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The Herp and HRD1-dependent degradation of TRPP2

by

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Abstract

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a prevalent genetic disorder where multiple fluid-filled cysts destroy kidney architecture, eventually requiring hemodialysis or kidney transplant. Approximately 15% of cases of ADPKD cases are caused by defects in TRPP2, a calcium permeable, non-selective cation channel protein in the ER and plasma membrane. Studies by our lab indicate that TRPP2 immunoprecipitates with ER stress-regulated proteins Herp and HRD1. Herp is an endoplasmic reticulum (ER) integral membrane protein involved in the ubiquitylation and degradation of substrates by ER associated degradation (ERAD). HRD1 functions as an E3 ubiquitin ligase and is important in retrotranslocation of ERAD substrates. This study sought to examine the interaction between TRPP2 and Herp/HRD1. Our findings suggest that Herp cleavage affects its TRPP2 binding affinity, but not ubiquitylation. We also confirmed that HRD1 interacts with TRPP2, and may be involved in its ubiquitylation.

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Table of Abbreviations

- aa - Amino acid
- ADPKD - Autosomal dominant polycystic kidney disease
- ALS - Amyotrophic lateral sclerosis
- AMFR - Autocrine motility factor receptor (also GP78)
- ATF4/6 - Activating transcription factor 4/6
- ATP - Adenosine triphosphate
- BAK - BH antagonist or killer
- BAX - BCL2-associated X protein
- BCL-2 - B cell lymphoma 2
- BH3 - BCL-2 homology 3
- BIM - BCL-2-interacting mediator of cell death
- BiP/GRP78 - binding immunoglobulin protein/glucose regulated protein 78 kDa
- bZIP - Basic leu-zipper
- CASVM - Caspase support vector machine server
- CCT - Choline cytidylyltransferase
- CDC48p - Cold-sensitive cell-division-cycle protein 48 (yeast VCP homolog)
- CD3δ - Cluster of differentiation 3δ
- CHOP - C/EBP homology protein (also GADD153)
- CK2 - Casein kinase 2
- COPII - Coat protein II

- DMSO - Dimethyl sulphoxide
- DNA - Deoxyribonucleic acid
- EDEM1 - Endoplasmic reticulum degradation-enhancing alpha-mannosidase-like protein 1
- EDTA - Ethylenediaminetetraacetic acid
- EGF - Epidermal growth factor
- EGFR - Epidermal growth factor receptor
- eIF2α - Eukaryotic initiation factor 2α
- Endo H - Endoglycosidase H
- ER - Endoplasmic reticulum
- ERAD - Endoplasmic reticulum associated degradation
- ERK - Extracellular signal-regulated protein kinase (also MAPK)
- ERSE - Endoplasmic reticulum stress element
- ESRD - End stage renal disease
- FBS - Fetal bovine serum
- GADD153 - Growth arrest and DNA damage-inducible protein 153 kDa (also CHOP)
- GFP - Green fluorescent protein
- GFR - Glomerular filtration rate
- GM130 - Golgi matrix protein 130 kDa
- GP78- Glycoprotein 78 kDa (also AMFR)
- GSK - Glycogen synthase kinase

- HCL - Hydrochloric acid
- HEK-293 - Human embryonic kidney 293rd experiment
- HeLa - Henrietta Lack's cells
- Herp - Homocysteine-inducible endoplasmic reticulum resident protein
- HRD1 - Hydroxymethylglutaryl-CoA reductase degradation 1
- HRP - Horseradish peroxidase
- HSP-60 - Heat shock protein 60 kDa
- HUVEC - Human umbilical vein endothelial cells
- IP_3R - Inositol (1,4,5) triphosphate receptor
- IRE1α - Inositol-requiring protein 1α
- kDa - kilodaltons
- MAM - Mitochondrial associated membrane
- MAPK - Mitogen associated protein kinase (Also ERK)
- MAP1LC3B - Microtubule-associated proteins 1A/1B light chain 3
- MDCK - Madine Darby canine kidney
- MG-132 - N-(benzyloxycarbonyl)leucinylleucinylleucinal (Z-Leu-Leu-Leu-al)
- MHC1 - β2-microglobulin heavy chain class 1
- mTOR - Mammalian target of rapamycin
- NHK - alpha1-antitrypsin null Hong Kong
- NS1 κ LC - Immunoglobulin NS1 κ light chain
- Orai - Protein family named after the keepers of the gates of heaven in Greek mythology

- OS-9 - Osteosarcoma amplified 9, endoplasmic reticulum lectin
- PACS - Phosphofurin acidic cluster sorting proteins
- Pael - Parkin-associated endothelin receptor-like
- PAGE - Polyacrylamide gel electrophoresis
- PBS - Phosphate buffered saline
- PC12 - Pheochromocytoma 12 cells
- PDI - Protein disulphide isomerase
- PERK - Protein kinase RNA-like endoplasmic reticulum (ER) kinase
- PIGEA-14 - Polycystin-2 interactor, golgi- and endoplasmic reticulum-associated protein - 14 kDa
- PKC/A - Protein kinase C/A
- PKD(REJ) - Polycystic kidney disease (receptor for egg jelly)
- PKHD1 - Polycystic kidney and hepatic disease 1
- PNGase F - Peptide: N-glycosidase F
- PrKD - Protein kinase D (different abbreviation to differentiate from PKD genes)
- PUMA - P53 upregulated modulator of apoptosis
- P53 - Protein 53 or tumor protein 53
- P97/VCP- Protein 97/valosin containing protein
- RCF - Relative centrifugal force
- RING - Really interesting new gene
- RPE1 - Retinal pigmented epithelial cell line 1

- RPM - Revolutions per minute
- RyR - Ryanodine receptor
- $SCF^{\beta\text{-}TRCP}$ - Skp1, cullin 1, F-box containing complex with substrate recognition subunit β-transducin repeat containing protein
- SEL1L - Suppressor of lin-12-like protein 1
- SERCA - Sarcoplasmic/endoplasmic reticulum calcium ATPase
- SOCE - Store operated calcium entry
- STIM1 - Stromal interaction molecule 1
- TM - Transmembrane domain
- TRP(V,A,N,C,M,ML,P) - Transient receptor potential (vanilloid, ankyrin, no mechanoreceptor potential C (NOMPC), canonical, melastatin, mucolipin, polycystin)
- UBC7 - Ubiquitin conjugating enzyme 7
- UBL - Ubiquitin-like domain
- uORF - Upstream open reading frames
- UPR - Unfolded protein response
- UTR - Untranslated region
- VCP/P97 Valosin containing protein/protein 97
- VIMP - VCP interacting membrane protein
- V2RA - Vasopressin-2 receptor antagonist
- WT - Wild type
- XBP1 - X-Box binding protein 1

- xCT - Cystine/glutamate antiporter system subunit
- XTP3-B - XTP3-transactivated gene B protein
- ZVAD-FMK - carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

1 Introduction

1.1 Polycystic Kidney Disease

Polycystic kidney diseases (PKDs) are a group of diseases that through multiple causes lead to development of multiple fluid-filled lesions in the kidney, referred to as cysts. These cysts destroy kidney architecture and usually lead to renal dysfunction. PKDs can be caused non-genetically or genetically. Among genetic PKDs, autosomal recessive PKD (ARPKD) has been distinguished from autosomal dominant PKD (ADPKD) because of its tendency to have an earlier onset (usually in infancy (1)). In ADPKD, cyst development occurs later, usually leading to renal dysfunction by the fourth decade (2). Since PKDs were first described, there have been a many changes in PKD classification and nomenclature. As a result, it is useful to examine the history of our understanding of PKD.

1.1.1 History

Although PKDs have been described since antiquity (3), their first description as an independent clinical entity was first described by Pierre Rayer in 1841 (3). “Polycystic” was first used as a descriptive term of the disease in the doctoral thesis of Felix Lejars, published in 1888 (4). By the end of the 19th century, adult onset PKD (later referred to as autosomal dominant PKD or ADPKD) could be diagnosed separately from other renal cystic diseases, such as infantile PKD (later referred to as autosomal recessive PKD or ARPKD), multicystic renal dysplasia and acquired renal cystic disease (3). This

improvement in diagnosis paved the way for recognition of the molecular underpinnings of genetic PKDs. Before we get into that discussion, however, it is useful to examine some non-genetic causes in contrast.

There are many reported causes of non-genetic PKDs. Among these are multicystic renal dysplasia, acquired renal cystic disease, and echinococcosis (3,5). Multicystic renal dysplasia is non-genetic condition that is usually sporadic, but may be familial. It is usually diagnosed by the presence of one or more cystic kidneys present in a developing fetus upon ultrasound imaging. The disease may present as the bilateral or unilateral presence of multicystic kidneys. The bilateral condition is usually fatal, whereas infants born with unilateral cysts have a better prognosis. Unilateral cyst formation can be graded according to severity and the nature of observed structural defects, and is the most common cause of flank mass in an infant (6). Acquired renal cystic disease usually denotes bilateral renal cyst development in patients with non-genetic end stage renal disease (ESRD) who have been long-term dialysis (though the term has been used in the past to denote a broader spectrum of renal cystic disorders (3)). This condition affects 40-90% of patients depending on dialysis duration, though they usually regress upon renal transplantation (6). Echinococcosis refers to infection by tapeworms of the genus *Echinococcus*. Ingestion of these tapeworm larvae causes long-term growth of hyatid cysts usually in the lungs and/or liver, but is also found in the kidney (3,5). There have been reported many other non-genetic causes in addition to those described here (6), but in the interest of brevity they have been omitted. Next we

turn our attention to genetic PKDs and in particular the most commonly found and studied, ADPKD and ARPKD.

The first genetic PKD gene locus to be discovered was that of autosomal dominant Adult PKD (Later called ADPKD). It was mapped to chromosome 16p13.3 (7) in 1985 by Stephen Reeders in the laboratory of Sir David Weatherall at Oxford (8). Although that locus (later referred to as the PKD1 locus) accounted for only about 86% of observed cases of this disease in 1993 (9), it was not until 1993 that a second locus (later referred to as the PKD2 locus) was mapped to chromosome 4q21-23 (7,9). Together these two loci account for nearly all cases of ADPKD, although there is still suspected to be a third locus present in a small number of unlinked families (10). Though a partial sequence of the PKD1 locus appeared in 1994 (11), the first complete PKD1 sequence was reported in 1995 (12). At that time, the product of this gene (named Polycystin-1) was established to be a 4302 amino acid membrane protein with eleven predicted transmembrane domains, a long extracellular N terminus and a relatively short intracellular C terminus (12,13). The location and sequence of the PKD2 locus soon followed in 1996 (7). It is of note that the gene product of the PKD2 locus was named Polycystin-2, and has since also been called PKD2, PC2, or TRPP2. The PKD2 gene encodes a 968 amino acid protein with six predicted transmembrane domains, a long extracellular loop, and intracellular N and C termini. Mutations in Polycystin-1 and Polycystin-2 account for the development of ADPKD in

approximately 85% and 15% of cases, respectively (10). In addition to ADPKD, there has also been a fair amount of study done on a related disorder, ARPKD.

The genetic locus of human infantile PKD or ARPKD was first mapped in 1994 to 6p21 (14), and the human ARPKD gene (termed PKHD1) was independently cloned by 3 groups and reported in 2002 (15,16). The PKHD1 gene encodes a receptor-like 4074 aa protein that is referred to as Fibrocystin or polyductin (16,17). Fibrocystin possesses a small C-terminal cytoplasmic domain, a single transmembrane helix, and an extremely long extracellular N-terminal domain that possesses multiple domains that are thought to bind to as of yet unknown ligands (15,16). In addition to ADPKD and ARPKD, there have also been identified other genetic causes of PKD, including many associated with fetal birth defects (6), but in the interest of brevity they have been omitted. Having identified the history of the most common genetic PKDs, we now turn to a more detailed look into their clinical manifestation.

1.1.2 Clinical Manifestation of Genetic PKDs

Since the two most common genetic PKDs ADPKD and ARPKD are the most relevant to this study, we will focus on the clinical aspects of those two alone, starting with ADPKD. ADPKD is one of the most common genetic diseases, with an estimated occurrence of 2 in every 1000 live births (6). Most ADPKD cases are first diagnosed on the discovery of hypertension, the sudden onset of renal pain or hematuria, or the inadvertent discovery of nephromegaly on

physical or radiological examination. Renal insufficiency can appear at any age, but most often after the fourth decade. In the late stages of the disease, kidneys are massively enlarged and have a substantially altered appearance, including thick fibrotic bands and severely decreased functioning renal parenchyma (2). Besides the above described signs, an ADPKD patient may also have nephrolithiasis, and/ or exhibit glomerular hyperfiltration, loss of urine concentrating capacity, or mild albuminuria. Although there is a severe and constantly increasing loss of functioning renal parenchyma, the remaining healthy tissue compensates via hyperfiltration to maintain GFR at a substantially normal rate until it finally declines at a rate of about 5 ml/min/1.73 m^2/year (2) until culminating in ESRD. ESRD is considered to occur when GFR declines below a rate of 3 ml/min (18). Once ESRD has begun, the patient's only current options are dialysis or kidney transplant. As both of these options have have a significant effect on the patient's quality of life, alternative means of treatment are highly desirable. Although there has been some work done on therapeutic agents meant to slow disease progression such as vasopressin receptor antagonist V2RA (19), and mTOR inhibitors everolimus and sirolimus (20-22), none of these have so far proven to be clinically effective. Therefore, future treatment of this disease depends on an increased understanding of the functioning of molecular disease determinants such as Polycystin-1 and 2.

ARPKD is less common than ADPKD, with an estimated occurrence of approximately 1 in every 20,000 live births (6). It is usually diagnosed during the

perinatal period, with almost half of affected newborns dying shortly after birth due to pulmonary hypoplasia (14,17). Ultrasound imaging presents enlarged, bilaterally echogenic kidneys and enlarged, echogenic livers with biliary ectasia (bile duct widening). Severity of the disease is variable, and dependent on the nature of the mutation of PKHD1, with truncations being more deleterious than point mutants (17). Infants and children develop hypertension, and 20% - 45% of all cases progress to ESRD by age 15 (6). For those that reach adulthood with some renal function, liver disease becomes a dominant complication (6). This exploration of the nature of these two diseases naturally progresses into an interest in wanting to understand them better in the hopes of discovering a cure, or in the case of ADPKD an interest in delaying the onset of symptoms. Though many aspects of the molecular underpinnings of these diseases have been studied, this study set out to examine ADPKD in particular, and the role of TRPP2 in that disease specifically. As there have been studies linking TRPP2 to the endoplasmic reticulum and endoplasmic reticulum stress pathways (23-25), we chose to focus on that aspect of TRPP2.

1.2 Endoplasmic Reticulum (ER) Stress

The endoplasmic reticulum (ER) is a dynamic tubular network involved in many cellular processes, such as gluconeogenesis, lipid synthesis, and protein folding (26). It is also a major intracellular calcium store, giving it an important role in signaling (See section 1.3). It also participates in the biogenesis of

lysosomes and autophagosomes (26). In addition to calcium storage, the ER is also an important site for protein folding, glycosylation, disulphide bond formation, and synthesis of secretory proteins. These ER functions are essential to the life of multicellular organisms - in fact, proteins in the secretory pathway have been estimated to contribute to as much as 30% of the proteome of eukaryotic cells (26). When in the ER, these proteins rely on a specific set of intralumenal conditions to be able to operate correctly, including appropriate calcium and ATP concentrations and oxidation/reduction state (Figure 1.1). In addition to these factors, it is also important to maintain the correct balance of chaperones and degradative proteins in proportion to the amount of proteins being synthesized and prepared for secretion. To ensure the greatest efficiency and fidelity of protein folding, these factors are constantly adjusted by the cell through the integration of protein responses and signaling pathways to multiple cellular and environmental signals (26). In spite of this signaling network, cells still frequently encounter disruptions in the homeostatic conditions that are ideal for correct folding of secretory and membrane proteins in the ER. These perturbations can include an overabundance of protein expression, a perturbation of intralumenal calcium concentration, and inhibition of essential proteins in the secretory pathway. Examples of chemicals that alter ER homeostasis are thapsigargin (A sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) inhibitor (28)), and tunicamycin (an N-glycosylation inhibitor (29)). Perturbation of these lumenal factors important to folding is referred to as ER stress. ER stress is sensed

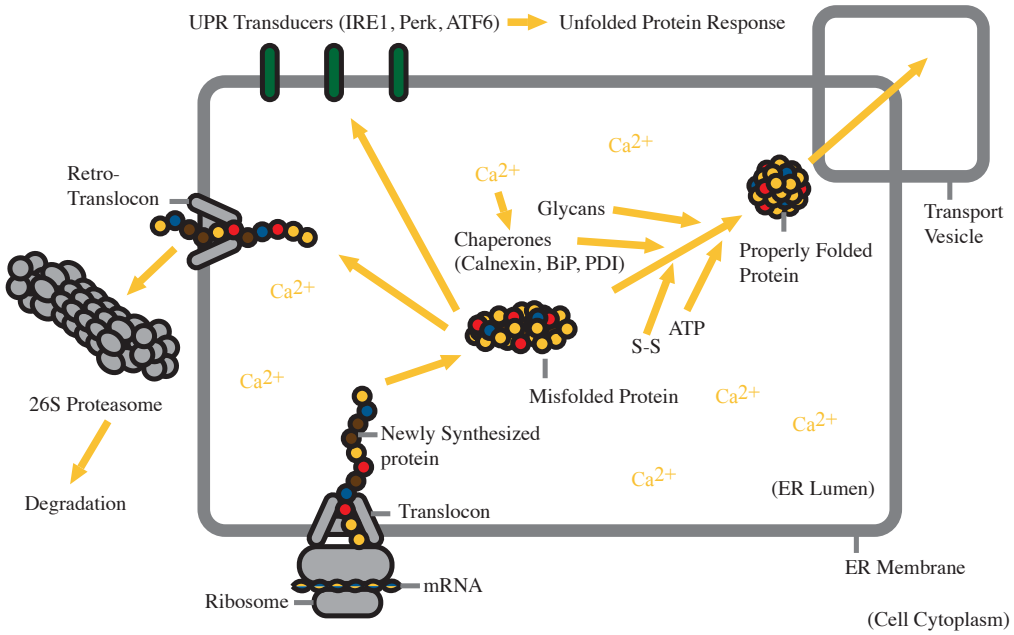

Figure 1.1: ER Stress. Nascent proteins are produced by the ribosome and translocated into the ER lumen. There, they are folded and matured through the action of many factors. Proteins that are properly folded are sensed by the ER, and are transported out of the ER to other cell localizations through the use of transport vesicles. Proper folding can be disrupted through the imbalance of many ER homeostatic factors. Examples of such a state are calcium depletion, glycosylation inhibition, ER redox alteration, and ATP depletion. In the case of disruption to any of these factors, unfolded proteins accumulate in the ER lumen, and initiate a signaling system termed the Unfolded Protein Response (UPR). The UPR leads to upregulation of proteins and initiation of cell signaling in an attempt to correct the problem. Unfolded proteins are also degraded by the ER-associated degradation mechanism through retrotranslocation and proteasomal degradation. Based on (27).

by proteins involved in what is termed the unfolded protein response (UPR). The UPR is the name given to the cellular process where signaling proteins such as IRE1α, PERK, or ATF6 sense the accumulation of unfolded proteins in the ER. This sensing activates signaling pathways seeking to restore normal secretory homeostasis through protein modulation and/or expression. Among the proteins that are upregulated are those that fall into two categories: those involved in proper folding/maturation (generally termed chaperones), and those involved in the degradation of unfolded proteins. Proteins that function in this degradation are considered to be part of the ER associated degradation, or ERAD pathway (see section 1.2.2). Under normal cell conditions, there is a dynamic equilibrium between the actions of chaperones and ERAD pathway members so that there is a maximization of proteins being properly folded and secreted. Under conditions of ER stress, both chaperones and ERAD pathway members are upregulated to try to compensate for deficiencies in the intralumenal environment, and to rescue the cell from the potentially lethal accumulation of misfolded proteins. Should these remediation efforts fail to relieve ER stress over a reasonable time period, pro-apoptotic pathways are initiated. This process is implicated in the development and progression of many degenerative diseases, such as Parkinson's, Alzheimers, amyotrophic lateral sclerosis (ALS) (30), and polycystic kidney disease (23,24,31). Un-alleviated ER stress is also linked to non-degenerative diseases, such as early onset torsion dystonia (32). This has led to an extensive amount of study being done on proteins in this pathway. This introduction will focus on three

aspects of ER stress: ER stress signaling and UPR, the ER associated degradation pathway, and the initiation of ER stress-associated apoptosis.

1.2.1 ER Stress Signaling and the Unfolded Protein Response

As previously mentioned, the initiation of ER stress in a cell leads to the activation of a number of complementary adaptive mechanisms that seek to remedy the imbalance through signaling, a process termed the unfolded protein response (UPR) (26). In addition to the remedying of ER stress-specific imbalances, these proteins participate in a number of important cellular processes. These processes include lipid and cholesterol metabolism, energy homeostasis, inflammation, and cell differentiation (26). Three ER lumenal membrane proteins in particular have been identified to be key players in this system: inositol-requiring protein 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum (ER) kinase (PERK), and activating transcription factor 6 (ATF6) (33). For the sake of relevance, each of these will be discussed in the context of the proteins most directly involved in this thesis.

IRE1α is an ER-resident membrane RNAse with a binding immunoglobulin protein (BiP, also known as GRP78) - binding luminal sensor domain, and a cytosolic domain with kinase and endoribonuclease activity (26,34,35). Upon initiation of ER stress, BiP dissociation drives the oligomerization of individual IRE1α subunits. This, coupled with unfolded protein

binding at the lumenal domain of IRE1α leads to activation of endoribonuclease activity (protein binding initiates endoribonuclease activity, whereas oligomerization increases the rate of cleavage (36)). Since IRE1α possesses autophosphorylation activity, and since phosphorylation of IRE1α monomers increases the rate of endoribonuclease catalytic activity, IRE1α is activated in proportion to the degree of ER stress the protein senses (37). Once activated, IRE1α cleaves several target mRNAs possessing a consensus binding sequence. Among the sequences cleaved is the well characterized X-box binding protein 1 (XBP1) (35). The protein product of cleaved XBP1 mRNA (termed XBP1s) is a transcription factor with greater activity than the protein product of the uncleaved mRNA (termed XBP1u). The XBP1s proceeds to the nucleus where it is involved in the transcriptional activation of genes involved in protein folding and quality control, ER-associated degradation (ERAD), and phospholipid synthesis (26). Among those proteins upregulated are BiP (38), homocysteine-inducible ER resident protein (Herp) (39), hydroxymethylglutaryl-CoA reductase degradation 1 (HRD1) (40), and choline cytidylyltransferase (CCT) (41,42).

PERK is an ER resident type 1 membrane protein with autophosphorylation and eukaryotic initiation factor 2α (eIF2α) kinase activity upon homodimerization (24). It possesses a luminal ER stress sensing N-terminal domain, and a large C terminal cytoplasmic kinase domain (24). PERK is involved in the phosphorylation of its target eIF2α (43,44). eIF2α phosphorylation inhibits global protein synthesis, represses cell proliferation (45), and induces the

translational upregulation of proteins such as C/EBP homology protein (CHOP, also known as GADD153) and activating transcription factor 4 (ATF4) (46). ATF4 is constitutively expressed at low levels, due to the presence of upstream open reading frames (uORF's) in its 5' untranslated region (UTR) (47). These uORF's occupy the translational machinery under normal conditions, preventing efficient translation of the CHOP and ATF4 genes (47,48). Upon induction of ER stress and PERK-mediated phosphorylation of eIF2α, the translational machinery bypasses translation of the upstream sequences, resulting in increased protein translation, and therefore expression of many proteins, such as ATF4 and CHOP (48). ATF4 then translocates to the nucleus, where it is involved in the transcriptional activation of genes involved in autophagy such as microtubule-associated proteins 1A/1B light chain 3 (MAP1LC3B) (49). It is also involved in the transcriptional activation of apoptosis genes such as CHOP (50) and amino acid metabolism such as asparagine synthase (46). Finally, it also transcriptionally activates genes involved in the antioxidant response such as the cystine/glutamate antiporter system subunit xCT (26,51,52). In addition to these, ATF4 is also involved in the transcriptional activation of proteins common to the IRE1 pathway, such as Herp (53). It achieves this transcriptional activation through binding at alternate promoters within the promotor region of these proteins. In particular, ATF4 binds to the the C/EBP-ATF composite site of Herp, as opposed to the ERSE I and II sites that bind XBP1 and ATF6 respectively (39,53).

