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Ubiquitin-mediated degradation via UPS and lysosome

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Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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UBIQUITIN-MEDIATED DEGRADATION VIA UPS AND LYSOSOME

(Thesis format: Integrated Article)

By

Qizhi Sun

Graduate program in Biology

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract

Ubiquitination plays a fundamental role in determining protein fate. Once ubiquitinated, the cargo is directed to the proteasome for partial or complete degradation or lysosome for complete degradation. Failing to eliminate these cargos results in the accumulation of toxic proteins that contribute to neurodegenerative and immunological diseases, cancer and other human maladies. Thus, identifying proteins subject to ubiquitin-mediated degradation and characterizing the mechanisms governing these processes underscores their importance to human health. The calcium/calmodulin-dependent serine protein kinase (CASK) is one such protein that is required for brain development. However, mutations that cause CASK to accumulate are correlated to X-linked mental retardation and autism spectrum disorder in humans. I have investigated CASK protein turnover and found that the protein is constantly degraded. This degradation, however, is only partial suggesting that the peptides generated have functions distinct from the full-length polypeptide. Subsequent analyses revealed that these peptides form as a result of CASK being first phosphorylated and then ubiquitinated prior to its limited degradation in the proteasome. During these investigations I identified poly ADP-ribosyl transferase-like 2 (PARP-2) as a protein also degraded through the ubiquitin-proteasome system. PARP-2 degradation occurs when cells are grown in the presence of serum. Ro52, an E3 ubiquitin ligase interacting with PARP-2 was identified as a candidate required for its ubiquitination. Interestingly, when cells are serum-starved, PARP-2 was sequestered to an SDS-insoluble fraction by a yet-to-be identified mechanism. Finally, further investigations with Ro52 revealed that when ectopically expressed, cells develop large circular structures, which I identified to be autophagosomes, the intermediate organelles in autophagy that selectively target
ubiquitinated cargo for lysosomal degradation. The RING finger domain of Ro52 and its E3 ligase activity are, however, not required for this process and the deletion of the RING domain does not affect the inclusion and targeting of ubiquitinated proteins to the autophagosomes. Together, these results from studying two disparate proteins, CASK and PARP-2, not only highlight the selective ability of ubiquitination to specify the limited or complete degradation of proteins, but also shed new light on Ro52 as an adaptor involved in the autophagic turnover of proteins in the lysosome.

**Keywords:** ubiquitin, proteasome, lysosome, CASK, PARP-2, Ro52, protein degradation, posttranslational modifications
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1.1 GENERAL INTRODUCTION

It is well known that a myriad of proteins acting in a very structured and organized manner participate in signaling cascades in every cell in the body. Proteins responsible for the organization of these signaling hubs are called scaffold proteins, which bind to at least two other signaling proteins simultaneously, thereby promoting communication while localizing signaling molecules to specific areas of the cell (Shaw and Filbert, 2009). Furthermore, by increasing local concentrations of these proteins, these scaffolds enhance the message passing capability of the signaling pathway (Ferrell, 2000). Despite these commonalities, scaffold proteins in general share limited sequence homology, which is evident by the diversity of the numerous domains contained within the polypeptide. This diversity also reflects the plethora of signaling molecules they transduce, which has hampered the systematic identification and characterization of scaffold proteins based on sequence similarity alone. There are, however, exceptions as evident by the SH3 and PDZ domains that are found in variety of scaffold proteins. These domains, together with the extensive interactome data and advent of the field of proteomics, have provided investigators with a foothold towards the identification of the entire mouse and human scaffold proteomes. Proteomic analysis has also been facilitated through the use of mathematical simulations and engineered scaffold protein studies (Tian and Song, 2012; Zeke et al., 2009). These in silico analyses have provided insight into the functions and mechanisms provided by the scaffolds in signaling events, although most have yet to be validated by in vivo experiments with tissue cultured cells and whole animal model systems.

Based on the established models, scaffold proteins play a more active role than was once
thought. The basic function of these scaffolds is to provide a platform on which signaling molecules can assemble. Forming such a signaling complex enriches the concentration of participants in one locale, where the close proximity of the signaling components result in an increase of specificity. A good example is the well-studied mitogen-activated protein kinase (MAPK) signaling pathways. The MAPK pathways respond to various stimuli such as growth factors, hormones, cytokines and cellular stresses to control gene expression, metabolism, cytoskeletal functions and other cellular regulatory events. The core unit of these pathways is composed of a three-kinase module that includes MAPKs, MAPK kinases (M KKs) and MAPK kinase kinases (MKKKs). Different combinations of these three kinases as well as the scaffold proteins that orchestrate specific protein interactions define the specific response to diverse signals transduced through MAPK pathways (Pearson et al., 2001; Seger and Krebs, 1995). For instance, a MAPK pathway, organized into a module that includes Ste11 (MKK K), Ste7 (MKK) and Fus3 (MAPK) by the scaffold protein Ste5, mediates the mating response of haploid yeast cells (Bhattacharyya et al., 2006; Malleshaiah et al., 2010). Dimerization of Ste5 is pivotal in activating Ste11 and the subsequent activation of this pathway. When the Ring-H2 domain of Ste5 is mutated, dimerization is disrupted and although Ste11, Ste7 and Fus3 still bind Ste5, yeast cells no longer respond to mating pheromone (Yablonski et al., 1996; Inouye et al., 1997). In contrast, another MAPK signaling complex, containing Sho1 (an osmosensor), Ste11, Pbs2 (a MKK and also a scaffold protein) and Hog1 (a MAPK), is involved in the high osmolarity response in yeast (Posas and Saito, 1997). Despite the commonality of Ste11 in the two MAPK pathways, there is no crosstalk between them, with Ste11 activation controlled in one case by Ste5 interacting with the Gβγ subunit complex of the pheromone receptor, and in the other where activation is by the p21-activated kinase family member Ste20 (Posas and Saito, 1997; Raitt et
al., 2000). In other words, scaffold proteins act as an insulator between the proteins that would otherwise interact if not segregated to ensure the signaling specificity.

Establishing a platform to assemble signaling components is one aspect important to scaffold proteins, but equally important is their ability to regulate the activation or inactivation of the signaling components. Many of the scaffold proteins identified to date are catalytic enzymes. For example, The JNK interacting protein-1 (JIP-1), which is the scaffold at the center of the JNK MAP kinase module that includes MLK3, M KK7 and JNK, enhances the activation of JNK when co-expressed with M KK7 and MLK3 (Whitmarsh et al., 1998). Another example is MEK partner 1 (MP1), a scaffold protein that when over-expressed enhances ERK1 activation (Schaeffer et al., 1998). Finally, the kinase suppressor of Ras (KSR) binds to MEK1 and ERK and when mutated, it attenuates Ras-mediated activation of the ERK pathway. Activation of Ras induces the translocation of KSR to the plasma membrane where it activates Raf-1 activity, a property of KSR that is independent of its intrinsic kinase activity (Sundaram and Han, 1995; Yu et al., 1998).

Much attention has focused on how scaffold proteins regulate the activities of the proteins in a signaling hub, and much of this regulation may be the result of modulating the turnover or changing the availability of the scaffold without the need of individually regulating the active components. This form of regulation is facilitated by posttranslational modifications such as phosphorylation and ubiquitination of protein in the scaffold. As an example, the yeast MAPK scaffold protein Ste5 resides primarily in the nucleus of naïve cells and low levels are maintained due to its phosphorylation- and ubiquitination-dependent degradation. Ste5, exported from the nucleus in response to mating pheromone stimulation, serves to organize and stabilize the MAPK complex at the mating projection. Even if the nuclear degradation of Ste5 is slightly
compromised, the cells demonstrate MAPK activation-related responses to pheromone induction, namely, growth inhibition, G1-specific arrest and shmoo formation in the absence of pheromone (Garrenton et al., 2009).

### 1.2 POSTTRANSLATIONAL MODIFICATIONS

Following the central dogma, the structural gene is transcribed first into mRNA, which is then translated into a polypeptide. While proteins carrying out the vast majority of physiological functions are encoded by 20,000 to 25,000 genes, an estimated 25,000 to 30,000 human genes encode non-translated RNAs. This limitation in the number of proteins in vertebrates has raised the question of how the complex activities are performed by the relative paucity of genes in their genomes. Global mass spectrometric analysis has revealed the high density and variety of posttranslational modifications (PTMs) in the mammalian proteome. In addition to alternative splicing, the extensive use of posttranslational modifications to generate multiple, functionally-distinct proteins from a single gene product is thought to be the strategic plan compensating for a scarcity of genes.

Many types of posttranslational modifications (PTMs) are known including, but not limited to, phosphorylation, acetylation, methylation, O-GlcNacylation, ubiquitination, SUMOylation, NEDDylation, biotinylation, ADP ribosylation and glycosylation. These are typically reversible processes regulating protein activity (Bedford and Richard, 2005; Chapman-Smith and Cronan, 1999; Ciechanover, 1998; Geiss-Friedlander and Melchior, 2007; Hassa et al., 2006; Kouzarides, 2000; Kurosawa, 1994; Love and Hanover, 2005; Rabut and Peter, 2008; Spiro, 2002). Posttranslational modifications regulate protein activity by affecting the interaction of target proteins with other proteins, or through allosteric effects. Depending on the physiological context,
different types of PTMs can modify the target protein solely or combinatorially. Since the outcome of each PTM is different, the combination of multiple modifications to a single polypeptide would increase the information content significantly and thereby generate numerous outcomes. Two types of PTMs that have been studied extensively include phosphorylation and ubiquitination. Both of these mechanisms use a wide spectrum of targets and each is involved in almost every aspect of cellular physiology. Another key PTM pertinent to this thesis involves ADP-ribosylation, which regulates physiological processes such as the maintenance of DNA integrity, gene transcription, cell division and cell death.

1.2.1 PROTEIN PHOSPHORYLATION

Protein phosphorylation is a ubiquitous regulatory mechanism used by eukaryotes and prokaryotes. In eukaryotes a single phosphoryl group from the $\gamma$ position of ATP is catalytically transferred to the hydroxyls of serine, threonine and tyrosine (Ubersax and Ferrell, 2007). Histidine and aspartic acid are the frequent residues phosphorylated by protein kinases in prokaryotes. Regardless of the residue itself, the modification has profound effects on protein conformation and function. In reference to the former, the covalent attachment of a phosphate group to the R group of an amino acid residue changes the hydrophilicity of a protein, thereby introducing a conformational change in the structure of the protein via interaction with other hydrophobic or hydrophilic residues in that protein. With regard to activity, phosphorylation acts in either a positive or negative manner, and together this phosphorylation-dependent regulation of a substrate is largely dependent on the conformational change in protein structure following the addition of the phosphate group (Taylor et al., 2004).

Most biological processes are regulated through phosphorylation by protein kinases (Nolen et
al., 2004). Protein kinases facilitate the transfer of phosphoryl groups to their substrates, resulting in the modulation of protein activity. Typically, a kinase contains a catalytic core that binds a substrate and ATP in the presence of a divalent metal ion, usually Mg$^{2+}$. Sequence analysis has revealed three conserved motifs in the catalytic domain of kinases that are essential for catalysis: the Val-Ala-Ile-Lys (VAIK) motif in subdomain II that anchors and positions the ATP through the interaction of a lysine residue with the $\alpha$ and $\beta$ phosphates of ATP; the His-Arg-Asp (HRD) motif in subdomain VIb that functions as a base receptor to achieve proton transfer; and the Asp-Phe-Gly (DFG) motif in subdomain VII, that binds the Mg$^{2+}$ ions (Hanks et al., 1988; Knighton et al., 1991a; Knighton et al., 1991b; Taylor and Radzio-Andzelm, 1994). Several conserved residues including aspartate, lysine, glutamate and asparagine are most often found within the core and are thought to be essential for kinase activity. However, approximately 10% of proteins containing a kinase-like domain lack one or more of these residues, and are considered catalytically inactive (Boudeau et al., 2006). These kinases are classified as pseudokinases, which were probably derived from active kinases following evolutionary changes to the conserved residues. Due to the lack of kinase activity, pseudokinases are often functionally relegated to a scaffolding role. Growing evidence, however, suggests that although lacking the ability to phosphorylate substrates, pseudokinases are pivotal in regulating diverse cellular processes (Boudeau et al., 2006). Recently, Mukherjee and colleagues discovered a novel mechanism on how the presumed pseudokinase CASK (Calcium/Calmodulin-dependent serine protein kinase), phosphorylates its substrate. In their study, the membrane-associated guanylate kinase (MAGUK) protein CASK, which contains a CaM kinase-like domain in its N-terminus, phosphorylates neurexin in an Mg$^{2+}$-independent manner (Mukherjee et al., 2010; Mukherjee et al., 2008). These results have led to a resurgence in the field as investigators are now
reexamining pseudokinases and their ability to phosphorylate substrates. Towards that end, another “active” pseudokinase ErbB3/HER3 has since been found that uses a distinct mechanism to catalyze autophosphorylation (Shi et al., 2010).

Like many other posttranslational modifications, phosphorylation is a reversible process. Phosphatases are enzymes that hydrolyze the phosphate ester bond in the phosphorylated protein. Although their numbers are estimated to be one tenth that of kinases (Moorhead et al., 2007), phosphatases are just as important as their counterparts in cellular regulation. In fact, the balance between kinases and phosphatases must be maintained precisely to keep cellular physiology from failing. Based on substrate specificity, phosphatases are generally divided into two groups, namely the Ser/Thr phosphatases and the Tyr phosphatases (Shi, 2009; Tonks, 2006). Both groups hydrolyze phosphoester bonds, but each uses distinct catalytic mechanisms during the process. Regardless of the mechanism, the importance of phosphorylation in cellular regulation lies in three interrelated categories. First, phosphorylation alters the activity of target proteins either directly or indirectly in diverse cellular processes. Second, phosphorylation induces specific protein-protein interactions. Third, phosphorylation affects the subcellular localization of proteins as a result of altered intra- or intermolecular interactions. Given the diversity and importance that these three categories impart, it is easy to see the significance and requirement for dephosphorylation.

1.2.1.1 REGULATION OF PROTEIN ACTIVITY BY PHOSPHORYLATION

Approximately one third of the proteins present in a typical mammalian cell are covalently bound to phosphate at one time or another (Chesnik et al., 2011). As noted above, the addition or removal of a phosphate from a protein changes its properties in many ways, including its activity.
The MAP kinase family of enzymes play a major role in phosphorylation (Dhanasekaran and Johnson, 2007). One member, MAP kinase-activated protein kinase 2 (MK2 or MAPKAP K2), is a stress-activated enzyme that acts downstream of p38 MAPK (Ono and Han, 2000). MK2 in its inactive form is localized to the nucleus, but under stress conditions it is activated by p38 MAPK, where phosphorylation facilitates the translocation of MK2 to the cytoplasm together with p38 MAPK (Ben-Levy et al., 1998; Engel et al., 1998). In vitro and in vivo FRET analyses have shown that the phosphorylation-dependent transition of inactive to active MK2 is the result of a conformational change (Neininger et al., 2001).

There are an overwhelming number of studies detailing how phosphorylation leads to protein activation, but an equal number report on the opposite consequence, where phosphorylation leads to inactivation. For instance, Glycogen synthase kinase 3 (GSK-3) phosphorylates a broad range of substrates including the transcription factors c-myc, c-Jun and c-myb (Plyte et al., 1992) and the translation factor eIF2B (Welsh et al., 1996). Insulin stimulation or signaling by growth factors causes the rapid phosphorylation of Ser21 in GSK-3α and Ser9 in GSK-3β, resulting in the inhibition of GSK-3 kinase activity (Cross et al., 1995; Welsh and Proud, 1993). Inhibition of GSK-3 activity stabilizes PTEN (phosphatase and tensin homologue) and counteracts PI3K/Akt signalling during the gastrulation of zebrafish (Finkieltsztein and Kelly, 2009).

1.2.1.2 REGULATION OF PROTEIN-PROTEIN INTERACTIONS BY PHOSPHORYLATION

Large peptide domains within proteins can mediate constitutive protein-protein interactions, while stimulus-regulated protein-protein interactions often depend on small posttranslational modifications (PTM) such as phosphorylation-induced conformational changes. In the latter case,
these changes translate into a change of activity either for the interacting proteins or their substrates. For instance, the intermediate effector protein Smad3 of the TGF-β signaling pathway is in an autoinhibitory state when its N and C termini bind through intramolecular interactions. Smad3 is phosphorylated at its extreme C terminus (SSVS) by the type I TGF-β receptor following stimulation by TGF-β. This PTM introduces a conformational change that leads to the dissociation of the N- and C-terminal domains, leaving the now active Smad3 to bind the coactivator p300/CREB-binding protein, which activates transcription of TGF-β target genes (Macias-Silva et al., 1996; Shen et al., 1998; Zhang et al., 1996).

While much attention has focused on phosphorylation being the driving influence facilitating protein-protein interactions, one must also consider that phosphorylation can also negatively regulate these interactions. One example is the N-type calcium channel α1B subunit, that following PKC or CaM KII-dependent phosphorylation of its synaptic protein interaction site, is unable to interact with the SNARE protein complex (Yokoyama et al., 1997). In another example, the ubiquitination and downregulation of p53 is prevented by the Cdk-5-dependent phosphorylation of Hdm2, an E3 ubiquitin ligase that interacts with p53 to regulate its expression (Lee et al., 2007).

### 1.2.1.3 REGULATION OF PROTEIN LOCALIZATION BY PHOSPHORYLATION

Posttranslational modifications are key modulators of proteins and they influence where the protein may be localized in the cell. The subcellular distribution of a protein is also determined by its primary amino acid sequence, the presence of protein motifs and domains in its secondary structure, and the ability of the protein to interact with other proteins, lipids or DNA. Proteins are actively trafficked within cells in order to respond to the physiological dynamics within and
placed upon cells. Protein trafficking relies heavily on posttranslational modifications, especially phosphorylation, which can expose intrinsic localization signals within the protein or affect interactions with other proteins that direct the protein to different locales or serve to tether the protein at these signaling centres. For instance, the MAPK protein ERK5 is localized in the cytoplasm in quiescent cells, but translocates to the nucleus when a constitutively active form of the MEK5 kinase is co-expressed (Kato et al., 1997; Yan et al., 2001). MEK5 phosphorylates the TEY sequence in the N-terminus of ERK5, which disrupts the intramolecular binding between the N- and C-termini of ERK5. Once “open” the nuclear export signal is inhibited and the nuclear localization signal activated, thereby facilitating translocation into the nucleus (Kondoh et al., 2006).

Localization of proteins in different compartments can also be affected by altered protein-protein interactions induced by phosphorylation. For instance, microtubule-associated protein 2 (MAP2), is a microtubule binding protein that plays a role in stabilizing microtubules and accelerating microtubule growth. However, phosphorylation at KXGS motifs disrupts MAP2-microtubule interactions and MAP2 becomes localized to the actin cytoskeleton (Ozer and Halpain, 2000).

Although a vast amount of literature exists on the importance of phosphorylation in modifying the function and/or localization of a protein, other forms of protein posttranslational modification use phosphorylation as the initiator for further protein regulation. This is the case for ubiquitination, where a target protein is first phosphorylated and then is covalently bound to ubiquitin, prior to its degradation by the proteasome.

1.2.2 AN OVERVIEW OF UBIQUITINATION AND ITS FUNCTION
Until the discovery of organelles and pathways governing proteolysis, proteins were considered inert, if not immortal (Ciechanover, 2005). Protein turnover is just as important as protein synthesis in that it not only provides a self-renewal mechanism for any particular protein, but also serves as a regulatory tool in many cellular processes. Several proteins, complexes and organelles participate in protein degradation, including the proteases/protease complexes and the modulators, such as ubiquitin and ubiquitin-like proteins working with lysosomal and proteasomal organelles.

Ubiquitination is a reversible PTM, in which a 76–amino acid polypeptide, ubiquitin, is covalently attached to the ε-amino group of lysines or the N-terminus of the target protein. A group of enzymes, including E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase, catalyze this ATP-dependent process, leading to the attachment of a single moiety or polymeric ubiquitin chains to the substrates. Ubiquitination plays major roles in regulating a broad host of cellular processes, including cell proliferation, differentiation, cell signaling, protein trafficking, and quality control. Aberrant ubiquitination of proteins has been reported in the pathogenesis of some diseases, certain malignancies, neurodegenerative disorders, and pathologies of the inflammatory immune response. The regulatory roles ubiquitination play in different processes are largely associated with ubiquitination-dependent protein turnover, although many are carried out through its non-proteolytic functions.

Ubiquitination was first recognized as a means of targeting substrates for proteasomal degradation (Komander, 2009). The proteasome is a multi-subunit, 26S particle composed of a 20S cylindrical chamber and two 19S regulatory complexes. The 20S cylinder has intrinsic proteolytic activity, which degrades substrates transferred into the proteasome interior, while the 19S regulatory complexes recognize, bind and unfold the polyubiquitinated substrates (Voges et
Proteins targeted for proteasomal degradation are usually hydrolyzed to small peptides, although limited proteolysis has been reported in some cases (Orian et al., 1995; Palombella et al., 1994). The mechanism of proteolytic degradation is very efficient and the ubiquitin chain attached to the substrates is recycled through the activity of de-ubiquitinating enzymes.

In addition to the ubiquitin-proteasome pathway, many membrane proteins are delivered to the lysosome for degradation following ubiquitination. In this manner, ubiquitin serves as a signal to sort proteins from different membrane compartments. For example, ubiquitination of a growth factor receptor following ligand binding leads to its endocytosis. Under different circumstances, damaged membrane proteins are ubiquitinated and sorted at the Trans-Golgi Network to the endosome, before being delivered to the lysosome.

Protein degradation relies on ubiquitination, but the extent of this modification, as in the number of ubiquitin residues that are attached to the target protein, would indicate that distinct mechanisms are at work. Monoubiquitination is the attachment of a single ubiquitin to a protein, whereas multiubiquitination occurs when a single ubiquitin molecule modifies several lysine residues. In each case these forms of ubiquitination are required for the internalization of membrane proteins. The addition of polyubiquitin chains to lysine residues leads to the polyubiquitination of a protein, and its subsequent degradation in the proteasome. Since there are seven internal lysine residues in ubiquitin, each can form a linkage with another ubiquitin to produce a variety of different polyubiquitin linkages. Among them, Lys48-linked polyubiquitination leads to proteasomal degradation of the target proteins, whereas linkage through Lys63 is crucial in DNA damage repair, the inflammatory response, the endocytic pathway and ribosomal protein synthesis (Pickart and Fushman, 2004). Functions for other types
of polyubiquitination linkage through Lys6, Lys11, Lys27, Lys29 and Lys33 have also been reported (Al-Hakim et al., 2008; Chastagner et al., 2006; Ikeda and Kerppola, 2008; Nishikawa et al., 2004; Xu et al., 2009b).

The ability of monoubiquitin and different polyubiquitin chains to signal in different ways depends largely on the specificity and function of proteins that serve as ubiquitin receptors because they contain ubiquitin-binding domains (UBDs). To date at least 20 individual UBDs have been identified, many of which interact with hydrophobic regions on the surface of the ubiquitin molecules (Hurley et al., 2006; Kirkin and Dikic, 2007). Proteins containing UBDs have diverse roles in a different number of cell types. Ubiquitinated proteins targeted for proteasomal degradation need ubiquitin binding adaptor proteins to dock to the site of degradation (Elsasser and Finley, 2005; Hishiya et al., 2006; Young et al., 1998). Endocytic adaptor proteins containing UBDs serve as vehicles and sorting tools for the endocytosis of cell-surface receptors, transporters and ion channels (Di Fiore et al., 2003; Haglund et al., 2003; Hicke and Dunn, 2003). DNA repair and chromatin remodeling ubiquitin-binding proteins also play a significant role in the nucleus (Flick et al., 2006; Haglund and Dikic, 2005; Muratani and Tansey, 2003). Together, these results highlight the importance ubiquitination has in both proteolytic and non-proteolytic roles.

Ubiquitination, like phosphorylation, is a reversible PTM. A superfamily of isopeptidases known as deubiquitinases (DUBs) opposes the function of E3 ligases to remove ubiquitin chains from ubiquitinated substrates. Nearly eighty DUBs have been identified; a number that suggests some may exhibit substrate specificity. In fact DUBs can recognize specific substrates and also particular ubiquitin chain types. The function of DUBs includes cleaving linear multiple ubiquitin molecules into free ubiquitin, removing ubiquitin chains from ubiquitinated proteins.
and proofreading ubiquitin chain formation (chain type specificity). Given these specialized roles, it is not surprising that many deubiquitinases harbor the UBDs that mediate the interaction with their ubiquitin containing substrates. The function(s) of protein ubiquitination are summarized in the following four sections.

1.2.2.1 REGULATION OF PROTEIN STABILITY AND ACTIVITY BY UBIQUITINATION

Ubiquitination targets substrate proteins for degradation either through the proteasomal or lysosomal pathway. Misfolded proteins, short-lived proteins and many long-lived proteins are degraded in the proteasome following ubiquitination. However, integral membrane proteins with tyrosine kinase activity, which are ubiquitinated or associated with ubiquitinated proteins, are constitutively degraded in the lysosome or degraded following stimulation by growth factors. The mechanism(s) behind such selectivity for degradation is defined by the pattern in which the ubiquitin moiety is covalently attached to the target proteins. Different ubiquitin linkages result in various conformations of the ubiquitin chain and create a range of molecular signals in the cell. In general, proteins modified by the Lys48-linked polymeric ubiquitin chain are subject to the proteasomal degradation, whereas monomeric ubiquitin modification of the proteins leads to lysosomal degradation. In addition to regulating protein stability in vivo, ubiquitination also alters the activity of its target proteins. The ability to alter protein activity is carried out through the attachment of atypical ubiquitin chains, including all variations of the multimeric ubiquitin structure except for the classic Lys48 polyubiquitination. Recently, the non-proteolytic function of ubiquitination has attracted much attention and is now a key topic in the field of cellular regulation (Chen and Sun, 2009).
The c-Myc proto-oncoprotein is a transcription factor that has a half-life of 20 to 30 minutes (Gregory and Hann, 2000). c-Myc expression is tightly controlled at different levels because it plays a central role in regulating cellular proliferation (Marcu et al., 1992; Spencer and Groudine, 1991). One mechanism involved in c-Myc regulation is ubiquitination and proteasomal degradation, which contributes directly to the short half-life of the protein. The stability of c-Myc in some human cancers, e.g. Burkitt’s lymphoma, is significantly increased, which underscores the essential role for ubiquitin-dependent proteolysis in preventing human disease (Gregory and Hann, 2000). Another example involves the tumor-suppressor protein p53, which plays a pivotal role in regulating the cell cycle, apoptosis and genomic stability. Under normal conditions and in concert with at least five ubiquitin ligases, the ubiquitin-proteasome pathway regulates the stability of p53. However, in response to DNA damage the activity, but not the stability, of p53 is affected by the ubiquitination, leading to the activation of target genes associated with growth arrest rather than cell death (Le Cam et al., 2006).

