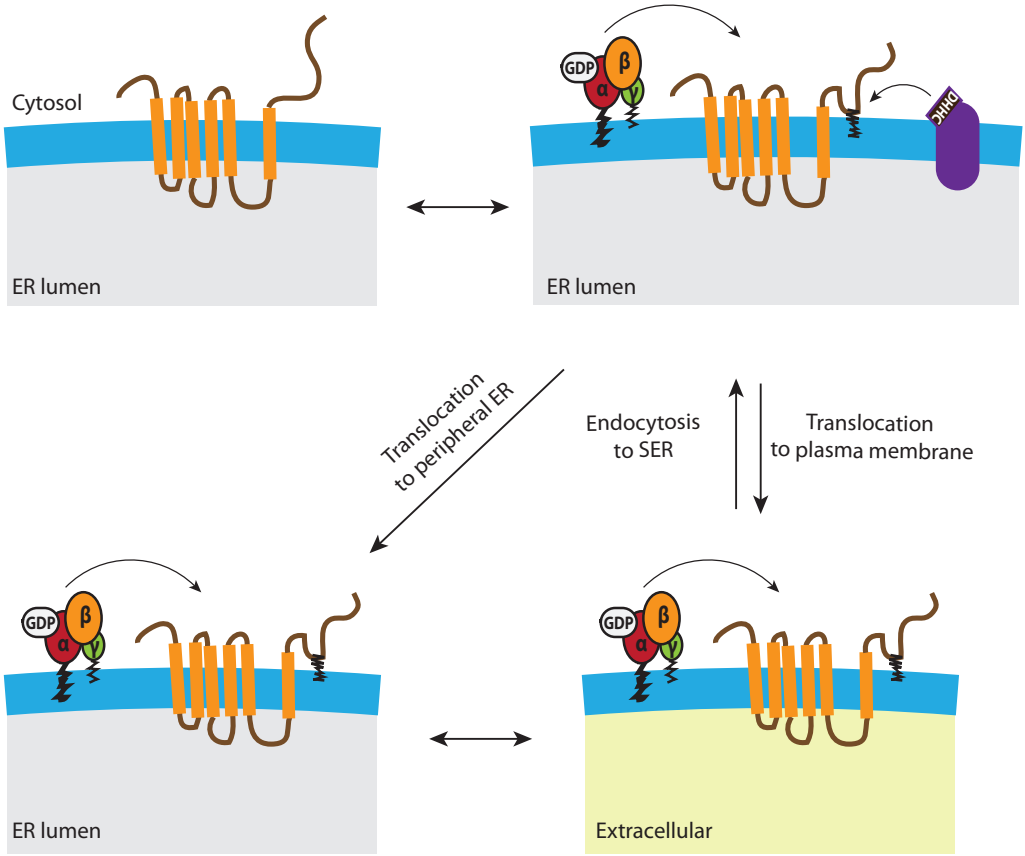
palmitoylation on receptor conformation, protein-protein interactions (i.e. Gα proteins), substrate affinities and RING finger function in the ERAD pathway.

Next, we look at the Gα subunit and show in chapter 3 that the E3 ubiquitin ligase gp78/AMFR interacts with and recruits several G proteins to the ER, namely Gαi1 and Gαs. In order to reinforce the possibility that gp78/AMFR is a novel ER-localized GPCR, we need to confirm that gp78/AMFR directly interacts with one or both Gα subunits using a purified protein-protein binding assay. Finally, it is critical to study conditions whereby gp78/AMFR discriminates between Gαi1 and Gαs (i.e. ligand binding and endocytosis, cell surface expression vs. ER localization), supplying us with hints of the physiological significance of G protein binding to gp78/AMFR. Finally, the study of G proteins led us to investigate a novel role for the Gαs subunit in the ER where we demonstrate in chapter 4 that Gαs is present within an ERAD protein complex, and is able to induce substrate polyubiquitylation and protect against ER stress. Here, we propose a novel function for Gαs within the ER domain, however we still need to identify its direct protein binding partner(s), as well as describe in detail the mechanism(s) by which Gs signals in both the ERAD pathway and the ER stress response.

ERQC represents a complex assembly of pathways that are vital in maintaining proper cellular function and homeostasis. Taken together, the findings described in this manuscript broaden our knowledge and most importantly, question the physiological significance of E3 ubiquitin ligase palmitoylation, G protein binding to gp78/AMFR in the ER, and finally, Gαs-mediated substrate polyubiquitylation and protection against ER stress, in ERQC mechanisms.