ATF6 is an integral membrane protein that possesses a basic leu-zipper (bZIP) domain (similar to the bZIP domain in ATF4) in its C terminus, and an ER stress sensing domain on its N terminus (26). In unstressed cells, ATF6 is primarily found in the ER, but upon initiation of stress it is translocated to the Golgi through interaction with coat protein II (COPII), where it is processed by golgi-resident proteases S1P (site-1 protease) and S2P (26). This protealytic cleavage results in the cytoplasmic release of a C-terminal domain fragment (termed ATF6f) with transcription factor activity. ATF6f translocates to the nucleus where it is involved in the transcriptional activation of chaperones such as BiP (54,55), folding factors such as the protein disulphide isomerase (PDI), (54), ERAD components such as ER degradation-enhancing alpha-mannosidase-like protein 1 (EDEM1) (55,56), and XBP1 (57,58). Since Herp and HRD1 are both targets of the XBP1 pathway, these proteins are also considered to be downstream of ATF6 signaling (58). Though Herp contains an ERSE II promotor element (59) that binds ATF6 (53), there is some evidence that ATF6 is not an important direct transcriptional activator of HRD1 (60). However, there is still evidence of HRD1 induction in IRE1/XBP1 knockout cells (60). This suggests that either ATF4 or -6 may still bind to the promotor region (there is evidence for this possibility (61)). However, if ATF4 or -6 activate HRD1 induction, the effect is not as pronounced as that caused by XBP1 (60). Having examined signaling pathways that allow the cell to respond to ER stress stimulus, we now examine a downstream aspect of that pathway, ER associated degradation.

1.2.2 ER associated Degradation

ER associated degradation (or ERAD) refers to the process where substrates in the ER lumen or membrane are targeted for degradation, usually via the ubiquitin-proteasomal system (62). Proteins are synthesized and secreted into the ER lumen in an unfolded state. Once there, the folding process is a dynamic one where chaperones and other folding factors attempt to fold their substrates correctly. While this is taking place, folding factors compete with ERAD proteins that work to identify and selectively degrade substrates that are terminally misfolded, or would be too energy intensive to fold properly (62). The balance of these two processes is usually in favour of correct folding, and is determined by the half life of proteins from both systems (63). Chaperones such as BiP, calnexin, calreticulin, and PDI usually have a long half life and stability compared to ERAD proteins such as Herp, EDEM1 and OS-9 (63,64). As ERAD proteins are constantly degraded, but chaperones are not, chaperones have a greater opportunity to bind unfolded substrates, biasing the process in favour of proper folding. The first step in admission to the ERAD pathway is the recognition of a terminally unfolded substrate. After this recognition step, chaperones are removed and the substrate is ubiquitylated and delivered to the ERAD retro-translocation complex (Figure 1.2) (62,63). That substrate is then retrotranslocated out of the ER lumen in an energy dependent manner, and finally degraded by the 26S proteasome in the cytosolic compartment (62). Treatment with proteasomal

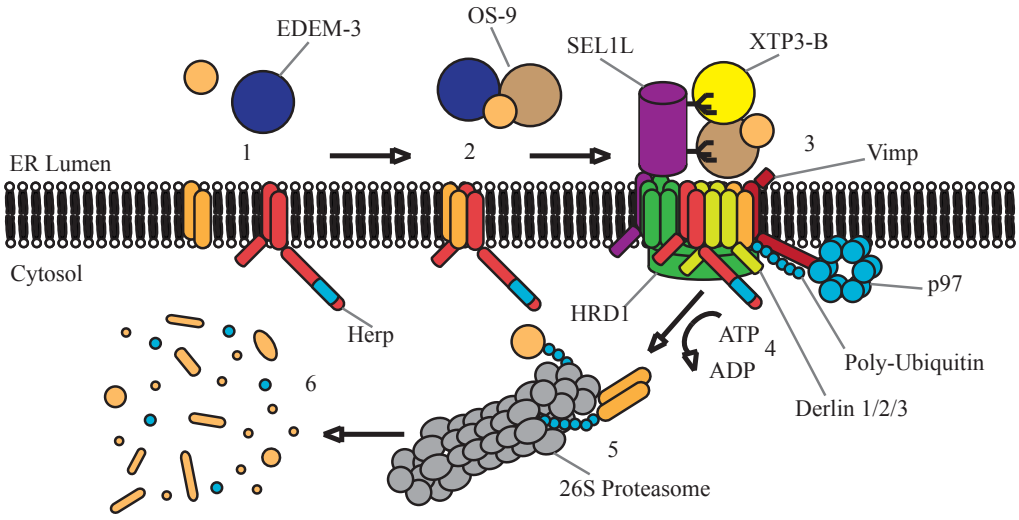

Figure 1.2: ER Associated Degradation. 1) Unfolded proteins in the ER membrane or lumen are recognized by and bind to recognition factors such as EDEM-3, or potentially Herp. 2) The unfolded substrates are shuttled to the retrotranslocation complex either directly by the recognition factors or after being passed on to shuttling factors such as Os-9 and XTP3-B 3) Shuttling/recognition factors dock to receptors on the retrotranslocation complex such as SEL1L, and mediate the ubiquitylation and retrotranslocation of substrates through a complex that includes an E3 ligase such as HRD1. Additional proteins that may compose the retrotranslocation pore in mammalian cell systems include VIMP, p-97 and the derlins. Once retrotranslocated, unfolded substrates are recognized by the 26S proteosome complex and degraded into their constituent amino acids for further metabolism by the cell. Based on (65).

inhibitors, such as MG-132 (66), cause a buildup of unfolded, ubiquitylated substrates in the ER lumen. If increased expression of both the chaperone system and ERAD system are unable to cope with the ER stress for a sustained period of time, the cell initiates apoptotic signaling through the UPR system (26). The following section will examine in more depth the initiation of apoptosis that follows prolonged, unmitigated ER stress.

1.2.3 ER-stress Associated Apoptosis

In conditions of prolonged, unmitigated ER stress and irreversible ER damage, the cell initiates pro-apoptotic pathways. This occurs through the core mitochondrial apoptosis pathway regulated by proteins in the B cell lymphoma 2 (BCL-2) family (26). The first step is the activation of UPR proteins such as PERK and IRE1α. These proteins then cause the activation of transcription factors such as CHOP and ATF4 (activated by IREα and PERK respectively), and P53 (whose mechanism of activation is not well defined (26,46,67). Once activated, these transcription factors upregulate what are referred to as the BCL-2 homology 3 (BH3)-only proteins. Among these BH3-only proteins are BCL-2-interacting mediator of cell death (BIM) (a CHOP-upregulated protein (68)), p53 upregulated modulator of apoptosis (PUMA), and cell death sensitizer NOXA (latin for damage). Of these PUMA and NOXA are regulated by ATF4, p53 and possibly CHOP (26,69)). These proteins serve to conformationally activate proteins such as BCL2-associated X protein (BAX), and BH antagonist or killer (BAK) (26),

whose activation is a key step in the establishment of mitochondrial membrane permeability and release of cytochrome C. Cytochrome C release then initiates caspase activation, and the related stimulation of apoptosis (70). Among the caspases that are implicated in ER stress are caspase 12 (in mice), and caspase 4 (in humans). Of these, caspase 12 is able to activate apoptosis independently of cytochrome C (71). Among the many substrates of activated caspase cleavage are ERAD proteins such as Herp (72) and multiple E3 ligases (73,74), as well as many other proteins including eIF2α (75), the inositol (1,4,5) triphosphate (IP_3) Receptor (IP_3R) type 1 (76), p53 (77), and alpha-actinin (78) (a TRPP2 interacting partner (79)) (80,81). In addition to this pathway, ER stress activates apoptosis through other complementary mechanisms, such as that mediated by the BH3-only protein BH3-interacting domain death agonist (BID) (82), or by ER calcium release. Of these, alterations in ER calcium release may sensitize mitochondria to activate apoptosis (26,83). Now that we have discussed the general principles underlying ER stress and ADPKD, we turn our attention to the general exploration of a topic related to both polycystic kidney disease and ER stress, and of particular relevance to the function of TRPP2, calcium signaling.

1.3 ER Calcium Signaling

The ER has a foundational role in cell calcium signaling. Generally, the highest calcium concentration intracellularly is found in the ER lumen, with estimates having been made from 100 μm to 800 μm, compared with a basal

cytosolic concentration of ~50 nM and an extracellular concentration of ~1 mM (84,85). This calcium store is filled through the operation of what is called the "store operated calcium entry" (SOCE) system. SOCE is a molecular system that fills calcium-depleted ER or SR vesicles with calcium in a mechanism that is dependent on the ER membrane protein STIM1 and plasma membrane Orai calcium channels. Briefly, when the calcium concentration of the ER falls below a certain threshold, STIM1 molecules sense this change through conformational changes in their lumenal EF hand (calcium sensing) domain. This causes oligomerization of STIM1 molecules, and subsequent microtubule-dependent migration of STIM1-enriched ER tubules towards the plasma membrane (86). There, STIM1 complexes associate with Orai calcium-selective plasma membrane channels, and cause their activation. Calcium moves across the plasma and ER membrane from relatively high extracellular concentrations (again, usually about 1mM (85)) to refill the calcium-depleted ER. Once the ER lumenal calcium concentration has reached a sufficiently high level, channel activity is quickly inactivated in a mechanism that is related to the selectivity filter of the Orai channel (87).

Once in the ER, calcium plays an important role in ER lumenal processes, notably protein folding. ER chaperones such as BiP, calnexin and calreticulin all bind calcium, and are functionally regulated by calcium (84,88-90). Though much of the calcium found in the ER is present as free calcium, there is still a significant calcium buffering capacity through calcium binding domains on ER lumenal

proteins. Examples of these include the previously mentioned chaperones calnexin and calreticulin, as well as calsequestrin, reticulocalbin, 55-kDa ER Ca^{2+}-binding protein, reticulocalbin-3, 45-kDa Ca^{2+}- binding protein, and calumenin (84). Cytosolic calcium is generally kept at very low concentrations (again, with a basal concentration of around 50nM (85)), partially through the use of high capacity cytosolic calcium buffering proteins. Cytosolic calcium concentration is kept so low to facilitate cytosolic calcium signaling. Cytosolic calcium signaling is then important for many cell processes such as cell cycle progression, cell proliferation and division (87).

The two most important protein families for ER calcium release and signaling are the IP_3R and ryanodine receptor (RyR) families. These two protein families release ER calcium and activate downstream signaling pathways in response to IP_3 and cytosolic calcium, respectively. ER released calcium plays an important role in gene transcription and other signal transduction pathways such as the extracellular signal-regulated protein kinase (ERK) / mitogen-activated protein kinase (MAPK) pathway (91,92). It also modulates ER mobility and associated cytoskeletal remodeling (84). Cytoskeleton remodeling in general may also be regulated by cytosolic calcium, as calcium influx stimulates actin polymerization, and high levels of cytosolic calcium cause microtubule depolymerization (93). The intense calcium buffering capacity of the cytosol means that cytosolic calcium diffusion is limited. This allows there to be very large differences in local calcium concentration, such as a reported >100 μM at

the mouth of the IP_3R, versus 1 μM only a few micrometers away (94). It follows that there is a great range of temporal and spatial sensitivity to calcium signals, a property fully exploited by the biochemical nature of ER calcium release channels (84).

This calcium signaling is also extensively modified by the presence of a growing group of intracellular calcium channels that release or uptake calcium in the ER and other membrane bound organelles (95). Among these are several members of the transient receptor potential (TRP) family of proteins, which will be discussed in more detail later. Of particular relevance is the channel TRPP2, whose overexpression increases the intensity of IP_3R-mediated intracellular calcium transients (96). As more is known about the action of other proteins functional in intracellular calcium release, a better understanding of intracellular and ER calcium signaling will develop.

Once released from the ER, cytosolic calcium is continually taken back into the ER in an energy dependent manner through the activity of SERCA (84). This protein action ensures that there is always a stable pool of ER lumenal calcium present for signaling purposes, and prevents excessive loss of cytosolic calcium out of the cell. When the normal functioning of SERCA is inhibited (a condition present in some disease states or through pharmacological inhibition such as that of thapsigargin), cytosolic calcium is allowed to reach sufficiently high concentrations as to initiate downstream apoptotic pathways (84). ER calcium release is also the primary mechanism of mitochondrial calcium filling

(84). Mitochondria closely associate with the ER at so-called “mitochondrial associated membranes” (or MAMs). In conditions favourable to mitochondrial calcium uptake, a high local cytosolic concentration of ER-released calcium allows mitochondrial calcium uniporters to import calcium into the mitochondria. Once there, calcium plays a role in mitochondrial functions such as the activation of dehydrogenase enzymes in the Krebs cycle, mitochondrial metabolite transport, mitochondrial motility and apoptosis (84,97,98). During the activation of the intrinsic apoptotic pathway, mitochondria are permeabilized, permitting apoptosome components such as cytochrome C to be released. Although the precise mechanism of this action is unclear, numerous lines of evidence suggest that the most important factor in this process is mitochondrial calcium (98). Accordingly, the ER may signal mitochondrial-dependent apoptosis through this calcium-signaling dependent pathway. Therefore ER calcium signaling plays an important role in cell functioning and communication, and is highly relevant to the functioning of the proteins described in this thesis. Having examined the basic processes that are most relevant to this study, we turn to a family of channels that play an important role in ER calcium signaling, and which contain the primary focus of this study, TRPP2.

1.4 TRP Channels

The (transient receptor potential) TRP channels are an extended family of integral membrane-protein encoding genes that were first described in the fruit fly

Drosophila melanogaster (99). They have been divided according to homology into seven subfamilies, TRPV, TRPA, TRPN, TRPC, TRPM, TRPML, and TRPP (100). It is thought that most TRPs function as homotetramers, although the formation of heteromultimeric complexes between members of the same family or of different subfamilies has been described (77,101,102). A typical TRP protein possesses six putative transmembrane segments with a pore forming re-entrant loop between segments 5 and 6 (100). Intracellular amino and carboxyl termini are variable in length and generally possess a variety of functional domains (100). In animals, TRP channels are expressed in almost every cell type and in both excitable and non-excitable tissues (100). Within the cell, they are present in every cellular membrane except for the nuclear envelope and mitochondria (100). Most TRP channels localize to the plasma membrane, where they play an important role in the influx/transport of Ca^{2+}, Mg^{2+}, and trace metal ions (100). This function is important for many physiological processes. These processes include pheromone signaling (TRPC2), taste transduction (TRPM5/TRPP3), nociception (TRPV1/ TRPA1), temperature sensation (TRPV1-4). TRP channel function is also important for many cellular homeostatic and signaling functions, such as cation reabsorption (TPRPM6/7), osmotic regulation (TRPV1/4) (103), muscle contraction (TRPC1/TRPA1), and vasomotor control (TRPV4) (100). TRP channel function is often regulated by association with accessory proteins involved in many different intracellular pathways and functions (104). An example of this is TRPP2's association with components of the actin cytoskeleton

(79,105). Several TRP genes have been implicated in the development of human disease. Diseases that have some kind of channel deficiency as their cause are described by the term “channelopathies” (100). See Table 1.1 for examples of channelopathies and their linked TRP channels. TRP channel activity has been reported to be regulated by membrane phospholipids such as phosphatidylinositol 4,5-bisphosphate (113,114). TRP channels are also regulated by phosphorylation, including PKC and PKA phosphorylation. Both PKC and PKA phosphorylate TRPV1 and TRPM8 (115-118). Another example is glycogen synthase kinase 3 (GSK3), that phosphorylates TRPP2 (119). TRP channels are also regulated by ligand binding such as the well characterized binding of capsaicin to TRPV1 (115), and of menthol to TRPM8 (118). Because of their physiological importance and disease relevance, ongoing research on these channels is expected to contribute to the understanding of and subsequent development of effective therapies for associated disorders. Having described the general characteristics of the TRP family, we now examine in more detail the most relevant subfamily, that of the TRPP’s.

1.4.1 The TRPP Subfamily

The TRPP, or the transient receptor potential polycystin subfamily, is currently considered to contain three canonical members, based on channel activity. These members are identified as TRPP2, TRPP3, and TRPP5 (70,100). However, the TRPP family could also be said to contain as many as eight

Table 1.1: Channelopathies and linked proteins.

Channelopathy	Linked Protein	Reference
focal and segmental glomerulosclerosis	TRPC6	(106)
autosomal dominant polycystic kidney disease	TRPP2	(7)
skeletal dysplasias termed brachyolmia	TRPV4	(107)
spinal muscular atrophy	TRPV4	(108)
mucolipidosis type IV	TRPML1	(109)
congenital stationary night blindness	TRPM1	(110)
autosomal dominant familial heart block type 1	TRPM4	(111)
hypomagnesemia with secondary hypocalcemia	TRPM6	(112)

members when including non-canonical members (70). Based on homology, it is possible to divide the extended subfamily into two groups, those similar to PKD1 (polycystin-1 or also sometimes called TRPP1), including PKD1, PKDREJ, PKD1L1, PKD1L2, and PKD1L3, and those similar to PKD2 (polycystin-2 or TRPP2), including PKD2/TRPP2, PKD2L/TRPP3 and PKD2L2/TRPP5 (70). Because the PKD1-like proteins lack channel activity when expressed alone (70), the use of TRPP nomenclature for these proteins has not been developed, though it is well accepted in the case of the canonical TRPP channels. In view of this and for the sake of simplicity, those proteins that are part of the PKD1 grouping will be referred to by their PKD1 name, and those that are part of the PKD2 grouping will be referred to by their TRPP name. Because the TRPP grouping (the canonical TRPP's, those similar to PKD2) is more directly relevant to this study, this section will focus on those proteins.

1.4.2 The Canonical TRPP's

Like the other TRPs, the canonical TRPP members possess six transmembrane domains and intracellular N and C termini. However, like the TRPML subfamily but unlike any of the other subfamilies, the TRPP's are also characterized by a long extracellular loop (70). This loop is glycosylated in TRPP2 (120), is predicted to be glycosylated in TRPP3 (121), and is also likely glycosylated in TRPP5, based on homology to the other TRPP members and glycosylation sequence prediction (122,123). Besides TRPP2, for which disease

causing mutants alter glycosylation (120), to date no extensive studies have been done to investigate the role of glycosylation and extracellular ligand binding in TRPP function. Besides this feature, the canonical TRPP's all possess well characterized C-terminal domains including an EF hand domain (a calcium binding structure originally identified comprising the E and F helices of Carp parvalbumin protein (124,125)). In TRPP2, the EF hand domain mediates structural changes upon calcium binding (126). TRPP C terminals also contain an ER retention sequence. In TRPP2 this sequence is important for localization and therefore functional regulation (87). The canonical TRPP's also contain a coiled-coil domain on their c-terminus. In TRPP2 this domain is important for protein-protein interactions and is also involved in localization and function (127,128). In addition, all the TRPP's are homologous to calcium channel α1 subunits (33,129). Of the TRPPs, TRPP2 has undoubtably been the most studied and best characterized, and as it is the most directly relevant has been given its own chapter in this introduction. After TRPP2, the next most studied protein in this family is TRPP3.

The TRPP3 gene was first identified in 1998, shortly after the identification of the TRPP2 gene product, independently by the laboratories of Jing Zhou and Stephan Somlo (33,121). Those studies demonstrated a more restricted expression pattern for TRPP3 than TRPP2. TRPP3 was demonstrated to be calcium activated, calcium permeable non-specific cation channel (130). Since then, TRPP3 expression was demonstrated in a subset of taste receptor cells (131),

and together with PKD1L3 proposed to be a candidate sour taste receptor (132). Mice with the TRPP3 gene knocked out showed a marked reduction in the ability to sense sour taste stimuli, but not a complete elimination (133). This suggests that other molecular players besides TRPP3 are also involved in the sensing of sour taste. The third and last member of the canonical TRPP family is TRPP5.

The TRPP5 gene was first characterized in 1999 by a database homology search for the sequences of both PKD1 and TRPP2 (134), then cloned and characterized the following year (122). It is predominantly expressed in the heart and testis, where in the heart it functions in conjunction with TRPP2 as a large conductance cation channel (135) In the testis, TRPP5 functions in calcium signaling during spermatogenesis (136). Having examined the other canonical TRPP family members, we now move to the most relevant protein to this study, TRPP2.

1.5 TRPP2

As mentioned previously, of the three canonical TRPP channels the greatest body of work has been performed on the structure and function of the TRPP2 channel. As this channel is the most relevant to this study, the most space will be dedicated to it. The most relevant aspects of TRPP2 involve its structure, function, and interacting partners.

1.5.1 TRPP2 Protein Structure and Trafficking

TRPP2 is a 968 amino acid (aa) protein with predicted six transmembrane (TM) domains, a large extracellular loop containing several predicted N-glycosylation sites between TM's 1 and 2, and intracellular N and C termini (Figure 1.3) (7). TRPP2 is expressed in the kidney of fetal and adult humans, and fetal liver, though with little or no apparent expression in human fetal brain (137). In the adult, TRPP2 is expressed in a number of tissues to varying degrees, including red blood cell precursors, smooth, skeletal and cardiac muscle, pancreatic islets, testes, and ovaries. It is of note that there is an apparent increase in relative expression of TRPP2 in neonatal tissues compared with adult tissues (138). In the kidney, TRPP2 is present in both the medulla and cortex, with almost double the medullary expression compared to cortical (139). TRPP2 was found in all segments of the kidney nephron except potentially the glomerulus, however with greater apparent expression in the distal convoluted tubule and cortical collecting duct compared with other segments (139).