1.2.2.2 REGULATION OF PROTEIN-PROTEIN INTERACTIONS BY UBIQUITINATION

As mentioned earlier, proteins with ubiquitin-binding domains interact with the hydrophobic groups on the surface of the ubiquitin moiety bound to a target protein. Thus, ubiquitination not only mediates protein-protein interactions between the ubiquitinated protein and the ubiquitin-binding proteins, but in some cases this interaction will enhance the binding of a non-ubiquitinated protein to ubiquitin-binding proteins (Garrus et al., 2001). Monoubiquitination of the ubiquitin-binding domain-containing protein is also a mechanism used to negatively regulate the interaction between itself and the ubiquitinated protein. An example is the UBD-containing
protein Sts2, which when bound to ubiquitinated EGFR complexes, inhibits the endocytosis of the receptor. Monoubiquitination of Sts2 leads to an intramolecular interaction between itself and ubiquitin, thereby preventing Sts2 from interacting with other ubiquitin residues and promoting its dissociation from EGFR (Hoeller et al., 2006).

1.2.2.3 UBIQUITIN REGULATION OF PROTEIN SUBCELLULAR LOCALIZATION

As described earlier, the intracellular localization of a protein is primarily determined by localization signals in the primary sequence of the polypeptide, and its ability to translocate to different locales is affected by protein-protein interactions and/or PTMs including phosphorylation and ubiquitination. In regards to the latter, many cell surface proteins use monoubiquitination as a mechanism for internalization into the endocytic pathway (Haglund et al., 2003). The ubiquitinated cargo is recognized by ubiquitin-binding proteins of the endocytic machinery acting as adaptor proteins to mediate vesicle budding (Hicke and Dunn, 2003; Katzmann et al., 2002). It is interesting to note that some of these proteins may not themselves be ubiquitinated, but instead require the interaction with a ubiquitinated transport modifier for protein trafficking (Myat et al., 2002; Shenoy and Lefkowitz, 2003).

The translocation of NF-κB into the nucleus is another example of ubiquitination-regulated protein trafficking. NF-κB is a transcription factor comprised of two subunits, p50 and p65. In the cytoplasm, NF-κB is sequestered by IκBα, IκBβ, IκBε and other related proteins (Baldwin, 1996; Karin, 1999). Following stimulation of the cell by stress, interleukin-1 (IL-1) or tumor necrosis factor (TNF), a signaling cascade results in phosphorylation of IκBs by IκB kinase (IKK) complexes, leading to their subsequent ubiquitination and degradation by the 26S proteosome (Desterro et al., 2000). De-repression of the IκBs unmask the nuclear localization signals of
p50 and p65, allowing the heterodimer to translocate to the nucleus where the complex regulates the expression of genes containing NF-κB responsive elements (Baldwin, 1996). A different but ubiquitin-related regulation of protein translocation involves β-catenin, which is tethered at the plasma membrane to mediate cell-cell adhesion. When Wnt ligands are absent, non-membrane-bound β-catenin in the cytosol is first phosphorylated by CK1, making it a primed substrate for multiple phosphorylation by GSK-3β. These posttranslational modifications to β-catenin are necessary for its ultimate ubiquitination and degradation in the proteasome (Xu et al., 2009a). However, cytoplasmic levels of β-catenin increase following Wnt stimulation, allowing β-catenin to translocate to the nucleus, where it acts with TCF-1/LEF-1 to regulate gene expression (MacDonald et al., 2009).

1.2.2.4 UBIQUITIN-MEDIATED AUTOPHAGIC DEGRADATION

Autophagy is another major intracellular degradation pathway that has been characterized extensively in eukaryotes. There are three types of autophagy including: 1) chaperone-mediated autophagy (CMA); 2) microautophagy; and 3) macroautophagy (Klionsky, 2005; Massey et al., 2004). CMA selectively targets substrate proteins into the lysosomal lumen for degradation, whereas with microautophagy, cytoplasmic materials are directly invaginated into lysosomes without any selection. Compared with the other types of autophagy, macroautophagy (referred hereafter simply as autophagy) is the most prevalent and sophisticated form. During autophagy, small portions of cytoplasm are sequestered by double-membrane structures to form autophagosomes. These autophagosomes will later fuse with lysosomes to form autolysosomes and the constituents are degraded by hydrolases in the lysosome and recycled.
Autophagy is an evolutionarily conserved process that can be mechanistically divided into induction, cargo packaging, fusion with lysosome or vacuole, hydrolysis of cargo and autophagic lysosome reformation (in mammalian cells). More than thirty-two autophagy-related (Atg) proteins have been found to participate in this process, 16 of which are involved in all three types of autophagy (Pyo et al., 2012). Although cells undergo constitutive basal autophagy at very low levels under normal conditions, significant autophagy is induced when cells are stressed. In the end, the outcome of autophagy is to reconfigure the cellular resources to sustain cell survival or induce apoptosis (Maiuri et al., 2007). Although the signal of environmental stress is often transduced through the mTOR (mammalian target of rapamycin) signaling complex (He and Klionsky, 2009), TOR-independent induction has also been documented (Kanazawa et al., 2004; Mordier et al., 2000; Stephan et al., 2009). The formation of Atg1-Atg13-Atg17 scaffold following TOR inhibition by starvation is essential in autophagy initiation (Mizushima, 2010). The former recruits autophagy proteins to the pre-autophagosomal structure (PAS) for the assembly of autophagosomes, while the latter functions in both the recruitment of autophagy proteins and phosphorylation of downstream proteins that participate in autophagosome formation (Mizushima, 2010). The packaging of cargo starts from a structure called the phagophore, or isolation membrane. The nucleation and assembly of the initial phagophore membrane is mediated by the class III PI3K (phosphatidylinositol 3-kinase) complex, including the PI3K (Vps34), Beclin1 (Atg6) and p150 (Vps15) core complex (Marino and Lopez-Otin, 2004). Numerous proteins participate in the regulation of the nucleation through interactions with the core complex proteins (He and Klionsky, 2009; Pyo et al., 2012). The PI3K complex produces PI3P (phosphatidylinositol 3-phosphate) and recruits two interrelated ubiquitin-like conjugation systems to the phagophore. The elongation of the phagophore depends on these two
ubiquitination-like reactions. The first reaction is the conjugation of Atg12 (ubiquitin-like molecule) to Atg5 (Kuma et al., 2002), which is catalyzed by Atg7 (E1-like enzyme) and Atg10 (E2-like enzyme) (Mizushima et al., 1998; Shintani et al., 1999; Tanida et al., 2001). Subsequent interaction of Atg12-Atg5 with Atg16L leads to the formation of a tetrameric Atg12-Atg5.Atg16 protein complex by self-oligomerization of Atg16 (Mizushima et al., 1999) and its attachment to the phagophore (Mizushima et al., 2003). The second reaction is the conjugation of LC3 (Atg8) to the phospholipid phosphatidylethanolamine (PE). Before the conjugation, LC3 is first cleaved at the C-terminus by Atg4 (a cysteine protease) to expose a glycine residue and then conjugated to PE by Atg7 (E1), Atg3 (E2) and Atg12-Atg5 (E3). The lipidated LC3 (LC3-II) associates with the phagophore to expand its membrane (Fujita et al., 2008; Hanada et al., 2007; Kirisako et al., 2000), and the autophagosome forms following the closure of the phagophore. Although many proteins are involved in the maturation and fusion of the autophagosome and the lysosome (Eskelinen, 2005; He and Klionsky, 2009; Pyo et al., 2012), one protein, TECPR1, seems to be pivotal in the autophagosome-lysosome fusion event in mammalian cells. TECPR1, which resides on and recruits Atg5 to the autolysosome membrane, binds to the Atg12-Atg5 conjugate in a mutually exclusive manner with Atg16. Depending on the Atg12-Atg5 conjugate, TECPR1 binds to PI3P and therefore it plays a tethering role in the autophagosome-lysosome fusion (Chen et al., 2012). The fusion occurs at the outer membrane of the autophagosome, and when complete, the contents within the autophagosome and the inner membrane are degraded by the acidic hydrolases from the lysosome. The degradation products are subsequently exported to the cytosol for reuse, but the mechanism as to how this occurs is still largely unknown. Recently, however, a process called autophagic lysosome reformation (ALR) has been described for what occurs following lysosomal degradation in mammalian cells (Tong et al., 2010). The degradation
products released from the autolysosome reactivate mTOR, which inhibits further autophagy after prolonged starvation. As a result, proto-lysosomal tubules and vesicles extruding from the autolysosomes form and mature into functional lysosomes (Yu et al., 2010). The lysosomal efflux permease, sprinter, is essential in the reactivation of mTOR and ALR (Rong et al., 2011), and the budding and fission of the proto-lysosome from the reformation tubules requires clathrin and phosphatidylinositol-4,5-bisphosphate (PIP2) (Rong et al., 2012). Overall, it is clear that ALR is important to maintaining lysosome homeostasis following its consumption in autophagy.

Although autophagy in response to nutrient starvation appears to be non-selective in cargo recognition, there is also selective autophagy in cells grown under nutrient-rich conditions. In yeast, prApe1 is specifically targeted to the vacuole through the cytosol-to-vacuole targeting (Cvt) pathway for processing and generation of mature Ape1. A receptor protein, Atg19, and the adaptor protein, Atg11, mediate this selectivity of cargo into a vacuole. Atg19 binds to the vacuolar-targeting signal of prApe1 and the complex is recruited to the PAS through the interaction between Atg19 and Atg11. The cargo is finally packaged into Cvt vesicles by Atg8-PE conjugate, which interacts with Atg19 in the complex (He and Klionsky, 2009; Scott et al., 2001; Shintani et al., 2002). In mammalian cells, single proteins, protein aggregates, organelles and pathogens can be specifically engulfed by autophagosomes. Despite the difference in the cargo types, a common mechanism exists that involves ubiquitination of the substrates. As discussed previously, ubiquitination marks proteins for proteasomal degradation as well as serving as signals for various non-degradation functions. Due to the structure of the 26S proteasome, the 20S cylinder with proteolytic activity can only allow the single, unfolded protein to enter. This feature of the proteasome limits the type of substrate it can degrade. The double-membrane autophagosome, however, is malleable in its size and thus it can accommodate
various cargos ranging from proteins to organelles. Central to the selectivity of these cargos are the autophagic adaptor proteins, which are a group of proteins that bind ubiquitinated substrates and/or the autophagy specific LC3/GABARAP modifier on the inner sheath of autophagosomes. The common domain structure of selective autophagy adaptors contains domain mediating recognition and binding of the ubiquitin moieties on the substrates and/or LC3/GABARAP interacting region (LIR) motif. Three different ubiquitin-binding domains have been identified so far in the selective autophagy adaptors, including ubiquitin-associated (UBA), ubiquitin-binding zinc-finger (UBZ) and ubiquitin binding in A20-binding inhibitor of NF-κ-B and NF-κ-B essential modulator (UBAN) domains (Behrends and Fulda, 2012). The LIR motif is a short hydrophobic sequence with the consensus X₃-X₂-X₁-W/F/Y-X-X-L/I/V where X₃-X₂-X₁ are usually acidic residues (Johansen and Lamark, 2011) (Fig 4). Interestingly, the LIR motif is also present in several autophagy-related non-adaptor proteins facilitating their interactions with Atg8/LC3 during autophagy (Johansen and Lamark, 2011). In addition to those proteins playing direct adaptor roles in selective autophagy, there are other, non-adaptors, which mediate autophagosome targeting through indirect association with ubiquitinated proteins or with LC3.

1.2.3 PROTEIN ADP-RIBOSYLATION

Although the focus up to now on protein regulation has been dedicated to phosphorylation and ubiquitination, another form of PTM involves protein ADP-ribosylation, which has profound effects on substrate function. The addition of an ADP-ribose moiety to acceptor proteins is mediated by mono-ADP-ribosyltransferases (MARTs) or poly-ADP-ribose polymerases (PARPs). During ADP-ribosylation, NAD⁺ is used as the donor of ADP-ribose, and the energy comes from hydrolysis of the high energy bond between the nicotinamide and ribose moieties of NAD⁺. Two
families of enzymes termed mono-ADP-ribose-protein hydrolases (MARHs) and poly-ADP-ribose glycohydrolases (PARGs) reverse the ADP-ribosylation modification of the substrate by cleaving the protein-ADP-ribose bond and ribose-ribose bonds, respectively. Growing evidence indicates that ADP-ribosylation is pivotal in many cellular processes such as cell signaling, immune response, cell division, DNA damage sensing and repair, transcription regulation and cell death or survival determination.

Intracellular and extracellular mono-ADP-ribosylation often results in the inactivation of the target proteins. This modification is phylogenetically ancient and is seen in viruses, prokaryotic and eukaryotic species. Several bacterial toxins with MART activity, including diphtheria, cholera, pertussis and clostridial toxins, hijack hosts by mono-ADP-ribosylating some crucial proteins such as the heterotrimeric GTP-binding proteins, Rho, monomeric actin and translation elongation factor 2 (Corda and Di Girolamo, 2003). Similar enzymes are also detected in eukaryotes. These are ectoenzymes that are primarily membrane associated or secretory proteins. Intracellular mono-ADP-ribosylation has also been documented (Frye, 1999; Liszt et al., 2005), which suggests that a group of structurally different enzymes with MART activity may be present in the cytoplasm and nucleus. Two groups of putative intracellular mono-ADP-ribosyl transferases, including the SIRT (sirtuin) family and PI-MART (PARP-like MART) family, have been found which possess this activity in vitro (Hassa et al., 2006). At least one member of the SIRT family and two members of the PI-MART family have bona fide intracellular MART activity (Fahie et al., 2009; Kleine et al., 2008; Loseva et al., 2010). However, further investigation is needed to elucidate the roles of the other enzymes in these families.

Mono-ADP-ribosylation is not only a mechanism of pathogenesis used by some bacteria, but also a regulatory tool to control metabolic enzymes. For instance, the activity of dinitrogenase
reductase of the nitrogen-fixing bacterium *R. rubrum* is under the control of an arginine-specific MART (Ludden, 1994). In eukaryotes, mono-ADP-ribosylation is involved in the innate immune response (Paone et al., 2006; Paone et al., 2002; Stevens et al., 2009), G protein signaling modulation (Lupi et al., 2000; Lupi et al., 2002) and other cellular processes (Corda and Di Girolamo, 2003).

In contrast to mono-ADP-ribosylation, protein poly-ADP-ribosylation involves synthesis of a poly-ADP-ribose polymer using NAD⁺ as the precursor and an immediate substrate. Unlike the polyubiquitin modification, the long poly-ADP-ribose polymer branches irregularly. This structural heterogeneity is postulated to specify the functional outcomes of the modified substrates (Hassa et al., 2006), and the covalent modification of proteins with poly-ADP-ribose is mediated by poly-ADP-ribose polymerases. This family of enzymes, whose founding member is PARP-1, contains 17 members. The structural signature of the PARP superfamily is the conserved β-α-loop-β-α NAD⁺ fold, and a highly conserved glutamate residue (Glu988 in PARP-1) in this region seems critical to the elongation of poly-ADP-ribose chain. It is interesting to note that members with substituted non-conserved residues show variations in their enzymatic activity. One member, BAL 1/PARP-9 is reported to be inactive (Aguiar et al., 2005), whereas TiPARP/PARP-7 demonstrates intact poly-ADP-ribosylating activity (Ma et al., 2001). PARP-10, on the other hand may use an alternative catalytic mechanism to function as a transferase rather than a polymerase during ADP-ribosylation (Kleine et al., 2008). All together, these members with MART activity are also grouped with the PARP-like mono-ADP-ribosyl transferase (Pl-MART) family.

With the discovery of novel PARPs and their substrates, our understanding of the biological functions of poly-ADP-ribosylation has expanded from surveillance and maintenance
of genome integrity to the regulation of transcription, maintenance of telomere homeostasis, cell division, cell signaling and energy metabolism (Juarez-Salinas et al., 1979; Krishnakumar and Kraus, 2010; Beneke et al., 2008; Kidwell and Burdette, 1974; Xu et al., 2006; Bai et al., 2011). The DNA damage sensor proteins PARP-1 and PARP-2 detect DNA strand breaks and recruit DNA repair proteins to the lesions. Thus, they are involved in various aspects of DNA metabolism such as DNA repair, replication and recombination during which DNA strand breaks or base excisions may arise. In addition to the conventional functions in DNA damage repair, PARP-1 affects gene expression to regulate a variety of physiological processes such as EMT (epithelial-mesenchymal transition), memory consolidation and circadian clocks in peripheral organs (Asher et al., 2010; Fontan-Lozano et al., 2010; Rodriguez et al., 2011). PARP-1 activation leads to neuronal death due to NAD⁺ depletion (Alano et al., 2010) and signals AIF (Apoptosis Inducing Factor) release from mitochondria, resulting in caspase-independent cell death (Yu et al., 2002). Poly-ADP-ribosylation of centromeric proteins by PARP-1 and PARP-2 would indicate a role of PARP in cell division (Saxena et al., 2002a; Saxena et al., 2002b). Two tankyrases of the PARP family are involved in telomere length regulation. Poly-ADP-ribosylation of TRF1 (Telomeric Repeat Binding Factor-1) by tankyrase-1 and tankyrase-2 diminishes its ability to bind to telomeric DNA (Smith et al., 1998) and allows telomerase to access the telomeric complex, therefore promoting telomere elongation (Cook et al., 2002). Tankyrase-1 and tankyrase-2 also stimulate proteasome-mediated axin degradation, thereby implicating them in regulating canonical Wnt signaling (Huang et al., 2009).

1.2.4 CROSSTALK BETWEEN PHOSPHORYLATION AND UBIQUITINATION

Although phosphorylation and ubiquitination regulate a wide array of disparate cellular
processes, there is crosstalk in signaling between these two mechanisms as evident by the fact that both positively and/or negatively regulate each other (Hunter, 2007). Phosphorylation regulates ubiquitination of a protein by either altering the activity of its E3 ligase, creating a phosphodegron, affecting the subcellular compartmentalization of the substrate and/or the ubiquitin enzymes or modulating the activity of a deubiquitinase. Conversely, ubiquitination regulates phosphorylation by influencing the stability or the activity of the protein kinase and phosphatase.

1.2.4.1 PHOSPHORYLATION REGULATES E3 UBIQUITIN LIGASE

During ubiquitination an E3 ligase is essential in that it not only catalyzes/assists the transfer of ubiquitin from an E2-Ub conjugate to a lysine residue on the substrate, but it also facilitates substrate selection. Thus, like any enzyme, modulating E3 ligase activity affects the ubiquitination of a particular substrate and this regulation comes in the form of phosphorylation, which plays either a positive or negative role. For instance, phosphorylation of Ser166 and Ser188 in Mdm2 (Mouse double-minute 2), a RING finger ligase acting as an adaptor between E2-Ub and its target protein p53, increases its ligase activity towards p53 (Feng et al., 2004). In converse, phosphorylation of Mdm2 by c-Abl inhibits its ligase activity (Goldberg et al., 2002).

Phosphorylation of E3 ligases also influences their compartmentalization, as demonstrated by the HECT domain ligase Nedd4-2. Although localization of the epithelial Na⁺ channel on the cell surface is influenced by Nedd4-2 (Ke et al., 2010), this ligase also serves in the ubiquitination-dependent degradation of this channel. This negative regulation is lifted due to a serum- and glucocorticoid-inducible protein kinase (Sgk1) (Ichimura et al., 2005), which phosphorylates a specific serine (468) on Nedd4-2, thereby inactivating the ligase. The activity of HECT domain
ligases is also affected through conformational changes induced by phosphorylation. Itch is one such ligase, whose activity in multiple signaling pathways and pathological conditions (Melino et al., 2008) is either regulated in a positive manner by JNK phosphorylation (Gallagher et al., 2006) or inhibited by Fyn-mediated Tyr phosphorylation (Yang et al., 2006). These examples highlight how modulating E3 ligase activity by phosphorylation can influence protein ubiquitination. Phosphorylation, however, also modifies the electrostatic and/or the conformation of the target protein, thereby creating recognition signals that allow E3 ligases to ubiquitinate its substrate.

1.2.4.2 PHOSPHORYLATION CREATES A PHOSPHODEGRON

A degradation signal, or “degron”, is usually defined as a minimal element within a protein that is sufficient for recognition and degradation by the proteolytic machinery (Varshavsky, 1991). A variety of degradation signals, including N-degron, phosphodegron, oxygen-dependent degron, hydrophobic degron and chitodegron, have been integrally linked to the ubiquitin-proteasome pathway (Ravid and Hochstrasser, 2008). Among them, the phosphodegron is one that requires a post-translational modification in order to be recognized by E3 ligases. This phosphorylation-dependent ubiquitination and degradation is best exemplified by the destruction of Sic1, an inhibitor of the cyclin B-regulated kinase that blocks cell cycle progression (Barberis et al., 2005). Elimination of Sic1 by the SFCdc4 ubiquitin ligase requires that it be phosphorylated on at least 6 of its 9 Cdc4-phosphodegron sites (Orlicky et al., 2003). In this case, phosphorylation leads to the subsequent degradation of an inhibitor, thereby allowing the cell cycle to proceed.
1.2.4.3 PHOSPHORYLATION REGULATES UBIQUITINATION BY CHANGING THE SUBCELLULAR LOCALIZATION OF SUBSTRATES

As discussed previously, the subcellular localization of a protein is influenced by phosphorylation, and ubiquitin enzymes and their substrates take advantage of this mechanism for the same purpose. Protein ubiquitination is affected by the compartmentalization of proteins, which leads to the segregation of the ubiquitin enzymes and their substrates or access of the enzymes to their substrates. In the non-phosphorylated state, the tumor suppressor protein PTEN (Phosphatase and Tensin Homolog) is subject to rapid degradation in vivo through a ubiquitin-proteasome pathway. Phosphorylation at the C-terminal domain of PTEN changes its conformation and membrane localization, which greatly reduces its ubiquitination and degradation (Tolkacheva et al., 2001; Torres and Pulido, 2001; Vazquez et al., 2000; Wu et al., 2003). Since phosphorylation of PTEN does not affect its subsequent ubiquitination in vitro, it is clear that the change in the subcellular localization of PTEN is attributed to the inhibition of ubiquitination in vivo (Maccario et al., 2010). Conversely, insulin-induced phosphorylation of FKHR (Forkhead transcription factor) leads to its nuclear export, where in the cytoplasm it is ubiquitinated and degraded through the ubiquitin-proteasome pathway. Maintaining FKHR in the nucleus results in less ubiquitination, despite the fact that it is phosphorylated (Matsuzaki et al., 2003).

1.2.4.4 PHOSPHORYLATION MODULATES DEUBIQUITINASE ACTIVITY

As noted above, deubiquitinases reverse protein ubiquitination by removing the ubiquitin chain from the target protein. The substrate and chain type specificity of each deubiquitinase makes it a potent regulator in many cellular processes that rely on ubiquitination. For instance,
by cleaving the Lys48-linked polyubiquitin chain attached to the substrate, Lys48 deubiquitinase stabilizes its substrate targeted for proteasomal degradation (Schwickart et al., 2010). The Lys63 deubiquitinase specifically hydrolyses Lys63-linked polyubiquitin chains from their substrates and therefore reverses Lys63 ubiquitination-mediated cellular processes that are known to be involved in Wnt and NF-κB signalling (Heyninck and Beyaert, 1999; Tauriello et al., 2010). That many deubiquitinases are substrates for different kinases indicates their activity is subject to the regulation by phosphorylation. For example, NF-κB activity is positively regulated by Lys63-linked polyubiquitination of NF-κB regulators including RIP, TRAF6, TRAF2, and NEMO (Chen, 2005; Krappmann and Scheidereit, 2005). The Lys63 deubiquitinase A20 interacts with these regulators and removes the polyubiquitin chain from them, thus inhibiting the NF-κB signaling pathway (Heyninck and Beyaert, 1999; Song et al., 1996; Wertz et al., 2004; Zhang et al., 2000). However, A20 itself can be phosphorylated by IKKβ, an essential activator of the NF-κB signaling pathway. Phosphorylation of A20 enhances its deubiquitinase activity and further inhibits NF-κB signaling (Hutti et al., 2007). In contrast to A20, the enzyme activity of the Lys63 deubiquitinase CYLD is decreased when phosphorylated by IKKε, leading to cell transformation (Hutti et al., 2009). Thus, regulation by and between phosphorylation and ubiquitination is by no means unidirectional. Instead, both the activity and stability of kinases and phosphatases are under the tight control of the ubiquitination machinery.

1.2.4.5 UBIQUITINATION REGULATES KINASE/PHOSPHATASE STABILITY AND ACTIVITY

In many cases, phosphorylation of a protein and the formation of a phosphodegron are required before its ubiquitination. There is no evidence, however, for the opposite where
ubiquitination of a protein is a prerequisite for its phosphorylation. Based on our current understanding, ubiquitination regulates phosphorylation/dephosphorylation by affecting the stability and activity of protein kinases and phosphatases. As seen in the previous example, the phosphorylation of the deubiquitinase A20 down regulates NF-κB signaling. In contrast, activation of the same pathway relies on ubiquitination-dependent activation of the IκBα kinase (IKK). Ubiquitination and degradation of IκBα is regulated by its phosphorylation at Ser32 and Ser36 (Brockman et al., 1995; Brown et al., 1995; Chen et al., 1995; Traenckner et al., 1995; Whiteside et al., 1995), which leads to the release of the sequestered NF-κB to translocate into the nucleus. The kinase responsible for IκBα phosphorylation is a protein complex possessing E3 ligase activity. Activation of IκBα kinase requires the ubiquitination of the IKKγ subunit at Lys63. The subsequent interaction of ubiquitinated IKKγ with the IKKα and IKKβ subunits activates IKK kinase activity, which in turn phosphorylates IκBα at Ser32 and Ser36. Therefore, in this example ubiquitination serves a regulatory role in phosphorylation, rather than in proteolysis (Chen et al., 1996; Shambharkar et al., 2007; Sun et al., 2004).

In addition to activating kinases, ubiquitination also regulates the stability and activity of phosphatases. Protein phosphatase 2A (PP2A) is a serine/threonine phosphatase that dephosphorylates Akt at Thr308, thus terminating Akt signaling (Tremblay and Giguere, 2008; Trotman et al., 2006). Ubiquitination of the catalytic c subunit of PP2A (PP2Ac) by the E3 ligase MID1 (Midline-1) leads to its proteasome-dependent degradation. This degradation is necessary as evident in the human X-linked Opitz G/BBB syndrome, where elevated PP2Ac levels cause hypophosphorylation of MAPs (microtubule-associated proteins) (Trockenbacher et al., 2001).

1.2.5 CROSSTALK BETWEEN UBIQUITINATION AND ADP-RIBOSYLATION
Like several other proteins discussed so far, the enzymes involved in ADP-ribosylation are subject to ubiquitination and deubiquitination. ADP-ribosylation or ubiquitination of a protein can affect each other mutually in a variety of distinct ways. Furthermore, the identification of the conserved WWE domain, which mediates protein-protein interactions in both E3 ligases and some PARPs, has raised the possibility that these proteins share common binding partners, modifying them to impart different functional consequences (Aravind, 2001). This notion has some validity as evident by the PARP family member Tankyrase-1, which is recruited to the lateral membrane of polarized epithelial cells, but following the increase of its enzymatic activity and auto-poly-ADP-ribosylation, it translocates to the cytosol where it is ubiquitinated and targeted for proteasomal degradation (Yeh et al., 2006). A similar process is seen with TRF1, which first dissociates from telomeres following poly-ADP-ribosylation, and then is ubiquitinated and degraded in the proteasome, which ultimately leads to telomere elongation (Chang et al., 2003). That PARP-1 itself is modified at its N-terminus with the Lys48-linked polyubiquitin chain would indicate that polyubiquitination plays a regulatory role in poly-ADP-ribosylation of nuclear proteins (Wang et al., 2008).