Scheme 5.5.1 RING finger palmitoylation in the modulation of gp78/AMFR topology

Here, we illustrate the potential role for RING finger palmitoylation in terms of gp78/AMFR membrane topology and trafficking between the plasma membrane and the smooth ER. Based on the assumption that both the N-terminus and the C-terminus are cytosolic, gp78/AMFR is a six transmembrane receptor. In the central ER, gp78/AMFR undergoes palmitoylation within the RING finger domain by a DHHC enzyme and is then translocated to the peripheral ER and potentially to the plasma membrane. RING finger palmitoylation tethers the cytosolic tail to the plasma membrane creating an additional intracellular loop that may induce interactions with G proteins.



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APPENDIX: Supplementary data

Figure A.1 The N-terminal transmembrane domain of gp78/AMFR undergoes non-conventional palmitoylation

A. Distribution of 9 cysteine residues within the N-terminal transmembrane domain (TMD) of Flag-gp78/AMFR. B and C. Using site-directed mutagenesis, cysteines were systematical substituted for alanines in the N-terminal Flag-tagged transmembrane domain of gp78/AMFR that lacks the C-terminal domain (TMD). Cos7 cells were transiently transfected with different Flag-gp78/AMFR TMD mutant constructs and metabolic labeling with ^{3}H-palmitate was done to detect protein palmitoyation. Cell lysates were collected for anti-Flag immunoprecipitation and immunoblotting. Films were exposed for 1 week to detect palmitoylation signal and anti-Flag immunoblots were done to determine total protein expression (1% input). The immunoblots represent three independent experiments.

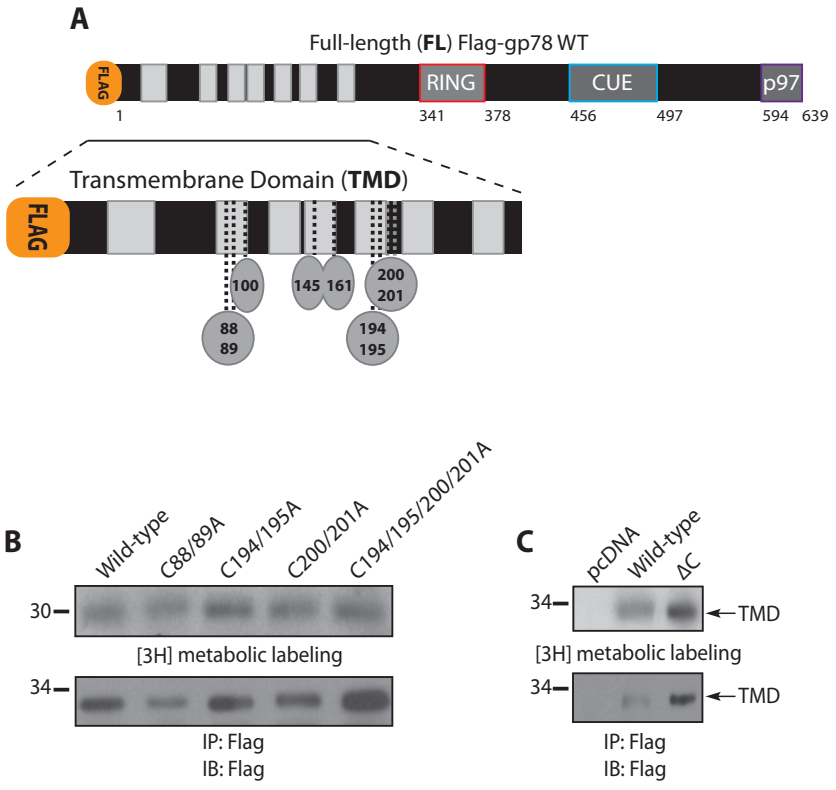


Figure A.2 Distribution of DHHC enzymes that modulate RING finger gp78/AMFR palmitoylation

Cos7 cells were transiently transfected with one Myc-tagged DHHC enzyme and labeled for the ER using the anti-calnexin antibody. Cells were fixed with methanol/acetone and images were acquired with the confocal microscope. Approximately 30 cells (for each condition) were imaged in three independent experiments. Scale bar = 20 micrometers.