TRPP2 contains at least three phosphorylation sites, one on the N terminus (Ser76), phosphorylated by GSK3 (119), and two on the C terminus. Of these, Ser812 is phosphorylated by casein kinase 2 (CK2), regulating TRPP2 trafficking (87) and modulating calcium-dependent channel activity (140). TRPP2 Ser801 is phosphorylated protein kinase D (PrKD). This phosphorylation modulates TRPP2 ER calcium release and associated downstream pro-cell proliferation signaling (141) (Figure 1.3, also chapter 1.5.2). As previously mentioned, TRPP2 contains a

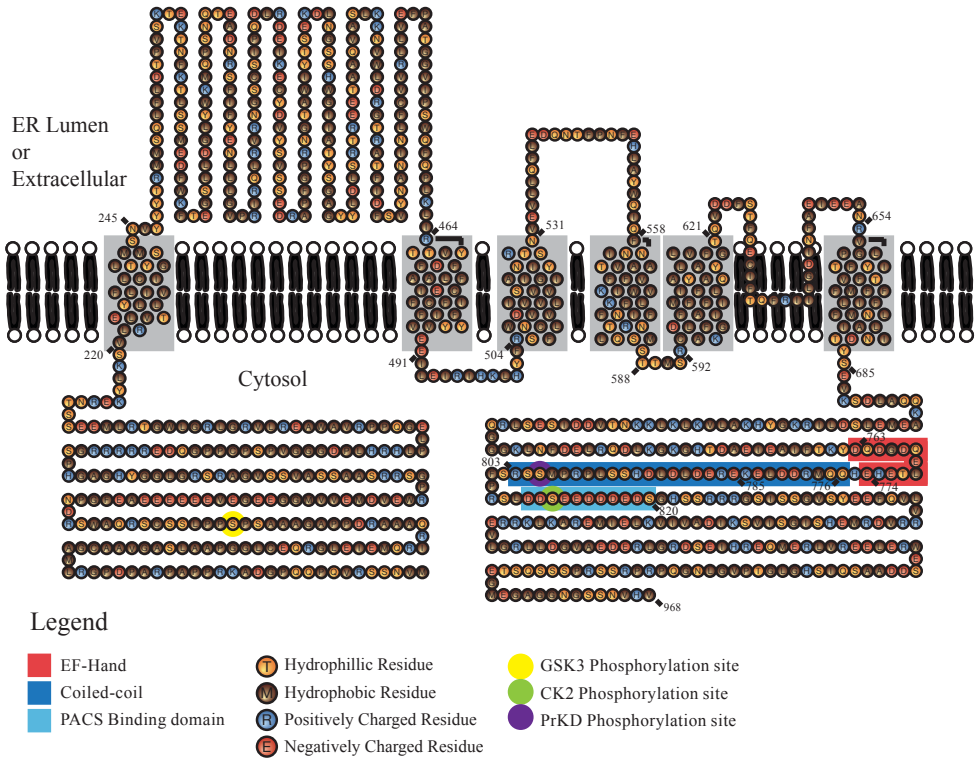

Figure 1.3: Membrane topology of TRPP2. Amino acid structure of the gene product of PKD2 (based on NP_000288.1). Structure shows the predicted cytosolic N and C termini, ER lumen/extracellular loop between TM's 1 and 2, and the predicted pore-forming loop between TM's 5 and 6. Structure also shows the location of the EF hand domain, coiled-coil domain, and PACS-protein binding domain. The PACS-binding domain has also been called the ER-targeting domain. Structure also includes the location of three demonstrated TRPP2 phosphorylation sites.

coiled-coil domain in its cytoplasmic C terminus. This domain interacts with numerous proteins, including PKD1. PKD1 interaction affects TRPP2 trafficking and function, and is one of the factors that leads to TRPP2 ciliary localization (127). Another protein that interacts with TRPP2's coiled-coil domain is PIGEA-14. PIGEA-14 interacts with GM130, a cis-Golgi marker. This interaction regulates TRPP2 trafficking to the cis-Golgi compartment (128). In addition to the coiled-coil domain of TRPP2, there also exists a so-called ER retention motif of acidic amino acids located immediately after the coiled-coil on the C-terminus. This site binds to phosphofurin acidic cluster sorting proteins (PACS) 1 and 2 in a process that is affected by the phosphorylation state of previously mentioned Ser812 (87). PACS protein binding then modulates TRPP2 trafficking. This same site binds the N terminus of IP_3R (142), suggesting that binding of TRPP2 to IP_3R may occlude the site and promote ER retention. TRPP2 also possesses a canonical calcium binding EF-hand domain and a divergent non-calcium binding helix-loop helix motif. This divergent motif may have evolved from a canonical EF hand, such as one found at an analogous site found in invertebrate TRPP2 homologs (126). Calcium binding to the EF hand domain induces conformational changes that may lead to functional modulation (126,143). As mentioned previously, little work has been done to assess the role of ER lumenal/extracellular glycosylation of TRPP2 on protein function. Initial characterization of TRPP2 glycosylation using PNGase F and Endo H (enzymes that cleave N-linked oligosaccharides and high mannose oligosaccharides respectively) showed that TRPP2 is in fact

glycosylated, and that truncation at Leu703 causes differential glycosylation. This differential glycosylation is believed to be linked to the differential trafficking observed between WT and Leu703 truncated TRPP2 (120). However, the observation that TRPP2 is differentially glycosylated in different tissues (144), and that even in systems where TRPP2 is plasma membrane expressed it remain sensitive to Endo H (usually used as a marker of intracellular retention (145,146)) suggest that further studies on this aspect of TRPP2 may be of scientific value.

TRPP2 is homologous to other TRP channels, interacts and oligomerizes with TRPC1 (77), and is homologous to the α1 units of voltage gated calcium channels (though they do not associate (77)). All TRPs are also homologous to other types of voltage gated channels, such as the bacterial voltage gated potassium channels. Although TRPs are generally not considered to be voltage sensitive, some of them have voltage sensitive-like properties under certain circumstances (specifically showing gating at non-physiological membrane potentials (147)). TRPP2 is not thought to be voltage gated, although its TM4 domain (homologous to the voltage sensing domain of voltage gated channels) does contain positive amino acid residues. TRPP2 possesses three positive residues, two lysines and one arginine, (Figure 1.3) versus the canonical five to nine positive residues for a voltage gated channel (148). TRPP2 open probability is voltage and calcium sensitive, though also not calcium gated (96). However, this does not exclude that it may be gated by some other physiological stimulus, or under specific conditions the way that TRPP3 is activated by calcium (130).

Because of the observation that some TRP channels are constitutively active and therefore functionally regulated by intracellular localization and trafficking (70), several studies have investigated this area of TRPP2 molecular biology (87,149). As trafficking is dependent to a large extent on amino acid sequence and structure, I have included it in this section. Discussion about TRPP2 trafficking has been controversial, primarily because of apparent discrepancies in observations of TRPP2 localization between different groups (87,140). While TRPP2 is primarily located in the endoplasmic reticulum (96), there is also a significant body of evidence that it can also be found in the plasma membrane, particularly in the plasma membrane of primary cilia (145). Differences in localization are mediated by a complex interaction between TRPP2 and a number of other proteins binding to different TRPP2 motifs that combined decide TRPP2 location in any given system. Aside from the acidic ER retention motif, the N terminal Ser76 phosphorylation site, and the coiled-coil domain-binding proteins previously mentioned (87,119,127,128), there are other factors that influence TRPP2 trafficking. Among these is the finding that TRPP2 interacts with the epidermal growth factor receptor (EGFR) at the ciliary membrane, and that this interaction is important for EGF-stimulated TRPP2 plasma membrane trafficking and channel activity at the ciliary plasma membrane (150). This finding may point to a role for EGFR in stimulating TRPP2's transport to the cilia. A recent paper also argues that TRPP2 takes separate routes to the plasma membrane and the cilia, and that one of the factors involved is the LysPheIle575-577 sequence on the

cytoplasmic side of the fourth transmembrane domain. This sequence may serve as a plasma membrane localization signaling sequence (151). Therefore, TRPP2 localization is the result of a complex interplay of different proteins and protein transport systems that yields very different subcellular localization depending on cell type and situation.

1.5.2 TRPP2 Function

TRPP2 is a non-selective (152) calcium permeable cation channel with a pore size of approximately 11 Å (153) and unique properties, including frequent transitions between open and closed states over large voltage differences, and a tendency for the open probability to increase with the increase of intracellular calcium (154). Plasma membrane expressed TRPP2 was demonstrated to be insensitive to nifedipine, an inhibitor of voltage gated calcium channels, IP_3, which as mentioned previously stimulates intracellular calcium release, and ryanodine, a chemical that inhibits the RyR (154).

In the ER, TRPP2 operates as a high conductance calcium-activated calcium release channel (96) when regulated in concert with the IP_3R system. Vasopressin, a chemical that causes ER calcium release, activates stably expressed ER-localized TRPP2 activity, but not stably expressed TRPP2 truncation mutant L703X or point mutant D511V activity. In contrast, 2-aminoethyl diphenyl borate, an IP_3R inhibitor, inhibited the vasopressin-mediated effect, but does not inhibit TRPP2 in a lipid bilayer system (96)). The regulation of TRPP2 by the IP_3R

system is mediated through a direct interaction between TRPP2 and IP_3R type 1 (142,155). This interaction occurs specifically by IP_3R interaction with the PACS binding domain of TRPP2 (87). In a human kidney proximal tubule expression system, co-expression of PKD1 was also required to observe TRPP2-mediated increases in calcium release upon addition of IP_3 (156). In addition to these, TRPP2 channel activity is stimulated by alpha-actinin, an actin bundling cytoskeletal protein. Addition of purified alpha-actinin stimulated the single-channel activity of purified TRPP2 reconstituted in a planar lipid bilayer electrophysiology system (79). This suggests that TRPP2 may be subject to regulation by the structure of the cytoskeleton. This regulation may then influence its role in cell adhesion, proliferation and migration (79).

In addition to TRPP2's functional interaction with IP_3R, TRPP2 also interacts with the RyR of heart cells. RyR works similarly to IP_3R in that it also functions to release calcium from the ER/Sarcoplasmic reticulum (SR) (157). Knockout of TRPP2 leads to decreased ER calcium release upon stimulation by caffeine, a RyR agonist, in murine embryonic cardiomyocytes, and a reduced apparent SR calcium storage (157).

There has been some evidence that phosphorylation of TRPP2 at Ser812 increases vasopressin-induced ER calcium release (as overexpression of the TRPP2 S812A mutant decreases vasopressin-induced TRPP2-mediated TRPP2 calcium release (140)). However, this phosphorylation takes place at a residue that is important in TRPP2 trafficking (87,149), and more work needs to be done to

reconcile the data present in both papers that provide different viewpoints in the role of phosphorylation at that site. In addition to Ser812, phosphorylation at Ser801 by PrKD regulates TRPP2 ER calcium release and associated downstream pathways (141). Specifically, ATP-stimulated intracellular calcium transients in Madine Darby canine kidney (MDCK) cells were greater in cells overexpressing TRPP2 compared with control cells, measured by fluorescence of a calcium sensitive dye (141). This effect was also observed when overexpressing the phosphorylation mimic Ser801Asp, but not the phosphorylation inactive Ser801Gly (141). This study also showed that the application of both serum and endothelial growth factor (EGF) in both kidney HEK293 and MDCK cells lead to an increase in phosphorylation at Ser801. This increase in phosphorylation led to a decrease in cell proliferation, assessed by a bromodeoxyuridine (BrdU) incorporation assay (141). This suggests that TRPP2 is involved in cell proliferation signaling through functional release of calcium, and that TRPP2 calcium release is regulated by phosphorylation at the Ser801 residue (141).

Aside from function in the ER, TRPP2 localizes to and potentially operates at various other parts of the cell, including the primary cilia (158), the apical membrane of syncytiotrophoblasts (159,160), lamellipodia (104), and at the centrosomes (161). In the primary cilia, TRPP2 is thought to co-localize with PKD1 on the ciliary membrane. Once there, they both participate in calcium signaling in a manner that is dependent on the RyR (158). TRPP2 is also believed to colocalize on the cilium with other proteins such as fibrocystin, cystin, and

polaris (158,162). As all of these proteins can show cystic phenotypes when mutated, it is suggested that the primary cilia plays an important role in cyst formation (158). In human syncytiotrophoblasts, TRPP2 is expressed in the primary cilia-containing apical membranes (160). In a preparation of reconstituted human syncytiotrophoblasts, TRPP2 functional activity was dependent on microtubular state, with microtuble disruptor colchicine inhibiting TRPP2 channel function and microtubule stabilizer taxol stimulating TRPP2 activity in a lipid bilayer system (160). Also, the addition of chemicals such as hydrogen peroxide that generate reactive oxygen species also inhibit TRPP2 (159). As TRPP2 is hypothesized to regulate the uptake of calcium into the placenta, the presence of oxidative species may decrease the amount of maternal calcium accessible to the fetus during gestation (159). In the lamellipodia, TRPP2 colocalizes with proteins Hax-1 and cortactin, and resides in the same protein complex (104). As cortactin is an important protein in cell motility and invasion, TRPP2 is thought to contribute to that process, though its precise function has not been studied (163). It is of note that another TRPP2 interacting partner, CD2-AP, also binds cortactin, increasing the evidence that TRPP2 is involved in processes related to the actin cytoskeleton (163,164). Finally, TRPP2 is present in the basal bodies and centrosomes of primary cilia, where its presence there is dependent on its interaction with the protein pericentrin (161). siRNA-mediated knockdown of pericentrin is sufficient to prevent localization ofTRPP2 with the basal body of retinal pigmented epithelial (RPE1) cells (161). Though it is possible that TRPP2

carries out some function at this location, it may also be that centrosomal association through pericentrin is merely part of normal ciliary assembly. Having examined TRPP2 in detail we now turn our attention to the two other proteins that are most relevant to this study, proteins in the ERAD pathway: Herp and HRD1.

1.6 Relevant Interacting Partners of TRPP2

Although TRPP2 interacts with a wide range of partners that mediate a very wide range of cellular functions (165), the partners most directly relevant to this study are those ERAD proteins that are upregulated in response to ER stress via the UPR pathway (section 1.2.1). The two proteins most directly involved are the ubiquitin-domain like containing protein Herp (23), and the E3 ubiquitin ligase HRD1 (166).

1.6.1 Herp

Herp is the short form of the gene name HERPUD1 (homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1) (167). The HERPUD1 gene encodes a 361 amino acid, ER resident, integral membrane protein with cytosolic N and C termini, and an ubiquitin-like domain on the N terminus (Figure 1.4) (72,168). Herp is strongly induced in response to ER stress, where it plays a role in ubiquitylation of substrates for proteasomal degradation by the ERAD pathway (169,170). Herp was first identified as being significantly upregulated in response to homocysteine treatment of human

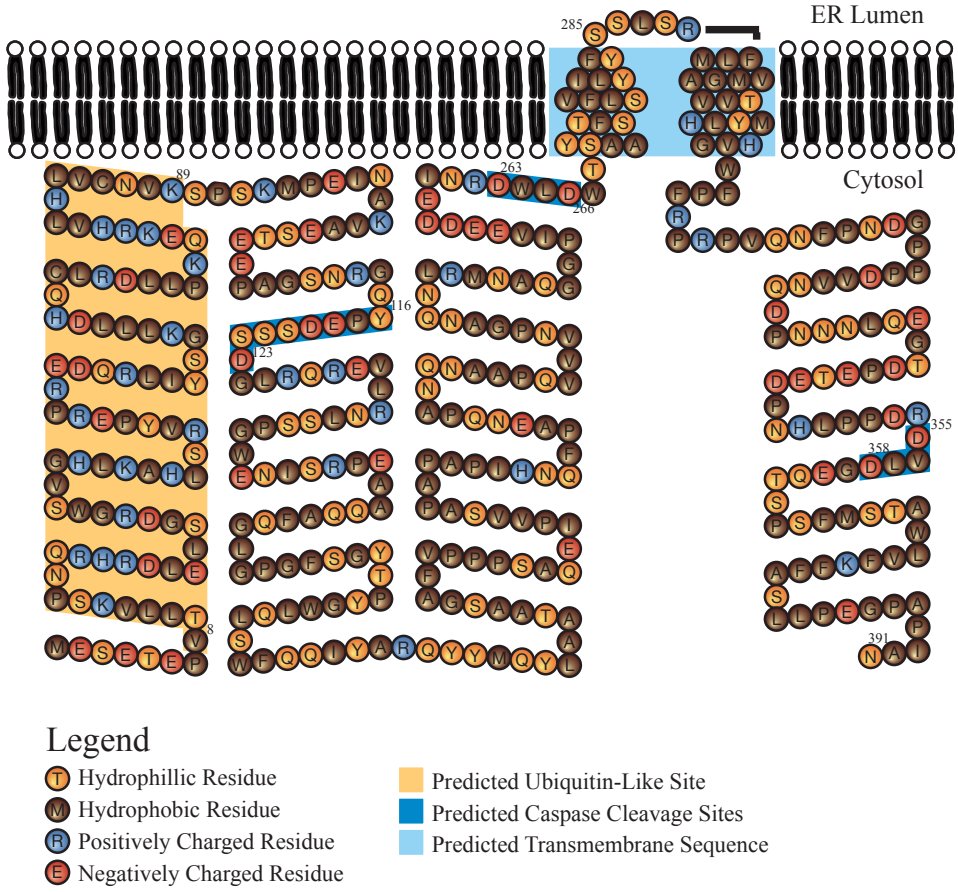

Figure 1.4: Membrane topology of Herp. Amino acid structure of the gene product of HERPUD1 (Isoform 1, based on NP_055500.1). Assignation of the ubiquitin-like domain based on PDB 1WGD. Predicted caspase cleavage sites based on predictions made by CASVM server. Transmembrane domain based on Uniprot sequence annotation and hydrophobicity index. (See section 2.9 for references) Structure shows the predicted cytosolic N and C termini, predicted transmembrane region, and location of ubiquitin-like domain and predicted caspase cleavage sites.

umbilical vein endothelial cells (HUVEC's) (168). Immunofluorescence microscopy suggests that Herp is associated with the ER membrane, and proteinase protection assay results suggest that little if any of Herp resides in the ER lumen (168). The designation of Herp as an integral membrane protein is tentative, based on the presence of 2 ~21 aa domains with hydrophobic and helical character that are presumed to be transmembrane helices (50,171). Herp has been characterized as an anti-apoptotic protein, based on the observation that knockdown of Herp sensitizes neural PC12 cells to apoptosis (72), and the finding that knockdown of Herp leads to an increase in CHOP expression and ER calcium depletion (172). Herp's function to stabilize ER calcium levels is dependent on a functioning ERAD system, suggesting its role is to contribute to the degradation of calcium modulating proteins in the ER (172). Herp forms a complex with other members of the ERAD/retrotranslocation complex, including HRD1, P97 (also known as VCP), derlin-1, and VCP interacting membrane protein (VIMP) (173,174). Overexpression of Herp increases both global ubiquitylation, and ubiquitylation of ERAD substrates (170,175). Herp's interaction with HRD1 regulates HRD1-mediated ubiquitylation of substrates in a way that is dependent on the ubiquitin-like domain of Herp (166). Herp also interacts with ubiquilins, proteins that function as shuttle factors that deliver substrates to the ERAD complex for degradation. This interaction suggests Herp participates in a shuttling role (166,176). Herp is a substrate of caspase cleavage at the $DWLD_{263-266}$ site. This was demonstrated in vivo through treatment with ZVAD-FMK, a broad spectrum

caspase inhibitor, as well as in vitro through digestion of radio-labeled Herp by purified recombinant caspase 3 (72). Herp interacts with the C and N terminus of TRPP2, and is involved in its ubiquitylation and ERAD-dependent degradation (23,25). The last protein needing mentioning in this study is HRD1.

1.6.2 HRD1

HRD1 (hydroxymethylglutaryl(HMG)-CoA reductase degradation 1), also known as synoviolin-1, is an ER resident, 617 amino acid integral transmembrane protein. HRD1 has E3 ubiquitin ligase activity and is upregulated in response to induction of ER stress through treatment with thapsigargin, tunicamycin, and brefeldin A (177). Human HRD1 was identified based on homology to the yeast HRD1p, though the two only share homology in the N-terminal (transmembrane) domain, and the mammalian homolog is not directly involved in the degradation of mammalian HMG-CoA (177-179). Human HRD1 possesses a cytosolic really interesting new gene (RING)-H2 domain in its cytosolic C terminus that is conjugated in vitro by the ubiquitin conjugating enzyme UBC7, and that possesses in vitro ubiquitylation activity at Lys48 (180). HRD1 forms a complex with other proteins involved in the ERAD pathway, including derlin-1, VIMP, P97/VCP, SEL1L, XTP3-B, and Herp (174,181-183). The yeast homolog of HRD1, Hrd1p, is able to mediate the retrotranslocation of a model ERAD substrate (yeast HMG-CoA reductase) in vitro, requiring only a few proteins. Among these proteins are CDC48p (the yeast homolog of P97), Ubx2p (a protein

that anchors CDC48p to the yeast ER membrane, analogous to VIMP-P97 binding in mammals (173)), and proteasomal coupling factors Dsk2p and Rad23p (184). Surprisingly, the N-terminal transmembrane helices of Hrd1p were not required for retrotranslocation. This suggests that it is the cytosolic C-terminal domain of Hrd1p, and therefore potentially human HRD1, that functions in retrotranslocation of ER membrane and lumenal ERAD substrates for proteasomal degradation (184). Human HRD1 binds to and mediates the degradation of human P53 protein. This degradation regulates the biological functions of P53, including transcriptional activation (185,186). In addition to P53, human HRD1 regulates the ERAD pathway dependent degradation of a number of important substrates, examples of which are summarized in Table 1.2. Though apparently not required for retrotranslocation, the transmembrane domain of HRD1 is important for its stability and binding to partner protein SEL1L, whose association ensures the optimal degradation kinetics of ERAD substrates (193,194). HRD1 binds to Herp, whose ubiquitin-like domain regulates HRD1 ubiquitylation of substrates (166). Though no previous studies indicated that TRPP2 and human HRD1 interact, preliminary studies in our lab were confirmed by my data showing their interaction at least in the case of overexpressed, tagged protein. My preliminary results suggest that the TRPP2-HRD1 interaction is involved in TRPP2 ubiquitylation.

Table 1.2: Degradation Substrates of HRD1

Substrate	References	Notes
α1-antitrypsin	(3,182)	
(Pael)-receptor	(187)	Implicated in Parkinson's disease
polyglutamine-expanded huntingtin	(188,189)	Product of the ITI5 gene, implicated in Huntington's disease
GP78	(190)	E3 ligase
cholera toxin	(191)	
neuroserpin	(192)	implicated in the development of familial encephalopathy

1.7 Rationale, Hypothesis and Objectives

Prior to the start of this work, a preliminary co-immunoprecipitation experiment was done by my predecessor Genquing Liang suggesting that TRPP2 and HRD1 interact with each other. This coupled with the previously published data (23) showing that TRPP2 interacts with and is regulated by Herp suggested that the TRPP2-Herp and TRPP2-HRD1 interactions may play an important role in the regulation of TRPP2 degradation. TRPP2 degradation was deemed to be important because both upregulation and knockout of TRPP2 causes kidney dysfunction (195-197). Those studies suggest that small changes in the steady-state protein expression level of TRPP2 have an important effect on its function. In addition, experiments where mice kidneys have been artificially subjected to reperfusion/ischaemia injury show that there is a marked upregulation of renal TRPP2 under those conditions, in a manner that is regulated by TRPP2 dosage (139,198,199). This suggests that TRPP2 is an important player in kidney injury and disease. As TRPP2 is primarily an ER membrane protein, and participates in ER-stress signaling, and because ER stress pathways are very important for cell function, it is suggested that TRPP2's ER function affects kidney injury and disease. Also, since both Herp and TRPP2 are involved in ER calcium release (50,96), and therefore potentially ER-mediated cell proliferation (141,200), differentiation (149), and apoptosis signaling (83), we hypothesized that their interaction is relevant and interesting in the context of TRPP2's contribution to the

development of ADPKD. To this end, we established the following research objectives:

Objective 1: **Further characterize the nature and consequences of the Herp-TRPP2 interaction** -Produce truncation and caspase cleavage mutants of Herp for overexpression in mammalian cells to study the functional effect of their overexpression on TRPP2

Objective 2: Confirm the binding of HRD1 and TRPP2 through Co-immunoprecipitation -Reproduce and confirm earlier findings of HRD1-TRPP2 interaction through Co-IP

Objective 3: Establish the functional consequences of HRD1-TRPP2 binding -Perform experiments to assess if the HRD1-TRPP2 interaction is important for ubiquitylation and/or ERAD- dependent degradation of TRPP2

Objective 4: Establish if Herp is involved in the HRD1-mediated ubiquitylation of TRPP2 -Perform experiments overexpressing both Herp and HRD1 to see if together the effect is distinct from what is observed if either are overexpressed alone.

2 Materials and Methods

2.1 Materials

The mHerpf plasmid (23) was full length Herp with an N-terminal c-Myc tag and a C-terminal flag tag in a pcDNA backbone. The pcDNA TRPP2 plasmid was of a full length TRPP2 cDNA purchased from Thermo Scientific inserted into the pcDNA backbone by Wang Zheng in our lab. GFP-TRPP2 is full-length TRPP2 with a GFP tag on the N terminus in a pEGFP backbone (23). The alpha-1 antitrypsin null Hong Kong (NHK) -HA was a generous gift from Mauricio Molinari (Institute for Research in Biomedicine, Bellinzona, Switzerland), and is full length alpha-1 antitrypsin with the NHK mutation and an HA tag. The HA-Ub was a generous gift from Gerd Walz (University of Freiburg, Freiburg, Germany, though originally by Dirk Bohmann at the University of Rochester Medical Center, Rochester, NY, USA), and is full-length ubiquitin with an HA tag. The HRD1-M and HRD1-M C329S were a generous gift from Michael Seeger (Charité - Universitaetsmedizin Berlin, Berlin, Germany), and are full-length HRD1 with or without the C329S mutation with a c-Myc tag. The mHerpf vector mutants mHerpf D119N, D123N, D266N, D358N, I280-N391, R313X, G359X and S90-N391 (Figure 1.5) were produced by ATGCell in Edmonton, Alberta, Canada and are all c-Myc tagged on the N terminus and Flag tagged on the C terminus.

The ZVAD-fmk used in this study was obtained from Cedarlane, the thapsigargin from Sigma and the DMSO they were dissolved in from Sigma.