1.3 OBJECTIVES OF STUDY

The goal of this study was to explore the scope of posttranslational modifications in the regulation of CASK, a multi-function scaffolding protein. Extensive research has revealed that CASK serves many roles, including the organization of supramolecular protein complexes that serve as signaling hubs (Borg et al., 1998; Butz et al., 1998), as transcription co-activators to modulate reelin and NR2b expression (Hsueh et al., 2000; Wang et al., 2004a; Wang et al., 2004b), as a bona fide kinase to phosphorylate neurxin (Mukherjee et al., 2010; Mukherjee et al.,
and as binding protein required in many cellular processes (Hong and Hsueh, 2006; Hsueh, 2009; Lozovatsky et al., 2009). In addition to these identified functions, CASK is thought to be a substrate for Cdk5 (Samuels et al., 2007) and PKA (Huang et al., 2010), which would regulate its subcellular localization and Tbr-1 binding, respectively. Given the diversity in the roles offered by CASK, some of which relying on phosphorylation, it is not unreasonable to postulate that the protein may be subject to other types of post-translational modification. Likewise, the possibility also exists that crosstalk between these modifications will have a significant impact on the function of CASK in different cell types. The characteristic degradation pattern of endogenous and exogenous CASK in my experiments together with the in silico sequence analysis prompted me to hypothesize that CASK is subject to ubiquitin modification and proteasome-mediated degradation. Furthermore, and although CASK was previously reported to be a phosphoprotein, I propose that its ubiquitination and subsequent degradation is initiated by phosphorylation. While testing the CASK hypothesis, I serendipitously found that the regulation of PARP-2 is mediated by proteasome-dependent degradation. I also found that PARP-2 transformed into a SDS-insoluble form, sequestered in cells soon after serum was removed from the media and this change was reversed when cells were grown in serum. The fact that the structure of PARP-2 resembles PARP-1, which is ubiquitinated and degraded in the proteasome (Masdehors et al., 2000; Wang et al., 2008), and has similar functions (Oliver et al., 2004), led me to hypothesize that PARP-2 is also regulated in a ubiquitin-proteasome-dependent manner. Furthermore, in an affinity-purification mass spectrometry study of PARP-1, PARP-2 and PARG interactomes, Ro52/TRIM21 was identified as an interacting protein with both PARP-1 and PARP-2 (Isabelle et al., 2010). Ro52 is a cytoplasmic RING finger domain E3 ligase, but it also translocates to the nucleus when cells are under stress (Espinosa et al., 2008; Nobuhara et al., 2007; Oke et al., 2009;
Strandberg et al., 2008). Interestingly, the poly-ADP-ribose polymerase activity of PARP-1 and PARP-2 is also activated by stress-induced DNA strand breaks. The coincident responses of PARPs and Ro52 to cellular stress and their interactions suggest they may be functionally related, which led to my hypothesis that Ro52 is the E3 ligase of PARP-1 and PARP-2 ubiquitination. While testing this hypothesis, I found that overexpressing Ro52 resulted in the formation of large membrane-bound structures within the cytosol. The morphology of these structures resembled the structures formed during autophagy, the self-eating process described earlier. These observations have not been documented although an uncharacterized structure called a cytoplasmic body that forms following Ro52 overexpression was reported (Tanaka and Kamitani, 2010; Tanaka et al., 2010). Moreover, Ro52 cytoplasmic bodies are actively transported along the microtubule network (Tanaka et al., 2010), a feature in autophagosome trafficking.

Ro52, an autoantigen in several autoimmune diseases such as Sjögren’s syndrome (SS) and systemic lupus erythematosus (SLE), modulates the production of cytokines in response to viral and bacterial infection and participates in the innate immune response by targeting internalized antibody-bound viruses to the UPS for degradation (McEwan et al., 2013). Another feature of Ro52 is its ability to monoubiquitinate active IKKβ, which leads to the autophagic degradation of the latter (Niida et al., 2010). In addition, Ro52 interacts with the autophagy receptor protein p62, which is involved in selective autophagy (Kim and Ozato, 2009). These lines of evidence together with my observation that Ro52 overexpression resulted in the formation of autophagosome-like structures suggest Ro52 also participates in autophagy, and experiments were designed to address this hypothesis.
**1.4 LITERATURE CITED**


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CHAPTER 2 POST-TRANSLATIONAL MODIFICATION OF CASK LEADS TO ITS PROTEASOME-DEPENDENT DEGRADATION


2.1 INTRODUCTION

CASK (Ca\(^{2+}\)/calmodulin-dependent serine protein kinase) is a MAGUK (membrane-associated guanylate kinase) protein that is essential in mice (Atasoy et al., 2007; Dimitratos et al., 1997; Hoskins et al., 1996; Mukherjee et al., 2008). In humans CASK mutations are implicated in X-linked optic atrophy, microcephaly, cerebellar hypoplasia and X-linked dominant mental retardation (Dimitratos et al., 1998; Froyen et al., 2007; Hayashi et al., 2008; Najm et al., 2008; Piluso et al., 2009). CASK contains numerous domains that act as platforms for the binding of other proteins (Butz et al., 1998; Cohen et al., 1998; Fallon et al., 2002; Hata et al., 1996), which together participate in membrane–cytoskeleton scaffolding complexes, in synaptic targeting, transcriptional co-activation and regulation of epidermal progenitor cells (Bass and Humphries, 2002; Biederer and Sudhof, 2001; Cohen et al., 1998; Hsueh et al., 2000; Lee et al., 2002; Martinez-Estrada et al., 2001; Ojeh et al., 2008; Wang et al., 2004a). Recently, CASK was reported to phosphorylate neurexin, despite the fact that its calmodulin (CaM) kinase domain lacks a motif thought to be indispensable for kinase activity (Mukherjee et al., 2008). Finally, CASK is present in the nuclei of undifferentiated myoblasts and basal keratinocytes in newborn rodent skin and developing hair follicles, but is predominantly cytoplasmic when cells...
differentiate (Gardner et al., 2006; Ojeh et al., 2008). These findings are significant in that not unlike β-catenin they endow nuclear function to a cytoplasmic protein. Since the carboxy-terminal guanylate kinase-like (GK) domain of CASK has the comparable effect on regulating gene expression as the full-length protein, a model was proposed to explain its nuclear translocation (Bredt, 2000). In the case of cerebral cortex development, CASK moves into the nucleus after it is either phosphorylated or proteolytically processed (Wang et al., 2004a).

Here we show for the first time that CASK binds ubiquitin and is proteolytically processed in a characteristic fashion in diverse cell types. While the attachment of ubiquitin on target proteins modulates apoptosis, the cell cycle, cell signaling, transcription and DNA replication (Adhikari et al., 2007; Branzei and Foiani, 2008; Steller, 2008; Weake and Workman, 2008), ubiquitination is intricately linked to protein degradation (Ciechanover, 1980; Ciechanover, 1978; Piper and Luzio, 2007; Thompson et al., 2008). The presence of three putative PEST sequences in CASK prompted us to hypothesize that degradation was calpain or proteasome-dependent. Chemical treatments and inhibitors revealed that phosphorylation and ubiquitination precede CASK degradation in the proteasome. Given the widespread, characteristic nature of this degradation, we propose that this processing serves as a mechanism to either dismantle multimeric scaffolding complexes and/or to generate fragmented polypeptides involved in cell signaling.

2.2 MATERIALS AND METHODS

2.2.1. PLASMIDS, ANTIBODIES AND REAGENTS

pRK5 myc-CASK (Full-length, FL), pRK5 myc-CASK (1–612) and pRK5 myc-CASK (576–898) were provided by Dr. B. Margolis (University of Michigan), MT123-ubiquitin-HA by Dr. D.
Bohmann (University of Rochester), and pcDNA 3.1 myc/his CASK constructs (FL, S51A, S395A, S51/395A) by Dr. L.H. Tsai (MIT). myc-CASK was cloned into pLitmus for in vitro transcription/translation. Staurosporine and antibodies against CASK (H-107, targeting amino acids 353–459 and C-19, targeting the C-terminus), HA (Y-11 and F-7), myc (9E10), ubiquitin,-actin, ERK and phospho-ERK were from Santa Cruz Biotechnology, anti-β-catenin from Sigma and anti-phospho-Serine from Abcam and Chemicon. Secondary antibodies were from Pierce. BAPTA-AM, A23187, cycloheximide, MG132, ATP, creatine and creatine phosphate were from Sigma, calpeptin from Calbiochem and ubiquitin and ubiquitin aldehyde from Boston Biochem.

2.2.2 CELL CULTURE, TRANSFECTION AND TREATMENT

Rat inner medullary collecting duct (IMCD), NIH3T3 fibroblasts, COS-7, HeLa, U87MG glioblastoma and MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin. Cells were transfected with CASK and/or ubiquitin constructs using Fugene 6 according to the manufacturer’s recommendations. Cells were treated with MG132 or NH₄Cl to inhibit proteasome and lysosome activity, respectively. COS-7 cells were also treated with CaCl₂, A23187, BAPTA-AM, calpeptin or EGTA to observe their effects on CASK degradation. DMSO was the vehicle control.

2.2.3. IN VITRO TRANSCRIPTION, TRANSLATION AND DEGRADATION ASSAY
The TNT T7 kit (Promega) and $^{35}$S-methionine were used to in vitro transcribe and translate radiolabeled CASK for in vitro degradation assays (Carrano et al., 1999). Assays were performed in a buffer containing 40 mM Tris–HCl, pH 7.6, 5mM MgCl$_2$, 1 mM DTT, 10% glycerol, 1 mg/ml ubiquitin, 1µM ubiquitin aldehyde, 10 mM phosphocreatine, 100µg/ml creatine phosphokinase and 0.5mM ATP. COS-7 hypotonic lysate was prepared as previously described (Yudkovsky et al., 2000), with minor modifications. Briefly, cells were resuspended in 500µl of buffer (20 mM HEPES pH 7.6, 1.5 mM MgCl$_2$, 0.5 mM KCl, 1 mM DTT, 10µg/ml leupeptin, 10µg/ml pepstatin, 1 mM PMSF and 17µg/ml aprotinin) for 5 min on ice, and then homogenized, centrifuged and the supernatant stored at −80°C. Approximately 12µg of lysate and 3µl of the in vitro translated $^{35}$S-CASK reaction was added to make a final 10µl volume. Reactions were performed at 30°C for 2 h and then terminated with 2x Laemmli buffer and boiling. Samples were resolved on 8% denaturing gels, which were dried and exposed to X-ray film for 4 days.

2.2.4. EXTRACT PREPARATION, IMMUNOPRECIPITATION AND IMMUNOBLOTTING

COS-7 cells were transfected with pRK5 myc-CASK (FL, 1–612 or 576–898), MT123-ubiquitin-HA or both and grown for 24 h. For immunoprecipitation cells were lysed in RIPA buffer supplemented with Minicomplete protease inhibitor cocktail. Lysates (800µl) were boiled for 5 min and then pre-cleared for 3 h at 4°C using protein A/G plus beads (Santa Cruz Biotechnology). The myc 9E10 or the CASK C-19 antibody was added to the lysate and incubated overnight at 4°C, and then 20µl of bead slurry was added to the lysate and incubated overnight at 4°C. Beads were pelleted, washed extensively in RIPA buffer, and then mixed with
2x Laemmli buffer and boiled. Proteins were resolved on 6% or 8% denaturing gels and then transferred to membranes, which were probed with HA, myc or phospho-Ser antibodies. For immunoblotting, cells were lysed in 1x Laemmli buffer supplemented with protease inhibitor cocktail and the quantity of protein determined using Bradford assays. Proteins were resolved on 8% gels and then transferred to membranes, which were incubated in 5% skim milk, Tris-buffered saline/Tween 20 (TBS/T) buffer. Overnight primary antibody (1:2500) incubation at 4°C was followed by washing in TBS/T. Membranes were incubated with HRP-conjugated secondary antibodies (1:4000), and then washed with TBS/T. Signals were detected by enhanced chemiluminescence detection (Pierce). Films were scanned and densitometric analysis performed using FluorChem 8900 software.

2.2.5. IMMUNOCYTOCHEMISTRY

Cells grown on coverslips were transfected with constructs as described above. At 24 h cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, and then washed extensively in PBS. Cells were permeabilized with 0.2% Triton X-100/PBS, blocked with 3% BSA/PBS and then incubated overnight at 4°C with anti-CASK (C-19), ubiquitin, myc (9E10) or HA antibodies diluted 1:50 in 3% BSA/PBS. Cells were washed in PBS and incubated at 37°C with the secondary antibody (1:100–1:200) conjugated to either FITC or TRITC. Following additional PBS washes, coverslips were mounted using glycerol/PBS. Images, captured using a Zeiss Axiovert 100 laser scanning confocal microscope, were processed into plates with Adobe Photoshop/Illustrator CS3.
2.3 RESULTS

2.3.1 CHARACTERISTIC CASK DEGRADATION IN VARIOUS CELL LINES

CASK was expressed in IMCD, NIH3T3, COS-7, U87MG, MCF-7 and HeLa cells (Fig. 2.3.1A). Although reported to have an ∼110–112 kDa molecular mass (Cohen et al., 1998; Leonoudakis et al., 2004), results with the H-107 antibody showed a consistent degradation pattern and a prominent band at ∼70 kDa. This consistency led to the hypothesis that CASK is degraded by a common mechanism. Protein lysates from cell lines transfected with myc-tagged, CASK (pRK5 myc-CASK) were probed with anti-CASK (H-107) or myc (9E10) antibodies and differences between endogenous and exogenous CASK resolved using the 9E10 antibody (middle panel, Fig. 2.3.1B). Myc-positive signals were not seen in lysates isolated from untransfected cells (data not shown). The H-107 antibody recognized numerous bands ranging from 112 to 36 kDa, and especially a prominent one between 36 and 50 kDa (boxed region) (Fig. 2.3.1B). The lower signals correspond to N-terminal fragments, which harbor the epitope tag (middle panel, Fig. 2.3.1B). The C-terminal-specific C-19 CASK antibody only recognized full-length CASK or a C-terminal-containing fragment of ∼70 kDa (Fig. 2.3.1B). These characteristic degradation patterns and its role as a transcriptional co-activator (Hsueh et al., 2000), suggested CASK might be a short-lived protein.

2.3.2 SEQUENCE ANALYSIS AND PROTEIN TURNOVER

Proteins that have short half-lives often contain PEST sequences rich in Pro (P), Glu (E), Ser (S) and Thr (T) (Barnes and Gomes, 1995; Belizario et al., 2008). A PEST search algorithm
Figure 2.3.1. CASK degradation in mammalian cells. (A) Immunoblots of protein from IMCD, NIH3T3, COS-7, U87MG, MCF-7 and HeLa cells probed with the H-107 CASK antibody. (B) Lysates from cells expressing pRK5 Myc-CASK (FL) were immunoblotted with anti-myc, or the CASK H-107 or C-19 antibodies. Unless stated otherwise, blots are representative of three independent experiments.
(http://emb1.bcc.univie.ac.at/embnet/tools/bio/PESTfind/) revealed three putative regions in CASK: 316–345 (score +5.12), 458–487 (score +6.77) and 594–611 (score +7.37) (underlining, Fig. 2.3.2A). Epestfind (http://emboss.bioinformatics.nl/) also identified three motifs: 316–345 (score 5.77), 458–487 (score 6.76) and 594–611 (score 7.37). This bioinformatic information supported our hypothesis that CASK is a candidate for proteolytic degradation at the PEST sequences. Surprisingly, levels of CASK and ERK 1/2 in COS-7 cells treated with cycloheximide did not change appreciably after 24 h of treatment (Fig. 2.3.2B). The relative level of phospho-ERK1/2, however, declined and signals corresponding to p44 were absent by 3 h (Fig. 2.3.2B). The major degradation fragment of CASK (70 kDa; N-terminus) was stable over the course of the experiment (arrow, Fig. 2.3.2B). β-catenin (92–94 kDa) levels in cycloheximide-treated cells were also stable (Fig. 2.3.2B), although a major band at approximately 75 kDa was present.

2.3.3 CASK DEGRADATION IS MEDIATED THROUGH THE PROTEASOME PATHWAY

The putative PEST sequences, as in proteins targeted by Ca2+-dependent calpain proteases (Rechsteiner and Rogers, 1996), and the ability of the CaM kinase domain to bind calmodulin in a Ca2+-dependent manner (Hata et al., 1996; Mukherjee et al., 2008; Ohno et al., 1984), prompted us to propose that CASK degradation involved Ca2+. COS-7 cells grown in the presence of extracellular (EGTA) or intracellular calcium chelators (BAPTA-AM) had no affect on CASK degradation relative to DMSO controls, and at least two bands with one corresponding to full-length CASK (arrow) was seen in each lane (Fig. 2.3.3A). Similarly, the calpain inhibitor
Figure 2.3.2. Putative PEST sequences and relative stability of CASK. (A) Three putative PEST sequences, between amino acids 316–345, 458–487 and 594–611, are underlined. (B) COS-7 cells were treated for different intervals with cycloheximide (62.5µg/ml), and then protein lysates collected for immunoblot analysis with antibodies against CASK, β-catenin, p-ERK and ERK.
(A) 

..RKFNARRKLKGAVLAAVSSHKFNFSYGDPP 325 
EELPDFSEDPTSSGLLAERAARVSQVLDSLE 355 
EIHALTDCEKDLDFLHSVQDQHHLHTLD 385 
LYDKINTKSSPQIRNPSPDAVQRAKEVLEE 415 
ISCYPENNDAKEKLRILTQPHFMALLQTTHD 445 
VVAHEVYSDEALRVTTPPTSPYLNGDSPES 475 
ANGDMDMENVTRVRLVFQKNTDEPMGITL 505 
KMNELNHCIVARAMHGGMHRQGLHVGEDE 535 
IREINGISVANQTVEQLQKMLREMRSITF 565 
KIVPSYRTQSSCSERDSPSTSRQSPANGHS 595 
STNNSSVSDLPSTTQPGRQIVYVRAQFEYDP.. 625

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- CASK
- β-catenin
- p-ERK
- ERK
calpeptin did not reduce, and raising the extracellular Ca\(^{2+}\) concentration did not enhance, CASK degradation (Fig. 2.3.3A). Increasing intracellular Ca\(^{2+}\) levels using A23187 had a modest effect, as evident by the band immediately below that at 112 kDa (Fig. 2.3.3A). Since ubiquitinated proteins can be degraded by the lysosome, it was necessary to test if this organelle was responsible for the degradation of CASK. Proteins from COS-7 cells treated with NH\(_4\)Cl to neutralize lysosomal pH (Dean et al., 1984), were immunoblotted and probed with anti-CASK and ERK antibodies. Results showed there was no appreciable difference (1.22 units each) in CASK levels in untreated or 30 mM NH\(_4\)Cl-treated cells (Fig. 3B, N=3 biological replicates), and degradation continued even at the 60 mM concentration.

Ruling out calpain and lysosomal involvement in CASK processing, our attention turned to the proteasome. COS-7 cells treated with the proteasome inhibitor MG132 showed reduced levels of degraded CASK relative to ERK (arrow, Fig. 2.3.3B), and reduction was dose-dependent (1.90 units vs. 1.18 units in DMSO alone, Fig. 2.3.3B). In vitro transcribed/translated CASK and an in vitro degradation assay gave similar results (Fig. 2.3.3C), where the 112 kDa, \(^{35}\)S-labeled CASK (arrow, Fig. 2.3.3C) was degraded within 2 h in the presence of EGTA or calpeptin. Interestingly, CASK was also degraded in the presence of MG132, but the ratio of full-length protein that remained relative to the input was greater (0.74) than that in other treatments (arrow, Fig. 2.3.3C).

2.3.4 IMMUNOLOCALIZATION OF CASK AND UBIQUITIN
Figure 2.3.3. Proteasome degradation of CASK. (A) COS-7 cells were treated with chemicals either individually or in combination as indicated. DMSO was the vehicle control. The concentration of each chemical was: CaCl₂, 5 mM; DMSO, 0.2%; A23187, 5µM; BAMPTA-AM, 20µM; calpeptin, 30µM; EGTA, 1.5 mM. Following 24 h (4 h for EGTA), protein extracts were probed with anti-CASK (H-107) and ERK antibodies. (B) Immunoblots with anti-CASK and ERK antibodies of proteins from cells treated with varying concentrations of NH₄Cl (top) or for 24 h with different concentrations of MG132 (bottom). Densitometric quantification below each lane represents the ratio between the bands denoted by arrows. (C) In vitro CASK degradation assay performed in the presence of EGTA, calpeptin or MG132. Densitometric quantification for each treatment represents the ratio of the signal (2 h vs. 0 h) for the band denoted by the arrow.
The endogenous CASK and ubiquitin expression was examined by immunocytochemistry before testing our hypothesis that CASK is ubiquitinated prior to degradation (Fig. 2.3.4A–C). Confocal microscopy revealed homogeneous CASK staining throughout the cell (Fig. 2.3.4A), whereas ubiquitin staining appeared punctate around the nucleus (Fig. 2.3.4B). Merging the images revealed the degree of overlap (yellow dots, Fig. 2.3.4C). Immunocytochemistry was also used to detect ectopically expressed HA-ubiquitin and myc-CASK (FL) (Fig. 2.3.4D–F). Myc staining was almost exclusively cytosolic and no obvious nuclear staining was seen (Fig. 2.3.4D). The absence of myc staining in some cells (arrow, Fig. 2.3.4D) indicated that the antibody was not cross-reacting with endogenous myc. HA staining of the ubiquitin fusion protein was similar to CASK, with the exception that nuclear staining was seen occasionally (arrow, Fig. 2.3.4E). Merged images show the extent of co-localization (Fig. 2.3.4F).

2.3.5 UBIQUITINATED CASK IS DEGRADED IN THE PROTEASOME

Proteins from COS-7 cells co-transfected with myc-CASK (FL) and HA-ubiquitin were immunoprecipitated with the CASK C-19 antibody and then probed with anti-HA antisera. Results revealed a characteristic protein smear between 100 and 250 kDa, especially from MG132-treated cells (Fig. 2.3.5). Blots stripped and reprobed with the myc antibody showed approximately equal levels of CASK in MG132-treated and untreated samples. While the ectopic expression approach provided evidence that CASK was ubiquitinated, we also tested whether endogenous CASK can be ubiquitinated. Proteins from HA-ubiquitin transfected cells were immunoprecipitated with CASK antibodies and smears were seen on anti-HA probed blots (not shown).
**Figure 2.3.4.** Immunolocalization of CASK and ubiquitin. (A–C) COS-7 cells grown on coverslips were processed for immunocytochemistry using C-19 anti-CASK and anti-Ub antibodies. Secondary antibodies against CASK and ubiquitin were labeled with FITC and TRITC, respectively. Scale bar equals 5µm. (D–F) COS-7 cells expressing myc-tagged, full-length CASK (D) and HA-tagged ubiquitin (E) were examined by confocal microscopy. (C) and (F) are merged images highlighting the expression of the endogenous proteins and those expressed ectopically from the transfected plasmid, respectively. Scale bar equals 15µm.
2.3.6 N- AND C-TERMINI OF CASK ARE UBIQUITINATED AND DEGRADED IN A PROTEASOME-DEPENDENT MANNER

An immunoblot of proteins isolated from DMSO-treated COS-7 cells transfected with myc-CASK (FL) and probed with the myc antibody, showed multiple bands below 112 kDa (Fig. 2.3.6A). A similar pattern was seen in MG132-treated transfected cells, but there was less degradation as evident by the prominent band at ~60 kDa, as well as the reduced amount of total protein in this lane (actin, Fig. 2.3.6A). MG132 also inhibited the degradation of the ~70 kDa CASK 1–612 (missing the GK domain) and ~40 kDa 576–898 (containing the C-terminal-most PEST sequence) deletion constructs, suggesting they may also be ubiquitin-bound. To test this, cells were co-transfected with HA-ubiquitin and either myc-CASK 1–612 or 576–898, then prepared for immunoprecipitation using the myc or C-19 antibody or incubated with protein A/G beads alone as a control (empty lanes, Fig. 2.3.6B). Immunoblots with an anti-HA antibody showed high molecular weight, HA-ubiquitin-positive bands/smears in samples from both deletion constructs (Fig. 2.3.6B). Staining was not seen in samples when the antibody was omitted during immunoprecipitation. Likewise, myc staining of proteins in lysates, prior to immunoprecipitation, confirmed the expression of the epitope-tagged proteins (right panel, Fig. 2.3.6B).

2.3.7 PHOSPHORYLATION OF CASK IS REQUIRED FOR ITS UBIQUITINATION

The phosphorylation of CASK (Mukherjee et al., 2008; Samuels et al., 2007), prompted us to hypothesize that it, like β-catenin, is regulated by phosphorylation before degradation. To
Figure 2.3.5. Ubiquitinated CASK is degraded in the proteasome. COS-7 cells cotransfected with myc-CASK and HA-ubiquitin (lanes 1 and 2) or transfected with myc-CASK only (lanes 3 and 4), and grown in the presence of MG132, were lysed and proteins immunoprecipitated with C-19 CASK antisera. Immunoblots were probed with an anti-HA or myc (9E10) antibody.
Figure 2.3.6. N and C-termini of CASK are ubiquitinated and degraded by the proteasome. (A) Protein lysates of COS-7 cells transfected with myc-CASK-FL, myc-1–612 CASK or myc-576–898 CASK and treated with MG132, were probed with anti-myc (9E10) or actin antibodies. (B) Proteins from cells co-transfected with HA-ubiquitin and either myc-1–612 or myc-576–898 were immunoprecipitated with the mouse myc or the goat C-19 CASK antibody and then analyzed on blots with an anti-HA antibody. Empty lanes represent samples incubated with beads alone. Lysates prior to immunoprecipitation were also probed with the myc antibody to detect ectopic CASK expression (right panel).
address whether or not kinase inhibition influences CASK ubiquitination, cells were co-transfected with myc-CASK (FL) and HA-ubiquitin, and then treated with the broad spectrum kinase inhibitor staurosporine (O’Brian and Ward, 1990). Immunoblots of proteins immunoprecipitated using the C-19 antibody and probed with an HA antibody showed cells treated with DMSO having more ubiquitinated CASK compared to those treated with staurosporine (Fig. 2.3.7A). The size of the ubiquitinated CASK polypeptides (112 to >250 kDa) in both samples was comparable to that seen in Fig. 5 and when blots were stripped and reprobed for myc, approximately equal amounts of CASK were seen in each treatment (middle panel, Fig. 2.3.7A). Immunoprecipitated fractions were also blotted and probed with phospho-Ser antibodies and although signals were consistently weak, the DMSO-treated, 112 kDa form of CASK appeared more prominent compared to that following staurosporine treatment (bottom panel, Fig. 2.3.7A). While phosphorylation of Ser 51 and 395 by Cdk5 is involved in CASK regulation (Samuels et al., 2007), Cdk5 is not expressed in COS-7 cells (Michel et al., 1998), which indicates that phosphorylation involves another kinase. Constructs encoding full-length CASK-myc, or CASK-myc mutated with serine to alanine at position 51, 395 or both, were transfected into COS-7 cells and grown in the presence or absence of MG132. Immunoblot analysis with anti-myc antisera showed that altering the Ser 51 and/or 395 sites to alanine did not influence proteolytic cleavage or proteasome activity as similar staining patterns were seen in MG132-treated cells (Fig. 2.3.7B). Likewise, only one band, corresponding to the 112 kDa protein, was present when the inhibitor was omitted.