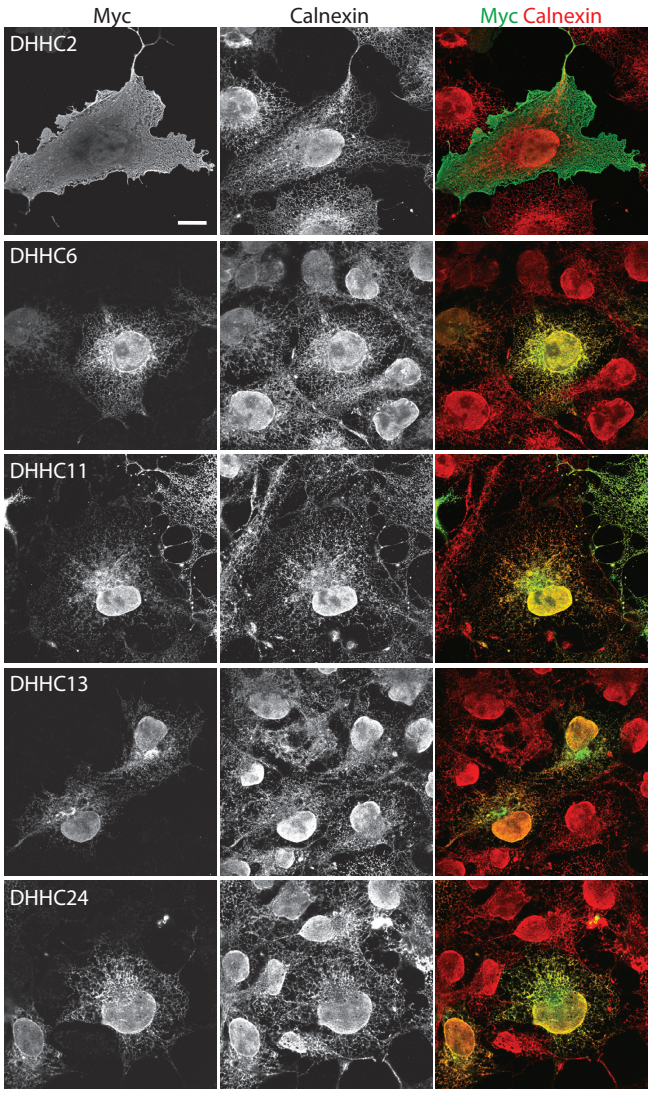


Figure A.3 Gp78/AMFR RING finger cysteine mutants are all sequestered within the central ER

A and B. Full-length Flag-gp78/AMFR WT and RING finger cysteine mutants (C352S and a cysteine-free RING finger mutant, ΔC RING) were transiently transfected into Cos7 cells. Cells were either labeled for the ER with the anti-calnexin antibody (A) or transiently cotransfected with the tubular ER marker Myc-reticulon (Rtn4a) (B). Cells were fixed with methanol/acetone and images were acquired with the confocal microscope. Arrows indicate protein accumulation in the central ER. A. The percent intensity of Flag-gp78/AMFR constructs within the calnexin-labeled ER was determined. A One-way ANOVA followed by Tukey's multiple comparison tests was done. $^{**}P < 0.01$ compared to Flag-gp78/AMFR WT. B. Pearson's coefficients were calculated between Myc-Rtn4a and each Flag-gp78/AMFR construct. $^{*}P < 0.05$ compared to Flag-gp78/AMFR WT. Approximately 30-40 cells (for each condition) were imaged in three independent experiments. Scale bar = 20 micrometers; zoom scale bar = 0.3 micrometers.