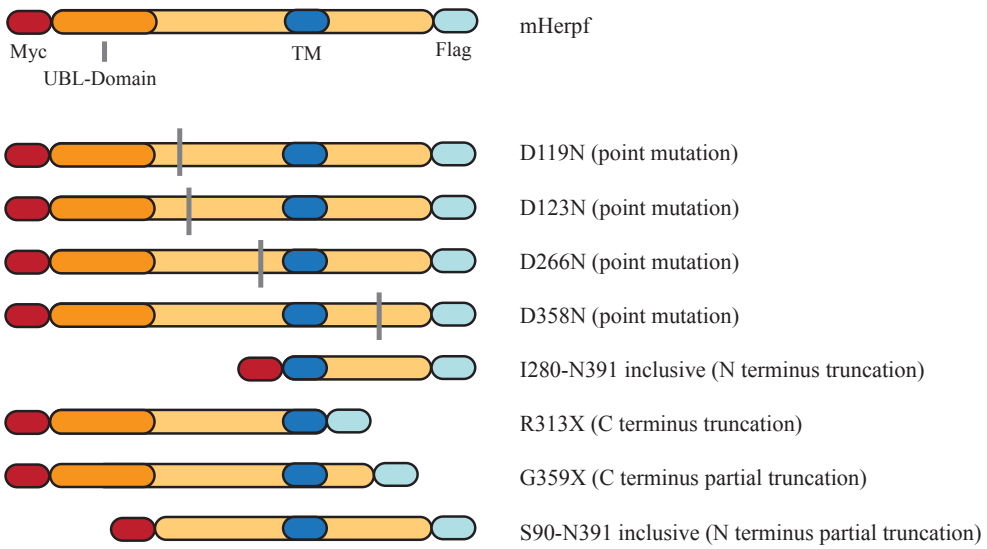

Figure 1.5: Herp mutants. Schematic demonstrates mutations produced in the mHerpf backbone, and the resulting nomenclature describing them.

The primary antibodies directed against c-Myc were from both Cell Signaling (mouse, 9B11) and Santa Cruz (mouse, 9E10). TRPP2 (rabbit, h-280), β-actin (mouse, C4), and HA (rabbit, Y11), and HSP-60 (mouse, H1) were all obtained from Santa Cruz. Anti-ubiquitin (mouse, P4D1) was purchased from Cell Signaling. HRD1 antibody (rabbit, ap2184a) was purchased from Abgent. The endogenous Herp (rabbit) was a generous gift from Koichi Kokame (National Cerebral and Cardiovascular Center, Osaka, Japan). Anti-rabbit (NA934V) and anti-mouse (NA931V) HRP-conjugated secondary antibodies were GE healthcare, and anti-goat HRP-conjugated secondary antibody was obtained from Santa Cruz (sc-2020).

2.2 Cell Lines

Human embryonic kidney (HEK) 293 and HeLa cells were grown in Thermo Scientific water jacketed CO_2 cell culture incubators. During the course of this work, I became aware of the history of HEK 293 cells, and made the decision in consultation with my supervisor to discontinue their use in this project on the grounds of a moral objection. All representative data in this document were obtained using HeLa cells, but HEK293 were used during the repetition of some experiments, as indicated in the appropriate figure. Both cell lines were maintained in Dulbecco's modified Eagle medium (Gibco/Sigma) with 10% fetal bovine serum (FBS) (Sigma), 1% (100 units/ml) penicillin/streptomycin (Gibco), and during periods of acute mycoplasma or white mold contamination of our

incubators, with 2.5 μg/ml Plasmocin (Cedarlane) and 50 μg/ml Normocin (Cedarlane). Cells were subcultured upon confluency using 3 ml 0.25% trypsin EDTA (Gibco) for five minutes, and HeLa cells were cell counted using a Hausser Scientific hemacytometer and seeded for transfection in six-well plates at 250,000 cells/well or 500,000 cells per well depending on when transfection was planned to take place (in two days or one day, respectively).

2.3 Plasmid Amplification

Briefly, plasmids were amplified by streaking a thawed glycerol stock culture on a 10 cm LB-agar plate with the appropriate selection markers (either ampicillin or kanamycin), or by transformation of purified plasmid into chemically competent e-coli cells according to the manufacturer's directions (Invitrogen). Selection marker resistant colonies were selected and incubated in 5 ml LB with the same selection marker for 12-18 hours, then plasmid DNA was purified from bacterial lysates using miniprep kits from Avegene (PD300), Promega (A1222), Sigma (PLN350), and midi/maxiprep kits from Qiagen (12143/12181). Purified DNA was stored at -20 °C until needed.

2.4 Site-Directed Mutagenesis

The mHerpf mutants D119N, D123N, D266N, D358N, I280-N391, R313X, G359X and S90-N391 were produced using by introducing the following changes:

-For the D119N, 355 (nucleotides from the 5' beginning of Herp, with 1 being the A of ATG) 5' GAT 3' was changed to 5' AAC 3'.

-For D123N, 367 5' GAT 3' was changed to 5' AAT 3'.

-For D266N, 796 5' GAT 3' was changed to 5' AAT 3'.

-For D358N, 1072 5' GAT 3' was changed to 5' AAT 3'.

-For I280-N391, the addition of:

"ATGGAGCAGAAGCTGATCTCCGAGGAGGACCTG"

was made prior to 838 5' TAG 3'.

-For R313X, the addition of

"GACTACAAGGACGATGACGATAAGTGA"

was made after 936 5' CCG 3'.

-For G359X, the addition of

"GACTACAAGGACGATGACGATAAGTGA"

was made after 1072 5' GAT 3'.

-For S90-N391, the addition of

"ATGGAGCAGAAGCTGATCTCCGAGGAGGACCTG"

was made prior to 270 5' AGT 3'.

2.5 Transient Expression in HEK293 and HeLa Cells

HEK 293 and HeLa cells were transiently transfected with Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer's directions. Briefly, cells were seeded into six-well plates according to the above

concentrations. At approximately 70% confluency, 0.2-1 µg plasmid DNA and 5 µl Lipofectamine 2000 was diluted into two separate 250 µl aliquots of Optimem (31985, Gibco) media, and incubated at room temperature for 5 minutes. The two aliquots were then mixed thoroughly and incubated at room temperature for an additional 20 minutes. The 500 µl reagent mix was then added to a single well of a six-well plate (Santa Cruz), where the media had been changed to 1.5 µl Optimem. Cells were left with reagent 4-16 hours before reagent was replaced by normal media.

2.6 Cell Collection and Western Blotting

After 48 hours of transient transfection, media was aspirated using suction, and replaced by 2 ml PBS at 4 °C. The PBS was then aspirated and replaced by 500-800 µl (depending on protocol) cell lysis buffer (C2978 Sigma) with 300 µl protease inhibitor (1861279, Fisher). Six-well plates were then placed on a room temperature rocking incubator (Rose scientific/Fisher) for 20 minutes, then harvested into a 1.5 ml microcentrifuge (Fisher/Santa Cruz) tube using a disposable plastic cell scraper (Fisher) for HeLa, or through vigorous shaking for HEK 293. Microcentrifuge tubes containing the lysate were then briefly vortexed and put in a room temperature rotating incubator (Barnstead) for 20 minutes, then vortexed for 1 additional minute. Lysates were then centrifuged at 10,000 RPM (9615RCF) for 10 minutes to pellet the nuclear and cytoskeletal proteins, then the

supernatant was transferred to a fresh 1.5 ml microcentrifuge tube and frozen at -80 °C.

PAGE gels were hand cast using the BioRad miniPROTEAN casting station and the suggested concentrations and proportions. Tris HCL was purchased from Fisher, SDS and Bis/Acrylamide was purchased from BioRad. Unless otherwise indicated, all gels used were 8% Bis/Acrylamide. Protein concentration of samples was assessed using a Bradford assay (using #500-0006 BioRad protein dye) and a Smartspec 3000 BioRad spectrophotometer. 5X loading dye was added, and samples were denatured at 69 °C for 5 minutes prior to loading, then either cooled on ice for two minutes, or frozen at -20 °C overnight. Samples were loaded using Fisher gel loading tips (02707181), and run in running buffer made according to the BioRad protocol at 80 mV for 2 hours using the miniprotean system with a PowerPac Basic power supply. Gels were then soaked in methanol - containing transfer buffer (made according to the BioRad protocol) for 10 minutes, before stacking with BioRad 1620115 0.45 μM nitrocellulose membranes in the transfer assembly. Proteins were transferred onto the nitrocellulose membranes at 110 mV for two hours, transferred to PBS with 1% Tween purchased from Fisher (BP377-500), then blocked one hour using PBST with 3% milk. Primary and secondary antibodies were diluted in maximum 8 ml PBST with 3% milk, at concentrations varying from 1:1000 to 1:4000. Membranes were incubated with milk containing primary antibodies overnight at 4 °C, washed three times with PBST, then incubated with secondary antibodies for

1-4 hours at room temperature. Membranes were then washed an additional 3 times with PBST, then developed using either GE healthcare RPM2106 ECL, or RPN2132 ECL plus on film purchased from ThermoFisher (34091). Films were scanned using a desktop scanner, and analyzed using ImageJ and Sigmaplot V12.0.

2.7 Chemical Treatments

ZVAD-fmk was dissolved in DMSO at a concentration of 7134 μM, and incubated in 1 ml of media with a final concentration of 100 μM. Although the results obtained with this dilution were acceptable, the final DMSO concentration was a bit high at 7%. Thapsigargin was dissolved in DMSO at a concentration of 1000 μM and incubated in media with a final concentration of 0.5 μM.

2.8 Co-immunoprecipitation

Cell lysates were either immunoprecipitated using Dynabeads protein G beads (100.07D Invitrogen) according to the manufacturer's directions, or using protein G beads (17061801 GE healthcare). For the GE healthcare beads, lysates were pre-incubated with 25-50 μL of beads (and for endogenous TRPP2, also with 2 μg rabbit normal rabbit IGG) for 1 hour at room temperature. The antibody (1-10 μl depending on the antibody) was then added and incubated at 4 °C for 6-24 hours. 75-100 μl of beads were then added, and incubated on a rotating incubator for an additional 24 hours. Beads were then washed four times with an

NP-40 substitute-containing lysis buffer (I7771 Sigma), then processed according to standard western blot preparation.

2.9 Figure Preparation

For the Herp amino acid structure (Figure 1.5), assignation of the ubiquitin-like domain based on PDB 1WGD (201). Predicted caspase cleavage sites based on predictions made by CASVM server (202-204). Transmembrane domain based on Uniprot sequence annotation and hydrophobicity index (168,171).

3 Results

3.1 Caspase Cleavage of Herp

Previous work in our lab determined that TRPP2 and disease causing mutants of TRPP2 interact with Herp (23), and this interaction increases the ubiquitylation of TRPP2 (25). Furthermore, Herp is a caspase cleavage substrate of caspase 3, and Herp cleavage has functional implications on the survival of a neuronal cell lineage (72). We set out to examine if caspase cleavage, and generally Herp truncation affected the interaction between Herp and TRPP2, and if this change in interaction had any functional consequences. Overexpression studies of mHerpf showed that in HeLa cells multiple N-terminal truncation fragments of Herp are produced and retained by the cell ((23), also Figure 1.6). These fragments are not produced immediately upon mHerpf expression, but are the result of continual proteolytic cleavage of overexpressed mHerpf. To examine the effect of mHerpf expression and cleavage at various timepoints, we expressed mHerpf in HeLa cells, and harvested at various timepoints (Figure 1.6). In lanes 1-4, only non-specific bands are present, whereas in lanes 5-12 there is observed the expression of full length mHerpf as early as 12 hours (lane 5). In addition, there could also be observed the presence of an ~54 kDa cleavage band of herp as early as 12 hours (lane 5, c-Myc (mHerpf) 15 minute exposure panel), as well as two more cleavage band beginning to appear in lane 7, 36 hours (also in the c-Myc (mHerpf) 15 minute exposure panel). Given this data, we set out to further characterize the nature of Herp caspase cleavage.

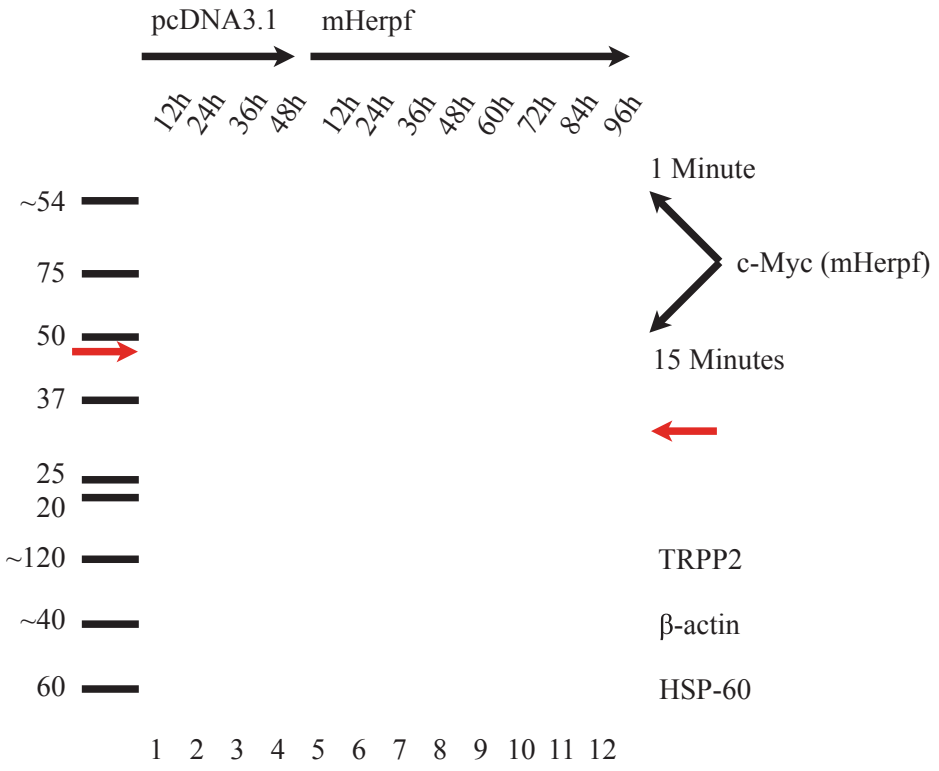

Figure 1.6: mHerpf expression. Expression time course of mHerpf in HeLa cells. HeLa cells were transfected with 1 μg of mHerpf cDNA or empty vector DNA at 0 hours, and lysates were collected at 12, 24, 36, 48, 60, 72, 84, or 96 hours post transfection. Lanes 1-4 represent untransfected HeLa cells collected at the indicated points. Note the presence of 2 non-specific bands (indicated by red arrows) in the untransfected controls. Lanes 5-12 represent HeLa cells transfected with mHerpf. Note the appearance of full length mHerpf at approximately 54 kDa, and caspase cleavage bands at approximately 48 kDa, 30 kDa, and 20 kDa. Lysates were electrophoresed on an 8% PAGE gel and subjected to western blot. Of note is the observation that mHerpf caspase cleavage fragments appear at different intensities and at different times, suggesting that Herp caspase cleavage is regulated in a time-dependent manner.

Although it has previously been suggested that Herp is cleaved at $DWLD_{263-266}$ in PC12 neuronal cells (72), the pattern of cleavage in that system was distinct from the one we observed in HeLa. We also sought to replicate and expand the findings that system. To this end we treated HeLa cells with the same broad spectrum caspase cleavage inhibitor, ZVAD-fmk, as used in the previous paper (Figure 1.7). HeLa cells overexpressing mHerpf treated with ZVAD-fmk for 24 and 48 hours (lanes 3/8 and 5/10 respectively), showed a significant decrease in the appearance of mHerpf N terminal cleavage bands compared with the vehicle controls (lanes 2/7 and 4/9 respectively) at the ~48 and ~20 kDa positions, relative to full length mHerpf at ~54 kDa (black arrows denote the relevant bands for each panel). This was in good agreement with the reported caspase cleavage of endogenous Herp in PC12 cells, resulting in two fragments at approximately ~30 kDa and ~48 kDa from a full length Herp of ~54 kDa. In the previous study, this cleavage occurred both in vivo, and in vitro upon incubation of ^{35}S-labeled, in vitro transcribed/translated Herp with purified recombinant caspase 3 (72). Of note is that the ~48 and ~30 kDa fragments of endogenous Herp reported in that study correlate with the ~48 and ~30 kDa fragments of mHerpf when overexpressed in HeLa cells (Figure 1.8). Although the authors produced, stably expressed, and functionally characterized the caspase cleavage point mutant D266E, no work was done to ascertain if this mutation correlated to one of the observed fragments of Herp (72).

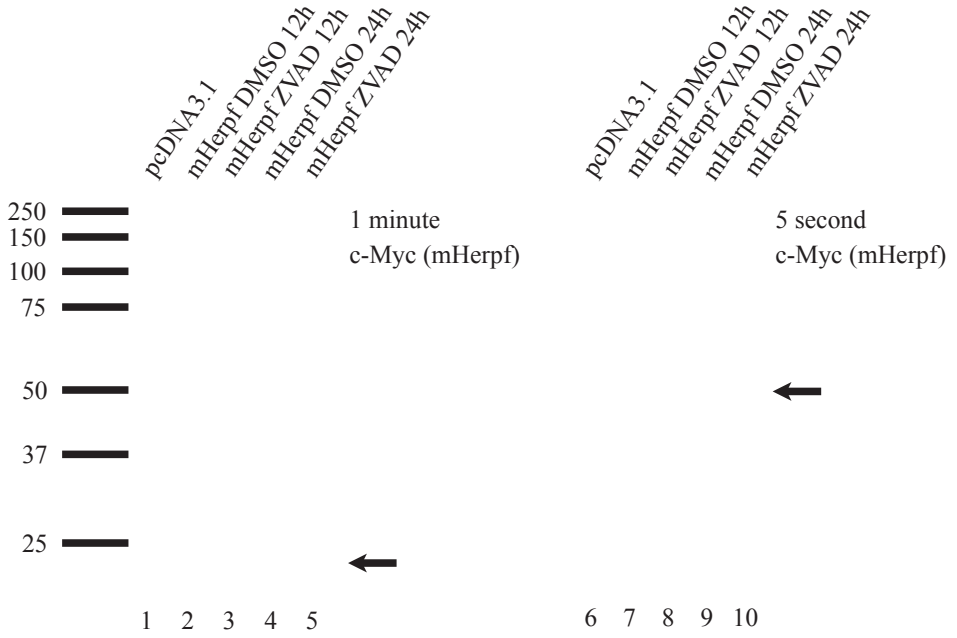

Figure 1.7: Effect of ZVAD-FMK on mHerpf caspase cleavage. Effect of ZVAD-fmk treatment on mHerpf cleavage fragment expression. HeLa cells were transfected with 1 μg of mHerpf cDNA and after 48 hours post-transfection were treated with 45 μg/ml (100 μM) ZVAD-FMK for 12 or 24 hours. Lysates were collected, electrophoresed on an 8% PAGE gel, and subjected to western blot. Lanes 1-5 were all exposed for one minutes, whereas 6-10 were exposed for 5 seconds. Lanes 1 and 6 had the transfection of pcDNA3.1 as an empty vector control. Lanes 2 and 4 (7 and 9) were treated with DMSO as a vehicle control and harvested at the indicated times. Lanes 3 and 5 (8 and 10) were treated with DMSO with broad spectrum caspase inhibitor ZVAD-fmk and harvested at the indicated times. Of note is the observation that under ZVAD-fmk treatment there is a substantial reduction in the appearance of caspase cleavage fragments of mHerpf.

To confirm that the fragments we were observing in HeLa cells were indeed produced by caspase cleavage, and to ascertain what cleavage sites produced the observed fragments, we asked local biotechnology company ATGCell to produce mHerpf point and truncation mutants (Figure 1.5). Overexpression of the D358N point mutant was observed to significantly reduce the appearance of the ~48 kDa fragment upon overexpression compared with WT mHerpf, suggesting that this fragment is produced by caspase cleavage of Herp at the $DVLD_{355\text{-}358}$ site. Our preliminary studies also suggest that the ~20 kDa fragment is produced by caspase cleavage at the D_{123} site, but only N=1 has been performed for this experiment (data not shown). Based on these results and those of the previously published results of Herp caspase cleavage (72), it is also probable that caspase cleavage at the $DWLD_{263\text{-}266}$ site corresponds to the ~30 kDa fragment of mHerpf. However, although a D266N mutant of mHerpf was produced, its effect on the expression of the ~30 kDa fragment was not studied (In HeLa cells, the ~30 kDa fragment shows significantly reduced expression compared to ~48 and ~54 kDa mHerpf compared with cleavage of endogenous Herp in PC12 cells).

To briefly characterize the function of produced Herp caspase cleavage and truncation mutants, we overexpressed those mutants in conjunction with mutated alpha1-antitrypsin NHK, a Herp/HRD1 client (Figure 1.8). We observed a detectable alteration in NHK steady state levels (compare the band intensity of the bands in the HA 10 second panel between lanes 1 and 2 or between lanes 1

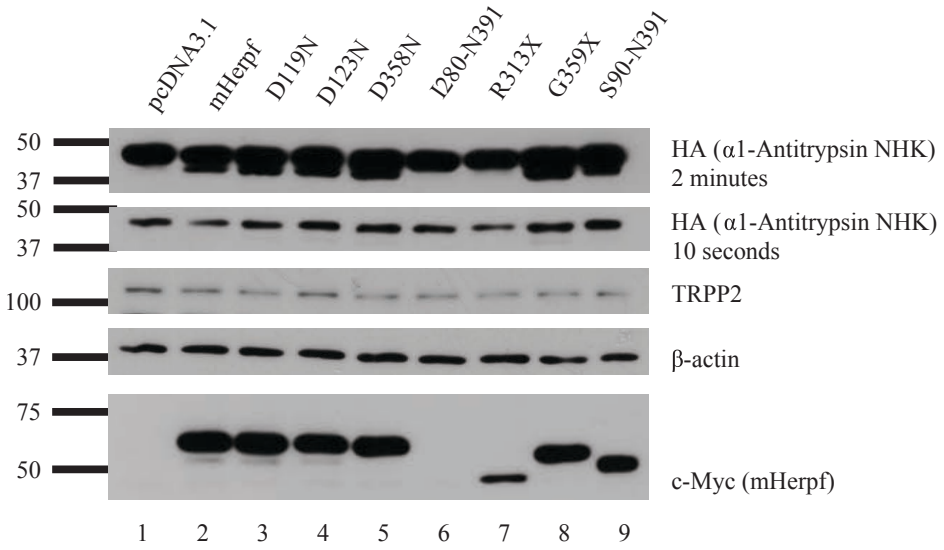

Figure 1.8: Effect of Herp mutants on NHK expression. HeLa cells were transfected with 0.4 μg alpha1-antitrypsin null Hong Kong-HA (NHK-HA) cDNA and 0.4 μg of the indicated mHerpf mutant cDNA. Lysates were collected after 48 hours, electrophoresed on an 8% PAGE gel, then subjected to western blot. Lane 1 represents the empty vector transfected control. Lanes 2-9 represent transfection with one of the produced mHerpf mutants (Figure 1.5). Note the appearance of the predicted deglycosylation band of lower molecular weight than the larger band in lanes 2-5, 8 and 9 of the 2 minute Ha (α1-Antitrypsin NHK) data. Also note the absence of a band in lane 6 of the c-Myc (mHerpf) data.

and 7) and apparent glycosylation (note the appearance of a lower molecular weight band on 2-5, 8 and 9 in the HA 2 minutes panel), though not enough experiments were performed to ascertain statistical significance. Of note, we were never able to obtain a detectable band for Herp I280-N391 (see lane 6, c-Myc panel), though the plasmid had been sequenced by ATGCell prior to our using it, and the DNA concentration was comparable to the other plasmid stocks. This suggests that the S90-I280 segment of Herp is essential for its stability in the cell, a finding supported by the knowledge that C-terminal fragments of Herp are quickly degraded (23). Together, these results suggest that the ~48, ~30, and ~20 kDa cleavage band of Herp correspond to caspase cleavage at the $DVLD_{355-358}$, $DWLD_{263-266}$, and $SSSD_{120-123}$ sites respectively, that Herp caspase cleavage happens at different sites at different times, and that the S90-I280 and R313-D358 segments of Herp are important for NHK de-glycosylation and degradation, but do not affect TRPP2 expression.