2.4. DISCUSSION
Figure 2.3.7. CASK ubiquitination is phosphorylation-dependent. (A) Proteins from cells cotransfected with myc-CASK and HA-ubiquitin in the presence of staurosporine or DMSO were immunoprecipitated with the C-19 antibody and then analyzed on blots with an anti-mouse HA antibody. Stripped blots were reprobed with the myc antibody to detect ectopic CASK. Anti-CASK immunoprecipitated proteins were also probed with a phospho-serine antibody (lower panel). (B) Proteins from cells transfected with pcDNA 3.1 myc/his CASK constructs encoding full-length CASK (CASK-Myc) or CASK with mutated Ser to Ala residues at position 51 (S51A) and/or 395 (S395A), grown in the presence or absence of MG132 were probed with the myc or anti-actin antibody, the latter as a loading control.
CASK is influenced by phosphorylation, which affects its activity, subcellular distribution (Mukherjee et al., 2008; Samuels et al., 2007) and its degradation (this study). The degradation issue caught our attention after seeing consistent patterns of degradation in numerous, disparate cell types. CASK degradation was first reported in a study on sperm maturation (Burkin et al., 2004), and although a mechanism was not elucidated, the authors proposed that proteolysis into proteins of ∼55–65 kDa would allow CASK to participate in sperm binding, the acrosome reaction and fertilization. CASK fragments at ∼50 kDa were reported in differentiating myogenic cells, but they are attributed to a skeletal muscle isoform expressed from the CASK-A splice variant (Gardner et al., 2006). Recently, a ∼75 kDa CASK fragment in COS-7 cells was reported and postulated to be the smaller isoform (Sanford et al., 2008). These same authors report a pattern of degradation in muscle from CASK transgenic mice, indicating that the over-expressed protein is subject to degradation similar to that by the endogenous protein. This also supports our overexpression results seen in various epithelial and fibroblast cells. Incidentally, there is also a CASK-C isoform, with a predicted molecular mass ∼102 kDa (Weng et al., 2009). We, however, are confident that the lower molecular weight bands are products degraded from the 112 kDa CASK-B isoform because none of the cell types we examined were muscle-specific and more importantly, the antibody derived from the C-terminus and unique to CASK-B, detected these fragments. Bioinformatic evidence supported our hypothesis that CASK is a candidate for proteolysis at PEST sequences, which in the case of transcription factors occurs rapidly in a calpain or proteasome pathway (Rechsteiner and Rogers, 1996), the latter constituting the major route (Fuertes et al., 2003). Although CASK is a co-activator of transcription (Hsueh et al., 2000; Wang et al., 2004a), it is categorized as a MAGUK protein rather than a transcriptional modulator. Interestingly, the CASK binding protein CINAP, which
modulates transcription, contains two PEST sequences and is degraded in the proteasome (Hsueh, 2006; Wang et al., 2004a). Although evidence from the cycloheximide treatments indicate that CASK is not a short-lived protein, unlike phospho-ERK (Moos et al., 1996), its degradation would nevertheless add another level of regulation to CINAP-dependent gene regulation. Presently, it is not known how or if the PEST sequences contribute to CASK degradation, but ruling out Ca\(^{2+}\) and calpain involvement altered the focus to the proteasome. MG132 reduced CASK degradation, but the reduction was not as striking as expected and we attribute this to the low turnover rate of CASK and the short exposure to MG132. The fact that proteins targeted for proteasome degradation are generally, although not exclusively, modified by ubiquitin (Janse et al., 2004), prompted us to ask if ubiquitin associates with CASK prior to degradation.

Immunocytochemistry data provided circumstantial evidence for a CASK–ubiquitin interaction in the cytosol, since unlike CASK endogenous ubiquitin staining was not seen in the nucleus. In contrast, ectopically expressed HA-ubiquitin staining seen in the nucleus, and reported previously (Cummings et al., 1998), may be the result of protein over-expression and not physiologically relevant. In fact, given the role of CASK in the nucleus it may be unfavorable to have it ubiquitinated if this association precedes degradation. In reference to ectopic CASK expression, although we and others have not seen nuclear localized myc-CASK (Marble et al., 2005), others have (Wang et al., 2004a). Despite this discrepancy the more important issue is the presence of endogenous CASK in the nucleus, which warrants further investigation in light of the studies showing nuclear CASK in non-neuronal cells (Gardner et al., 2006; Ojeh et al., 2008). Evidence for a biochemical interaction between CASK and ubiquitin came from the immunoprecipitation data, and extending these results with those from the NH\(_4\)Cl and MG132 treatments indicated that CASK degradation is proteasomal. Interestingly, the
deletion constructs, one of which encodes only one putative PEST sequence, revealed that different regions of the protein are also subject to ubiquitination and proteasomal degradation.

CASK degradation, as with β-catenin, is preceded by phosphorylation. Although the kinases involved in degradation are unknown, Cdk5 phosphorylates CASK at Ser 51 and Ser 395 (Samuels et al., 2007). Phosphorylation at these sites, however, is not required for ubiquitination and proteasome degradation. We did note a contradiction in the banding patterns between COS-7 cells expressing CASK-myc (Fig. 2.3.7B) and those expressing myc-CASK (Fig. 2.3.6A). Possibly owing to differences in expression efficiency between the two vectors, we favor the idea that differences are due to the position of the epitope, and with the predicted cleavage sites being more toward the N-terminus. This would explain why multiple bands ranging from 30 to 75 kDa are seen in lysates from the myc-CASK-, rather than the CASK-myc-transfected cells (Fig. 2.3.6A). Differences were also seen in the MG132 treatments, which are also explained by the position of the epitope. Another plausible explanation relates to exposure time of the chemiluminescence detection, which when kept short to highlight multiple bands seen following MG132 treatment was not sufficient to see faint bands in the inhibitor’s absence (Fig. 2.3.7B). Although other reasons may exist, results nevertheless support the contention that CASK phosphorylation is an obligatory step required for its subsequent proteasomal degradation. Towards that end, a search is underway for the kinase(s) involved in CASK phosphorylation.

Confident that CASK is degraded in a characteristic fashion in a number of different cell lines, we provide new evidence for a mechanism of degradation involving phosphorylation and a ubiquitin–proteasome pathway. It is tempting to suggest that CASK degradation contributes to sperm maturation (Burkin et al., 2004) and although this remains to be tested, there is evidence for the importance of ubiquitin and proteolysis in sperm remodeling (Rodriguez and Stewart,
2007; Sutovsky, 2003). With the assignment of a nuclear function to CASK in neurons (Hsueh et al., 2000; Wang et al., 2004b), muscle (Gardner et al., 2006) and in keratinocytes (Ojeh et al., 2008), it is clear that degradation and clearance of CASK would serve a pivotal role in cell signaling. One example is in cerebral cortex, where CASK translocates to the nucleus and forms a Tbr-1/CINAP complex to affect reelin and NR2b expression (Hsueh et al., 2000; Wang et al., 2004b). A model was proposed suggesting phosphorylation and proteolytic processing causes CASK or part of it to translocate into the nucleus (Bredt, 2000), and with the ability of the GK domain itself to bind Tbr-1 and enhance reelin expression (Hsueh et al., 2000), the partial degradation of CASK and release of its carboxy-terminal tail would facilitate signaling. CASK fragments in the nucleus have not been reported, but with evidence that the C-terminus of CASK is preferentially degraded, we have reopened the search. That CASK has no transcriptional co-activation motif (Hsueh, 2006), and unlike transcription factors is relatively stable, invites questions regarding degradation. Does the dismantling of CASK scaffolding complexes in the cytoplasm ultimately provide the “source” of the GK domain that is responsible for influencing gene expression? If so, our evidence that CASK appears as multiple fragments in a number of different cell types, follows not only the original idea that proteolytic processing allows CASK to participate in several roles (Burkin et al., 2004), but also fits the model on how processing facilitates nuclear translocation (Bredt, 2000). In either case, the eventual phosphorylation and ubiquitination would serve to remove these polypeptides and hence attenuate signaling. The fact that we identified endogenous CASK in the nucleus of COS-7 cells using the same C-19 antibody that detected either the 70 or 112 kDa protein on blots is of special interest and current investigation is underway to determine if one, or both, forms of the protein translocates to the nucleus.
In summary, CASK phosphorylation dictates its function (Mukherjee et al., 2008; Samuels et al., 2007) and ultimately its degradation. Proteolysis, like phosphorylation, may provide another mechanism allowing CASK to have different functions and/or subcellular localizations. When no longer required and prior to degradation, we propose that these processed polypeptides are further phosphorylated and subsequently ubiquitinated. Although this remains to be tested, our study demonstrates a new role for phosphorylation of CASK and provides insight on how this regulation could impact on CASK function(s) in a number of disparate roles and cell types. With the crucial role CASK plays in CNS development (Fallon et al., 2002; Najm et al., 2008) and possibly also in fertility (Burkin et al., 2004), the identification of proteins that regulate its function(s) will ultimately shed new light on the etiology of certain human diseases.
2.5 LITERATURE CITED


CHAPTER 3 SERUM-DEPENDENT AND INDEPENDENT REGULATION OF PARP-2 LEVELS

3.1 INTRODUCTION

Poly-ADP-ribose polymerase (PARP) enzymes were first described as playing an integral part in DNA repair (Jeggo, 1998), but they are now known to have a much broader role and have been linked to the inflammatory response, tumorigenesis and to mitochondrial function and oxidative metabolism (Bai and Canto, 2012; Krishnakumar and Kraus, 2010). In response to a variety of physiological and pathophysiological conditions, PARPs catalyze the formation of free poly-ADP-ribose polymers (Nicolas et al., 2010) as well as poly-ADP-ribosylated proteins using NAD$^+$ as an immediate substrate. PARP-2 shares similar crystal structure and function with the founding member PARP-1 (Oliver et al., 2004), and both proteins homo- and heterodimerize and poly ADP-ribosylate each other (Schreiber et al., 2002). Although their functions are partially redundant, as indicated by the embryonic viability when one or the other gene is knocked out, both proteins are indispensable as the embryos die in the double knockout mice (Menissier de Murcia et al., 2003; Nicolas et al., 2010). One of these important roles assigned to PARP proteins is their involvement in the maintenance of telomere integrity (Dantzer et al., 2004) and genome stability through recruiting DNA repair factors to DNA-strand breaks and base-excision lesions that result from DNA damage (Ame et al., 1999; Schreiber et al., 2002). These activities are suspended during apoptosis by caspase-8, which serves to inactivate PARP-2 (Benchoua et al., 2002). PARP-2 also functions in a manner distinct from PARP-1, and this can be seen in PARP-2$^{-/-}$ mice, which show specific traits including increased energy expenditure due to the upregulation of NAD$^+$-dependent SIRT1 deacetylase (Bai et al., 2011), impaired synergistic interaction with p53 in tumor suppression (Nicolas et al., 2010), hypofertility, impaired
spermatogenesis and sperm maturity (Dantzer et al., 2006b; Jha et al., 2009; Tramontano et al., 2007) and hepatic cholesterol accumulation (Szanto et al., 2014). In addition, PARP-2 is known to contribute substantially to the translocation of apoptosis-inducing factor from the mitochondria to the nucleus during excessive oxidative damage to DNA (Li et al., 2010). PARP-2, together with the PPARgamma/RXR transcription machinery, is also important in adipocyte differentiation (Bai et al., 2007), and in the regulation of surfactant protein B expression in pulmonary cells (Maeda et al., 2006). Recently, it was reported that PARP-2 is essential for hematopoietic stem/progenitor cells (HSPC) survival under steady-state conditions and in response to radiation stress (Farres et al., 2013). However, the loss of PARP-2 in this HSPC study led to an increase in apoptosis, which contradicts the focal cerebral ischemia report where PARP-2 nulls show a suppression of apoptosis-inducing factor (Li et al., 2010). Despite these contradictions that may be the result of cell specificity, these studies provide evidence that PARP-2 is not entirely functionally redundant with PARP-1. Furthermore, they also underscore the importance of PARP-2 in not only limiting genomic instability, but also in maintaining a number of key cellular physiological processes.

The importance of PARP-2 in a diverse number of biological roles led us to hypothesize that mechanisms might be in place to regulate its levels and hence its activity at different times in the cell cycle. While, as noted above, caspase-dependent deregulation is one mechanism involved during apoptosis (Benchoua et al., 2002), other methods of proteolytic inactivation involving the lysosome and proteasome are expected to be in place under normal conditions. To investigate how PARP-2 levels are regulated, we first surveyed several cell types for the appearance of the protein and all, with the exception of NIH3T3 cells, showed robust levels that were relatively stable over several hours of cycloheximide treatment. Interestingly, PARP-2 was not seen when
cells were grown in serum free media and in fact they fell precipitously within 15 min following serum removal, which prompted further investigation. Results showing that the PARP-2 gene expression was not affected by serum removal would indicate that the putative serum response element in the PARP-2 promoter that was previously reported (Ame et al., 2001) was not functional and the decrease in protein levels resulted from a post-translational event. Analysis using the customary group of proteolytic inhibitors failed to identify the protease responsible for PARP-2 degradation under serum free conditions. Finally, focus turned to the proteasome and the post-translational modification of the target protein by ubiquitination that precedes proteolytic degradation.

The ability of the ubiquitin-proteasome system (UPS) to regulate PARP-1 levels and the structural similarity between PARP-2 and PARP-1 (Masdehors et al., 2000; Wang et al., 2008), led us to investigate whether or not a similar mechanism of regulation was in place for PARP-2. Having established that PARP-2 and ubiquitin (Ub) co-localized in cells was suggestive, but not unequivocal proof, of PARP-2 being degraded by the UPS. In vitro and in vivo assays provided evidence that PARP-2 was in fact ubiquitinated and we identified Ro52, an E3 ubiquitin ligase as a candidate involved in the process. Results also revealed that when cells were grown in complete medium, PARP-2 ubiquitination led to its degradation in the proteasome. Surprisingly, however, inhibiting proteasome activity in cells grown in serum free media failed to restore PARP-2 level, which, together with the protease inhibition experiments, suggested PARP-2 was sequestered to a detergent-insoluble compartment rather than degraded by protease following serum deprivation. We tested this notion by inhibiting protein synthesis with cycloheximide in cells grown in serum free media and returned to complete media to see if PARP-2 level recovers. Results showed PARP-2 disappeared on the blot in serum-starved cells and restored when cells
were returned to complete media even if protein synthesis was halted. Our findings strongly support the contention that PARP-2 is sequestered to a detergent resistant compartment in the absence of serum. Given the two means by which PARP-2 is regulated, when serum is present and cells are stimulated to grow, PARP-2 levels are maintained to fulfill its numerous roles in maintaining normal cellular physiology. When nutrients are not favorable, through a yet-to-be identified mechanism, PARP-2 is converted to an SDS-insoluble form which reverts to the SDS-soluble form upon addition of serum. At present we are speculating that this might either serve to reduce global ADP-ribosylation enzyme activity thus to minimize energy expenditure under adverse conditions, or if one of its functions is to behave like PARP-1, then this sequestration would promote cell cycle arrest (Kashima et al., 2012).

3.2 MATERIALS AND METHODS

3.2.1 ANTIBODIES, PLASMIDS AND REAGENTS

PARP-1/2 (H250), β-actin and ERK antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti-mouse PARP-2 (Yucatan) antibody was from Enzo Life Sciences. Rabbit anti-human PARP-2 antibody was from AXXORA. GST and HA antibodies were purchased from GenScript. HRP-conjugated secondary antibodies were from Pierce. pBC GST-PARP-2, pBC GST-NLS and pEGFP-PARP-2 plasmids were gifts of Dr. V. Schreiber (École Supérieure de Biotechnologie de, France). The mRFP-ub plasmid was a gift of Dr. N. Dantuma (Karolinska Institutet, Sweden) (Addgene plasmid number 11935). The pMT123 HA-ubiquitin plasmid was kindly provided by Dr. D. Bohmann (University of Rochester, U.S), and the p3xFLAG-Ro52 and p3xFLAG-Ro52 ΔRING plasmids were gifts of Dr. S. Hatakeyama (Hokkaido University, Japan). MG-132 and cycloheximide were from Sigma. Caspase inhibitor
III (B-D-FMK), calpeptin and pepstatin A methyl ester (PME) were from Calbiochem. Caspase-8 inhibitor (Z-IETD-FMK) was from BD Biosciences. Alpha-2-macroglobulin was from Enzo Life Sciences. Leupeptin was from Bio Basics Inc. Halt protease inhibitor cocktail was from Pierce. GST-PARP-2 human recombinant protein was from BPS Bioscience. Protein fraction II, HA-ubiquitin, ubiquitin aldehyde and ubiquitin conjugation reaction buffer kits were purchased from Bostonbiochem. The transfection reagent XtremeGene 9 was from Roche Applied Sciences. Glutathione Sepharose 4B beads were purchased from GE Healthcare Life Sciences. Power SYBR Green PCR master mix was purchased from Invitrogen Life Science Technologies.

3.2.2 CELL CULTURE, TREATMENT AND TRANSFECTION

COS-7, MCF-7, HeLa, NIH3T3, MEF F20 and IMCD cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) /F-12 or DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin in 5% CO₂ humid atmosphere at 37°C. Cells were treated with different protease inhibitors at the concentration and duration as indicated in the figures. Cells were subject to serum starvation or maintained in media containing different concentrations of sera where it is stated. Transfections were carried out using XtremeGene 9 transfection reagent as per manufacturer’s recommendation.

To test if PARP-2 was sequestered to a detergent-insoluble fraction, MCF-7 cells were grown to approximately 90% confluence in complete media and then media were removed and replaced with serum free media containing 50µg/ml cycloheximide to inhibit protein synthesis. The cells were incubated for seven hours then media were replaced with complete media containing 50µg/ml cycloheximide. The cells were allowed to grow for one hour until being lysed in sample buffer. Cells serum-starved for seven hours with or without cycloheximide treatment or
remained in complete media with or without cycloheximide were also lysed in sample buffer in the meantime. All samples were resolved by 10% SDS-PAGE gel and followed by western blotting with PARP-2 antibody.

3.2.3 END POINT RT-PCR AND qRT-PCR

MCF-7 cells were grown in complete medium until 90% confluence. At this point the medium was removed and for one plate it was replaced with serum free medium, while cells on the other plate were maintained in complete medium. Following 30 minutes cells on both plates were lysed in TRIZol (Invitrogen) and total RNA extracted following the manufacturer’s instructions. Following preparation of first strand cDNA by reverse transcription with (+) or without reverse transcriptase (-) (control), PCR was performed using primers specific to human PARP-2, which were forward 5’- GAATCTGTGAAGGCCTTGCTG-3’ and reverse 5’- TTCCCAACCCAGTTACTCATCC-3’. PCR products were resolved by 1% agarose gel and the image captured with a FluorChem 8900 Imager (Alpha Innotech).

For qRT-PCR, 90% confluent MCF-7 cells grown in complete media or serum-starved for 15, 30 and 60 minutes were used to extract total RNA with RNeasy mini kit (QIAGEN). First strand cDNA was prepared using qScript cDNA supermix (Quanta Bioscience) following the manufacturer’s instructions. qRT-PCR was performed using the abovementioned PARP-2 primers and GAPDH primers and SYBR Green master mix (Applied Biosystem) with Rotor-Gene 3000 (Corbett Research). qPCR data were processed with the software of the manufacturer and normalized to GAPDH control.

3.2.4 IN VIVO UBIQUITINATION ASSAY AND GST PULL-DOWN
COS-7 cells were transfected with pBC GST-PARP-2, or pBC GST-NLS (control) alone or with pMT123 HA-ubiquitin plasmids by XtremeGene 9 transfection reagent (Roche). 24 hours after transfection, one plate of COS-7 cells transfected with both pBC GST-PARP-2 and pMT123 HA-ubiquitin was treated with 40μM MG-132, the other with DMSO as a vehicle control. After 20 hours of treatment, cells were harvested by lysing in 1x RIPA buffer (pH 7.5) supplemented with protease inhibitor cocktail on ice after a PBS wash. The cell lysates were stored at -80ºC for the GST-pull down assay.

HeLa cells were transfected with pBC GST-PARP-2 alone, or pBC GST-PARP-2 and p3xFLAG-Ro52, or pBC GST-PARP-2 and p3xFLAG-Ro52ΔRING plasmids using XtremeGene 9 reagent. Twenty-four hours after transfection, one plate of HeLa cells transfected with both pBC GST-PARP-2 and p3xFLAG-Ro52 or p3xFLAG-Ro52ΔRING, was treated with 20µM MG132 for 7 hours. After a brief wash in PBS, the cells were lysed on ice in 1x RIPA buffer (pH 7.5), supplemented with protease inhibitor cocktail. Cell lysates were stored at -80ºC for GST-pull down assay. Centrifuging for 10 min at 18,500g at 4ºC precleared the RIPA lysates, and protein concentrations were then determined using a Bradford assay. Different volumes of each supernatant, used to ensure the same amount of total protein was present in each sample, were incubated with beads that were rotated overnight in Eppendorf tubes at 4ºC. Beads were washed 4 x 5 min with RIPA buffer and resuspended in 2x sample buffer. Pull-down proteins were resolved by 8% SDS-PAGE, then subject to immunoblot analysis.

3.2.5 IN VITRO UBIQUITINATION ASSAY

GST-PARP-2 human recombinant protein (0.4μg) was added to a 20μl final volume reaction mix containing 1x ubiquitin conjugation reaction buffer, 0.5mM MG-132, 1x ubiquitin aldehyde,
2mM HA-ubiquitin, 1x Mg-ATP and 1μl HeLa protein fraction II (protein fraction of HeLa S3 cell extract that binds to anion exchange resin). For control, 1μl HeLa protein fraction II was omitted from the reaction mix and supplemented with 1μl H2O. Reactions were carried out at 37°C for 2 hours and then stopped by adding 2 μl 10x E1 stopping buffer, 4μl 5x sample buffer and 1.5 μl β-mercaptoethanol, then boiled for 5 min. Proteins were resolved by 8% SDS-PAGE, then subject to immunoblot analysis.

3.2.6 IMMUNOBLOTTING

After a PBS (phosphate buffered saline) wash, cells were lysed in 1x sample buffer (without bromophenol blue) supplemented with protease inhibitor cocktail on ice. Lysates were sonicated for 10 sec and boiled for 5 min, then centrifuged for 10 min at 18,500g at 4°C. Aliquots of samples were diluted 200 fold to minimize SDS interference before protein concentrations were determined by a Bradford assay. Equal amount of proteins were resolved by 8% or 10% SDS-PAGE and then transferred to nitrocellulose membranes, which were then blocked in 5% skim milk/Tris-buffered saline/Tween 20 (TBS/T) buffer for 2 hours at room temperature. Following an overnight incubation in primary antibody (1:2500 diluted in blocking buffer) at 4°C, membranes were washed in TBS/T buffer and then incubated one hour with a HRP-conjugated secondary antibody (1:4000 diluted in blocking buffer) at room temperature. Membranes were washed extensively with TBS/T buffer and signals were detected by enhanced chemiluminescence substrate kit (Pierce). Densitometric analyses were performed using ImageJ software (NIH).

3.2.7 CONFOCAL MICROSCOPY
HeLa cells grown on glass cover slips were transfected with pEGFP-PARP-2 and mRFP-ub plasmids. Cells at 24 hours post-transfection were fixed with 4% PFA (paraformaldehyde) in PBS for 30 min at room temperature. After 3 x 10 min washes with PBS, cells were mounted with ProLong Gold anti-fade mounting medium (Invitrogen) and viewed with Zeiss LSM 510 Duo Vario confocal microscope.

3.3 RESULTS
3.3.1 PARP-2 EXPRESSION IS SERUM RESPONSIVE BUT IS NOT REGULATED BY ITS PUTATIVE SRE

PARP-2 is ubiquitously expressed in mammalian tissues and a putative serum response element (SRE) within the promoter of PARP-2 gene suggests its expression may be subject to serum stimulation (Ame et al., 1999; Ame et al., 2001). To test whether this SRE is functional, we first assayed PARP protein levels in COS-7, MCF-7, HeLa, inner medullary collecting duct (IMCD) cells and NIH3T3 cells grown in complete and serum free (SF) media. Immunoblot analysis with the PARP H-250 antibody revealed a prominent 116kD band corresponding to PARP-1 in all cell lines (Fig. 3.3.1A). A band at 89kD, corresponding to the C-terminal fragment of PARP-1 (Chaitanya et al., 2010), was detected in COS-7 cells and weakly in MCF-7 cells. The appearance of this and the 116kD band did not change significantly when cells were grown in complete or in SF media. The H-250 antibody also recognized a 62kD band, corresponding to PARP-2, in all cells grown in complete media (Fig. 3.3.1A). This band, however, was absent when COS-7 or MCF-7 cells were grown in SF media. To verify the identity of the 62kD band, two PARP-2 specific antibodies were used to probe MCF-7 and MEF cell lysates. Consistently, a 62kD band corresponding to PARP-2 was absent when cells were grown in SF media,
confirming the specificity of the H-250 antibody to PARP-2 (Figs. 3.3.3B and 3.3.7A). ERK staining, used as a loading control in other studies (Fernandez-Garcia et al., 2007; Rygiel et al., 2008; Xu et al., 2012) showed that protein was present in all samples, with approximately equal amounts assayed in the COS-7 and MCF-7 lanes under the different culturing conditions.