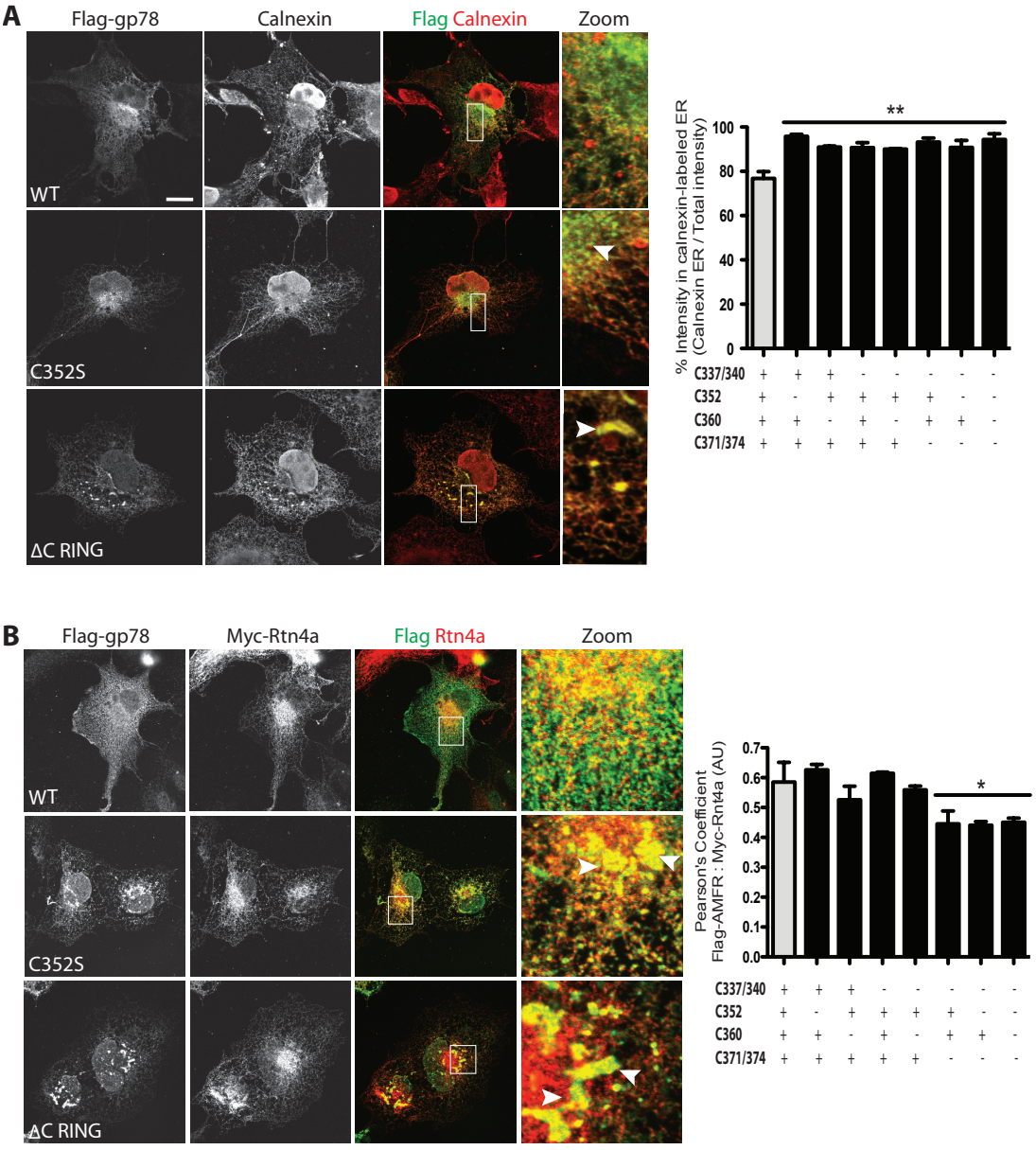
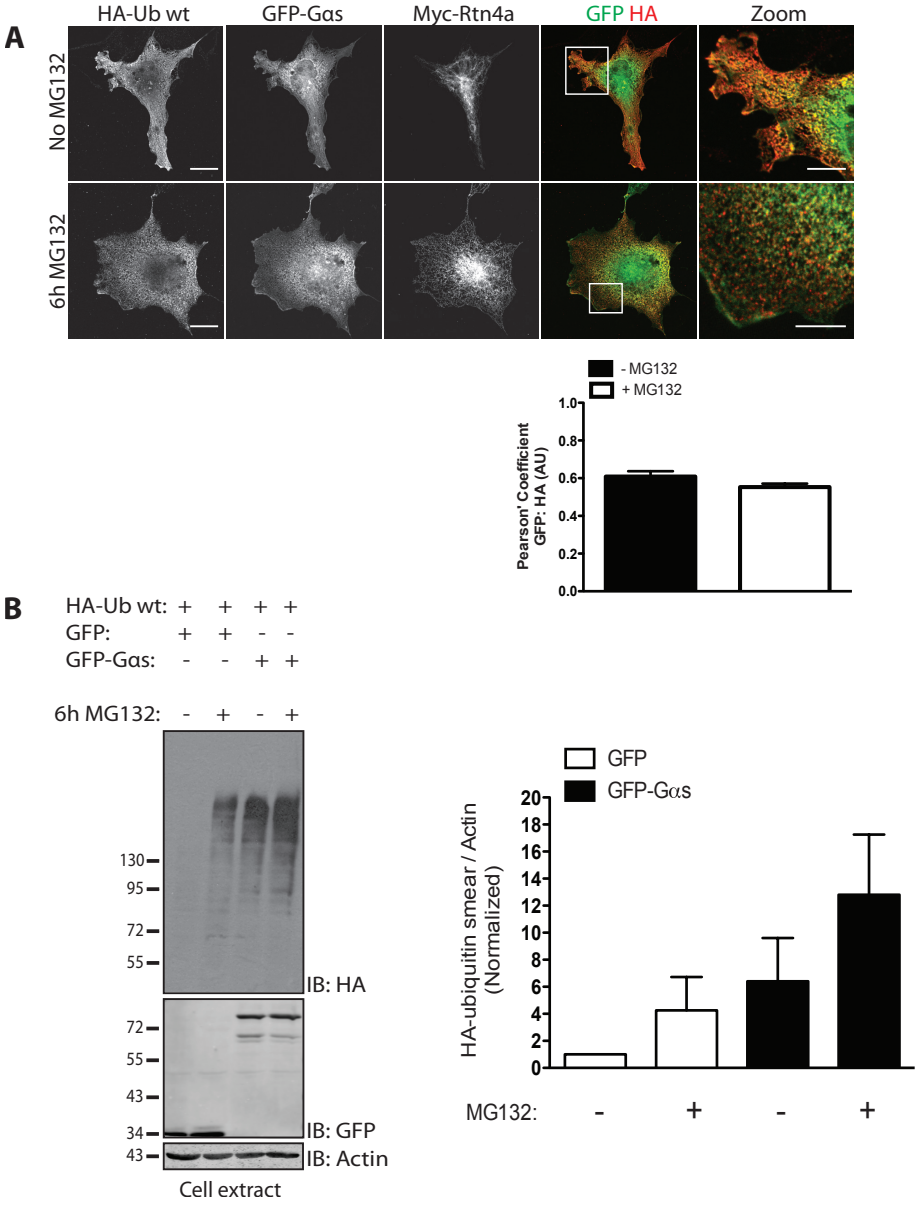