3.2 The TRPP2-Herp Interaction

Besides TRPP2 (23), Herp contributes to the degradation of a number of other degradation substrates, among them alpha1-antitrypsin NHK (166), immunoglobulin NS1 κ light chain (NS1 κ LC) (170), cluster of differentiation 3 δ (CD3δ) (176), and nicastrin (205). Of these, Herp interacts directly with TRPP2 (23), nicastrin (205) and NS1 κ LC, but not NHK (170), and is involved in the ubiquitylation of TRPP2 (25), nicastrin (205), and NHK (166). Having produced

truncation and caspase cleavage mutants of Herp, we were interested in seeing if this truncation or cleavage affects the interaction between TRPP2 and Herp, and also the ubiquitylation of TRPP2. To detect if point mutation or truncation of Herp affects TRPP2 interaction, we overexpressed the Herp mutants with GFP tagged TRPP2 in HeLa and HEK cells, then collected lysates and co-immunoprecipitated using an anti-GFP primary antibody (Figure 1.9). mHerpf G359X, a mutant simulating cleavage at the $DVLD_{355\text{-}358}$ site, interacts significantly more strongly with TRPP2 compared with WT mHerpf (compare the intensity of the ~48 kDa band in lane 8 to the analogous one in lane 4 versus the ~54 kDa band in lane 6 compared to the same one in lane 2). The ~48 kDa fragment of WT mHerpf also interacts more strongly with TRPP2 compared with WT (~54 kDa) Herp (compare the ~48 kDa band of lane 6 to 2). Also, the D358N mutant interacts similarly to WT Herp, but not enough experiments were performed to confirm statistical significance in these cases (N=3). In addition to these mutants, we also saw interaction between TRPP2 and Herp R313X and S90-N391, and all of the point mutants except D119N (for which the experiment was not performed), but also did not perform sufficient experiments to arrive at a conclusion regarding interaction strength (data not shown). That being said, interaction between TRPP2 and both the full-length (~54 kDa) and ~48 kDa bands of the point mutants (with the exception of D358N) is similar to WT Herp. The interaction strength was assessed using the following formula: interaction strength = (IP mHerpf band density normalized for TRPP2 immunoprecipitated and relative exposure)/(input

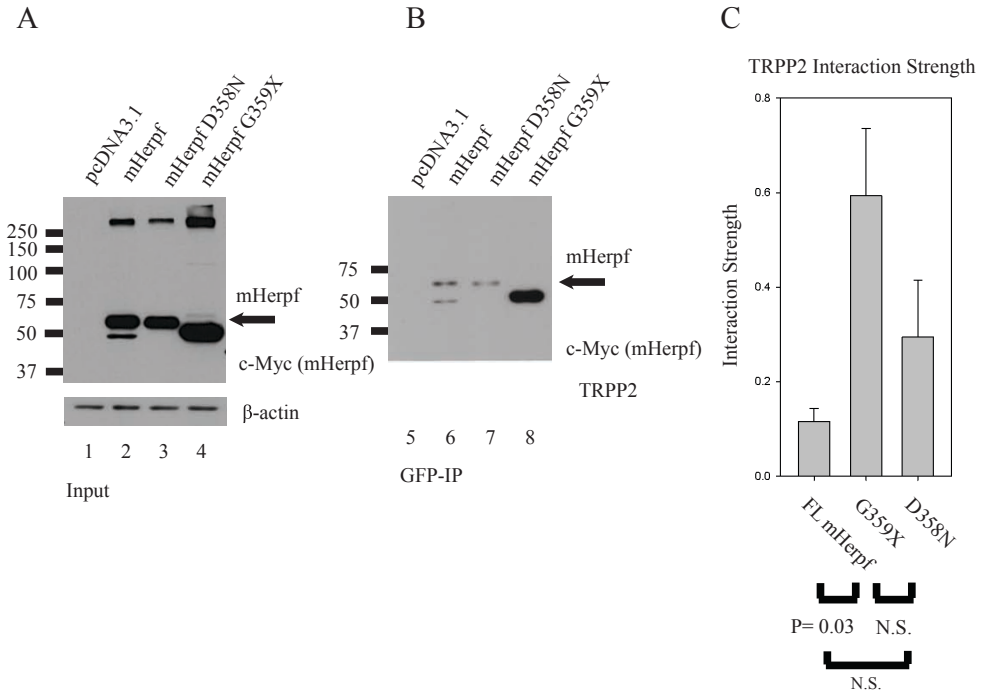

Figure 1.9: The interaction between mHerpf G359X and TRPP2. Relative interaction between TRPP2 and mHerpf or mHerpf fragments. HeLa or HEK293 cells were transfected with 1 µg GFP-TRPP2 and 1 µg of the indicated Herp mutant or empty vector. Lysates were collected at 48 hours and subjected to co-immunoprecipitation using an anti-GFP primary antibody and protein G beads. Input lysates and immunoprecipitates were then electrophoresed on an 8% PAGE gel and subjected to western blotting with the indicated primary antibodies. Band density was calculated using ImageJ, the background subtracted, and interaction strength was calculated according to the formula: Interaction strength = IP mHerpf band density normalized for TRPP2 Immunoprecipitated and relative exposure / Input mHerpf band density normalized for β-actin loaded and relative exposure (IS = (Myc(IP)*TRPP2(IP)/(Myc(input)*β-actin(input))). Statistical significance was calculated with a 2-tailed t-test using the Sigmaplot program. Lanes 1-4 represent the blotting pattern of the 3 mHerpf plasmids indicated, and lanes 5-8 represent the same lysates after having gone through a GFP (TRPP2) IP. Note that the relative intensity of the bands in lanes 6-8 varies in proportion to the intensity of the same bands in lanes 2-4. Also note absence of the ~48 kDa band of D358N (Lanes 3 and 7). A and B are respresentative of the data used to calculate C (N=3).

mHerpf band density normalized for β-actin loaded and relative exposure). This formula can be simplified as (Myc(IP)*TRPP2)/(Myc(input)*β-actin). For clarity, a brief explanation of the methodology follows. Raw band density data was obtained from the ImageJ program using the measure command and a selection box of the same size for every band on a given scanned film and the background. This data was then entered into a spreadsheet. Once on the spreadsheet, the background was subtracted, and the density was adjusted based on the β-actin (for the input) or TRPP2 (for IP) bands. All six data sets were then normalized so that the average density was the same between different experiments of the same result. This was done because the exposure for all three datasets was not the same, which made them difficult to compare. After all normalizations, the IP was divided by the input, resulting in the final numbers used for "interaction strength" in Figure 1.9.

Once the interaction was ascertained, we also sought to discover whether or not the increased interaction resulted in an increased ubiquitylation of TRPP2 by Herp (Figure 1.10). Our findings suggest there is no increase in Herp G359X TRPP2 ubiquitylation relative to WT Herp (compare the intensity of the ubiquitylation band in lane 3 to that of 4, or 11 to that of 12). Note that the ubiquitylation band of both lanes 11 and 12 are still darker than that of lane 10, suggesting that G359X is still functional in increasing TRPP2 ubiquitylation. In summary, TRPP2 interacts with Herp somewhere between S90 and R313, and interacts with all point mutants, though does not interact with I280-N391

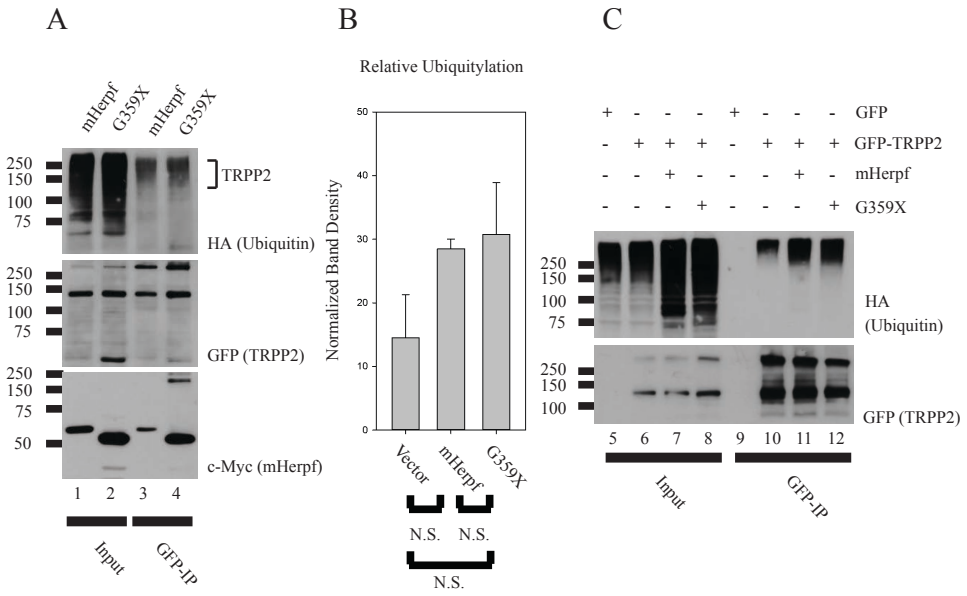

Figure 1.10: Ubiquitylation of TRPP2 by mHerpf G359X. Relative ability of mHerpf or mHerpf G359X mutant to increase ubiquitylation of TRPP2. HeLa or HEK 293 cells were transfected with 1 µg HA-Ub cDNA, 1 µg GFP or GFP-TRPP2 cDNA and 1 µg of the indicated Herp mutant cDNA. Lysates were collected at 48 hours and subjected to co-immunoprecipitation using an anti-GFP primary antibody and protein G beads. Input lysates and immunoprecipitates were then electrophoresed on an 8% PAGE gel and subjected to western blotting with the indicated primary antibodies. For ubiquitylation, band density for the area from approximately 100 kDa to the top of the observable band was calculated using ImageJ, and background subtracted and normalized for TRPP2 precipitated. The three data sets were then normalized for exposure and mHerpf G359X IP ubiquitylation density was compared to mHerpf ubiquitylation density using a 2 tailed t-test and the Sigmaplot program. None of the resulting data sets were statistically significant compared to the others (bottom of part B) Lanes 1 and 2 represent input lysates of the mHerpf plasmid and G359X mutant, whereas lanes 3 and 4 represent the same lysates after GFP-IP. Lanes 7 and 8 reprise the same conditions from 1 and 2, as do 11 and 12 the same data as 3 and 4, only the two data sets are the result of different expriments. Lanes 5 and 6 represent control lanes, with 5 having GFP only transfected (negative control for IP), and lane 6 having GFP-TRPP2 transfected (negative control for mHerpf ubiquitylation). Lanes 9 and 10 are the results of IP of the controls. A contains representative data showing the relative mHerpf expression and the effect on TRPP2 ubiquitylation. B is the analysis of N=3 data sets, with C being representative data from one of the data sets.

(potentially because it was not expressed). TRPP2 interacts more strongly with the ~48 kDa cleavage band of WT Herp, and with G359X, compared to full length Herp, though statistical significance was only found for G359X. Though cleavage at D358 increased Herp-TRPP2 interaction, it did not increase ubiquitylation of TRPP2, though ubiquitylation was observed to be at the same level as WT Herp.

3.3 The TRPP2-HRD1 Interaction

HRD1 ubiquitylates a number of substrates, including the previously mentioned alpha1-antitrypsin NHK (166), Neuroserpin (192), β2-microglobulin heavy chain class 1 (MHC1) (206), GP78 (179,190), P53 (186), and the Pael receptor (187). Though TRPP2 is ubiquitylated and degraded via the ERAD pathway (23), and ubiquitylated by the E3 ligase $SCF^{\beta\text{-}TRCP}$ (207), whether or not it is the only E3 ligases that is responsible for its ubiquitylation was unclear. As multiple E3 ligases can ubiquitylate the same substrate (192), and unpublished studies by our lab suggest that TRPP2 co-immunoprecipitates with HRD1, we sought to confirm the TRPP2-HRD1 interaction, and see whether or not this interaction is involved in the ubiquitylation of TRPP2. To confirm the previous studies, we overexpressed GFP tagged TRPP2 and Myc tagged HRD1 in HeLa cells, and immunoprecipitated using Myc and GFP antibody (Figure 1.11). Both GFP-TRPP2 immunoprecipitates with anti-HRD1 antibody, and Myc-HRD1 with anti-GFP antibody, suggesting that they interact in HeLa cells (note the presence of both bands when immunoprecipitated by both antibodies in lanes 3 and 5

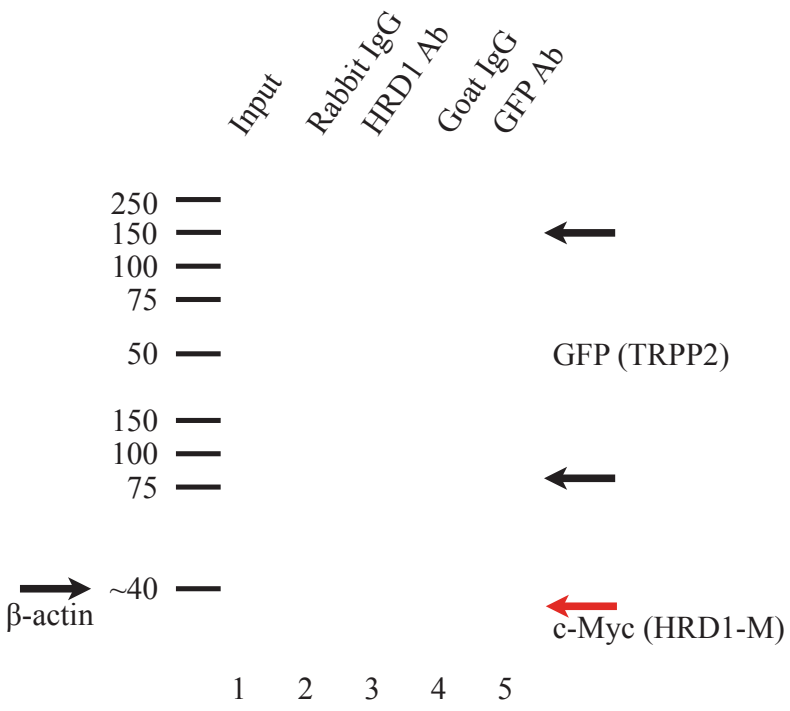

Figure 1.11: Co-immunoprecipitation of HRD1 with TRPP2. Overexpressed, GFP tagged TRPP2 was co-immunoprecipitated with c-Myc tagged HRD1. HeLa cells were transfected with 1 μg of cDNA encoding GFP-TRPP2 and 1 μg of cDNA encoding Myc-HRD1. Lysates were collected at 48 hours post-tranfection. Lysates were then co-immunoprecipitated using the indicated antibodies and Protein-G beads. Input lysates and immunoprecipitates were then electrophoresed on an 8% PAGE gel and subjected to western blotting with the indicated primary antibodies. Lane 1 represents the input lysates for lanes 2-5. Lane 2 represents the negative control (rabbit IgG) for co-immunoprecipitation with the rabbit HRD1 primary antibody. Note in lane 3 the presence of an HRD1 band in the lower panel (confirmation that the co-IP was successful), and the presence of a TRPP2 band in the upper panel (confirmation that TRPP2 co-precipitated with HRD1). Lanes 4 and 5 represent the reciprocal IP, with lane 4 being the negative control (goat IgG) for co-immunoprecipitation with the goat GFP primary antibody. Note in lane 5 the presence of a TRPP2 band in the upper panel (confirmation that the reciprocal co-IP was successful), and the presence of an HRD1 band in the lower panel (confirmation that HRD1 co-precipitated with TRPP2). Red arrow denotes a non-specific band.

compared with the absence of the same bands in the IgG controls in lanes 2 and 4). These experiments were also carried out using endogenous TRPP2 and HRD1 (N=2, data not shown), but the results are difficult to interpret and were not included in this thesis. In the endogenous experiments, HRD1 antibody precipitated a lower molecular weight TRPP2-reacting band than expected (data not shown). This band may represent deglycosylated TRPP2 (to be expected if the interaction is associated with retrotranslocation - retrotranslocation requires prior deglycosylation (208)), or it may represent a non-specific band. The second is more likely as overexpressed, tagged TRPP2 interacts with HRD1 in its fully glycosylated form. To assess if this interaction has any functional relevance, we sought to see whether HRD1 ubiquitylates TRPP2 by overexpressing Myc tagged HRD1. We then immunoprecipitated with TRPP2 antibody, and blotted for ubiquitin (Figure 1.12). Our preliminary results show that overexpression of both HRD1-M, and the ubiquitin ligase activity inactive C329S mutant increases the amount of ubiquitylated TRPP2 relative to the untransfected control (compare the 2 minute ubiquitin band of lanes 10-13 to that of 8 and 9 - 8 being the co-IP negative control and 9 being the ubiquitylation negative control). Interestingly, though overexpression of both Herp and HRD1 increase TRPP2 ubiquitylation, overexpression of the ubiquitylation dominant negative HRD1 also increases TRPP2 ubiquitylation. This suggests that either the plasmid we used for transfection is not the plasmid that we believe it to be (which could be confirmed through sequencing of the plasmid), or that HRD1 increases TRPP2 ubiquitylation

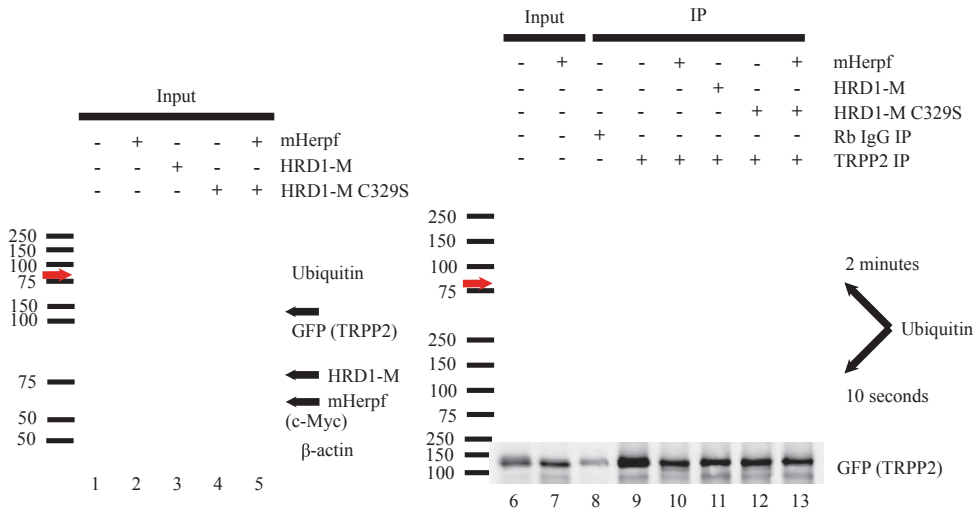

Figure 1.12: Effect of HRD1 overexpression on TRPP2 ubiquitylation. Immunoprecipitation of ubiquitin with overexpressed TRPP2. HeLa cells were transfected with 0.2 μg cDNA encoding TRPP2, and 0.4 μg of cDNA encoding mHerpf or HRD1-M/C329S as indicated. Lysates were collected at 48 hours and subject to co-immunoprecipitation using TRPP2 antibody. Input lysates and immunoprecipitates were then electrophoresed on an 8% PAGE gel and subjected to western blotting with the indicated primary antibodies. Lanes 1-5 indicate the input lysates prior to co-immunoprecipitation, with lanes 1 and 2 being the same lysates as lanes 6 and 7 (but run on separate gels). The lysate in lane 1 was split into two fractions, with one fraction co-immunoprecipitated with rabbit IgG (lane 8) to serve as a negative control for immunoprecipitation, and one fraction co-immunoprecipitated with GFP antibody (lane 9) to serve as a negative control for mHerpf ubiquitylation. Lanes 10-13 are the GFP precipitates of lanes 2-5. Note that all conditions lead to similar ubiquitin bands in the input lysates (lanes 1-5), though with a modest increase with both Herp and HRD1 overexpression. In the GFP precipitates however, not all conditions lead to an equivalent increase in TRPP2 ubiquitylation (lanes 9-13, top panel). Lighter TRPP2 band in the bottom panel of lane 8 compared to the band in the bottom panel of lane 9 confirms the success of the co-immunoprecipitation. Black arrow on the GFP (TRPP2) panel of lanes 1-5 denotes GFP-TRPP2. Red arrows denote non-specific bands, though in lanes 2, 4, and 5 this band may correspond to HRD1-M (based on the observation that it is the correct size and only appears upon HRD1 overexpresion).

through some means other than direct ubiquitylation. It may also be that the HRD1-TRPP2 interaction is primarily for retrotranslocation of TRPP2, with an increase in ubiquitylation being a result of increased retrotranslocation complexes being available. In summary, TRPP2 co-immunoprecipitates with HRD1 in HeLa cells, and preliminary results suggest HRD1 is somehow involved in the ubiquitylation of TRPP2.

3.4 Effect of Herp on the HRD1-Mediated Ubiquitylation of TRPP2

As stated in 3.2, of the Herp substrates mentioned studies were done to show a Herp-mediated increase of substrate ubiquitylation only for NHK (166), nicastrin (205), and TRPP2 (25), though it is probable that Herp is involved in the ubiquitylation of the other substrates as well. In particular, Herp regulates the HRD1-dependent ubiquitylation of NHK in a manner that depends on Herp's UBL domain (166), suggesting that Herp may regulate the HRD1-mediated ubiquitylation of other substrates. Having already confirmed the interaction between Herp-TRPP2 and HRD1-TRPP2, and obtaining data suggesting that the HRD1-TRPP2 interaction is important for the ubiquitylation of TRPP2, we sought to also find whether or not Herp regulates the HRD1-mediated ubiquitylation of TRPP2. To this end we overexpressed mHerpf at the same time as we were performing our experiments on the ubiquitylation of TRPP2 by HRD1 (Figure 1.12). Interestingly, the increase in TRPP2 ubiquitylation was reduced when both

mHerpf and catalytically inactive (C329S) HRD1 was overexpressed together (lane 13 of Figure 1.12) compared to just mHerpf, HRD1-M or HRD1-MC329S overexpressed alone (lanes 10-12 of Figure 1.12). This suggests that either Herp overexpression inhibits HRD1 ubiquitylation of TRPP2, or that there is some interaction between the two overexpressed proteins that mitigates TRPP2 ubiquitylation. This is unusual in the light that similar expression studies showed a different result for Herp and HRD1 substrate alpha1-antitrypsin NHK (166), though it is notable that there, Herp overexpression alone does not increase NHK ubiquitylation the way it does for TRPP2, and Herp and HRD1 were not overexpressed together. In summary, our preliminary results suggest that HRD1-mediated ubiquitylation of TRPP2 is regulated by the expression level of Herp, but it is unclear whether or not that is the result of the Herp-HRD1 interaction, or the result of some other mechanism. Our proposed model of the Herp and HRD1-mediated degradation of TRPP2 can be found in Figure 1.13.

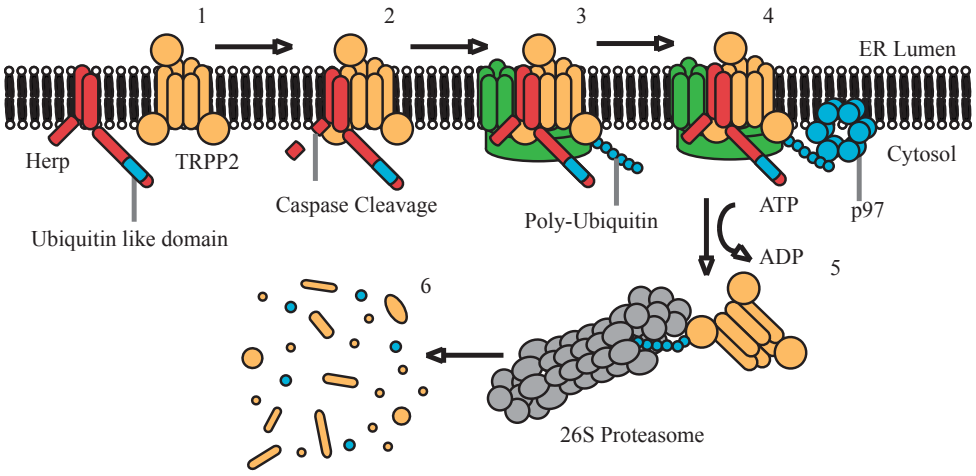

Figure 1.13: Proposed model of TRPP2 degradation. 1) TRPP2 is recognized by and binds to Herp. 2) Herp may be protealytically cleaved by caspases in a manner that increases binding to TRPP2. 3) TRPP2 is delivered by Herp to the retrotranslocation complex, which contains HRD1, and is poly-ubiquitylated. 4) TRPP2 is retrotranslocated into the cytosol in an energy dependent manner that requires p97. 5) TRPP2 is recognized by the 26S proteasome. 6) TRPP2 is degraded into its constituent amino acids.