The loss of the PARP-2 signals in cells grown in serum free media was not due to cell death and in fact within one hour, when grown in complete media these previously serum-starved COS-7 cells had reacquired a PARP-2 signal (Fig. 3.3.1B). Furthermore, signals were comparable to those in cells that had been continually growing in complete media. These results showing that PARP-2 levels were influenced greatly by the presence or absence of serum implied that regulation was either at the level of the gene, the protein or both. To address whether or not serum had an effect on altering the activity of the PARP-2 gene, and specifically the SRE in its promoter, MCF-7 cells at 90% confluence were grown in complete or SF media for 30 minutes and then total RNA was extracted and used for cDNA synthesis. MCF-7 cells were chosen for this study since PARP-2 expression in these cells responded to serum in the same manner as in COS-7 cells (Fig. 3.3.1A), and unlike the monkey sequence the human PARP-2 sequence was available for PCR primer design. Endpoint PCR using these specific primers showed that PARP-2 mRNA was present regardless of serum being present or not (+ or - lanes). Immunoblot analysis of MCF-7 cells grown under the identical conditions as those used for the PCR analysis, showed PARP-2 signals in cells grown in complete media, but not in those that were serum-starved (Fig. 3.3.1D). Quantitative RT-PCR was used to confirm that serum starvation had no effect on the expression of the PARP-2 gene. Results showed that there were no significant differences in the expression levels of PARP-2 when cells were serum starved (Fig. 3.3.1C). Together these observations indicating that PARP-2 expression was not affected by
**Figure 3.3.1.** Loss of PARP-2 expression in serum-deprived cells (A) Immunoblots of protein lysates from IMCD, NIH3T3, COS-7, MCF-7 and HeLa cells probed with the H-250 PARP-1/2 and ERK antibodies. (B) COS-7 cells were grown in complete media until 90% confluent, and then the media was replaced with serum free media (Lanes 1 and 2) for 1 hour. Cells in lane 1 were lysed and those in lane 2 were cultured for an additional hour in complete medium. Cells grown for the entire time in complete media served as controls. Immunoblots were probed with antibodies against PARP-1/2 and ERK. (C) MCF-7 cells were grown in complete media until 90% confluent and then cultured for 30 min in either serum free media or maintained in complete media. RNA was extracted from each sample and endpoint RT-PCR was done with human PARP-2 specific primers. The + lane had reverse transcriptase added to the first strand reaction, and the - lane was the control for genomic contamination. (D) Proteins collected from MCF-7 cells cultured as in (C), were used for immunoblot analysis with antibodies against PARP-1/2 and ERK. CM, complete media. SF, serum free media. Unless stated otherwise, blots for this and other figures are representative of three independent experiments.
### A)

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### B)

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### D)

- **CM**
  - PARP-1
  - PARP-2
  - ERK

- **SF**
  - PARP-1
  - PARP-2
  - ERK

**qRT-PCR**

- **CM**
  - 1.5
  - 1.6
  - 1.7
  - 1.8
  - 1.9

**SF**

- 1.0

- **Relative Expression**
  - 0.0
  - 0.5
  - 1.0
  - 1.5
  - 2.0
  - 2.5

- Time Points:
  - CM: 15, 30, 60 minutes
  - SF: 15, 30, 60 minutes
serum deprivation brought into question the significance of the putative SRE. More importantly, these results strongly suggested that the decline in PARP-2 levels could be the consequence of the accelerated degradation of the protein itself or its sequestration to a detergent insoluble compartment following serum starvation.

3.3.2 PARP-2 IS A LONG-LIVED PROTEIN IN CELLS GROWN IN COMPLETE MEDIA

Since results indicated that PARP-2 might be a short-lived protein when cells were grown in serum free media, an *in silico* analysis was done to identify PEST sequences (mobyle.pasteur.fr), which are present in and responsible for the rapid turnover of many short-lived proteins (Belizario et al., 2008). When no putative sites were identified in PARP-2, COS-7 cells were grown in complete media to determine if there was a rapid turnover and replacement under these conditions. Cells were grown in the presence of cycloheximide (50µgml⁻¹) for 1-7 hr to inhibit protein synthesis, and then processed for immunoblot analysis to detect PARP-2 (Fig. 3.3.2A). Contrary to the rapid disappearance seen in serum free culture, PARP-2 appeared relatively stable over the 7 hr period. Similar results were seen for PARP-1 and ERK, which together would indicate that the rate by which PARP-2 gets downregulated was serum-dependent.

The loss of the 62kD PARP-2 band in cells cultured in SF media prompted us to undertake a more detailed investigation on the relationship between serum treatment and PARP-2 expression (Fig. 3.3.2B). COS-7 cells were grown in SF media or in media containing increasing amounts of serum. Immunoblot analysis showed a relatively similar staining pattern for full-length PARP-1 and its C-terminal fragment, regardless of whether serum was present or not (Fig. 3.3.2B). In contrast, increasing the serum concentration from 0 to 10% resulted in the concomitant increase
**Figure 3.3.2.** PARP-2 turnover under complete and serum free conditions (A) COS-7 cells were grown in complete media until 90% confluent. Cycloheximide (50 mg ml$^{-1}$) was then added (Lane 2, 3, 4 and 5), and cell lysates collected after 1, 3, 5 or 7 hours. Immunoblots were probed with antibodies against PARP-1/2 and ERK. (B) COS-7 cells were grown in serum free media or media containing increasing amounts of serum as indicated. Lysates were collected for immunoblot analysis with antibodies against PARP-1/2 and ERK. (C) COS-7 cells were grown in complete media until 90% confluent, and then the media was replaced with serum free media. Cells were harvested at 15 min intervals for 60 minutes and lysates collected for immunoblot analysis with antibodies against PARP-1/2 and ERK.
in the appearance of the 62kD band. Increasing the serum concentration had no apparent effect on ERK staining and approximately equal amounts of signal were seen in each lane. Together these results showed that PARP-2, but not PARP-1, levels increased in cells in response to serum. Furthermore, the evidence would suggest that this increase is the result of protein accumulation rather than increased gene activity.

Having determined that PARP-2 was regulated in a serum dependent manner, the next question was to address how fast the protein was downregulated when cells were deprived of serum. To determine this, COS-7 cells were grown in complete media for 24 hr and to approximately 90% confluence, and then the media was removed and replaced with SF media. Cell lysates were collected at 15, 30, 45 and 60 min and then processed for immunoblot analysis with the H-250 PARP antibody. Results showed that PARP-1 levels were unaffected by the serum conditions, and comparable signals were seen in all lanes (Fig. 3.3.2C). The opposite result, however, was seen with PARP-2, as signals were absent within 15 minutes after serum deprivation (Fig. 3.3.2C). This finding suggested serum starvation activated a rather efficient mechanism which could be either proteolytic degradation or sequestration to a detergent-insoluble fraction of PARP-2. Both could lead to the disappearance of the PARP-2 signal from the blot.

3.3.3 PARP-2 DOWNREGULATION FOLLOWING SERUM DEPRIVATION IS NOT MEDIATED BY PROTEASE

Since caspase-8 is known to cleave PARP-2 in apoptotic neurons in a murine model of acute ischemia (Benchoua et al., 2002), and caspase activation is seen in osteoblastic cells following serum deprivation (Mogi et al., 2004), this group of proteases was the first to be investigated.
COS-7 cells were treated with 40µM of either the caspase-8-specific inhibitor Z-IETD-FMK (IETD) or the broad-spectrum caspase inhibitor B-D-FMK (CI III), and whole cell lysates were collected for immunoblot analysis to look for changes in PARP-2 expression (Fig. 3.3.3A). PARP-1 levels in cells grown in serum free media and treated with either of the two inhibitors were comparable to those in cells grown in complete media. PARP-2 downregulation, however, was unaffected by the caspase inhibitors (Fig. 3.3.3A). These results, together with those seen in Fig. 1A, led us to dismiss the notion that PARP-2 downregulation in cells growing in serum free media is a caspase-dependent, apoptotic-related event.

Since the caspase inhibitors had no effect on preventing PARP-2 disappearance when cells were deprived of serum, the next step was to broaden the search for other proteases that might be involved in the process. Mouse embryonic fibroblast F20 (PARP-1+/+) cells were selected for these studies since the Yucatan PARP-2 antibody provides little to no consistent signal with human PARP-2, but is robust in detecting mouse PARP-2. Furthermore, and as described above, the expression of PARP-2 in these F20 cells responded to serum in the same manner as in COS-7 and MCF-7 cells. F20 cells were grown in complete media until 90% confluence and were pretreated for 5 hr with 20-250µM leupeptin, 20-30µM pepstatin A methyl ester (PME), 20-30µM calpeptin, or 50µgml⁻¹ α-2-macroglobulin, and then the media were replaced with serum free media containing the corresponding protease inhibitor. In another experiment, cells were treated with the 1x Halt Protease Inhibitor Cocktail (Pierce), which contains AEBSF-HCl, Aprotinin, Bestatin, E-64, Leupeptin, Pepstatin A and EDTA, and is optimized to inactivate a broad spectrum of endo- and exopeptidases. After fifteen minutes of incubation, cell lysates were collected and processed for immunoblot analysis with a Yucatan PARP-2 antibody. Results showed that the inhibitors, either alone or in a cocktail (Halt), were not effective in preventing
Figure 3.3.3. PARP-2 degradation is not affected by proteolytic inhibitors (A) COS-7 cells were grown in complete media until 90% confluent and then treated for 5 hr in: DMSO vehicle control, (Lanes 1 and 2), IETD (40µM, Lane 3), or Caspase Inhibitor III (40µM, Lane 4). Following treatment, the media was replaced with DMSO in complete (Lane 1) or serum free media (Lane 2), or serum free media containing IETD (Lane 3) or Caspase Inhibitor III (Lane 4). Cells were cultured for 15 minutes and then lysates collected for immunoblot analysis with antibodies against PARP-1/2 and ERK. (B) Mouse embryonic fibroblast F20 cells were grown in complete media until 90% confluent, and then for 5 hr in complete media containing Leupeptin (Leu, 250µm), pepstatin A methyl ester (PME, 30µM), calpeptin (Cal, 30µM), α2-macroglobulin (α2-M, 50µg/ml) or 1x Halt protease inhibitor cocktail containing 1.25µM EDTA. Following treatment, the media was replaced with serum free media containing the same protease inhibitors and cells were cultured for an additional 15 minutes. Cells continually grown in complete media served as controls. Cell lysates from all treatments were collected for immunoblot analysis with an antibody to PARP-2 (Enzo) or β-actin. CM, complete medium; SF, serum free medium; IETD, Z-IETD-FMK; CI III, B-D-FMK. The asterisk denotes non-specific staining.
PARP-2 downregulation (Fig. 3.3B). The data led us to conclude that under serum deprivation, PARP-2 was not being degraded by an amino, serine, cysteine, metallo- and aspartic acid proteases. Confounded by these unexpected results and after exhausting a broad spectrum of candidate proteases thought to be responsible for degrading PARP-2, our attention turned to the ubiquitin-proteasome system (UPS).

3.3.4 PARP-2 IS UBIQUITINATED

Since the UPS is involved in degrading PARP-1 (Masdehors et al., 2000; Wang et al., 2008), we considered the same might be true for PARP-2 and began by using confocal microscopy with HeLa cells transfected with pEGFP-PARP-2 and mRFP-ub to see if the proteins would co-localize at the level of this method. Results showed that PARP-2 was present in the nucleus, while Ub was present in the nucleus and cytoplasm (Fig. 3.3A). Although the co-localization of these proteins in the nucleus was only suggestive that PARP-2 was ubiquitinated, further analysis was necessary to provide conclusive evidence. An in vitro assay using human recombinant GST-PARP-2 and immunoblot analysis with a GST antibody showed a smear of higher molecular weight PARP-2 when proteasome Fraction II was present (lane 2, Fig. 3.3B). Omitting the ubiquitination enzymes served as a negative control (lane 1, Fig. 3.3B). That PARP-2 can be ubiquitinated in vitro prompted us to test if the same would occur in vivo.

To determine if PARP-2 could be ubiquitinated in vivo, COS-7 cells were co-transfected with pBC GST-PARP-2 and pMT123 HA-ubiquitin plasmids, and then grown in complete media. Cells transfected without pMT123 HA-ubiquitin served as a negative control. Cell lysates were collected and GST pull down assays were performed prior to immunoblot analysis with an anti-HA antibody. Results showed that this ectopically expressed mouse PARP-2 was ubiquitinated
Figure 3.3.4. PARP-2 association with ubiquitin (A) HeLa cells were transfected with pEGFP-PARP-2 and RFP-ubiquitin constructs and then grown in complete media for 24hr. Cells were fixed in 4% paraformaldehyde in PBS, mounted in ProLong Gold antifade medium, and viewed on a Zeiss LSM 510 confocal microscope. (B) Human GST-PARP-2 recombinant protein was incubated with Mg-ATP +/- Ubiquitin/Protein Fraction II and resolved on by SDS-PAGE, and then transferred to blots and probed with a GST-specific antibody. The input lane was protein from COS-7 cells overexpressing GST-PARP-2. (C) COS-7 cells were transfected with pBC GSTPARP-2 or pBC GST-NLS, with or without pMT123 HA-ubiquitin and then treated with MG-132 (40µM) or left untreated as a control. Cells were lysed in 1x RIPA and the lysate used in a GST-pull down assay. Proteins were electrophoresed and transferred to blots, which were probed with antibodies against HA or GST. The input lane was protein from COS-7 cells that were overexpressing GST-PARP-2 and HA-ubiquitin. The asterisk represents GST fragments from the recombinant proteins.
in vivo (lane 1, Fig. 3.3.4C), and the amount of ubiquitin (smear) accumulated following MG-132 treatment (lane 2, Fig. 3.3.4C). As expected, no HA-ubiquitin signal was detected in cells transfected with pBC GST-PARP-2 alone (lane 3, Fig. 3.3.4C). A simian virus 40 nuclear localization signal epitope tagged to GST served as a positive control and was also ubiquitinated (lane 4, Fig. 3.3.4C). Thus, when serum was present, exogenously expressed PARP-2 is ubiquitinated and despite being a relatively long-lived protein under these conditions, this post-translational modification may be the prerequisite for targeting to the proteasome.

3.3.5 Ro52 (TRIM21), A CANDIDATE E3 UBIQUITIN LIGASE INVOLVED IN PARP-2 UBIQUITINATION

Having shown PARP-2 was ubiquitinated in vitro and in vivo, the search began for the E3 ligase responsible for this post-translational modification. A previous interactome study had identified Ro52/TRIM21 as a candidate that interacts with PARP-1 and PARP-2 (Isabelle et al., 2010). Ro52 is a RING finger domain E3 ubiquitin ligase that is located primarily in the cytoplasm, but is also detected in the nucleus especially when it is ectopically expressed or when cells are under oxidative stress (Espinosa et al., 2008; Strandberg et al., 2008). To confirm that the PARP-2 and Ro52 interaction leads to the ubiquitination and degradation of PARP-2 through the UPS, we transiently transfected GST-PARP-2 with FLAG-Ro52 or FLAG-Ro52ΔRING in HeLa cells. The ΔRING construct is a truncated form of Ro52 lacking E3 ligase activity (Takahata et al., 2008). Cells were cultured with or without 20µM MG132 for 7 hours after transfection, and lysates collected for immunoblot analysis to confirm the expression of GST-PARP-2, FLAG-Ro52 and FLAG-Ro52ΔRING (data not shown). Having confirmed the expression of epitope-tagged proteins, samples were subjected to a pull down assay with
**Figure 3.3.5.** PARP-2 interacts with Ro52 E3 ligase. HeLa cells were transfected with plasmids encoding GST-PARP-2 (Lane 1), GST-PARP-2 and FLAG-Ro52 (Lane 2 and 3), or GST-PARP-2 and FLAG-Ro52ΔRING (Lane 4 and 5). After 24 hr, cells were grown for 7 hr in complete media (lanes 1, 2 and 4) or complete media containing 20µm MG-132 (lanes 3 and 5), and then lysed in 1x RIPA buffer. Lysates were collected and subjected to a GST-pull down assay (A). Isolated GST proteins were analyzed on immunoblots and an anti-FLAG antibody. The lower panel in (A) shows the presence of GST-PARP-2 in all lanes.
A)

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GST-Pull Down
IB: FLAG

IB: GST

B)

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Glutathione (GSH)-bound beads and then blotted for the epitope (Fig. 3.3.5A). Results showed that both forms of Ro52 had bound with GST-PARP-2. Inhibiting proteasome activity with MG-132 also resulted in a higher ratio of FLAG-Ro52/GST-PARP-2 being pulled down (Fig. 3.3.5B). MG-132, relative to the untreated, did not seem to have an effect on the amount of FLAG-Ro52ΔRING/GST-PARP-2 that was isolated (Fig. 3.3.5B). These results would indicate that the E3 ligase activity of Ro52 contributes to the ubiquitination of PARP-2.

3.3.6 PARP-2 IS DEGRADED BY THE PROTEASOME IN THE PRESENCE OF SERUM

The in vitro and in vivo ubiquitination results and the identification of a candidate E3 ubiquitin ligase, together with the results showing that the MG-132 proteasome inhibitor prevented ectopically expressed PARP-2 from degrading, strongly suggested that the UPS was the mechanism used to regulate endogenous PARP-2 levels in cells grown in serum free media. Unexpectedly, however, results showed that MG-132 had no effect on blocking PARP-2 degradation under these conditions (Fig. 3.3.6A). Likewise, MG-132 had no effect on altering the levels of PARP-1 or ERK, which all together brought into question our ubiquitination assays with the recombinant and ectopically expressed PARP-2. To address these observations COS-7 cells were then grown in complete media and treated with MG-132, and results from immunoblot analysis showed reduced PARP-2 degradation (Fig. 3.3.6B). In fact, PARP-2 levels increased with increasing concentrations of MG-132. A similar trend was seen with MCF-7 cells (data not shown). PARP-1 levels did not change appreciably after MG-132 treatment, which mimicked that seen under serum free conditions, possibly because of the low turnover rate of PARP-1. Together this data would indicate that when cells are grown in complete media, PARP-2 is
Figure 3.3.6. The UPS is involved in PARP-2 degradation when cells are grown in complete media (A) COS-7 cells were grown in complete media until 90% confluent and then transferred and grown for 5 hr in complete media containing MG-132 (45µM). Cells were then cultured for 15 minutes in serum free media containing MG-132, and lysates collected for immunoblot analysis with antibodies against PARP-1/2 and ERK. (B) COS-7 cells were grown in complete media for 24hr and then in serum free media (SF, Lane 1), complete media (CM, Lane 2), or CM containing different concentrations of MG-132 (Lane 3, 4 and 5). Cells were maintained for 24 hr under these conditions and then lysates were collected for immunoblot analysis with antibodies against PARP-1/2 and ERK.
ubiquitinated and degraded in the proteasome. The data also points to the fact that different mechanisms of PARP-2 downregulation exist and that these mechanisms are activated in a manner that depends on the conditions under which the cells are grown.

3.3.7 PARP-2 IS SEQUESTERED TO AN SDS-INSOLUBLE FRACTION AFTER SERUM DEPRIVATION

Having shown PARP-2 is degraded through the UPS in complete media and excluded the involvement of protease in its absence after serum withdrawal, we set out to find if PARP-2 was sequestered to an SDS-insoluble compartment following serum deprivation, which could lead to the disappearance of PARP-2 signal from the western blot when samples were prepared with Laemmli SDS sample buffer. We deduced if PARP-2 is degraded after serum withdrawal, its level would not resume as indicated in Figure 1B after serum is added to the media when protein synthesis is inhibited. To this end, MCF-7 cells were grown and treated as described in the Materials and Methods. Results from immunoblotting analysis with a PARP-2 antibody (Axxora) showed even though protein synthesis was inhibited by cycloheximide, serum-starved MCF-7 cells reacquired PARP-2 signal after grown in complete media for one hour (Fig. 3.3.7A, lane 5). The signal strength was almost identical to cells without cycloheximide treatment (Fig. 7A, lane 6). Cells transferred to serum free media with or without cycloheximide lost PARP-2 signal (Fig. 3.3.7A, lane 2 and 3). These results suggested serum starvation led to sequestration of PARP-2 to an SDS-insoluble compartment, which could recycle back to a soluble form once serum was present rather than proteolytic degradation. To further understand the capacity of this sequestration, we overexpressed GST-PARP-2 in MCF-7 cells and cultured the cells in complete media or serum free media for 15, 30 and 60 minutes when the cells have reached approximately
**Figure 3.3.7.** PARP-2 is sequestered to an SDS-insoluble fraction upon serum starvation (A) MCF-7 cells were grown in complete media until 90% confluent then media were replaced with serum free media containing 50µgml⁻¹ cycloheximide (lanes 2 and 5) or not (lanes 3 and 6). In lane 4, 50µgml⁻¹ cycloheximide was added to the complete media at the same time. After seven hour incubation, cells in lane 1, 2, 3 and 4 were lysed in 1x sample buffer, and serum free media were replaced with complete media containing 50µgml⁻¹ cycloheximide (lane 5) or not (lane 6). The cells were incubated for one hour until being lysed in 1x sample buffer. All samples were resolved with 10% SDS-PAGE and analyzed with antibodies against PARP-2 (Axxora) and ERK.

(B) MCF-7 cells were transfected with GST-PARP-2 plasmid. 24 hour after transfection, complete media were replaced with serum free media (lane 2, 3 and 4). After serum starved for 15, 30 or 60 minutes, cells were lysed in 1x sample buffer. Lysates were resolved with 10% SDS-PAGE and analyzed with antibodies against GST and ERK. The asterisk denotes non-specific staining.
90% confluence. Immunoblotting analysis with a GST antibody indicated the GST-PARP-2 signal was comparable between the cells grown in complete media and serum free media (Fig. 3.3.7B), suggesting the sequestration capacity was limited and most ectopically expressed PARP-2 was free from the SDS-insoluble compartment. The minor bands on the blot were likely the fragments from ubiquitin-mediated proteasomal degradation in complete media. Compared with the minor bands in the CM lane, these bands in SF lanes were not significantly enhanced, supporting the notion that serum withdrawal did not induce GST-PARP-2 degradation. The ERK staining showed approximately equal loading (Fig. 3.3.7B).

3.4 DISCUSSION

PARP enzymes were first identified as being key players in DNA repair (Jeggo, 1998) and this involves initiating the poly ADP-ribosylation polymerase activity of these enzymes (Barzilai and Yamamoto, 2004; Dantzer et al., 2006a; Huber et al., 2004). In addition to this crucial role, PARP proteins also serve many other roles including, but not limited to the regulation of gene activity, cell death, the immune system and cellular metabolism. Given this diversity, the mechanisms to control the level and activity of these enzymes must be tightly regulated. In the case of PARP-1 and PARP-2, basal activities are very low, while in response to DNA damage this changes rapidly (Bai and Canto, 2012; Krishnakumar and Kraus, 2010). At the level of the gene, the presence of a putative serum response element (SRE) in the PARP-2 promoter region (Ame et al., 2001), suggests that transcriptional activity may be influenced by growth factor and/or mitogen stimulation. To investigate this we grew different cell lines in the presence or absence of serum and assayed for PARP-2 expression. The loss of the PARP-2 protein in cells grown in serum free media (Fig. 3.3.1A), and its return when serum was replaced (Fig 3.3.1B),
suggested that the gene was serum responsive. Given this on-off response at the protein level, and the presence of the putative SRE, we had expected to see similar results in PARP-2 mRNA expression. This, however, was not the case and the presence of a PARP-2 amplicon in the serum free media and the fact that the q-RT-PCR results showed no significant differences in expression in cells grown under the different conditions, indicated that the regulation of PARP-2 in cells deprived of serum was at the level of the protein rather than direct regulation of the gene in the time frame of our investigation. Furthermore, the presence of PARP-2 mRNA under these conditions indicates that the message was available (Fig. 3.3.1C) but did not seem to contribute to the fast return (within one hour) of PARP-2 signal after serum was added (Fig. 3.3.1B), as evidenced by the fact that cycloheximide inhibition of protein synthesis had no effect on PARP-2 return in the course of serum deprivation and re-addition (Fig. 3.3.7A, lane 5).

Serum withdrawal in different cultured cells is known to activate many proteolytic, and proteolytic-related proteins including caspases, calpains, autophagy-related proteins, ubiquitin, and proteasome subunits (Fuertes et al., 2003; Kilic et al., 2002; Nakashima et al., 2005; Schamberger et al., 2005). Caspases, a family of enzymes that cleave a variety of cellular substrates leading to apoptosis, are activated in cells following serum deprivation (Mogi et al., 2004). This information and the fact that studies have shown that they can cleave PARP-2 (Benchoua et al., 2002), placed them at the forefront of our candidates responsible for the possible serum-dependent degradation noted in the different cell types (Fig. 3.3.1). Unfortunately, our analysis using a caspase-specific and a broad-spectrum caspase inhibitor (Fig. 3.3.4A) as well as a proteolytic inhibitor cocktail (Fig. 3.3.4B), ruled out the possibility that caspases were responsible for the proteolysis. Subsequent experiments were designed to explore the involvement of serine, cysteine, metallo- and aspartic proteases in PARP-2 degradation (Fig.
As with the caspase inhibitors, those routinely employed to prevent proteolysis failed to prevent PARP-2 from disappearing (Fig. 3.3.4B). Furthermore, the reports that the cysteine protease cathepsin L is present in the nucleus, like PARP-2, and is involved in cell-cycle progression (Puchi et al., 2010) prompted us to block its activity to see what effect it would have on endogenous PARP-2 levels in serum-deprived cells. Leupeptin, an inhibitor of endosomal trypsin-like serine and cysteine proteases (Simmons et al., 2005), had no effect, which ruled out cathepsin L. Blocking other candidate proteases had similar negative effects (Fig. 3.3.4B), suggesting the disappearance of PARP-2 signal may not be the result of proteolytic degradation but transformation to an SDS-insoluble form, which could be reversed upon addition of serum to the culture media.