A
Flag-gp78
Calnexin
Flag Calnexin
Zoom
WT
C352S
ΔC RING
% Intensity in calnexin-labeled ER
(Calnexin ER / Total intensity)
**
100
80
60
40
20
0
C337/340 + + + - - - - -
C352 + - + + + + - -
C360 + + - + - + + -
C371/374 + + + + + - - -
B
Flag-gp78
Myc-Rtn4a
Flag Rtn4a
Zoom
WT
C352S
ΔC RING
Pearson's Coefficient
Flag-AMFR : Myc-Rnf4a (AU)
*
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
C337/340 + + + - - - - -
C352 + - + + + + - -
C360 + + - + - + + -
C371/374 + + + + + - - -

Figure A.4 GFP-Gαs-mediated substrate polyubiquitylation is unaffected by the proteasome inhibitor MG132

A. Cos7 cells were transiently cotransfected with HA-Ub wt, GFP-Gαs and the smooth tubular ER marker Myc-reticulon (Rtn4a). 24 h post-transfection, cells were treated for 6 h with the proteasome inhibitor MG132 (final concentration 30 μM) and fixed with methanol/acetone. Images were acquired with the confocal microscope. Colocalization between proteins was determined by Pearson's coefficient. Mean ± S.E.M. Scale bar = 20 micrometers; zoom scale bar = 0.3 micrometers. Approximately 30 cells were imaged (for each condition) and the experiment was repeated three times. B. In the *in vitro* ubiquitin ligase activity assay, Cos7 cells were transiently cotransfected with HA-Ub wt and either GFP protein (negative control) or GFP-Gαs. 24 h post-transfection, cells were treated for 6 h with 30 μM MG132. Cell lysates were collected and immunoblots were probed for anti-HA, anti-GFP and anti-actin. C. Formation of polyubiquitylated substrates in the presence and absence of MG132 treatment was determined in total cell extracts by the HA smear, normalized to actin expression. Later, all conditions were normalized to the non-treated GFP + HA-Ub wt control. The graph represents three independent experiments.


A
HA-Ub wt
GFP-Gαs
Myc-Rtn4a
GFP HA
Zoom
No MG132
6h MG132
- MG132
+ MG132
Pearson' Coefficient GFP: HA (AU)
1.0
0.8
0.6
0.4
0.2
0.0
B
HA-Ub wt: + + + +
GFP: + + - -
GFP-Gαs: - - + +
6h MG132: - + - +
130
95
72
55
IB: HA
72
55
43
34
IB: GFP
43
IB: Actin
Cell extract
GFP
GFP-Gαs
HA-ubiquitin smear / Actin (Normalized)
20
18
16
14
12
10
8
6
4
2
0
MG132: - + - +