4 Discussion and Future Experiments

4.1 Discussion

Despite a significant body of work done to elucidate the effect of TRPP2 on cell proliferation, differentiation, and apoptosis, details of its role in these pathways remains elusive (156). One promising avenue of research focuses on its role in ER calcium release (156). As overexpression of TRPP2 affects ER calcium release (96), this function may be regulated by ER associated degradation similar to the mechanism that regulates HMG-CoA reductase (184). Among the many proteins that participate in ER associated degradation, human Herp and HRD1 possess unique properties. Herp is unique because of its prompt and dramatic upregulation in response to ER stress (169), and HRD1 because of its apparent importance to the retrotranslocation of ERAD substrates (184). It was with this interest that we examined the interaction between TRPP2 and these proteins.

TRPP2 possesses remarkable stability in cells (both in our experience and the published observations of others (151)). This character is not surprising, given that both downregulation and upregulation of TRPP2 results in kidney defects (195,196). Thus, the study of its functional regulation by ER-associated degradation represents an important way we can understand its role in development of ADPKD. In the present study we show that not only does Herp bind TRPP2, but this binding is subject to regulation by caspase cleavage. Also, Herp regulation occurs not only through the apparent removal of Herp from the ER membrane but also potentially by covalently modifying the binding site of Herp to bind TRPP2 more strongly. Caspase cleavage of Herp occurs at different

sites at different times over a relatively long time frame. This suggests a mechanism of either progressive regulation, or regulation then inactivation of Herp. This complex regulation may fine-tune the degradation of TRPP2 and other substrates based on how far along a given cell is in the gradual progression from adaptive to chronic to terminal ER stress. Though caspase cleavage of Herp at the $DVLD_{355\text{-}358}$ site increases binding to TRPP2, it does not increase ubiquitylation. This suggests that either there is a saturation of possible TRPP2 ubiquitylation at the respective E3s (potentially including HRD1), or that increased Herp-TRPP2 binding only increases the rate of shuttling to the ERAD complex (a possibility not tested), or it participates in some other function of Herp that requires binding.

The purpose of the ubiquitin-like domain of Herp remains enigmatic, as although it is important in the functional increase in ubiquitylation of substrates by Herp (166), it did not play an important role in TRPP2 binding. This study does however confirm the observation (176) that C-terminal fragments missing large portions of the N-terminus while retaining the transmembrane domain are substantially less stable without it. (Non-membrane associated flag-tagged mHerpf fragments are generally not seen in western blots (data not shown), and the Herp N-terminal truncation mutants we overexpressed showed remarkable instability. In particular, the S90-N391 mutant showed low expression and the I280-N391 mutant no apparent expression). This is also interesting given that Herp degradation is decreased in the absence of the UBL (175). It has been suggested that this domain is involved in a sort of modular assembly, where

complex chains and branches of ubiquitin-like molecules are added and removed by their respective ligases and proteases. This complex modular modification of ubiquitin-like molecules has been demonstrated in many systems, and affects virtually every cell signaling pathway (209).

Given that Herp caspase cleavage at $DWLD_{263-266}$ and $SSSD_{120-123}$ creates N-terminal fragments that do not contain the transmembrane domain, it is expected that fragments cleaved there would no longer be membrane associated. Indeed, our experiments show that cleavage likely occurs at these sites (refer to the 30 kDa and 20 kDa cleavage bands of Figure 1.6). If this is true, one of the roles of caspase cleavage of Herp may be to liberate it from membrane association. Because the effect of Herp on TRPP2 appears membrane associated, what the function of non-membrane associated fragments of Herp is, and whether or not that has any importance to its functional interaction with TRPP2 remains to be seen. (Our experiments suggest that the Herp TM is necessary for TRPP2 interaction, data now shown. For data showing that TRPP2 does not associate with Herp N-terminal fragments, see (23)) One possibility is that cleaved Herp may participate in cytosolic protein signaling, potentially through covalent addition to a protein or pre-existing ubiquitin-like chain (209). What portion of Herp binds TRPP2 remains elusive. It remains this way because although some Herp fragments bound TRPP2 and some did not, the character of TRPP2-binding fragments suggests that TRPP2-Herp binding is complex. Interestingly, TRPP2 WT, N truncation or C truncation all bind Herp, but the truncation of both TRPP2

N and C termini eliminates binding (23). Therefore, either a similar binding site is present on both termini, or Herp may bind to TRPP2 and other proteins through a recognition of a specific substrate character. If so, Herp binding would be similar to how many chaperones function by binding to exposed hydrophobic portions of their substrates (210).

A general rule for ubiquitylation of substrates is that while there are relatively few E1 and E2 proteins (E1's and E2's activate and conjugate ubiquitin to the E3 ligase respectively), there are relatively many E3 ligases with strong apparent affinity for their particular substrates (209). That being said however, there is strong evidence that any given substrate may be ubiquitylated my multiple E3s (192). This functional redundancy ensures that no potential substrates are missed, an important consideration in preventing the accumulation of unfolded substrates (192). Thus, while $SCF^{\beta\text{-}TRCP}$ is involved in the ubiquitylation of TRPP2 (207), it may represent but one of many E3 ligases that together ensure that TRPP2 is ubiquitylated and degraded in an appropriate manner. Although HRD1 may not directly ubiquitylate TRPP2, it does play a role in TRPP2 ubiquitylation. As the ubiquitylation of TRPP2 represents an important means of its regulation, the identity of this role merits further study. Of note is the finding that it is the cytosolic domain of Hrd1p is most important to the retrotranslocation of substrates, but that this domain in human HRD1 bears little homology to the yeast (184). It could be however, that HRD1 is homologous to Hrd1p in the same way as Herp is homologous to the yeast Usa1p protein. For Herp/Usa1p, although

they bear little detectable sequence homology, Herp is able to functionally replace Usa1p in a yeast deletion mutant (211). In a similar way, the cytosolic domain of human HRD1 may be functionally equivalent to the cytosolic domain of yeast Hrd1p in spite of little remaining sequence homology. That HRD1 ubiquitylates and retrotranslocates some substrates (166), but does not ubiquitylate other retrotranslocation substrates remains an interesting phenomena (188). It is possible that what one of these two functions are exercised by HRD1 depends on the particular sequence and character of the substrate. How TRPP2 is processed by HRD1 may be in reference to TRPP2's integral membrane nature, or differential glycosylation, or particular sequence compared to other HRD1 substrates.

Another enigmatic feature of this study is that co-expression of Herp and HRD1 C329S decreased the amount of TRPP2 ubiquitylation relative to either overexpressed alone. As this experiment has only been carried out twice, it requires further repetition to confirm that this surprising result accurately reflects the normal situation. To the best of my knowledge there is no previous record of the effect of such a co-expression on the ubiquitylation of a substrate being published. That and the observation that different substrates do tend to show quite different ubiquitylation patterns under different conditions (166,188) suggests that this could be an underlying feature of the Herp/HRD1 complex. Just as it is unclear whether or not HRD1 ubiquitylates TRPP2 directly, it is also unclear whether or not Herp requires functional HRD1 to ubiquitylate TRPP2. As the

effects seen were dependent on co-overexpression of Herp or HRD1, identifying this particular aspect of Herp-mediated increases of TRPP2 ubiquitylation would be better suited to the use of HRD1 siRNA. Of note is that although siRNA was used in some of the experiments of this study (data not shown), the use of HRD1 siRNA in particular was not. It is interesting that in previously published results (166) and in our findings even catalytically dead HRD1 C329S was able to increase the amount of global ubiquitylation (though not HRD1 substrate-specific ubiquitylation). This suggests that the regulation of ubiquitylation in HRD1 overexpression systems is complex and may be carried out by other E3s through HRD1. Among the factors that may affect this system is the observation that overexpression of HRD1 induces sufficient ER stress to upregulate endogenous Herp (166). As this suggests that ER-stress signaling pathways are activated, this may be a confounding factor in this study.

In addition to their role in TRPP2 degradation, it is worthy of mentioning that the interaction of both HRD1 and Herp with TRPP2 may have some other cell function. Indeed, TRPP2 interacts with numerous other proteins in a wide variety of pathways that may implicate TRPP2-ERAD protein binding in other cell functions (165). In particular TRPP2 participates in the ER calcium signaling of apoptosis (212). The subsequent activation of caspases may then feed back to inactivate or modulate TRPP2 through its interaction with Herp. This modulation/ inactivation may also participate in more wide-scale cell remodeling - suggesting that caspase cleavage of TRPP2-bound Herp may be important for cell

remodeling processes (80). To the best of my knowledge, no studies have been done to ascertain whether or not HRD1 possesses caspase cleavage sites and/or is subject to caspase cleavage. Use of the CASVM caspase cleavage prediction server predicts at least 2 caspase cleavage sites on the long C-terminal cytosolic domain of HRD1: $MPED_{595}$ and $GEPD_{599}$ (202-204)). Since many other E3 ligases are regulated through cleavage (73,74), it would not be unusual to find that HRD1 is also regulated in this manner. Further study of the role of caspase cleavage in regulation of proteins such as Herp, HRD1, and TRPP2 will help us better understand how they contribute to cell functions and the development of ADPKD.

4.2 Future Experiments

Much remains to be done to fully profit from the findings of this study. Our findings indicate that Herp possesses at least one but as many as three caspase cleavage sites. Though from our findings and preliminary studies it seems probable that each of those sites matches one of our point mutants, further repetitions and confirmations are required. Once that is done there remains the functional characterization of the mutants. Our preliminary findings with NHK can easily be expanded to see if Herp caspase cleavage and/or truncation affect NHK ubiquitylation. Although it was always a desire of this study to provide evidence of a Herp mutation differentially regulating TRPP2's steady state expression level versus wild type Herp, this proved difficult because of the

relatively small effect Herp overexpression had on TRPP2 expression level. This was true even under the influence of translational disruption such as by the use of translation inhibitor cycloheximide or during analysis after radioactive ^{35}S metabolic labeling. There has been reported some evidence that cycloheximide induces upregulation of specific substrates through inducing the phosphorylation of eIF2α (213). As eif2α is regulated by TRPP2 (24), this suggests that cross-talk between proteins involved in translational modulation may affect TRPP2 expression in the presence of cycloheximide. In the context of this study, this may have contributed to the demonstrated high stability of TRPP2. As more is known about the regulation of TRPP2 transcription and translation, further studies of TRPP2 degradation will be able to isolate degradation from other regulatory effects, and be able to report with more clarity the function of ERAD pathway members on TRPP2. Of note is that although there was generally a noticeable decrease in TRPP2 expression upon mHerpf overexpression, the effect was very small. This made comparisons between WT and different mHerpf mutants difficult. Of more promise were studies using Herp siRNA, which consistently showed a more pronounced TRPP2 expression increase (data not shown).

Although this study showed that Herp G359X had a statistically significant increase in interaction with TRPP2 versus WT Herp, a few other mutants, such as the D358N mutant, also showed promising results. Confirmation of interaction strength for these mutants surely would have only required a few more repeats to result in statistical significance, but time did not permit those repetitions. This

information on relative binding strengths would have been useful in determining the relative effect of different mutations of Herp. This information would then have shed some more light on the influence of caspase cleavage on Herp function. Although preliminary results were obtained assessing the effect of ubiquitylation of TRPP2 by all of the Herp mutants, experimental errors throw the results in doubt. Repetition of this experiment could also lead to a better perspective on the functional effect of Herp cleavage on its interaction with TRPP2. Preliminary data suggest that altering the Herp-TRPP2 interaction by greatly overexpressing the C terminus of TRPP2 relative to WT TRPP2 may also affect TRPP2 ubiquitylation (data not shown). Though this line of thinking was interesting, we were unable to prove that this overexpression in fact affects interaction, or repeat the experiment sufficiently to use the results.

In spite of efforts to prove that native TRPP2 interacts with native HRD1, our two experiments in that direction were unsuccessful. This could have been a result of low apparent HRD1 expression coupled with low affinity of the HRD1 antibody to HRD1. For the antibody we used, endogenous HRD1 was detectable, but at a level much lower than that of non-specific bands. In the second experiment, we attempted to induce HRD1 levels with ER stress inducing agent thapsigargin, but without apparent improvement to the results. Purchase of another HRD1 antibody or refinement in the IP technique in response to HRD1-specific difficulties may be necessary to bring final confirmation to this experiment.

Finally, further repetition of the initial results suggesting that HRD1 increases ubiquitylation of TRPP2 is required to confirm the finding. Once completed, these further studies could significantly improve our understanding of the Herp-TRPP2 and HRD1-TRPP2 interaction. More knowledge of these interactions should then shed some light on TRPP2 function and regulation. Once more is known about the role of TRPP2 protein function in the molecular development of cysts, a more comprehensive picture of the role of TRPP2 in ADPKD pathology will surely follow.

5 Bibliography

1. Dell, K.M. (2011) The spectrum of polycystic kidney disease in children. *Adv Chronic Kidney Dis,* **18,** 339-347.
2. Grantham, J.J., Mulamalla, S., and Swenson-Fields, K.I. (2011) Why kidneys fail in autosomal dominant polycystic kidney disease. *Nat Rev Nephrol,* **7,** 556-566.
3. Torres, V.E., and Watson, M.L. (1998) Polycystic kidney disease: antiquity to the 20th century. *Nephrol Dial Transplant,* **13,** 2690-2696.
4. de Almeida, E.A.F., and Prata, M.M. (2008) First description of polycystic kidney disease in a Portuguese journal. *Nephrol Dial Transplant,* **23,** 1831-1833.
5. McManus, D.P., Gray, D.J., Zhang, W., and Yang, Y. (2012) Diagnosis, treatment, and management of echinococcosis. *BMJ,* **344,** e3866.
6. Bisceglia, M., Galliani, C.A., Senger, C., Stallone, C., and Sessa, A. (2006) Renal cystic diseases: a review. *Adv Anat Pathol,* **13,** 26-56.
7. Mochizuki, T., Wu, G., Hayashi, T., Xenophontos, S.L., Veldhuisen, B., Saris, J.J., Reynolds, D.M., Cai, Y., Gabow, P.A., Pierides, A., Kimberling, W.J., Breuning, M.H., Deltas, C.C., Peters, D.J., and Somlo, S. (1996) PKD2, a gene for polycystic kidney disease that encodes an integral membrane protein. *Science,* **272,** 1339-1342.
8. Reeders, S.T., Breuning, M.H., Davies, K.E., Nicholls, R.D., Jarman, A.P., Higgs, D.R., Pearson, P.L., and Weatherall, D.J. (1985) A highly

polymorphic DNA marker linked to adult polycystic kidney disease on chromosome 16. *Nature,* **317,** 542-544.

9. Peters, D.J., Spruit, L., Saris, J.J., Ravine, D., Sandkuijl, L.A., Fossdal, R., Boersma, J., van Eijk, R., Nørby, S., Constantinou-Deltas, C.D., Pierides, A., Brissenden, J., Frants, R., van Ommen, G., and Breuning, M. (1993) Chromosome 4 localization of a second gene for autosomal dominant polycystic kidney disease. *Nat Genet,* **5,** 359-362.

10. Tan, Y.C., Blumenfeld, J., and Rennert, H. (2011) Autosomal dominant polycystic kidney disease: genetics, mutations and microRNAs. *Biochim Biophys Acta,* **1812,** 1202-1212.

11. Ward, C.J., Peral, B., Hughes, J., Thomas, S., Gamble, V., MacCarthy, A.B., Sloane-Stanley, J., Buckle, V.J., Kearney, L., Higgs, D.R., Ratcliffe, P.J., Harris, P.C., Roelfsema, J.H., Spruit, L., Saris, J.J., Dauwerse, H.G., Peters, D.J., Breuning, M.H., Nellist, M., Brook-Carter, P.T., Maheshwar, M.M., Cordeiro, I., Santos, H., Cabral, P., Sampson, J.R., Janssen, B., Hesseling-Janssen, A.L., van den Ouweland, A.M., Eussen, B., Verhoef, S., Lindhout, D., and Halley, D.J. (1994) The polycystic kidney disease 1 gene encodes a 14 kb transcript and lies within a duplicated region on chromosome 16. The European Polycystic Kidney Disease Consortium. *Cell,* **77,** 881-894.

12. Hughes, J., Ward, C.J., Peral, B., Aspinwall, R., Clark, K., San Millán, J.L., Gamble, V., and Harris, P.C. (1995) The polycystic kidney disease 1 (PKD1)

gene encodes a novel protein with multiple cell recognition domains. *Nat Genet,* **10,** 151-160.

13. Glücksmann-Kuis, M.A., Tayber, O., Woolf, E.A., Bougueleret, L., Deng, N., Alperin, G.D., Iris, F., Hawkins, F., Munro, C., Lakey, N., Duyk, G., Schneider, M.C., Geng, L., Zhang, F., Zhao, Z., Toroslan, S., Zhou, J., Reeders, S.T., Bork, P., Pohischmidt, M., Löhning, C., Kraus, B., Nowicka, U., Leung, A.L., and Frischauf, A. (1995) Polycystic kidney disease: the complete structure of the PKD1 gene and its protein. The International Polycystic Kidney Disease Consortium. *Cell,* **81,** 289-298.
14. Zerres, K., Mücher, G., Bachner, L., Deschennes, G., Eggermann, T., Kääriäinen, H., Knapp, M., Lennert, T., Misselwitz, J., and von Mühlendahl, K.E. (1994) Mapping of the gene for autosomal recessive polycystic kidney disease (ARPKD) to chromosome 6p21-cen. *Nat Genet,* **7,** 429-432.
15. Ward, C.J., Hogan, M.C., Rossetti, S., Walker, D., Sneddon, T., Wang, X., Kubly, V., Cunningham, J.M., Bacallao, R., Ishibashi, M., Milliner, D.S., Torres, V.E., and Harris, P.C. (2002) The gene mutated in autosomal recessive polycystic kidney disease encodes a large, receptor-like protein. *Nat Genet,* **30,** 259-269.
16. Zhang, J., Wu, M., Wang, S., Shah, J.V., Wilson, P.D., and Zhou, J. (2010) Polycystic kidney disease protein fibrocystin localizes to the mitotic spindle and regulates spindle bipolarity. *Hum Mol Genet,* **19,** 3306-3319.

17. Deltas, C., and Papagregoriou, G. (2010) Cystic diseases of the kidney: molecular biology and genetics. *Arch Pathol Lab Med,* **134,** 569-582.

18. Franz, K.A., and Reubi, F.C. (1983) Rate of functional deterioration in polycystic kidney disease. *Kidney Int,* **23,** 526-529.

19. Meijer, E., Gansevoort, R.T., de Jong, P.E., van der Wal, A.M., Leonhard, W.N., de Krey, S.R., van den Born, J., Mulder, G.M., van Goor, H., Struck, J., de Heer, E., and Peters, D.J. (2011) Therapeutic potential of vasopressin V2 receptor antagonist in a mouse model for autosomal dominant polycystic kidney disease: optimal timing and dosing of the drug. *Nephrol Dial Transplant,* **26,** 2445-2453.

20. Walz, G., Budde, K., Mannaa, M., Nürnberger, J., Wanner, C., Sommerer, C., Kunzendorf, U., Banas, B., Hörl, W.H., Obermüller, N., Arns, W., Pavenstädt, H., Gaedeke, J., Büchert, M., May, C., Gschaidmeier, H., Kramer, S., and Eckardt, K.U. (2010) Everolimus in patients with autosomal dominant polycystic kidney disease. *N Engl J Med,* **363,** 830-840.

21. Serra, A.L., Poster, D., Kistler, A.D., Krauer, F., Raina, S., Young, J., Rentsch, K.M., Spanaus, K.S., Senn, O., Kristanto, P., Scheffel, H., Weishaupt, D., and Wüthrich, R.P. (2010) Sirolimus and kidney growth in autosomal dominant polycystic kidney disease. *N Engl J Med,* **363,** 820-829.

22. Grantham, J.J., Bennett, W.M., and Perrone, R.D. (2011) mTOR inhibitors and autosomal dominant polycystic kidney disease. *N Engl J Med,* **364,** 286-7; author reply 287-9.

23. Liang, G., Li, Q., Tang, Y., Kokame, K., Kikuchi, T., Wu, G., and Chen, X.Z. (2008) Polycystin-2 is regulated by endoplasmic reticulum-associated degradation. *Hum Mol Genet,* **17,** 1109-1119.

24. Liang, G., Yang, J., Wang, Z., Li, Q., Tang, Y., and Chen, X.Z. (2008) Polycystin-2 down-regulates cell proliferation via promoting PERK-dependent phosphorylation of eIF2alpha. *Hum Mol Genet,* **17,** 3254-3262.

25. Gao, H., Wang, Y., Wegierski, T., Skouloudaki, K., Pütz, M., Fu, X., Engel, C., Boehlke, C., Peng, H., Kuehn, E.W., Kim, E., Kramer-Zucker, A., and Walz, G. (2010) PRKCSH/80K-H, the protein mutated in polycystic liver disease, protects polycystin-2/TRPP2 against HERP-mediated degradation. *Hum Mol Genet,* **19,** 16-24.

26. Hetz, C. (2012) The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol,* **13,** 89-102.

27. Ma, Y., and Hendershot, L.M. (2004) The role of the unfolded protein response in tumour development: friend or foe? *Nat Rev Cancer,* **4,** 966-977.

28. Thastrup, O. (1990) Role of Ca^{2+}-ATPases in regulation of cellular Ca^{2+} signalling, as studied with the selective microsomal Ca2(+)-ATPase inhibitor, thapsigargin. *Agents Actions,* **29,** 8-15.

29. Huet, G., Gouyer, V., Delacour, D., Richet, C., Zanetta, J.P., Delannoy, P., and Degand, P. (2003) Involvement of glycosylation in the intracellular trafficking of glycoproteins in polarized epithelial cells. *Biochimie,* **85,** 323-330.

30. Doyle, K.M., Kennedy, D., Gorman, A.M., Gupta, S., Healy, S.J., and Samali, A. (2011) Unfolded proteins and endoplasmic reticulum stress in neurodegenerative disorders. *J Cell Mol Med,* **15,** 2025-2039.

31. Okada, S., Misaka, T., Tanaka, Y., Matsumoto, I., Ishibashi, K., Sasaki, S., and Abe, K. (2008) Aquaporin-11 knockout mice and polycystic kidney disease animals share a common mechanism of cyst formation. *FASEB J,* **22,** 3672-3684.

32. Chen, P., Burdette, A.J., Porter, J.C., Ricketts, J.C., Fox, S.A., Nery, F.C., Hewett, J.W., Berkowitz, L.A., Breakefield, X.O., Caldwell, K.A., and Caldwell, G.A. (2010) The early-onset torsion dystonia-associated protein, torsinA, is a homeostatic regulator of endoplasmic reticulum stress response. *Hum Mol Genet,* **19,** 3502-3515.

33. Nomura, H., Turco, A.E., Pei, Y., Kalaydjieva, L., Schiavello, T., Weremowicz, S., Ji, W., Morton, C.C., Meisler, M., Reeders, S.T., and Zhou, J. (1998) Identification of PKDL, a novel polycystic kidney disease 2-like gene whose murine homologue is deleted in mice with kidney and retinal defects. *J Biol Chem,* **273,** 25967-25973.

34. Li, J., and Lee, A.S. (2006) Stress induction of GRP78/BiP and its role in cancer. *Curr Mol Med,* **6,** 45-54.

35. Oikawa, D., Tokuda, M., Hosoda, A., and Iwawaki, T. (2010) Identification of a consensus element recognized and cleaved by IRE1 alpha. *Nucleic Acids Res,* **38,** 6265-6273.

36. Pincus, D., Chevalier, M.W., Aragón, T., van Anken, E., Vidal, S.E., El-Samad, H., and Walter, P. (2010) BiP binding to the ER-stress sensor Ire1 tunes the homeostatic behavior of the unfolded protein response. *PLoS Biol,* **8,** e1000415.

37. Korennykh, A.V., Egea, P.F., Korostelev, A.A., Finer-Moore, J., Zhang, C., Shokat, K.M., Stroud, R.M., and Walter, P. (2009) The unfolded protein response signals through high-order assembly of Ire1. *Nature,* **457,** 687-693.

38. Hirota, M., Kitagaki, M., Itagaki, H., and Aiba, S. (2006) Quantitative measurement of spliced XBP1 mRNA as an indicator of endoplasmic reticulum stress. *J Toxicol Sci,* **31,** 149-156.