The solubility in detergents of different constituents within a cell is different and is determined by the chemical properties of the substrates as well as the detergents. Some cellular entities are naturally resistant to extraction of detergents (Horigome et al., 2008; Takata et al., 2009), while other proteins may be converted to detergent-soluble or insoluble forms upon various stimulations (Peters et al., 2012; Reis-Rodrigues et al., 2012). Serum starvation has been shown to induce translocation of dynein to a more detergent soluble compartment in NRK cells and this change is reversed by addition of serum (Lin et al., 1994). Serum withdrawal also leads to sequestration of caspase-9 to detergent-insoluble cytoskeletal structures in rat 423-cells (Schamberger et al., 2005). In IM-9 cells, growth hormone (GH) induces detergent insolubility of GH receptors through the formation of cross-linked disulfide bonds (Goldsmith et al., 1997). These reports and the negative results from our protease inhibition experiments prompted us to investigate if serum deprivation could lead to the change of SDS solubility of PARP-2 in cells. By inhibiting protein synthesis during serum deprivation, we ruled out the possibility that PARP-
2 was degraded since PARP-2 restored to the normal level after the cells were grown in complete media for one hour in the presence of cycloheximide (Fig. 3.3.7A). The disappearance of PARP-2 from the blots following serum withdrawal apparently attributed to a transformation to an SDS-insoluble form. This transformation was reversed by addition of serum and was dose-dependent (Fig. 3.3.1B, Fig. 3.3.2B and Fig. 3.3.7A). It is interesting to note that despite the similarities between the structures of PARP-1 and PARP-2, PARP-1 solubility in SDS didn’t change following serum deprivation, indicating serum withdrawal activated a PARP-2-specific mechanism. PARP-1 changes its solubility in NP-40 when modified by sumoylation upon heat shock (Martin et al., 2009) and is found in lamin-enriched or DNA-bound detergent-resistant fractions (Frouin et al., 2003; Vidakovic et al., 2004). Several other proteins e.g. PCNA, p21, OGG1, XRCC1 and CAF-1 p150 are also present in the DNA-bound detergent-resistant fractions to regulate DNA replication and repair (Amouroux et al., 2010; Campalans et al., 2013; Frouin et al., 2003; Okano et al., 2003). Given one of the functions of PARP-2 is to repair DNA damage, its association with DNA could also result in detergent resistance like PARP-1. However, the association with DNA would only enhance its resistance to some nonionic detergents such as Triton and NP-40 but not the anionic detergent SDS. Therefore, despite the fact that serum starvation activates DNA damage response pathways in some cancer cells and induces DNA fragmentation in normal cells (Lu et al., 2008; Shi et al., 2012), it is unlikely that the transformation of PARP-2 to an SDS-insoluble form is through the binding with DNA lesions induced by serum starvation. Proteins transformed to SDS-insoluble forms have been reported in some neurodegenerative diseases. In the mouse model of Alzheimer’s disorder (AD), amyloid β protein (Aβ) changes to SDS-insoluble forms of Aβ42 and Aβ40 from 6-7 months (Kawarabayashi et al., 2001). In Huntington’s disease (HD), mutated Huntingtin with
polyglutamine [poly (Q)] repeat expansions in the first exon is resistant to SDS extraction (Heiser et al., 2000; Scherzinger et al., 1999). These conversions to SDS-insoluble forms are pathologic processes and may be the combinatorial effects of conformational change, oligomerization and fibril formation and appear irreversible in AD and HD patients (Cruz et al., 1997; Diaz-Hernandez et al., 2005; Dolev and Michaelson, 2004; Wong et al., 2008). However, the conversion of PARP-2 between soluble and insoluble forms under different serum conditions is reversible, suggesting it may adopt a physiological conformation in either condition. The accumulation of SDS-insoluble proteins in cells is also an indication of aging. In yeasts and nematodes, an assembly of SDS-insoluble proteins accumulates during aging (Peters et al., 2012; Reis-Rodrigues et al., 2012). Moreover, the accumulation of SDS-insoluble proteins can be induced by nitrogen starvation even in young yeast cells (Peters et al., 2012). Tor1 kinase plays a regulatory role in this accumulation which is deemed a novel autophagic cargo preparation process (Peters et al., 2012). However, we don’t think the transformation of PARP-2 to SDS-insoluble forms is to prepare PARP-2 for autophagic degradation since inhibiting lysosomal enzymes with leupeptin had no effects on PARP-2 level (Fig. 3.3.3B) and as a nuclear protein, it is unlikely PARP-2 is degraded by a cytosolic organelle. Besides association with chromatin and forming aggregates, cellular proteins change their detergent solubility when associating with glycosylphosphatidylinositol (GPI) enriched microdomain or cytoskeleton or induced conformational change by posttranslational modifications (Brown and Rose, 1992; Fujita et al., 2011; Ledesma et al., 1994; Paladino et al., 2002; Refolo et al., 1991; Waelter et al., 2001). These changes are physiological or pathophysiological responses to extra- or intracellular stimuli. How PARP-2 undergoes such changes and the physiological significance of this transformation in response to serum deprivation remain to be clarified and our investigation is on the way.
Since the in silico analysis did not detect PEST sequences that participate in the degradation of short-lived proteins (Sun and Kelly, 2010), two other motifs present in rapidly-turned over proteins were considered. The first was the lysosome-targeting KFERQ motif (Massey et al., 2006) and second, the R-X-X-L-X-X-X-N destruction (D) box (Glotzer et al., 1991). A sequence similar to the lysosome-targeting motif (KSERQ) is present in mouse PARP-2, however, the side-chain polarity of the serine residue conflicts with that of phenylalanine and was ruled out. The more promising candidate for the turnover of PARP-2 was the D-box, which is recognized by the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase activated by the WD-40 proteins Cdc20 and Cdh-1 (Qiao et al., 2010). PARP-1 is known to interact with several of the proteins in the APC/C (Isabelle et al., 2010), and while this complex is normally considered as being primarily involved in mitotic exit, several reports exist that in conjunction with Cdh-1, it is active in cells in G0 (Listovsky et al., 2004). Since, however, the interaction of PARP-2 with Ro52 and not APC/C was reported in the proteomic screen (Isabelle et al., 2010), our studies focused on Ro52/TRIM21 as the E3 ubiquitin ligase, responsible in part, for PARP-2 degradation.

Ro52, is a TRIM family member that translocates to the nucleus during autoimmune inflammation (Oke and Wahren-Herlenius, 2012), or when cells are treated with the oxidative stressor hydrogen peroxide (Nobuhara et al., 2007). Ro52-dependent ubiquitination occurs on a number of proteins including interferon regulatory factors (IRFs) (Bolland and Garcia-Sastre, 2009; Jonsson et al., 2011), IgG1(Takahata et al., 2008), and the p27 cyclin-dependent kinase inhibitor (Sabile et al., 2006), and our results would suggest PARP-2 is also a substrate (Fig. 3.3.5). Ubiquitination occurs in vitro (Fig. 3.3.4B) and in vivo (Fig. 3.3.4C), and it appears that the interaction between Ro52 and PARP-2 is independent of the RING domain on the former
(Fig. 3.3.5). Ultimately, this post-translational modification in cells grown under favourable conditions leads to PARP-2 degradation in the proteasome (Figs. 3.3.6B). While managing PARP-2 levels in this manner would ensure that excessive PARP activity would not result in energy depletion from increased NAD$^+$ consumption or accumulation of free poly-ADP-ribose polymers, causing cell death in a caspase-independent manner (Koh et al., 2004; Siegel and McCullough, 2011), it does not explain why PARP-2 are so dramatically transformed to an SDS-insoluble form when cells are depleted of serum.

The multiple roles assigned to PARP-2 suggest different regulators may be present in the places where it functions. The possible involvement of Ro52, a molecule with diverse functions, in the regulation of PARP-2 makes deciphering its role even more difficult. While the ubiquitination of the IRFs that regulate the expression of proinflammatory cytokines and type 1 interferon point to Ro52 in having a key role in the normal physiological immune response, that of p27 points to Ro52 being an important mediator of S-phase progression through the mammalian cell cycle (Sabile et al., 2006). Results from our study would indicate that Ro52 is not using PARP-2 to promote cell cycle progression when cells are grown in serum-depleted media. Instead, the increase in Ro52 activity leading to PARP-2 ubiquitination and degradation may mimic what is known for PARP-1, where its ubiquitination and degradation promotes cell cycle arrest (Kashima et al., 2012). Although it is not known whether PARP-2 is involved in cell cycle arrest, the failure of MG-132 to prevent the disappearance of the protein in serum-deprived cells (Fig. 3.3.7A), suggest it is not targeted for proteasomal degradation. Furthermore, serum depletion had no effect on PARP-1 levels, thus contradicting the CHFR and cell cycle arrest report (Kashima et al., 2012). Nevertheless, the fact that we did see PARP-2 ubiquitination when cells were grown in complete media (Fig. 3.3.4), and the results showing PARP-2 being a long-
lived protein (Fig. 3.3.2A), point to the gradual and continual turnover expected of an enzyme that would maintain steady levels available to regulate poly-ADP-ribosylation of proteins.

### 3.5 CONCLUSION

In summary, in the presence of serum, PARP-2 is continually degraded by the ubiquitin proteasome system, but levels remain relatively high since we have shown it to be a long-lived protein. In the case of cell stress, when serum is removed, PARP-2 is converted to an SDS-insoluble form and whether this is a prelude to apoptosis or to some other physiological requirement in the stress response is currently not known. Likewise, the means by which PARP-2 is transformed to SDS insoluble form also remains elusive. Nevertheless, alleviating this cellular stress by the addition of serum recycles PARP-2 to SDS-soluble form, thereby allowing the protein to resume its enzymatic role involved in the poly-ADP-ribosylation of target substrates.
3.6 LITERATURE CITED


CHAPTER 4 INVOLVEMENT OF Ro52/TRIM21 IN UBIQUITIN-MEDIATED SELECTIVE AUTOPHAGY

4.1 INTRODUCTION

Ro52/TRIM21 (Tripartite motif protein 21) is a ubiquitously expressed intracellular protein that contains a tripartite motif, the signature of TRIM proteins, which include a RING finger domain, a B-box domain and a coiled-coil (CC) region and a C-terminal B30.2/PRYSPRY domain. As an autoantigen Ro52 has clinical importance in several autoimmune diseases including Sjögren's syndrome (SS), cutaneous lupus erythematosus and systemic lupus erythematosus (SLE) (Oke and Wahren-Herlenius, 2012). Although autoantibodies against Ro52 in the sera often present in patients diagnosed with these diseases, the pathological processes of how an autoimmune reaction against Ro52 is initiated in these diseases is unknown. In mammalian cells, Ro52 is a component of Ro ribonucleoprotein (Ro RNP) complexes comprising Ro60, La autoantigens and small Y RNA (Slobbe et al., 1992). Through homomultimerization Ro52 forms rod-like structures called cytoplasmic bodies, which are excluded from mitochondria, endosomes, lysosomes and proteasosomes (Reymond et al., 2001). The function of these bodies is poorly understood, but it is known they are dynamic structures that are transported along the microtubule network (Rhodes et al., 2002; Tanaka et al., 2010; Wada et al., 2006). Though it is still not clear how Ro52 associates with microtubules it is well known that it, like several other TRIM proteins, has E3 ubiquitin ligase activity due to the presence of its Zn\(^{2+}\) binding RING finger (Wada and Kamitani, 2006a). As a result, Ro52 mediates polyubiquitination and degradation of interferon regulatory transcription factor (IRF) 3, 5, 7 and 8 and subsequent down-regulation of type 1 interferon and multiple cytokines after bacterial and
viral infection (Espinosa et al., 2009; Higgs et al., 2010; Higgs et al., 2008; Kim and Ozato, 2009; Yoshimi et al., 2009). In the case of IRF3 degradation and following stimulation of TLRs (Toll-like Receptors), the ligase activity of Ro52 is regulated by tyrosine phosphorylation at Y388 and Y393 within its B30.2 domain (Stacey et al., 2012). In addition to its role in polyubiquitination, Ro52 can monoubiquitinate proteins, including IKKβ (Wada et al., 2009), which leads to its translocation and degradation in the autophagosome/lysosome resulting in the down-regulation of NF-κB signaling (Niida et al., 2010). Furthermore, Ro52 participates in the formation of a Skp1, Skp2 and Cul1 complex to target the cyclin-dependent kinase inhibitor p27 for ubiquitin-mediated proteasomal degradation, leading to S-phase progression (Sabile et al., 2006). Although many proteins serve as substrates for Ro52, including UnpEL/Usp4 (Wada and Kamitani, 2006b), TRIM5α (Yamauchi et al., 2008) and Ro52 itself (Fukuda-Kamitani and Kamitani, 2002), the biological relevance for these interactions has not been defined. As noted above and in addition to the RING finger, the two other motifs present in Ro52 are the B-box domain and the coiled-coil region.

B-box domains also bind Zn$^{2+}$ and in the case of Ro52, the domains are separated by a ~ 40-residue linker peptide to the N-terminal RING (Hennig et al., 2008). The authors of this study have reported that this RING-B-box linker not only appears to confer functional and structural importance to Ro52, but it is also recognized by autoantibodies from patients with rheumatic disease. In the case of other TRIM proteins, including TRIM5α and TRIM63/MuRF1, the B-box domains may mediate self-association (Diaz-Griffero et al., 2009; Li and Sodroski, 2008; Mrosek et al., 2008). Replacing TRIM5α’s B-box domain and flanking linker regions with Ro52’s abolishes the HIV-1 restriction capability of TRIM5α (Li et al., 2006). While homomultimerization of TRIM proteins facilitated by the coiled-coil region (Reymond et al, 2001)
plays an integral role in the cytoplasmic localization of Ro52 (Espinosa et al., 2008), the same region is responsible for the hetero-dimerization to the apoptosis-related protein FLASH (Tanaka and Kamitani, 2010). These authors report that the C-terminal B30.2 domain of Ro52 also binds to Daax, another apoptosis-related protein, and together with FLASH the complex relocates to the cytoplasm. The B30.2 domain also possesses a high affinity for IgG (Rhodes and Trowsdale, 2007). The latter is significant in regards to intracellular immunity following infection, whereby antibody-bound viruses are targeted for proteasomal degradation (James et al., 2007; Keeble et al., 2008; Mallery et al., 2010; McEwan et al., 2012). Other functions of the B30.2 domain of Ro52 include its binding to and proteasomal targeting of IRF3, IRF7 and IRF8 (Higgs and Jefferies, 2008; Higgs et al., 2010; Kong et al., 2007), and its requirement for the translocation of Ro52 into the nucleus following exposure to the inflammatory mediator nitric oxide (Espinosa et al., 2008).

As noted above the autoantigenicity of Ro52 has been investigated extensively due to its clinical significance pertaining to several autoimmune diseases. Antibodies that develop against different epitopes of Ro52 have distinct outcomes towards the pathogenesis of these diseases. For instance those specific to amino acids 200-239 within the coiled-coil region induce congenital heart block in rats (Ambrosi et al., 2012), whereas antibodies against the RING domain inhibit E3 ligase activity (Espinosa et al., 2011). Both linear and conformational epitopes within Ro52 have been proposed, and this is evident in Sjögren's syndrome patients where although the coiled-coil domain posts more antigenicity than the B-box, RING or B30.2 domain, it is still less than that posted by the full-length Ro52 polypeptide (Burbelo et al., 2010). Another study has reported that the RING motif and linker region to a B-box is the conformation-dependent antigenic epitope in SS patients (Hennig et al., 2008). In addition to the autoimmunity
issue, these patients experience dry mouth and eyes due to lymphocytic infiltration of the salivary and lachrymal glands (Sandilands et al., 2012). Major changes to normal architectural salivary organization occurs as a result of this infiltration (Barrera et al., 2013), and in an animal model the atrophy of the salivary gland was accompanied by the activation of the mammalian target of rapamycin (mTOR) autophagy and appearance of autophagy-related proteins (Silver et al., 2010).

Autophagy is a catabolic process during which a portion of cytoplasm or an aberrant organelle is enclosed by a double membrane to form an autophagosome (Feng et al., 2014; Shibutani and Yoshimori, 2014). The autophagosome eventually fuses with the lysosome and the content is hydrolyzed by lysosomal proteases and the products recycled. This self-eating process is conserved from yeast to human and is usually initiated to cope with adverse conditions such as nutrient deprivation and oxidative stress. So far more than 35 proteins have been identified to be autophagy-related (Zirin et al., 2013). However, only one protein Atg8/LC3 is found to associate with the autophagosome membrane throughout the process and is therefore used as the unique marker (Kabeya et al., 2000). Studies in yeast have revealed part of the molecular mechanisms underlying autophagy (Yang and Klionsky, 2010). During canonical autophagy, environmental stresses are transduced through inactivating mTOR, resulting in the formation of Atg1/ULK1-Atg13 complexes, which are a part of a larger protein complex required for induction of autophagy (Yang and Klionsky, 2010). Subsequent nucleation of the isolation membrane is mediated through a class III phosphatidylinositol 3-kinase complex. Following nucleation of vesicles, two ubiquitination-like conjugation reactions lead to the expansion of the autophagosome membranes. One is the conjugation of the ubiquitin-like molecule Atg8 to its receptor phosphatidylethanolamine (PE) by Atg7, an E1-like enzyme, and Atg3, an E2-like
enzyme. Binding of Atg8-PE to the autophagosome depends on a second series of reactions, including the conjugation of Atg12, a ubiquitin-like molecule, to Atg5 by Atg7 (E1-like enzyme) and Atg10 (E2-like enzyme). Following the closure of the autophagosome, its outer membrane fuses with the lysosome to form an autolysosome and the inner membrane and contents are degraded by hydrolases. Through tubulization and budding, the remaining autolysosomes will regenerate lysosomes to maintain cellular homeostasis (Rong et al., 2012; Rong et al., 2011; Yu et al., 2010). Although autophagy has long been regarded as a bulk degradation mechanism, accumulating evidence has demonstrated that under nutrient-rich conditions, a specific selection of cargo is targeted to the autophagosome. This type of autophagy is since termed selective autophagy (Yorimitsu and Klionsky, 2005). Despite non-selective and selective autophagy both using common machinery to degrade their cargos, selective autophagy is facilitated by a group of autophagy receptor proteins including p62 and NBR1, which are able to bind both ubiquitinated proteins and autophagosome membrane-associated LC3. As autophagy is involved in many aspects of cell physiology, dysregulated autophagy is correlated to developmental defects, tumorigenesis and neurodegenerative disease (Chen and Klionsky, 2011; Jiang and Mizushima, 2014).

As noted above, studies have shown that Ro52-mediated suppression of NF-κB signaling is the result of the downregulation of active IKK beta through autophagy (Niida et al., 2010). This led us to test whether or not Ro52 is an autophagy-related protein. In this study we present evidence that Ro52 overexpression leads to the formation of autophagosomes in COS-7 and HeLa cells. Furthermore, the specific targeting of ubiquitinated proteins into these autophagosomes suggests the selective nature of this process. Although Ro52 binds to β-actin and α-tubulin, depolymerization of actin cytoskeleton but not the microtubule network, disrupts
Ro52-driven autophagosome formation. In addition, GFP-actin overexpression results in the up-regulation of endogenous Ro52 and LC3B-II. Overexpressing Ro52 by itself is also sufficient to cause the increased lipidation of LC3B. Finally, our results show that Ro52 is not associated with autophagosomes in basal and starvation-induced autophagy at physiological concentrations. Together, the data strongly suggests that Ro52 is an autophagy-related protein, but only when its levels are increased.

4.2 MATERIALS AND METHODS

4.2.1 ANTIBODIES, PLASMIDS AND REAGENTS

Anti-Ro52 (D-12) and anti-actin (C4) mouse monoclonal antibodies were purchased from Santa Cruz Biotechnology. Anti-LC3A/B (4108) antibody was purchased from Cell Signaling and anti-LC3B (NB100-2220) from Novus. An anti-tubulin antibody (Sigma, B512) was provided by Dr. Jim Karagiannis (Western University, Canada). Anti-FLAG (A00170), anti-GFP (A01704) and anti-RFP (A00682) antibodies were purchased from GenScript. HRP-conjugated secondary antibodies were purchased from Pierce. Fluorescent dye-conjugated secondary antibodies were from Invitrogen and Pierce. Plasmids p3xFLAG-Ro52 and p3xFLAG-Ro52ΔRING plasmids were kindly provided by Dr. Shigetsugu Hatakeyama (Hokkaido University, Japan) and pEGFP-p62 was a gift from Dr. Terje Johansen (University of Tromsø, Norway). pEGFP-actin plasmid was purchased from Clonetech. YFP-LC3 (Addgene plasmid #24989), mCherry-tubulin (Addgene plasmid #26767), LAMP-1-RFP (Addgene plasmid #1817), GFP-Ub (Addgene plasmid #11928), GFP-Ub KO (Addgene plasmid #11934) and mRFP-ub (Addgene plasmid #11935) plasmids were gifts from Drs. Isei Tanida, Torsten Wittmann, Walther Mothes and Nico Dantuma, respectively. Rhodamine-phalloidin (Invitrogen) was kindly
provided by Dr. Alexander Timoshenko (Western University, Canada). Protein A/G magnetic beads were purchased from Pierce. Chloroquine was from Tocris Bioscience, EBSS from Hyclone, and the XtremeGene 9 transfection reagent from Roche.

4.2.2 CELL CULTURE, TRANSFECTION AND TREATMENT

COS-7 and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% FBS (Gibco), 100 units/ml penicillin and 100 mg/ml streptomycin (Lonza) in 5% CO2 humid atmosphere at 37°C. Transfections were performed according to the manufacturer’s recommendation when cells were about 60-70% confluent. Cells were treated with chemicals as indicated in their respective figure legends.

4.2.3 IMMUNOSTAINING AND CONFOCAL MICROSCOPY

Since Ro52 has high affinity to the Fc region of antibodies (Rhodes and Trowsdale, 2007), it could pose a problem in the double and triple immunostaining procedures. Our preliminary experiments demonstrated that Ro52 cross-reacted with non-Ro52 antibodies during immunofluorescent staining, but not on immunoblots (unpublished data). Thus, we co-transfected cells with plasmids expressing proteins with either a fluorescent protein tag or with FLAG. COS-7 and HeLa cells grown on glass coverslips were transfected with the plasmids as indicated. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde/PBS for 30 min at room temperature. After three 10 min washes with PBS, cells were permeabilized with 0.1% Triton X-100/PBS for 10 minutes and blocked in 3% BSA/PBS for 1 hour. Anti-FLAG or anti-Ro52 antibodies diluted at 1:50-1:100 in blocking buffer were applied to cells and the incubation was carried out at room temperature for 2 hours. Cells were washed
three times 5 minutes each with PBS and then incubated with fluorescent dye conjugated secondary antibodies at room temperature for 1.5 hours. After three 5 minutes washes with PBS, the cover slips were mounted with ProLong Gold anti-fade mounting medium (Invitrogen) and viewed with Zeiss LSM 510 Duo Vario confocal microscope.

In the experiments where the endogenous proteins were co-stained with FLAG-Ro52 or FALG-Ro52ΔRING, an extra step was performed following blocking with 3% BSA/PBS. Briefly, cells were blocked for an additional hour at room temperature in normal serum of mouse or rabbit origin, which is different from the origin of the primary antibody to the endogenous proteins. Rhodamin-Phalloidin staining was performed after the secondary antibody staining for 30 minutes at room temperature.

4.2.4 IMMUNOBLOTTING

Cells were washed in PBS and then lysed on ice in 1x sample buffer supplemented with protease inhibitor cocktail (Roche). Lysates were sonicated for 10 seconds and boiled for 5 minutes, then centrifuged for 10 minutes at 18,500g at 4°C. Protein concentrations were determined by a Bradford assay. Equal amount of proteins were resolved by 8% or 10% SDS-PAGE and then transferred to nitrocellulose membranes, which were then blocked in 5% skim milk/Tris-buffered saline/Tween 20 (TBS/T) buffer for 2 hours at room temperature. Following an overnight incubation in primary antibody (1:2500 diluted in blocking buffer) at 4°C, membranes were washed in TBS/T buffer and then incubated one hour with an HRP-conjugated secondary antibody (1:4000 diluted in blocking buffer) at room temperature. Membranes were washed extensively with TBS/T buffer and signals were detected using an enhanced chemiluminescence substrate kit (Pierce).
4.2.5 CO-IMMUNOPRECIPITATION

HeLa cells transiently expressing FLAG-Ro52 and YFP-LC3, LAMP-1-RFP, GFP-actin or mCherry-tubulin, were lysed on ice with 1x radioimmune precipitation assay (RIPA) buffer (pH 7.56) containing protease inhibitor cocktail. The cell lysates were centrifuged at 18,500g for 15 minutes at 4°C to pellet cell debris. Anti-FLAG antibodies (approx. 4 μg) were added to the supernatant in each Eppendorf tube and incubation was carried out at 4°C overnight with rotation. Next morning, 20μl of magnetic protein A/G beads were added to each tube and incubated for 5 hours at 4°C with rotation. Beads were pelleted and washed 4 times with TBS/T buffer, and then resuspended in 1x sample buffer containing protease inhibitor cocktail. The samples were stored at -20°C for later immunoblot analysis.

4.3 RESULTS

4.3.1 ECTOPICALLY EXPRESSED Ro52 IS INVOLVED IN THE FORMATION OF AUTOPHAGOSOME-LIKE STRUCTURES

Several studies report that Ro52 assembles into dot- or rod-like structures called cytoplasmic bodies (Niida et al., 2010; Reymond et al., 2001; Rhodes et al., 2002; Tanaka and Kamitani, 2010; Tanaka et al., 2010). The mechanism for this assembly is unknown, but evidence indicates that these bodies do not colocalize with mitochondria, endosomes, lysosomes or proteasomes (Rhodes et al., 2002; Tanaka et al., 2010). The size and shape of these cytoplasmic bodies, however, suggest they are complexes formed by protein aggregation or association with highly organized compartments such as the intracellular membrane system. To further investigate these Ro52 cytoplasmic bodies, we transiently expressed an epitope tagged Ro52 plasmid (FLAG-
Ro52) in COS-7 cells and HeLa cells (data not shown). Following indirect immunofluorescent staining using antibodies against the FLAG tag or Ro52 (data not shown) and confocal microscopy analysis, we identified the formation of distinct structures, resembling what appears to be a series of sequential steps resembling different stages of autophagosome formation (Fig 4.3.1A). The staining pattern in various cells changes from relatively small densely packed particles throughout the cytoplasm, to much larger, vacuolated vesicles, which appeared to coalesce into an even larger, filamentous network (Fig 4.3.1A). Based on the staining pattern of these FLAG-Ro52-positive structures, we assigned each of them a number representing the progression in the formation of this filamentous network. At stage 1, FLAG-Ro52 staining was diffuse throughout cytoplasm in the form of puncta and rods (Fig. 4.3.1A-1). In cells where there were few small puncta, the FLAG-Ro52 signal appeared in large round (arrows) and much smaller rod-like structures (Fig. 4.3.1A2; stage 2). These round structures were nearly flat and uniformly stained as evident by Z-stack analysis of confocal images (data not shown). Cup-like vesicles, believed to have formed from these densely stained structures (arrows, Fig. 4.3.1A-3, stage 3), acquired a distinct circular form (Fig. 4.3.1A-4, stage 4). Small spikes first appeared protruding from these circular vesicles (Fig. 4.3.1A-5, arrows; stage 5), but then elongated in what appeared to be the concomitant coalescence of different vesicles. Eventually, the distinct circular nature of these vesicles was lost (Fig. 4.3.1A-6, arrows; stage 6) and staining revealed an extensive network of filamentous structures (Fig. 4.3.1A-7; stage 7).

The appearance of these Ro52-positive vesicles, culminating in a filamentous network throughout the cytoplasm was reminiscent of the staining patterns reported for LC3, a known marker of autophagy (Kabeya et al., 2000). To address whether or not the staining of the ectopically expressed Ro52 protein was highlighting autophagic structures, nutrient-deprived
Figure 4.3.1: Formation of autophagosome-like structures by Ro52. (A) COS-7 cells were transiently transfected with FLAG-Ro52 construct for 24 hours then fixed and stained with anti-FLAG antibody and TRITC-conjugated secondary antibody. The cells were viewed under confocal microscope. Different stages of autophagosome-like structures were identified (arrow). Scale bar=4µm. (B) HeLa cells grown in EBSS with or without 20µM chloroquine (CHQ) were stained with anti-LC3B antibody and TRITC-conjugated secondary antibody and examined under confocal microscope. Different stages of autophagosome were shown (arrow). Scale bar=3µm. (C) COS-7 cells transiently transfected with FLAG-Ro52ΔRING construct for 24 hours were stained as in (A) and examined under confocal microscope. The circular structures formed by FLAG-Ro52ΔRING fused to form large circles. Scale bar=4µm.
(EBSS) HeLa cells were treated with or without chloroquine (CHQ), a drug that inhibits lysosome function rather than autophagosome formation (Shintani and Klionsky, 2004; Yoon et al., 2010). LC3B staining, possibly denoting the double-membrane phagophore sacs associated with autophagy-related proteins at the phagophore assembly site (Xie and Klionsky, 2007) in cells (Fig. 4.3.1B-1), was similar to the homogenous FLAG-Ro52 pattern designated as Stage 1, prior to or at the onset of autophagy (Fig. 4.3.1A-1). Compared to the untreated group, however, punctate LC3B staining was more intense when cells were treated with CHQ (Fig. 4.3.1B-2). Cup-like structures representing expanding phagophores marked by the LC3B staining (Fig. 4.3.1B-3; arrow) were similar to those stained by the anti-FLAG antibody (Fig. 4.3.1A-3). Likewise, the positive-LC3B stained circular vesicles (Fig. 4.3.1B-4, arrows), representing the autophagosome (Kabeya et al., 2000), were similar to those stained positively with the FLAG antibody described above (Fig. 4.3.1A-4). Finally, LC3B stained rods, representing lysosome reformation tubules generated from the autolysosomes after autophagosome and lysosome fusion, appeared in nutrient deprived cells (Fig. 4.3.1B-5), but were not present when cells were treated with CHQ. These tubules were similar to the filamentous structures stained positively with the FLAG antibody (Fig. 4.3.1A-7). Although the formation of tubules emanating from the autolysosomes was not seen, it is documented in a previous report (Rong et al., 2012).