39. Yamamoto, K., Yoshida, H., Kokame, K., Kaufman, R.J., and Mori, K. (2004) Differential contributions of ATF6 and XBP1 to the activation of endoplasmic reticulum stress-responsive cis-acting elements ERSE, UPRE and ERSE-II. *J Biochem,* **136,** 343-350.

40. Kaneko, M., Yasui, S., Niinuma, Y., Arai, K., Omura, T., Okuma, Y., and Nomura, Y. (2007) A different pathway in the endoplasmic reticulum stress-

induced expression of human HRD1 and SEL1 genes. *FEBS Lett,* **581,** 5355-5360.

41. Sriburi, R., Bommiasamy, H., Buldak, G.L., Robbins, G.R., Frank, M., Jackowski, S., and Brewer, J.W. (2007) Coordinate regulation of phospholipid biosynthesis and secretory pathway gene expression in XBP-1(S)-induced endoplasmic reticulum biogenesis. *Journal of Biological Chemistry,* **282,** 7024-7034.
42. Brewer, J.W., and Jackowski, S. (2012) UPR-Mediated Membrane Biogenesis in B Cells. *Biochem Res Int,* **2012,** 738471.
43. Harding, H.P., Zhang, Y., and Ron, D. (1999) Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature,* **397,** 271-274.
44. Marciniak, S.J., Garcia-Bonilla, L., Hu, J., Harding, H.P., and Ron, D. (2006) Activation-dependent substrate recruitment by the eukaryotic translation initiation factor 2 kinase PERK. *J Cell Biol,* **172,** 201-209.
45. Sequeira, S.J., Ranganathan, A.C., Adam, A.P., Iglesias, B.V., Farias, E.F., and Aguirre-Ghiso, J.A. (2007) Inhibition of proliferation by PERK regulates mammary acinar morphogenesis and tumor formation. *PLoS One,* **2,** e615.
46. Su, N., and Kilberg, M.S. (2008) C/EBP homology protein (CHOP) interacts with activating transcription factor 4 (ATF4) and negatively

regulates the stress-dependent induction of the asparagine synthetase gene. *J Biol Chem,* **283,** 35106-35117.

47. Lange, P.S., Chavez, J.C., Pinto, J.T., Coppola, G., Sun, C.-W., Townes, T.M., Geschwind, D.H., and Ratan, R.R. (2008) ATF4 is an oxidative stress–inducible, prodeath transcription factor in neurons in vitro and in vivo. *The Journal of Experimental Medicine,* **205,** 1227-1242.

48. Palam, L.R., Baird, T.D., and Wek, R.C. (2011) Phosphorylation of eIF2 facilitates ribosomal bypass of an inhibitory upstream ORF to enhance CHOP translation. *J Biol Chem,* **286,** 10939-10949.

49. Rzymski, T., Milani, M., Singleton, D.C., and Harris, A.L. (2009) Role of ATF4 in regulation of autophagy and resistance to drugs and hypoxia. *Cell Cycle,* **8,** 3838-3847.

50. Belal, C., Ameli, N.J., El Kommos, A., Bezalel, S., Al'khafaji, A.M., Mughal, M.R., Mattson, M.P., Kyriazis, G.A., Tyrberg, B., and Chan, S.L. (2012) The homocysteine-inducible endoplasmic reticulum (ER) stress protein Herp counteracts mutant α-synuclein-induced ER stress via the homeostatic regulation of ER-resident calcium release channel proteins. *Hum Mol Genet,* **21,** 963-977.

51. Harding, H.P., Zhang, Y., Zeng, H., Novoa, I., Lu, P.D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D.F., Bell, J.C., Hettmann, T., Leiden, J.M., and Ron, D. (2003) An integrated stress response regulates

amino acid metabolism and resistance to oxidative stress. *Mol Cell,* **11,** 619-633.

52. Lewerenz, J., Sato, H., Albrecht, P., Henke, N., Noack, R., Methner, A., and Maher, P. (2012) Mutation of ATF4 mediates resistance of neuronal cell lines against oxidative stress by inducing xCT expression. *Cell Death Differ,* **19,** 847-858.

53. Ma, Y., and Hendershot, L.M. (2004) Herp is dually regulated by both the endoplasmic reticulum stress-specific branch of the unfolded protein response and a branch that is shared with other cellular stress pathways. *J Biol Chem,* **279,** 13792-13799.

54. Okada, T., Yoshida, H., Akazawa, R., Negishi, M., and Mori, K. (2002) Distinct roles of activating transcription factor 6 (ATF6) and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) in transcription during the mammalian unfolded protein response. *Biochem J,* **366,** 585-594.

55. Wu, J., Rutkowski, D.T., Dubois, M., Swathirajan, J., Saunders, T., Wang, J., Song, B., Yau, G.D., and Kaufman, R.J. (2007) ATF6alpha optimizes long-term endoplasmic reticulum function to protect cells from chronic stress. *Dev Cell,* **13,** 351-364.

56. Hosokawa, N., Wada, I., Hasegawa, K., Yorihuzi, T., Tremblay, L.O., Herscovics, A., and Nagata, K. (2001) A novel ER alpha-mannosidase-like protein accelerates ER-associated degradation. *EMBO Rep,* **2,** 415-422.

57. Chakrabarti, A., Chen, A.W., and Varner, J.D. (2011) A review of the mammalian unfolded protein response. *Biotechnol Bioeng,* **108,** 2777-2793.

58. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell,* **107,** 881-891.

59. Kokame, K., Kato, H., and Miyata, T. (2001) Identification of ERSE-II, a new cis-acting element responsible for the ATF6-dependent mammalian unfolded protein response. *J Biol Chem,* **276,** 9199-9205.

60. Yamamoto, K., Suzuki, N., Wada, T., Okada, T., Yoshida, H., Kaufman, R.J., and Mori, K. (2008) Human HRD1 promoter carries a functional unfolded protein response element to which XBP1 but not ATF6 directly binds. *J Biochem,* **144,** 477-486.

61. Smith, S.E., Granell, S., Salcedo-Sicilia, L., Baldini, G., Egea, G., Teckman, J.H., and Baldini, G. (2011) Activating transcription factor 6 limits intracellular accumulation of mutant α(1)-antitrypsin Z and mitochondrial damage in hepatoma cells. *J Biol Chem,* **286,** 41563-41577.

62. Smith, M.H., Ploegh, H.L., and Weissman, J.S. (2011) Road to ruin: targeting proteins for degradation in the endoplasmic reticulum. *Science,* **334,** 1086-1090.

63. Bernasconi, R., Galli, C., Noack, J., Bianchi, S., de Haan, C.A., Reggiori, F., and Molinari, M. (2012) Role of the SEL1L:LC3-I Complex as an ERAD Tuning Receptor in the Mammalian ER. *Mol Cell,* **46,** 1-11.

64. Hori, O., Ichinoda, F., Yamaguchi, A., Tamatani, T., Taniguchi, M., Koyama, Y., Katayama, T., Tohyama, M., Stern, D.M., Ozawa, K., Kitao, Y., and Ogawa, S. (2004) Role of Herp in the endoplasmic reticulum stress response. *Genes Cells,* **9,** 457-469.

65. Määttänen, P., Gehring, K., Bergeron, J.J., and Thomas, D.Y. (2010) Protein quality control in the ER: the recognition of misfolded proteins. *Semin Cell Dev Biol,* **21,** 500-511.

66. Pajonk, F., van Ophoven, A., Weissenberger, C., and McBride, W.H. (2005) The proteasome inhibitor MG-132 sensitizes PC-3 prostate cancer cells to ionizing radiation by a DNA-PK-independent mechanism. *BMC Cancer,* **5,** 76.

67. Vuong, L., Conley, S.M., and Al-Ubaidi, M.R. (2012) Expression and role of p53 in the retina. *Invest Ophthalmol Vis Sci,* **53,** 1362-1371.

68. Tabas, I., and Ron, D. (2011) Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol,* **13,** 184-190.

69. Woehlbier, U., and Hetz, C. (2011) Modulating stress responses by the UPRosome: a matter of life and death. *Trends Biochem Sci,* **36,** 329-337.

70. Pedersen, S.F., Owsianik, G., and Nilius, B. (2005) TRP channels: an overview. *Cell Calcium,* **38,** 233-252.

71. Rao, R.V., Castro-Obregon, S., Frankowski, H., Schuler, M., Stoka, V., del Rio, G., Bredesen, D.E., and Ellerby, H.M. (2002) Coupling endoplasmic

reticulum stress to the cell death program. An Apaf-1-independent intrinsic pathway. *J Biol Chem,* **277,** 21836-21842.

72. Chan, S.L., Fu, W., Zhang, P., Cheng, A., Lee, J., Kokame, K., and Mattson, M.P. (2004) Herp stabilizes neuronal Ca^{2+} homeostasis and mitochondrial function during endoplasmic reticulum stress. *J Biol Chem,* **279,** 28733-28743.

73. Dix, M.M., Simon, G.M., and Cravatt, B.F. (2008) Global mapping of the topography and magnitude of proteolytic events in apoptosis. *Cell,* **134,** 679-691.

74. Mahrus, S., Trinidad, J.C., Barkan, D.T., Sali, A., Burlingame, A.L., and Wells, J.A. (2008) Global sequencing of proteolytic cleavage sites in apoptosis by specific labeling of protein N termini. *Cell,* **134,** 866-876.

75. Satoh, S., Hijikata, M., Handa, H., and Shimotohno, K. (1999) Caspase-mediated cleavage of eukaryotic translation initiation factor subunit 2alpha. *Biochem J,* **342,** 65-70.

76. Hirota, J., Furuichi, T., and Mikoshiba, K. (1999) Inositol 1,4,5-trisphosphate receptor type 1 is a substrate for caspase-3 and is cleaved during apoptosis in a caspase-3-dependent manner. *J Biol Chem,* **274,** 34433-34437.

77. Tsiokas, L., Arnould, T., Zhu, C., Kim, E., Walz, G., and Sukhatme, V.P. (1999) Specific association of the gene product of PKD2 with the TRPC1 channel. *Proc Natl Acad Sci,* **96,** 3934-3939.

78. Thiede, B., Treumann, A., Kretschmer, A., Söhlke, J., and Rudel, T. (2005) Shotgun proteome analysis of protein cleavage in apoptotic cells. *Proteomics,* **5,** 2123-2130.

79. Li, Q., Montalbetti, N., Shen, P.Y., Dai, X.Q., Cheeseman, C.I., Karpinski, E., Wu, G., Cantiello, H.F., and Chen, X.Z. (2005) Alpha-actinin associates with polycystin-2 and regulates its channel activity. *Hum Mol Genet,* **14,** 1587-1603.

80. Crawford, E.D., and Wells, J.A. (2011) Caspase substrates and cellular remodeling. *Annu Rev Biochem,* **80,** 1055-1087.

81. CASBAH. http://bioinf.gen.tcd.ie/cgi-bin/casbah/casbah.pl. Accessed June 25, 2012.

82. Upton, J.P., Austgen, K., Nishino, M., Coakley, K.M., Hagen, A., Han, D., Papa, F.R., and Oakes, S.A. (2008) Caspase-2 cleavage of BID is a critical apoptotic signal downstream of endoplasmic reticulum stress. *Mol Cell Biol,* **28,** 3943-3951.

83. Grimm, S. (2012) The ER-mitochondria interface: the social network of cell death. *Biochim Biophys Acta,* **1823,** 327-334.

84. Stutzmann, G.E., and Mattson, M.P. (2011) Endoplasmic reticulum Ca^{2+} handling in excitable cells in health and disease. *Pharmacol Rev,* **63,** 700-727.

85. Collins, S.R., and Meyer, T. (2011) Evolutionary origins of STIM1 and STIM2 within ancient Ca^{2+} signaling systems. *Trends Cell Biol,* **21,** 202-211.

86. Prakriya, M. (2009) The molecular physiology of CRAC channels. *Immunol Rev,* **231,** 88-98.

87. Köttgen, M., Benzing, T., Simmen, T., Tauber, R., Buchholz, B., Feliciangeli, S., Huber, T.B., Schermer, B., Kramer-Zucker, A., Höpker, K., Simmen, K.C., Tschucke, C.C., Sandford, R., Kim, E., Thomas, G., and Walz, G. (2005) Trafficking of TRPP2 by PACS proteins represents a novel mechanism of ion channel regulation. *EMBO J,* **24,** 705-716.

88. Lakkaraju, A.K., Abrami, L., Lemmin, T., Blaskovic, S., Kunz, B., Kihara, A., Dal Peraro, M., and van der Goot, F.G. (2012) Palmitoylated calnexin is a key component of the ribosome-translocon complex. *EMBO J,* **31,** 1823-1835.

89. Feng, M., Gu, C., Ma, S., Wang, Y., Liu, H., Han, R., Gao, J., Long, Y., and Mi, H. (2011) Mouse FKBP23 mediates conformer-specific functions of BiP by catalyzing Pro117 cis/trans isomerization. *Biochem Biophys Res Commun,* **408,** 537-540.

90. Bernard-Marissal, N., Moumen, A., Sunyach, C., Pellegrino, C., Dudley, K., Henderson, C.E., Raoul, C., and Pettmann, B. (2012) Reduced calreticulin levels link endoplasmic reticulum stress and Fas-triggered cell death in motoneurons vulnerable to ALS. *J Neurosci,* **32,** 4901-4912.

91. Li, W., Llopis, J., Whitney, M., Zlokarnik, G., and Tsien, R.Y. (1998) Cell-permeant caged InsP3 ester shows that Ca^{2+} spike frequency can optimize gene expression. *Nature,* **392,** 936-941.

92. Carrasco, M.A., Jaimovich, E., Kemmerling, U., and Hidalgo, C. (2004) Signal transduction and gene expression regulated by calcium release from internal stores in excitable cells. *Biol Res,* **37,** 701-712.

93. Mattson, M.P. (1992) Calcium as sculptor and destroyer of neural circuitry. *Exp Gerontol,* **27,** 29-49.

94. Allbritton, N.L., Meyer, T., and Stryer, L. (1992) Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate. *Science,* **258,** 1812-1815.

95. Taylor, C.W., and Dale, P. (2012) Intracellular Ca^{2+} channels - a growing community. *Mol Cell Endocrinol,* **353,** 21-28.

96. Koulen, P., Cai, Y., Geng, L., Maeda, Y., Nishimura, S., Witzgall, R., Ehrlich, B.E., and Somlo, S. (2002) Polycystin-2 is an intracellular calcium release channel. *Nat Cell Biol,* **4,** 191-197.

97. Dorn, G.W., and Maack, C. (2012) SR and mitochondria: Calcium cross-talk between kissing cousins. *J Mol Cell Cardiol,*

*9*8. Rizzuto, R., De Stefani, D., Raffaello, A., and Mammucari, C. (2012) Mitochondria as sensors and regulators of calcium signalling. *Nat Rev Mol Cell Biol,*

*9*9. Cosens, J., and Manning, A. (1969) Abnormal Electroretinogram from a Drosophila Mutant. *Nature,* **224,** 285-287.

100. Nilius, B., and Owsianik, G. (2011) The transient receptor potential family of ion channels. *Genome Biol,* **12,** 218.

101. Hofmann, T., Schaefer, M., Schultz, G., and Gudermann, T. (2002) Subunit composition of mammalian transient receptor potential channels in living cells. *Proc Natl Acad Sci,* **99,** 7461-7466.

102. Zhang, P., Luo, Y., Chasan, B., González-Perrett, S., Montalbetti, N., Timpanaro, G.A., Cantero, M.R., Ramos, A.J., Goldmann, W.H., Zhou, J., and Cantiello, H.F. (2009) The multimeric structure of polycystin-2 (TRPP2): structural-functional correlates of homo- and hetero-multimers with TRPC1. *Hum Mol Genet,* **18,** 1238-1251.

103. Liedtke, W., Choe, Y., Martí-Renom, M.A., Bell, A.M., Denis, C.S., Sali, A., Hudspeth, A.J., Friedman, J.M., and Heller, S. (2000) Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell,* **103,** 525-535.

104. Gallagher, A.R., Cedzich, A., Gretz, N., Somlo, S., and Witzgall, R. (2000) The polycystic kidney disease protein PKD2 interacts with Hax-1, a protein associated with the actin cytoskeleton. *Proc Natl Acad Sci,* **97,** 4017-4022.

105. Li, Q., Dai, Y., Guo, L., Liu, Y., Hao, C., Wu, G., Basora, N., Michalak, M., and Chen, X.Z. (2003) Polycystin-2 associates with tropomyosin-1, an actin microfilament component. *J Mol Biol,* **325,** 949-962.

106. Winn, M.P., Conlon, P.J., Lynn, K.L., Farrington, M.K., Creazzo, T., Hawkins, A.F., Daskalakis, N., Kwan, S.Y., Ebersviller, S., Burchette, J.L.,

Pericak-Vance, M.A., Howell, D.N., Vance, J.M., and Rosenberg, P.B. (2005) A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. *Science,* **308,** 1801-1804.

107. Rock, M.J., Prenen, J., Funari, V.A., Funari, T.L., Merriman, B., Nelson, S.F., Lachman, R.S., Wilcox, W.R., Reyno, S., Quadrelli, R., Vaglio, A., Owsianik, G., Janssens, A., Voets, T., Ikegawa, S., Nagai, T., Rimoin, D.L., Nilius, B., and Cohn, D.H. (2008) Gain-of-function mutations in TRPV4 cause autosomal dominant brachyolmia. *Nat Genet,* **40,** 999-1003.
108. Deng, H.X., Klein, C.J., Yan, J., Shi, Y., Wu, Y., Fecto, F., Yau, H.J., Yang, Y., Zhai, H., Siddique, N., Hedley-Whyte, E.T., Delong, R., Martina, M., Dyck, P.J., and Siddique, T. (2010) Scapuloperoneal spinal muscular atrophy and CMT2C are allelic disorders caused by alterations in TRPV4. *Nat Genet,* **42,** 165-169.
109. Bach, G. (2005) Mucolipin 1: endocytosis and cation channel--a review. *Pflugers Arch,* **451,** 313-317.
110. Audo, I., Kohl, S., Leroy, B.P., Munier, F.L., Guillonneau, X., Mohand-Saïd, S., Bujakowska, K., Nandrot, E.F., Lorenz, B., Preising, M., Kellner, U., Renner, A.B., Bernd, A., Antonio, A., Moskova-Doumanova, V., Lancelot, M.E., Poloschek, C.M., Drumare, I., Defoort-Dhellemmes, S., Wissinger, B., Léveillard, T., Hamel, C.P., Schorderet, D.F., De Baere, E., Berger, W., Jacobson, S.G., Zrenner, E., Sahel, J.A., Bhattacharya, S.S., and Zeitz, C.

(2009) TRPM1 is mutated in patients with autosomal-recessive complete congenital stationary night blindness. *Am J Hum Genet,* **85,** 720-729.

111. Kruse, M., Schulze-Bahr, E., Corfield, V., Beckmann, A., Stallmeyer, B., Kurtbay, G., Ohmert, I., Schulze-Bahr, E., Brink, P., and Pongs, O. (2009) Impaired endocytosis of the ion channel TRPM4 is associated with human progressive familial heart block type I. *J Clin Invest,* **119,** 2737-2744.
112. Walder, R.Y., Landau, D., Meyer, P., Shalev, H., Tsolia, M., Borochowitz, Z., Boettger, M.B., Beck, G.E., Englehardt, R.K., Carmi, R., and Sheffield, V.C. (2002) Mutation of TRPM6 causes familial hypomagnesemia with secondary hypocalcemia. *Nat Genet,* **31,** 171-174.
113. Karashima, Y., Prenen, J., Meseguer, V., Owsianik, G., Voets, T., and Nilius, B. (2008) Modulation of the transient receptor potential channel TRPA1 by phosphatidylinositol 4,5-biphosphate manipulators. *Pflugers Arch,* **457,** 77-89.
114. Stein, A.T., Ufret-Vincenty, C.A., Hua, L., Santana, L.F., and Gordon, S.E. (2006) Phosphoinositide 3-kinase binds to TRPV1 and mediates NGF-stimulated TRPV1 trafficking to the plasma membrane. *J Gen Physiol,* **128,** 509-522.
115. Bhave, G., Hu, H.J., Glauner, K.S., Zhu, W., Wang, H., Brasier, D.J., Oxford, G.S., and Gereau, R.W. (2003) Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). *Proc Natl Acad Sci,* **100,** 12480-12485.

116. Premkumar, L.S., Raisinghani, M., Pingle, S.C., Long, C., and Pimentel, F. (2005) Downregulation of transient receptor potential melastatin 8 by protein kinase C-mediated dephosphorylation. *J Neurosci,* **25,** 11322-11329.

117. Jeske, N.A., Diogenes, A., Ruparel, N.B., Fehrenbacher, J.C., Henry, M., Akopian, A.N., and Hargreaves, K.M. (2008) A-kinase anchoring protein mediates TRPV1 thermal hyperalgesia through PKA phosphorylation of TRPV1. *Pain,* **138,** 604-616.

118. De Petrocellis, L., Starowicz, K., Moriello, A.S., Vivese, M., Orlando, P., and Di Marzo, V. (2007) Regulation of transient receptor potential channels of melastatin type 8 (TRPM8): effect of cAMP, cannabinoid CB(1) receptors and endovanilloids. *Exp Cell Res,* **313,** 1911-1920.

119. Streets, A.J., Moon, D.J., Kane, M.E., Obara, T., and Ong, A.C. (2006) Identification of an N-terminal glycogen synthase kinase 3 phosphorylation site which regulates the functional localization of polycystin-2 in vivo and in vitro. *Hum Mol Genet,* **15,** 1465-1473.

120. Cai, Y., Maeda, Y., Cedzich, A., Torres, V.E., Wu, G., Hayashi, T., Mochizuki, T., Park, J.H., Witzgall, R., and Somlo, S. (1999) Identification and characterization of polycystin-2, the PKD2 gene product. *J Biol Chem,* **274,** 28557-28565.

121. Wu, G., Hayashi, T., Park, J.H., Dixit, M., Reynolds, D.M., Li, L., Maeda, Y., Cai, Y., Coca-Prados, M., and Somlo, S. (1998) Identification of PKD2L,

a human PKD2-related gene: tissue-specific expression and mapping to chromosome 10q25. *Genomics,* **54,** 564-568.

122. Guo, L., Schreiber, T.H., Weremowicz, S., Morton, C.C., Lee, C., and Zhou, J. (2000) Identification and characterization of a novel polycystin family member, polycystin-L2, in mouse and human: sequence, expression, alternative splicing, and chromosomal localization. *Genomics,* **64,** 241-251.
123. NetNGlyc 1.0 Server. http://www.cbs.dtu.dk/services/NetNGlyc/. Accessed June 18, 2012.
124. Richardson, J.S., and Richardson, D.C. (1988) Helix lap-joints as ion-binding sites: DNA-binding motifs and Ca-binding "EF hands" are related by charge and sequence reversal. *Proteins,* **4,** 229-239.
125. Kretsinger, R.H., and Nockolds, C.E. (1973) Carp muscle calcium-binding protein. II. Structure determination and general description. *J Biol Chem,* **248,** 3313-3326.
126. Petri, E.T., Celic, A., Kennedy, S.D., Ehrlich, B.E., Boggon, T.J., and Hodsdon, M.E. (2010) Structure of the EF-hand domain of polycystin-2 suggests a mechanism for Ca^{2+}-dependent regulation of polycystin-2 channel activity. *Proc Natl Acad Sci,* **107,** 9176-9181.
127. Qian, F., Germino, F.J., Cai, Y., Zhang, X., Somlo, S., and Germino, G.G. (1997) PKD1 interacts with PKD2 through a probable coiled-coil domain. *Nat Genet,* **16,** 179-183.

128. Hidaka, S., Könecke, V., Osten, L., and Witzgall, R. (2004) PIGEA-14, a novel coiled-coil protein affecting the intracellular distribution of polycystin-2. *J Biol Chem,* **279,** 35009-35016.

129. "Basic Local Alignment Search Tool". http://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed June 18, 2012.

130. Chen, X.Z., Vassilev, P.M., Basora, N., Peng, J.B., Nomura, H., Segal, Y., Brown, E.M., Reeders, S.T., Hediger, M.A., and Zhou, J. (1999) Polycystin-L is a calcium-regulated cation channel permeable to calcium ions. *Nature,* **401,** 383-386.