As noted earlier, one of the prominent features of Ro52 is its E3 ligase activity. To test whether or not the RING finger domain and E3 ligase activity of Ro52 are required for the formation of autophagosome-like structures, we transiently expressed a RING domain-truncated plasmid (FLAG-Ro52ΔRING) in COS-7 cells and HeLa cells (data not shown). Chemically fixed cells stained with an antibody to detect the FLAG epitope showed staining patterns similar to those in cells expressing full length Ro52 (Fig. 4.3.1C). Thus, and although it would appear
that the RING domain interaction and E3 ligase activity are not necessary in the formation of the autophagosome-like structures, there were obvious differences between the development and morphology of FLAG-Ro52- and FLAG-Ro52ΔRING-positive structures. Firstly, no discernible cytoplasmic bodies at any stages were seen in cells expressing the FLAG-Ro52ΔRING construct. Secondly, it appeared that the autophagosome-like structures formed in FLAG-Ro52ΔRING expressing cells were more round and with smoother, thinner walls (Fig. 4.3.1C-2) than their FLAG-Ro52 counterparts (Fig. 4.3.1A-4). Thirdly, the fusion of these circular structures, prevalent in FLAG-Ro52ΔRING-expressing cells, resulted in the formation of relatively large vesicles (Fig. 4.3.1C-3). Finally, FLAG-Ro52ΔRING-expressing cells appeared to have been unable to form tubules from the circular-structures as the formation of the extensive network described above (Fig. 4.3.1A-5-7) was never seen in these cells.

4.3.2 LC3 CO-LOCALIZES WITH Ro52-POSITIVE STRUCTURES AND INTERACTS WITH Ro52 IN A RING-INDEPENDENT MANNER

A hallmark feature of the autophagosome is the association of the LC3 protein to the double membrane of the structure (Kabeya et al., 2000). Superficially, the LC3 and Ro52 staining patterns appear similar, which prompted us to determine if LC3 localizes to the FLAG-Ro52-positive structures. To test this HeLa cells were transiently transfected with FLAG-Ro52 and YFP-LC3 constructs and then fixed and immunostained with an anti-FLAG antibody for confocal microscopy analysis. Results show YFP-LC3 co-localized with FLAG-Ro52-positive structures, with labeling conspicuously in circular puncta (Fig. 4.3.2A; arrow). Since deleting the RING domain of Ro52 had no apparent effect on the formation of these circular structures (Fig. 4.3.1C), we predicted that the localization of LC3 to these structures should not be influenced if
in fact they are autophagosomes. That YFP-LC3 co-localized with FLAG-Ro52ΔRING in HeLa cells transiently expressing these constructs (Fig. 4.3.2A, lower panels), was evidence for Ro52 as a component of the autophagosome.

Having shown that ectopically expressed YFP-LC3 localized to Ro52-positive structures, we next tested if the endogenous LC3 protein would also localize to the Ro52-positive structures. HeLa cells transiently transfected with either FLAG-Ro52 or FLAG-Ro52ΔRING plasmids were fixed and stained with FLAG and LC3 antibodies prior to confocal microscopy analysis. Results show that endogenous LC3 staining co-localized to the Ro52- and Ro52ΔRING-positive structures (Fig. 4.3.2A), and a co-IP assay was used to determine if this was a direct interaction. Lysates of HeLa cells transiently expressing YFP-LC3 and FLAG-Ro52 were collected and Ro52 was immunoprecipitated using the FLAG antibody (Fig. 4.3.2B). Subsequent immunoblot analysis showed that YFP-LC3 was pulled down with FLAG-Ro52 (Fig. 4.3.2B). Endogenous LC3, however, was not detected in these pull-down experiments. That FLAG-Ro52 did not appear in the whole cell lysate lane in the reprobed blot, would suggest that this amount of lysate (4%) comprises only a small fraction of the ectopically expressed protein, and one that is below the detection limit of the antibody (Fig. 4.3.2B, bottom panel). Taken together, these results would suggest that the Ro52-positive structures are autophagosomes and that an interaction between the autophagosome marker LC3 and Ro52 are independent of the RING domain in the latter protein.

4.3.3 Ro52-POSITIVE STRUCTURES FUSE WITH LYSOSOMES

The lysosome-associated membrane protein-1 (LAMP-1) is involved in maintaining lysosomal structure and functions in the lysosomal degradation of autophagic vacuoles
**Figure 4.3.2:** Ro52-positive structures colocalize with autophagosome marker LC3. (A) HeLa cells transfected with YFP-LC3 and FLAG-Ro52 or FLAG-Ro52ΔRING plasmids were stained with anti-FLAG antibody and Dylight 405-conjugated secondary antibody and examined by confocal microscopy. YFP-LC3 colocalized with Ro52-positive structures (arrow, upper two panels). HeLa cells transfected with FLAG-Ro52 or FLAG-Ro52ΔRING were stained with anti-FLAG and anti-LC3A/B antibodies and Dylight 405-conjugated and TRITC-conjugated secondary antibodies, respectively, and imaged. Endogenous LC3 colocalized with Ro52-positive structures (lower two panels). Scale bar=6µm. (B) HeLa cells transfected with FLAG-Ro52 and YFP-LC3 plasmids were lysed in RIPA buffer and subjected to co-IP assay with anti-FLAG antibody and then immunoblotted with anti-LC3A/B antibody. 4% whole cell lysates (WCL) was used as the input. The blot was reprobed with anti-FLAG antibody. FLAG-Ro52 was not shown in the input lane due to the small amount of lysate used. Note that YFP-LC3 was post-translationally modified as indicated by the higher molecular bands.
(Eskelinen, 2006). To confirm that the Ro52-positive structures are autophagosomes and are able to fuse with lysosomes to form autolysosomes, we transiently expressed FLAG-Ro52 and LAMP-1-RFP in HeLa cells. Following fixation, cells were labeled with an anti-FLAG antibody and the secondary antibody conjugated with Alexa Fluor 488. Results from the confocal microscopy analysis showed LAMP-1-RFP co-expression largely decreased the number of the cells containing distinct FLAG-Ro52-positive structures (Fig. 4.3.3, top panels). In these cases, FLAG-Ro52 staining was relatively homogenous throughout the cell. LAMP-1-RFP, however, co-localized with FLAG-Ro52 in cells that exhibited Ro52-positive structures (Fig. 4.3.3 arrow, middle panels). Similarly, in HeLa cells transiently expressing FLAG-Ro52ΔRING and LAMP-1-RFP (Fig. 4.3.3 arrow, lower panels), the number of the circular structures formed was less than in cells only transfected with the FLAG-Ro52ΔRING plasmid. Although FLAG-Ro52 or FLAG-Ro52ΔRING and LAMP-1-RFP co-localized at these structures, the co-IP experiments didn’t confirm a protein-protein interaction (data not shown). These results would suggest that Ro52 or Ro52ΔRING and LAMP-1 only reside in close proximity. Nevertheless, these observations would indicate the Ro52-positive structures fuse with the lysosomes, possibly facilitated by the increased LAMP-1 levels, which would accelerate autophagic degradation.

4.3.4 Ro52-POSITIVE STRUCTURES AND THE ACTIN CYTOSKELETON

Actin microfilaments play a key role in selective autophagy in yeast and mammals (Lee et al., 2010; Reggiori et al., 2005), and our results would indicate that Ro52 is also involved. As noted above, Ro52 associates with the microtubule network (Tanaka et al., 2010), but no report has documented a link between Ro52 and actin. To address this COS-7 cells transiently expressing FLAG-Ro52 and GFP-actin were fixed and stained with an anti-FLAG antibody and a secondary
**Figure 4.3.3:** Ro52-positive structures colocalized with lysosome marker LAMP-1. HeLa cells transfected with LAMP-1-RFP and FLAG-Ro52 or FLAG-Ro52ΔRING were stained with anti-FLAG antibody and Alexa Fluor 488-conjugated secondary antibody and examined by confocal microscopy. LAMP-1 overexpression disrupted Ro52-positive structure formation in some cells (top panels) but colocalized with remaining Ro52-positive structures in other cells (arrow, middle and bottom panels). Scale bar=6µm.
antibody conjugated with TRITC, and then examined by confocal microscopy (Fig. 4.3.4). Results show that GFP-actin not only co-localized with the Ro52-positive structures, but was also enclosed in these structures (Fig. 4.3.4A, top panel). COS-7 cells transiently expressing FLAG-Ro52ΔRING and GFP-actin also displayed GFP staining within these circular structures (Fig. 4.3.4A, middle panel). Staining for endogenous actin was also performed to determine if the co-localization to and internalization of GFP-actin in the Ro52-positive structures was an artifact due to the overexpression. COS-7 cells transfected with FLAG-Ro52 or FLAG-Ro52ΔRING plasmids were labeled with an anti-FLAG antibody and a Dylight405-conjugated secondary antibody, and then stained with rhodamin-phalloidin to detect filamentous (F-) actin (Fig. 4.3.4A, lower two panels). Confocal microscopy analysis showed F-actin co-localized with FLAG-Ro52 and FLAG-Ro52ΔRING-positive structures, but it did not appear that the Ro52-positive structures contained F-actin. Co-IP assays were then performed to test for a bona fide interaction between F-actin and FLAG-Ro52. Lysates from HeLa cells transfected with plasmids encoding FLAG-Ro52 and GFP-actin were collected and proteins immunoprecipitated using a FLAG antibody. Subsequent immunoblot analysis with an anti-GFP antibody showed that actin had been pulled down with FLAG-Ro52 (Fig. 4.3.4B).

Since GFP-actin or endogenous actin associates with FLAG-Ro52, we asked whether an intact actin cytoskeleton was required for the formation of Ro52-positive structures. COS-7 cells were transfected with FLAG-Ro52 or FLAG-Ro52ΔRING and GFP-actin plasmids and 24 hours later they were treated with 20μM cytochalasin B for 5 hours to depolymerize F-actin. As shown in Figure 4.3.4C, disrupting the actin cytoskeleton had no affect on the co-localization of GFP-actin with FLAG-Ro52 or FALG-Ro52ΔRING (top and middle panels). More importantly,
Figure 4.3.4: Actin cytoskeleton affects Ro52-positive structure formation and LC3 lipidation. (A) COS-7 cells were transfected with FLAG-Ro52 or FLAG-Ro52ΔRING with or without GFP-actin plasmids then stained with anti-FLAG antibody and TRITC-conjugated or Dylight 405-conjugated secondary antibody and rhodamine-phalloidin for endogenous actin then imaged. GFP-actin (upper two panels) or actin (lower two panels) colocalized with Ro52-positive structures (arrow). Scale bar=10µm. (B) HeLa cells transfected with FLAG-Ro52 and GFP-actin plasmids were subjected to co-IP assay with anti-FLAG antibody then immunoblotted with anti-GFP antibody. GFP-actin was pulled down with FLAG-Ro52. 4% whole cell lysate (WCL) were used as input. FLAG-Ro52 was not shown in the input lane due to small amount of lysate used. Note that GFP-actin was post-translationally modified as indicated by the higher molecular weight bands. (C) COS-7 cells transfected with FLAG-Ro52 or FLAG-Ro52ΔRING with or without GFP-actin were treated with 20µM cytochalasin B for 5 hours then stained as in (A). GFP-actin (upper two panels) or actin (lower two panels) colocalized with Ro52 although Ro52-positive structures were disrupted by actin depolymerization with cytochalasin B. Scale bar=10µm. (D) HeLa cells transiently expressing GFP-actin were analyzed by immunoblotting with the antibodies shown. Endogenous Ro52 and LC3B-II were upregulated by GFP-actin overexpression. (E) HeLa cells transfected with FLAG-Ro52 plasmid were analyzed by immunoblotting with the antibodies shown. Endogenous LC3B-II was upregulated by FLAG-Ro52 overexpression.
however, was the noticeable absence of Ro52-positive structures. Similar results were obtained from COS-7 cells transfected with FLAG-Ro52 or FLAG-Ro52ΔRING plasmids and stained with an anti-FLAG antibody and rhodamine-phalloidin (Fig. 4.3.4C, lower and bottom panels). Together, these results would suggest that an intact actin cytoskeletal network is important in the formation and possibly maintenance of Ro52-positive structures.

The involvement of microfilaments and autophagy noted above, and the identified relationship between the actin cytoskeleton and Ro52-positive structures, was an invitation to determine if altering actin levels would affect Ro52 and autophagy. Towards that end, immunoblot analysis was used to compare endogenous Ro52 and LC3 levels in HeLa cells with those in HeLa cells expressing GFP-actin. It is interesting to note that the overexpression of actin, as evident on the reprobed blot using the anti-GFP antibody (Fig. 4.3.4D, lower panel), resulted in an increase of Ro52 and lipidated LC3B (Fig. 4.3.4D, upper and middle panels). FLAG-Ro52 was then expressed in HeLa cells and results show that this was sufficient to increase LC3B-II levels (Fig. 4.3.4E). Thus, the evidence suggests that increasing the load of an ectopically expressed protein, in this case GFP-actin, leads to a subsequent increase in Ro52, and as a consequence this promotes the lipidation of LC3B that accompanies autophagy.

### 4.3.5 DISRUPTING MICROTUBULES DOES NOT AFFECT THE FORMATION OF Ro52-POSITIVE STRUCTURES

Although the role for microtubules in autophagosome formation is controversial, it is known they facilitate autophagosome trafficking (Monastyrska et al., 2009). This, together with the report indicating Ro52 cytoplasmic bodies are located along the microtubule network and are highly motile (Tanaka et al., 2010), prompted us to examine if microtubules are involved in the
Figure 4.3.5: α-tubulin colocalized with Ro52-positive structures but did not affect their formation. (A) HeLa cells transfected with FLAG-Ro52 or FLAG-Ro52ΔRING with or without mCherry-tubulin plasmids were stained with anti-FLAG antibody and Alexa Fluor 488-conjugated (upper two panels) or TRITC-conjugated (lower two panels) secondary antibody, respectively. The lower two panels were also stained with anti-tubulin and Dylight 405-conjugated secondary antibody. mCherry-tubulin and endogenous tubulin colocalized with Ro52-positive structures (arrow). Scale bar=5µm. (B) HeLa cells transfected with FLAG-Ro52 and mCherry-tubulin plasmids were subjected to co-IP assay with anti-FLAG antibody then immunoblotted with anti-RFP antibody. mCherry-tubulin was pulled down with FLAG-Ro52. The higher molecular weight bands indicate mCherry-tubulin was post-translationally modified. (C) HeLa cells transfected with FLAG-Ro52 or FLAG-Ro52ΔRING plasmids were treated with 1μM colchicine for 17 hours then stained with anti-FLAG and anti-tubulin antibodies and TRITC-conjugated and Dylight 405-conjugated secondary antibodies, respectively. Disrupting microtubules did not affect Ro52-positive structure formation and colocalization of tubulin and Ro52 (arrow).
formation and/or maintenance of the Ro52-positive structures. HeLa cells were transiently transfected with mCherry-tubulin and FLAG-Ro52 plasmids and the ectopically expressed proteins analyzed by confocal microscopy. FLAG-Ro52 staining appeared in autophagosome-like structures, which co-localized with mCherry-tubulin (Fig. 4.3.5A, top panels). Deleting the RING domain of Ro52 did not perturb this co-localization (Fig. 4.3.5A, middle panels), and endogenous α-tubulin also localized to the Ro52-positive structures (Fig. 4.3.5A, lower and bottom panels). As with the actin analysis, we used co-IP assays to examine the mCherry-tubulin interaction with FLAG-Ro52. As shown in Figure 5B, mCherry-tubulin was pulled down with FLAG-Ro52. Reprobing the blot with the anti-FLAG antibody detected the presence of the ectopically expressed Ro52 in the immunoprecipitated complex as well as in the whole cell lysate (Fig. 4.3.5B, bottom panel).

Following from the actin data and given the results showing co-localization between mCherry-tubulin and FLAG-Ro52, we predicted that a disrupted microtubule network would affect the formation or positioning of the Ro52-positive structures. HeLa cells transfected with FLAG-Ro52 or FLAG-Ro52ΔRING plasmids were treated 17 hours after transfection with 1μM colchicine to inhibit microtubule polymerization. The cells were stained with antibodies against FLAG and α-tubulin and then analyzed by confocal microscopy. Results show that Ro52-positive structures were not affected by colchicine (Fig. 4.3.5C), which was unexpected and favors the notion that although microtubules participate in the trafficking of autophagosomes to the sites of lysosomes (Monastyrska et al., 2009), they are not involved in the formation of the autophagosomes.

4.3.6 Ro52 CO-LOCALIZES WITH p62 CYTOPLASMIC BODIES
p62 (Sequestosome 1) is a scaffold/adaptor protein involved in various cellular processes and modulation of several signaling pathways (Moscat and Diaz-Meco, 2012). In selective autophagy it facilitates binding to the ubiquitin moiety of ubiquitinated proteins as well as to LC3 (Johansen and Lamark, 2011), while in macrophages it interacts with Ro52 and IRF8 leading to ubiquitination and degradation of IRF8 through the proteasome and subsequent down-regulation of cytokine gene expression (Kim and Ozato, 2009). Together this information prompted us to investigate if the formation of Ro52-positive structures was influenced by p62. HeLa cells transfected with FLAG-Ro52 and GFP-p62 plasmids were fixed and stained with an anti-FLAG antibody and a secondary antibody conjugated with TRITC. Confocal microscopy analysis indicated that the ectopic expression of FLAG-Ro52 and GFP-p62 formed characteristic structures (Fig. 4.3.6A). In most cells where the Ro52-positive structures were in the early stages of autophagosome formation, FLAG-Ro52 co-localized to the aggregates formed by GFP-p62 (Fig. 4.3.6A, upper panels, arrow head). Much more co-localization was seen in cells containing late stage Ro52-positive structures (Fig. 4.3.6A, upper panels, arrow). Interestingly, almost no GFP-p62 localized to the mid-stage Ro52-positive structures (Fig. 4.3.6A, upper panels). Likewise, in HeLa cells transiently expressing FLAG-Ro52ΔRING and GFP-p62, FLAG-Ro52ΔRING was localized to GFP-p62 aggregates (Fig. 4.3.6A, lower panels, arrow), but GFP-p62 was not present inside the circular structures formed by FLAG-Ro52ΔRING (Fig. 4.3.6A, lower panels, arrowhead).

These localization results would suggest that the interaction between Ro52 and p62 does not appear to be essential for the formation of Ro52-positive structures, while the recruitment of Ro52 to the p62 aggregates is likely due to the ability of the latter to aggregate polyubiquitinated proteins. To address this hypothesis, we transiently expressed FLAG-Ro52, GFP-p62 and RFP-
**Figure 4.3.6:** Ro52 localized to p62 aggregates but not vice versa. (A) HeLa cells transfected with FLAG-Ro52 or FLAG-Ro52ΔRING with GFP-p62 plasmids were stained with anti-FLAG antibody and TRITC-conjugated secondary antibody then imaged. Ro52 was localized to p62 aggregates (arrow) but p62 was not localized to Ro52-positive structures (arrow head). (B) HeLa cells transfected with GFP-p62, RFP-ubiquitin and FLAG-Ro52 or FLAG-Ro52ΔRING plasmids were stained with anti-FLAG antibody and Dylight 405-conjugated secondary antibody then imaged. Ro52 localized to p62 aggregates with RFP-ubiquitin. Note that RFP-ubiquitin localized to Ro52-positive structure too (arrow, upper panel). Scale bar=8μm.
ubiquitin in HeLa cells and subjected them to confocal microscopy analysis. As shown in Figure 4.3.6B (upper panels), RFP-ubiquitin co-localizes to the FLAG-Ro52-GFP-p62 aggregates. The same staining pattern was seen in cells co-expressing FLAG-Ro52ΔRING, GFP-p62 and RFP-ubiquitin, but not vice versa (Fig. 4.3.6B, lower panels). It is also noteworthy that the RFP-ubiquitin signal was also detected inside the Ro52-positive structures, which would suggest the contents of these structures are ubiquitinated (Fig. 4.3.6B, arrow, upper panels). Together, these results would indicate that p62 does not participate in the formation of Ro52-positive structures, and these structures are not protein aggregates containing p62.

4.3.7 PROTEINS TARGETED TO THE Ro52-POSITIVE STRUCTURES ARE UBIQUITINATED

Our previous experiments revealed that the ectopic expression of different proteins results in their internalization within Ro52 autophagosomes. The localization of RFP-ubiquitin to the Ro52-positive structures seen in Figure 6B led us to propose that proteins targeted to these structures are ubiquitinated. To examine this further, we transiently expressed FLAG-Ro52, YFP-LC3 and RFP-ubiquitin in HeLa cells. The cells were fixed, stained and then analyzed by confocal microscopy. As expected, YFP-LC3 co-localized with Ro52-positive structures and RFP-ubiquitin was concentrated at these structures (Fig. 4.3.7A arrow, upper panels). This observation was consistent with the immunoblot results showing what appears to be the post-translational modification of YFP-LC3 (Fig. 4.3.2B, higher MW bands). Since Ro52 is an E3 ubiquitin ligase, it was tempting to speculate that its ligase activity contributes to the ubiquitination of the contents of the Ro52-positive structures. To test this, we transfected HeLa cells with the RING-deletion construct FLAG-Ro52ΔRING, YFP-LC3 and RFP-ubiquitin and
Figure 4.3.7: Proteins targeted to Ro52-positive structures were ubiquitinated. (A) HeLa cells transfected with YFP-LC3, RFP-ubiquitin and FLAG-Ro52 or FLAG-Ro52ΔRING were stained with anti-FLAG antibody and Dylight 405-conjugated secondary antibody then imaged. YFP-LC3 localized to Ro52-positive structures with RFP-ubiquitin (arrow). (B) HeLa cells transfected with mCherry-tubulin, GFP-ubiquitin and FLAG-Ro52 or FLAG-Ro52ΔRING were stained as in (A) then imaged. mCherry-tubulin localized to Ro52-positive structures with GFP-ubiquitin (arrow). (C) HeLa cells transfected with FLAG-Ro52 or FLAG-Ro52ΔRING and GFP-ubiquitin KO were stained with anti-FLAG antibody and TRITC-conjugated secondary antibody then imaged. GFP-ubiquitin KO was almost excluded from Ro52-positive structures. Scale bar=10µm.
results show that YFP-LC3 and RFP-ubiquitin co-localized to the FLAG-Ro52ΔRING-positive circular structures (Fig. 4.3.7A, arrow, bottom panels). Thus, these experiments would indicate the ubiquitination of the protein content is carried out not by Ro52, but by other E3 ligase(s). If so, we wondered if the other ectopically expressed cargo found inside the Ro52-positive structures (described above), was ubiquitinated. mCherry-tubulin, a protein enclosed in the Ro52-positive structures (this study), was co-expressed with FLAG-Ro52 and GFP-ubiquitin in HeLa cells and the staining pattern examined by confocal microscopy. Results show that the ectopically expressed proteins co-localized (Fig. 4.3.7B), and this was not dependent on the RING domain (Fig. 4.3.7B, arrow, bottom panels). To rule out the possibility that the ubiquitin signal seen inside Ro52-positive structures was enclosed independently, we took advantage of GFP-ubiquitin KO, a mutant ubiquitin-encoding construct, in which all 7 lysines are mutated to arginines, and therefore not able to form polyubiquitin chains on the substrates (Bergink et al., 2006). HeLa cells transfected with FLAG-Ro52 or FLAG-Ro52ΔRING and GFP-ubiquitin KO were stained with an anti-FLAG antibody and a TRITC-conjugated secondary antibody, and then analyzed by confocal microscopy. Results showing the GFP-ubiquitin KO signal outside the Ro52-positive structures (Fig. 4.3.7C), would suggest ubiquitin could only be enclosed in Ro52-positive structures when it is covalently attached to a substrate. Taken together, these results indicate that proteins within the Ro52-positive structures are ubiquitinated, while ubiquitination of the cargo itself is independent to the Ro52 ligase activity.

4.3.8 ENDOGENOUS Ro52 DOES NOT PARTICIPATE IN BASAL OR STARVATION-INDUCED AUTOPHAGY

Our experimental evidence would indicate that Ro52 overexpression leads to the formation of
**Figure 4.3.8:** Ro52 was not associated with autophagosome in basal and starvation-induced autophagy. (A) HeLa cells grown in complete medium (CM) or EBSS with or without 20μM chloroquine were stained with anti-Ro52 and anti-LC3B antibodies and Alexa Fluor 488-conjugated and TRITC-conjugated secondary antibodies, respectively, and imaged. Inhibiting autophagy with 20 μM chloroquine (CHQ) did not change the localization and expression level of Ro52 but LC3B. (B) HeLa cells of the same treatment as in (A) were subjected to immunoblotting analysis with the antibodies shown. Inhibiting the basal or starvation-induced autophagy did not change Ro52 expression levels. Scale bar=10μm.
autophagosome-like structures that contain ubiquitinated proteins. Since targeting of ubiquitinated protein to the autophagosome is the hallmark of selective autophagy, then endogenous Ro52 might play a role in basal or starvation-induced autophagy. HeLa cells grown in the complete medium or nutrient-deprived EBSS were treated with chloroquine (20μM) or left untreated and then stained with an anti-Ro52 and anti-LC3B antibody and subjected to confocal microscopy analysis. Surprisingly, Ro52 was not found to co-localize with LC3B regardless of the nutrient conditions. The addition of chloroquine did not change the staining pattern of Ro52, while the LC3 pattern appeared as puncta in the cytosol (Fig. 4.3.8A). HeLa cells with the same treatments were also analyzed by immunoblotting. As shown in Figure 4.3.8B, inhibiting autophagy with chloroquine in nutrient-rich (complete medium) or under nutrient-deprived (EBSS) conditions did not change steady state levels of endogenous Ro52, whereas endogenous levels of LC3B-II increased. These results contradict the ectopic expression data, suggesting that endogenous Ro52 is not associated with autophagosomes in basal or starvation-induced autophagy.