131. LopezJimenez, N.D., Cavenagh, M.M., Sainz, E., Cruz-Ithier, M.A., Battey, J.F., and Sullivan, S.L. (2006) Two members of the TRPP family of ion channels, Pkd1l3 and Pkd2l1, are co-expressed in a subset of taste receptor cells. *J Neurochem,* **98,** 68-77.

132. Ishimaru, Y., Inada, H., Kubota, M., Zhuang, H., Tominaga, M., and Matsunami, H. (2006) Transient receptor potential family members PKD1L3 and PKD2L1 form a candidate sour taste receptor. *Proc Natl Acad Sci,* **103,** 12569-12574.

133. Horio, N., Yoshida, R., Yasumatsu, K., Yanagawa, Y., Ishimaru, Y., Matsunami, H., and Ninomiya, Y. (2011) Sour taste responses in mice lacking PKD channels. *PLoS One,* **6,** e20007.

134. Veldhuisen, B., Spruit, L., Dauwerse, H.G., Breuning, M.H., and Peters, D.J. (1999) Genes homologous to the autosomal dominant polycystic kidney disease genes (PKD1 and PKD2). *Eur J Hum Genet,* **7,** 860-872.

135. Volk, T., Schwoerer, A.P., Thiessen, S., Schultz, J.H., and Ehmke, H. (2003) A polycystin-2-like large conductance cation channel in rat left ventricular myocytes. *Cardiovasc Res,* **58,** 76-88.

136. Chen, Y., Zhang, Z., Lv, X.Y., Wang, Y.D., Hu, Z.G., Sun, H., Tan, R.Z., Liu, Y.H., Bian, G.H., Xiao, Y., Li, Q.W., Yang, Q.T., Ai, J.Z., Feng, L., Yang, Y., Wei, Y.Q., and Zhou, Q. (2008) Expression of Pkd2l2 in testis is implicated in spermatogenesis. *Biol Pharm Bull,* **31,** 1496-1500.

137. Foggensteiner, L., Bevan, A.P., Thomas, R., Coleman, N., Boulter, C., Bradley, J., Ibraghimov-Beskrovnaya, O., Klinger, K., and Sandford, R. (2000) Cellular and subcellular distribution of polycystin-2, the protein product of the PKD2 gene. *J Am Soc Nephrol,* **11,** 814-827.

138. Markowitz, G.S., Cai, Y., Li, L., Wu, G., Ward, L.C., Somlo, S., and D'Agati, V.D. (1999) Polycystin-2 expression is developmentally regulated. *Am J Physiol,* **277,** F17-F25.

139. Zhao, Y., Haylor, J.L., and Ong, A.C. (2002) Polycystin-2 expression is increased following experimental ischaemic renal injury. *Nephrol Dial Transplant,* **17,** 2138-2144.

140. Cai, Y., Anyatonwu, G., Okuhara, D., Lee, K.B., Yu, Z., Onoe, T., Mei, C.L., Qian, Q., Geng, L., Wiztgall, R., Ehrlich, B.E., and Somlo, S. (2004)

Calcium dependence of polycystin-2 channel activity is modulated by phosphorylation at Ser812. *J Biol Chem,* **279,** 19987-19995.

141. Streets, A.J., Needham, A.J., Gill, S.K., and Ong, A.C. (2010) Protein kinase D-mediated phosphorylation of polycystin-2 (TRPP2) is essential for its effects on cell growth and calcium channel activity. *Mol Biol Cell,* **21,** 3853-3865.

142. Sammels, E., Devogelaere, B., Mekahli, D., Bultynck, G., Missiaen, L., Parys, J.B., Cai, Y., Somlo, S., and De Smedt, H. (2010) Polycystin-2 activation by inositol 1,4,5-trisphosphate-induced Ca^{2+} release requires its direct association with the inositol 1,4,5-trisphosphate receptor in a signaling microdomain. *J Biol Chem,* **285,** 18794-18805.

143. Celic, A.S., Petri, E.T., Benbow, J., Hodsdon, M.E., Ehrlich, B.E., and Boggon, T.J. (2012) Calcium-induced Conformational Changes in C-terminal Tail of Polycystin-2 Are Necessary for Channel Gating. *J Biol Chem,* **287,** 17232-17240.

144. Obermüller, N., Gallagher, A.R., Cai, Y., Gassler, N., Gretz, N., Somlo, S., and Witzgall, R. (1999) The rat pkd2 protein assumes distinct subcellular distributions in different organs. *Am J Physiol,* **277,** F914-F925.

145. Witzgall, R. (2005) Polycystin-2--an intracellular or plasma membrane channel? *Naunyn Schmiedebergs Arch Pharmacol,* **371,** 342-347.

146. Newby, L.J., Streets, A.J., Zhao, Y., Harris, P.C., Ward, C.J., and Ong, A.C. (2002) Identification, characterization, and localization of a novel kidney polycystin-1-polycystin-2 complex. *J Biol Chem,* **277,** 20763-20773.

147. Nilius, B., Talavera, K., Owsianik, G., Prenen, J., Droogmans, G., and Voets, T. (2005) Gating of TRP channels: a voltage connection? *J Physiol,* **567,** 35-44.

148. Liu, Y., Li, Q., Tan, M., Zhang, Y.Y., Karpinski, E., Zhou, J., and Chen, X.Z. (2002) Modulation of the human polycystin-L channel by voltage and divalent cations. *FEBS Lett,* **525,** 71-76.

149. Fu, X., Wang, Y., Schetle, N., Gao, H., Pütz, M., von Gersdorff, G., Walz, G., and Kramer-Zucker, A.G. (2008) The subcellular localization of TRPP2 modulates its function. *J Am Soc Nephrol,* **19,** 1342-1351.

150. Ma, R., Li, W.P., Rundle, D., Kong, J., Akbarali, H.I., and Tsiokas, L. (2005) PKD2 functions as an epidermal growth factor-activated plasma membrane channel. *Mol Cell Biol,* **25,** 8285-8298.

151. Hoffmeister, H., Babinger, K., Gürster, S., Cedzich, A., Meese, C., Schadendorf, K., Osten, L., de Vries, U., Rascle, A., and Witzgall, R. (2011) Polycystin-2 takes different routes to the somatic and ciliary plasma membrane. *J Cell Biol,* **192,** 631-645.

152. Hanaoka, K., Qian, F., Boletta, A., Bhunia, A.K., Piontek, K., Tsiokas, L., Sukhatme, V.P., Guggino, W.B., and Germino, G.G. (2000) Co-assembly of

polycystin-1 and -2 produces unique cation-permeable currents. *Nature,* **408,** 990-994.

153. Anyatonwu, G.I., and Ehrlich, B.E. (2005) Organic cation permeation through the channel formed by polycystin-2. *J Biol Chem,* **280,** 29488-29493.

154. Vassilev, P.M., Guo, L., Chen, X.Z., Segal, Y., Peng, J.B., Basora, N., Babakhanlou, H., Cruger, G., Kanazirska, M., Ye, C., Brown, E.M., Hediger, M.A., and Zhou, J. (2001) Polycystin-2 is a novel cation channel implicated in defective intracellular Ca^{2+} homeostasis in polycystic kidney disease. *Biochem Biophys Res Commun,* **282,** 341-350.

155. Li, Y., Wright, J.M., Qian, F., Germino, G.G., and Guggino, W.B. (2005) Polycystin 2 interacts with type I inositol 1,4,5-trisphosphate receptor to modulate intracellular Ca^{2+} signaling. *J Biol Chem,* **280,** 41298-41306.

156. Mekahli, D., Sammels, E., Luyten, T., Welkenhuyzen, K., van den Heuvel, L.P., Levtchenko, E.N., Gijsbers, R., Bultynck, G., Parys, J.B., De Smedt, H., and Missiaen, L. (2012) Polycystin-1 and polycystin-2 are both required to amplify inositol-trisphosphate-induced Ca^{2+} release. *Cell Calcium,* **51,** 452-458.

157. Anyatonwu, G.I., Estrada, M., Tian, X., Somlo, S., and Ehrlich, B.E. (2007) Regulation of ryanodine receptor-dependent calcium signaling by polycystin-2. *Proc Natl Acad Sci,* **104,** 6454-6459.

158. Nauli, S.M., Alenghat, F.J., Luo, Y., Williams, E., Vassilev, P., Li, X., Elia, A.E., Lu, W., Brown, E.M., Quinn, S.J., Ingber, D.E., and Zhou, J. (2003) Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. *Nat Genet,* **33,** 129-137.

159. Montalbetti, N., Cantero, M.R., Dalghi, M.G., and Cantiello, H.F. (2008) Reactive oxygen species inhibit polycystin-2 (TRPP2) cation channel activity in term human syncytiotrophoblast. *Placenta,* **29,** 510-518.

160. Montalbetti, N., Li, Q., Wu, Y., Chen, X.Z., and Cantiello, H.F. (2007) Polycystin-2 cation channel function in the human syncytiotrophoblast is regulated by microtubular structures. *J Physiol,* **579,** 717-728.

161. Jurczyk, A., Gromley, A., Redick, S., San Agustin, J., Witman, G., Pazour, G.J., Peters, D.J., and Doxsey, S. (2004) Pericentrin forms a complex with intraflagellar transport proteins and polycystin-2 and is required for primary cilia assembly. *J Cell Biol,* **166,** 637-643.

162. Wu, Y., Dai, X.Q., Li, Q., Chen, C.X., Mai, W., Hussain, Z., Long, W., Montalbetti, N., Li, G., Glynne, R., Wang, S., Cantiello, H.F., Wu, G., and Chen, X.Z. (2006) Kinesin-2 mediates physical and functional interactions between polycystin-2 and fibrocystin. *Hum Mol Genet,* **15,** 3280-3292.

163. Kirkbride, K.C., Sung, B.H., Sinha, S., and Weaver, A.M. (2011) Cortactin: a multifunctional regulator of cellular invasiveness. *Cell Adh Migr,* **5,** 187-198.

164. Lehtonen, S., Ora, A., Olkkonen, V.M., Geng, L., Zerial, M., Somlo, S., and Lehtonen, E. (2000) In vivo interaction of the adapter protein CD2-associated protein with the type 2 polycystic kidney disease protein, polycystin-2. *J Biol Chem,* **275,** 32888-32893.

165. Chen, X.Z., Li, Q., Wu, Y., Liang, G., Lara, C.J., and Cantiello, H.F. (2008) Submembraneous microtubule cytoskeleton: interaction of TRPP2 with the cell cytoskeleton. *FEBS J,* **275,** 4675-4683.

166. Kny, M., Standera, S., Hartmann-Petersen, R., Kloetzel, P.M., and Seeger, M. (2011) Herp regulates Hrd1-mediated ubiquitylation in a ubiquitin-like domain-dependent manner. *J Biol Chem,* **286,** 5151-5156.

167. HERPUD1. http://www.ncbi.nlm.nih.gov/gene?term=9709. Accessed June 26, 2012.

168. Kokame, K., Agarwala, K.L., Kato, H., and Miyata, T. (2000) Herp, a new ubiquitin-like membrane protein induced by endoplasmic reticulum stress. *J Biol Chem,* **275,** 32846-32853.

169. Liang, G., Audas, T.E., Li, Y., Cockram, G.P., Dean, J.D., Martyn, A.C., Kokame, K., and Lu, R. (2006) Luman/CREB3 induces transcription of the endoplasmic reticulum (ER) stress response protein Herp through an ER stress response element. *Mol Cell Biol,* **26,** 7999-8010.

170. Okuda-Shimizu, Y., and Hendershot, L.M. (2007) Characterization of an ERAD pathway for nonglycosylated BiP substrates, which require Herp. *Mol Cell,* **28,** 544-554.

171. Uniprot Herp1 Human. http://www.uniprot.org/uniprot/Q15011. Accessed June 26, 2012.

172. Chigurupati, S., Wei, Z., Belal, C., Vandermey, M., Kyriazis, G.A., Arumugam, T.V., and Chan, S.L. (2009) The homocysteine-inducible endoplasmic reticulum stress protein counteracts calcium store depletion and induction of CCAAT enhancer-binding protein homologous protein in a neurotoxin model of Parkinson disease. *J Biol Chem,* **284,** 18323-18333.

173. Christensen, L.C., Jensen, N.W., Vala, A., Kamarauskaite, J., Johansson, L., Winther, J.R., Hofmann, K., Teilum, K., and Ellgaard, L. (2012) The human selenoprotein VCP-interacting membrane protein (VIMP) is non-globular and harbors a reductase function in an intrinsically disordered region. *J Biol Chem,* **287,** 26388-26399.

174. Schulze, A., Standera, S., Buerger, E., Kikkert, M., van Voorden, S., Wiertz, E., Koning, F., Kloetzel, P.M., and Seeger, M. (2005) The ubiquitin-domain protein HERP forms a complex with components of the endoplasmic reticulum associated degradation pathway. *J Mol Biol,* **354,** 1021-1027.

175. Sai, X., Kokame, K., Shiraishi, H., Kawamura, Y., Miyata, T., Yanagisawa, K., and Komano, H. (2003) The ubiquitin-like domain of Herp is involved in Herp degradation, but not necessary for its enhancement of amyloid beta-protein generation. *FEBS Lett,* **553,** 151-156.

176. Kim, T.Y., Kim, E., Yoon, S.K., and Yoon, J.B. (2008) Herp enhances ER-associated protein degradation by recruiting ubiquilins. *Biochem Biophys Res Commun,* **369,** 741-746.

177. Kaneko, M., Ishiguro, M., Niinuma, Y., Uesugi, M., and Nomura, Y. (2002) Human HRD1 protects against ER stress-induced apoptosis through ER-associated degradation. *FEBS Lett,* **532,** 147-152.

178. Nadav, E., Shmueli, A., Barr, H., Gonen, H., Ciechanover, A., and Reiss, Y. (2003) A novel mammalian endoplasmic reticulum ubiquitin ligase homologous to the yeast Hrd1. *Biochem Biophys Res Commun,* **303,** 91-97.

179. Ballar, P., Ors, A.U., Yang, H., and Fang, S. (2010) Differential regulation of CFTRDeltaF508 degradation by ubiquitin ligases gp78 and Hrd1. *Int J Biochem Cell Biol,* **42,** 167-173.

180. Kikkert, M., Doolman, R., Dai, M., Avner, R., Hassink, G., van Voorden, S., Thanedar, S., Roitelman, J., Chau, V., and Wiertz, E. (2004) Human HRD1 is an E3 ubiquitin ligase involved in degradation of proteins from the endoplasmic reticulum. *J Biol Chem,* **279,** 3525-3534.

181. Ye, Y., Shibata, Y., Kikkert, M., van Voorden, S., Wiertz, E., and Rapoport, T.A. (2005) Recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane. *Proc Natl Acad Sci,* **102,** 14132-14138.

182. Christianson, J.C., Shaler, T.A., Tyler, R.E., and Kopito, R.R. (2008) OS-9 and GRP94 deliver mutant alpha1-antitrypsin to the Hrd1-SEL1L ubiquitin ligase complex for ERAD. *Nat Cell Biol,* **10,** 272-282.

183. Hosokawa, N., Wada, I., Nagasawa, K., Moriyama, T., Okawa, K., and Nagata, K. (2008) Human XTP3-B forms an endoplasmic reticulum quality control scaffold with the HRD1-SEL1L ubiquitin ligase complex and BiP. *J Biol Chem,* **283,** 20914-20924.

184. Garza, R.M., Sato, B.K., and Hampton, R.Y. (2009) In vitro analysis of Hrd1p-mediated retrotranslocation of its multispanning membrane substrate 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase. *J Biol Chem,* **284,** 14710-14722.

185. Krieg, A.J., Hammond, E.M., and Giaccia, A.J. (2006) Functional analysis of p53 binding under differential stresses. *Mol Cell Biol,* **26,** 7030-7045.

186. Yamasaki, S., Yagishita, N., Sasaki, T., Nakazawa, M., Kato, Y., Yamadera, T., Bae, E., Toriyama, S., Ikeda, R., Zhang, L., Fujitani, K., Yoo, E., Tsuchimochi, K., Ohta, T., Araya, N., Fujita, H., Aratani, S., Eguchi, K., Komiya, S., Maruyama, I., Higashi, N., Sato, M., Senoo, H., Ochi, T., Yokoyama, S., Amano, T., Kim, J., Gay, S., Fukamizu, A., Nishioka, K., Tanaka, K., and Nakajima, T. (2007) Cytoplasmic destruction of p53 by the endoplasmic reticulum-resident ubiquitin ligase 'Synoviolin'. *EMBO J,* **26,** 113-122.

187. Omura, T., Kaneko, M., Okuma, Y., Orba, Y., Nagashima, K., Takahashi, R., Fujitani, N., Matsumura, S., Hata, A., Kubota, K., Murahashi, K., Uehara, T., and Nomura, Y. (2006) A ubiquitin ligase HRD1 promotes the degradation of Pael receptor, a substrate of Parkin. *J Neurochem,* **99,** 1456-1469.

188. Yang, H., Zhong, X., Ballar, P., Luo, S., Shen, Y., Rubinsztein, D.C., Monteiro, M.J., and Fang, S. (2007) Ubiquitin ligase Hrd1 enhances the degradation and suppresses the toxicity of polyglutamine-expanded huntingtin. *Exp Cell Res,* **313,** 538-550.

189. Mühlau, M., Winkelmann, J., Rujescu, D., Giegling, I., Koutsouleris, N., Gaser, C., Arsic, M., Weindl, A., Reiser, M., and Meisenzahl, E.M. (2012) Variation within the Huntington's disease gene influences normal brain structure. *PLoS One,* **7,** e29809.

190. Shmueli, A., Tsai, Y.C., Yang, M., Braun, M.A., and Weissman, A.M. (2009) Targeting of gp78 for ubiquitin-mediated proteasomal degradation by Hrd1: cross-talk between E3s in the endoplasmic reticulum. *Biochem Biophys Res Commun,* **390,** 758-762.

191. Bernardi, K.M., Williams, J.M., Kikkert, M., van Voorden, S., Wiertz, E.J., Ye, Y., and Tsai, B. (2010) The E3 ubiquitin ligases Hrd1 and gp78 bind to and promote cholera toxin retro-translocation. *Mol Biol Cell,* **21,** 140-151.

192. Ying, Z., Wang, H., Fan, H., and Wang, G. (2011) The endoplasmic reticulum (ER)-associated degradation system regulates aggregation and degradation of mutant neuroserpin. *J Biol Chem,* **286,** 20835-20844.

193. Iida, Y., Fujimori, T., Okawa, K., Nagata, K., Wada, I., and Hosokawa, N. (2011) SEL1L protein critically determines the stability of the HRD1-SEL1L endoplasmic reticulum-associated degradation (ERAD) complex to optimize the degradation kinetics of ERAD substrates. *J Biol Chem,* **286,** 16929-16939.

194. Omura, T., Asari, M., Yamamoto, J., Kamiyama, N., Oka, K., Hoshina, C., Maseda, C., Awaya, T., Tasaki, Y., Shiono, H., Shimizu, K., and Matsubara, K. (2012) HRD1 levels increased by zonisamide prevented cell death and caspase-3 activation caused by endoplasmic reticulum stress in SH-SY5Y cells. *J Mol Neurosci,* **46,** 527-535.

195. Wu, G., Markowitz, G.S., Li, L., D'Agati, V.D., Factor, S.M., Geng, L., Tibara, S., Tuchman, J., Cai, Y., Park, J.H., van Adelsberg, J., Hou, H., Kucherlapati, R., Edelmann, W., and Somlo, S. (2000) Cardiac defects and renal failure in mice with targeted mutations in Pkd2. *Nat Genet,* **24,** 75-78.

196. Burtey, S., Riera, M., Ribe, E., Pennekamp, P., Passage, E., Rance, R., Dworniczak, B., and Fontés, M. (2008) Overexpression of PKD2 in the mouse is associated with renal tubulopathy. *Nephrol Dial Transplant,* **23,** 1157-1165.

197. Park, E.Y., Sung, Y.H., Yang, M.H., Noh, J.Y., Park, S.Y., Lee, T.Y., Yook, Y.J., Yoo, K.H., Roh, K.J., Kim, I., Hwang, Y.H., Oh, G.T., Seong, J.K., Ahn, C., Lee, H.W., and Park, J.H. (2009) Cyst formation in kidney via B-Raf signaling in the PKD2 transgenic mice. *J Biol Chem,* **284,** 7214-7222.

198. Obermüller, N., Cai, Y., Kränzlin, B., Thomson, R.B., Gretz, N., Kriz, W., Somlo, S., and Witzgall, R. (2002) Altered expression pattern of polycystin-2 in acute and chronic renal tubular diseases. *J Am Soc Nephrol,* **13,** 1855-1864.

199. Prasad, S., McDaid, J.P., Tam, F.W., Haylor, J.L., and Ong, A.C. (2009) Pkd2 dosage influences cellular repair responses following ischemia-reperfusion injury. *Am J Pathol,* **175,** 1493-1503.

200. Aguiari, G., Banzi, M., Gessi, S., Cai, Y., Zeggio, E., Manzati, E., Piva, R., Lambertini, E., Ferrari, L., Peters, D.J., Lanza, F., Harris, P.C., Borea, P.A., Somlo, S., and Del Senno, L. (2004) Deficiency of polycystin-2 reduces Ca^{2+} channel activity and cell proliferation in ADPKD lymphoblastoid cells. *FASEB J,* **18,** 884-886.

201. RCSB Protein Data Bank 1WGD. http://www.rcsb.org/pdb/explore/remediatedSequence.do?structureId=1WGD. Accessed June 29, 2012.

202. Wee, L.J., Tan, T.W., and Ranganathan, S. (2006) SVM-based prediction of caspase substrate cleavage sites. *BMC Bioinformatics,* **7,** S14.

203. CASVM. http://casbase.org/casvm/index.html. Accessed June 27, 2012.

204. Wee, L.J., Tan, T.W., and Ranganathan, S. (2007) CASVM: web server for SVM-based prediction of caspase substrates cleavage sites. *Bioinformatics,* **23,** 3241-3243.

205. Marutani, T., Maeda, T., Tanabe, C., Zou, K., Araki, W., Kokame, K., Michikawa, M., and Komano, H. (2011) ER-stress-inducible Herp, facilitates the degradation of immature nicastrin. *Biochim Biophys Acta,* **1810,** 790-798.

206. Burr, M.L., Cano, F., Svobodova, S., Boyle, L.H., Boname, J.M., and Lehner, P.J. (2011) HRD1 and UBE2J1 target misfolded MHC class I heavy chains for endoplasmic reticulum-associated degradation. *Proc Natl Acad Sci,* **108,** 2034-2039.

207. Tian, Y., Kolb, R., Hong, J.H., Carroll, J., Li, D., You, J., Bronson, R., Yaffe, M.B., Zhou, J., and Benjamin, T. (2007) TAZ promotes PC2 degradation through a SCFbeta-Trcp E3 ligase complex. *Mol Cell Biol,* **27,** 6383-6395.

208. Spiro, R.G. (2004) Role of N-linked polymannose oligosaccharides in targeting glycoproteins for endoplasmic reticulum-associated degradation. *Cell Mol Life Sci,* **61,** 1025-1041.

209. Schaefer, A., Nethe, M., and Hordijk, P.L. (2012) Ubiquitin links to cytoskeletal dynamics, cell adhesion and migration. *Biochem J,* **442,** 13-25.

210. Kubota, H. (2009) Quality control against misfolded proteins in the cytosol: a network for cell survival. *J Biochem,* **146,** 609-616.

211. Carvalho, P., Goder, V., and Rapoport, T.A. (2006) Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell,* **126,** 361-373.

212. Wegierski, T., Steffl, D., Kopp, C., Tauber, R., Buchholz, B., Nitschke, R., Kuehn, E.W., Walz, G., and Köttgen, M. (2009) TRPP2 channels regulate apoptosis through the Ca^{2+} concentration in the endoplasmic reticulum. *EMBO J,* **28,** 490-499.

213. Aparna, G., Bhuyan, A.K., Sahdev, S., Hasnain, S.E., Kaufman, R.J., and Ramaiah, K.V. (2003) Stress-induced apoptosis in Spodoptera frugiperda (Sf9) cells: baculovirus p35 mitigates eIF2 alpha phosphorylation. *Biochemistry,* **42,** 15352-15360.