4.4 DISCUSSION

The presence of anti-Ro52 antibodies in the sera of patients with SS and SLE has prompted investigations into the involvement of Ro52 in the etiology of these autoimmune diseases. Ro52 forms cytoplasmic bodies when overexpressed in a variety of cell lines, and efforts to characterize these bodies have ruled out their co-localization with mitochondria, endosomes, proteasomes, caveolae and lysosomes (Reymond et al., 2001; Rhodes et al., 2002; Tanaka et al., 2010; Wada et al., 2006). In this study, we found that Ro52 cytoplasmic bodies developed into a series of morphologically different structures that resembled the succession into the
autophagosome, an organelle that develops during autophagy (Fig. 4.3.1A, B). Numerous fusions between these autophagosome-like structures occurred throughout this process, which is typical of autophagosomes formation (Nakatogawa et al., 2007). We have provided morphological and biochemical evidence that these autophagosome-like structures were indeed autophagosomes. Targeting of the ubiquitinated cargos into Ro52 autophagosomes indicated Ro52 is specifically involved in the selective type of autophagy. Moreover, the induction of the autophagosome-like structures by ectopic Ro52 expression in cells grown under nutrient-rich conditions was clearly different from the starvation-induced autophagosome formation and suggests that different mechanism are involved. This notion became evident when endogenous Ro52 levels were examined and found to remain steady after cells were treated with chloroquine in the nutrient-rich or starvation conditions (Fig. 4.3.8). Thus, endogenous Ro52 does not likely participate in the basal or starvation-induced autophagy. Interestingly, and with the exception of the final tubulization step, the stage when nascent lysosomes are regenerated from the autolysosomes (autophagic lysosome reformation) (Rong et al., 2012; Yu et al., 2010), the formation of autophagosome-like structures induced by ectopic Ro52 was not compromised by the deletion of the RING-finger domain (Fig. 4.3.1C). These results highlighted the fact that neither the E3 ligase activity of Ro52 nor its RING domain interaction was required for initiating the formation of the autophagosome.

mTOR signaling is involved in initiating autophagosome formation during canonical autophagy (Codogno et al., 2012; Jung et al., 2010), and this involves the cooperation of a few autophagy-related protein complexes including ULK1 (Atg1) and the Class III PI3K. Subsequent ubiquitination-like conjugation reactions result in membrane elongation and sequestration of cytosol to form double membrane-bound autophagosomes (Pyo et al., 2012). Whether or not
Ro52 interacts with the components within these complexes was heretofore not known. Results from our study, however, especially the ectopic expression data showing that Ro52 promotes the formation of autophagosomes in nutrient-rich condition, would indicate that Ro52 plays a major role in autophagy induction. Another noteworthy feature of the ectopic expression data was the extent of the fusion seen between Ro52 autophagosomes, which is a prevalent characteristic in canonical autophagy (Nakatogawa et al., 2007). Since Ro52 can oligomerize into a trimer through self-association, even in the absence of the RING domain (Rhodes and Trowsdale, 2007), the question remains whether or not Ro52 oligomerization is the catalyst for autophagosome formation, or does it facilitate the fusion between them? The data in this study does not address either situation and other experiments are necessary to elucidate the exact role of endogenous Ro52 in autophagy. Another aspect about autophagosome formation that needs to be clarified pertains to the source of the double-membrane. So far various origins for the autophagosomal membrane have been proposed including that from the mitochondria, endoplasmic reticulum (ER), plasma membrane and Golgi complex (Hailey et al., 2010; Hayashi-Nishino et al., 2009; Nishida et al., 2009; Ravikumar et al., 2010; Yla-Anttila et al., 2009). Despite being outside the scope of this study, it is interesting to note that our data showing the robust autophagosome induction by Ro52 would provide an ideal scenario by which to study the origin of the autophagosomal membrane. Moreover, it could also be an ideal model for studying autophagosome-lysosome fusion and autophagic lysosome reformation.

The localization of the autophagosome marker LC3 to Ro52-positive structures was evidence in support that ectopic expression of Ro52 induced autophagosome formation (Fig. 4.3.2A). LC3 not only localized to the membrane of Ro52 autophagosomes, but when ectopically expressed it became enclosed within the autophagosome. In addition, LC3 associated with Ro52-positive
structures throughout the process of autophagosome formation, and this interaction was bona
fide as evident from the co-IP experiments (Fig. 4.3.2B). While it is interesting to note that this
interaction has not been previously reported, it is still unclear whether Ro52-LC3 interaction was
direct or mediated by another protein(s). Towards that end, it is known that Ro52 interacts with
the LC3-binding protein p62 (Kim and Ozato, 2009). p62 is an adaptor protein that plays a key
role in selective autophagy by targeting ubiquitinated cargo into the autophagosome for
lysosomal degradation (Ichimura et al., 2008; Pankiv et al., 2007). Initially, the previous report
detailing the interaction between Ro52 and p62 prompted us to hypothesize that the selectivity of
the Ro52 autophagosome contents is mediated by the adaptor function of p62. It turned out,
however, from the co-expression experiments that p62 was not localized to Ro52
autophagosomes (Fig. 4.3.6A). Thus, the selective localization of ubiquitinated proteins to Ro52
autophagosomes was not mediated by p62. The inclusion of YFP-LC3 in Ro52 autophagosomes
indicated LC3 was not only an effector protein in autophagy, but when ectopically expressed it
too is the cargo and is ubiquitinated (Fig. 4.3.7A). That deletion of the RING domain of Ro52
did not affect the localization of ubiquitinated LC3 to the Ro52ΔRING-induced autophagosome
(Figs. 4.3.2A and 7A), suggests that this domain is dispensable for cargo sequestration.

Digestion of autophagosome cargoes is carried out by lysosomal enzymes. This process
would require fusions between autophagosomes and lysosomes. Besides autophagosomes,
lysosomes also fuse with endosomes. However, one study has ruled out the possibility that Ro52
cytoplasmic bodies are endosomes (Tanaka et al., 2010). Our results showing the fusion between
Ro52-positive structures and lysosomes, indicative by the LAMP-1-RFP co-localization (Fig.
4.3.3), was taken as evidence for Ro52-positive structures being autophagosomes. That the
number of Ro52-induced autophagosomes diminished when LAMP-1 was co-expressed with
Ro52, is possibly the result of increased lysosome formation and subsequent autophagosome-lysosome fusion and degradation (Fig. 4.3.3, top panels). The lysosome markers LAMP-1 and LAMP-2 were previously used to characterize Ro52 cytoplasmic bodies (Rhodes et al., 2002; Tanaka et al., 2010). Interestingly, neither group found evidence to support the notion that these cytoplasmic bodies co-localize with lysosomes. Since fusions between autophagosomes and lysosomes take place when autophagosomes are mature and Ro52 cytoplasmic bodies seem to be intermediate structures, it is reasonable to predict that Ro52 cytoplasmic bodies and lysosomes are in different compartments. The localization of LAMP-1 to the late stage tubule-like structures seen during autophagy is of particular interest, since we saw similar apparently hollow structures that appeared to have budded from the Ro52-positive structures (Figs. 4.3.1A 5, 6 and 7; Fig. 4.3.3, middle panels). The tubulation from autolysosomes is the hallmark of autophagic lysosome reformation (ALR) in the late stage of autophagy (Tong et al., 2010). These tubules will later form nascent lysosomes, which maintain cellular lysosome homeostasis following their consumption in autophagy. LAMP-1 is one of the proteins that resides at these tubules extruding from autolysosomes (Rong et al., 2012). In addition to LAMP-1, two other proteins are also present until the tubulation stage. One protein is LC3, which associates with the inner and outer membrane of autophagosomes after lipidation (Rong et al., 2012). The inner LC3 is degraded with the contents after fusion with lysosomes and the outer LC3 is later recycled from the membrane through the protease activity of ATG4 (Kabeya et al., 2000; Satoo et al., 2009). The second protein is α-tubulin (Rong et al., 2012), which when assembled into microtubules, plays a key role in the trafficking of autophagosomes to facilitate their fusion with lysosomes (Monastyrska et al., 2009; Xie et al., 2010). YFP-LC3 and mCherry-tubulin were found to localize to these tubules (Figs. 2A and Fig 5A, top panels). Our results were clearly a snapshot of
autophagic lysosome reformation after the fusion of Ro52 autophagosomes with lysosomes. Tubulation was abolished when the RING domain was deleted despite the fact that the fusion between the Ro52ΔRING autophagosomes and lysosomes were not affected (Fig. 4.3.3, bottom panels). Although these results suggest that the RING domain plays a pivotal role in the budding event, more work involving the identification and characterization of the protein(s) interacting with Ro52 RING domain is needed to better understand the budding mechanism.

Actin, a major component of the cytoskeleton, is required for initial membrane remodeling at the early stage of starvation-induced autophagy in HeLa cells (Aguilera et al., 2012) and cargo selection during selective autophagy in yeast (Reggiori et al., 2005). Furthermore, depolymerizing microfilaments interferes with the formation of the autophagosome in rat kidney cells (Aplin et al., 1992). Our studies provide new evidence that actin interacts with Ro52 (Fig. 4B), and this interaction is specific for G-actin (Fig. 4.3.4A). Having an intact actin cytoskeleton, however, is very important in stabilizing the Ro52 autophagosome since F-actin depolymerization led to its decomposition (Fig. 4.3.4C). Although the mechanism is still not clear, our data are in agreement with the observation in rat kidney cells (Aplin et al., 1992) and in HeLa cells (Aguilera et al., 2012). Another major finding of our study is the regulatory role of actin in both Ro52 expression and LC3 lipidation. While overexpressing actin increased Ro52 levels and resulted in the accumulation of lipidated LC3 (Fig. 4.3.4D), the same effect on LC3 was seen when Ro52 was ectopically expressed (Fig. 4.3.4E). At present it is not known how the levels of Ro52 are regulated by actin, but given the reports regarding Ro52 and ubiquitination (Espinosa et al., 2008; Espinosa et al., 2006; Fanelli et al., 2004; Fukuda-Kamitani and Kamitani, 2002), it is tempting to speculate that the interaction between actin and Ro52 helps to sequester and protect Ro52 from polyubiquitination and proteasomal degradation. If that were the case,
then the increase in Ro52 levels would in turn promote the formation of autophagosomes, which requires the conversion of LC3-I to LC3-II. While our data clearly indicated that endogenous actin is a natural cargo in the Ro52 autophagosome (Fig. 4.3.4A, bottom panels), subsequent studies are needed to examine actin levels in cells induced to undergo autophagy as well as in those where autophagy is inhibited. Whatever the mechanism, the interplay between actin and Ro52 revealed in this study would suggest that a feedback mechanism is in place to function in balancing the levels of these two proteins.

Another cytoskeletal element involved in mammalian autophagy is the microtubule (Kochl et al., 2006). Although microtubules are known to facilitate autophagosome trafficking, their role(s) in autophagosome formation is less clear (Monastyrska et al., 2009), and one question that remains pertains to how microtubules connect to autophagosomes. Our results showing Ro52 interacting with α-tubulin might provide an answer to that question, but the “motor” necessary for this movement is still unknown. The fact that Ro52-induced autophagosomes were not disrupted in the microtubule depolymerization studies would suggest that microtubules do not participate in autophagosome formation and as such the involvement of microtubules in autophagosome trafficking or fusion with lysosomes remains to be determined. Nevertheless, α-tubulin, like that described earlier for actin, not only appears to be a natural cargo in these Ro52 autophagosomes (Fig. 4.3.5A, bottom panels), but is also a ubiquitinated protein (Fig. 4.3.7B), which is expected for those destined for autophagy via this mechanism (Kraft et al., 2010).

Adaptor proteins mediate the highly selective targeting of ubiquitinated proteins to the autophagosome (Kraft et al., 2010). We demonstrated that the proteins ectopically expressed in our studies that get enclosed inside Ro52 autophagosomes were ubiquitinated (Figs. 4.3.7A and 4.3.7B). Unexpectedly, ubiquitination was not due to Ro52 since deleting its RING domain did
not affect the formation of the Ro52-induced autophagosome or the inclusion of ubiquitinated proteins. The selective nature of the cargos in these autophagosomes would suggest that a sorting mechanism exists to facilitate this process. An autophagy receptor, p62, was considered to be involved in sorting, but our results would state otherwise (Figs. 4.3.6A and 4.3.6B). If so, Ro52 may play the adaptor role between LC3 and ubiquitinated cargos, and although it lacks an obvious LC3 interacting region and ubiquitin binding domain(s), Ro52 did bind LC3 (Fig. 4.3.2B). Although it is still not known if Ro52 binds directly to ubiquitin, if it does then binding must fall outside of the RING domain since the RING truncation did not affect the overall targeting of ubiquitinated cargos into Ro52ΔRING autophagosomes. Nonetheless, that Ro52 binds to ubiquitinated proteins through the mediation of another ubiquitin-binding protein cannot be ruled out at this point. Again, if this were the case, the binding region must reside outside the RING domain.

Finally, we were surprised that although the overexpression of Ro52 led to the formation of autophagosomes, endogenous Ro52 did not appear to participate in basal or starvation-induced autophagy. These findings indicate that different mechanisms may be required in the formation of the autophagosomes induced by the overexpression of Ro52 and that each lead to autophagosomes having different functions. To maintain cellular homeostasis, basal autophagy ensures that selective mechanisms are in place to rid the cell of aberrant proteins and organelles. The fact that Ro52 is not involved in these housekeeping processes suggests it plays a more specific role in autophagy, where an Ro52 autophagosome only forms when Ro52 levels increase as the result of some form of cellular stress. This notion is further supported by the fact that Ro52 is not part of the conventional autophagy machinery (this study). To put this in context, Ro52 is up-regulated in cells following the stimulation of interferons (IFNs) (Rhodes et al., 2002;
Strandberg et al., 2008) or exposure to UV (Oke et al., 2009). Interferons are cytokines that are produced and secreted by host cells in response to viral or bacterial infections (Fensterl and Sen, 2009). While Ro52 targets endocytotic antibody-bound viruses for proteasomal degradation (Mallery et al., 2010; McEwan et al., 2012), invaded bacteria are cleared by ubiquitin-mediated selective autophagy (Cemma et al., 2011; Mallery et al., 2010; Zheng et al., 2009). This correlation between the IFN-induced Ro52 up-regulation and pathogen invasion would suggest that the Ro52 autophagosome has a special function in clearing intracellular pathogens, therefore contributing to innate immunity. As for the UV-induced Ro52 up-regulation, this would be an adaptive mechanism to combat and clear UV-damaged proteins and organelles through Ro52-mediated selective autophagy.

In summary, increasing the steady-state levels of Ro52 leads to the formation of autophagosomes. Moreover, there is an interaction between actin and Ro52 that regulates the intracellular levels of Ro52 and the lipidation of LC3. Together these results would suggest that as Ro52 levels increase within the cell, the induced autophagosomes specifically target ubiquitinated proteins for lysosomal degradation.
4.5 LITERATURE CITED


CHAPTER 5 GENERAL DISCUSSION

5.1 LESSONS FROM THE UBIQUITIN-MEDIATED DEGRADATION OF CASK AND PARP-2

CASK and PARP-2 are two distinct substrates of ubiquitination. The former is mainly a cytoplasmic protein that serves as a scaffold for an evolutionarily conserved protein complex containing Mint 1 and Velis (Tabuchi et al., 2002). In some instances, however, CASK can translocate to the nucleus and serve as an adaptor for transcription modulators in the brain. In contrast, PARP-2 is a nuclear enzyme functioning in DNA strand break repair and cellular energy expenditure management. Despite the difference in their loci and functions, both are constantly ubiquitinated and degraded through the UPS. Although in both cases the stability of the protein is affected by ubiquitination, the rationale for their degradation may not be the same.

Normally, proteins subjected to proteasomal degradation are cleaved into small peptides of about 5-20 amino acid residues in length (Nussbaum et al., 1998; Piwko and Jentsch, 2006) to avoid creating active fragments without the scrutiny of the regulatory domains. The consistent degradation pattern of CASK across different cell lines suggests it is more like partial degradation, at least at some point, than complete destruction of the protein. PARP-2 on the other hand appears to be turned over completely. That partial degradation of CASK generates larger fragments with the same pattern in different cell lines raises interesting questions about how the specificity of the cleavage sites is achieved in the 26S proteasome-mediated degradation, and about the consequence/physiological function of this partial degradation. The regulated ubiquitin proteasome-dependent processing (partial degradation) has previously been reported for several proteins including the transcription factors NF-κB, Sp1, the yeast NF-κB-related Spt23 and
Mga2 and *Drosophila* Cubitus interruptus (Ci) (Fan and Maniatis, 1991; Hoppe et al., 2000; Palombella et al., 1994; Rape and Jentsch, 2002; Su et al., 1999; Tian et al., 2005). The biologically active protein fragments of these proteins are generated from the precursors by proteasomal processing. One structural cue for partial degradation is the polyglutamine stretch, where polyglutamine-containing proteins such as mutant Huntingtin are partially degraded and trapped within proteasomes (Holmberg et al., 2004). Similar to the polyglutamine stretch, the Gly-Ala repeat (GAr) sequence of EBNA1 of Epstein-Barr virus is a transferable element, which leads to the partial degradation of the substrates containing GAr by disrupting the unfolding of the substrates in a position- and substrate-specific manner (Daskalogianni et al., 2008; Heessen et al., 2002; Zhang and Coffino, 2004). Notably, both the polyglutamine stretch and GAr are sequences with low compositional complexity. Tian et al. proposed that an amino acid sequence of low complexity in conjunction with a tightly folded domain in the direction of proteasome movement comprises the stop signal for proteolysis in these proteins and suggested that this rule may be applied to other regulatory proteins (Tian et al., 2005). The model addresses how proteins are degraded from their ends (either N- or C-terminus) and is called the end-first model. Apparently, proteins processed following this rule would only generate one fragment no matter how many stop signals there are in these proteins, such as NF-κB and Ci. However, the end-first model cannot explain the partial degradation of some membrane-bound proteins, including Spt23.

An alternative model called the endoproteolysis or loop model was proposed based on the studies of several native proteasome substrates (Liu et al., 2003; Piwko and Jentsch, 2006). Degradation of proteins such as p21, α-synuclein (α-syn), Spt23 and Mga2 is initiated from a loop-like internal site, followed by bidirectional degradation towards both ends until encountering a tightly folded domain. The initial sites of proteolysis are thus determined by the positions of...
ubiquitination sites and by the most flexible domains of the protein, e.g. both ends of the protein and the internal loops (Piwko and Jentsch, 2006). The loop model addresses the partial processing of the ER membrane-anchored Spt23 protein and is also applicable to the complete degradation of other proteins. As noted earlier, CASK is constantly cleaved into fragments regardless of cell type. Despite some smaller fragments that might form from the intermediate product of proteolysis, one prominent band around 70kD has a longer half-life and is very likely a biologically active fragment generated by partial degradation. This band overlaps with the N-terminal half of CASK as indicated by the myc-CASK expression and subsequent anti-myc staining results, suggesting the degradation starts from its C-terminus (Figs. 2.3.1 and 2.3.5). Interestingly, when CASK-myc is expressed in COS-7 cells in the presence of MG132, smaller bands corresponding to the C-terminus of CASK are detected by anti-myc staining (Fig. 2.3.7). These results suggest the degradation initiates from its N-terminus. Together with the data showing that the degradation starts at the C-terminus (Figs. 2.3.1 and 2.3.5), it is likely that the degradation site is within the full-length CASK protein, which is in agreement with the loop model. CASK contains three PEST motifs between amino acids 316-611. PEST motifs are conformationally flexible sequences that are rich in hydrophilic amino acids (Rechsteiner and Rogers, 1996). The dominance of PEST amino acids in the sequence also indicates the low compositional complexity of the sequences. In addition, PEST sequences are usually the sites where phosphorylation and ubiquitination modifications occur (Radivojac et al., 2010). These features of the PEST motifs fulfil the requirement of forming internal loops where proteolysis occurs in the 20S proteasomes. The subsequent bidirectional degradation leads to the complete degradation of the C-terminus and generation of a ~70kD N-terminal fragment. The weak C-terminal band present when myc-CASK is overexpressed would suggest the C-terminus is
normally turned over relatively quickly. The ~70kD fragment suggests the PEST sequence (amino acids 594-611) between the PDZ and SH3 domain is the preferred degradation initiation site and when this occurs the fragment contains the N-terminal domains including CaM-kinase domain, L27N, L27C and PDZ domain. While L27N, L27C and PDZ domains mediate protein-protein interactions in CASK, the CaM-kinase domain phosphorylates itself and neurexin-1 in an Mg\(^{2+}\)-independent manner (Mukherjee et al., 2008). Since the CaM-kinase domain adopts a constitutively active conformation (Mukherjee et al., 2008), it is unlikely that this processing of CASK is to activate/unmask its kinase activity, and instead it might be a mechanism to produce a dominant-negative regulator. This notion, however, requires further investigation.

In contrast to CASK, PARP-2 appears to be completely degraded by the UPS since in our studies, no persistent smaller fragments were observed in cells under stress-free conditions. Therefore, PARP-2 degradation may not serve as a means of generating physiologically active fragments. In addition to the UPS-mediated degradation, when serum is deprived from the media, PARP-2 is transformed into an SDS-insoluble form by a yet-to-be identified mechanism. This transformation, however, is reversible and does not require protein synthesis following serum replacement. The shift between the soluble and insoluble form of PARP-2 reflects the importance of PARP-2 under these different conditions.

Another noted difference between the proteasomal degradation of CASK and PARP-2 is the rate of turnover. While the endogenous level of CASK does not change appreciably following MG-132 treatment, PARP-2 levels increase 2-3 fold in the same period of time and when the same concentration of MG-132 is used. Thus, PARP-2 is degraded faster than CASK. Interestingly, cellular proteins are maintained at steady levels with roughly equal synthesis and turnover rate and these dynamics depend on the organism’s developmental stage and its
environment (Rothman, 2010). Changes in either synthesis or degradation would of course lead to an increase or decrease in protein levels (Doherty and Beynon, 2006; Rothman, 2010). The half-lives of proteins in cells vary between a few minutes to days, where those having slow degradation rates tending to have longer half-lives (Eden et al., 2011). Although the mechanism governing the degradation rates of proteins is still not completely understood, several sequential signals affecting protein turnover have been identified including N-terminal residue, PEST sequence, destruction-box and KEN-box. Despite the fact that proteins containing these signals are prone to degradation, there is no apparent correlation between the motifs and the half-life of a protein (Tompa et al., 2008). This notion is supported by our studies, where we reported that despite the fact that CASK contains three PEST motifs in its sequence, it has a relatively longer half-life than PARP-2, which does not possess any known degradation signals. Other factors proposed to be related to protein half-life include the length of the polypeptide chain and structural disorder (Tompa et al., 2008), the number of ubiquitination sites (Radivojac et al., 2010) and the subcellular localization of the protein (Eden et al., 2011). When these factors are examined in CASK and PARP-2 degradation, it seems the number of ubiquitination sites may contribute to the difference in their degradation rates. Using an algorithm developed to predict the ubiquitination sites within substrates (http://www.ubpred.org), CASK and PARP-2 score 10 (5 sites are of medium confidence) and 15 (11 sites are of medium to high confidence), respectively. Although suggestive, the higher number of putative ubiquitination sites in PARP-2 would correlate with its higher degradation rate. In general, ubiquitination sites and flanking sequences are conformationally flexible and disordered, which are features that may help protein unfolding before it enters the proteasome chamber, thus facilitating the increase in degradation rate.
5.2 UBIQUITIN-MEDIATED PROTEASOMAL DEGRADATION vs. LYSOSOMAL DEGRADATION

Proteasomes and lysosomes are two distinct types of organelles that degrade proteins. Although ubiquitination does not necessarily lead to protein degradation, it generally facilitates protein targeting, and thus selective degradation in these organelles. Proteasomes, which are distributed in both cytoplasm and nucleus, are protein complexes comprising of one 20S core with protease activity and two 19S regulatory subunits. In contrast, lysosomes are membrane-bound cytoplasmic vesicles containing pH-sensitive hydrolases. The sheer size of the proteasome and lysosome defines the size of their substrates that they can process. With a small pore of 53Å and a narrow entrance of 13Å, the proteasome is only able to degrade unfolded proteins one at a time. Lysosomes, however, can degrade proteins and even excessive organelles in bulk. One feature of the proteasome that is unavailable to the lysosome is its ability to reside in the nucleus, conveniently positioned to degrade nuclear proteins. Since both proteasome and lysosome can process ubiquitinated proteins, it raises an interesting question about how ubiquitinated proteins are discerned and diverted to one of them, but not another. For instance, we found CASK is degraded through the UPS, but not the lysosome pathway. It is now known that there are many ubiquitin-binding proteins playing adaptor roles in recognition and targeting of ubiquitinated proteins. The common feature of these adaptor proteins is the presence of domains, which can bind to ubiquitin moieties or the substrate protein as well as the proteasome or autophagosome. In this way, these domains provide a means by which to sort ubiquitinated proteins to different destinations. It is noteworthy that the proteasome has intrinsic ubiquitin binding activity so many proteins targeted here may not need an adaptor protein. Our data does not show whether CASK
or PARP-2 is sorted to proteasomes by adaptors, but the enclosure of ubiquitinated mCherry-tubulin into Ro52 autophagosomes suggests an adaptor protein may be involved in this process. α- and β-tubulin are reported to be ubiquitinated by parkin and degraded in the proteasome (Ren et al., 2003), which may be a cell-type specific route for tubulin degradation. The targeting of α-tubulin to Ro52 autophagosomes is apparently another route. Since lysosomal degradation is more efficient to rid a cell of excessive protein, we expect this is the preferred mechanism when tubulin is over-expressed. Moreover, the fusion of the autophagosome/lysosome isolates the cargo proteins from other cellular compartments therefore minimalizing the impact these excess proteins would have on normal cellular physiology. As with other ubiquitinated proteins targeted for lysosomal degradation, the selective nature of targeting tubulin to the autophagosome is very likely facilitated by an adaptor protein. So far, several adaptor proteins have been found to participate in the selective type of autophagy, including p62, NBR1, NDP52, OPTN, NIX, Atg19p, Atg32p and Atg34p (Behrends and Fulda, 2012). That Ro52 interacts with the autophagosome membrane protein LC3 and ubiquitinated proteins including α-tubulin and β-actin, suggests it may be a novel autophagic adaptor protein. If Ro52 is an adaptor, then it would be expected to possess domain(s) that facilitate these interactions. The identification and characterization of these domains require further investigation.

5.3 SUMMARY AND CONCLUSIONS

Ubiquitination is a highly regulated process that imposes different outcomes to the substrate proteins. Identifying proteins subject to ubiquitination leads to the question of whether their quantity, subcellular localization or activity is affected as a result of ubiquitination. In addition, ubiquitinated cargos can be diverted to different cellular compartments, and players involved in
this process have been and continue to be identified. The substrate-specificity of E3 ubiquitin enzymes reflects the delicateness of ubiquitination and underscores the importance of identifying E3 ligases responsible for protein ubiquitination. The continued identification of protein substrates and their corresponding E3 ligases not only aid in our understanding of the regulatory mechanisms that govern normal cellular physiology but also, in general, help to provide the basis as to what could happen when these interactions between substrates and E3 ligases are prevented. Under normal conditions ubiquitinated cargos are diverted to either the proteasome or lysosome for degradation and to maintain normal physiology, a large group of ubiquitin-binding proteins is needed to play decisive roles in the recognition and sorting of these proteins. Towards that end, the data herein not only provides new data as to how very disparate proteins are degraded under normal and stress-induced conditions, but also sheds new light and a new role for one protein, Ro52, that has previously been linked to human autoimmune diseases.
5.4 LITERATURE CITED


Appendix

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